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STUDIES INTO THE MAP KINASE PATHWAY OF CELLS TRANSFORMED BY THE FBR MURINE SARCOMA VIRUS AND THEIR REVERTANTS

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Dissertation submitted to the Faculty of Medicine of the

University of Glasgow

in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

Cancer Research Campaign Beatson Laboratories

The Beatson Institute for Cancer Research

Glasgow, December 1998

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ABSTRACT

Neoplastic transformation by the FBR *fos* oncogene is associated with aspects of the transformed phenotype such as morphological alterations, anchorage-independent growth and metastasis. The products of the FBR and FBJ *fos* oncogenes are members of the transcription factor AP-1, which operates downstream of the growth factor - *ras* - MAP Kinase pathway in mammalian cells. The constituents of this pathway, such as the protein kinases Raf-1 and MEK-1 are necessary for normal and oncogenic cell growth.

By contrast to other oncogene transformed fibroblasts, cell transformed by the FBR *fos* display a reduced intensity of MAPK signalling. This suggested to us that *fos*-induced transformation might be independent of upstream signalling elements.

To investigate the role of MAP Kinase signalling and its requirement during *fos*-induced transformation dominant negative mutations that previously have been reported to interfere with transformation by other oncogenes were employed. A transfection approach was used to introduce TAM-67, a dominant negative deletion mutant of the c-*jun* protooncogene, and kinase inactive mutants of Raf-1 and MEK-1. The cell lines isolated as stable clones after transfection had the properties of the revertant, but nevertheless all contained the FBR v-*fos* oncogene.

This investigation suggested that although the MAP Kinase pathway might not be efficiently activated in v-*fos* transformed fibroblasts, it is required for some aspects of the transformed phenotype. This finding extends previous observations that growth factor - *ras* signalling is important for oncogenesis by various oncogenes and that feedback mechanisms are of biological relevance in transformation by nuclear oncogenes.

To the memory of my father

ΑΝΤΙΚΡΥΣ ΓΑΡ ΕΙΠΕΝ ΟΤΙ ΤΟ ΑΛΗΘΕΣ ΚΑΙ ΤΟ ΦΑΙΝΟΜΕΝΟΝ ΤΑΥΤΟΝ ΕΣΤΙ
ΚΑΙ ΟΥΔΕΝ ΔΙΑΦΕΡΕΙ ΤΗΝ ΑΛΗΘΕΙΑΝ ΚΑΙ ΤΟ ΤΗΙ ΑΙΣΘΗΣΕΙ ΦΑΙΝΟΜΕΝΟΝ.

ΑΛΛΑ ΚΑΙ ΤΟ ΦΑΙΝΟΜΕΝΟΝ ΕΚΑΣΤΩΙ ΚΑΙ ΤΟ ΔΟΚΟΥΝ ΤΟΥΤΟ ΚΑΙ ΕΙΝΑΙ ΑΛΗΘΕΣ, ΩΣΠΕΡ ΚΑΙ ΠΡΩΤΑΓΟΡΑΣ ΕΛΕΓΕ,
ΚΑΤΑ ΓΕ ΤΟΝ ΟΡΘΟΝ ΛΟΓΟΝ ΔΙΑΦΕΡΟΝΤΩΝ, ΚΑΙ ΤΗΣ ΜΕΝ ΑΙΣΘΗΣΕΩΣ ΚΑΙ ΤΗΣ ΦΑΝΤΑΣΙΑΣ ΠΕΡΙ ΤΟ ΦΑΙΝΟΜΕΝΟΝ ΕΧΟΥΣΗΣ,
ΤΟΥ ΔΕ ΝΟΥ ΠΕΡΙ ΤΗΝ ΑΛΗΘΕΙΑΝ.

ΔΗΜΟΚΡΙΤΟΣ 113. ΦΙΛΟΠ. Π. ΨΥΧ. 71, 19.

ACKNOWLEDGEMENTS

I would like to thank Professor Brad Ozanne for suggesting the basic questions that lead to the formation of this thesis and for leadership throughout my graduate studies. Teaching me the hard task of repetition and the art of disappointment when facing the unexpected.

During my work at the Beatson Institute I have been helped by many of its scientific staff.
I would like to thank: *Drs* John Pitts, David Gillespie, Chris Barthlomew, Margaret Frame, Richard Lamb, Agelliki Malliri, Una FitzGerald, Heather Spence ; Dr. Joe Winnie, Dr Liz Black, Bill Clark, Val Fincham, Robert McFarlane helped always with any laboratory problems; Peter McHardy with the IT requests.
Especially I would like to thank *Dr.* Genevieve Stapleton for her excellent English and her thorough criticisms *Dr.* Walter Kolch for many discussions and for technical advice regarding signal transduction, and *Dr.* Vladimir Grigoriev for showing me molecular biology,

During the course of these studies I was a recipient of a Cell Biology studentship from the Greek National Scholarship Foundation (IKY).

I also like of thank my friends: Al for his support, Adam for his great intelligence and Lynn for moving me with her car whenever I needed.

Finally I would like to thank the Director, Professor John Wyke for his long term support: making the impossible possible.

DECLARATION

The experimental work that is included in this Thesis is performed by myself unless otherwise stated.
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ABBREVIATIONS

A-MuLV :	Abelson Murine Leukemia Virus
AP-1 :	Activator Protein-1
ATP :	Adenosine 5-triphosphate
BSA :	Bovine serum albumin
cAMP :	cyclic AMP
cAMP DPK :	cAMP-Dependent Protein Kinase (PKA)
C^0 :	Centigrade
Ci :	Curie
DMEM :	Dulbecco's Modified Eagle's Medium
DMSO :	Dimethyl sulfoxide
DNA :	Deoxyribonucleic acid
EGF :	Epidermal growth factor
EGFR :	Epidermal Growth Factor Receptor
ERK1 :	Extracellular (signal) Regulated Kinase 1
ERK2 :	Extracellular (signal) Regulated Kinase 2
FBJ MuSV :	FBJ Murine Sarcoma Virus
FBR MuSV :	FBR Murine Sarcoma Virus
FCS :	Foetal calf serum
FITC :	Fluorescein Isothiocyanate
g :	gram
G-protein :	GTP-binding protein
GRB2 :	Growth Factor Receptor-Bound protein 2
GTP :	Guanosine triphosphate
Ha-MuSV :	Harvey Murine Sarcoma Virus
JNK :	Jun N-terminal Kinase
IGF-I :	Insulin-like Growth Factor I
IRS :	Insulin Receptor Substrate
k :	Kilo
kD :	kilodalton(s)
Ki-MuSV :	Kirsten Murine Sarcoma Virus
l :	litre (10^3 cm^2)
LPA :	Lysophosphatidic acid
LTR:	Long Terminal Repeat
M :	Molar
m :	milli (10^{-3})
μ :	micro (10^{-6})
MAPK :	Mitogen Activated Protein Kinase
MAPKK :	Mitogen Activated Protein Kinase Kinase
MAPKKK :	Mitogen Activated Protein Kinase Kinase Kinase
MEKK :	MEK Kinase
Mo-MuLV :	Moloney Murine Leukemia Virus
n :	nano (10^{-9})
P:	P value (student's Test in statistics)
PAGE :	Polyacrylamide Gel Electrophoresis
PBS :	Dulbecco's Phosphate Buffered Saline
PDGF :	Platelet derived growth factor
PKA :	Protein Kinase A
PKC :	Protein Kinase C
PLCγ1 :	Phospholipase Gamma 1
*ras*GAP :	*ras* GTPase Activating Protein
rpm :	revolutions per minute
RT-PCR :	Reverse Transcriptase-Polymerase Chain Reaction
SAPK :	Stress Activated Protein Kinase
SD:	Standard Deviation
SV40 :	Simian Virus 40
c-*jun* TAM-67 :	Transactivation Mutant-67 of the c-*jun* protooncogene
TPA :	Tetradecanoyl-phorbol-12-myristate-13 acetate
TRITC :	Tetramethyl Rhodamine Isothiocyanate
v/v :	volume for volume

v/w :	volume for weight
wt :	wild type

CHAPTER 1 INTRODUCTION

CHAPTER 1.

INTRODUCTION

1.1 The FBJ and FBR RNA-containing Murine Sarcoma Viruses

1.1.1. The role and origin of retroviral oncogenes

The early observations of Rous, Gross and Harvey implicated a transmissible agent as the cause of murine and avian neoplasms (Rous, 1911; Gross, 1951; Harvey, 1964). These agents were identified as RNA tumour viruses containing cellular-derived oncogenes using molecular hybridisation and mutants of the transforming genes (Martin, 1972; Scolnick *et al.,*1973; Stehelin *et al.,*1976).

Diverse types of viruses can induce a neoplastic phenotype including the large and small DNA viruses, such as adenoviruses and papovaviruses respectively, and the retroviruses which are RNA-containing viruses. The retroviruses are formed after the capture of cellular genes involved in cell proliferation and acquire oncogenic properties through interaction with the host genome (Bishop, 1983).

The oncogene carrying retroviruses elicit a specific series of events that lead to tumourigenicity and to the transformed phenotype *in vitro.* The induction of malignancy follows a pattern of high selectivity for certain tissues with remarkable specificity. Bishop (1982) suggested that retroviral oncogenes are considered to be evolutionary luxuries of the retroviruses, since they are not required for the viral cycle of infection and they play no role in replication of the retrovirus. The role oncogene products, the oncoproteins are usually present in high levels and that seems to be necessary for the induction and maintenance of the transformed phenotype *in vitro* and *in vivo* (Collett *et al.,*1978b). When the levels of retroviral oncogenes decrease cells revert to a normal phenotype (MacPherson, 1965; Deng *et al.,*1974; Deng *et al.,*1977). The proteins encoded by retroviral oncogenes are components of mitogenic signalling (Bishop, 1985, 1991; Cantley *et al.,*1991; Hatakeyama *et al.,*1994) with diverse functions such as : Protein kinases (Collett and Erikson,1978a; Levinson *et al.,*1978), Guanine nucleotide binding proteins (Scolnick *et al.,*1979), Growth factors (Doolittle *et al.,*1983; Waterfield *et al.,*1983), Growth factor Receptors (Downward *et al.,*1984; Ullrich *et al.*,1984) and Transcriptional activators (Weinberger *et al.,*1985; Sap *et al.,*1986; Bohmann *et al.,*1987).

The identification of the *fos* oncogene of the Finkel-Biskis-Jinkins (FBJ) and Finkel-Biskis-Reilly (FBR) Murine Sarcoma Viruses (Finkel *et al.*,1966; Finkel *et al.*,1975) and the establishment of its relationship to the transcription factor AP-1, opened a new area in the molecular biology of experimental cancer. The v-*fos* oncogene exists in two different forms: as a truncated transforming oncogene and as a gag-*fos* oncogene with multiple alterations (van Beveren *et al.*,1983; van Beveren *et al.*,1984). The *fos* oncogenes are involved in the generation of murine osteosarcoma-like tumours and transform cells of mesenchymatic origin *in vitro* (Jenuwein *et al.*,1985).

1.1.2. Isolation of the FBJ and FBR Murine Sarcoma Viruses

The FBJ-MuSV was isolated from a radiation-induced spontaneous mouse osteosacroma in a CF1 mouse (Finkel *et al.*,1966). Extracts derived from such tumours subsequently showed tumourigenic activity *in vivo.* Subcutaneous injection of tumours extracts into newborn mice resulted in the production of tumours with a short incubation time (Finkel *et al.*,1966).

This virus showed specificity towards bone tissue with no other tumours reported to occur at other sites. Electron microscopy showed viral particles similar to those of Moloney (Mo-MuLV), Rauscher (Ra-MuLV) and Friend Murine Leukemia viruses, demonstrating evolutionary relationships among different types of retroviruses, prior to the molecular cloning of the FBJ virus specific oncogene (Van Beveren *et al.*,1983).

A second osteosarcoma-inducing virus was also isolated from a ^{90}Sr-induced osteosarcoma in a X/Gf mouse, termed FBR-Murine Sarcoma Virus (Finkel *et al.*,1975). The FBR-MuSV Virus is an acute oncogenic, type-C replication-defective retrovirus, with high competence single-hit transformation kinetics. The FBR-MuSV induces morphological transformation of all rodent fibroblastic-like cells of mesodermal origin. In tissue culture assays FBR-MuSV caused complete transformation of cells in a rapid manner which was enhanced by the presence of corticosteroids (Lee *et al.*,1979).

The FBJ-MuSV and FBR-MuSV are according to Finkel some of the ubiquitous naturally occurring Murine Sarcoma Viruses (Lee *et al.*,1979). This is due to the fact that they comprise natural viral isolates and not viral strains that have been recovered by other means, such as the injection of Murine Leukemia Viruses in recipient animals (Scolnick *et al.*,1973).

1.1.3. Histological Characteristics of the FBJ-induced neoplasms

Early studies showed that specificity towards bone tissue was prevalent among different strains of mice after infection with the FBJ-MuSV. Locally invasive tumours attached to bone tissue were common in the infected mice. All the locally dividing and invasive cells were of mesoderm origin suggesting that a cell of fibroblastic origin is the target of the FBJ Virus. Electron microscopy studies suggested that a variety of cell types was present (Yumoto *et al.,*1970). The more malignant populations of these cells show differences in the amount of organelles, a phenomenon that is observed also in tissue culture cells infected with the FBJ-MuSV. Collagen fibres were also observed between cell layers in the tumours. These tumours displayed chondrogenic and fibrosarcomatic characteristics with undifferentiated cell types and shared homology, but not identity with human osteosarcomas, where cellular pleiomorphism is restricted.

Further investigation in CBA mice showed that a statistically significant number of mice infected with the FBJ virus developed bone tumours in a short incubation time (Price *et al.,*1972). According to microscopic examination, a variety of cell types was present with a low proliferative mitotic index, low invasiveness, minimal vascularisation, presence of collagen fibres and rich in alkaline phosphatase staining.

Another study with the FBJ-MuSV showed (Ward and Young, 1976) that bone and muscle tumours were formed after a relatively long incubation period in Swiss NIH mice. Small neoplastic foci with periosteal cells of spindle shape with a rounded morphology appeared attached to bone tissue. Neoplastic regions were covered by stroma and extracellular components. Locally invasive regions were identified and no metastases were detected consistent with the previous study. The tumours that are formed by the FBJ-MuSV are mainly localised in the periosteum, in contrast to the human osteosarcomas that arise in the deep bone cortex.

The histological variation, the low degree of metastasis and the cellular pleiomorphism found in the tumours derived from infection with the FBJ-MuSV suggested that no real osteosarcoma is observed after injection with the virus, but that the tumours formed are of a parosteal type of bone tumour as suggested by Verma (Verma and Graham, 1987).

1.1.4. FBJ and FBR Murine Sarcoma Viruses can induce focus formation

Studies with cultured cells showed that the FBJ-MuSV was a replication defective retrovirus, that was present in the infectious extracts as a mix of a transforming virus and a non-pathogenic, helper Leukaemia Virus (Levy *et al.,*1973). In tissue culture assays the FBJ-MuSV induced the formation of discrete foci of rat and mouse cells in tissue culture, whereas the FBJ-MuLV did not. These foci contained a large number of flat elongated Non-Producer (NP) cells that were different from those infected by Mo-MuSV and Ha-MuSV. The non-producer cells are cells that retain their oncogenic potential without releasing viral particles, and they were proven to be a valuable tool in the isolation of the v-*fos* gene encoded by FBJ MuSV.

The transformation kinetics reported by this group had a single-hit profile for the FBJ-MuSV. However another group later showed that the FBJ-MuSV is not so potent in terms of its transforming ability, and serial passaging of the cell lines was required to achieve morphological transformation, and to produce tumours upon injection into new-born mice (Rhim *et al.,*1979).

More recent studies suggest a different role for the two viruses: FBJ-MuSV causes transformation but not immortalisation of cells in tissue culture whereas FBR-MuSV is a potent inducer of both functions (Jenuwein *et al.,*1985). The induction of immortalisation and transformation of tissue culture cells as a property of the FBR-MuSV is unique among retroviral oncogenes.

The v-*fos* oncogene can transform other cell types such as those of epithelial origin and lymphocytes (Lee *et al.,*1993; Valge-Archer *et al.,*1990). These findings raise new questions in the role of v-*fos* issues in cellular transformation, since the identified targets for v-*fos* are cells of mesenchymal origin, a rather restricted cell type. The v-*abl* oncogene also has been shown to be able to induce transformation of diverse cell types, such as lymphoid cells and fibroblasts *in vitro* (Prywes *et al.,*1983).

1.1.5. Identification and cloning of Viral and Cellular fos genes

Curran and Teich (1982a) first reported the isolation of the FBJ MuSV. The establishment of a non-producer cell (NP) line was a prerequisite to isolate and to study the FBJ viral complex. From transformed foci and colonies in soft agar, colonies enriched in transformed cells were

obtained, using the 208F cell line, a thioguanine-resistant derivative of Rat-1 cells (Quade, 1979).

Injection of syngeneic mice with NP cells gave rise to tumours. Tumour Bearing Rat Sera (TBRS) derived from these mice was tested for precipitation of material from [^{35}S] labelled FBJ-MuSV NP cells. This anti-sera precipitated a 39 Kd protein of cellular origin. This protein seemed to be specific for the FBJ-MuSV NP cells since it was not precipitated from 208F uninfected and cells transformed by Kirsten-MuSV (Ki-MuSV), Moloney-MuSV (Mo-MuSV), Abelson-MuLV (A-MuLV) or Rausher-MuLV (Ra-MuLV), and also not by sera that recognise structural proteins of the virus. *In vivo* labelling and tryptic peptide studies showed that this protein was structurally unrelated to envelope proteins of the FBJ-MuSV.

Further investigation identified the transforming component of the FBJ-MuSV as a protein of 55 Kd (Curran and Teich, 1982b). In these experiments TBRS precipitated two different proteins: one of 39 Kd and a novel protein of 55 Kd. The 55 Kd protein is specific for the FBJ Virus Non Producer cells and is not detected in cells transformed by Ki-MuSV, Ab-MuLV and Mo-MuSV. Two dimensional separation of [^{35}S] labelled fingerprints from cell extracts showed that these two proteins are different and distinct from those of *gag* and *env* encoded products (Curran and Teich, 1982b). Studies with *in vitro* translated viral RNA in a reticulocyte lysate and subsequent peptide mapping and immunoprecipitation showed that only the 55 Kd protein was specific for the FBJ complex. Phosphoamino acid analysis showed that this protein is phosphorylated on Serine residues, but not Tyrosine residues and that it does not possess protein kinase activity.

Molecular cloning of a restriction fragment that contained the FBJ-MuSV proviral DNA established the relationship between the *fos* viral genes to those of human and rodent origin (Curran *et al.,*1983). Sequence analysis showed that the FBJ v-*fos* oncogene is derived from the mouse homologue with a major substitution at the 3' end. The fourth exon, which is the last of the mouse c-*fos* protooncogene, is deleted during the biogenesis of the FBJ-MuSV (Van Beveren *et al.,*1983). The product of the FBJ-MuSV is a 381 amino acid protein while the normal c-Fos protein is composed of 380 amino acids with a molecular weight of 55 kD. The two proteins are identical in the first 332 amino acids with the exception of 5 amino acid substitutions. The C-terminus of the FBJ-MuSV-encoded Fos protein however is different compared to the p55$^{c\text{-}Fos}$, due to a alternative reading frame.

The mouse c-*fos* protooncogene, an intron containing gene, is highly conserved between humans and mice (Van Straaten *et al.*,1983). This high evolutionary conservation suggests an important role for the c-*fos* gene in normal cellular physiology. The human and mouse fos genes are 70% homologous at the nucleotide level with higher conservation at the 3' Untranslated Region (UTR) (Van Straaten *et al.*,1983).

The FBR v-*fos* oncogene encodes the $p75^{v\text{-}Fos}$, a gag-Fos fusion protein (Curran and Verma, 1984). This protein has a significant number of alterations. It is fused to *gag* viral sequences at the N-terminus and has a significant substitution of eight amino acids at the C-terminus, which are derived from cellular genomic sequences termed *fox*. FBR $p75^{v\text{-}Fos}$ also lacks also the first 24 and the last 98 amino acids of the mouse c-Fos protein and has three in-frame deletions: one in the N-terminus and two in the main *fos* region (Van Beveren *et al.*,1984) [Fig.1a and b].

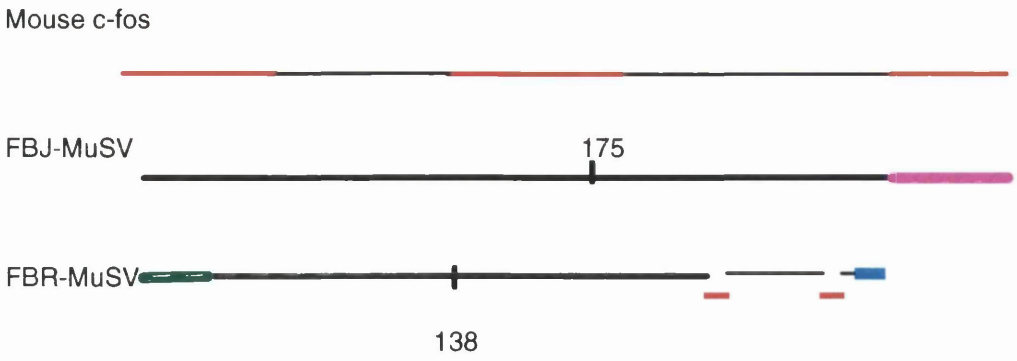


Figure 1a. Structure of cellular and Viral fos genes (Vingron *et al.*,1988).

In the mouse c-fos gene the red regions represent conserved regions through evolution. The red domain in the middle represents the basic-zipper region (bZip), that is required for transformation and transactivation.
In the FBJ-MuSV DNA, amino acid 175 represents a single amino acid change that acts to suppress transformation. The magenta bar in the FBJ C-terminus represents the region that is deleted during the biogenesis of the FBJ-MuSV.
In the FBR-MuSV DNA the green N-terminal fragment represents the gag region of the gene. Amino acid 138 is a point mutation that activates the immortalising potential of the FBR oncogene. The two red C-terminal deletions in the FBR DNA present in the c-fos gene have a negative role in transformation and the blue line represents the cellular fox sequences.

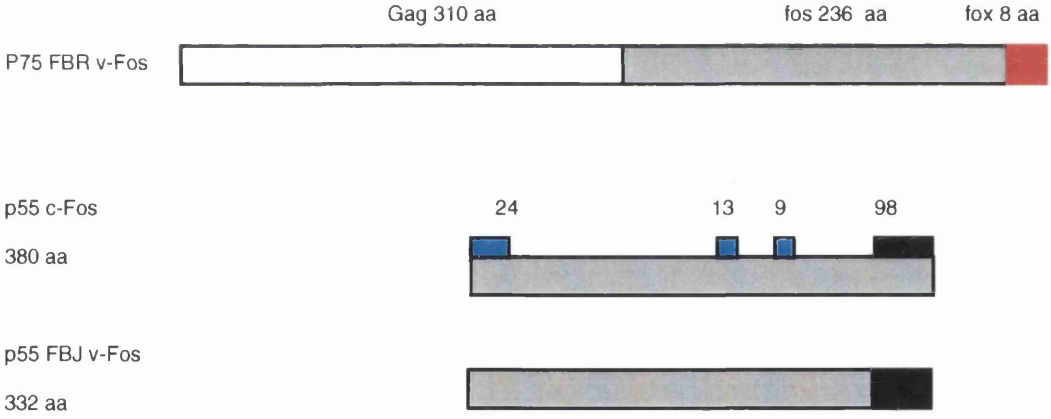


Figure.1b. Structure of the mouse Fos protein and of the products of the FBR and FBJ oncogenes (Muller, 1986) [See 1.1.5. and 1.3.5]. In the FBR v-Fos protein the white region represents the gag moiety, and the red bar in the C-terminus the cellular fox sequences. In the c-Fos protein the three blue bars represent deletions that occur in these particular regions in the sequence of the FBR v-Fos and the black bar in the C-terminus represents the in frame deletion that occurs in the FBJ v-Fos. The same alteration is presented in the FBJ v-Fos protein by a black bar in the C-terminus of the protein.

1.1.6 Genes with similarities with fos: fra-1 and fosB

Genes with homology to c-*fos* have been identified and molecularly cloned. These genes have not been identified as retroviral oncogenes but instead their isolation was performed by antibody screening of phage libraries or by using oligonucleotide probes with homology to c-*fos*. The genes that show homology to *fos* are the *fos*-Related Antigen-1: *fra*-1 (Cohen and Curran, 1988), *fos*B (Zerial *et al.*,1989) and the *fos*-Related Antigen-2: *fra*-2 (Matsui *et al.*,1990). Figure 2 shows the homologies of the two more extensively studied proteins FosB and Fra-1.

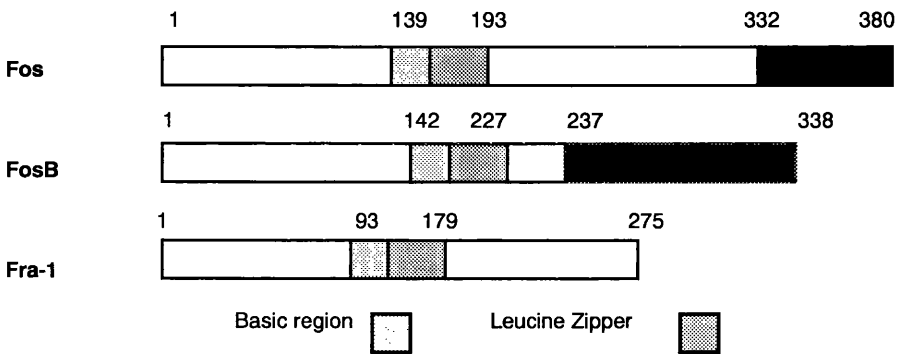


Figure 1.2. Structure of the Fos and Fos-related proteins.
The light grey boxes represent the basic region, the dark grey boxes represent the Leucine zipper domain. The black box in Fos represents the region that is missing from the C-terminus of the FBJ v-Fos protein. The black box in FosB protein represents the region that is missing from the FosB/short form which is an alternatively spliced form of the fosB gene resulting in a protein truncated at the C-terminus

The proteins were firstly identified in experiments using two-dimensional high resolution gel electrophoresis and immunoprecipitation with Fos-specific antibodies from [^{35}S]-labelled extracts (Franza *et al.*,1987 , 1988; Rauscher *et al.*,1988b). Screening of a cDNA library with antibodies to a conserved region of Fos lead to the isolation of a *fos*-related antigen named *fra*-1 (Cohen and Curran, 1988). This gene showed similarity to Fos at the amino acid level, but not at the nucleotide acid sequences. The Fra-1 protein is induced by serum and its kinetics are delayed compared to the kinetics of the induced p55$^{c\text{-}Fos}$ which is very rapid. A functional region mostly conserved between *fra*-1 and c-*fos* was shown previously to correspond to a functional

domain of the GCN4 yeast trans-activator and to sequences of the Jun protein (Vogt *et al.*,1987; Bohmann *et al.*,1987) that are required for DNA-binding.

Bergers *et al.* (1995) have shown that the *fra*-1 gene is up-regulated in fibroblasts transfected with viral or conditional mutants of the c-*fos,* *fos*B, *fra*-1 and c-*jun* genes. Their study shows that the *fra*-1 gene is under positive control by the transcription factor AP-1 in fibroblasts transfected with a Fos-ER fusion protein, and that upon estrogen induction there is a dramatic up-regulation of the *fra*-1 mRNA compared to other AP-1 family members such as *fra*-2, c-*jun*, *jun*B, and *jun*D. *fra*-1 mRNA is also induced at high levels after ectopic expression of the FBJ v-*fos* and *fos*B transforming genes in *rat*-1 fibroblasts. In soft agar assays the *fra*-1 gene induces colony formation to a level similar to that induced by c-*fos,* and the fra-1 clones showed tumourigenic potential when injected into nude mice (Bergers *et al.* 1995).

In *ras* transformed fibroblasts *fra*-1 is over-expressed and the Fra-1 protein is modified by a post-translational mechanism, most probably by phosphorylation, as shown by its retarded mobility after SDS-polyacrylamide electrophoresis (Mechta *et al.*,1997). In FBR v-*fos* transformed fibroblasts the *fra*-1 gene and Fra-1 protein are also up-regulated. Results from this laboratory show that Fra-1 protein levels are higher in the FBR v-*fos* transformed fibroblasts compared to the normal 208F cells (Dr. K. Hawker, L. McGarry and B.W. Ozanne, unpublished observations).

The *fos*B gene was originally identified by screening of a mouse cDNA library using a *fos*-specific probe. This gene displayed considerable homology to *fos*, and the *fos*B mRNA displayed similar kinetics to c-*fos* mRNA. After stimulation of fibroblasts with serum and purified growth factors the *fos*B gene is rapidly induced similar to other immediate early genes (Zerial *et al.*,1989). The product of *fos*B, the FosB protein can form *in vitro* dimers with products of the *jun* family genes such as Jun and JunB, in the presence or in the absence of an oligonucleotide containing an AP-1 site. *fos*B can transform rodent fibroblasts *in vitro* and it encodes a protein with the properties of a transactivator (Schuermann *et al.*,1991).

The cloning of a short form of the *fos*B, (Nakabeppu and Nathans, 1991; Mumberg *et al.*,1991; Yen *et al.*,1991; Dobrzanski *et al.*,1991) was reported which is a natural occurring form of *fos*B resulting from alternative mRNA splicing. The resulting protein lacks the C-terminus of FosB, and is shorter by 100 amino acids in comparison to the parental FosB protein. This

feature is not novel for an oncogene, since a similar transcription event has been described for the c-*abl* protooncogene (Ben-Neriah *et al.*, 1986; Bernards *et al.*, 1988). The truncated version of *fos*B is a negative regulator of cellular transformation and suppresses *fos*-induced transformation (Mumberg *et al.*, 1991).

2. Structure of c-Fos and v-Fos Proteins

2.1. Structure of Fos proteins

2.1.1. Functional Domains in the Fos protein

The p55 c-Fos protein is composed of distinct domains which are responsible in functions including transactivation, dimerization with Jun family members and DNA-binding, and also cellular transformation. A representation of these domains is presented in Figure 1.3.

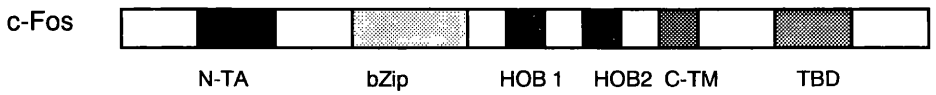


Figure 1.3. Structure of the $p55^{c\text{-}Fos}$ protein
The $p55^{c\text{-}Fos}$ protein contains a N-terminal transactivation domain (N-TA: dark grey box) and the Leucine zipper/basic region (light grey box).Towards the C-terminus is found the recently identified C-Terminal domain (C-TM) and the TATA-binding domain (TBD).

The Fos protein contains an N-Terminal Activation domain (NT-A) which is an independent domain involved in transactivation and transformation (Jooss *et al.*,1994). Adjacent to the N-TA domain is the basic/zipper region (BZip region) which is involved in DNA-binding and dimerisation with Jun family of proteins. The BZip domain includes the basic region which contacts DNA and the Leucine repeat which forms a characteristic helical structure that stabilises protein dimers.

In the C-terminus the HOB domains are involved in transactivation and are conserved between different AP-1 family members. The HOB 1 motif is absent from the FBR $p75^{v\text{-}Fos}$ protein (Sutherland *et al.*,1992). At the C-terminus of the Fos protein is found a TATA-binding motif which is absent from the FBJ and FBR v-Fos proteins (Metz *et al.*,1994a; 1994b). The C-TM domain is present in the c-Fos protein and in the FBJ $p55^{v\text{-}Fos}$, but deleted from the FBR $p75^{v\text{-}Fos}$. In the case of the FBR $p75^{v\text{-}Fos}$, its transforming potential is rescued by the presence of the cellular *fox* sequences, that partially substitute the C-terminus of the wild type Fos protein (Funk *et al.*,1997). In the following sections we give describe in detail the function and the role of these regions in transformation by Fos proteins.

2.1.2. The N-termini of Fos proteins have a role in transformation

Wisdom and Verma (1993a) demonstrated that the region between amino acids 41 and 73 in the N-terminus of FosB and FosB/sf is highly conserved between all Fos family members. Deletion of these amino acids results in a compromised transforming ability of FosB. These studies suggested that in addition to the Leucine zipper/basic an intact region N-terminus of Fos is required for transformation.

Similar findings exist for the c-Jun oncoprotein. Angel *et al.* (1989) identified different regions in the c-Jun protein that could affect the transcriptional activity of the protein in a sequence-specific manner. Region II that spans amino acids 68 to 81 in the c-Jun protein is essential for transcriptional activation by Jun in an Jun-independent and Fos-dependent transcriptional activation assays. This region, is homologous to the N-terminal transactivation domain (N-TA): a Fos-specific domain that was identified in deletion analysis of Fos proteins (Jooss *et al.*,1994). The NT-A domain, located between amino acids 72 to 84 was found to have an important role in transformation, and is highly conserved throughout all Fos cellular and viral proteins and also in Fra proteins. N-TA contains Proline and Glutamine residues that usually are present in transactivation domains :

c -Jun human	ASPELER LIIQ
Fos / 66-79	SPDLQW LVQP
c-Fos human	TSPDLQWLVQP
Fos B human	TSQDLQWLVQP
Fra-1 rat	TSQELQWMVQP
Fra-2 human	TSQDLQWMVQP
FBJ-MuSV	TSPDLQWLVQP
FBR-MuSV	TSPDLQWLVQP

Fig. 1.4. The N-termini of Fos and Jun proteins contain Serine residues that could be phosphorylated by MAP Kinases. The underlined protein sequences represent conserved motifs found in the various Fos proteins (Jooss *et al.*,1994).

This region of Fos shares homology with the N-terminal domain of the c-Jun oncoprotein, in a region that has been found to be regulated by Serine phosphorylation. The Serine 70 in Fos proteins corresponds to the Serine 73 in the c-Jun protein which is phosphorylated by Stress Activated Protein Kinases (SAPK/JNK) and is required for transactivation and transformation (Alani *et al.*,1991; Binetruy *et al.*,1991; Smeal *et al.*,1991; Pulverer *et al.*,1991). This suggests

that the transactivation potential of Jun and Fos proteins can be regulated by phosphorylation *in vivo*, affecting also the transformation potential of various Fos and Jun members.

2.1.3. The Leucine zipper is required for dimerisation and DNA-binding

Fos and Jun oncoproteins act in a co-operative fashion both in trans-activation and DNA-binding to an AP-1 specific binding site termed TRE (TPA-Resposive Element) (Angel *et al.*,1988; Chiu *et al.*,1988; Sassone-Corsi *et al.*,1988b). This demonstrated the existence of a physical interaction between the products of the *fos* and *jun* protooncogenes. Studies with *in vitro* translation products were employed in order to identify the DNA-binding potential of the Fos and Jun proteins, as well as the nature of the dimers that were formed during *in vitro* translation. Jun was found to bind to the TRE forming dimers with itself and with other *jun* family members such as JunB and JunD, and as heterodimers with *fos* family members (Kouzarides and Ziff, 1988; Halazonetis *et al.*,1988; Nakabeppu *et al.*,1988; Rauscher *et al.*,1988c). The Fos-Jun heterodimers display an increased binding affinity for the TRE compared to Jun homodimers, due possibly to a reduced stability of the Jun-Jun homodimer. In this case the stability of the heterodimer might be important its capability to transform primary cells (Schutte *et al.*,1989b).

The interaction between all the members of *fos* and *jun* protooncogenes is mediated by the Leucine zipper, a helical structure first identified in the liver CCAAT Enhancer binding Protein by Steven McKnight and his colleagues (Landschulz *et al.*,1988). The Leucine zipper forms a coiled coil in which the Leucine residues are located in a canonical structure spaced every seven amino acids along the polypeptide chain.

Jun ESQERIKAERKRMRNRIAASKCRKRKLERIARLEEKVKTLKAQNSELASTANMLREQVAQL

Fos SPEEEEKRRIRRERNKHAAAKCRNRRRELTDTLQAETDQLEDEKSALQTEIANLLKEKEKL

Basic Motif **Leucine Repeat**

Figure 1.5 . The structure of the Bzip domain in Fos and Jun proteins
A, Alanine; C, Cysteine; D, Aspartic Acid; E, Glutamic Acid; F, Phenylalanine; G, Glycine; H, Histidine; I, IsoLeucine; K, Lysine; L,Leucine; M, Methionine; N, Asparagine; P, Proline; Q, Glutamine; R, Arginine; S, Serine; T, Threonine; V, Valine; W, Tryptophane; Y, Tyrosine

This canonical structure favours interactions between two Leucine repeats from two different polypeptides and allows them to dimerize. In all the Jun and Fos family members and

other transactivators such as GCN4, CREB and C/EBP these Leucine residues are found in the same conserved positions, supporting the importance of these residues through the evolution of DNA-binding domains. Additionally site-directed mutagenesis of the Leucine zipper has shown that this region is also important for DNA-binding (Kouzarides and Ziff, 1988; Turner and Tjian, 1989; Gentz *et al.,*1989; Neuberg *et al.,*1989a).

Fos proteins can bind to a TRE when the Fos Leucine zipper is replaced by the Jun Leucine zipper (Neuberg *et al.*,1989b). These results show that the Leucine zipper somehow affects the DNA-binding activity of individual Fos and Jun proteins (Neuberg *et al.*,1989b) and that is highly specific in terms of the recognition of another Leucine zipper, for instance the Leucine zipper of Fos does not interact with other Leucine zippers (Kouzarides and Ziff, 1988; 1989).

2.1.4. The Leucine zipper is required for transformation

Characterisation of the regions of the FBR v-*fos* oncogene has shown that the region between amino acids 111 to 220 is crucial for the induction of transformation. This region contains an acidic domain, the basic domain that contacts DNA, and the Leucine zipper (Jenuwein and Muller, 1987). Sequences important for transformation by the FBJ v-*fos* were also determined by deletion analysis and span amino acids 111 to 206, in a domain which is homologous to the FBR v-*fos* region mentioned above (Yoshida *et al.,*1989).

Analysis of the function of Fos proteins in terms of their interaction with the Jun proteins showed that most of the Leucine residues contribute to the formation of an intact, functional zipper. In the case of a single Leucine mutant residue (Leu-179) in the highly transforming v-Fos protein E300, showed the least important functional effect, whereas mutation of the Leu-165 completely abolishes binding to c-Jun and represses the transforming properties of the protein. From this analyses it is apparent that an intact Leucine zipper is necessary for the induction of transformation *in vitro* (Schuermann *et al.,*1989).

Using Fos proteins with various mutations domains, including the Leucine zipper it was shown that the several Leucine zipper mutants had the ability of inhibiting transformation by FBR v-*fos* to a significant level (Wick *et al.,*1992). It should be noted that although the transforming properties of the v-Fos protein were partially suppressed, the proliferative properties of the cell lines expressing the dominant negative mutants were not altered. These Leucine Zipper/Basic

region mutant Fos proteins were found to inhibit transformation by other oncogenes, such as the EJ Ha-*ras* oncogene by 50%. These results suggest that the retroviral oncogene v-*fos* is a part of the *ras* signal transduction pathway and correlates with microinjection experiments, were injection of *ras* proteins could induce c-*fos* expression in cultured cells (Stacey *et al.,*1987).

2.1.5. An altered C-terminus determines the transforming potential of FBJ v-Fos

The c-*fos* gene is undetected in quiescent cells and is induced to high levels after the addition of growth factors or serum; its levels decline soon after mitogenic stimulation (Greenberg and Ziff, 1984; Kruijer *et al.,*1984; Muller *et al.,*1984). The FBJ v-*fos* escapes from this tight regulation and has alterations in its coding domain that account for its increased transforming potential. The FBJ v-Fos has a different C-terminus as a result of an out of frame deletion during the generation of the transforming FBJ v-*fos* sequences (Van Beveren *et al.,*1983; Van Stratten *et al.,*1983). This different carboxyl terminus is absent in the product of the mouse and human c-*fos* gene.

Using plasmids encoding c-*fos* genes with altered C-termini and under strong transcription signals, Miller *et al* (1984) and Meijlink *et al* (1985) demonstrated that the altered 3' sequences from the coding region of the v-*fos* gene are sufficient for the induction of transformation. This shows that a nuclear protooncogene can possess oncogenic potential after structural modifications. Additional evidence showed that sequences at the 3' end of the c-*fos* gene destabilise the message (Lee *et al.,*1988 ; Wilson and Treisman, 1988). Replacement of the c-*fos* terminal sequences with those of the FBJ v-*fos* stabilise the hybrid c-*fos*/v-*fos* mRNA.

2.1.6. Point mutation and structural alterations determine the transforming potential of FBR v-Fos

Along with its transforming potential, FBR v-*fos* has an immortalisation function, essential during the passage of rodent cultures towards a neoplastic stage. The product of the FBJ v-*fos* gene transforms early passage cells, but it is unable to immortalise them (Jenuwein *et al.,*1985). Extensive detailed analysis of the FBJ-FBR-c-*fos* chimeras showed that two different alterations determine the transforming potential of the FBR v-Fos protein (Jenuwein and Muller, 1987). The first mutation that is involved in the induction of immortalisation is an amino acid change from a

polar Glutamine to Valine at position 138. When this mutation is introduced into the FBJ v-Fos protein activates its immortalising potential. This point mutation suggests that in the FBR v-Fos protein this single exchange is sufficient for the induction of immortalisation. Replacement of this mutation in the c-Fos protein results in an increased transactivation potential (Lucibello *et al.*,1991). The presence of another point mutation, a Glutamine 175 to Lysine in the FBJ v-Fos protein, when introduced to the FBR v-Fos, results in a decrease in the transforming potential of the protein approximately 80%.

It should be noted that under strong transcriptional signals oncogenes such as mutated human Ha-*ras*, v-*myc* and v-*src* they can induce transformation and immortalisation of primary fibroblast cultures *in vitro* without the assistance of other co-operating oncogenes (Spandidos and Wilkie, 1984; Vennstrom *et al.*,1984; Helle *et al.*,1988).

A second alteration that contributes to the transforming potential of FBR v-Fos is the presence of two internal deletions which exist in the C-terminus. If these deletions are replaced by the equivalent c-*fos* sequences in the FBR v-Fos, the resulting v-Fos chimeric protein has a reduced transforming potential (Jenuwein and Muller, 1987). Analysis using a 5X TRE construct, which allows monitoring of the transcriptional activity of Fos proteins, showed that there is a co-operation phenomenon between the FBR-specific point mutations and the two C-terminal deletions. Replacement of the deletions does not affect the transactivation potential but dramatically suppresses transformation (Lucibello *et al.*,1991). This observation is important, since the transactivation domain of Fos is located at the C-terminus of the protein (Lucibello *et al.*,1988; Sassone-Corsi *et al.*,1988c) and an expression plasmid encoding a C-terminally truncated FBR v-Fos protein inhibited transactivation of a TRE-containing construct (Lucibello *et al.*,1988).

Domains which play a positive regulatory role in transcriptional activation by Fos such as the HOB1 and the TATA-Binding Domain (TBD) are deleted in the FBR v-Fos (Sutherland *et al.*,1992; Metz *et al.*,1994). The contribution to the transforming potential is rescued by two alternative domains: the N-Terminal Transactivating domain, N-TA and C-TM (Jooss *et al.*,1994; Funk *et al.*,1997) and by replacement of the C-terminus by cellular *fox*-sequences (Jenuwein and Muller, 1987). Detailed analysis of the C-terminus identified the novel C-TM domain in c-Fos, FosB and FBJ v-Fos proteins which shows close similarity with the transcriptional activation domain of the viral transactivator VP16 at conserved hydrophobic amino acid residues (Funk *et*

al., 1997). The C-TM module can activate transcription in a co-operative fashion with the N-TA module.

2.2. FOS Proteins and the Transcription Factor c-Jun/AP-1

"There are likely to be epigenetic influences in tumourigenesis, as well, but we know little of these
Bishop (1991)"

2.2.1. Fos is related to Transcription Factor AP-1

Early experiments demonstrated that Fos is a protein which had DNA-binding properties under certain conditions suggesting a putative role of cellular and viral Fos proteins in the regulation of transcription. In transfection assays FBJ and FBR encoded v-*fos* oncogenes were found to induce transcriptional activation(Sambucetti and Curran 1986; Renz *et al.*,1987). They were shown to induce the a_1 (III) Collagen promoter and the Rous Sarcoma Virus Long Terminal Repeat, but not the SV40 or β-actin promoter regions (Setoyama *et al.*,1986).

Direct evidence that Fos is a member of a nuclear factor which activates transcription came from studies on a gene implicated in adipocyte differentiation termed aP-2, which encodes for a lipid-binding protein. A 7-base pair DNA sequence in the promoter region of the aP-2 gene termed Fat Specific Element: FSE, was found to be a binding site for Fos. These experiments demonstrated a correlation with specific binding of factors in this element that was abolished by Fos-specific antibodies in gel retardation assays, and by immunoprecipitation of covalently-linked proteins to the FSE (Distel *et al.*,1987; Rauscher *et al.*,1988a).

The FSE shows homology to a *cis*-acting element that was identified in promoter regions of diverse genes and is involved in the transcriptional response by TPA, a tumour promoter phorbol ester. This element was found to be required for the basal and induced transcriptional activity of the promoter region of human Metallothionein IIα (hMTIIα) and human collagenase (Karin *et al.*,1984; Angel *et al.*,1987).

This *cis*-acting element, termed TRE for TPA-Responsive Element was identified in the promoter regions of various genes such as: hMTIIα (Karin *et al.*,1984), human Collagenase (Angel *et al.*,1987), IL-2 (Fujita *et al.*,1986) and Viral Enhancers as : SV40 (Imbra and Karin 1987), and Polyoma Virus enhancer (Wasylyk *et al.*,1987). In 1987 the laboratories of Robert

Tjian and Michael Karin isolated a TPA-inducible factor, which showed sequence specific DNA-binding to TRE found in the promoter region of hMtIIα and Human Collagenase, as demonstrated by footprinting assays. This sequence specific transactivator was termed Activator Protein-1: AP-1 (Lee *et al.*,1987a; Angel *et al.*,1987).

2.2.2 The v-jun oncogene is an activator of transcription

The transcription factor AP-1 shares a common DNA-binding sequence with the transcriptional activator GCN4, which is required for amino acid biosynthesis in yeast (Struhl, 1987). A database computer search showed sequence homology between GCN4 and a retroviral oncogene, v-*jun* (Maki *et al.*,1987; Vogt *et al.*,1987).The v-*jun* oncogene was isolated and cloned by Peter Vogt and its colleagues from a retrovirus that caused fibrosarcomas in chickens (Cavallieri *et al.*,1985). A combination of experimental approaches showed the relationship between Jun and Fos oncoproteins to the transcription factor AP-1.

2.2.3. Identification of the c-jun protooncogene

Studies using high resolution two-dimensional gel electrophoresis showed that Fos proteins and Fos-related antigens (FRA's) were complexed with a basic 39-kDa protein (Franza *et al.*,1987). This cellular protein was first identified by Curran and co-workers (Curran and Teich, 1982a; Curran *et al.*,1985). The identification of Fos-associated p39 as the product of c-*jun* protooncogene contributed significantly to our understanding of the molecular mechanisms that are involved in transcriptional regulation by AP-1(Bohmann *et al.*,1987; Rauscher *et al.*,1988b; Angel *et al.*,1988b; Sassone-Corsi *et al.*,1988b).

The cellular homologue of v-*jun*, c-*jun* was identified using an oligonucleotide derived from the coding sequence of v-*jun* oncogene to screen a genomic DNA library (Bohmann *et al.*,1987). The isolated c-*jun* cDNA was inserted into a bacterial expression vector in order to express the protein in bacterial lysates. After purification of the c-Jun fusion protein expressed in bacteria it was shown that this protein could bind the same SV40 enhancer elements with the specificity of AP-1 demonstrating that c-*jun* had essentially the properties of AP-1.

Using similar screening procedures Karin and co-workers cloned the c-*jun* protoncogene from a human fibroblast cDNA library (Angel *et al.*,1988b). By DNA-transfection

experiments It was also shown that the v-*jun* protooncogene acts as a sequence specific transactivator in F9 cells, which contain very low levels of endogenous AP-1 activity.

Curran and co-workers using an elegant biochemical approach, in DNA-affinity precipitation assays followed by high-resolution two-dimensional electrophoresis showed that Fos proteins and Fos-related antigens share a common binding site with AP-1 in the promoters of cellular and viral genes (Franza *et al.*,1988). This was followed by electrophoretic mobility shift analysis and photoaffinity cross-linking and showed that the FSE could bind efficiently Fos proteins (Rauscher *et al.*,1988a) and that *fos*-associated protein p39 is directly related to c-*jun* protooncogene (Rauscher *et al.*,1988b).

p-39/Jun was subsequently identified as an AP-1-associated protein in immunoprecipitation experiments using Fos-specific and Jun-specific antibodies (Sassone-Corsi *et al.* 1988b). Using immunoprecipitated material from [^{35}S]-labelled cells, it was shown that Jun/AP-1 and p39 share identical two dimensional tryptic maps and that in an electrophoretic mobility shift assay gel purified p39 bound a TRE.

These findings were the first demonstration that *fos* and *jun* oncogenes form the AP-1 complex and, as a result of this association could activate transcription *in vitro* and *in vivo*. This illustrates the necessity of transactivation or deregulation of transcription during oncogenic transformation. It also suggests that this interaction is a co-operative event between the actions of the *fos* and *jun* protooncogenes.

3. Regulation of AP-1 activity

3.A. Transcriptional regulation of the AP-1 components

3.A.1. Transcriptional regulation of the c-fos protooncogene

If serum is withdrawn from tissue culture cells then a decrease in various metabolic processes takes place. This metabolic response includes events which are important for cell proliferation and growth, such as RNA and protein synthesis. This suggests that serum contains a component which is required for cell proliferation and growth. When serum is added to quiescent cultures, a rapid increase in total RNA synthesis takes place (Todaro *et al.*, 1966). This type of stimulation is a very rapid event, with RNA synthesis ensuing within minutes, whereas protein synthesis and DNA synthesis occurs much later. These researchers first suggested that these events might be due to the presence of a macromolecular substance in the serum, a serum growth factor.

A number of cellular genes are acutely transcribed after the addition of purified growth factors to serum-deprived fibroblasts. These sequences were identified as Immediate Early response elements of gene transcription *in vitro*, comparable with the products of the Early genes of DNA tumour viruses (Cochran *et al.*, 1983; Linzer and Nathans, 1983; Zullo *et al.*, 1985; Lau and Nathans, 1985, 1987). Among the sequences that can be induced after growth factor stimulation are those of c-*myc* and c-*fos* protooncogenes. c-*myc* is induced by PDGF and lymphocyte mitogens in blood cells (Kelly *et al.*, 1983) and the c-*fos* protooncogene is induced rapidly within minutes after the addition of PDGF or EGF to the quiescent cultures of NIH 3T3 mouse fibroblasts (Greenberg and Ziff, 1984; Kruijer *et al.*, 1984; Muller *et al.*, 1984). The induction of c-*fos* is followed by the rapid synthesis of the 55kD c-Fos protein. In a attempt to understand the regulation of the c-*fos* expression site-directed mutagenesis of the 5' regulatory region of c-*fos* identified a minimal region that was responsive to growth stimulation (Treisman, 1985). This sequence, the Serum Response Element (SRE), contains a 56 base pair region that responds to a variety of extracellular stimuli. Most of this sequence is highly homologous with sequences in the human c-*fos* promoter. A factor that binds to this region was identified in electrophoretic mobility shift analysis experiments (Treisman, 1986). DNAase I protection studies showed that the region of factor binding is a region of dyad symmetry (DSE) which is

the shorter sequence within the SRE that is required for induction. The DNA-binding protein that binds to this region was identified and cloned. This protein is called Serum Response Factor (SRF) and it is bound to the SRE in the absence of growth factor stimulation (Norman *et al.*,1988). The formation of a complex over the SRE seems to require another protein factor termed Ternary Complex Factor, TCF (Shaw *et al.*,1989). The c-*fos* SRE contains an Ets binding site that does not bind directly TCF, but only in the presence of the additional contacts with SRE-bound SRF (Treisman *et al.*,1992).

3.A.2. The ras Pathway

c-*fos* expression in mammalian cells and AP-1 activity is regulated by a signal transduction pathway that involves the small GTP-binding protein $p21^{ras}$ and kinases acting downstream of $p21^{ras}$ (Karin, 1995). The protein kinase Raf-1 is a direct target of the *ras* protooncogene (Leevers *et al.*,1994), and activated Raf-1 regulates the c-*fos* promoter in a signal-dependent manner (Jamal and Jiff, 1990). $p21^{ras}$ -induced activation of Raf-1 results in the phosphorylation of MEK-1 a MAP Kinase Kinase. MEK-1 is a dual specificity kinase and has the ability to phosphorylate Mitogen Activated Protein Kinases (MAP Kinases) on Tyrosine and Threonine residues. MAP Kinases were originally identified as insulin regulated kinases that were activated by phosphorylation on Tyrosine and Threonine residues (Ray and Sturgill, 1987). MAP Kinases have a variety of substrates, including nuclear transactivators. After mitogenic stumulation MAP Kinases translocate to the nucleus (Chen *et al.*,1992) where they phosphorylate transcriptional activators such as Elk-1. Elk-1 is a protein that belongs to the group of Ternary Complex Factors (TCF) which forms a complex with SRF (Hipskind *et al.*,1991). Phosphorylation of Elk-1 by MAP Kinases leads to the activation of its transcriptional properties (Gille *et al.*,1992; Hill *et al.*,1993; Marais *et al.*,1993; Janknecht *et al.*,1993).

In the next part of the introduction the role of phosphorylation in the post-translational modification of AP-1 activity is presented in more detail. The regulation of viral Fos and cellular Jun, Fos, and Fra-1 proteins by phosphorylation with a focus into the modulation of their transforming potential is discussed.

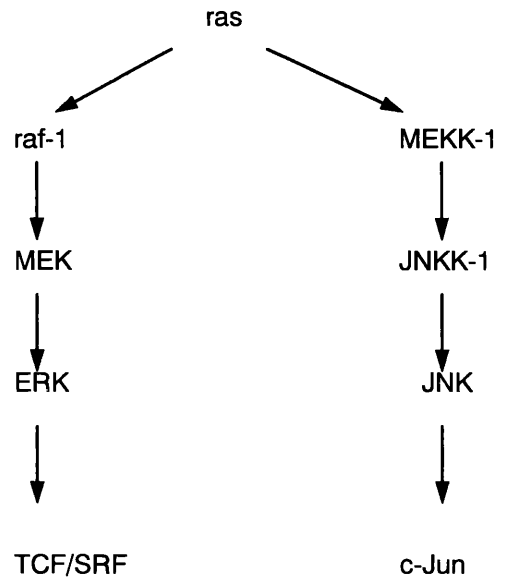


Figure 1.6. *ras* dependent regulation of AP-1 activity (Karin , 1995).
Phosphorylation of TCF/Elk-1 by MAP kinases (ERK) in the fos promoter stimulates its transcriptional activity, regulating fos induction. JNK mediated phosphorylation of c-Jun regulates *jun* promoter and c-*jun* induction.

3.B. Postranslational modification of the AP-1 components

"Initially, there was a concern that cooperation might be due to one of the oncogene products in a co-operating pair acting to stabilise or increase the expression of the other oncogene product, but where it has been examined this trivial explanation has been ruled out

-Hunter (1991)"

3.B.1. The Ha-ras oncogene potentiates the transcriptional activity of c-Jun by phosphorylation

Several lines of evidence are highly suggestive of phosphorylation as an important factor in modulating the regulation and function of the c-Jun oncoprotein. In co-transfection experiments it was found that c-Ha-*ras* modulated c-*fos* (Schontal *et al.*,1988) and c-*jun* (Binetruy *et al.*,1991; Smeal *et al.*,1991) expression and transcriptional activation function.

Transactivation by c-*jun* was enhanced 2 to 3-fold by co-transfection with the activated leu61 Ha-*ras* oncogene. This effect was independent of Fos, since a mutant Jun protein that is unable to heterodimerise with Fos was still transcriptionally active (Smeal *et al.*,1989). The c-Ha-*ras* induces phosphorylation of c-Jun oncoprotein as shown by immunoprecipitation analysis of [^{32}P] labelled cells and the incorporation of [^{32}P] into c-Jun protein. The amino acids that are modified are located at the c-Jun N-terminus as demonstrated by tryptic phosphopeptide analysis (Boyle *et al.*,1991). These N-terminal residues are required for transcriptional activation since a Jun protein lacking these sites can not be activated by Ha-ras (Binetruy *et al.*,1991).

Substitution of these phosphorylatable residues by Alanine resulted in the absence of the peptides in the two dimensional peptide maps, showing that these residues are subject to *ras* dependent phosphorylation. These mutants were ineffective in transcriptional activation assays and in focus forming assays, suggesting an important biological role of the phosphorylation of Serines 63 and 72 in transformation by the c-*jun* oncogene.

3.B.2. Identification of the Protein Kinases that phosphorylate c-Jun oncoprotein

c-Jun oncoprotein is a substrate for MAP Kinases *in vitro*, on residues which correlate with *in vivo* identified sites (Alvarez *et al.*,1991; Pulverer *et al.*,1991). The sites identified by these two

groups are not identical: Alvarez *et al.* (1991) have identified Serine 243 in the C-terminus and Pulverer *et al.* (1991) have identified Serines 63 and 73. These phosphorylation sites stimulate the transcriptional activation properties of c-Jun, thus they have a positive role in AP-1-mediated trans-activation.

Pulverer *et al* (1991) obtained convincing evidence regarding the identity and the *in vivo* role of the kinase that phosphorylates c-Jun. Using a combination of approaches such as peptide mapping, site-directed mutagenesis and synthetic peptides, it was shown that Serines 62 and 73 are the critical residues targeted by a protein kinase that is activated by phorbol esters. As shown by chromatographic methods this enzyme has a molecular weight similar to other MAP Kinases of approximately 40kD. In 1993 the laboratory of Michael Karin identified a kinase, belonging to the MAP Kinase family, that binds and potentiates the c-Jun N-terminal activation domain (Hibi *et al.,*1993). These kinases phosphorylate Serine or Threonine residues immediately preceding a Proline residue and so are often referred to as Proline-directed kinases; they are themselves activated by dual phosphorylation at Threonine and Tyrosine residues(Payne *et al.,*1991). However this enzyme has different activators since it was shown to be effectively activated by UV irradiation rather than by growth factors and serum as the typical MAP Kinases. Molecular cloning identified that this enzyme is related to the ERK1/ERK2 MAP family Kinases and is termed Jun-N terminal Kinase (JNK), since it is highly specific for the Jun N-terminus (Derijard *et al.*, 1994). This protein kinase is identical to a protein kinase that is activated by stress and protein synthesis inhibitors (Kyriakis and Avruch, 1990; Kyriakis *et al.,*1991; Kyriakis *et al.,*1994).

c-Jun can also be negatively regulated by phosphorylation. Casein Kinase II and a MAP Kinase negatively regulate DNA-binding to a TRE *in vitro*. Serine and Threonine residues located at the C-terminus are involved in this type of regulation *in vivo* (Lin *et al.,*1992; Chou *et al.,*1992).

3.B.3. Phosphorylation of the FBJ v-Fos oncoprotein

Barber and Verma (1987) studied the phosphorylation of c-Fos and $p55^{v\text{-}Fos}$ from cells labelled with [^{35}S] and [^{32}P] and have found that the c-Fos protein is extensively modified in serum stimulated fibroblasts compared to $p55^{v\text{-}Fos}$. This was confirmed by incubating immunoprecipitated proteins from labelled lysates with alkaline phosphatase and comparing the

mobilities of Fos proteins after SDS-polyacrylamide gel electrophoresis. Tryptic phosphopeptide analysis also showed that the c-Fos protein contains unique Serine residues, which are phosphorylated after stimulation of cultured cells.

A hybrid Fos protein that contained the main amino terminus of $p55^{v\text{-}Fos}$ and the carboxy terminus from the c-Fos protein had the same electrophoretic mobility and similar phosphopeptide map with wild type c-Fos protein. These results argue that the c-Fos protein is regulated by phosphorylation of the C-terminus. The c-Fos protein can also be phosphorylated *in vitro* by various protein kinases such as p34cdc2, PKA and PKC at regions that are required *in vivo* for transcriptional activation (Abate *et al.*,1991).

Studies with mutated proteins in the C-termini have shown that a C-terminus site of the c-Fos protein encompasses a PKA consensus phosphorylation site containing a Serine residue (Tratner *et al.*,1992). Mutation of this phosphorylation site to Alanine reduces the *in vitro* phosphorylation content of c-Fos and decreases its transforming potential in transfection assays. These data suggested that phosphorylation of the c-Fos protein contributes positively to its transforming potential.

3.B.4. Phosphorylation of the c-Fos protein interferes with transactivation

Sequence homology analysis showed that there is a similarity between the N-terminus of c-Jun and the C-terminus of c-Fos. These homology regions in c-Fos contain conserved amino acid modules termed HOB1 and HOB2 (Sutherland *et al.*,1992). The HOB1 motif contains a Threonine residue, the equivalent of Serine 73 of c-Jun oncoprotein that is involved in transcriptional activation (Sutherland *et al.*,1992). These motifs show independent transcriptional activation as well as co-operational modularity, and the HOB1 motif co-operates with the C-terminus of c-Fos protein to activate heterologous promoters.

Fos	HOB 1	^{226}**G L** P E **A** T $\underline{\text{T}}$ **P E** S **E** ^{236}
Jun	HOB 1	^{67} **G L** L K L **A** $\underline{\text{S}}$ **P E** L **E** ^{77}

Figure 1.7. HOB modules in the Fos protein contain a motif which is a target site for MAP Kinase. The underlined residues in the HOB motifs represent amino acids phosphorylated by MAP Kinases.

The c-Fos HOB1 motif can also be activated in a *ras*-dependent manner. Threonine 232 in the HOB1 module can be phosphorylated by ERK2 *in vitro*. Replacement in this motif by a PKA-responsive phosphorylation site activates the transcriptional properties of the protein by PKA phosphorylation (Bannister *et al.*,1994). Similar results were found for the the c-Jun oncoprotein *in vitro* and *in vivo* (Smeal *et al.*,1994).

Deng and Karin (1994) have demonstrated that Threonine 232 in the c-Fos HOB1 motif is a target for a kinase other than ERK2, in contrast to the study of Bannister *et al* (1994). Experiments with GST-fusion proteins and *in situ* kinase assays identified an 88 Kd kinase activity in A431 epidermoid carcinoma cells that is distinct from MAPK or SAPK/JNK family kinases. The identity of this protein kinase remains to be established (Deng and Karin, 1994).

3.B.5. Regulation of the transforming potential of Fos protein by phosphorylation

The C-terminus of the FosB protein is required for transformation (Wisdom and Verma, 1993b). This C-terminal function is localised at a region that is required for transactivation by c-Fos and FosB proteins and not by the *fos*-related antigens Fra-1 and Fra-2. GAL4 fusion proteins with the respective C-termini from different Fos proteins show transcriptional activation only in the case of c-Fos and FosB proteins but not for the *fos*-related antigens. Generation of various chimeric Fos proteins with C-termini from FosB show transactivation of AP-1 regulated transcription and transformation in a FosB dependent-manner (Wisdom and Verma, 1993b).

The FosB C-terminus contains two regions required for transactivation :a region between amino acids 256-275 and a Proline rich region between amino acids 265-275 (Wisdom *et al.*, 1992). The latter domain contains a cluster of Serines some of may represent target sites for MAP Kinases. Mutational analysis of individual Serines in this region showed that there was not a stringent requirement for phosphorylation by MAP Kinases but if more than one of these Serine residues are converted to Alanine then the transactivation potential and the transforming ability is dramatically reduced. This shows that multiple sites possibly are regulated by phosphorylation *in vivo* resulting in the modulation of the transactivation potential of FosB protein (Skinner *et al.*,1997). Indirect evidence such as stimulation with diverse agonists that stimulate MAP Kinases and JNK/SAPK family kinases suggests that the kinase that phosphorylates FosB protein might be distinct from ERK family or JNK/SAPK family of kinases (Skinner *et al.*,1997).

Work from another group was more conclusive regarding transcriptional activation of c-Fos by phosphorylation. The c-Fos C-terminus is absent from the transforming FBJ v-Fos oncoprotein, however the region studied by this group is conserved in Fos proteins and Fos-Related Antigens (Chen *et al.*, 1996).

c-Fos	AH **RKGSSSN**	EPSSDSLS**S**PTLLAL
FosB	GA **QRTSGS**	EQPSDPLN**S**PSLLAL
Fra-1	AH **RKSSSS** SG	DPSSDPLG**S**PTLLAL
Fra-2	AH **RRSSSSG**	DQSSDSLN**S**PTLLAL
	Ser 362	Ser 374

Figure 1.8. The C-termini of Fos proteins contain consensus sites for phosphorylation by ribosomal S6 kinase, $p90^{RSK}$ and MAP Kinase. Serine 362 is a Target site for $p90^{RSK}$ and Serine 374 for MAP Kinase.

Analysis of Fos proteins lacking N- and C-termini showed that two distinct kinases could phosphorylate c-Fos proteins in its C-terminus (Chen *et al.*, 1993). *In vitro* kinase reactions with purified S6 Kinase and MAP Kinase, together with tryptic phosphopeptide analysis showed that Serine 362 was a site for ribosomal S6 kinase, $p90^{RSK}$ and Serine 374 a site for MAP Kinase. Mutation of these Serine residues to Aspartic acid resulted in Fos proteins with increased transforming potential (Chen *et al.*, 1996) possibly due to increased stability of the modified c-Fos protein. This demonstrates that regulation of Fos protein by phosphorylation could contribute significantly to the induction of the transformed phenotype *in vitro*.

3.B.6 Regulation of Fos-Related Antigens by phosphorylation

Immunoprecipitation with antibodies against Fos from serum stimulated fibroblasts labelled with [^{35}S] identifies protein species of 35, 38 and 45 kD (Franza *et al.*, 1987). These proteins belong to the family of Fos-Related Antigens. A 38kD band could be specifically immunoprecipitated with Fra-1 specific antiserum was also identified by two dimensional high-resolution gel electrophoresis in samples from denatured and non-denatured cell lysates (Franza *et al.*, 1987).

During pulse-chase experiments, various protein species appeared to migrate between 35-40 kD, suggesting that differentially modified forms the Fra-1 proteins exist *in vivo* (Cohen *et al.*, 1989). [^{32}P] labelling showed a dramatic increase in the incorporation of phosphate into the

Fra-1 polypeptides. All these findings suggest that phosphorylation accounts for the post-translational modifications of Fra proteins *in vivo* (Cohen *et al.*,1989).

Experiments with [^{35}S] and [^{32}P] labelling and subsequent immunoprecipitation, and with *in vitro* synthesised proteins demonstrated that Fra-1 and Fra-2 proteins were post-translationaly modified by serum in stimulated Swiss 3T3 cells (Gruda *et al.*,1994). This effect could be reversed by treatment with a phosphatase *in vitro. In vivo* labelling with [^{32}P] showed that a large amount of phosphate was incorporated into these proteins after serum induction and resulted in higher migrating forms in SDS-Polyacrylamide gels suggesting phosphorylation by a MAP Kinase. Different kinases were found to stimulate the incorporation of [^{32}P] into Fra-1 and Fra-2, but the higher phosphorylated forms appeared only after incubation with purified MAP Kinase and phosphorylation was associated with increased DNA-binding activity of Fra polypeptides *in vitro*. These data argue for a role for MAP Kinase in the post-translational modifications and the increased *in vitro* DNA-binding activity of Fra-1 and Fra-2 proteins

1.4. Biological models of *fos* in the development of neoplasms

1.4.1. *fos* genes as regulators of cellular growth

1.4.1.1. Inhibition of cellular proliferation by antisense fos-RNA

Experiments with rodent fibroblasts argue for a role for *fos* genes in regulating proliferation and cell cycle progression. Holt *et al* (1986) showed that an expression vector containing antisense c-*fos* sequences under the control of an inducible promoter inhibited proliferation of Swiss 3T3 cells *in vitro*. This demonstration was supported further by experiments that showed that the same antisense-*fos* vectors inhibit the re-entry of quiescent fibroblastic cells into the cell cycle (Nishikura and Murray, 1987). Other groups have shown that the expression of *fos* in an anti-sense orientation, in *sis* (Mercola *et al.*,1988) and *ras* transformed cell lines (Ledwith *et al.*,1990) could reduce their proliferative potential and induce reversion of morphological transformation.

1.4.1.2. Inhibition of cellular proliferation by microinjection of Fos-specific antibodies

Microinjection of antibodies that recognised a conserved region of Fos proteins were found to inhibit DNA synthesis in quiescent cells stimulated with serum to re-enter the cell cycle, and also in asynchronously growing cells (Riabowol *et al.*,1988).

Detailed studies with a variety of different antibodies suggested that the regulation of cell cycle progression by Fos and Jun proteins might be of a more complex nature (Kovary and Bravo, 1991a; 1991b). These experiments demonstrated the requirement for Jun proteins in cell cycle progression of Swiss 3T3 cells in tissue culture. JunB is the predominant Jun protein in Swiss 3T3 cells. Its induction by serum is not attenuated and it is more stable compared to Fos proteins (Kovary and Bravo, 1991a). Antibodies against Fos, Fra-1 and FosB proteins were less effective in inhibiting DNA synthesis following serum stimulation, but if all the Fos-specific antibodies were combined then an additive effect was observed suggesting that individual Fos proteins have a redundant role in cell cycle progression. However it should be noted that these studies do not explain the role of *jun*B in cell cycle progression thoroughly since *jun*B is a negative regulator of c-*jun* and of AP-1 transcriptional activation (Chiu *et al.*,1989; Schutte *et al.*,1989b).

The Fos-related antigens Fra-1 and Fra-2 are not detected during quiescence but they appear to be persistent in cycling and serum-stimulated Swiss 3T3 fibroblasts, and remained at high levels after serum-stimulation (Kovary and Bravo, 1992). Immunoprecipitation analysis showed that after serum induction c-Jun is complexed with Fra-1 and JunB with Fra-2 proteins (Kovary and Bravo, 1992) and that these association are stable suggesting a co-operative effect in the action of Fos and Jun family members *in vivo*.

1.4.1.3. Cell growth studies with cells that lack c-fos or c-jun

The use of vectors that disrupted the genomic integrity of *fos* and *jun* loci made it possible to generate embryonic stem (ES) cells that lack one of the members of the AP-1 factor such as c-*fos* (Field *et al.*,1992; Brusselbach *et al.*,1995) and c-*jun* (Hilberg and Wagner, 1992). These knockout cells are tools in defining the role of AP-1 in cell cycle and cell proliferation.

The proliferative potential of ES *fos* -/- cells remained unaltered in comparison with wild type cells, both in terms of the saturation densities and doubling times. Serum induction of quiescent *fos* -/- cultures showed that they retained the same proliferative potential as the parental wild type *fos* +/+ cells (Field *et al.*,1992). Also the ES *fos* -/- cells retained the potential to differentiate *in vitro* in a spontaneous manner as do the wild type ES cells under the same culture conditions.

The study of Brusselbach *et al.* (1995) showed that proliferation and serum re-entry into the cell cycle was very similar between *fos* +/+ and *fos* -/- ES cells and fibroblastic-like cells. Analysis of proliferation using growth curves showed that there was no difference between *fos* +/+ and *fos* -/- fibroblasts. FACS analysis of these cell lines showed that cell cycle progression and re-entry was not impaired in the two different cell lines. Other important parameters for AP-1 activation such as DNA-binding to the TRE, and TRE-dependent trans-activation in transfection assays showed that there was no major difference in between *fos*-/- and wild type cells.

Hilberg and Wagner (1992) generated ES cells that lacked both alleles of the c-*jun* protooncogene. These cells had similar proliferative potential to wild type ES cells and their morphology and differentiation ability remained the same. In terms of the AP-1 gene expression of the *jun*, *jun*B and c-*fos* genes after serum stimulation, these were largely unaffected, except the c-*jun* message which was reduced by 2 to 5-fold. The c-*jun* -/- ES cells also induce tumours poorly upon injection into syngeneic mice, compared to the wild type ES cells that form tumours

efficiently. This evidence suggested that the c-*jun* gene is necessary at some stage of tumourigenesis by ES cells after injection into recipient animals but not for cell proliferation.

All this evidence converge upon the idea that the main AP-1 components, c-*fos* and c-*jun,* are not necessary for cell proliferation *in vitro*. This is in contrast with the previously published evidence that suggests that cell proliferation is indeed affected when the expression (Holt *et al.,*1986) or the function of *fos* and *jun* is impaired *in vitro* (Riabowol *et al.,*1988; Kovary and Bravo, 1991a, 1991b).

The evidence from the targeted disruption of *fos* and *jun* genes *in vivo* gave a more direct role for these genes in development and differentiation, and suggested that they are essential for mouse development. Targeted disruption of c-*jun in vivo* causes embryonic lethality (Johnson *et al.,*1993) and the absence of *fos* is associated with bone defects (Wang *et al.,*1992; Johnson *et al.,*1992).

1.4.2. In vivo models of fos and the development of oncogenesis

1.4.2.1. Studies with fos transgenic mice

The generation of transgenic mice carrying c-*fos* genes (Ruther *et al.*,1987) under a regulatable metallothionein promoter (MT) (Ruther *et al.,*1985) showed that high levels of *fos* could be induced in different cellular backgrounds. These mice surprisingly did not develop any neoplasms in their bones. The targeted expression of a c-*fos* transgene in mice under a H2-K^b MHC promoter impaired the function of the immune system but again no lymphoid or thymic neoplasms appeared (Ruther *et al.,*1988).

Wang *et al.* (1991) used ES cells transfected with a MT c-*fos* transgene to generate chimeric animals. High expression was maintained during development and these mice developed chondrogenic sarcomas irrespective of the degree of the chimerism. An important observation was the specificity of the tumour formation, since the MT *fos* transgene was expressed in other tissues apart from bone, but these tissues did not develop tumours. These results contrast with findings of the same group (Ruther *et al.,*1989), since they showed the main target cell is the osteogenic progenitor cells and not chondroblasts. However in the study of Wang *et al.* (1991) the *fos* genes were expressed ectopically early in development. This might reflect the potency of *fos* in regulating a different progenitor cell that leads to chondroblastic

tumours. These studies show that when a *fos* gene is expressed in early development it can transform specific cell types which apparently appear to be distinct from the usual targets of the v-*fos* oncogene.

When c-*fos* is expressed under the control of an MHC H-2K^b promoter with a 3' FBJ-MuSV LTR, then osteosarcomas could be induced with a short latency (Grigoriadis *et al.*,1993). These tumours grew rapidly into large masses in all bones with evidence of osteoblastic activity. The main cell types present in these tumours are osteoblasts and chondrocytes that stain rich for alkaline phosphatase. These neoplasms showed a large degree of neovascularisation and are highly invasive with an increased mitotic index. Cell lines established from these tumours exhibited transformed fibroblastic morphology and displayed the characteristics of osteoblastic gene expression. The same transgene was employed with the *fos*B or c-*jun* genes but in this case no osteosarcomas were formed (Grigoriadis *et al.*,1993). This however is in contrast to previous evidence that argue for a transforming role for the *fos*B gene when expressed under the transcriptional signals of the FBR-MuSV LTR (Schuermann *et al.*,1991).

1.4.2.2 Oncogene co-operation models in studies with fos oncogenes

The mouse epidermis has been used successfully to model the role of oncogenes in neoplastic development (Balmain *et al.*,1984; Quintanilla *et al.*,1986; Brown *et al.*,1986). Greenhalgh and Yuspa (1988) suggested that there is a role for the *fos* oncogene in the generation of epidermal tumours. Introduction of the v-*fos* oncogene into papilloma cell lines expressing an activated Ha-*ras* oncogene made them tumourigenic when injected into nude mice. Similarly papilloma cell lines expressing E1A or *myc* oncogenes did not form tumours, specifying the role of v-*fos* in the generation of these epidermoid malignancies. These investigators postulated that tumours could have formed through a co-operation event, between c-Ha-*ras* and a nuclear oncogene with the properties of a transcriptional activator, such a the v-*fos* oncogene. Later they reported that this co-operation was actual when they found that injected keratinocytes infected with v-*ras* and v-*fos* MuSV's into nude mice induced the formation of squamous cell carcinomas (Greenhalgh *et al.*,1990).

Taken together, these studies suggested a role for the *fos* oncogenes and AP-1 family members in experimental models of oncogenesis and a role for *ras* and *fos* oncogenes in this *in vivo* model of oncogene co-operation.

1.4.2.3. In vivo models with fos and Jun knockout mice : implications for malignancy

Disruption of c-*fos* in mice by homologous recombination (Johnson *et al.*,1992; Wang *et al.*,1992) resulted in mice which showed retarded growth and a low survival rate (Johnson *et al.*,1992). Additionally disruption of the *fos* gene resulted in abnormalities in the formation of bone, reduction of the proliferating cartilaginous tissues and hypertrophy of the chondrocytes (Wang *et al.*,1992). An important feature of the mice was the impaired function of the haemopoetic cells as demonstrated by FACS analysis.

Cells from *fos* -/- mice were used as a tool to investigate the role of the AP-1 family members in tumourigenesis. *In vitro* experiments with 3T3-like cells from *fos* -/- mice (Hu *et al.*,1994) showed that the growth rates of these cell lines are not substantially different from parental *fos* +/+ cells, arguing for a redundant role of AP-1 components in cellular proliferation in contrast to previously published observations (Holt *et al.*,1986; Nishikura and Murray, 1987; Riabowol *et al.*,1988). In *fos* -/- cells the expression of AP-1 members, such as *fra*-1 and *fra*-2 as well as *jun* family members showed variations after induction by growth factors and phorbol esters. The levels of the AP-1 binding activity did not show variations between the *fos* +/+ and the *fos*-/- cells indicating that *fos* has a questionable role to the *in vitro* AP-1 binding activity to the TRE. Gene expression studies showed that stromelysin, a tumour matrix degrading metalloprotease (Matrisian *et al.*,1986; Kerr *et al.*,1988), was absent and induced to very low levels by phorbol ester. Transformation studies with *fos* -/- cells showed that there was no significant difference in the focus formation induced by oncogenes upstream of AP-1 such as v-*raf*, v-*src* and v-*ras*, compared to the wild type *fos* +/+ cells. In fact in all cases there was a reduction in focus formation.

It was also suggested that the c-*fos* gene is an essential factor in the development of epidermal neoplasia, as shown in the chemically-treated mouse epidermis (Saez *et al.*,1995). In these studies, *fos* -/- mice were treated with phorbol esters which resulted in the formation of epidermal tumours. The c-*fos* -/- cells from these mice when transfected with a virus carrying a

ras oncogene and grafted into the skin of recipient animals, did not lead to tumour formation. This suggested that the *fos* null mutation impairs the initiating signals from the viral *ras* oncogene and that c-*fos* is required for tumourigenicity *in vivo* and that AP-1 lies downstream of the *ras* oncogene in this signalling pathway.

1.4.2.4. Co-operation models from the cells of jun knockout mice

Evidence obtained with cells from c-*jun* -/- mice (Johnson *et al.*,1993; Johnson *et al.*,1996) demonstrate that there is an important role for c-*jun* protooncogene in pathways downstream of *ras* protooncogene. c-*jun* -/- embryonic fibroblasts are unable to grow in the presence of growth factors, suggesting a blockage of the growth factor-*ras* pathway, due to lack of the c-*jun* protooncogene (Johnson *et al.*,1993). Johnson *et al.* (1996) introduced a SV40 T Antigen to restore the proliferation capacity of the *jun* -/- cells. The c-*jun* -/- cells are unable to grow in soft agar even after the transfection of a c-*jun* gene or after transfection of a retrovirus carrying a *ras* oncogene. Introduction of both *ras* and *jun* oncogenes restored the ability of the cells to grow in soft agar and to form tumours in mice (Johnson *et al.*,1996).

The introduction of a *ras* gene with *jun*B of *jun*D suprisingly led to the formation of tumours but in the case of the *jun*D no colony formation in soft agar appeared. This suggests that the absence of the c-*jun* protooncogene can be rescued by other *jun*-family members in tumourigenicity assays.

Taken in consideration that previously *jun*B and *jun*D are considered to be negative regulators of transformation and AP-1 activity (Chiu *et al.*,1989; Schutte *et al.*,1989b; Pfarr *et al.*,1994) these results are of a conflicting nature and the reason for the oncogenic ability of *junB* and *junD* remains to be explained.

Aims of this Thesis

There is evidence that the c-*jun* protooncogene is required for oncogenesis *in vivo* and morphological transformation *in vitro*. Therefore the trans-dominant deletion mutant of c-*jun* protooncogene TAM-67 was tested in its ability to inhibit AP-1 activity and subsequently v-*fos* transformation. While Interested in the effect of TAM-67 in *fos*-induced transformation we decided to extent our investigation by studying elements of upstream signal transduction pathways, and their requirement in morphological transformation.

It is established that components of the *ras* pathway such as Raf-1 (Kolch *et al.,*1991; Bruder et al., 1992) and MEK-1 (Pages et al., 1993; Cowley *et al.,*1994) are involved in a signalling pathway leading to AP-1 activation. To dissect the role of the constituents of the *ras* pathway in FBR v-*fos*-induced transformation dominant negative mutants have been transfected into FBR cells. The effect of the Raf-1 and MEK-1 dominant negative mutants in transformation and anchorage independence is discussed.

MATERIALS AND METHODS

CHAPTER 2 MATERIALS

CHAPTER 2. MATERIALS

2.1 Plasmids

pSV2neo provided by Dr. Liz Black, Beatson Institute

pCMV-TAM-67 (Brown et al., 1993) provided by Dr. M.J. Birrer, NIH, USA

pBABE puro (Cowley et al.,1994) provided by Prof. C.J. Marshall, Institute for Cancer Research, London

pBABE puro MEK-1 (Cowley et al.,1994) provided by Prof. C.J. Marshall, ICR, London

pBABE puro MEK-1 A221 (Cowley et al.,1994) provided by Prof. C.J. Marshall, ICR, London

pEXV NΔ Raf-1 (Schaap et al., 1993) provided by Prof. C.J. Marshall, ICR, London

2.2. Cell Culture Materials

2.2.1. Cell Culture Plasticware

Source : FALCON, Becton Dickinson Labware, Plymouth, Devon, England.

10 mm cell culture dishes

30mm cell culture dishes

24 well plates

12 well plates

6 well plates

Source : Nunc, Rocksilde, Denmark

Nunc cryotubes

2.2.2. Cell Culture Solutions

Source : Fisher Scientific UK, Ltd, Loughborough, Leicestershire, UK

Dimethyl Sulfoxide

Source : GIBCO Europe, Life Technologies Ltd, Paisley, Scotland.

Geneticin, G418, dessicate

Gentamycin 10 mg/ml, solution

L-Glutamine 200mM, solution

MEM Non-essential amino acids 100X, solution

Sodium Pyruvate 100mM, Solution

Trypsin 2,5%, Solution

Source : Oxoid Ltd., Basingstoke, England

PBS Tablets

Source : SIGMA Co Ltd., Poole, Dorset, England.

10X DMEM, solution

10X F-10 Ham's, Solution

Penicillin, dessicate

Pyromycin, dessicate

Streptomycin, dessicate

Source : TCS Biologicals

Foetal Calf Serum

Source : Fluka Chemie, Buchs, CH-9470

Methylcellulose 64630, dessicate

2.3. Antibodies

2.3.1. Primary Antibodies

Mouse **anti-ERK1,** Transduction Laboratories, KY, USA.

Mouse **anti-ERK2** (WB 1 : 5000), Transduction Laboratories, KY, USA.

Mouse **anti-FAK** (WB 1 : 5000), Transduction Laboratories, KY, USA.

Rabbit **anti-Fra-1** (WB - 1 : 250), Santa Cruz Biotechnology, CA, USA.

Rabbit **anti-Fos [K-25]** (WB - 1 : 400), Santa Cruz Biotechnology, CA, USA.

Rabbit **anti-c-Jun** (WB 1:400), Oncogene Science, UK.

Goat **anti-gag MuLV** (WB 1 : 1000), Dr Alan Rein, NCI Facility, Frederick, MD, USA.

Rabbit **anti-MAPK [Activated form]** (WB 1 : 10000), Promega Corp., Wisconsin, USA.

Rabbit **anti-MEK-1** (WB 1:500), Santa Cruz Biotechnology, CA, USA

Rabbit **anti-MEK-1 [Activated form]** (WB 1: 500), New England BioLabs, USA.

Mouse **anti-phosphotyrosine** [PY-20] (IF 1: 100), Affiniti Labs, UK.

Mouse **anti-Raf-1** (WB 1: 2000), Affiniti Labs, UK.

[Key : WB : Western Immunoblotting IF : Immunofluorescence]

2.3.2. Secondary Antibodies

Donkey Anti-Rabbit HRP-linked Whole IgG, Amersham, Bucks, England.

Sheep Anti-Mouse HRP-linked F(ab) fragment, Amersham, Bucks., England.

Goat Anti-Mouse FITC-linked, F0257, Sigma, Poole, Dorset, England.

2.4. Chemicals

2.4.1. Isotopes

Source : Amersham International PLC, Amersham, Buckinghamshire, England.

Redivue [γ-^{32}P] ATP 3000 Ci/mmol

2.4.2. Chemicals for Molecular Biology

Source : Boehringer Manheim UK Ltd., Lewes, East Sussex, England

HEPES, dessicate

Cesium Chloride, dessicate

DOTAP Transfection Reagent, solution

Source : Sigma Co Ltd., Poole, Dorset, England.

Ethidium Bromide, solution 10 mg/ml

Source : GIBCO Europe, Life Technologies Ltd, Paisley, Scotland.

Tris hydroxymethylaminomethane [TRIS], dessicate

2.4.3 General Chemicals

Source : FISCHER Scientific Equipment LtD, Loughborough, England

EDTA, dessicate

EGTA, dessicate

Glucose, dessicate

Glycine, dessicate

Hydrochloric Acid 11M, solution

Paraformaldehyde, dessicate

Potassium Chloride, dessicate

Potassium Hydroxide, dessicate

Sodium Dodecyl Sulfate, desiccate

Sodium Chloride, dessicate

Sodium Hydroxide, dessicate

2.4.4. Detergents

Source : Sigma Co Ltd., Poole, Dorset, England

Nonidet NP-40, solution

Triton X-100, solution

Tween-20, solution

Sodium Deoxycholate, dessicate

2.4.5. Enzyme Inhibitors

Source : Sigma Co Ltd., Poole, Dorset, England

Aprotinin, solution

Benzamidine, dessicate

Leupeptin, dessicate

Phenyl-Methyl-Sylfonyl-Fluoride (PMSF), dessicate

Sodium Fluoride, dessicate

Sodium Pyrophosphate, dessicate

Sodium Orthovanadate, dessicate

Source : Calbiochem, San Diego, CA, USA

PD 98059, MEK-1-specific inhibitor (dissolved in DMSO)

Source : Dr. David Heimbrook, Merck Co., West Point, PA, USA

L-779 450 Raf-1 kinase-specific inhibitor (dissolved in PBS)

2.4.6. Electrophoresis Reagents

Source :Severn Biotech Ltd., Kidderminster, Worcestershire, England

30% acrylamide/ 0.8% bisacrylamide solution

40% acrylamide / 1,5% bisacrylamide solution

Source : BDH Ltd, Poole, Dorset, England

Acrylamide ; Electran^{R} , dessicate

bis-Acrylamide ; Electran^{R}, dessicate

Source : Sigma Co. Ltd, Poole, Dorset, England

TEMED, solution

2.4.7. Reagents for Protein determination

Source : Sigma Chemical Ltd.Co, Poole, Dorset, England

Bichinoninic Acid, solution

Bovine Serum Albumin, dessicate

Copper (++), solution

2.5. Membranes, Paper and X-Ray Film

Source : Sigma Chemical Ltd.Co, Poole, Dorset, England

Paper for pH determination (pH 1-7, specific)

Source : Whatman International Ltd., Maidenstone,Kent, UK.

3MM, Chromatography paper

Source : Sartorius, Gmbh, Gottingen, Germany

Dialysis Tubes

Source : Millipore, UK Ltd, Watford, Hertfordshire, UK

Immobilon-P, Polyvinyl-Difluoride (PVDF) Membrane

Source : Eastman Kodak Co., Rochester, N.Y., USA

X-Ray Film (XAR-5)

Source : Fuji Photo Co, Ltd., Japan.

X-Ray Film (RX)

2.6. Molecular Biologicals

2.6.1 Media and Plasticware

Source : Difco Laboratories, Detroit, Michigan

Agar, Microbiological Grade

Source : GIBCO Europe, Life Technologies Ltd., Paisley, Scotland.

Agarose, dessicate

Agarose, Low Melting Point, dessicate

Source : Boehringer Manheim UK Ltd., Lewes, East Sussex, England.

Ampicillin

Source : Bibby Sterilin Ltd., Stone, Staffs., England.

Bacteriological Dishes

2.6.2 Molecular Weight Markers

Source : Life Tehnologies, Ltd., Paisley, Scotland

Bacteriophage I (Hind III Digested), solution (for DNA agarose gel electrophoresis)

Bacteriophage Φx 174 (Hind III Digested, solution (for DNA agarose gel electrophoresis)

Source : Amersham International PLC, Buckinghamshire, UK

Prestained Rainbow MW Markers, RPN 756, solution (for SDS polyacrylamide electrophoresis)

Source : BIO-RAD Labs, CA, USA

Molecular Weight Markers, High Range, solution (for SDS polyacrylamide electrophoresis)

2.6.3 Enzymes

Source : GIBCO, Europe, Life Tech., Ltd., Paisley, Scotland

Restriction Endonucleases and buffers

Source : NBL Northumbria Biochemicals

T4 Polynucleotide Kinase -T4 Kinase and 10X reaction Buffer

Alkaline Phosphatase - Alkaline Phosphatase and 10X reaction Buffer

2.7. Solvents and Dyes

Source : Fischer Scientific UK Ltd, Loughborough, Leicestershire, UK

Chloroform

Butanol

Formaldehyde

Isopropanol

Source : J. Burrough (FAD) Ltd., Witham, Essex, England.

Ethanol

Source : Fischer Scientific UK Ltd, Loughborough, Leicestershire, UK

Giemsa Stain, solution

Source : Sigma Chemical Ltd.Co, Poole, Dorset, England

Bromophenol Blue, dessicate

Xylene Cyanol, dessicate

Source : Sigma Chemical Ltd.Co, Poole, Dorset, England

TRITC-Phalloidin

2.8. Kits

Source : Amersham International PLC, Amersham, Buckinghamshire, England

Enhanced Chemiluminescence Reagent, solution

2.9. Growth Factors

Growth factors were provided by Dr. A. Malliri, Dr. Pierre Schembri-Wismayer, Jim O'Prey, and Dr. Val Brunton [Beatson Institute]. di-butiryl cAMP. EGF, IGF-I, LPA, PDGF-BB, Insulin were dissolved in PBS. TPA in DMSO. Their commercial origin is described below :

di-butiryl cAMP, from *Sigma Chemical Ltd.Co, Poole, Dorset, England*

EGF from *R&D Systems, Abingdon, OXON, UK*

IGF-I from *R&D Systems, Abingdon, OXON, UK*

Insulin from *Sigma Chemical Ltd.Co, Poole, Dorset, England*

LPA from *Sigma Chemical Ltd.Co, Poole, Dorset, England*

PDGF-BB from *Sigma Chemical Ltd.Co, Poole, Dorset, England*

TPA from *Sigma Chemical Ltd.Co, Poole, Dorset, England*

CHAPTER 3 METHODS

CHAPTER 3. METHODS

3.1. Cell culture

3.1.1. Cell lines

208F is a thioguanine derivative of Rat-1 cells (Quade, 1979). FBR cells are 208F cells transformed with the FBR v-*fos* oncogene under the control of the MuLV LTR.

3.1.2. Cell maintenance and storage

All cell lines were grown in DMEM supplemented with 10% FCS (500ml dd H_20, 50 ml DMEM, 5 ml NaPuryvate 100mM, 25 ml Sodium Bicarbonate, 5 ml 200mM Glutamine, 2.5 ml Gentamycin 10mg/ml). Cell lines were trypsinized in 1 ml of PE/trypsin and resuspended in DMEM with 10% FCS and divided to analogous number of dishes cells. Cell lines were kept in liquid Nitrogen for long term storage. For such a purpose cell lines were washed once with PBS and 1 ml of PE/trypsin solution was added to the plate. After trypsinization cells were centrifuged for 5 minutes at 1,000 g at room temperature, the tryspin-containing medium was aspirated and 1 ml freezing solution (90% FCS, 10 % DMSO) was added, mixed and the aliquot placed to a 2ml Nunc tube and put to - 70 ^{0}C. After 2-4 days the aliquots were placed in liquid nitrogen barrels. To recover cells from nitrogen, the nunc vials were thawed in 37 ^{0}C and resuspended in DMEM supplemented with 10% FCS, centrifuged for 5 minutes at 1,000 g at room temperature and the DMSO-containing media were aspirated. Immediately after this step the cells were resuspended in DMEM supplemented with 10% FCS and plated to 10 cm dishes.

3.1.2. Growth in semi-solid medium

Methylcellulose was used to prepare semi-solid medium. Initially 3g of methyllcellulose were mixed with 200 ml H_20. The solution was autoclaved and after sterilisation it was left to cool in room temperature and then stirred for 24 hours at 4 ^{0}C with the use of a mangetic stirrer. After methylcellulose was thoroughly mixed it was supplemented with the following:

22 ml 10X F10 Ham's Medium

4ml MEM minimal essential amino acids

4ml 0.1M NaPyruvate

5ml Na Bicarbonate

20ml FCS

5 ml 200mM Glutamine

For each experiment aliquots of $2x10^4$ cells were mixed with 10-14 ml of semi-solid medium and plated into duplicate or triplicate 10cm bacteriological grade dishes (Bibby, Sterilin). Fresh medium was added every 4 days. Cells were allowed to grow and colonies were counted after 3-4 weeks.

3.1.4. Immunofluorescence of actin stress fibres

Immunofluorescence was performed according to Nobes et al. (1994). $2x10^4$ cells were seeded on 16mm coverslips in 12-well plates for 48 hours. After the cells formed monolayers they were deprived from serum as following: the wells were washed three times with PBS and medium was changed to DMEM supplemented with 0.1 % FCS. The cultures were remained quiescent for a period of 7 days. After 7 days the cells were treated with agonists as long as required and fixation was performed by adding 3% Paraformaldehyde pH 7.0 for 20 minutes at 37 ^{0}C. After this step the coverslips were washed once with PBS, permabilisation buffer (PBS, 0.01% Triton X-100) was added for 5 minutes at room temperature. This step was repeated once and then samples were incubated with PBS containing Rhodamine-labelled Phalloidin for 45 minutes in an orbital shaker at room temperature covered with foil. After staining was completed the wells were washed three times in PBS over 5 minutes at room temperature and mounted in glass slides with Vectashield reagent (Vector Labs, Burlingame, CA, USA), sealed with nail polish and processed for fluorescence microscopy.

3.1.5. Immunofluorescence of Focal Adhesion Components

Cells were processed for fixing as in (3.1.4.)and after permabilisation was completed they were incubated with a mouse anti-phosphotyrosine antibody (PY-20) in a dilution 1:100 in blocking solution (1%FCS, PBS) for 18 hours at 4 ^{0}C. After the addition of the primary antibody the wells were washed three times with PBS over 5 minutes and incubated with an anti-mouse FITC-conjugated antibody in a dilution 1:100 in blocking solution (1%FCS, PBS) for 45 minutes at room temperature covered with parafilm. After this stage the samples were washed three times

with PBS over 5 minutes and the coverslips were transferred to glass slides, mounted with Vectashield Reagent, sealed with nail polish, and processed for immunofluorescence.

3.1.5. Preparation of conditioned media

Cells were passaged in DMEM supplemented with 10% FCS and grown into subconfluence. Then the growth medium was removed and the plates were washed 3 times with PBS. The medium was changed to serum-free DMEM and the dishes were incubated in a tissue culture incubator at 37 ^{0}C for 48 hours. After this 48 hours the medium was harvested, filtered with a 2.5 micron filter and used for replacing the medium of serum-deprived cultures of FBR and 208f cells.

3.2.Recombinant DNA Techniques

3.2.1 Transformation of competent E. Coli

Competent E.coli (DH5α E.Coli, Gibco, Life Tech., Paisley, Sco.) thawed on ice, and 50 μl aliquots were transferred to chilled polypropylene 5 ml Falcon tubes. Approximately 50 ng of plasmid DNA was added to the bacteria and the mix incubated on ice for 25 minutes. Then the cells heat-shocked for 45 seconds at 42 ^{0}C and immediately transferred to ice for 2 minutes. After this step 900 μl SOC medium (2% Bacto-tryptone, 0.5% bacto-yeast extract, 20 mM Glucose, 10 mM NaCl, 10 mM MgCl) was added and the beacterial solution was incubated for 1 hour in an orbital shaker at 37^{0}C. After completion of this step 50 μl of bacterial solution was plated to bacteriological grade dishes (Bibby Sterilin) containing 1.5% Agar and the appropriate selection antibiotic. The plates were incubated in an inverted position for 18 hours at 37^{0}C. Colonies were picked from the plates using an inoculating loop and transferred to 20 ml Universal tubes (Bibby Sterilin) containing L-Broth. The cultures were incubated overnight at 37^{0}C in a orbital shaking incubator for preparation of bacterial samples for small and large scale analysis of DNA.

3.2.2 Small scale plasmid DNA preparation

1ml of bacterial culture was pelleted in a eppendorf microfuge at 12000 g for 5 minutes at 4^{0}C. The supernatant was discarded and the pellet was resuspended in 200 μl of 2 mg/ml lysozyme

in Solution I (50mM Glucose, 25 mM Tris pH 8.0, 10 mM EDTA pH 8.0). The mix was vortexed briefly and placed for 5 minutes at room temperature. Then 400 μl Solution II (0.2 M NaOH, 1% SDS) was added to the tubes and the tubes were placed on ice. Then 300μl 3M NaAc pH 4.8 was added, the precipitate was vortexed immediately and the tubes were incubated for 10 minutes on ice. After this step tubes were centrifuged in a microfuge at 12000 g for 5 minutes at 4^0C. 800 ml of the supernatant was transferred to a new eppendorf tube and mixed with 480 μl of isopropanol and the tubes were placed in for 1 hour-70^0C to facilitate the precipitation of plasmid DNA. After this step tubes were centrifuged in a microfuge at 12000 g for 20 minutes at 4^0C. The supernatant was discarded and the pellet was washed with 1ml of 70 % v/v ethanol, the supernatant was discarded and the pellet was lyophilised for 20 minutes under vacuum. The DNA plasmid pellet was resuspended in 1X TE buffer pH 8.0 and stored at 4^0C.

3.2.3. Large scale plasmid DNA preparation

Large flasks with 500 ml of L-Broth were inoculated with 0.5 ml from cultures of bacterial stocks and incubated for 18 hours in an orbital shaker, at 37^0C. Cultures were divided into three Beckman 500ml bottles and centrifuged at 5000 g for 5 minutes at 4^0C in a Beckman J2-21 centrifuge. The supernatant was discarded and the pellet was resuspended in 20 ml of cold Solution I (50mM Glucose, 25 mM Tris pH 8.0, 10 mM EDTA pH 8.0). The bottles were placed on ice 2 ml of lysozyme (10mg/ml lysozyme in 10 mM Tris pH 8.0) was added, 80 ml of Solution II (0.2 M NaOH, 1% SDS) were added to the samples and the lysate was mixed by inversion carefully and incubated at room temperature for 10 minutes. After this step 30 ml of cold Solution III (60ml 5M Potassium Acetate, 11ml Glacial Acetic acid, 28 ml H_2O 28ml) was added and the lysate was mixed vigorously, the samples were incubated on ice for 10 minutes and centrifuged at 5000 g for 15 minutes at 4^0C in a Beckman J2-21 centrifuge . The supernatant was filtered through gauge, 0.6 vol. of isopropanol was added to the filtered solution, the solution was mixed well and left for 10 minutes at room temperature. Then the solution was centrifuged at 5000 g for 15 minutes in a Beckman J2-21 centrifuge. After centrifugation was completed the supernatant was removed very carefully and the pellet was washed with 70 % Ethanol (v/v) twice. The pellet containing the plasmid DNA of interest was dried at the 37^0C in a roller bottle tissue culture incubation cabinet.

3.2.4 Purification of plasmid DNA with CsCl ultracentrifugation

The dried pellet from the large scale of plasmid DNA preparation was resuspended in 12 ml of 1X TE Buffer pH 8.0. and 12.9 g CsCl was added to the pellet. 0.8 ml of 10mg/ml EtBr stock solution were added for every 10 ml of DNA solution. Optical density was adjusted to 1.39 with a densitometer, samples were aliquoted to polycarbonate Beckman tubes and centrifuged in a Beckman TL-100 ultracentrifuge in a TLA 100.3 rotor at 300000 g for 20 hours at 20^0C. The following day plasmid DNA was aspirated from the Beckman tubes under UV light using a 18 $^1/_2$ gauge needle. EtBr present in the plasmid solution was extracted 3 times with CsCl-saturated isopropanol. The DNA in solution was dialysed against against 700-800 ml of 1X TE Buffer pH 7.4 for 18 hours at 4^0C, with 3 or 4 changes of the buffer, using a dialysis bag (Sartorius Gmbh, Gottingen, Germany). After dialysis completed DNA solution volume was measured and precipitated with 10% 3M NaAc pH 4.8 and 2.5 volumes of 100% ethanol. The mix was centrifuged at 7000 g for 10 minutes at room temperature in a Beckman J2-21 centrifuge using. The supernatant was discarded and the plasmid DNA was washed twice with 70 % (v/v) Ethanol. Plasmid DNA was dried under vacuum for 10-20 minutes and resuspended in 50-100 µl of TE Buffer pH 8.0.

3.2.5 Restriction analysis of plasmid DNA

To identify the size of plasmid DNA after small and large scale preparations, restriction digests analysis was carried out. Digests of the plasmid DNA was performed as following: 250 ng - 500 ng of DNA was incubated with 10 Units of a restriction endonulease in a final volume sufficient to dilute the enzyme volume ten times, at 37^0 C for 1 hour. Digestion products were separated in a 0.8 % agarose gel for 1-2 hours at 80 volts. Fragments were visualised using a UV transilluminator.

3.2.6. Plasmid-DNA Transfections

Transfections were performed using the DOTAP lipofection protocol [Boehringer Manheim] with minor modifications.

Cell were plated 48 hours before transfection at 10 cm dishes. To prepare the transfection solution two 2ml nunc tubes containing the following were used:

1) 200μl of HEPES buffer solution and 50 μl of DOTAP solution and

2) 250 μl of HEPES buffer solution and 5-10 μg plasmid DNA.

Both solutions were mixed and the mix was incubated for 10 min at room temperature. The transfection mix was added directly to the culture dishes. After 24 hours the cells were divided to four 10 cm dishes and incubated another 24 hours at 37 ^{0}C. Then media aspirated and replaced with fresh DMEM supplemented with 10 % FCS plus the selection antibiotic.

After approximately 3-4 weeks of selection with G418 colonies appeared and cell clones were collected from the 10 cm dishes with sterile steel cylinders. To isolate colonies the dishes were washed carefully once with PBS, steel cylinders were attached to sterile vacuum grease, placed on the colonies, trypsin was added (50-80 μl) and the dishes were incubated for 15 min at 37^{0}C. Clones were isolated by pipetting the trypsin solution and plated at 24 well plates with DMEM supplemented 10% FCS plus the selection antibiotic. Clone growth were monitored daily and when a sufficient number of viable cells was present the cells were trypsinized and transferred to a 6 well plate. The individual cell clones were expanded and freezed in Liquid Nitrogen.

3.3. Biochemical Methods

3.3.1 Preparation of lysates from tissue culture cells

The protocol was provided by Dr. Liz Black (Beatson Institute for Cancer Research). Following appropriate culture conditions cell monolayers were washed twice with ice-cold PBS and 80-100 μl of lysis buffer (20 mM Hepes, 5mM EDTA, 10mM EGTA, 5 mM NaF, 1 mM DTT, 0.4 M KCL, 0.4% Triton-X100, 10% Glycerol, 5 μg/ml leupeptin, 1mM PMSF, 1mM Sodium Orthovanadate, 10mM β-Glycerophosphate, 1mM Benzamidine, 5 μg/ml Aprotinin) was added immediately to the monolayer. Cells were scraped with disposable cell scraper (Costar), the lysate was loaded to an eppendorf tube, samples were incubated on ice for 20-30 minutes and cleared by centrifugation at 12000 g in an eppendorf microfuge for 20 min at 4^{0}C. The supernatant which contains the detergent-soluble fraction which was measured with the Bichinoninic Acid method. If the samples were not used immediately they were frozen in a dry ice/ethanol bath and lysates were stored in -70^{0}C until use.

3.3.2. Determination of protein concetrations

Protein concentrations were determined using the Bichinoninic Acid method as following: an 10 μl aliquot of protein soluble fraction prepared as in *3.3.1.* was mixed with 200μl of bichinoninic solution (50 vol bichinoninic acid/1 vol 4% $CuSO_4$) in 96 well tissue culture plates and incubated for 45 minutes at 37^{0}C in a tissue culture incubator. Absorbance was measured at 590 nm wavelength in a Dynatech MR.7000 Spectrophotometer. The standard curve consists of 6 BSA standards (80, 100, 400, 800, 1000, 2000 μg/ml BSA/H_2O solutions).

3.3.3. Dephosphorylation of proteins in cell lysates using Alkaline Phosphatase

Protein concentration in cell lysates was determined and 50-100 μg of cell lysate was incubated with 20 U Alkaline Phosphatase and 10X dephosphorylation buffer for 2 hours at 37^{0}C (Lallemand et al., 1997). The reaction was stopped with the addition of 2X SDS sample buffer (62.5 mM Tris pH 6.8, 2% SDS, 10% Glycerol, 0.1% Bromophenol blue, 4% β-Mercaptoethanol) the samples were boiled for 5 minutes at 100^{0}C and loaded onto a 10% SDS-Polyacrylamide gel.

3.3.4. SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

For SDS-polyacrylamide gel electrophoresis an ATTO assembly apparatus and 2mm-thick glass plates were used. For 1 such SDS-PAGE gel of 2mm thickness the following solutions were used:

<u>10% SDS-PAGE Resolving Gel</u>

30% Acrylamide/ 0.8% Bisacrylamide	12 ml
4X RGB (1.5 M Tris, 0.4% SDS pH 8.8)	9 ml (RGB : Resolving Gel Buffer)
dd$H_2$0	15 ml
10% APS	360 μl
TEMED	36 μl

7.5% SDS-PAGE Resolving Gel

30% Acrylamide/ 0.8% Bisacrylamide	9 ml
4X RGB (1.5 M Tris 0.4% SDS pH 8.8)	9 ml (RGB : Resolving Gel Buffer)
ddH_20	18 ml
10% APS	360 µl
TEMED	36 µl

5% SDS-PAGE Stacking gel

30% Acrylamide/ 0.8% Bisacrylamide	2 ml
4X SGB (0.5 M Tris, 0.4% SDS pH 6.8)	3 ml (SGB : Stacking Gel Buffer)
ddH20	7 µl
10% APS	200 µl
TEMED	20 µl

First the resolving gel buffer/Acrylamide mix was poured into the space between the plates up to the marked line on the plastic apparatus. Then H_2O-saturated butanol with was layered over the gel solution and the gel was left to set at room temperature. When gel was set the layer of H_20-saturated butanol was washed extensively with water, the stacking gel solution was poured over the resolving gel and a 2mm-thick comb was inserted quickly and the gel was let to set at room temperature. After polymerisation was completed the comb was removed and the gel transferred to a gel tank containing 1X SDS-PAGE Running buffer (50 mM Tris, 1% Glycine, 0.1% SDS). Protein was quantified as in *3.3.2.* and samples were mixed with 2X SDS sample buffer (62.5 mM Tris pH 6.8, 2% SDS, 10% Glycerol, 0.1% Bromophenol blue, 4% β-Mercaptoethanol), boiled for 5 min at 100^0C, cooled briefly on ice, and loaded onto the gel. An LKB powerpack (Bromma, Sweden) was used and gels were run overnight at 45 volts.

3.3.4.1. Low-Bisacrylamide SDS Polyacrylamide Gel Electrophoresis

This protocol was provided by Bill Clarck (Beatson Institute for Cancer Research). For the detection of phosphorylated forms of MEK-1 with a phosphospecific antibody a 30% low bis-Acrylamide solution in SDS-polyacrylamide electrophoresis was used. The low bis-Acrylamide

solution contains 0.2% instead of 0.8% bis-Acrylamide. Low bis-Acrylamide containing gels have been used previously to separate phosphorylated proteins in their different isoforms by SDS-polyacrylamide gel electrophoresis (Marshall and Leevers, 1995). The solution was prepared in thefume hood to prevent Acrylamide and bis-Acrylamide toxicity and kept not more than two weeks at 4 ^{0}C due to the instability of its components.

SDS-PAGE, transfer to PVDF membranes and western immunoblotting was carried out as stated in *3.3.4., 3.3.5. and 3.3.6.1.*

3.3.5. Semi-Dry Transfer of SDS-PAGE gels onto PVDF Membranes

After electrophoresis was completed the two plates were separated and the gel that remained in the surface of the one plate was submerged into Dry Blot Buffer (1X DBB: 60mM Tris, 50 mM Glycine, 1.6 mM SDS, 20% Methanol v/v). The Polyvinylidene Di-Fluoride membrane (PVDF, Immobilon-P) was submerged briefly to 100% v/v Methanol, to dd H_20 for 3-5 minutes and then to 1X DBB. 12 pieces of Whatman paper were cut in a similar size to the size of the gel, and submerged into 1X DBB. The assembly of the dry blotter was as following: On the bottom of the apparatus six Whatman paper sheets followed by the PVDF membrane the gel and last six Whatman papers. The dry blotter was set to 200mA and 20V and electrotransfer proceeded for approximately for 1-2 hours.

3.3.6. Western Immunoblotting

Immediately after transfer the membrane was blocked in blocking solution (PBS, 0.1% Tween-20, 5% Nonfat Dry Milk) for 1-2 hours at room temperature or overnight at 4^{0}C, shaking at an orbital shaker. Primary antibodies were incubated were in blocking solution overnight at 4^{0}C After 16-18 hours incubation the membranes washed once with blocking buffer (PBS, 0.1% Tween-20, 5% Nonfat Dry Milk) for 15 minutes and 2 times with blocking buffer for 5 minutes at room temperature. After washes, a secondary Horse Radish Peroxidase-linked antibody (HRP-linked) diluted 1:5000 in blocking solution was employed for 1 hour at room temperature. Finally the membrane was washed two times with PBST (PBS, 0.1% Tween-20) for 10 minutes each at room temperature. The immunoreactive bands were detected with Enhanced Chemiluminecence system (Amersham), by submerging the membrane into reagent for 1

minute followed by different exposures on Fuji RX medical film (FUJI Photo, UK Ltd, London, England). Films were developed in a Kodak X-OMAT 480 RA Processor.

3.3.6.1. Western Immunoblotting with phospho-specific antibodies

The western immunoblotting protocol with phospho-specific antibodies was according to the published procedure from *New England Biolabs* with minor modifications. Immediately after transfer the membrane was blocked in TBST blocking solution (20 mM Tris pH 7.6, 150 mM NaCl, 0.1% Tween-20, 5% Nonfat Dry Milk) for 2 hours at room temperature in a plastic box in an orbital shaker. Phospho-specific antibodies against MAP Kinases (ERK1 and ERK2) and MEK-1 were used in 1:10000 and 1:1000 respectively and incubated in TBST-5%BSA buffer (20 mMTris pH7.6, 150 mM NaCl, 0.1% Tween-20, 5% BSA) at 4^0C. After 16-18 hours incubation the membranes were washed 3 times with TBST (20 mM Tris pH 7.6, 150 mM NaCl, 0.1% Tween-20) for 5 minutes each at room termperature and secondary HRP-linked antibody was added diluted 1:5000 in TBST blocking solution (20 mM Tris pH 7.6, 150 mM NaCl, 0.1% Tween-20, 5% Nonfat Dry Milk). Incubation with the secondary antibody was carried for 1 hour at room temperature. Then the membrane was washed three time with TBST for 5 minutes at room temperature. The immunoreactive bands were detected as in *3.3.6.*

3.3.6.2. Anti-phosphotyrosine Immunoblotting

After transfer the membrane was blocked in TBST-3%BSA blocking solution (20 mM Tris pH 7.6, 150 mM NaCl, 0.1% Tween-20, 3% BSA) for 1 hour in a plastic box shaking in an orbital shaker at room temperature. The mambrane was incubated with PY-20 mouse monoclonal anti-phosphotyrosine serum was diluted 1:1000 or 1:3000 depending on the experiment in TBST-3%BSA solution for 1 hour at room temperature. After incubation the membrane was washed 3X at room termperature with TBST 10 minutes each, and secondary HRP-linked antibody (anti-mouse 1:5000) was added diluted in TBST-3%BSA blocking solution. Incubation was carried out for 1 hour at room temperature. Finally the membrane was washed three times for 10 minutes each with TBST at room temperature. The immunoreactive bands were detected as in *3.3.6.*

3.3.7. Reprobing PVDF Membranes

For using the membranes second time the following protocol was used. The membrane was incubated in western pH 2.5 strip buffer (0.2 M Glycine, 1% SDS) for 1-2 hours at room temperature shaking at an orbital shaker, washed three times with TBST (20 mM Tris pH 7.6, 150 mM NaCl, 0.1% Tween-20) 20 minutes each at room temperature, shaking at an orbital shaker and incubated in blocking solution according to each protocol. Western immunoblotting was carried out depending on the nature of the primary antibody.

3.3.8. Immunoprecipitation and ERK Kinase assay

The protocol was provided by Dr. Walter Kolch (Beatson Insitutte for Cancer Research). 10cm dishes were lysed in 1 ml of ice-cold ERK lysis buffer (20 mM Tris pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1 mM PMSF, 1 mg/ml leupeptin, 5 mg/ml Aprotinin, 1mM Benzamidine, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate, 2 mM sodium fluoride, 2 mM sodium pyrophosphate) and the lysates were cleared by centrifugation at 12000 g for 10 minutes at 4^0C. The supernatant was transferred to a new eppendorf tube and protein concentration was determined as in *3.3.2.* 1000 μg of soluble lysate was used per one immunoprecipitation reaction.

Gamma-Bind Plus beads were used for immunopreciptation (Pharmacia). The beads were washed three times with each 0.8 ml ice-cold ERK lysis buffer and aliquots of 25 μl of beads were adjusted to 200 μl with ERK lysis buffer and mixed with 5 μl of ERK-1 anti-serum or with control serum for each immunoprecipitation reaction. The 800 μl of lysate was mixed with the 200 μl of beads and the reaction mix was incubated rotating for 16-18 hours at 4^0C.

The immunopreciptates were washed three times with ice-cold ERK lysis buffer, three times in ice-cold RIPA buffer supplemented with inhibitors (20 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5 % Sodium Deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate, 2 mM sodium fluoride, 2 mM sodium pyrophosphate) and 2 times with ice-cold kinase buffer (20 mM Tris pH 7.4, 20 mM NaCl, 10 mM $MgCl_2$) and the volume of each sample was adjusted to 25 μl with kinase buffer. The reaction was initiated by adding of 6 μg of MBP, 5 μCi of [γ-^{32}P] ATP and 6 μM ATP, per sample. Kinase reactions were incubated for 30

minutes at 37 ^{0}C. The reactions were terminated with 20 μl of 2X SDS sample buffer, boiling 5 minutes at 100 ^{0}C and the samples were separated by 10% SDS-Polyacrylamide gel electrophoresis. After electrophoresis the proteins were transferred onto PVDF nitrocellulose membranes using Towbin Transfer.

To verify the efficence of the immunoprecipitation the membrane was immunoblotted with an the anti-ERK-1 serum to determine that equal levels of the enzyme were isolated (*3.3.6.*).

3.3.8.1. Transfer of SDS-PAGE gels in Towbin Solution

This method is carried at 4 ^{0}C overnight. The PVDF membrane was submerged to 100% Methanol, to dd H_20 for 2-5 minutes and was immersed to Towbin transfer buffer pH 8.0 (25mM Tris, 192 mM Glycine, 20% Methanol). Four pieces of Whatman paper were cut in a larger size than the size of the gel, submerged to transfer buffer briefly, and placed two on the positive side of the tank and two in the negative. Then the membrane was placed in the and the gel in the. The assembly was placed in the tank (Bio-Rad Labs, CA, USA) and the proteins were transferred electrophoretically at 30mA.

After 12-18 hours of transfer the papers were separated from the membrane and the gel. The gel was discarded in a radioactive waste bin and the membrane was wrapped immediately in Saran Wrap in order not to dry, and exposed using intensifying screens at -70 ^{0}C for various lengths of time.

3.3.9. Immunoprecipitation and FAK anti-phosphotyrosine immunoblotting

Quiescent or stimulated FBR or 208F cultures were washed twice with ice-cold PBS, placed on ice and 100 μl of lysis buffer (20 mM Hepes, 5mM EDTA, 10mM EGTA, 5 mM NaF, 1 mM DTT, 0.4 M KCL, 0.4% Triton-X100, 10% Glycerol, 5μg/ml leupeptin, 1mM PMSF, 1mM Sodium Orthovanadate, 10mM β-Glycerophosphate, 1mM Benzamidine, 5 μg/ml Aprotinin) was added immediately to the monolayer. Cells were scraped with disposable cell scraper [Costar] and the lysate was loaded to an eppendorf tube. The samples were incubated on ice for 30 minutes. Then lysates were cleared by centrifugation in an eppendorf microfuge for 20 min at 12000 g in 4^{0}C. The supernatant protein content was measured with the Bichinoninic Acid method and

1000 μg of protein was diluted with ERK lysis buffer [3.3.8.] (20 mM Tris pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1 mM PMSF, 1 mg/ml leupeptin, 5 μg/ml Aprotinin, 1mM Benzamidine, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate, 2 mM sodium fluoride, 2 mM sodium pyrophosphate) to give 800 μl final volume of solution.

Gamma-Bind Plus beads were used for immunopreciptation (Pharmacia). The Beads were washed three times, each with 1 ml ERK lysis buffer [3.3.8.] and aliquots of 45 μl of beads were adjusted to 200 μl with ERK lysis buffer [3.3.8.] and mixed with 5 μl of anti-FAK serum for each corresponding immunoprecipitation sample. The 800 μl of lysate was mixed with the 200 μl of beads and the mix was incubated rotating for 16-18 hours at 4^0C. Next day the immunoprecipitates were washed 4 times with ERK lysis buffer [3.3.8.] and 40μl 2X SDS sample buffer was added and the samples were boiled for 5 minutes at 100^0C in a boiling waterbath. After this step the samples were centrifuged in a eppendorf centrifuge briefly, the supernatant was carefully loaded to an 7.5% SDS-PAGE gel, electrophoresed overnight, transferred to PVDF membranes [3.3.5. semi-dry transfer] and blotted with the PY-20 anti-phosphotyrosine antibody at 1:3000 as in 3.3.6.2.

To verify equal amounts of FAK in the immunoprecipitates the membrane was stripped, washed, blocked in blocking solution (PBS, 0.1% Tween-20, 5% Nonfat Dry Milk) and probed with the anti-FAK mouse monclonal antibody at 1:5000 diluted in the blocking solution.

RESULTS

RESULTS CHAPTER 4

GENERATION OF FBR REVERTANT CELL LINES FOLLOWING TRANSFECTION OF THE DOMINANT NEGATIVE MUTANT OF *c-jun*, TAM-67

RESULTS

CHAPTER 4. GENERATION OF FBR REVERTANT CELL LINES FOLLOWING TRANSFECTION OF THE DOMINANT NEGATIVE MUTANT OF *c-jun* TAM-67

4.1. The c-jun N-terminus is required for transformation

Full length human c-*jun* oncogene when it is transcriptionally driven under the strong signals of the Mo-MuSV LTR can transform established cell lines such as *rat*-1 as well as primary embryo cells when co-expressed with *ras* (Schutte *et al.*,1989a; 1989b).

Deletion analysis of c-*jun* showed that the N-terminus contains a transactivation domain (Angel *et al.*,1989). A series of different deletion mutants of c-*jun* was constructed (Alani *et al.*,1991) and the transactivation potential of these constructs was investigated in transactivation assays and in transformation assays. The results of these experiments demonstrated a direct correlation between c-*jun*-induced transformation and its N-terminal transactivation domain. A similar N-terminal deletion mutant of the v-*jun* oncogene reversed transformation by *ras, src*, Polyoma-mT, and *fos* oncogenes in tissue culture cells (Lloyd *et al.*,1991). This mutant efficiently suppressed transcriptional activation by oncogenes and phorbol esters and reduced the mRNA levels of cathepsin L.

These studies prompted us to use a dominant negative deletion mutant of c-*jun* termed TAM-67 to inhibit AP-1 activity in the FBR cells and to determine which attributes of the transformed phenotype in FBR cells are due to AP-1 function or which have arisen subsequently as a result of independent mutations. The TAM-67 dominant negative deletion mutant of c-*jun* protooncogene is identical to c-*jun* except that it lacks amino acids 1 to 123 which encode for the c-*jun* N-terminus transactivation domain. TAM-67 has been shown previously to suppress transformation by SV40 and *ras* and by *ras* and phorbol esters (Brown *et al.*,1993 ; Brown *et al.*,1994). This signifies a role for this truncated dominant deletion negative mutant of c-*jun* in the regulation of oncogenic transformation by the AP-1 transcription factor.

4.2. Transfection of TAM67 into FBR and to 208F fibroblasts

Lipofection was used to introduce p-CMV TAM-67 DNA into FBR and 208F cells. This vector contains the cDNA encoding the C-terminus of the c-*jun* protooncogene under the transcriptional

signals of the cytomegalovirus promoter (Brown *et al.*,1993). The p-CMV TAM-67 vector was transfected together with the pSV2neo, which confers G418 resistance as a means for selection of transfectants. Tissue culture dishes of approximate confluency 50-70 % were lipofected with the indicated amounts of plasmid DNA's (Table 4.1). 24 hours after lipofection the dishes were split to three or four plates and 24 hours later G418 selection with 200 mg/ml was initiated. After approximately three weeks, stable cell clones were isolated by cylinder cloning for phenotypic analysis.

After selection with G418 the transfection dishes were stained with Giemsa to assess colony formation (Table 4.1). Transfection of TAM-67 into FBR cells results in a 2-fold reduction in the colony forming ability when compared with FBR cells transfected only with the pSV2neo vector (Table 4.1). In the case of the 208F c-Jun TAM-67 transfected cells a 2-fold reduction in the colony formation compared to the 208F cells transfected with the pSV2neo vector alone (Table 4.1). This suggests that TAM-67 reduces the colony forming ability of FBR and in 208F cells.

The expression of TAM-67 in transfected clones was analysed by western immunoblotting to assess the efficiency of the transfection protocol (Figure 4.1). From growing cultures in DMEM supplemented with 10% FCS total cell lysates were prepared using a Triton X-100-containing lysis buffer. The dishes were washed twice with ice-cold PBS and lysis buffer was added. Then the cells were scraped and the lysates were incubated on ice and clarified by centrifugation. The protein content of the different samples was normalised, 50 μg of lysate was separated by 10% SDS-Polyacrylamide gel electrophoresis, transferred to PVDF membrane and blotted with an antibody specific for the Jun C-terminus. In the case of c-Jun TAM-67 the size of this protein (29 kD) allows its identification from the endogenous Jun protein (39 kD). TAM-67 migrates on SDS-PAGE gels as a doublet as shown by western immunoblotting (Figure 4.1B, lanes 3,4,5,6,7,8 and 9).

The expression of the transforming FBR p75 $^{v\text{-}Fos}$ oncoprotein was also analysed to ensure that it is still expressed and not lost during the selection process. The clones analysed by western immunoblotting for the expression p75 $^{v\text{-}Fos}$, all contained the transforming oncoprotein (Figure 4.1A, lanes 3, 4, 5, 6, 7, 8 and 9) suggesting that the expression levels of the p75 $^{v\text{-}Fos}$ and its posttranslational stability was not affected after introduction of the dominant negative deletion of c-Jun TAM-67. In these cell lines the expression of the c-Jun TAM-67 did not

interfere with the expression of the endogenous viral oncoprotein. Therefore both the p75 $^{v\text{-}Fos}$ and the dominant negative mutant TAM-67 are present in all cell lines that we examined.

208F TAM-67-transfected clones were also generated during this transfection experiment. 208F TAM-67 expressing cells exhibited a very flat phenotype. A representative clone of these transcfections is shown in Figure 4.2b. By western immunoblotting the presence of TAM-67 protein was identified in the 208F cells that have been selected for its expression with G418 (Figure 4.1C, lane 2).

Table 4.1. Colony formation in 10cm tissue culture dishes after transfection of TAM-67 into FBR and to 208F cells. Cells were transfected with the indicated amounts of CsCl-purified plasmid DNA using lipofection. After 3-4 weeks colonies of selection with 200 mg/ml G418 were fixed in 100% Methanol, washed with PBS, stained with 10% Giemsa solution (v/v in H_2O) and counted. [ND : Not Done].

Cell line	*Transfected plasmid*		*Colony number*
FBR	neo	1 μg	150 colonies / 1 μg
FBR	TAM-67 / neo	5 μg /1 μg	110 colonies / 1 μg
FBR	TAM-67 /neo	7 μg / 1 μg	ND
FBR	TAM-67 /neo	8 mg / 1 μg	85 colonies / 1 μg
208F	neo	1 μg	110 colonies / 1 μg
208F	TAM-67 / neo	10μg / 1μg	54 colonies / 1 μg

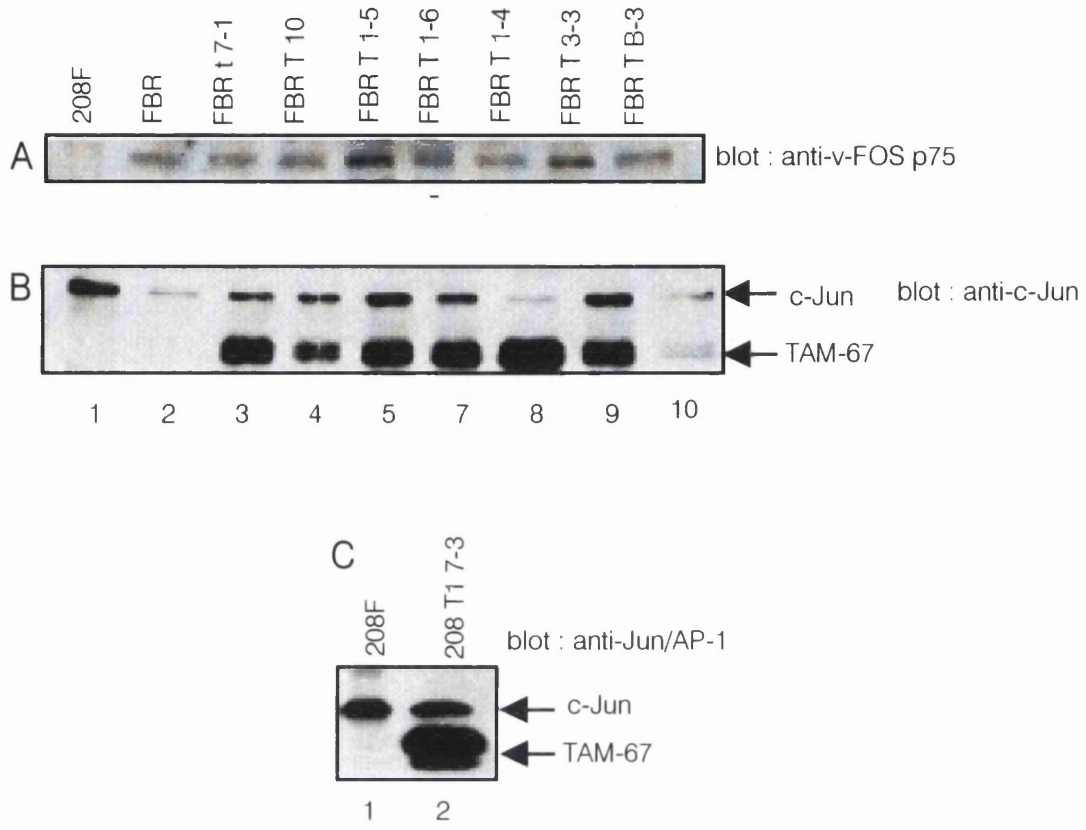


Figure 4.1. Expression of Fos and Jun proteins proteins in FBR, FBR TAM-67 revertant cell lines, 208F cells and 208F TAM-67 expressing cells

Cells were grown in DMEM supplemented with 10% FCS. From subconfluent cultures lysates were prepared and proteins were separated by 10% SDS-PAGE, transferred to PVDF membranes and probed with an antibody specific for
A) p75 v-Fos (anti-gag MuLV, Dr. Alan Rein, Frederick, USA)
B) c-Jun (Oncogene Science) and for 208F TAM-67 cells **C**) c-Jun (Oncogene Science).

A) and **B)** 1) 208F cells; 2) FBR cells; 3-9) FBR TAM-67 revertant cell lines.

C) 1) 208F and 2) 208F TAM-67 tranfectant.

4.3. Morphological characteristics of FBR TAM-67 transfectants

FBR cells have a bipolar, refractile shape, which is typical of the appearance of cell lines transformed by several viral oncogenes (Figure 4.2c). In contrast to this the FBR TAM-67-positive cell clones isolated they demonstrated a flat phenotype as shown in the (Figure 4.2d, e, and f) with few rounded cells to appear in the cultures. The characteristics of the FBR TAM-67 cells are reminiscent of revertant, and appeared after transfection of TAM-67 in FBR cells.

FBR TAM-67 clones demonstrated a flat morphology in approximately 70% of the clones isolated after transfection (Table 4.2). Also a number of FBR TAM-67 clones had a transformed morphology. This could be due to a genetic event that took place during the selection procedure that allowed individual clones to maintain the transformed phenotype and not to revert to a flat morphology. It is not known if this is related to a mutation in cellular genes or the $p75^{v\text{-}Fos}$ suffered a point mutation which might act in a dominant fashion. FBR cell lines resistant to the marker pSV2neo were also isolated. All the G418-resistant FBR clones were morphologically transformed similarly to the parental FBR cells. The FBR neo clones isolated and expanded in to mass cultures had a transformed morphology which remained stable through passaging.

The revertant phenotype of the FBR TAM-67 cells was more evident in higher cell densities (Figure 4.3 c). FBR TAM-67 revertants in low density show some of the properties of the parental FBR v-*fos* transformed fibroblasts, such as bipolar shape and extensions at the two edges of the cell (Figure 4.3a). In a higher cell density FBR TAM-67 display increased adherence to the substratum and form a monolayer in contrast to the parental FBR (Figure 4.2 c).

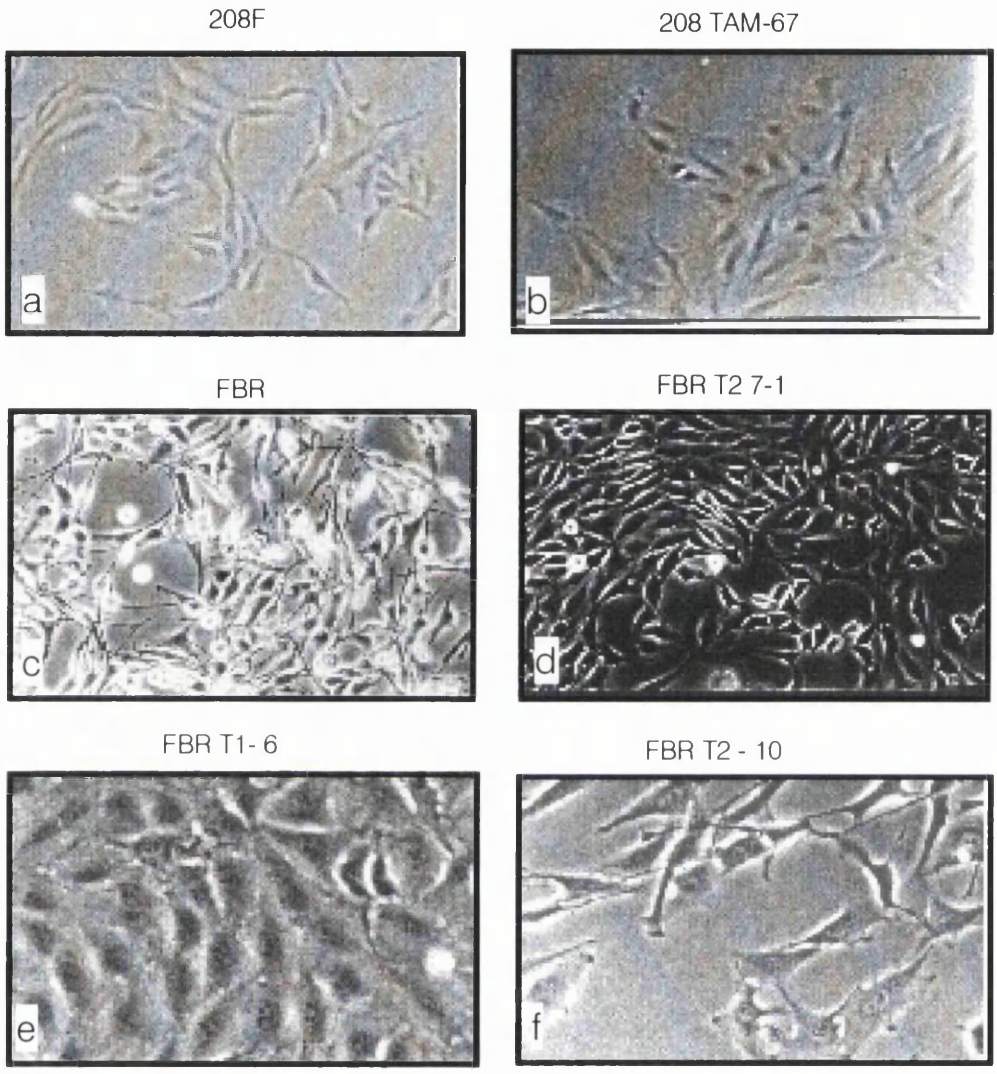


Figure 4.2. Morphology of FBR cells, 208F, FBR TAM-67 revertants and 208F cells transfected with TAM-67.

Phase-contrast pictures were taken with a Nikon camera attached to a Nikon inverted Diaphot microscope. **a)** 208F cells; **b)** 208F TAM-67 cells; **c)** FBR v-fos transformed fibroblasts; **d)** FBR TAM-67 revertant cloneT2-7-1; **e)** FBR TAM-67 revertant clone T1-6; **f)** FBR TAM-67 revertant clone 2-10.

Table 4.2. Morphology of FBR neo and FBR TAM-67 transfected cells lines.

Cell lines were plated in DMEM containing 10% FCS at 10cm tissue culture dishes and allowed to grow for 48 hours in order to adhere to substratum to identify the individual cell morphologies.

Cell line	*Transfected plasmid*	*Morphology*
FBR neo1	neo	transformed
FBR neo 2	neo	transformed
FBR neo 3	neo	transformed
FBR neo 4	neo	transformed
FBR neo 5	neo	transformed
FBR T1-2	TAM-67/neo	flat
FBR T1-11	TAM-67/neo	transformed
FBR T1-3	TAM-67/neo	transformed
FBR T1-4	TAM-67/neo	transformed
FBR T1-5	TAM-67/neo	flat
FBR T1-6	TAM-67/neo	flat
FBR T2-3	TAM-67/neo	flat
FBR T2-4	TAM-67/neo	flat
FBR T2-12	TAM-67/neo	transformed
FBR T2-2	TAM-67/neo	flat
FBR-T2-C2a	TAM-67/neo	transformed
FBR T2-2-1	TAM-67/neo	flat
FBR T2-2-4	TAM-67/neo	flat
FBR T2-5	TAM-67/neo	flat
FBR T2-6	TAM-67/neo	flat
FBR T-2-7a	TAM-67/neo	flat
FBR T2-7-1	TAM-67/neo	flat
FBR T2-7-2	TAM-67/neo	flat
FBR T2-7-3	TAM-67/neo	transformed
FBR T2-7-5	TAM-67/neo	transformed
FBR T2-15	TAM-67/neo	flat
FBR T3-6	TAM-67/neo	flat

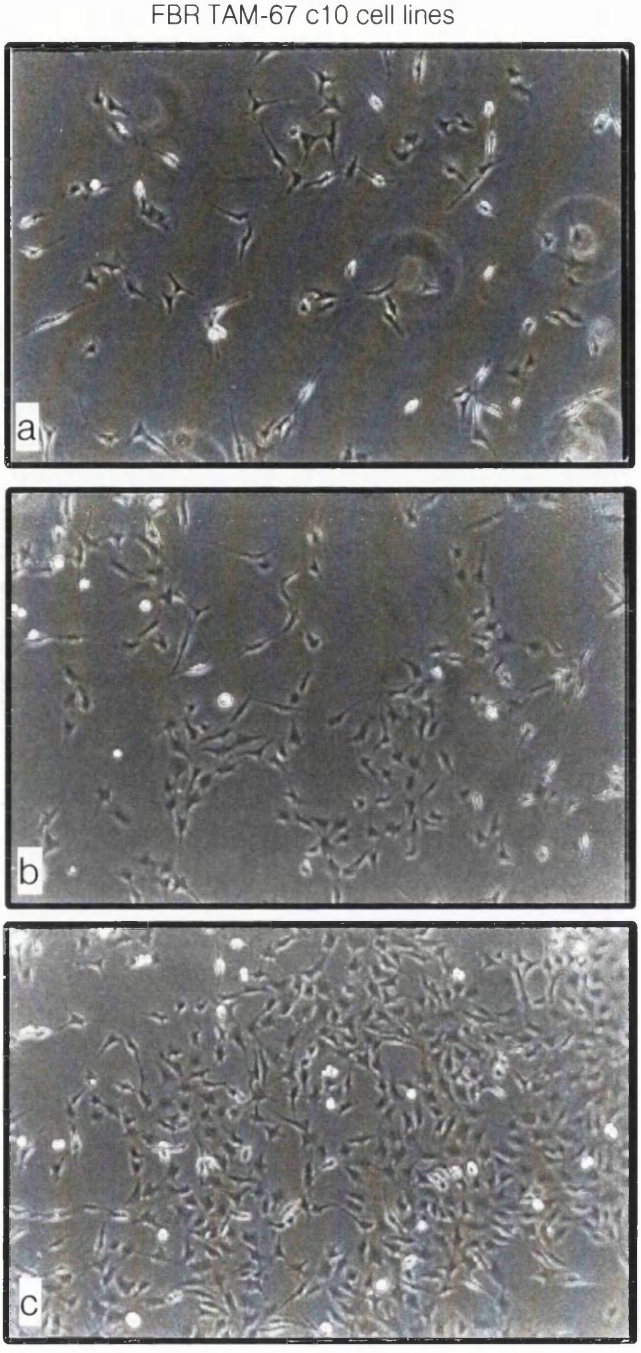


Figure 4.3 . Morphological reversion of FBR cells transfected with TAM-67 at different cell densities.

Cell lines were grown in DMEM supplemented with 10% FCS and photographed with a Nikon inverted Diaphot microscope.

4.4. TAM-67 inhibits FBR v-fos-induced anchorage independence

Flat revertants derived from RNA-containing Tumour Viruses do not grow efficiently in semi-solid medium (Ozanne and Vogel, 1974). Flat revertants isolated from FBJ-MuSV and FBR-MuSV transformed cell lines failed to form colonies in semi-solid medium and do not form tumours after injection in nude mice (Zarbl *et al.,*1987; Wisdom and Verma, 1991). It is interesting that one class of FBJ revertants could be re-transformed by Polyoma middle T and the *trk* oncogene and not by *ras*, *mos*, *abl* and *fos* oncogenes (Zarbl *et al.,*1987).

208F cells and 208F TAM-67 positive cells do not form colonies in semi-solid medium (Figure 4.4 a and b; Table 4.3) although in some cases the 208F cells line could form low numbers of small colonies under anchorage-independent conditions (not shown). The FBR cells formed colonies of a considerable size in the same assay (Figure 4.4 c; Table 4.3). The FBR TAM-67 revertant cells formed micro-colonies with reduced efficiency. When compared to the FBR-derived colonies the FBR TAM-67 colony forming ability was found to be 6- to 7-fold reduced (Figure 4.4, c and d; Table 4.3).

Another aspect of transformation that correlates with the oncogene suppression activity of TAM-67 is its ability to modulate invasion. Previously the invasiveness of FBR and 208F cells using an *in vitro* invasion assay was examined (Lamb *et al.,*1997). In this assay 208F cells require growth factors, such as EGF or PDGF, to invade efficiently; while FBR cells invade in a growth factor-independent fashion. Expression of TAM-67 was found to suppress invasion of 208F and of FBR cells (Lamb *et al.,*1997).

As shown in Figure 4.5 TAM-67 can suppress *in vitro* invasion of FBR cells. In this experiment FBR cells and FBR neo cells invade very efficiently the extracellular matrix whereas this property is severely impaired in FBR TAM-67 positive cells. This suggests that TAM-67 is involved through inhibition of AP-1 activity in growth factor and FBR v-*fos*-dependent invasion (Hennigan 1993; Lamb *et al.,*1997).

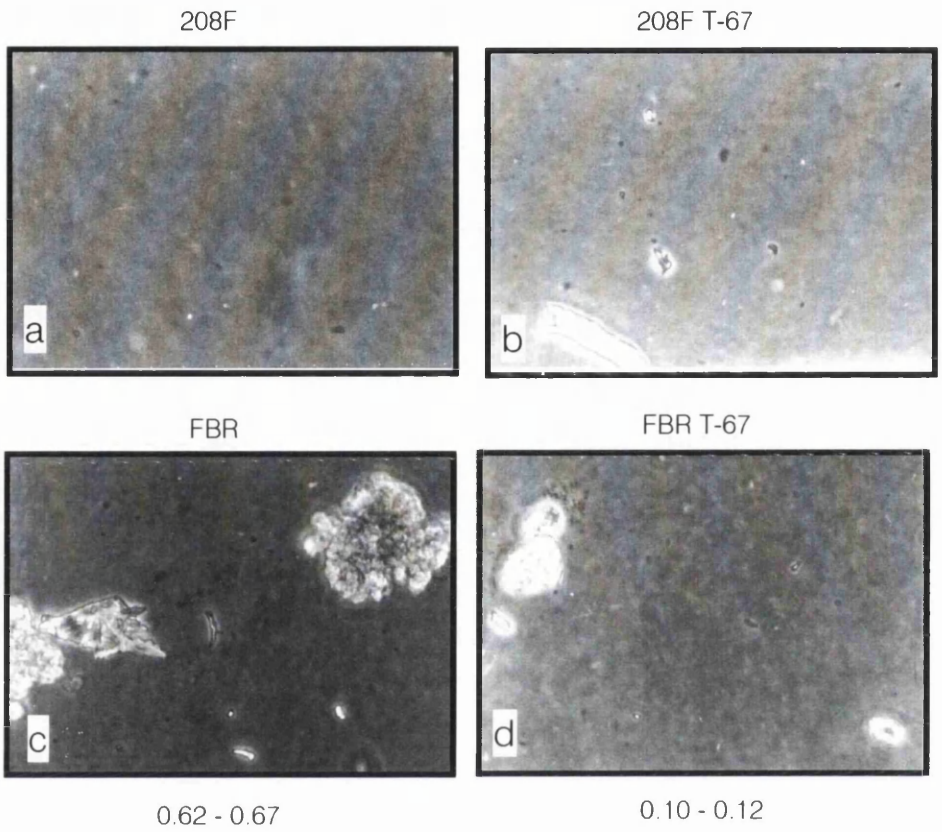


Figure 4.4 . Growth in semi-solid medium of FBR, 208F, and FBR and 208F transfected with c-Jun TAM-67.

Cells were grown in methylcellulose containing 10% FCS for 28 days, and photographed with an inverted Nikon Diaphot microscope. **a)** 208F cells; **b)** 208F TAM-67 transfected cells; **c)** FBR cells and **d)** FBR TAM-67 cells.

Table 4.3. *in vitro* Anchorage-Independent growth of FBR and FBR TAM-67 cells.

$2x10^4$ cells from each cell line were plated in Ham's media containing 1.5% Methylcellulose. Colonies were counted after 4 weeks. These results represent two independent experiments were duplicate samples were assayed.

Cell Line : FBR	*Experiment 1*	*Experiment 2*
Colony Number	14664 - 14444	17610 - 14352
Mean	14554	15756
Ratio	0.72	0.78

Cell Line : FBRTAM-67	*Experiment 1*	*Experiment 2*
Colony Number	2808 - 3120	2184 - 2184
Mean	2964	2184
Ratio	0.14	0.10

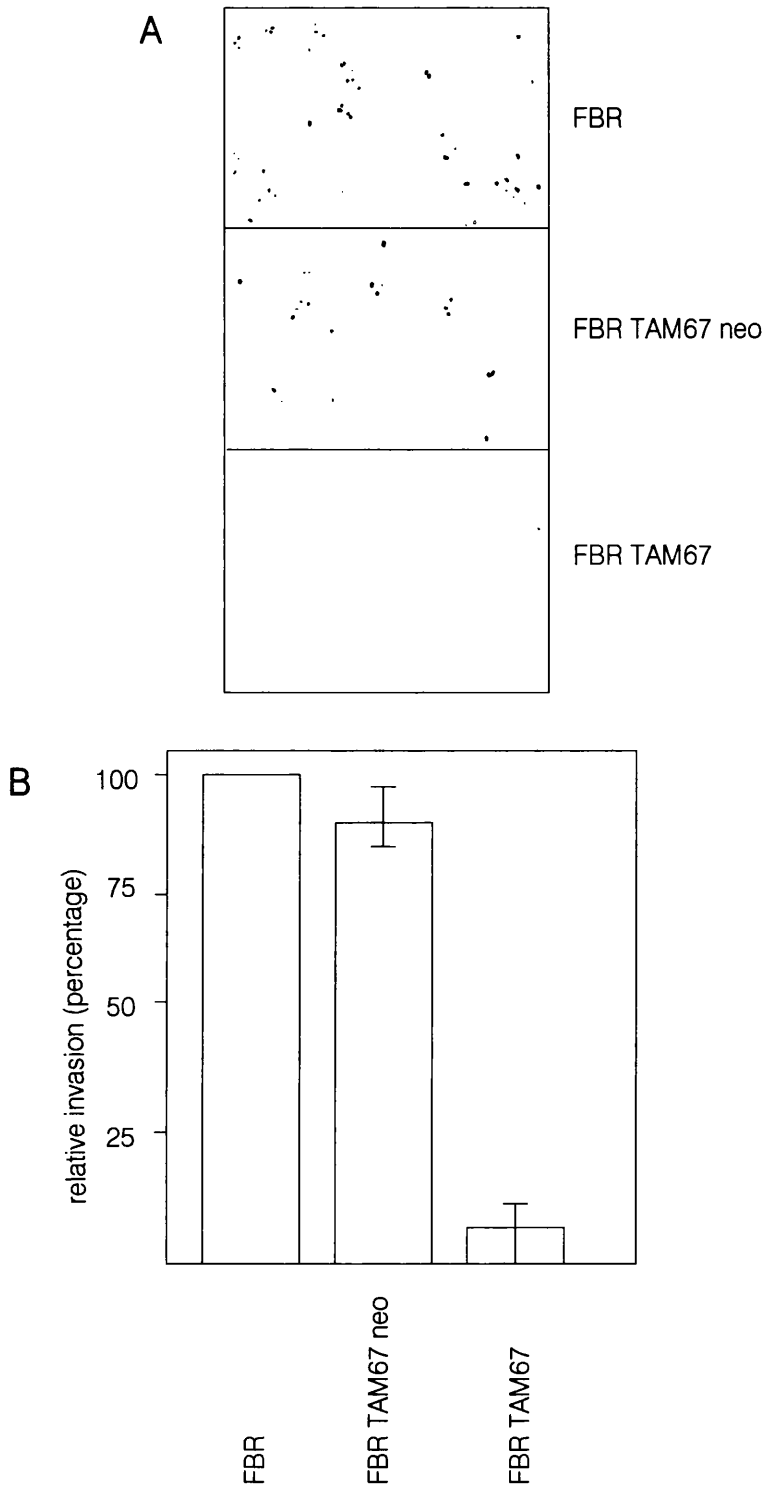


Figure 4.5. *in vitro* invasion assay of FBR and FBR TAM-67.

A) Migration of FBR , FBRneo and FBR TAM-67 above the filter in an *in vitro* invasion assay.
B) Relative invasion of FBR , FBR neo and FBR TAM-67 in an *in vitro* invasion assay.
Cells were allowed to attach to the underside of a transwell (Costar) and then chemoattracted across the 8 µm-pore-size filter. Cells were fixed, stained with propidium iodide and the nuclei visualised using a Bio-Rad MRC 600 Confocal illumination unit attached to a Nikon Diaphot inverted microscope.
Images of labelled cell nuclei were processed as tagged-image file format (TIFF) images, and pixels quantitated using a computer program described (Hennigan et al., 1994)
(This image is a representative of three experiments and was provided by Dr Richard Lamb and Prof. Brad Ozanne).)

4.5. FBR TAM-67 revertants are resistant to agonist-induced stress fiber formation

One of the major changes observed in cells during *ras* transformation is a decrease in polymerised actin associated with stress fibres and the corresponding increase in new cytoskeletal structures such as membrane ruffles (Bar-Sagi and Feramisco, 1986). These alterations are mainly ascribed to activation of small GTPases of the *ras* superfamily such as *rho,, rac* and *cdc*42 (Qiu *et al.,*1997).

Small GTPases do not only control the formation and distribution of actin in transformed but also in normal cells (Ridley and Hall, 1992). Adherent, serum-deprived Swiss 3T3 fibroblasts have a reduced actin content as shown by staining with Rhodamine-labelled Phalloidin (Ridley and Hall, 1992; Ridley *et al.,*1992). Microinjection of the small GTPase Rho or incubation with serum or Lysophosphatidic acid (LPA) induces a rapid re-formation of actin stress fibres in quiescent Swiss 3T3 cells. Membrane ruffling activity is specifically induced by other agonists such TPA, di-butyril-cAMP or by microinjection of the small GTPase Rac (Ridley and Hall, 1992, 1994).

Revertant cell lines have a flat phenotype mainly due to alterations in cytoskeletal components (Weber *et al.,*1974; Pollack *et al.,*1975). To identify if actin is involved in the flat phenotype of the FBR TAM-67 revertant cells, and to compare the actin content of the revertants with the parental FBR the cells were stained with Rhodamine-Phalloidin to visualise actin. The data were analysed using a Bio-Rad MRC Laser Confocal Illumination unit attached to Nikon Diaphot inverted microscope. This possibly would reveal a correlation between oncogenic transformation and the appearance of actin, and serve as an marker of reversion.

FBR cells growing in DMEM supplemented with 10% FCS were stained with Rhodamine-Phalloidin and processed for analysis with the confocal microscope. FBR cells cultured under these conditions have increased membrane ruffling at the leading end of the cells and the actin network is essentially lost except a very small number of stress fibres (Figure 4.6 a). In contrast 208F cells have multiple actin stress fibres with no evidence for membrane ruffling activity (Figure 4.6, b). The FBR v-*fos* TAM-67 revertants have a flat morphology quite similar to 208F cells with actin fibres present even when are cells are serum-deprived (Figure 4.9 a). This shows that FBR TAM-67 cells have a different cytoskeletal organisation compared to the FBR cells and defined actin stress fibres at quiescence. Our results are similar with the results of Weber and co-workers who showed that revertants from DNA-tumour virus

transformed cells have a flat morphology and contain stress fibres, although fewer compared to those found in normal fibroblasts (Weber *et al.*,1974; Pollack *et al.*,1975).

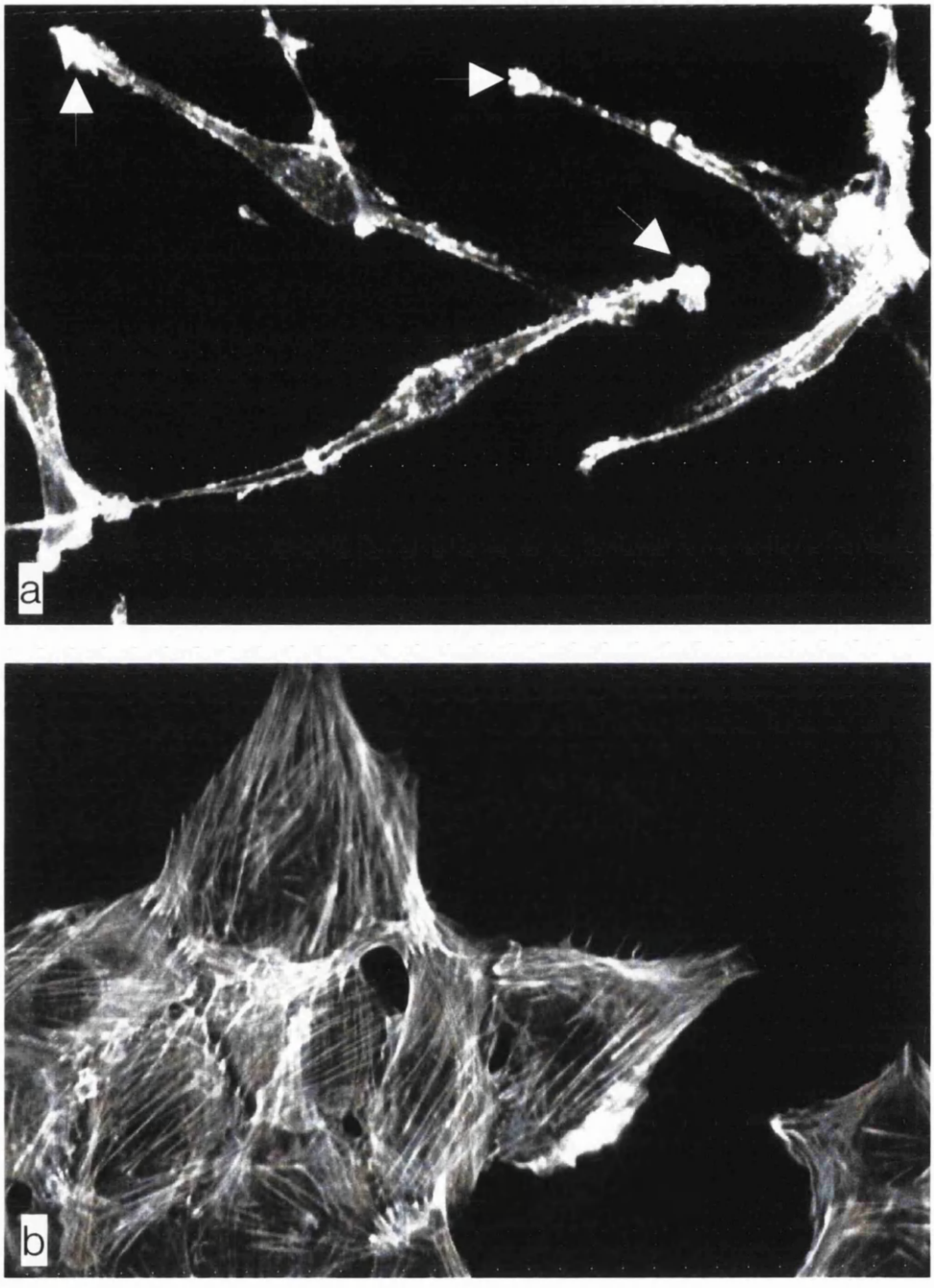


Figure 4.6. Actin distribution in FBR and 208F cells actively growing in DMEM supplemented with 10% FCS.

Cells were plated on coverslips, and grown in DMEM supplemented with 10% FCS. After 24 hours they were fixed, permeabilized and stained with TRITC-conjugated Phalloidin to visualise actin stress fibres.
a) FBR cells. The arrows point at the cell pseudopods were actin is concentrated locally.
b) 208F cells.

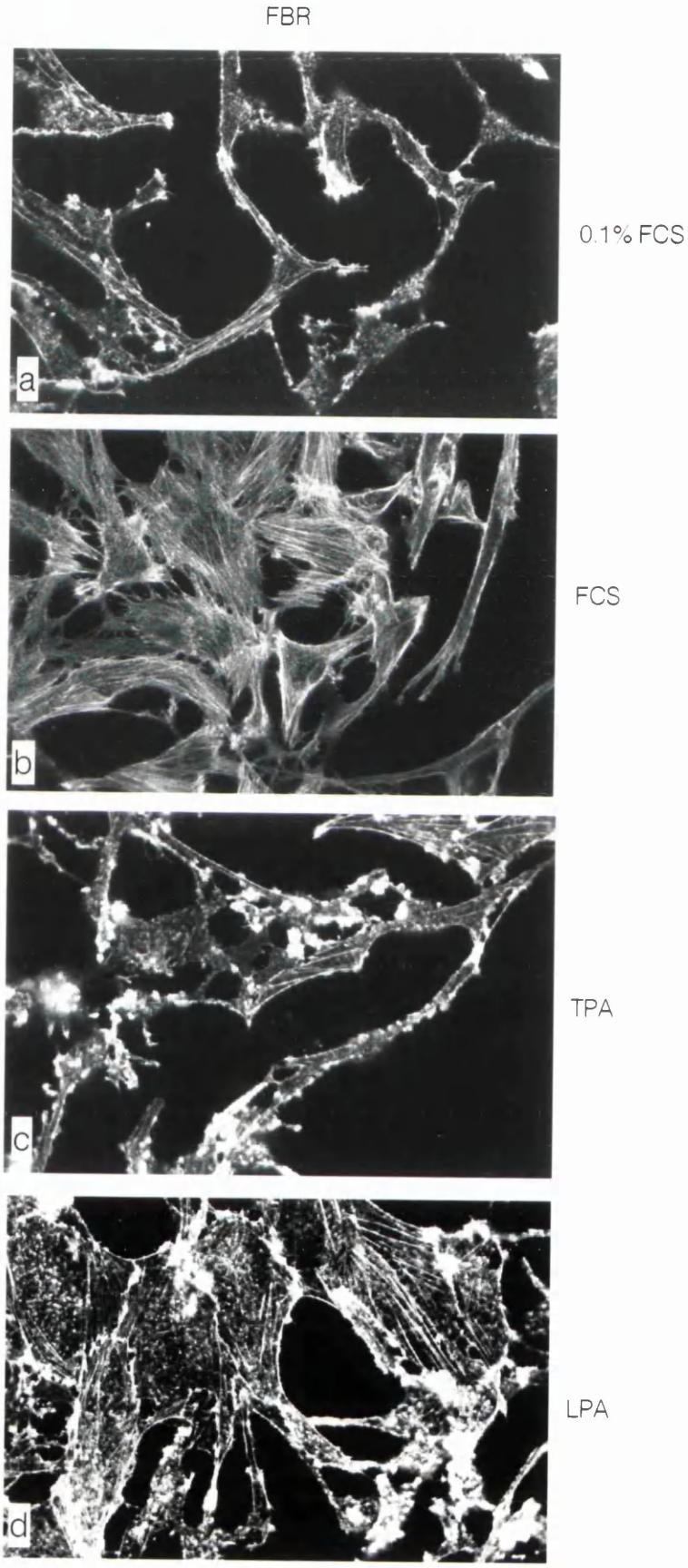
One question that appear interesting to ask after the landmark studies of Ridley and Hall (Ridley and Hall, 1992; Ridley *et al.,*1992) was whether stimulation of serum-deprived transformed fibroblasts with mitogens would have a direct effect on the reorganisation and distribution of actin. To our knowledge it has not been reported whether treatment with mitogens such as FCS induces actin stress fibres in serum-deprived oncogenically transformed cells.

When serum-deprived FBR cells stimulated with 10% FCS for 5 minutes they flatten out and the formation of peripheral actin and actin stress fibres becomes apparent (Figure 4.7 b). FBR cells also respond to other agonists by increased ruffling activity localised in the cell periphery in the case of treatment with 40 ng/ml phorbol ester TPA (Figure 4.7 c). Induction of quiescent FBR cells with 20 ng/ml LPA (Lysophosphatidic acid) results in the formation of a small number of stress fibres and membrane ruffling activity as shown in the Figure 4.7d. However it should be noted that the response of the FBR cells to LPA is clearly different from the response found in the 208F cells were the induction of abundant stress fibre formation is clear after stimulation with LPA.

Figure 4.7. Actin reorganization in FBR by serum, TPA and LPA.

Cell lines were plated on coverslips and grown in DMEM supplemented with 10% FCS and after 24 hours were serum deprived for 7 days. After this period of time, cells were stimulated for 5 minutes with 10% FCS, 40ng/ml TPA, 20 ng/ml LPA or remain untreated. To visualise actin stress fibres cells were fixed, permeabilized and stained with TRITC-conjugated Phalloidin. Images were analysed and processed using a Bio-Rad MRC 600 confocal microscope.
this experiment is a representative of four.

Figure 4.7

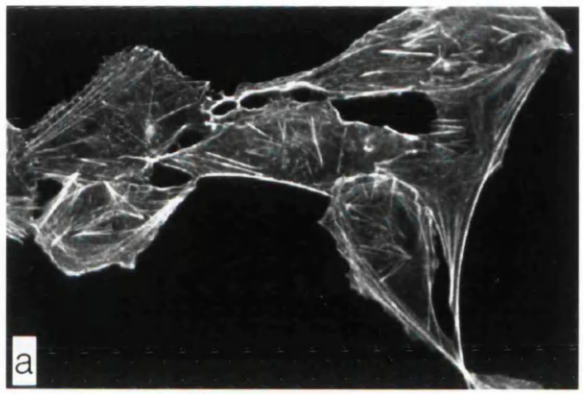


208F cells responded also to treatment with FCS, TPA and LPA. Serum-deprived 208F cells stimulated with 10% FCS show an increased number of stress fibres, that appear mainly at the cell periphery (Figure 4.8b). TPA induces effectively membrane ruffling activity in quiescent 208F cells (Figure 4.8c). Incubation of quiescent 208F cells with 20 ng/ml LPA acid resulted in the induction of stress fibres and also in cortical actin formation (Figure 4.8 d) which is similar to what observed in Swiss 3T3 cells (Ridley and Hall, 1992).

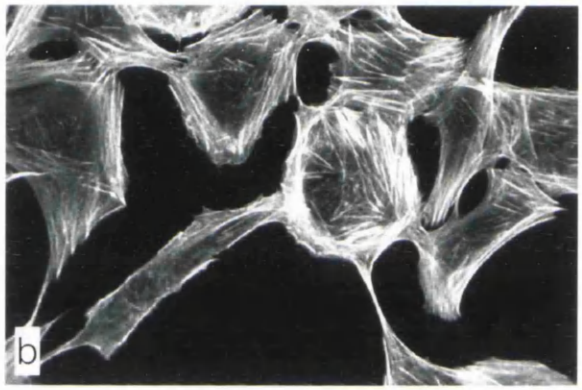
Figure 4.8. Actin reorganization in 208F by serum, TPA and LPA.

Cell lines were plated on coverslips and grown in DMEM supplemented with 10% FCS and after 24 hours were serum deprived for 7 days. After this period of time, cells were stimulated for 5 minutes with 10% FCS, 40ng/ml TPA, 20 ng/ml LPA or remain untreated. To visualise actin stress fibres cells were fixed, permeabilized and stained with TRITC-conjugated Phalloidin. Images were analysed and processed using a Bio-Rad MRC 600 confocal microscope.
This experiment is a representative of three experiments.

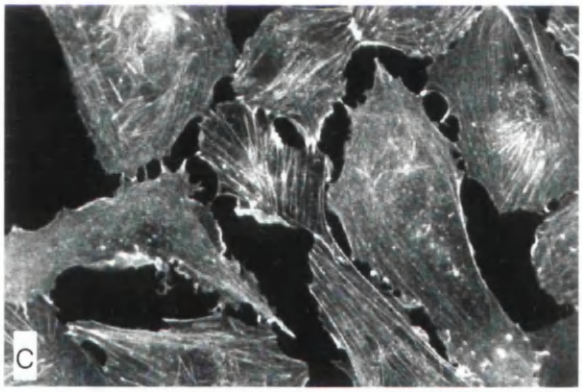
208F



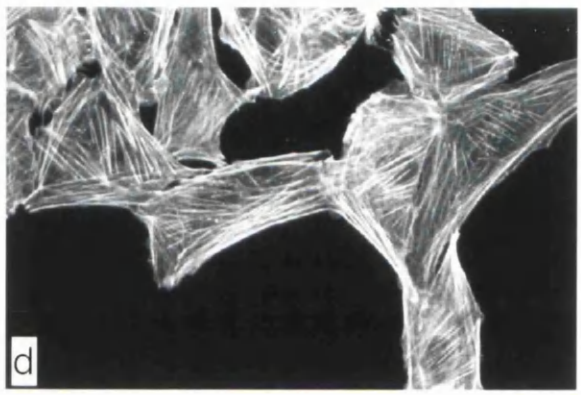
0.1% FCS



FCS



TPA



LPA

Figure 4.8

When quiescent FBR TAM-67 cells are induced with 10% FCS stress fibres form although less than those observed in serum-stimulated 208F cells. (Figure 4.9b). Treatment with 40 ng/ml TPA induces some ruffling activity which again is decreased when compared to the FBR cells following this treatment (Figure 4.9c compare to Figure 4.7c). Incubation of serum-deprived FBR TAM-67 cells with 20 ng /ml LPA induces the formation of a low number of stress fibres with this response also restricted when compared to FBR and 208F cells (Figure 4.8d).

These data show that TAM-67 inhibits to a significant extent the effect of serum-, TPA-, and LPA- in the stimulation of the stress fibre formation and membrane ruffling in FBR cells. Similar are the recently published observations by Malliri *et al.* (1998) regarding the role of TAM-67 in the actin reorganisation in the A431 epidermoid carcinoma cell line.

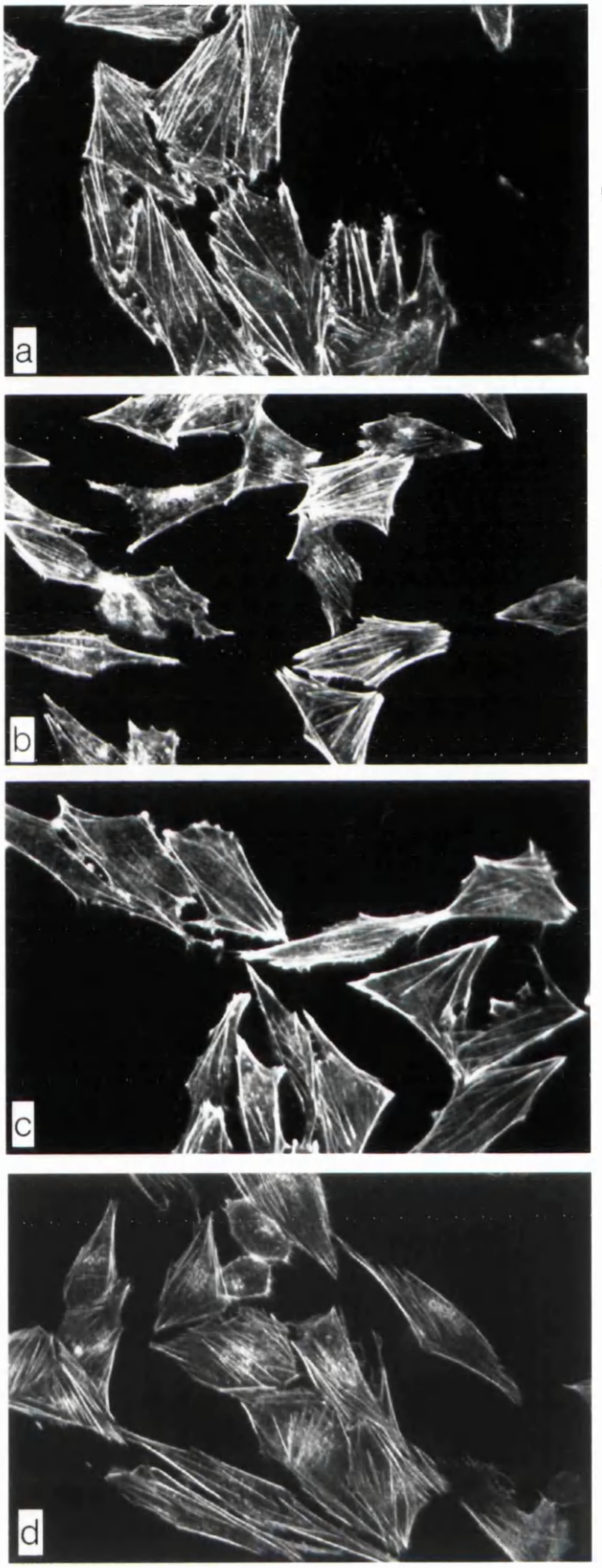
Membrane ruffling has been found to be downstream of the small GTPase *cdc*42 (Qiu *et al.,*1997). *cdc*42 is also associated with anchorage-independent cell growth in semi-solid medium. It remains to be seen if these changes in the actin distribution in FBR TAM-67 cells are directly attributed to the stimulation of *cdc*42, and if the introduction of TAM-67 in the FBR cells and the inhibition the transcription factor AP-1 has a role in the signalling pathways controlled by the *rho* family of small GTPases

Figure 4.9. Actin reorganisation in FBR TAM-67 revertants by serum, TPA and LPA.

Cell lines were plated on coverslips and grown in DMEM supplemented with 10% FCS and after 24 hours were serum deprived for 7 days. After this period of time, cells were stimulated for 5 minutes with 10% FCS, 40ng/ml TPA, 20 ng/ml LPA or remain untreated. To visualise actin stress fibres cells were fixed, permeabilized and stained with TRITC-conjugated Phalloidin. Images were analysed and processed using a Bio-Rad MRC 600 confocal microscope.
This experiment is a representative of three experiments.

FBR TAM-67

Figure 4.9



4.6. 208F TAM-67 transfectants are resistant to EGF-induced transformation

The AP-1 transcription factor and the c-*jun* protooncogene function downstream of the *ras* signal transduction pathway (Karin, 1995). AP-1 is regulated by mechanisms that involve the upstream network of Tyrosine Kinases (Devary *et al.*,1992) and small GTPases (Minden *et al.*,1995).

Members of the AP-1 transcription factor such as c-*jun,* play a role in the development of neoplasms. Disruption of the genomic integrity of the c-*jun* locus in ES cells results in less tumourigenic ES cell variants (Hilberg and Wagner, 1992) and fibroblasts lacking the c-*jun* gene have decreased proliferative capacity and reduced tumourigenic potential (Johnson *et al.*,1996)

The role of the dominant negative deletion mutant of c-*jun* TAM-67 has been well investigated in oncogene, growth factor and phorbol ester tumour promoter-induced transformation (Brown *et al.*,1993 -1994; Dong *et al.*,1994). Dong and co-workers have shown that EGF- and TPA-induced transformation of mouse epidermal cells could be suppressed by ectopic expression of TAM-67 (Dong *et al.*,1994).

It is well documented that high concentrations of serum (Peehl and Stanbridge, 1981; Gospodarowicz and Moran, 1974), transforming growth factors (de Larco and Todaro, 1978) and oncogenic viruses (Kaplan and Ozanne, 1983) are potent inducers of morphological transformation, anchorage independent growth, and dissolution of the stress fibre network (Ozanne *et al.*,1980). Such changes are mediated by a mechanism involving Receptor Tyrosine Kinases (RTK's). More analytically, adaptor proteins such as GRB2 (Matuoka *et al.*,1993) and the *ras*GTPase Activating Protein, *ras*GAP (McGlade *et al.*,1993) that bind to autophosphorylated RTK's mediate changes in the actin cytoskeleton implicating directly the growth factor receptors and their downstream effectors in EGF-induced transformation.

We hypothesised that if AP-1 and c-*jun* are downstream of an EGF-dependent signalling transforming mechanism then this mechanism could possibly be involved in regulating dissolution of the actin stress fibre network by transforming concentrations of EGF. This question was investigated in 208F cells and 208F TAM-67 transfectants by treating the cells with EGF and staining for Rhodamine-labelled Phalloidin to visualise actin cytoskeleton.

208F and 208F TAM-67 transfected cells were seeded in coverslips in DMEM supplemented with 10% FCS. 24 hours later in the plates 40ng/ml EGF was added and the cultures were incubated in 37 ^{0}C for an additional 24 hours. 208F cells growing in serum display a normal flat morphology with a significant number actin stress fibres extending throughout the

cell (Figure 4.10a). When 208F cells treated with 40ng/ml EGF for 24 hours were stained with Phalloidin then the number of stress fibres was dramatically reduced, the cells became bipolar, spindle-like with actin localised in membrane ruffles (Figure 4.10 c).

208F TAM-67 cells have a flat morphology and they contain stress fibres as shown by staining with Rhodamine-labelled Phalloidin (Figure 4.10 b). 208F TAM-67 cells treated for 24 hours with 40ng/ml of EGF did not show any evidence of morphological alterations in the actin cytoskeleton (Figure 4.10 d). This suggests that TAM-67 blocks downstream events of the EGF-induced signalling from the cell periphery to the nucleus through negative-regulation of AP-1transcriptionl activity.

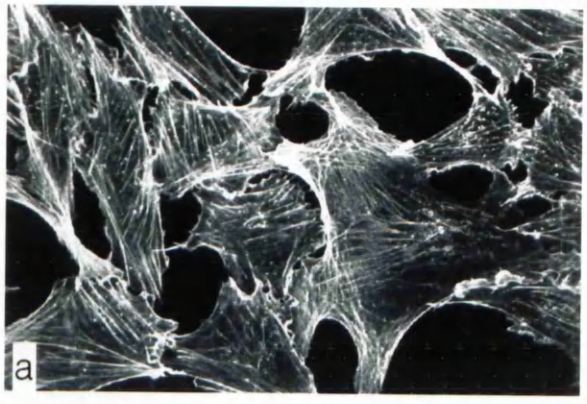
Figure 4.10. Inhibition of EGF-induced morphological transformation in 208F cells by TAM-67.

Cell lines were plated in DMEM supplemented with 10% FCS and after 24 hours they were incubated with 40 ng/ml of EGF for 24 hours or left untreated. To visualise actin stress fibres fixed, permeabilized and stained with TRITC-conjugated Phalloidin by indirect immunofluorescence microscopy.

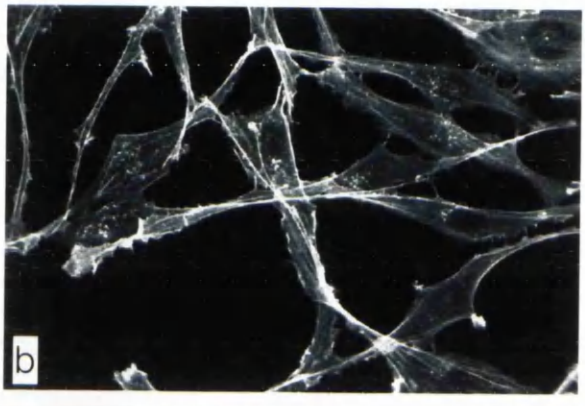
a) 208F control cells serum; **b)** 208F cells induced with EGF for 24 hours; **c)** 208F TAM-67 control; **d)** 208F TAM-67 cells induced with EGF for 24 hours

This experiment is a representative of three experiments.

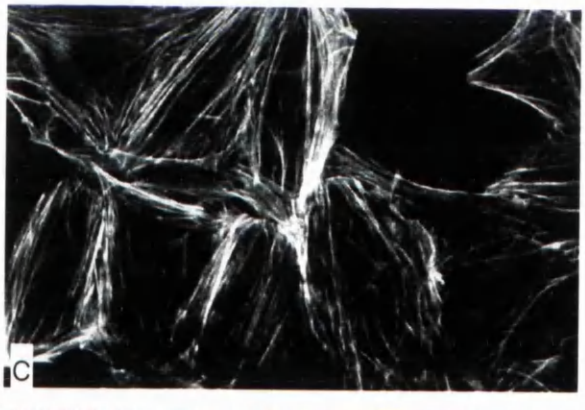
Figure 4.10



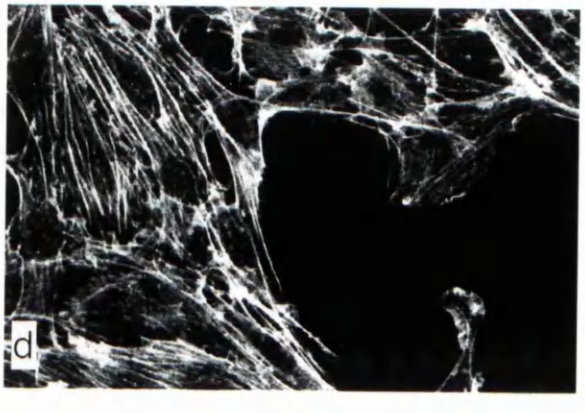
208F control



208F + EGF



208F TAM-67 control



208F TAM-67 + EGF

4.7. Conclusion

The c-*jun* protoncogene is a downstream effector of Receptor Tyrosine Kinase signalling pathways that lead to AP-1 transcription activation (Karin, 1995). It is also involved in transformation by the FBR v-*fos* oncogene (Neuberg *et al.*,1991; Lloyd *et al.*,1991; Hawker *et al.*,1993). This evidence make the c-*jun* protooncogene an interesting candidate in cellular transformation by upstream oncogenes.

The effect of the dominant negative mutant of c-*jun* TAM-67 in v-*fos*-induced transformation was tested. During this study several revertant cell lines were generated that might be useful to study transformation-relevant AP-1-mediated events during FBR v-*fos*-induced oncogenesis.

TAM-67 reversed *fos* transformation and the isolated revertant cell lines showed a stability through passaging, both in their morphological characteristics and the expression of TAM-67. The oncogene-suppressor activity of TAM-67 is associated with various parameters of transformation, such as anchorage independent growth in semi-solid media (Shin *et al.*,1975), actin stress fibre formation (Weber *et al.*,1974; Pollack *et al.*,1975) and AP-1 mediated transcriptional regulation of certain genes (Johnston *et al.*,1998, submitted for publication).

RESULTS CHAPTER 5

DOWNREGULATION OF MAPK SIGNALLING IN FBR CELLS

RESULTS

CHAPTER 5. DOWNREGULATION OF MAPK SIGNALLING IN FBR CELLS

5.1. Variations in the activation of the MAPK Pathway by oncogenes and growth factors

Extracellular signals activate signalling mechanisms through Receptor Tyrosine Kinases (RTK) and small GTP-binding proteins. This mode of activation results in the stimulation of cell proliferation and in other cases the induction of cell differentiation (Marshall, 1995).

One of the main pathways through which growth factors stimulate cell proliferation is the growth factor - *ras* - MAP Kinase pathway, which functions downstream of RTK's (Marshall, 1995). MAP Kinases were identified initially as insulin-stimulated kinases that phosphorylate microtubule-associated protein-2 and the translation-related Ribosomal S6 Kinase, (Ray and Sturgill, 1987; Sturgill *et al.*,1988). A number of MAP Kinase isoforms are known now, and the family of different genes that encode for homologous MAP Kinases includes enzymes such as the Extracellular Regulated Kinases ERK1 and ERK2 (Boulton *et al.*,1991), the Stress Activated Protein Kinases (SAPK) (Yan *et al.*,1994), and the inflammation-response related kinase p38 (Han *et al.*,1994).

Evidence form various laboratories provides a role for MAP Kinase signal transduction pathway in cellular transformation. Studies with the Raf-1 Serine/Threonine Kinase helped in identifying elements of this signal transduction pathway. The Raf-1 kinase is a target of activated GTP-bound $p21^{ras}$ and initially was identified as the product of the gene of the transforming retrovirus MC 3611 (Rapp *et al.*,1983). Raf-1 can activate MEK-1, a MAP Kinase Kinase which phosphorylates and activates MAP Kinases on Tyrosine and Threonine residues (Dent *et al.*,1992; Howe *et al.*,1992; Kyriakis *et al.*,1992).

MEK-1 also has a role in cellular transformation by the oncogenes acting upstream of this enzyme. Introduction of a kinase-inactive, dominant negative MEK-1 was shown to suppress v-*ras* and v-*src* transformation and to revert the transformed phenotype (Cowley *et al.*,1994; Mansour *et al.*,1994). Conversely a contitutively active MEK-1 was shown to activate MAP Kinase signalling (Seger *et al.*,1994), to bypass growth factor-dependent proliferation and to induce transformation of NIH 3T3 cells (Cowley *et al.*,1994; Mansour *et al.*,1994). The activated mutant of MEK-1 was also shown to induce differentiation of PC12 cells, expanding the

repertoire of biological roles of MEK-1 (Cowley *et al.,*1994). Collectively these data demonstrate that components of MAP Kinase pathway can be important modulators of cellular growth, mediating growth factor and oncogene stimulation of cell proliferation.

However in some cases there are variations from this paradigm, suggesting that oncogenic transformation is not necessarily associated with activation of MAP Kinase signalling. These findings emerge from studies with various oncogenes, mainly those located in the cytoplasm. In these studies oncogenes such as v-*raf* and activated *raf*-1 (Kizaka-Kondoh and Okayama, 1993; Samuels and McMahon, 1994), v-*src* (Stofega *et al.,*1997) and v-*crk* (Greulich *et al.,*1996) do not to induce activation of MAP Kinases as measured by a variety of methods.

5.2. Stimulation of MAPK signalling in FBR cells is not dependent on autocrine signals

The role of the *ras* oncogenes in cell growth and oncogenesis is well established (Barbacid, 1987). *ras* oncogenes can induce cell proliferation, through the production of growth factors in the culture media, resulting in an activation of an autocrine loop which activates signalling through Receptor Tyrosine Kinases. Autocrine activity secreted by cells transformed by the v-Ki-*ras* oncogene (Ozanne *et al.,*1980) or a constitutively active MEK-1 (Cowley *et al.,*1994) has been reported to enable them to proliferate in the absence of serum, and to grow in anchorage independent conditions (Kaplan and Ozanne, 1983 ; de Larco and Todaro 1978).

Cells transformed by the FBJ-MuSV do not produce any growth factors and display a moderate ability to grow in soft agar compared to FBR-MuSV transformed cells (Kaplan , 1983). It is already known from previous studies in this laboratory that FBR cells do not incorporate tritiated thymidine when cultured in low serum media (Hawker *et al.,*1993; Hennigan, 1993), suggesting that these cells do not secrete any autocrine activity sufficient to stimulate DNA synthesis (Hennigan, 1993).

If an autocrine activity similar to transforming growth factor-α (Ozanne et al., 1980; Kaplan and Ozanne, 1983) is secreted by the FBR cells, this should induce MAP Kinase activation. Using western immunoblotting the induction of MAP Kinases by conditioned media prepared from serum-deprived FBR cells was examined. In this assay phosphorylation of MAP kinases was examined using a phospho-specific antibody which recognises only the dually phosphorylated forms of ERK1 and ERK2 MAP Kinases. To prepare conditioned medium subconfluent FBR and 208F cell monolayers were rendered quiescent by serum deprivation for

48 hours and after this period of time the medium was collected and filtered through a 0.2 μ filter. The conditioned media were added directly to quiescent FBR and 208F serum-deprived cultures. The cells were washed with PBS; Triton X-100-containing lysis buffer was added and the cells were scraped and lysates clarified by centrifugation. The protein content was measured and 50 μg of total cell lysate was separated by 10% SDS-Polyacrylamide gel electrophoresis, transferred to a PVDF membrane and blotted with the MAP Kinase phosphospecific antibody. As a control for identifying MAP Kinase activation, lysates prepared from serum-stimulated FBR and 208F cells were also included in these experiments.

The activation of MAP Kinase isoforms, ERK1 and ERK2 is indicated by the intensity of the bands in the autoradiogram (Figure 5.1). These bands represent phosphorylated ERK1 and ERK2 (Figure 5.1). In these experiments no phosphorylation of ERK1 and ERK2 from either FBR or 208F cells treated with conditioned media was found (Figure 5.1 A, lane 3 and 4 ; Figure 5.1 B, lane 3 and 4). Stimulation with serum induces a 2-fold increase in MAPK phosphorylation in FBR cells and 10-fold in 208F cells (Figure 5.1 A2 and C2)

To ensure that equivalent amounts of protein were loaded to each sample the membrane was stripped and probed with an antibody that is specific for the C-terminus of ERK2.(Figure 5.1B and D, lanes 1, 2, 3, and 4)

This experiment shows that activation of MAP Kinases as shown by the phosphoreactive bands is induced by serum in FBR and 208F cells. In contrast to this finding, no induction of phosphorylation of MAP Kinases using conditioned media derived from serum-deprived FBR and 208F cells was detected. The inability of the conditioned media to activate MAP Kinase phosphorylation in FBR cells suggests that MAP Kinase signalling in this cell type is dependent upon mitogenic stimulation with serum, and that FBR cells do not secrete any autocrine activity as measured by this method. This result further investigation of activation mechanisms of MAP Kinase phosphorylation in FBR compared to normal 208F cells.

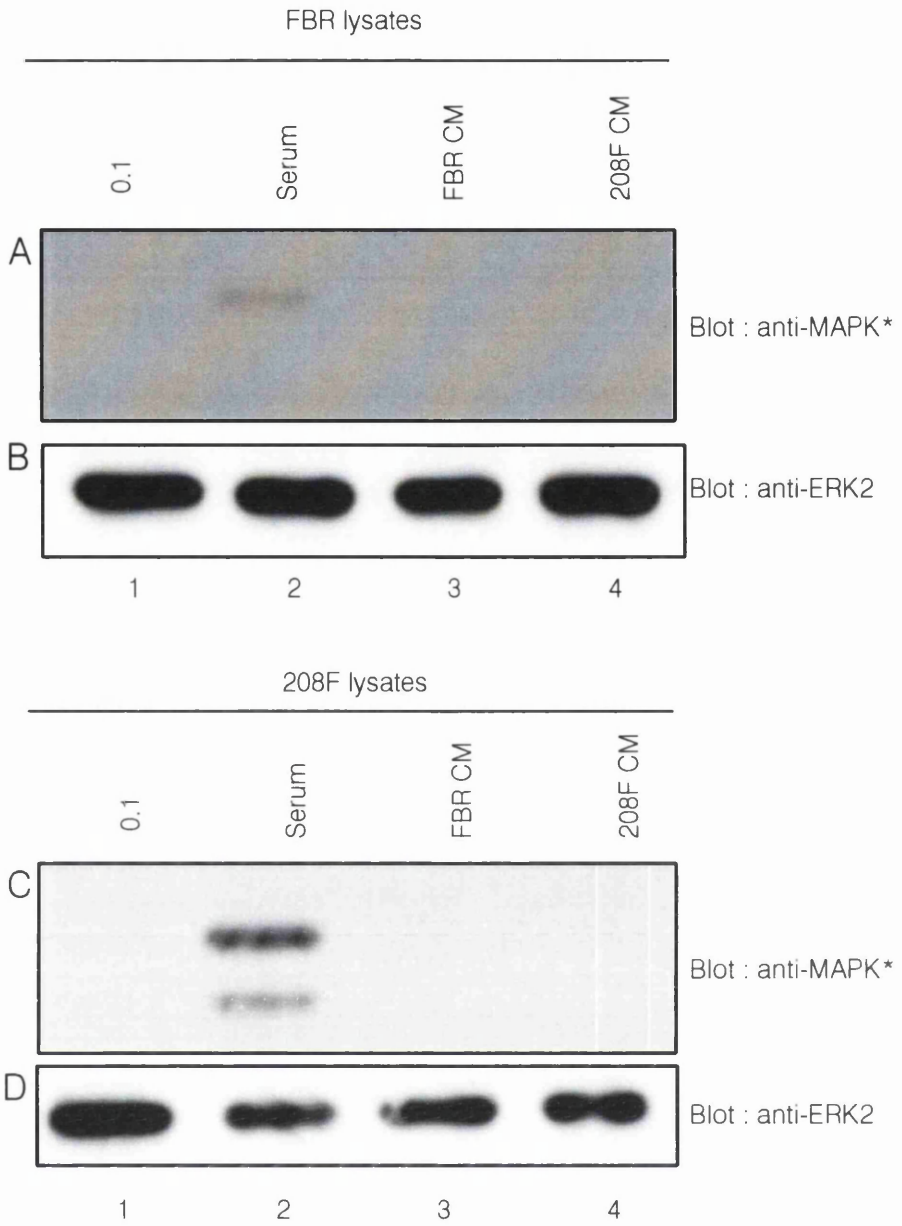


Figure 5.1 . Phosphorylation of MAP Kinases in FBR and 208F cells by serum and not by FBR- and 208F-derived conditioned medium.

Cells were grown in DMEM supplemented with 10% FCS followed by serum withdrawal for 48 hours (0.1% FCS). Stimulation was for 5 minutes with 10% FCS or with 10 ml of conditioned medium prepared from FBR and 208F cells. Cell lysates were prepared and proteins were separated by 10% SDS-PAGE, transferred to PVDF membranes and probed with an antibody specific for the phosphorylated ERK1 and ERK2 (MAPK*, Promega Corp., WI, USA). Blots were stripped and probed with an antibody specific for ERK2 as a loading control (ERK2, Transduction Labs, KE, USA).

A) 1) FBR serum-deprived cells; 2) FBR serum-stimulated cells; 3) FBR cells stimulated with FBR derived conditioned medium; 4) FBR cells stimulated with 208F derived conditioned medium.**B)** 1) 208F serum-deprived cells; 2) 208F serum-stimulated cells; 3) 208F cells stimulated with FBR derived conditioned medium; 4) 208F cells stimulated with 208F derived conditioned medium.

This experiment is a representative of two with identical results

5.3. Attenuated serum-induced MAPK signalling in FBR cells

To monitor activation of MAP Kinases in FBR and 208F cells by serum an immunocomplex-kinase assay. This assay is based on the isolation of ERK1 MAP Kinase from cultured cells by immunoprecipitation, followed by incubation with substrate Myelin Basic Protein and [γ-^{32}P] ATP as the source of phosphate. In this assay MAP Kinase activity is determined by autoradiography to detect phosphate incorporation into the substrate.

Cell lysates were prepared from serum-deprived and serum-stimulated FBR or 208F cells by using the ERK lysis buffer. 1000 μg of total cell lysate was incubated with a specific antibody for ERK1, or with pre-immune serum. The samples were incubated overnight and the following day the immunoprecipitates were washed and subjected to the *in vitro* kinase assay. The products of the kinase reaction were separated by 10% SDS-Polyacrylamide gel electrophoresis, transferred to PVDF membranes and exposed with intensifying screens to generate an autoradiograph. To verify the efficiency of the immunoprecipitation the membrane was subjected to western immunoblotting with an antibody to ERK1 to determine that equal levels of enzyme were immunoprecipitated (Figure 5.2 B).

This assay showed that phosphorylation of MBP was induced to a higher level in the immunoprecipitated samples from serum-induced 208F cells compared to the FBR serum-induced cells. (Figure 5.2 A lane 8 ; compare to lane 4 from serum-stimulated FBR fibroblasts). These data together with the results using the MAP Kinase phospho-specific antibody presented in Figure 5.1, show that there is a reduction in MAP Kinase activity in serum-stimulated FBR cells compared to the parental 208F non-transformed cells.

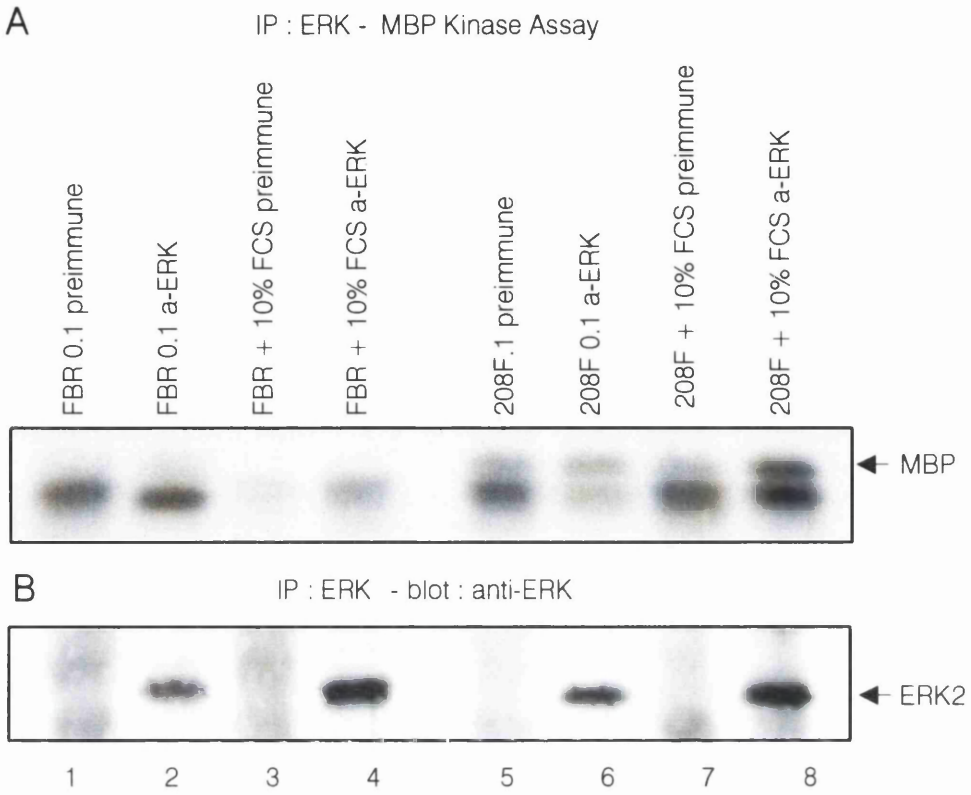


Figure 5.2. MAP kinase activity Assay.

Cells were plated in DMEM supplemented with 10 % FCS and when subconfluent serum-deprived for 48 hours (0.1% FCS). Stimulation was for 5 minutes with 10% FCS. Cell lysates were prepared and ERK1 activity was immunoprecipitated with an antibody against ERK1 (Transduction Labs, KY, USA). **A)** Kinase assay to determine ERK2 activity from cultured cells was performed with Myelin Basic Protein as a substrate in the presence of [γ-^{32}P]ATP. The products of the assay were resolved in 10% SDS-PAGE and transferred to a PVDF membrane. The membrane was exposed using intensifying screens and the autoradiograph shown was the result of a 45 minute exposure. **B)** The membrane was probed with an antibody specific for the ERK1/2 proteins (Transduction Labs) to verify immunoprecipitation of ERK2 activities.

1) Lysate from quiescent FBR immunoprecipitated with preimmune serum; **2)** lysate from quiescent FBR f immunoprecipitated with ERK-specific serum; **3)** lysate from stimulated FBR immunoprecipitated with preimmune serum; **4)** lysate from stimulated FBR immunoprecipitated with ERK-specific serum; **5)** lysate from quiescent 208F fibroblasts immunoprecipitated with preimmune serum; **6)** lysate from quiescent 208F fibroblasts immunoprecipitated with ERK-specific serum; **7)** lysate from stimulated 208F fibroblasts immunoprecipitated with preimmune serum; **8)** lysate from stimulated 208F fibroblasts immunoprecipitated with ERK-specific serum

5.4. Induction of MAP Kinases in FBR and 208F cells by Growth factors

To study phosphorylation of MAP Kinases by stimuli other than serum, growth factor induction of ERK1 and ERK2 activity was also investigated. This might yield insights into mitogenic signalling in these two distinct cells types and identify functional differences in the modes of MAP Kinase signalling and activation.

Serum deprived FBR and 208F cells were exposed for 5 minutes with 10% FCS, 100 ng/ml EGF, 20ng/ml PDGF, 50ng/ml Insulin, or 100ng/ml IGF-I. Lysates were prepared using Triton X-100-containing buffer and the protein content was normalised to 50 μg per sample. The proteins were separated by 10% SDS-PAGE, transferred to PVDF membranes and blotted with the MAP Kinase phosphospecific antibody. To demonstrate the specificity of the actions of these growth factors and the reproducibility of the results in Figure 5.3 two independent experiments are shown, where serum, EGF, PDGF, Insulin and IGF-I were used to induce MAP Kinase phosphorylation. The results from densitometric scanning of the western blots are summarised in the Table 5.1. In FBR cells serum-induced total ERK activity was 3-fold over the basal (0.16+/-0.049, n=3, Significant at P <0.05). EGF activated well ERK's (0.27+/-0.005, n=3, significant at P=0.0001). The response of FBR cells to Insulin and IGF-I was relatively low and not significant (0.03+/-0.03 for Insulin with P<0.6 and 0.08+/-0.02 for IGF-I with P<0.7). In this table the total ERK1 and ERK2 signal generated by western immunoblotting analysis is normalised for the ERK2 protein loading from the same autoradiographs.

Treatment of 208F cells with serum resulted in MAP Kinase activation approximately 10-fold over the basal levels (0.96+/-0.05, n=3 significant at P=0.006). EGF resulted in a similar mode and magnitude of activation in 208F cells when compared to serum (0.9+/-0.1, n=3, significant at P=0.0034). The response of 208F cells to Insulin was significant but very low ($0.06+/-9^{-10}$, n=3, P=0.001) and this to IGF-I low but not significant ((0.1+/-0.04, n=1, P=0.3).

Other reports have shown activation of MAP Kinases by Insulin in fibroblasts over-expressing the Insulin Receptor, using high concentrations up to 1000 μg/ml. After careful analysis of the literature, these concentrations are considered high for non-adipose like cells. The addition in high concentrations of growth factors to cultured fibroblasts could induce non-specific effects and activate multiple pathways. Alessi *et al.* (1995) also have shown that the magnitude of MEK-1 and MAP Kinase activation in tissue culture cells is dependent on the concentration of added growth factors. Here, growth factors for stimulation experiments were

administered at concentrations that by many groups have been shown to result in a specific response towards the activation of the MAP Kinase pathway.

The fact that in the case of induction with PDGF a similar response was observed in the FBR and 208F cells suggests a conserved mechanism for this particular growth factor and could be used as an internal standard. In the case of FBR cells the response to PDGF was approximately 5-fold over the basal (0.327+/-0.011, n=3, P=0.0007) and in the case of 208F cells approximately 3-fold over the basal (0.33+/-0.033, n=3, P=0.0034). The fact that the PDGF activation ERK1 and ERK2 is relatively conserved between transformed FBR and non-transformed 208F cells suggests that there might be a signal transduction pathway which somehow is not affected by the process of transformation by the FBR *fos* oncogene. Future more detailed studies could address the levels, the activity and the role of effectors of the PDGF mitogenic signal such as the PLC and Phoshoinositide 3-Kinase (Valius and Kazlauskas, 1993) in the FBR and 208F cells.

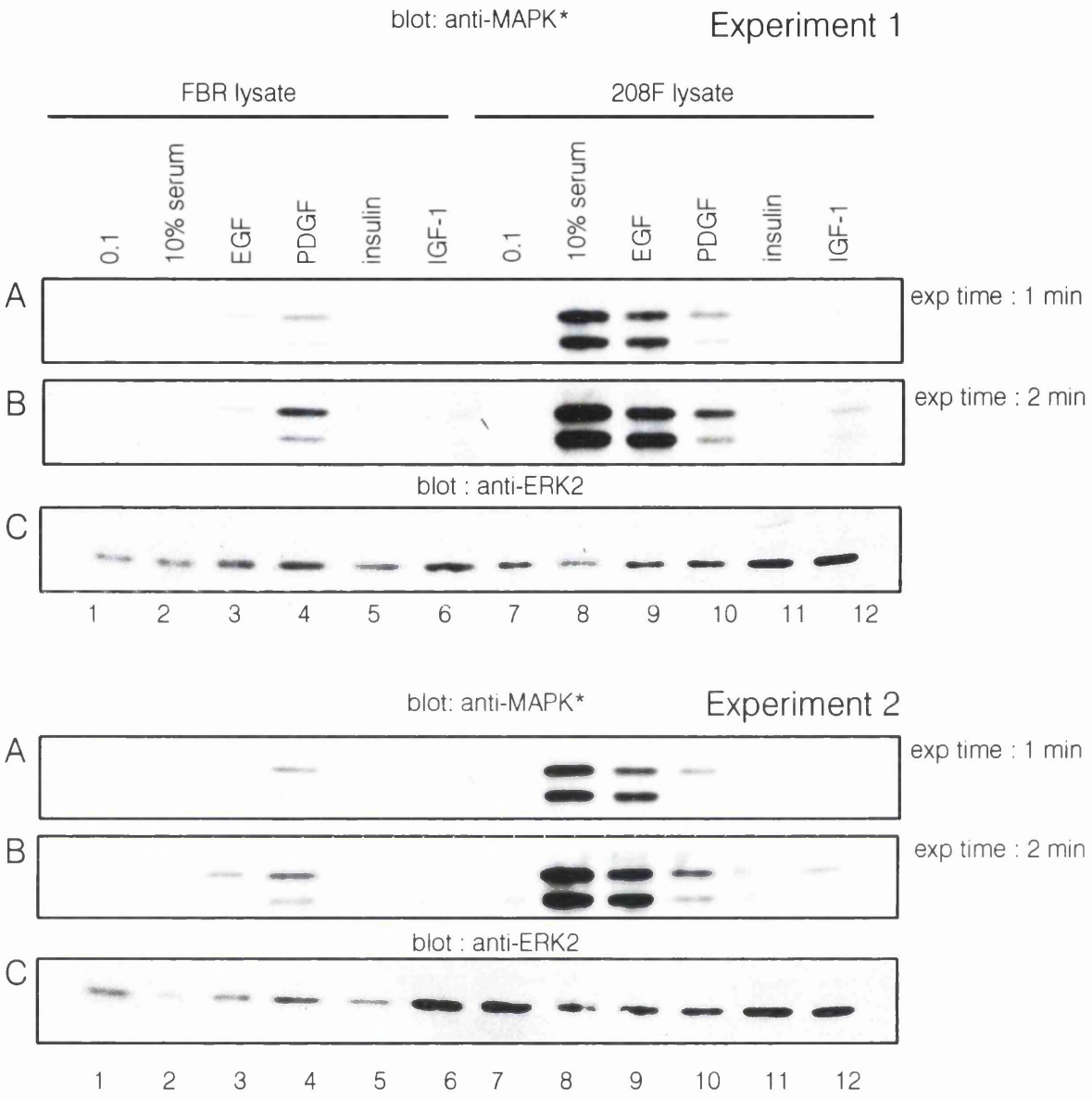
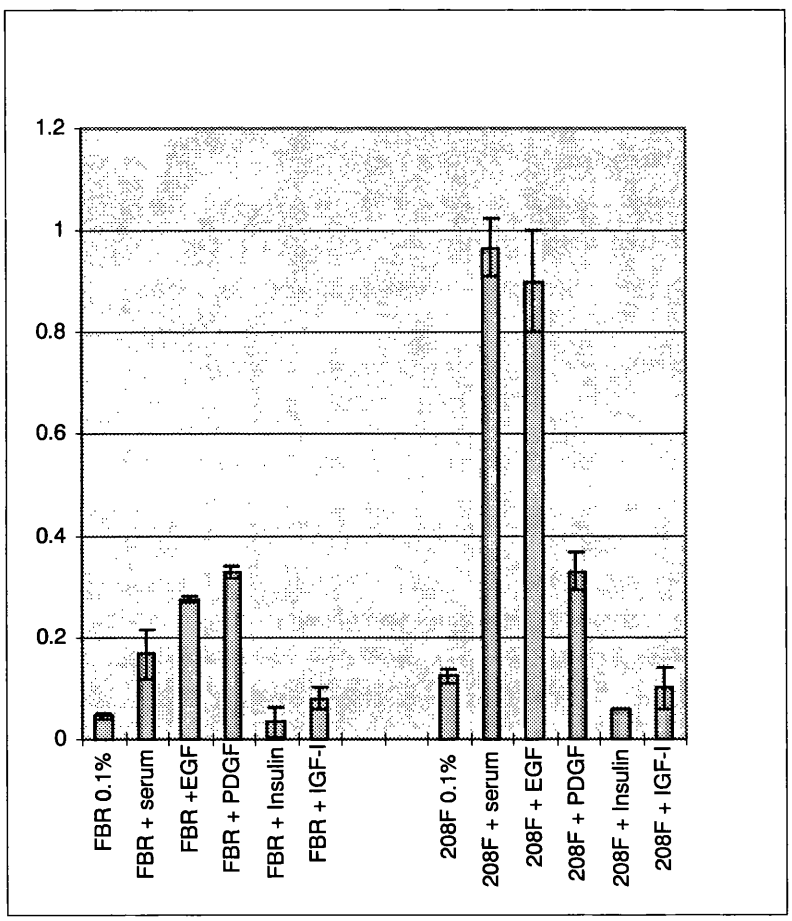


Figure 5.3. MAPK phosphorylation by serum in FBR and 208F cells by serum and purifed growth factors

Cells were grown in DMEM supplemented with 10% FCS and when subconfluent, serum-deprived for 48 hours (0.1% FCS) .Stimulation was for 5 minutes with 10% FCS, 100ng/ml EGF, 20ng/ml PDGF , 50ng/ml Insulin or 100ng/ml IGF-1.
Cell lysates were prepared and proteins were separated by 10% SDS-PAGE, transferred to PVDF membranes and probed **A)+B)** with an antibody specific for phosphorylated ERK1/2 (MAPK*, Promega labs, WI, USA).
Blots were stripped and reprobed with an antibody specific **C)** for ERK2 as a loading control (ERK2, Transduction labs)
1-6 : FBR cell lysates 1) serum deprived or stimulated with 2) 10% FCS, 3) EGF, 4) PDGF, 5), insulin, 6) IGF-1.
7-12 : 208F cells 7) serum deprived or stimulated with 8) 10% FCS, 9) EGF, 10) PDGF, 11), insulin, 12) IGF-1.
Two different exposure times of 1 and 2 minutes are shown.
These blots are representative of three different experiments.

Table 5.1. Activation of MAP Kinase by growth factors in FBR and 208F cells.

Quantification of the increase in phosphorylation of ERK1 and ERK2 was performed using scanning densitometry. The band of serum-stimulated 208F cells was taken as the maximal, 100% activation. The values were normalised for control loading of ERK2 for each individual experiment. The values are the mean +/- SD (standard deviation) of three independent experiments.



5.5. Induction of MAP Kinases in FBR and 208F cells by Lipid Singals

Apart from growth factors, phorbol esters such as TPA, a Protein Kinase C activator induces activation of ERK1 and ERK2 (de Vries Smits *et al.*,1992; Wood *et al.*,1992; Thomas *et al.*,1992). MAP Kinases also are activated in response to G protein-coupled Receptor activation. In this case the role of Lysophosphatidic Acid (LPA) is well documented (Van Corven *et al.*,1989). LPA can act as a mitogen for fibroblastic cells in culture by its ability to stimulate DNA synthesis via a pertussis toxin-sensitive pathway (Tokomura *et al.*,1994).

MAP Kinase phosphorylation after induction of quiescent FBR and 208F cells with TPA and LPA was examined. The results of these experiments are presented in Figure 5.4 and summarised in the table 5.2.

In the previous chapter, 20 ng/ml LPA were used to induce actin reorganisation in quiescent cells (Figure 4.7, 4.8, 4.9). Incubation of FBR cells with 20 ng/ml LPA did not enhance significantly phosphorylation of ERK1 and ERK2 MAP Kinases (0.07+/-0.06, n=3, P>0.05). Also stimulation with TPA of FBR cells resulted in a 2.8-fold increase of ERK phosphorylation, which was not significant (0.28+/-0.07, n=3, P>0.05). However a strong correlation between TPA stimulation and MAP Kinase phosphorylation was evident in 208F cells were a 3.2-fold activation over the basal levels appeared to be significant (0.46+/-0.05, n=3, P=0.01). It is apparent from the Figure 5.4 that in both FBR and 208F cells treatment with these low concentrations of LPA did lead to any significant activation of ERK1 and ERK2 phosphorylation. TPA was more potent in its role to activate MAP Kinase phosphorylation as shown in Figure 5.4 in the Experiments 1and 2, in lanes 10. Phorbol ester TPA is a potent activator of ERK's in normal tissue culture fibroblasts (de Vries Smits *et al.*,1992; L'Allemain *et al.*,1992; Thomas *et al.*,1992; Wood *et al.*,1992).

The results presented in here regarding the effect of LPA in FBR and 208F cells did not lead to any identification of phosphorylated MAP Kinases. It is known that low concentrations LPA do not activate very well MAP Kinases in tissue culture cells (Cook and McCormick, 1996). Since LPA did not induce activation of MAPK Kinases at low concentrations, LPA was used at the concentration of 1 μM which has been shown to stimulate non-mitogenic MAP Kinase signalling in rat-1 cells (Cook and McCormick. 1996).

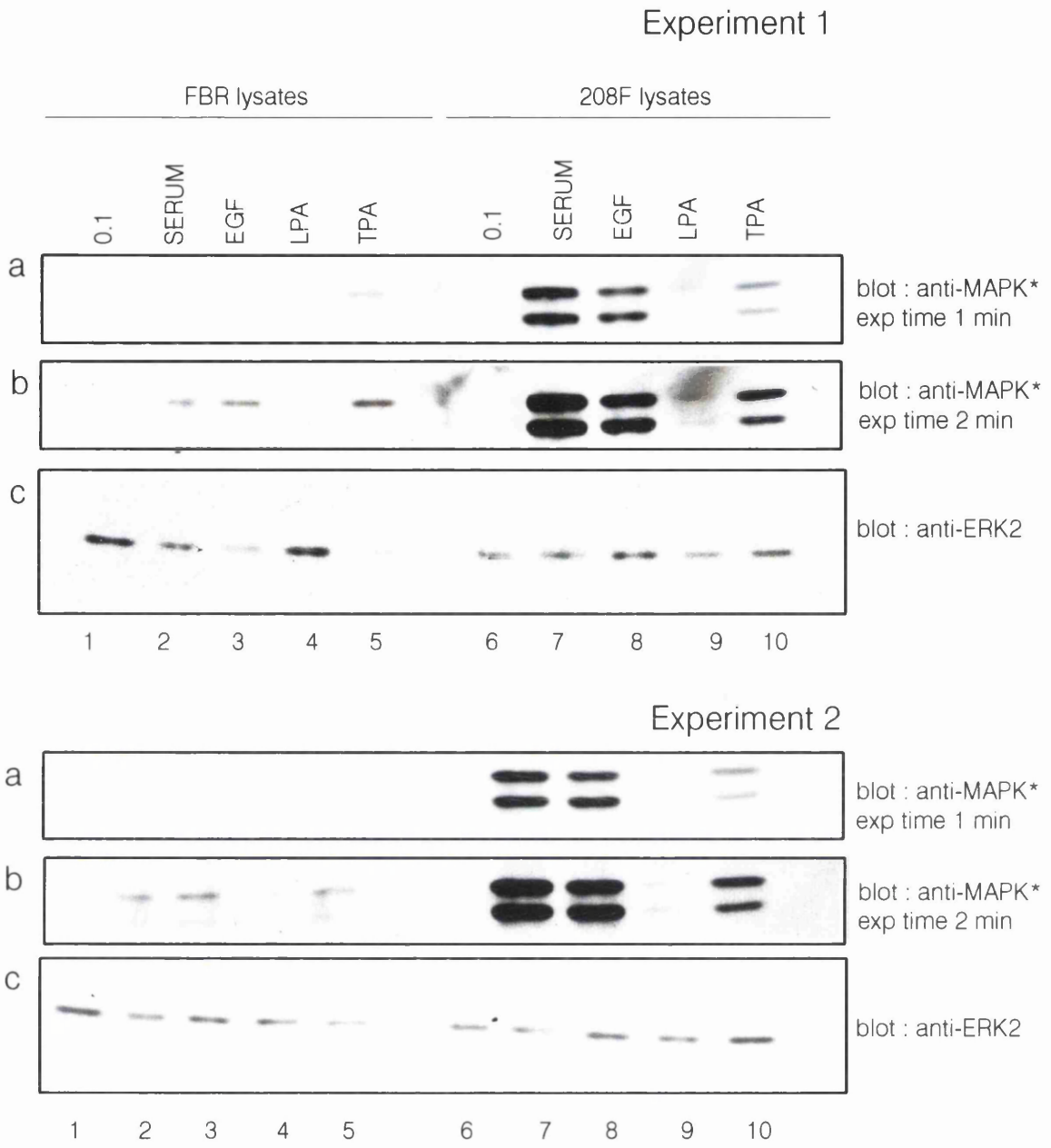
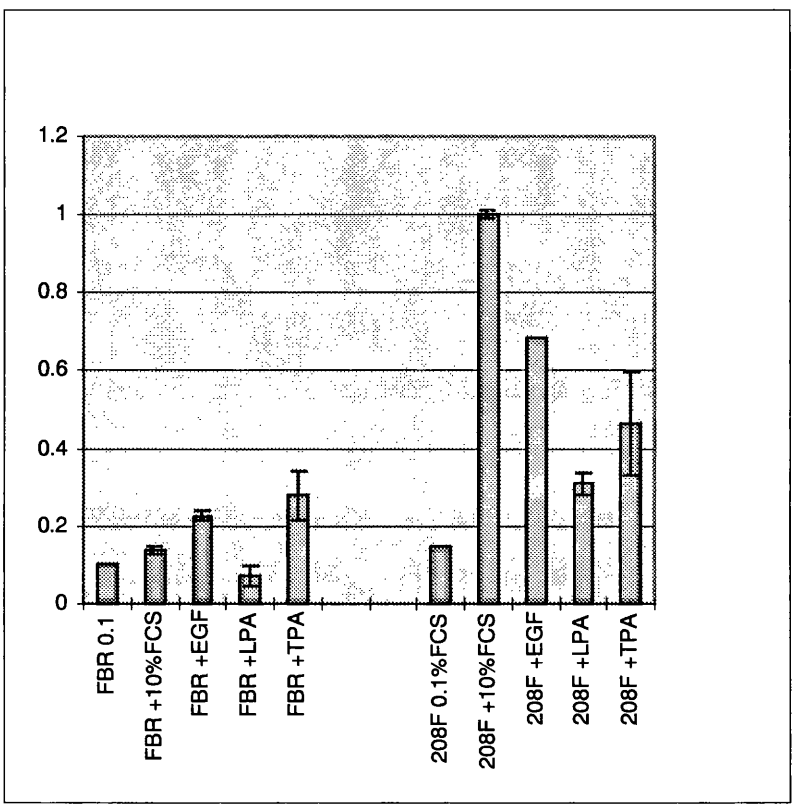


Figure 5.4. MAPK phosphorylation in FBR, 208F by serum, EGF and activators of PKC and G-coupled receptors.

Cells were grown in DMEM supplemented with 10% FCS and when subconfluent serum-deprived for 48 hours (0.1% FCS). Stimulation was for 5 minutes with 10% FCS, 100ng/ml EGF, 100ng/ml TPA and 20 ng/ml LPA or left untreated. Lysates were prepared and proteins were separated by 10% SDS-PAGE, transferred to PVDF membrane and probed with an antibody specific for the phosphorylated ERK1 and ERK2 (MAPK*, Promega Labs, WI, USA).
Blots were stripped and probed with an antibody specific for ERK2 as a loading control (ERK2, Transdcution Labs, KY, USA).
1-6 : FBR cell lysates 1) serum deprived or stimulated with 2) 10% FCS, 3) EGF, 4) LPA, 5), TPA.
7-12 : 208F cells 6) serum deprived or stimulated with 7) 10% FCS, 8) EGF, 9) LPA, 10), TPA.
Two different exposure times of 1 and 2 minutes are shown.
These blots are representative of three different experiments with similar results

Table 5.2. Activation of MAP Kinases by serum, EGF, LPA and TPA in FBR and 208F cells.

Quantification of the increase in phosphorylation of ERK1 and ERK2 was performed using scanning densitometry. The band of serum-stimulated 208F cells was taken as the maximal, 100% activation. The values were normalised for control loading of ERK2 for each individual experiment. The values are the mean +/- S.D. (standard deviation) of three independent experiments



5.6. Non-mitogenic doses of LPA induce MAP Kinases in 208F but not in FBR

A maximal response in MAP Kinase activation occurs with LPA concentrations of approximately 1 μM. At the higher concentration of 100 μM, LPA induces cell proliferation (Cook and McCormick, 1996). LPA levels in serum are between 1 and 5 μM (Eicholltz *et al.*,1993).

While the use of 20ng/ml of LPA in the preceding experiments had no effect on MAP Kinase activation (Figure 5.4) LPA at this concentration is sufficient to elicit biological responses such as re-formation of actin stress fibre in 208F cells or membrane ruffling in FBR cells (Figure 4.7 and Figure 4.8).

Basing further experiments on the results of Cook and McCormick (1996), the effect of 1 μM LPA was used to determine if this concentration is sufficient to induce MAP Kinase in 208F cells, a derivative of rat-1 cells and if similar signals can be elicited in FBR cells. Total cell lysates from FBR and 208F cells serum-deprived or stimulated with 10% FCS or 1 μM LPA for 5 minutes were prepared using the Triton X-100-containing lysis buffer. The proteins were separated by 10% SDS-PAGE, transferred to PVDF membranes and probed with the MAP Kinase phospho-specific antibody.

After treatment of 208F cells with 1 μM LPA, densitometric scanning of the autoradiogram showed that the total ERK signal is similar to the results obtained with serum. In the case of serum the result was significant (0.83+/-0.04, n=4, P=0.0001) and with LPA a similar response was evident (0.925+/-0.08, n=4, P=0.0003). This result is somewhat different from what others have observed, where a serum gives a higher response compared to the treatment with LPA. When ERK1 activation was measured by an immunoprecipitation kinase assay, 10% FCS gave a higher response than LPA (Cook and McCormick, 1996). The slight difference observed here could be due to the fact that in the present study examined MAP Kinase activation after 5 minutes of LPA, whereas Cook and McCormick (1996) treated cells for 10 minutes.

Consistently 1 μM LPA failed to induce MAPK phosphorylation in the FBR cells (0.0375+/-0.005, n=4) as shown in the two independent experiments as shown in the Figure 5.5. That was significant result with a P=0.003.For this section the results from 4 independent experiments are summarised in Table 5.3.

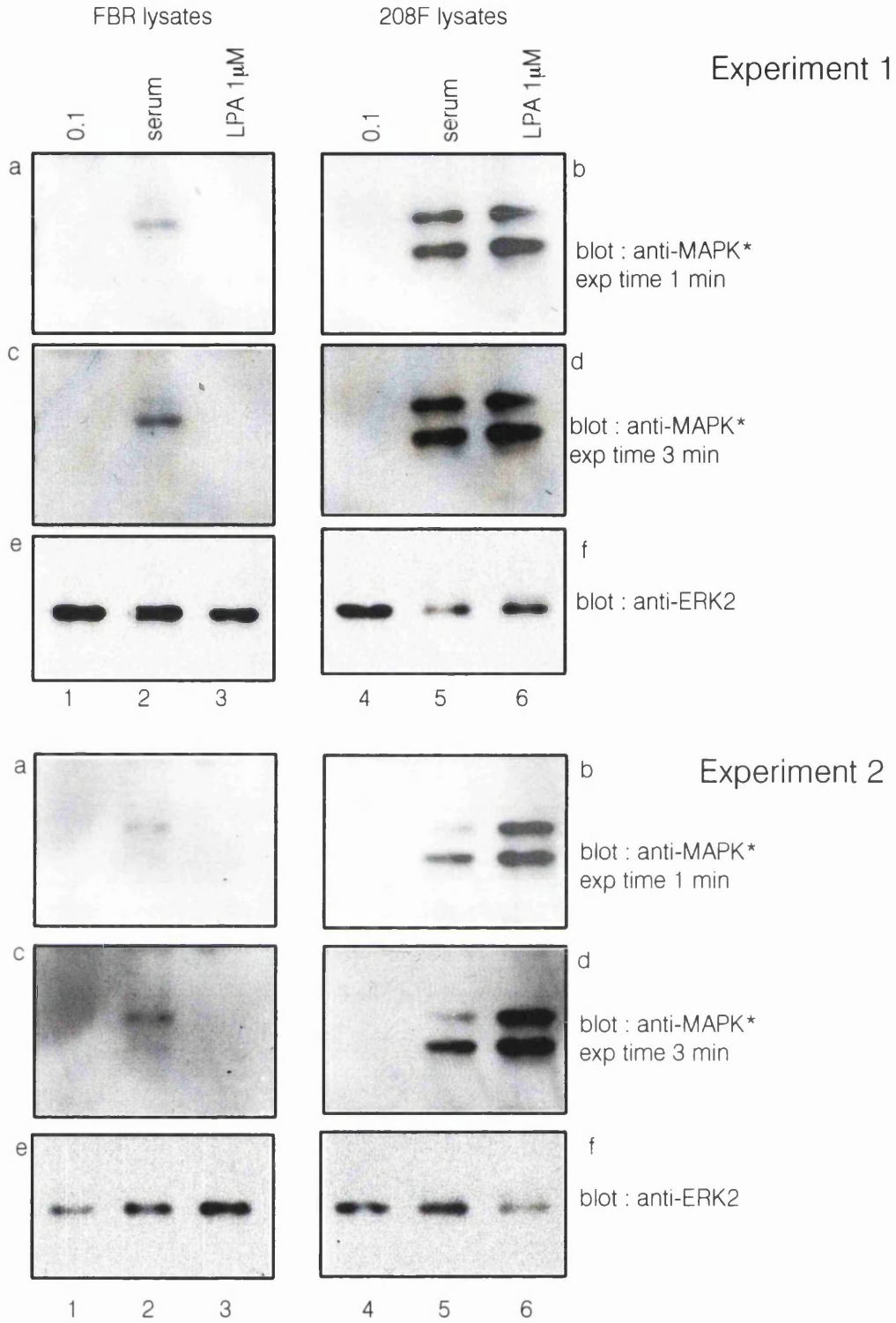
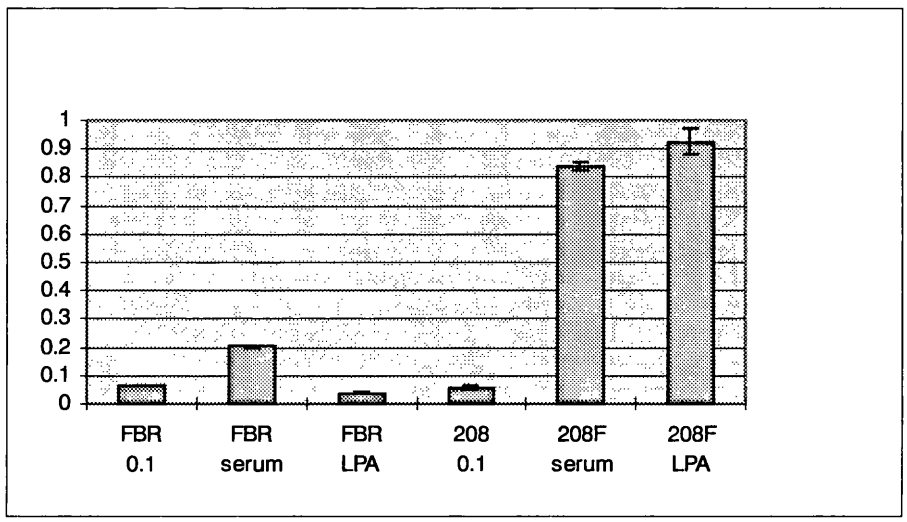


Figure 5.5. MAPK phosphorylation in FBR and 208F cells after induction with serum and 1 μM LPA.

Cell lines were grown in DMEM supplemented with 10% FCS and when subconfluent, serum-deprived for 48 hours (0.1% FCS). Stimulation was for 5 minutes with 10% FCS, 1μM LPA or remain untreated.
Cell lysates were prepared and proteins were separated by 10% SDS-PAGE, transferred to a PVDF membrane and probed with an antibody specific for
a), b), c) and d) phosphorylated MAPK* (Promega Labs , WI, USA).
e) and f) Blots were striped and probed with an antibody specific for ERK2 to verify equal loading (ERK2, Transduction Labs, KY, USA).
Two different exposures are shown for 1 and 3 minutes.
These blots are representative of four different experiments were similar results were obtained.

Table 5.3. Activation of MAP Kinases by serum and 1 μM LPA.

Quantification of the increase in phosphorylation of ERK1 and ERK2 was performed using scanning densitometry. The band of LPA-stimulated 208F cells was taken as the maximal, 100% activation. The values were normalised for control loading of ERK2 for each individual experiment. The values are the mean +/- standard deviation of four independent experiments.



5.6. Activation of MEK-1 in FBR and 208F cells

MEK-1 is the major activator of MAP Kinases in mammalian cells. Activation of MEK-1 in FBR and 208F cells is detected by western immunoblotting with antibodies that specifically recognise the major phosphorylation sites by Raf-1, Serines 217 and 221 (Alessi *et al.*, 1994).

From quiescent and serum induced FBR and 208F cells, lysates were prepared using the standard Triton X-100-containing lysis buffer. The lysates were clarified by centrifugation, the protein content was normalised and 300 μg of total cell lysate was separated by 10 % SDS-Polyacrylamide gel containing a low concentration of bis-Acrylamide (Marshall and Leevers, 1995). The reduction in the percentage of bis-Acrylamide has been found to be critical for the identification of phosphorylated protein iso-forms by western immunoblotting (Marshall and Leevers, 1995).

In the Figure 5.6 a representative experiment is shown where activation of MEK-1 by serum in FBR and 208F cells was investigated by western immunoblotting of total cell lysates with phospho-specific antibodies. By scanning densitometry the relative levels of phosphorylation MEK-1 were identified. Phosphorylation of MEK-1 was enhanced by 5-fold in serum-stimulated compared to serum-starved 208F cells. In the serum-stimulated FBR lysates, MEK-1 was activated 2-fold. Also the basal phosphorylation levels of MEK-1 in FBR and 208F were examined and found to be 4-fold higher in quiescent 208F cells compared to quiescent FBR cells (Figure 5.6 lanes 1 and 3).

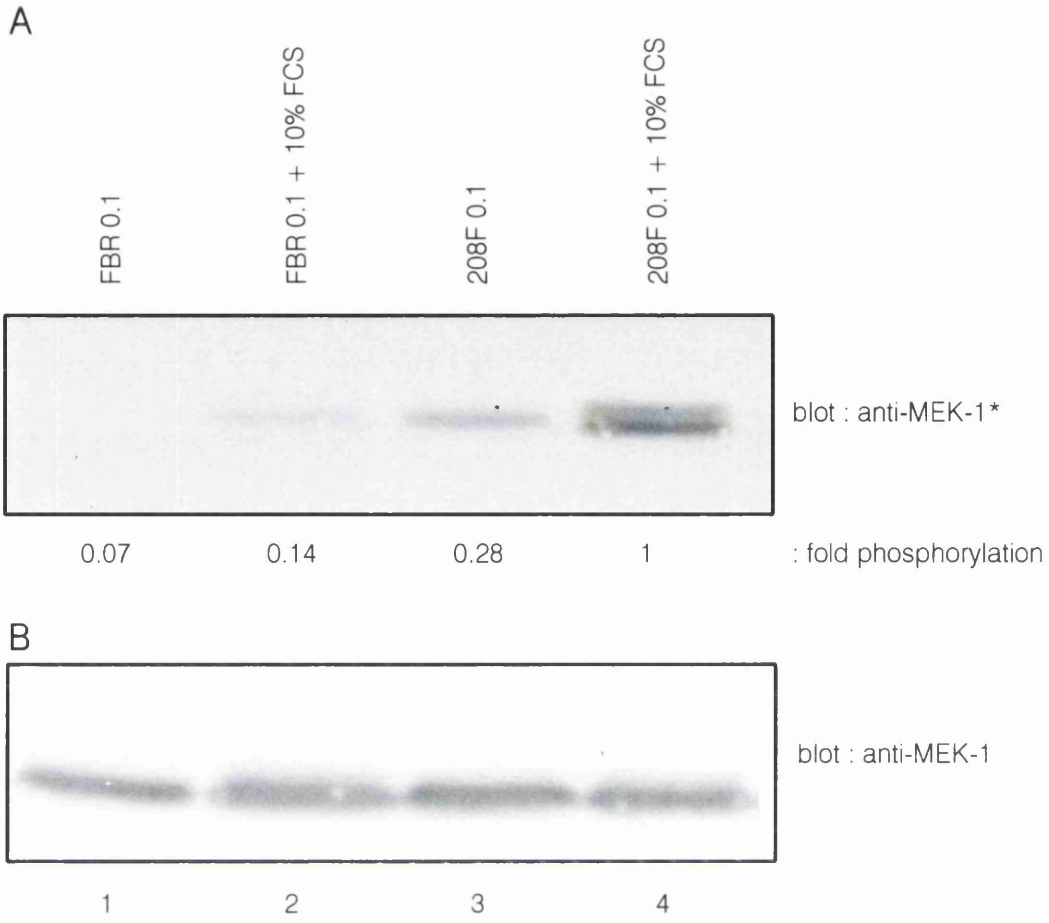


Figure 5.6. MEK-1 phosphorylation by serum in FBR and 208F cells.

Cells were grown to subconfluence in DMEM supplemented with 10% FCS and serum deprived for 48 hours (0.1% FCS). Stimulation was for 5 minutes with 10% FCS. Cell lysates were prepared and proteins were separated by 10% SDS-PAGE, transferred to a PVDF membrane and probed **A**) with an antibody specific for the phosphorylated MEK-1 (MEK-1*, New England Biolabs, MA, USA)
To confirm equal loading the blot was stripped and probed **B)** with an antibody specific for MEK-1 as a loading control (MEK-1, Santa Cruz Bio., CA, USA)
Using scanning densitometry the relative phosphorylation of MEK-1 in blot A was assessed. The protein content was normalised for the levels of MEK-1 using data presented in the blot 2. This experiment is a representative of two with similar results.

5.7. Conclusion

It is known that cells transformed by *raf, src* and *ras* oncogenes display an increase in MAP Kinase activity (Dent *et al.,*1992 ; Gallego *et al.* 1992 ; Leevers and Marshall, 1992). Also cellular transformation by v-*ras* and v-*src* requires MEK-1 (Cowley *et al.,*1994), and by v-*raf* requires MAP Kinases (Kortenjann *et al.* 1994). This evidence shows that MAP Kinase signalling is involved in transformation by oncogenes that function in upstream pathways from AP-1.

In this chapter evidence was presented which argued that MAP Kinase phosphorylation in FBR cells by various agonists is reduced compared to the parental 208F cells. This was an unexpected finding since in most cell lines transformed by viral oncogenes, MAPK signalling is enhanced and associated with transformation (Gallego *et al.* 1992; Troppmair *et al.,*1994) and proliferation (Kerkoff and Rapp, 1997 , 1998).

This suggests that FBR *fos* could transform cells without being dependent on any upstream component of the *ras* pathway such as Raf-1 and MEK-1 protein kinases. To investigate this further we have transfected dominant negative mutants of Raf-1 and MEK-1 in FBR cells and generated stable cell lines. The results of these transfections are presented in the two following chapters of results.

RESULTS CHAPTER 6

GENERATION OF FBR REVERTANT CELL LINES FOLLOWING TRANSFECTION OF THE DOMINANT NEGATIVE MUTANT OF MEK-1, A221

RESULTS

CHAPTER 6. GENERATION OF FBR REVERTANT CELL LINES FOLLOWING TRANSFECTION OF THE DOMINANT NEGATIVE MUTANT OF MEK-1, A221

6.1. MAP Kinase Kinase, MEK-1 is required for cellular transformation

A major pathway involved in the transmission of signals from the extracellular environment to the nucleus is the MAP Kinase pathway. Stimulation of cultured cells with mitogens results in activation of MAP Kinase (Anderson *et al.*, 1990a) by dual phosphorylation on Tyrosine and Threonine residues (Ray and Sturgill, 1988). MAP Kinases have multiple substrates including transcriptional activators, such as members of the transcription factor AP-1 (Karin, 1995; Gruda *et al.*, 1994).

The activation of this pathway involves the small GTP-binding protein $p21^{ras}$, which is activated by receptor tyrosine kinases and G-protein coupled receptors (Wood *et al.*, 1992; Gallego *et al.*, 1992). The activated GTP-$p21^{ras}$ binds to the N-terminus of the Raf-1 protein kinase (Vojtek *et al.*, 1993) and this event accounts for the activation of the MAP Kinase pathway. Raf-1 kinase initially was identified as a retroviral oncogene (Rapp *et al.*, 1983) and has been reported to activate a MAPK Kinase Kinase known as MEK-1 (Kyriakis *et al.*, 1992; Dent *et al.*, 1992; Howe., *et al.*, 1992). MEK-1 is a kinase with dual specificity, phosphorylating MAP Kinases on threonine and serine residues (Alessi *et al.*, 1994).

Experiments with oncogenes that lie upstream of MEK-1 showed that MEK-1 could indeed be at a convergent point of oncogene action. In these experiments the dominant negative kinase inactive MEK-1 A221 was shown to inhibit growth factor stimulation of MAP Kinase signalling (Cowley *et al.*, 1994). The same mutant when microinjected reversed transformation of v-*ras* and v-*src* transformed NIH 3T3 mouse fibroblasts. Conversely constitutively active MEK-1 has been shown to activate MAP Kinase signalling in the absence of growth factors and to induce cellular transformation in NIH 3T3 fibroblasts and differentiation of PC12 cells (Cowley *et al.*, 1994).

The AP-1 transcription factor and its components are regulated by extracellular signals. Here we have examined the role of the MEK-1, MAP Kinase Kinase Alanine 221 point mutant in

the context of transformation by the FBR v-*fos* oncogene, a member of the transcription factor AP-1.

Identification of serum-induced isoforms of MEK-1 in FBR by a phospho-specific antibody shows that phosphorylation of MEK-1 is low in FBR. This result makes MEK-1 an unattractive candidate for regulating *fos*-transformation. However since recently its function has been implicated in oncogenic growth we thought that it would still be interesting to test its role in *fos*-induced transformation, by examining the role of MEK-1 A221 in *fos*-induced anchorage independent growth. To investigate this we used a transfection approach by introducing an expression vector containing the MEK-1 A221 dominant negative cDNA into FBR. The cell lines that we have isolated had properties resembling those of revertants which indicated that some aspect of MAP Kinase signalling downstream of MEK-1 is important in transformation by v-*fos* oncogene.

6.2. Transfection of MEK-1 A221 into FBR and 208F cells

Lipofection was used to transfect FBR with pBABE *puro* expression vectors encoding wild type MEK-1 and MEK-1 A221. One day after transfection the cultures were divided into four 10cm dishes and 24 hours later selection with puromycin started. The results of this transfection assay are shown in Table 6.1. The number of puromycin resistant colonies is an indication of the effect of the transfected A221 and wild type MEK-1 on the growth of FBR and 208F cells. As a control a pBABE empty vector was transfected to FBR and 208F cells.

Transfection of 1 μg of wild type MEK-1 to FBR cells gave rise to a 1.5-fold increased number of colonies compared to FBR transfected with the empty pBABE *puro* vector. When FBR were transfected with 5 μg of wild type MEK-1 vector then a 3-fold decrease in the number of colonies was observed. This suggests that the wild type MEK-1 may confer a growth advantage to FBR in a low concentration but when the amount of the transfected plasmid was increased then this resulted in an inhibition of puromycin-resistant colony formation in FBR and in 208F. It is likely that an increase in the amount of the transfected wild type MEK-1 could result in cellular toxicity. This could be responsible for the appearance of low colony numbers after transfection of wild type MEK-1 into FBR cells.

When FBR were transfected with 1 μg of the pBABE MEK-1 A221 vector it was noticed that the number of puromycin resistant colonies was decreased 3-fold compared to an empty vector. When the amount of plasmid was increased to 5 μg, there was a 2-fold decrease in the amount of puromycin-resistant colonies suggesting a modest reduction of colony number of FBR after transfection of A221.

These transfection assays in FBR gave rise to a limited number of puromycin-resistant colonies after selection in DMEM supplemented with 10% FCS. This stringent selection process could probably be attributed to a specific effect of puromycin in FBR since in the same transfection assays in 208F cells we observed that puromycin did not limit the number of colonies after the selection process was completed and the plates were stained with Giemsa to identify individual colonies. In control experiments we have found that FBR are very sensitive to puromycin compared to 208F cells when concentrations of the antibiotic are higher than 2 μg/ml in the selection media.

Table 6.1. Colony formation in 10cm tissue culture dishes after transfection of of pBABE puro, pBABE MEK-1 wt and pBABE MEK-1 A221 into FBR and to 208F cells.

Cells were transfected with the indicated amounts of CsCl-purified plasmid DNA using lipofection. Colonies were counted after 3-4 weeks during selection with 2 μg/ml puromycin for the puro and wt MEK-1 vectors and 2.5 μg/ml puromycin for MEK-1 A221 vector followed by fixation in 100% Methanol, washed with PBS, stained with 10% Giemsa solution (v/v in H_2O). [ND : Not Determined].

Cell line	*Transfected plasmid*		*Colony formation efficiency*
FBR	puro	1 μg	24 colonies / 1μg
FBR	MEK wt	1 μg	36colonies / 1μg
FBR	MEK wt	5 μg	56 colonies / 5μg
FBR	MEK A221	1 μg	7 colonies / 1μg
FBR	MEK A221	5μg	17colonies / 5 μg
208F	puro	1 μg	220 colonies / 1μg
208F	MEK wt	1 μg	66 colonies / 1μg
208F	MEK A221	1 μg	150 colonies / 1μg

6.2. Morphological characteristics of FBR MEK-1 A221 transfectants

To investigate the morphological characteristics of FBR A221 clones, we isolated several cell lines by cylinder cloning. These clones were expanded into cultures and the morphology was scored by phase contrast microscopy.

Of the 17 FBR MEK-1 A221 revertant clones isolated by cylinder cloning, 14 clones had a flat morphology and this accounted for approximately 80 per cent of total colonies isolated from this experiment, designated Experiment 1 (Table 6.2). These pyromycin-selected FBR A221 clones maintained their morphological characteristics during passaging. In the control FBR transfectants with the vector pBABE *puro* alone, 7 of the 9 colonies isolated were transformed and 2 had a non-transformed morphology, resulting in 77% transformed colonies in the Experiment 1 (Table 6.2, first nine cell lines).

In a another transfection experiment, termed Experiment 2, with identical plasmid analogies as those in the Table 6.1, similar reversion data were obtained as those presented in Table 6.2. Table 6.3 summarises the morphological characteristics of the clones isolated from the Experiment 2. The percentage of reversion was 74%, with 17 out of 23 FBR A221 clones having a flat morphology. This result along with the results presented in Table 6.2 demonstrated that reversion after transfection of MEK-1 A221 into FBR is not a random, selection-dependent event but rather is related to the effect of this dominant negative mutant reversing *fos*-mediated transformation. When the phenotype of the revertant cells at different densities was examined, it was found that reversion occurred in FBR MEK-1 A221 transfectants at different densities. This is similar to that reported in FBR TAM-67 revertants (Figure 6.1). FBR appear to be bipolar in shape when growing in the presence of 10% FCS, and also a large number of cells appear to be rounded (Figure 6.1, a). In contrast to this 208F cells are flat when attached to plastic in the tissue culture dish, maintaining this property in higher densities and very rarely rounded cells appear in the cultures (Figure 6.1 b). FBR A221 clones 19 and 23 are two revertant clones we have isolated after transfection of MEK-1 A221 (Figure 6.1 c, d, e and f) which have a spindle-like morphology at low density which is reminiscent of FBR, whereas in a higher density they are flat (Figure 6.1 c and e ; and d and f respectively). Also the percentage of rounded cells which are present in FBR cultures (Figure 6.1, a) is reduced in the FBR A221 clones 19 and 23 (see Figure 6.1 d and f).

Table 6.2. Experiment 1. Morphological characteristics of FBR transfected with pBABE puro and pBABE MEK-1 A221.

FBR cells were transfected with 1 μg of pBABE puro and 5 μg of pBABE MEK-1 A221 using lipofection. The clones were selected with puromycin at a final concentration of 2 μg/ml and 2.5 μg/ml for the pBABE puro and pBABE MEK-1 A221 vectors respectively and maintained in culture using the same concentration.

Cell line	*Transfected plasmid*	*Morphology*
FBR puro 2	pBABE puro	transformed
FBR puro 3	pBABE puro	transformed
FBR puro 4	pBABE puro	transformed
FBR puro 5	pBABE puro	transformed
FBR puro 9	pBABE puro	flat
FBR puro 10	pBABE puro	transformed
FBR puro 11	pBABE puro	flat
FBR puro 14	pBABE puro	transformed
FBR puro 15	pBABE puro	transformed
FBR A2	MEK-1 A221	flat
FBR A3	MEK-1 A221	flat
FBR A4	MEK-1 A221	flat
FBR A5	MEK-1 A221	flat
FBR A6	MEK-1 A221	flat
FBR A8	MEK-1 A221	transformed
FBR A10	MEK-1 A221	flat
FBR A11	MEK-1 A221	flat
FBR A14	MEK-1 A221	transformed
FBR A16	MEK-1 A221	transformed
FBR A17	MEK-1 A221	flat
FBR A19	MEK-1 A221	flat
FBR A20	MEK-1 A221	transformed
FBR A21	MEK-1 A221	flat
FBR A22	MEK-1 A221	flat
FBR A23	MEK-1 A221	flat
FBR A28	MEK-1 A221	flat

Table 6.3 . Experiment 2. Morphological characteristics of FBR transfected with pBABE puro and pBABE MEK-1 A221.

FBR cells were transfected with 1 μg of pBABE puro and 5 μg of pBABE MEK-1 A221 using lipofection. The clones were selected with puromycin at a final concentration of 2 μg/ml and 2.5 μg/ml puromycin for the pBABE puro and pBABE MEK-1 A221 vectors respectively and maintained in culture using the same concentration.

Cell line	*Transfected plasmid*	*Morphology*
FBR puro 1	pBABE puro	transformed
FBR puro 2	pBABE puro	transformed
FBR puro 6	pBABE puro	transformed
FBR puro 9	pBABE puro	flat
FBR puro 10	pBABE puro	transformed
FBR puro 15	pBABE puro	transformed
FBR puro 19	pBABE puro	transformed
FBR puro 23	pBABE puro	transformed
FBR A221 1	MEK-1 A221	flat
FBR A221 2	MEK-1 A221	flat
FBR A221 3	MEK-1 A221	flat
FBR A221 4	MEK-1 A221	transformed
FBR A221 6	MEK-1 A221	transformed
FBR A221 7	MEK-1 A221	transformed
FBR A221 8	MEK-1 A221	flat
FBR A221 9	MEK-1 A221	flat
FBR A221 10	MEK-1 A221	flat
FBR A221 11	MEK-1 A221	transformed
FBR A221 12	MEK-1 A221	transformed
FBR A221 13	MEK-1 A221	flat
FBR A221 14	MEK-1 A221	flat
FBR A221 15	MEK-1 A221	flat
FBR A221 16	MEK-1 A221	flat
FBR A221 18	MEK-1 A221	flat
FBR A221 19	MEK-1 A221	flat
FBR A221 20	MEK-1 A221	flat
FBR A221 21	MEK-1 A221	flat
FBR A221 22	MEK-1 A221	flat
FBR A221 26	MEK-1 A221	transformed
FBR A221 27	MEK-1 A221	flat
FBR A221 28	MEK-1 A221	flat

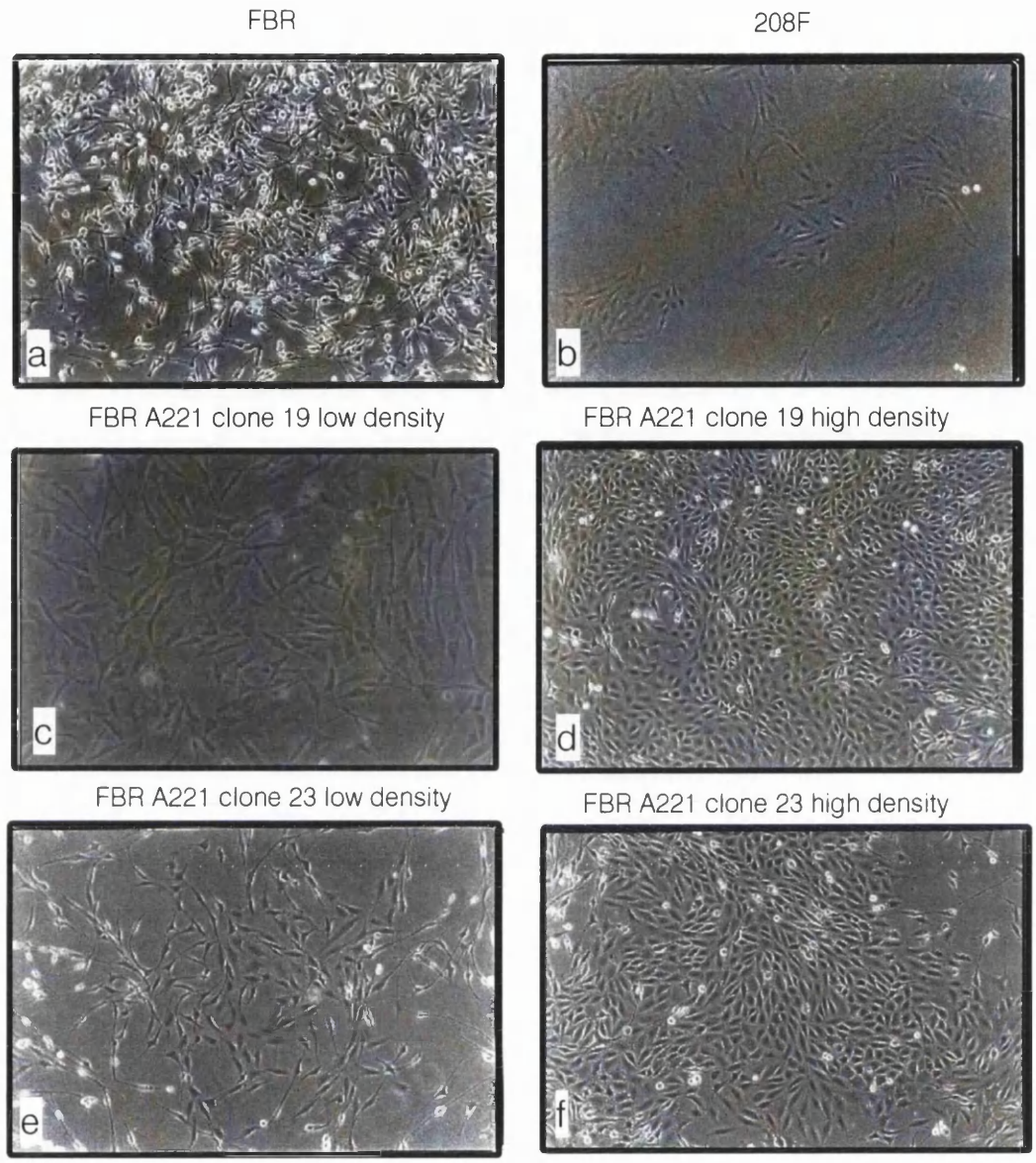


Figure 6.1. Morphological reversion at different cell densities.

Morphology of FBR, 208F and FBR transfected with the dominant negative mutant of MEK-1, MEK-1 A221 at low and high cell density.
Cell lines were plated in DMEM supplemented with 10% FCS, allowed to attach for 24-48 hours and photographed with a Nikon inverted Diaphot microscope. **a)** FBR; **b)** 208F; **c)** FBR A221 revertant clome 19 in low density; **d)** FBR A221 revertant clome 19 in high density; **e)** FBR A221 revertant clome 23 in low density; **f)** FBR A221 revertant clome 23 in high density.

6.3. MEK-1 A221 inhibits v-fos-induced anchorage independence

After isolation of FBR A221 clones these clones were tested for the ability to grow in methylcellulose, that is under anchorage-independent conditions. Cells from parental cell and from three revertant clones FA6, FA19 and FA23, were plated in Ham's media containing 1.5% methylcellulose and allowed to grow for 20 days. After this period of time colonies were observed using the light microscope. Clones FA6 and FA19 showed no colonies (Figure 6.2) and clone FA23 showed few colonies reduced in size compared to the parental FBR fibroblasts (Figure 6.2). The FBR cell line maintained under the same conditions gave rise to a high number of colonies of large size (Figure 6.2,a) and the 208F resulted in no colonies (Figure 6.2,b). As a positive control another transformed cell line Ki-Mu-SV transformed 208F cells (208F v-Ki-*ras*) was included. These cells formed colonies in methylcellulose with high efficiency of larger size than the FBR colonies (Figure 6.2,c). The data from three different experiments are summarised in Table 6.4. In this table it is shown that FBR cells form colonies with high efficiency but the FBR A221 6 and FBR A221 19 clones do not form any colonies in the semi-solid medium after approximately 3-4 weeks of growth in these media. FBR A221 23 clone formed colonies of decreased size and approximately 4-fold decreased in number when compared to FBR cells.

Previous reports highlight the role of a constitutively active form of MEK-1 in morphological transformation (Cowley *et al.*, 1994; Mansour *et al.*, 1994). In these published reports there is little evidence for MEK-1 A221 in oncogene-induced anchorage-independent growth (Cowley *et al.*, 1994). By transfection of the dominant negative mutant MEK-1 A221 into FBR cells we demonstrate that MEK-1 A221 has a role, in semi-solid media growth of FBR , as a parameter of cellular transformation *in vitro*.

Since the expression vectors for the wild type and A221 MEK-1 do not contain an epitope tag to identify the expressed proteins by western analysis or by immunoprecipitation experiments, a functional approach was employed to obtain evidence for biochemical activity of the mutant MEK-1 A221, as an indication of its presence in FBR A221 transfectants.

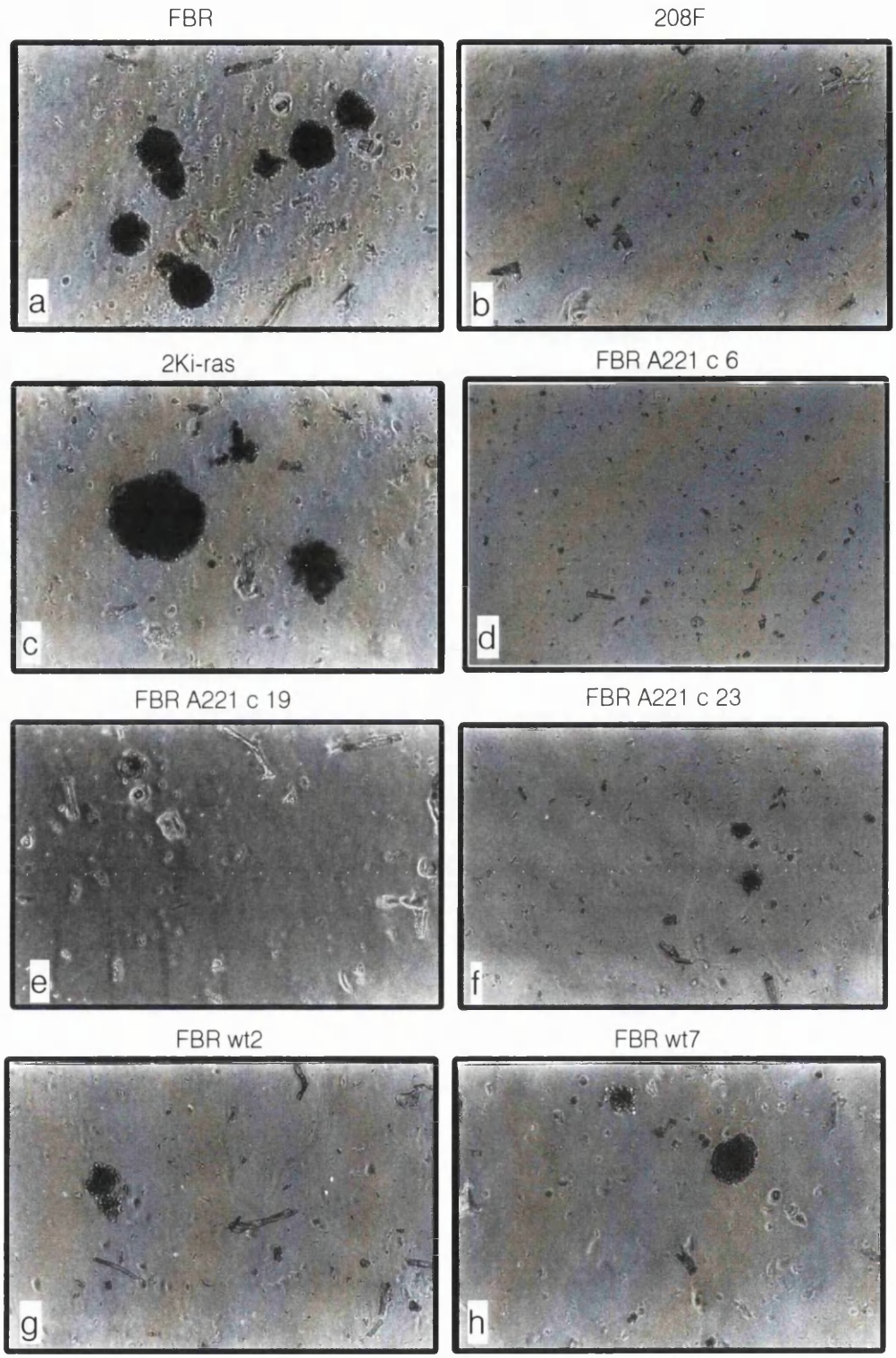


Figure 6.2. Growth in semi-solid medium of FBR, 208F and FBR transfected with wild type and dominant negative mutant of MEK-1 A221.

Cells grown in methylcellulose containing 10% FCS for 20 days and photographed with a Nikon Diaphot microscope. **a)** FBR cells; **b)** 208F cells; **c)** v-Ki-ras transformed 208F cells; **d)** FBR MEK-1 A221 revertant clone 6; **e)** FBR MEK-1 A221 revertant clone 19; **f)** FBR MEK-1 A221 revertant clone 23; **g)**FBR MEK-1 A221 clone wt2; **h)** FBR wild type MEK-1 clone wt7.

Table 6.4. *in vitro* Anchorage-Independent growth of FBR and FBR A221 cells.

Cell lines were passaged in DMEM supplemented with 10% FCS, trypsinized, counted with a cell chamber counter and $2x10^4$ cells were plated in Ham's media containing 1.5% Methylcellulose. Colonies were counted after 3-4 weeks by phase contrast microscopy. Three independent experiments are shown were duplicate samples were assayed

Cell line	*Experiment 1*	*Experiment 2*
FBR	12672 - 12096	12320 14227
Average	12384	13273
ratio	0.61	0.66

Cell line	*Experiment 1*	*Experiment 2*
FBR A221 23	3600 - 3264	ND
Average	3432	ND
ratio	0.17	ND

Cell line	*Experiment 3*
FBR	14784 - 12936
Average	13860
ratio	0.69

Cell line	*Experiment 3*
FBR A221 23	2669 - 4006
Average	3337
ratio	0.16

6.4. MEK-1 A221 suppresses serum-induced MAP-Kinase activation in FBR

MEK-1 mutants that have an Alanine substitution in the position of the Serine 221 have a reduced ability to phosphorylate MAP Kinase (Alessi *et al.*, 1994). Here the ability of MEK-1 A221 to suppress MAP Kinase activation by extracellular agonists was examined using phosphospecific antibodies in western immunoblotting experiments. Since the pBABE MEK-1 A221 vector (Cowley *et al.*, 1994) is not tagged, this method was chosen to demonstrate the function of the dominant negative MEK-1 in our cell lines. In previous experiments RT-PCR was not able to detect the MEK-1 A221 mutant. This could have been possibly due to the high homology at the nucleic acid level between the transfected rabbit enzyme (Ashworth *et al.*, 1992) and the endogenous rat MEK-1.

To test MEK-1 A221 the phospho-specific antibody that has affinity for the dually phosphorylated forms of MAP Kinases was used, ERK1 and ERK2 as it was used in the previous chapter to compare MAP Kinase phosphorylation between FBR and 208F cells. From quiescent and serum stimulated for 5 minutes cultures of FBR, 208F and FBR A211 clones 6 , 19 and 23 cell lysates were prepared using the standard Triton X-100 lysis buffer. The proteins were separated by 10% SDS-PAGE, transferred to PVDF membranes and blotted with the phosphospecific antibody.

208F cells induced with 10% foetal calf serum show a significant activation of MAP kinase phosphorylation approximately 10-fold over basal levels (maximal activation taken as 1, n=3, P=0.04; Table 6.5; Figure 6.3A, lane 8). In the case of FBR this response is 5-fold reduced (0.2+/-0.09, n=4 P<0.05; Figure 6.3A, lane 3). In the FBR cells the immunoreactivity of phospho-reactive MAP Kinase after serum stimulation showed reproducible decreased levels compared to the activation ERK1 and ERK2 in 208F cells.

In the FBR A221 clones 6, 19 and 23 after serum induction, a 2 to 3-fold decrease in the phosphorylation of MAP Kinases was observed when compared to FBR cells (Figure 6.3A lane 11 compare with lane 3/ and Figure 6.3B lanes 7 and 11 compare with lane 3). In clone 19 presented in the Table 6.5 the response to serum induction was approximately 2-fold reduced compared to FBR cells (In the FBR the response to serum was maximal at 0.2+/-0.05, n=3 , P<0.05, approximately 2 fold over the basal of FBR control and in clone 19 0.15+/-0.04, n=3, 1.3 fold reduced compared to FBR control, significant at P<0.05/ also look Table 6.5).

Other stimuli will also induce MAP kinase phosphorylation in fibroblasts. Stimulation for example with sodium orthovanadate, a general inhibitor of protein tyrosine phosphatases potentiates activation of ERK's possibly by enhancing the basal autophosphorylation activity of Receptor Tyrosine Kinases and by inhibiting the dual-specificity Tyrosine-Threonine phosphatases involved in MAP Kinase signalling. Recently there is evidence to support a positive role of vanadate in the activation of the MAP Kinase pathway (Zhao *et al.*, 1996) and the role of orthovanadate in ERK stimulation in Rat-1 fibroblasts has been well studied (Cook *et al.*, 1997).

The activation of MAP Kinases by orthovanadate in FBR, 208F and FBR A221 revertant cells was next addressed. As shown in Figure 6.4 the data are similar with the results of serum stimulation presented in Figure 6.3. In FBR serum induces MAPK phosphorylation whereas orthovanadate does not (Figure 6.4A lanes 2 and 3 and Figure 6.4B lanes 3 and 4, for serum and orthovanadate respectively; 0.6+/-0.2 activation for serum-treated FBR cells, n=3 not significant at $P>0.05$ and in FBR treated with orthovanadate activation was 0.09+/-0.01, n=3, not significant, $P>0.05$). In contrast, in 208F cells orthovandate stimulates a similar response to serum in activating ERK's which is significant (Figure 6.4A lanes 5 and 6 / 6.4B lanes 6 and 8 for serum and orthovanadate respectively; 208F serum treated activation was taken as maximal 1 and in the orthovanadate 208F treated samples 0.92+/-0.16, n=3, $P<0.05$). In the FBR A221 revertants the response to stimulation by orthovanadate was not significant (Figure 6.4A, lanes 9 and 12 and 6.4B lane 11; activation for clone 6 0.065+/-0.015, n=3, $P>0.05$; activation for clone 19 0.1+/-0.005, n=3, $P>0.05$; activation for clone 23 0.07+/-0.01, n=3, $P>0.05$). The serum induction in the FBR A221 clones was maintained in lower levels compared to FBR as shown in the Figure 6.3 (The activation by serum in FBR A221 clones was 2-3 fold reduced compared to FBR parental cells. More specifically for clone FBR A221 6 activation by serum was 0.15+/-0.04, n=3, $P<0.05$, for clone FBR A221 19 activation was 0.21+/-0.03, n=3, $P<0.05$ and for clone FBR A221 23 activation 0.33+/-0.011, n=3, $P<0.05$/).

These data suggest that MEK-1 A221-induced reversion of FBR possibly does sensitise the response to MAP Kinases to this inhibitor of protein tyrosine phosphatases. The phosphorylation of ERK's in response to this compound was maximal in 208F cells, a derivative of Rat-1 cells, but remained in lower levels in the FBR cells.

From these experiments is not possible to assess the expression of the MEK-1 dominant negative mutant A221, an alternative approach was used to try to identify its expression and function. This was to subject lysates prepared from FBR A221 derived clones and from control FBR and 208F cells to low bis-Acrylamide SDS-Polyacrylamide gel electrophoresis followed by western immunoblotting. In this experiment the phosphorylatable endogenous MEK-1 could be possibly distiguished from the transfected A221 mutant MEK-1 by mobility shift of the phosphorylated MEK-1. In the Figure 6.4a such an experiment is shown where the phosphorylation of MEK-1 in 208F cells is clearly indicated by the double band that appears after immunoblotting with the phospho-specific antibodies (Figure 6.4a A, lane 4). This pattern is several-fold less intense in the parental serum-induced FBR cells (Figure 6.4a A, lane 2). In the case of the FBR A221 clones 19 and 23 there is staining of MEK-1 that is shifting after western immunoblotting although majority of the MEK-1 is retained in the non-phosphorylated lower isoform. This experiment which is rather difficult to interpret suggests that the this approach although powerful did not gave clear evidence for the expression of the MEK-1 A221.

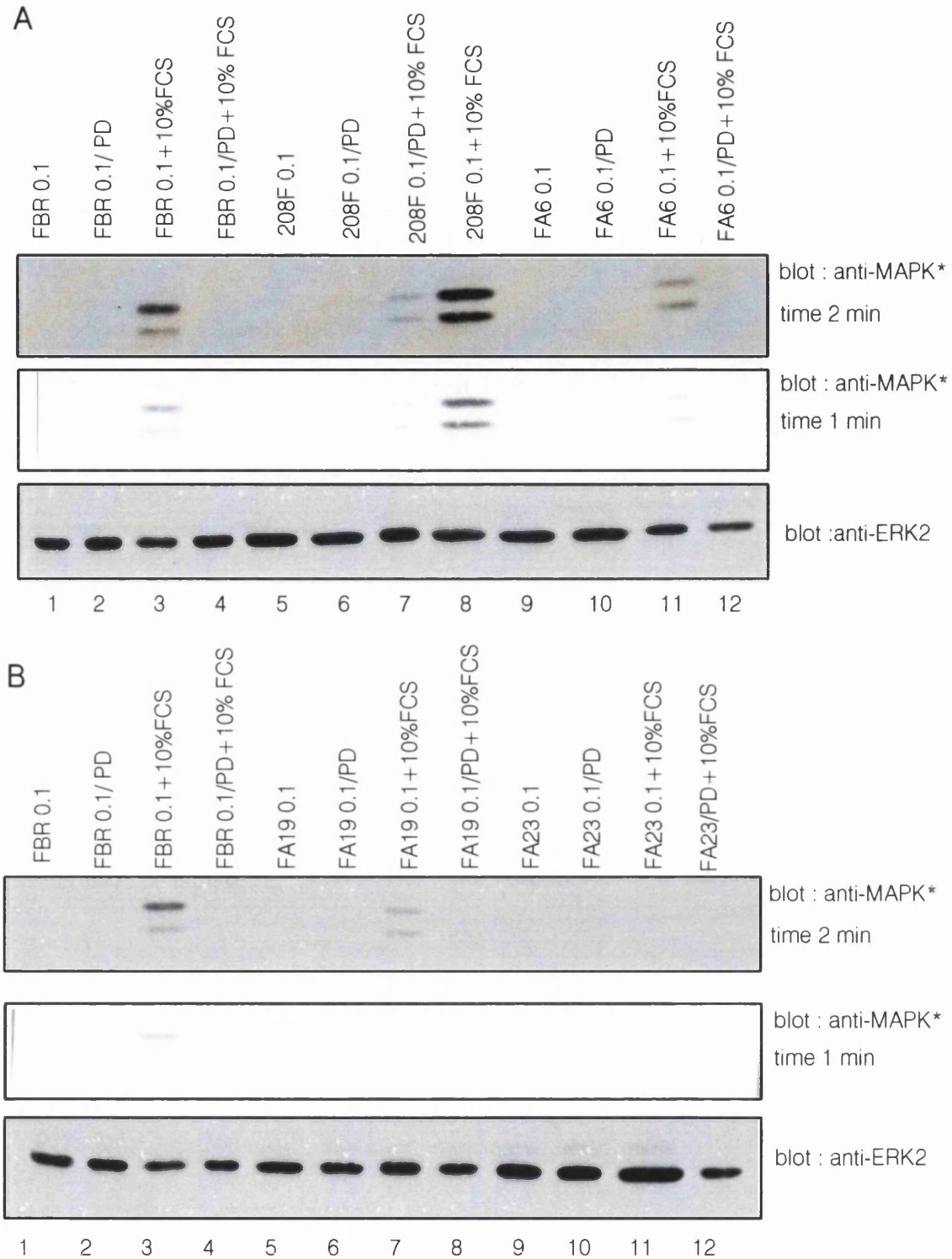


Figure 6.3 . Phosphorylation of MAPK by serum in FBR, 208F and FBR transfected with a dominant negative mutant of MEK-1 A221.

Cell lines were grown in DMEM supplemented with 10% FCS and when sub-cofluent serum-deprived for 48 hours (0.1% FCS).
Stimulation was for 5 minutes with 10% FCS. The pre-treatment with the MEK-1 inhibitor PD 98059 was for 30 minutes at a final concentration of 50μM. Cell lysates were prepared and proteins were separated by 10% SDS-PAGE, transferred to PVDF membranes and probed with an antibody specific for phosphorylated ERK1 and ERK2 (MAPK*, Promega labs, WI, USA).
Blots were stripped and probed with an antibody specific for ERK2 as a loading control (ERK2) (Trasnduction labs, KY, USA). The indicated times are the exposing times that the blots were exposed to the FUJI X-mat film after the ECL reagent was added to the membranes. This experiment is representative of two independent experiments.

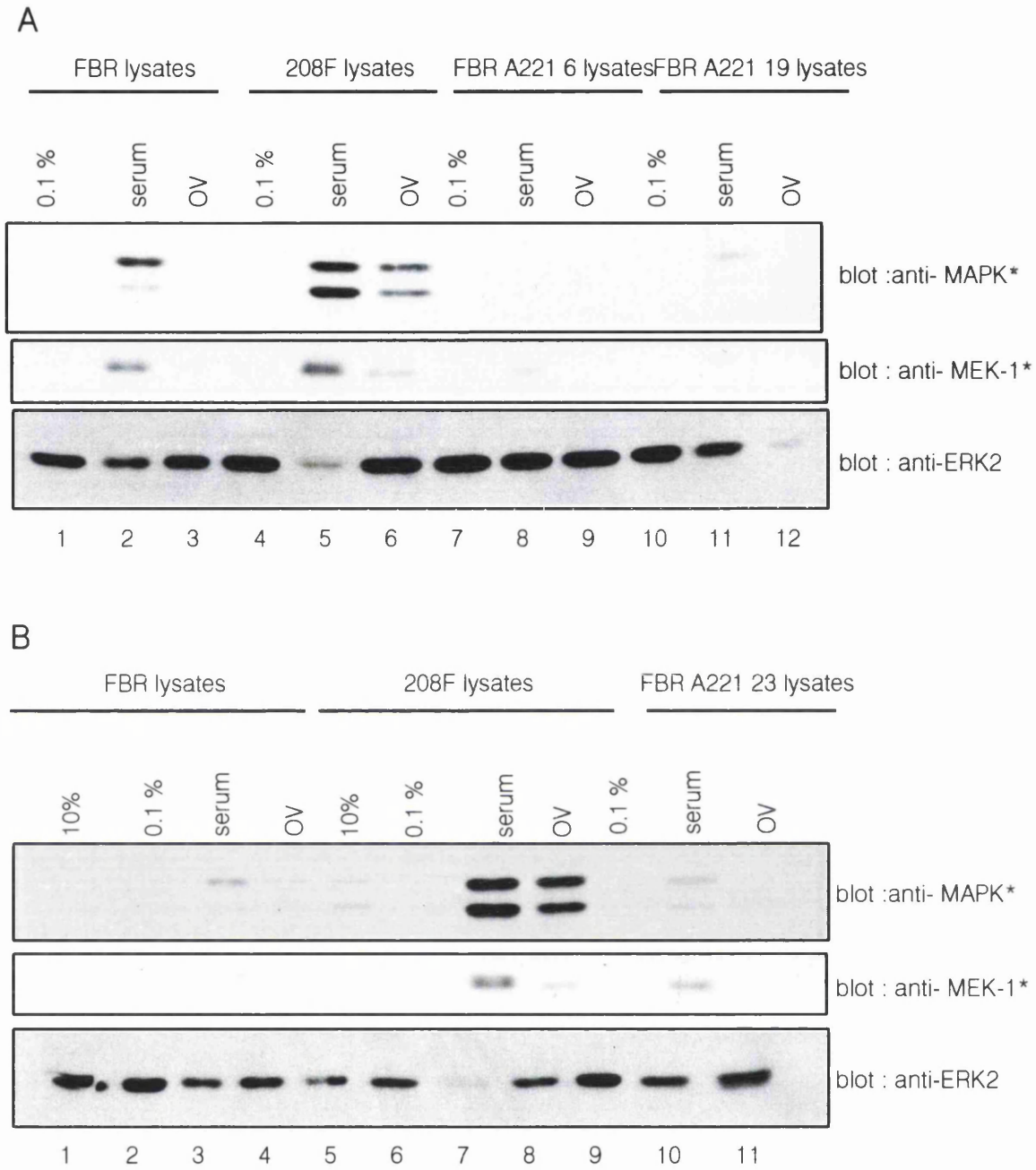


Figure 6.4. Phosphorylation of MAPK by serum, sodium orthovandate, and their comibination in FBR, 208F and FBR transfected with the dominant negative mutant of MEK-1 A221.

Cell lines were grown in DMEM supplemented with 10% FCS and when subconfluent serum-deprived for 48 hours (0.1% FCS).
Stimulation was for 5 minutes with 10% FCS, 100 µM sodium orthovanadate, their combination or the cells were left untreated. The pretreatment with the MEK-1 inhibitor PD 98059 was for 30 minutes at a final concentration of 50 µM. Cell lysates were prepared and proteins were separated by 10% SDS-PAGE, transferred to PVDF membranes and probed with an antibody specific for the phosphorylated ERK1 and ERK2 (MAPK*, Promega labs, WI, USA).
Blots were stripped and probed with an antibody specific for ERK2 as a loading control (ERK2, Transduction labs, KY, USA).

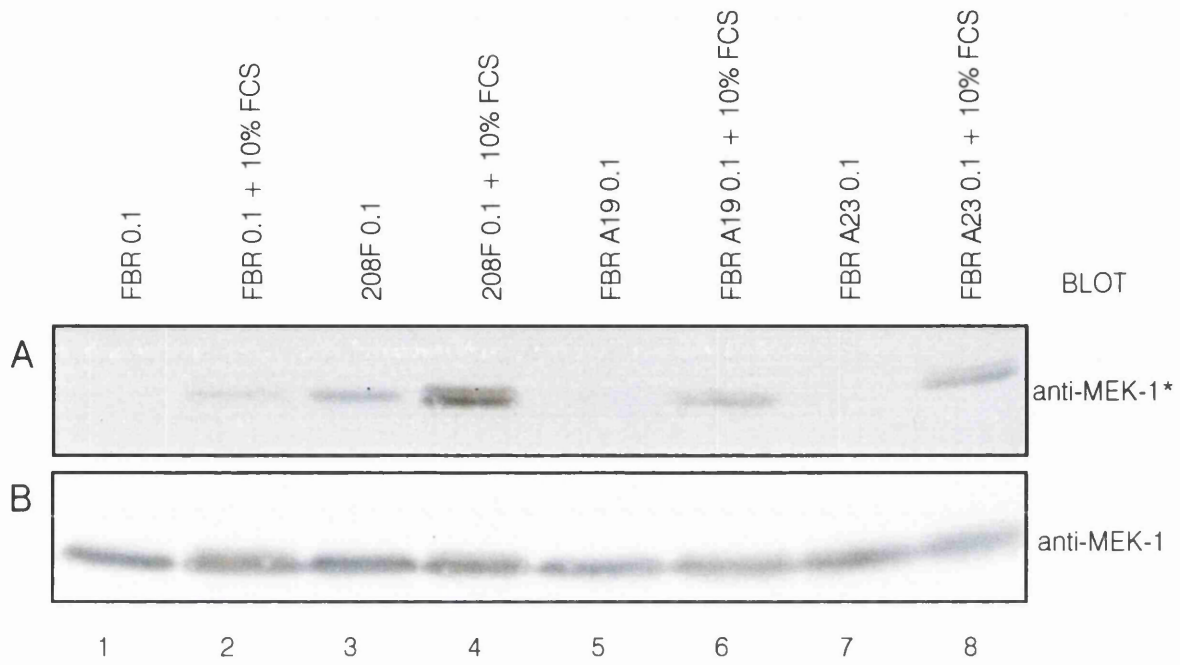


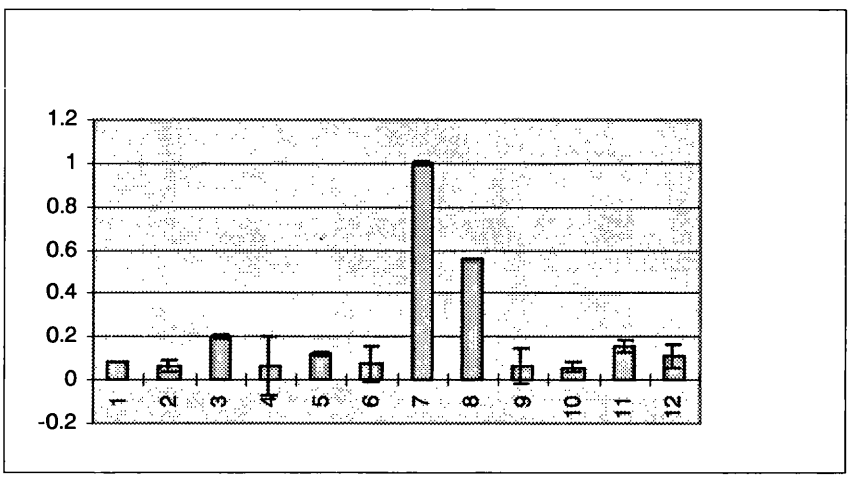
Figure 6.4a. Identification of MEK-1 phosphorylated isoforms by low-bisAcrylamide SDS-Polyacrylamide electrophoresis in lysates of -quiescent and serum-stimulated FBR, 208F and FBR transfected with a dominant negative mutant of MEK-1 A221.

Cell lines were grown to subconfluence in DMEM supplemented with 10% FCS and they were serum deprived for 48 hours (0.1% FCS). Stimulation was for 5 minutes with 10% FCS.
Cell lysates were prepared and the proteins were separated by 10% SDS-PAGE,, transferred to PVDF membrane and probed **A**) with an antibody specific for the dually phosphorylated MEK-1 (MEK-1*) (New England Bio., MA, USA). The same blot was stripped and probed with **B)** an antibody for MEK-1 (MEK-1) (Santa Cruz Bio., CA, USA) to verify equal loading.
A) and B) 1) FBR serum deprived cells; 2) FBR serum stimulated cells; 3) 208F serum deprived cells; 4) 208F serum stimulated cells; 5) FBR MEK-1 A221 revertant clone 19 serum deprived; 6) FBR MEK-1 A221 revertant clone 19 serum stimulated; 7) FBR MEK-1 A221 revertant clone 23 serum deprived; 8) FBR MEK-1 A221 revertant clone 23 serum stimulated.
This is a representative experiment of two with similar results

Table 6.5. Modulation of MAP Kinase by serum in FBR, 208F and FBR clone 19 transfected with MEK-1 A221.

The cells were pre-treated with 50 μM of PD98059 for 30 minutes and then left untreated or serum stimulated with 10% FCS for 5 minutes.

Quantification of the increase in phosphorylation of ERK1 and ERK2 was performed using scanning densitometry. The band of serum-stimulated 208F cells was taken as the maximal, 100% activation. The values were normalised for control loading of ERK2 for each individual experiment. The values are the mean of three independent experiments.



1) FBR serum-deprived cells
2) FBR cells treated with 50 μM of PD98059
3) FBR serum-stimulated cells
4) FBR cells pre-treated with 50 μM of PD98059 and serum-stimulated for 5 minutes
5) 208F serum-deprived cells
6) 208F cells treated with 50 μM of PD98059
7) 208F serum-stimulated cells
8) 208F cells pre-treated with 50 μM of PD98059and serum-stimulated for 5 minutes
9) FBR A221 clone 19 serum-deprived cells
10) FBR A221 clone 19 treated with 50 μM of PD98059
11) FBR A221 clone 19 serum-stimulated cells
12) FBR A221 clone 19 treated with 50 μM of PD98059 and serum-stimulated for 5 minutes

6.5. Growth factor activation of MAPK in FBR, 208F and FBR A221 revertants

To examine the effect of a purified growth factor on MAP Kinase activation, lysates of FBR, 208F and FBR A221 cells treated with EGF were analysed by western immunoblotting with the MAPK phosphospecific antibody. Activation was again determined by the intensity of the ERK1 and ERK2 in the autoradiograms. As shown in Figure 6.5, stimulation of FBR cells with 100 ng/ml EGF gave rise to a modest stimulation of MAP Kinase phosphorylation (Figure 6.5 lane 3; activation 0.15+/-0.02 , n=3, P<0.05/ Table 6.6). In the case of 208F cells, induction of ERK's with 100 ng/ml EGF resulted in maximal activation of ERK1 and ERK2 (Figure 6.5 lane 7; maximal activation taken as 1, n=3, P<0.05/ Table 6.6). FBR MEK-1 A221 revertants are sensitive to induction by serum of MAP kinases and the effect of serum is suppressed, as shown in Figures 6.3 and 6.4. However stimulation of quiescent FBR A221 revertant clones 6, 19 and 23 with 100 ng/ml EGF resulted in activation of ERK1 and ERK2 phosphorylation of approximately 2 to 3-fold over the response observed in the FBR cells (Figure 6.5A lane 11 and 6.5B lanes 7 and 11/ For the FBR A221 clone 6 activation was 0.4+/-0.02, n=3, P<0.05/ For clone FBR A221 19 activation was 0.23+/-0.07, n=3, P<0.05 and for clone FBR A221 23 activation 0.46+/-0.05, n=3, P>0.05/ look also Table 6.6).

Identical results were obtained by treating FBR A221 revertants with 100ng/ml EGF as shown in an independent experiment in Figure 6.6. Again there an induction of MAPK phosphorylation in the FBR A221 revertant clones by EGF was observed. A plausible explanation for the effect of EGF, according to the studies of Krebs and co-workers (Seger *et al.*, 1994) is that 100 ng/ml EGF is saturating concentration, and despite the presence of the inactive kinase the pathway is efficiently activated. These investigators have shown in transfection experiments that at 100 ng/ml EGF a maximal response in all the MEK-1 subtypes (wild type, dominant negative and constitutively active MEK-1 isoforms) occurs in NIH 3T3 cells. Marshall and co-workers also have suggested that the mutants of MEK-1 are effective in low concentrations of EGF, of approximately 0.2 nM (Cowley *et al.*, 1994). According to these investigators the MEK-1 A221 mutant at low concentrations of EGF is able to confer maximal 92% inhibition as a response to this particular growth factor in the activation of MAP Kinases.

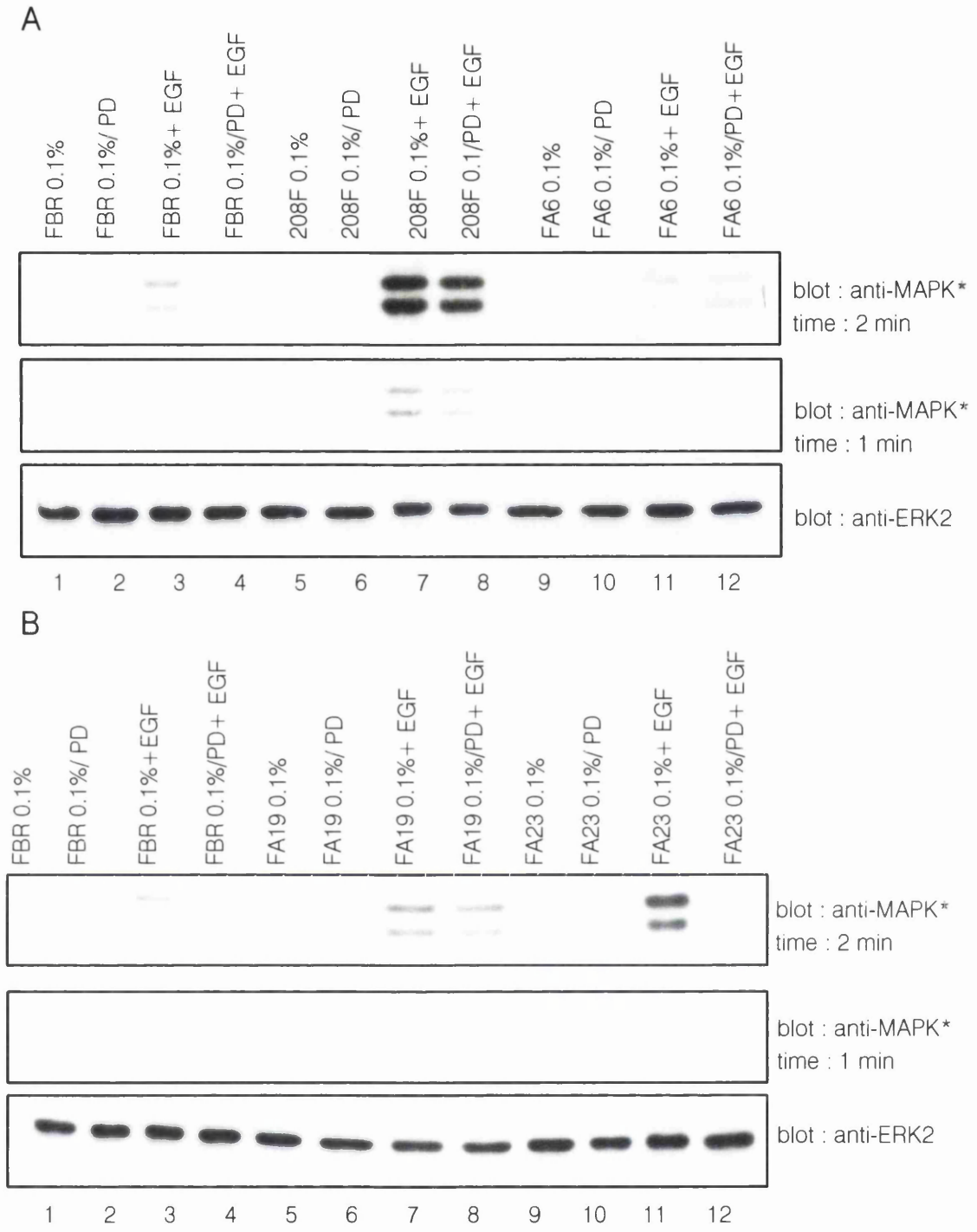


Figure 6.5. Phosphorylation of MAPK by EGF in FBR, 208F and FBR transfected with the dominant negative mutant of MEK-1 A221.

Cell lines were in DMEM supplemented with 10% FCS, and when subconfluent serum-deprived for 48 hours (0.1% FCS).
Stimulation was for 5 minutes with 100 ng/ml EGF. The pretreatment with the MEK-inhibitor PD 98059 was for 30 minutes at a final concentration of 50μM. Cell lysates were prepared and proteins were separated by 10% SDS-PAGE, transferred to PVDF membranes and probed with an antibody specific for phosphorylated ERK1 and ERK2 (MAPK*, Promega labs, WI, USA).
Blots were stripped and probed with an antibody specific for ERK2 as a loading control (ERK2, Transduction labs, KY, USA).

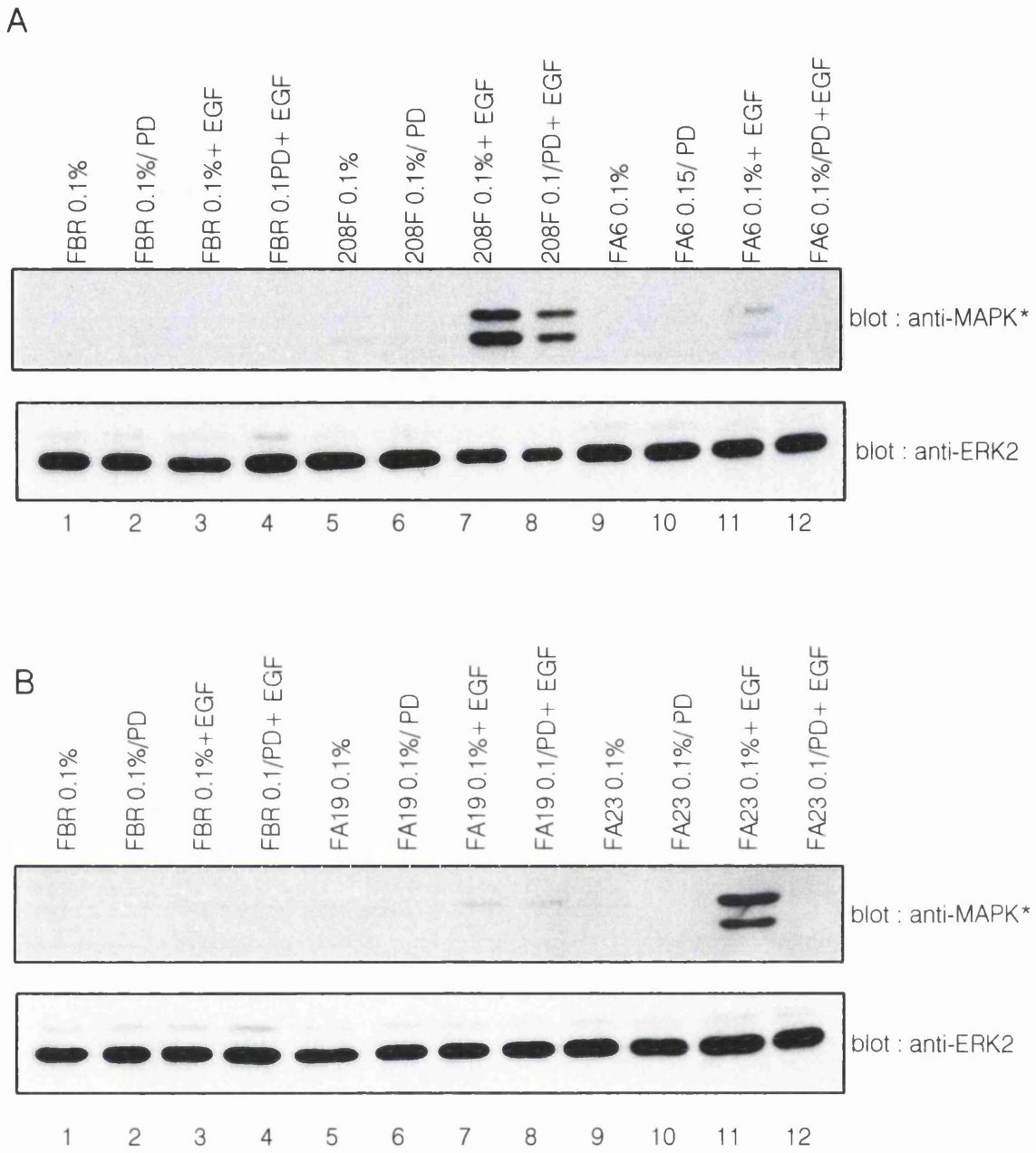


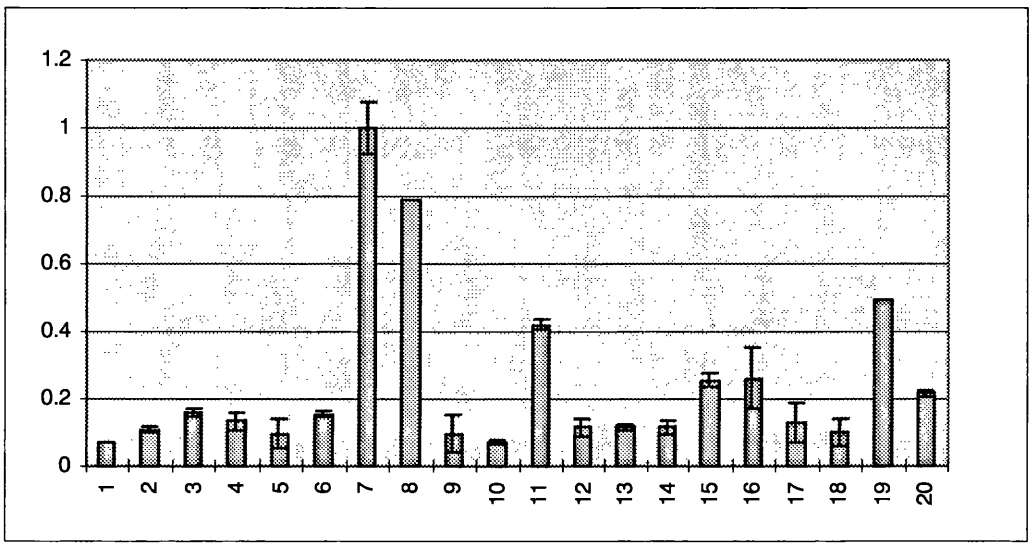
Figure 6.6. Phosphorylation of MAPK by EGF in FBR, 208F and FBR transfected with the dominant negative mutant of MEK-1 A221.

Cell lines were grown in DMEM supplemented with 10% FCS and when subconfluent serum-deprived for 48 hours (0.1% FCS).
Stimulation was for 5 minutes with 100 ng/ml EGF. The pre-treatment with the MEK-inhibitor PD 98059 was for 30 minutes at a final concentration of 50 µM. Cell lysates were prepared and proteins were separated by 10% SDS-PAGE, transferred to PVDF membranes and probed with an antibody specific for phosphorylated ERK1 and ERK2 (MAPK*, Promega Labs, WI, USA).
Blots were stripped and probed with antisera specific for ERK2 as a loading control (ERK2, Transduction labs, KY, USA). This experiment is a representative of two independent experiments.

Table 6.6. Modulation of MAP Kinase activation by 100 ng/ml of EGF FBR, 208F and FBR clones A221 6, 19 and 23 transfected with dominant negative MEK-1 A221.

The cells were pre-treated with 50 μM of PD98059 for 30 minutes and then left untreated or stimulated with 100 ng/ml of EGF for 5 minutes.

Quantification of the increase in phosphorylation of ERK1 and ERK2 was performed using scanning densitometry. The band of serum-stimulated 208F cells was taken as the maximal, 100% activation. The values were normalised for control loading of ERK2 for each individual experiment. The values are the mean of three independent experiments.



1) FBR serum-deprived cells
2) FBR cells treated with 50 μM of PD98059
3) FBR EGF-stimulated cells
4) FBR cells pre-treated with 50 μM of PD98059 and EGF-stimulated for 5 minutes
5) 208F serum-deprived cells
6) 208F cells treated with 50 μM of PD98059
7) 208F EGF-stimulated cells
8) 208F cells pre-treated with 50 μM of PD98059and EGF-stimulated for 5 minutes
9) FBR A221 clone 6 serum-deprived cells
10) FBR A221 clone 6 treated with 50 μM of PD98059
11) FBR A221 clone 6 EGF-stimulated cells
12) FBR A221 clone 6 pre-treated with 50 μM of PD98059 and EGF-stimulated for 5 minutes
13) FBR A221 clone 19 serum-deprived cells
14) FBR A221 clone 19 treated with 50 μM of PD98059
15) FBR A221 clone 19 EGF-stimulated cells
16) FBR A221 clone 19 treated with 50 μM of PD98059 and EGF-stimulated for 5 minutes
17) FBR A221 clone 23erum-deprived cells
18) FBR A221 clone 23treated with 50 μM of PD98059
19) FBR A221 clone 23 EGF-stimulated cells
20) FBR A221 clone 23 treated with 50 μM of PD98059 and EGF-stimulated for 5 minutes

6.6. PD98059 negatively regulates MAPK signalling in FBR

In this series of experiments (Figure 6.3, Figure 6.5, Figure 6.6) the recently described MEK-1-specific inhibitor PD 98059, developed by Alan Saltiel and co-workers (Dudley *et al.*, 1995) was also used. This inhibitor has been shown to be selective towards MEK-1, and acts to prevent extracellular stimulation of MAPK by growth factors (Dudley *et al.*, 1995 ; Alessi *et al.*, 1995).

Serum-starved FBR, 208F and FBR A221 revertant cells were treated with 50 μM PD 98059 for 30 minutes before the addition of 10% FCS serum or 100 ng/ml EGF. When FBR are pre-treated with PD 98059, then stimulated with 10% FCS, phosphorylation of MAP Kinases was abolished as shown in the western immunoblots. Statistical analysis showed that was a significant response (Figure 6.3A lane 4, Figure 6.3B lane 4; FBR activation 0.2+/-0.09 , n=3, $P<0.05$; and FBR pretreated with the PD98059 0.063+/-0.005, n=3, $P>0.05$).

A similar response was seen for PD 98059-pre-treated-FBR cells after induction with 100 ng/ml EGF (Figure 6.5A lane 4, Figure 6.5B lane 4 ; Figure 6.6A lane 4, Figure 6.6B lane 4; activation of FBR MAP Kinases by EGF was 0.15+/-0.02, n=3, $P<0.05$; inhibition by PD98059 of MAP Kinases in FBR treated with EGF was 0.13+/-0.05, n=3, not significant with $P>0.05$). These data suggest that in FBR, MAP Kinase phosphorylation is not very sensitive to pharmacological inhibition by the MEK-1 inhibitor PD 98059 although as it appears in the western immunoblotting experiments the levels of phosphorylated ERK's return to basal if FBR are treated with PD 98059 and then stimulated with serum or EGF.

In the case of 208Fcells there was a different pattern of response to the treatment with the PD 98059, both when treated with serum or EGF. 208F cells are more resistant to treatment with PD 98059, since after pretreament with PD 98059 and stimulation with serum the MAP Kinase phosphorylation is reduced 2 which was significant (Figure 6.3 A lane 7 maximal activation was taken as 1, n=3, $P<0.05$; compare to lane 8, activation 0.59+/-0.02, n=3, $P<0.05$). PD98059 reduces only by 20% the appearance of phosphorylated MAP Kinases in 208F cells after induction with 100 ng/ml EGF (significant with $P<0.05$, see Figure 6.4A lane 8 and Figure 6.5A lane 8, activation for 208F pre-treated with PD 98059 and treated with EGF: 0.79+/-0.04, n=3, $P<0.05$).

FBR A221 clones 6, 19 and 23 pre-treated with PD 98059 then stimulated with 10% FCS exhibited a reduction in the levels of phosphorylated ERK's (Figure 6.3A lane 12 ; Figure 6.3B lanes 8 and 12, e.g. clone 19, $P<0.05$). This suggests that: in the case of serum the FBR

A221 revertant clones are sensitive to the inhibition by PD 98059. When PD 98059-pretreated FBR A221 clones were stimulated with 100 ng/ml of EGF there was not a complete inhibition and still phosphorylated ERK1 and ERK2 bands appear in clones 6 and 19 (Figure 6.5A lane 12, Figure 6.5B lanes 8/ For example in the FBR A221 clone 6 the activation after pre-treatment with PD 98059 and subsequent EGF induction was 0.11+/-0.01, n=3, $P<0.05$; in the FBR A221 clone 19 the activation was 0.24+/-0.5, n=3, $P>0.05$ and in the FBR A221 clone 23 the activation was 0.21+/-0.01, n=3, $P<0.05$). These data show some similarities with the results from the 208F cells pre-treated with PD 98059 and stimulated with EGF where there is not an inhibition in the MAP Kinase phosphorylation (Figure 6.5A lane 8 ; Figure 6.6A lane 8)

The experiments with the MEK-1-specific inhibitor PD 98059 in 208F cells suggested that EGF and serum can activate MAP Kinase phosphorylation in the presence of the MEK-1 inhibitor PD 98059. This effect could be partially mediated by a non-MEK-1 signalling mechanism. When activation of MAP Kinases is measured by phosphospecific antibodies it was found to be increased in*rat*-1 fibroblasts compared to other cell lines such as NIH 3T3 and Hela cells (Yung *et al.*, 1997). Using MAP Kinase phosphospecific antibodies this group also showed that activation of the MAP Kinase pathway in *rat*-1 fibroblasts is elevated, sustained and maximally activated by purified growth factors such as EGF. Others have suggested that there might be alternative ways to activate MEK-1. These studies have demonstrated the existence of MEK-1 activators other than Raf-1 kinase (Zheng *et al.*, 1994; Pang et al., 1995; Pritchard *et al.*, 1995). Saltiel and co-workers have identified an alternative regulator of MEK-1 although have so far been unable to clone this kinase (Zheng *et al.*, 1994). McMahon and co-workers have identified that different forms of the Raf-1 kinase exert diverse responses in fibroblasts to the activation of MAP Kinases and the induction of DNA synthesis (Pritchard *et al.*, 1995), which may account for the results presented here.

6.7. FBR A221 revertants have increased content of F-actin

Cells transformed by oncogenic viruses have a decreased content of F-actin resulting from the reduction of number of stress fibres detected by staining with Rhodamine-labelled Phalloidin or actin-specific antibodies (Weber *et al.*, 1974; Pollack *et al.*, 1975).

To gain insight into the possible involvement of the dominant negative mutant of MEK-1 A221 into the reversion of *fos*-induced transformation, the actin distribution and actin content of

FBR A221 revertants in comparison to the parental FBR cells was investigated. In order to pursue this question a fluorescent immunostaining approach was used and the results analysed using a Bio-Rad MRC Laser Confocal Illumination unit attached to a Nikon Diaphot inverted microscope.

208F cells when actively growing in media with 10% FCS are well attached and they have actin stress fibres. Upon serum deprivation actin stress fibres in 208F cells do not disappear completely and the cells remain well attached to the plastic substratum (Figure 6.7a). Immunostaining with an monoclonal antibody for phosphotyrosine (P-tyr) to visualise focal adhesions showed that focal adhesions were detectable in 208F cells when starved and these are localised to the cell periphery (Figure 6.7a).

In contrast to 208F cells, FBR have a spindle-shaped morphology in monolayer culture and contain few actin stress fibres. Actin in these cells is disorganised (Figure 6.7c). FBR have a very small number of focal adhesions when quiescent, although these can still be detected by staining with the monoclonal antibody specific for phosphotyrosine (Figure 6.7c). In FBR focal adhesions are found in the cell periphery and actin is found concentrated to the end of the cell in the area of the pseudopod.

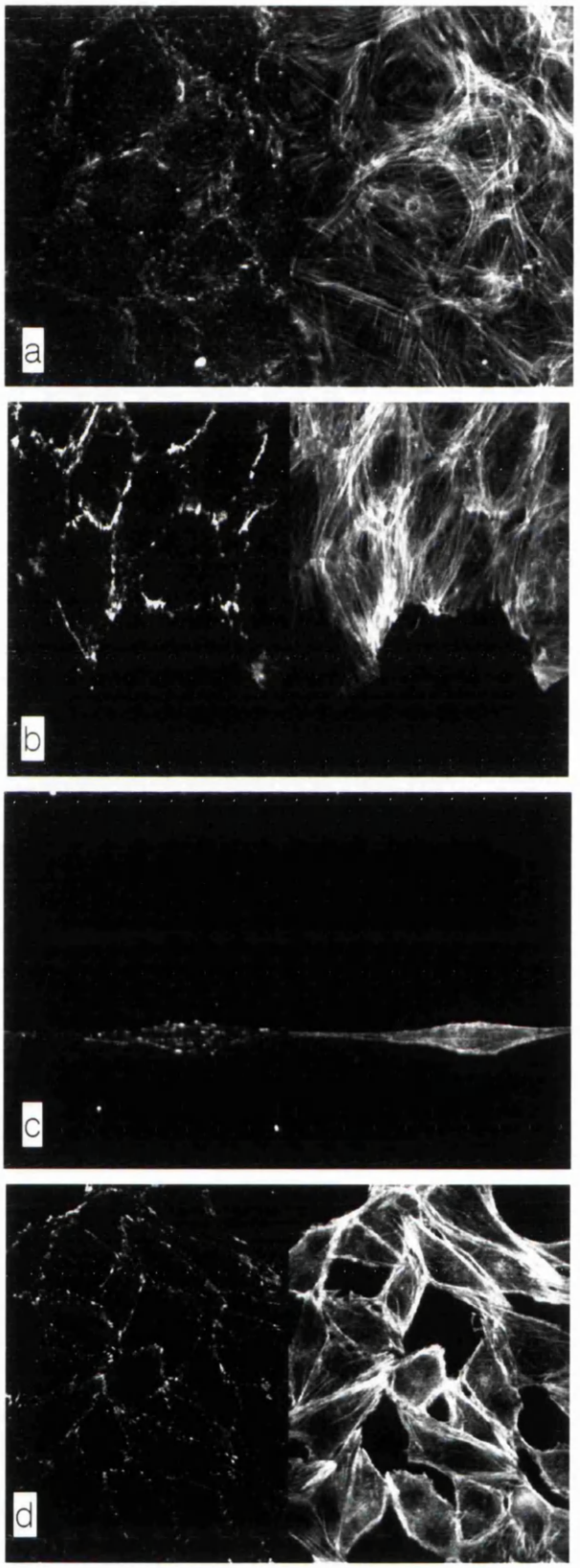
Treatment of quiescent 208F with 10% FCS results in a rapid reorganisation of the actin components into well defined actin stress fibres as shown in Figure 6.7b after immunostaining with Rhodamine-Phalloidin. Serum contains a component, most probably the lipid LPA, which is a potent inducer of actin reorganisation (Ridley and Hall, 1992).

In FBR the activation of MAPK by LPA is poor but serum and LPA are potent in their ability to induce actin filament redistribution upon stimulation for a short time (Figure 6.7d). This conserved function between normal and transformed fibroblasts suggest that serum might have a role in the anchorage-dependent growth, which might be dissociated from activation of MAP Kinase signalling.

Figure 6.7. Serum-induced Actin reorganisation in FBR and 208F cells.

Cell lines were plated in DMEM with 10% FCS and after 24-48 hours were serum deprived for 7 days. After this period of time cells were stimulated with 10% FCS for 5 minutes, fixed, permeabilized and stained with TRITC-conjugated Phalloidin to visualise actin and with a monoclonal antibody against phosphotyrosine to visualise focal adhesions. This is an experiment representative of at least three independent experiments.

Figure 6.7



208F 0.1% FCS

208F + 10% FCS

FBR 0.1 % FCS

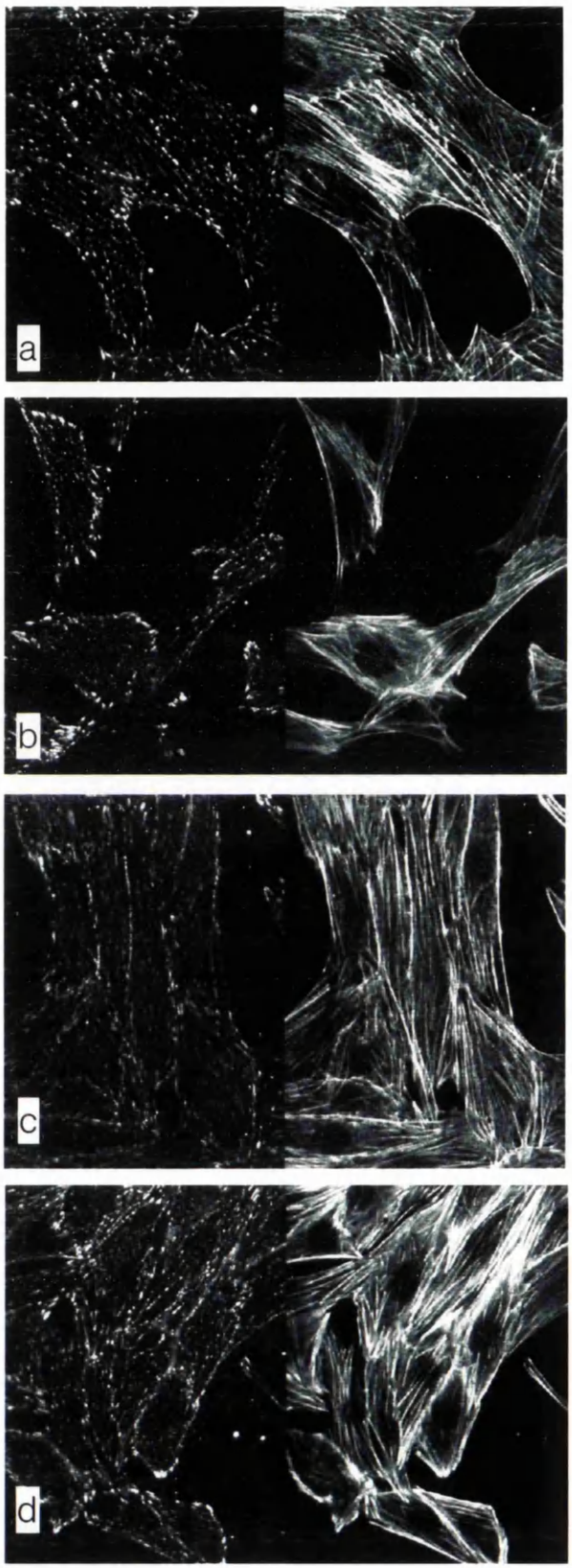
FBR + 10% FCS

In FBR A221 revertants the phalloidin-detectable F-actin filaments are increased, and the cytoskeleton is organised in a similar manner to 208F cells where actin is present in a large number of stress fibres (Figure 6.8 a and c). Staining for phosphotyrosine (P-tyr) to visualise focal adhesions showed that these structures are localised to the cell periphery and are abundant compared to 208F cells (Figure 6.8 a and c). Stimulation with serum resulted in a redistribution of F-actin to the periphery and an increased number of fibres (Figure 6.8 picture b and d) similar to 208F fibroblasts (Figure 6.8 b). In the FBR A221 revertant cells the focal adhesion components are reorganised by serum stimulation as shown by indirect immunofluoresence (Figure 6.8 c and d)

Figure 6.8. Serum-induced Actin reorganisation in FBR A221 transfectants

FBR A221 clones 19 and 23 were plated in DMEM with 10% FCS and after 24-48 hours were serum deprived for 7 days. After this period of time cells were stimulated with 10% FCS for 5 minutes fixed, permeabilized and stained with TRITC-conjugated Phalloidin to visualise actin and with a monoclonal antibody against phosphotyrosine to visualise focal adhesions. This is an experiment representative of at least three independent experiments.

Figure 6.8



6.8. Serum-dependent actin reorganisation in FBR A221 revertants

In an attempt to identify mechanisms of actin reorganisation, Ridley and Hall (1994) have examined the role of various activators of pathways involved in mitogenic signalling. Instead of serum and growth factors, they used activators of PKC and PKA to investigate changes in the actin distribution in Swiss 3T3 cells. Their data suggest that a requirement for a tyrosine kinase signal is important in the reorganisation of the actin component. Activators of PKA such as forskolin do not inhibit the formation of new stress fibres by LPA or by activated V14rho proteins. This suggests that although PKA signals disrupt stress fibre formation in Swiss 3T3 cells they do not lead to the prevention of formation of new actin fibres.

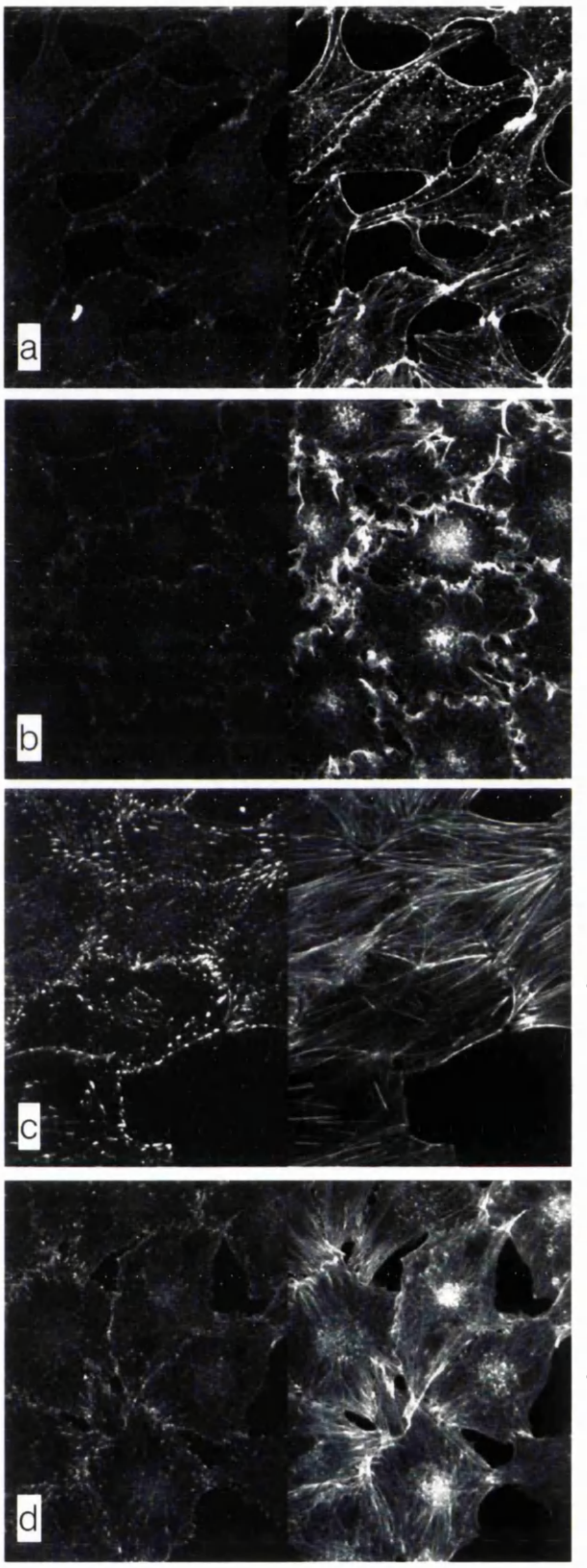
Incubation of serum-deprived 208F cells with 2 μM dibutyril-cAMP for 30 minutes to enhance endogenous PKA activity resulted in a dramatic reorganisation of the cytoskeleton in the form of peripheral actin resembling the effect of PDGF or Insulin on Swiss 3T3 cells (Figure 6.9b). Incubation of serum-starved 208F cells with 10% FCS resulted in the formation of stress fibres and new focal adhesions (Figure 6.9c). When these cells were pre-treated with dibutyril-cAMP and subsequently with 10% FCS for 5 minutes, the membrane ruffling activity due to PKA activation was to a large extent overcome by the effect of serum (Figure 6.9d) suggesting that serum was responsible for the formation of new stress fibres, in accordance with Ridley and Hall (1994).

Figure 6.9. Actin reorganisation in 208F cells by cAMP and serum.

Cells lines were plated in DMEM supplemented with 10% FCS and after 24 hours were serum deprived for 7 days. After this period, cells stimulated with 10% FCS, 2 μM dbcAMP, their combination or remained untreated. Cells were fixed, permeabilized and stained with TRITC-conjugated Phalloidin to visualise actin stress fibres and with a monoclonal antibody against phosphotyrosine to stain Focal Adhesions. This is an experiment representative of three independent experiments.

a) 208F fibroblasts in 0.1% FCS ; **b)** 208F fibroblasts stimulated for 5 minutes with 10% FCS ; **c)** 208F fibroblasts stimulated for 30 minutes with 2 μM of dbcAMP ; **d)** 208F fibroblasts stimulated for 20 minutes with 2 μM of dbcAMP and treated then with 10% FCS.

Figure 6.9


a
208F 0.1% FCS
b
208F + dbcAMP
c
208F +10% FCS
d
208F dbcAMP / 10% FCS
p-tyr
Actin

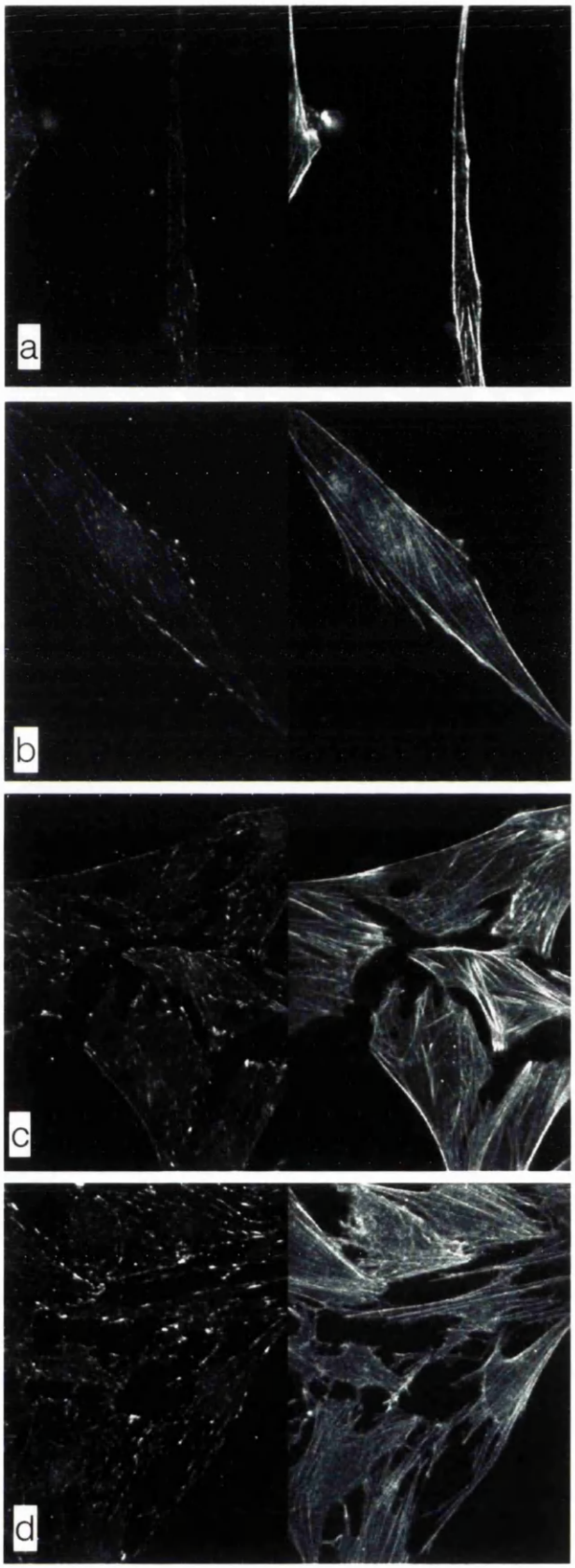
Treatment of quiescent FBR with dibutyril-cAMP did not lead to the induction of membrane ruffling and surprisingly induced the appearance of few stress fibres and an increase in cell volume, effects that are induced by the addition of foetal calf serum to the media. This treatment did not lead to the formation of new focal adhesions suggesting that the increase of the intracellular cAMP which opposes LPA and Rho signalling is not sufficient to mediate this event (Ridley and hall, 1992; Ridley and hall, 1994). Treatment with 10% FCS induced the reorganisation of the actin cytoskeleton and the reappearance of new focal adhesions (Figure 6.10c). Pre-treatment with dibutyril-cAMP for 30 mimutes and subsequent treatment with 10% FCS for 5 minutes lead to a similar situation to that observed in 208F's where focal adhesions and actin stress fibres reform, suggesting that the activation of PKA is not sufficient to lead to the dissolution of the reformed actin network and that the serum-induced response is sufficient for this effect (Figure 6.10d)

Figure 6.10. Actin reorganisation in FBR cells by cAMP and serum.

Cells lines were plated in DMEM supplemented with 10% FCS and after 24 hours were serum deprived for 7 days. After this period, cells stimulated with 10% FCS, 2 μM dbcAMP, their combination or remained untreated. Cells were fixed, permeabilized and stained with TRITC-conjugated Phalloidin to visualise actin stress fibres and with a monoclonal antibody against phosphotyrosine to stain Focal Adhesions. This is an experiment representative of three independent experiments.

a) FBR in 0.1% FCS ; **b)** FBR stimulated for 5 minutes with 10% FCS ; **c)** FBR stimulated for 20 minutes with 2 μM of dbcAMP ; **d)** FBR stimulated for 30 minutes with 2 μM of dbcAMP and treated then with10% FCS.

Figure 6.10


a
b
c
d
FBR 0.1% FCS
FBR + dbcAMP
FBR +10 % FCS
FBR + dbcAMP / 10% FCS
p-tyr
Actin

Tthe induction of actin reorganisation in the FBR A221 revertants by cAMP activation of PKA was also examined. Serum deprived FBR A221 cells display a flat morphology and have stress fibres (Figure 6.11a). Treatment with dibutyril-cAMP does not have any dramatic effect in the actin distribution neither nor on the formation of focal adhesions (Figure 6.11b). Treatment with 10% FCS results in reorganisation of the cytoskeleton and focal adhesions in the cell periphery (Figure 6.11c). This suggests that the pathway of serum induction of stress fibres remains unaltered by the transfection of the MEK-1 A221 expression vector. In the FBR A221 cells there is a constitutive appearance of actin stress fibres. This might be related to the presence of the dominant negative mutant MEK-1 A221.

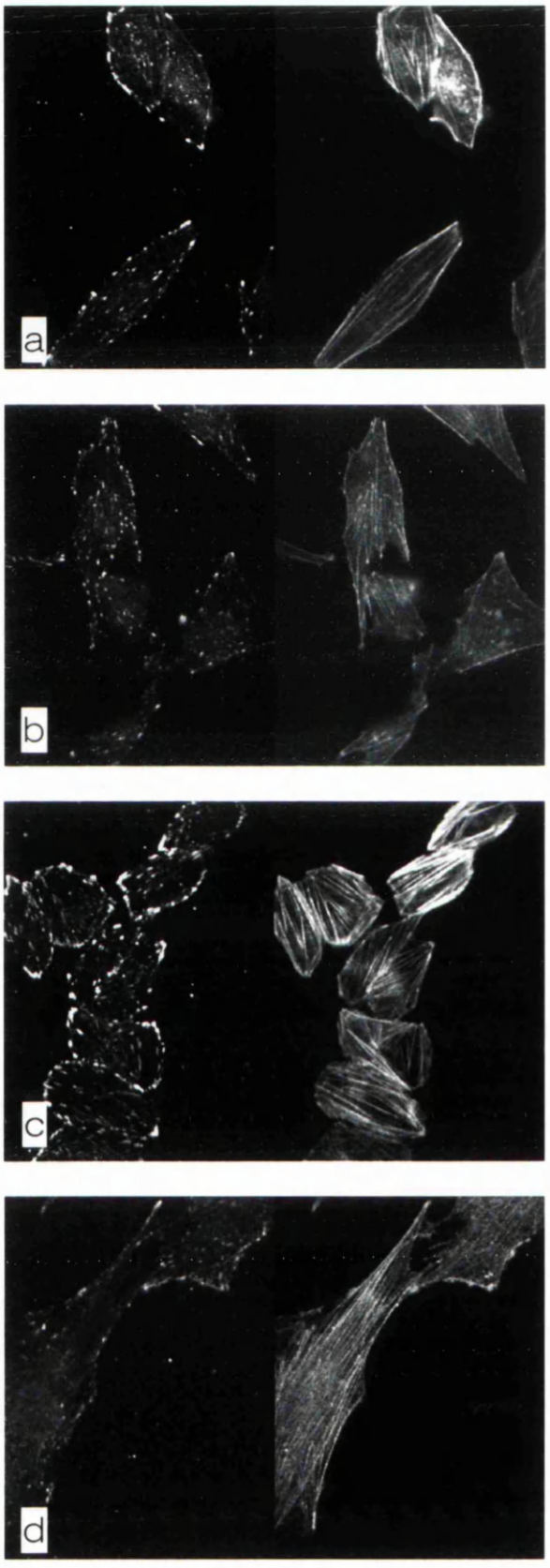
These data suggest that A221 is somehow related to the reversion of the transformed morphology through the induction of the microfilament network, but the presence of this dominant negative mutant does not prevent reorganisation of the actin network as in the case of FBR TAM-67 cells.

Figure 6.11. Actin reorganisation in FBR A221 cells by cAMP and serum.

Cells lines were plated in DMEM supplemented with 10% FCS and after 24 hours were serum deprived for 7 days. After this period, cells stimulated with 10% FCS, 2 μM dbcAMP, their combination or remained untreated. Cells were fixed, permeabilized and stained with TRITC-conjugated Phalloidin to visualise actin stress fibres and with a monoclonal antibody against phosphotyrosine to stain Focal Adhesions. This is an experiment representative of three independent experiments.

a) FBR A221 clone 19 in 0.1% FCS ; **b)** FBR A221 clone 19 stimulated for 5 minutes with 10% FCS ; **c)** FBR A221 clone 19 stimulated for 30 minutes with 2 μM of dbcAMP ; **d)** FBR A221 clone 19 stimulated for 20 minutes with 2 μM of dbcAMP and treated then with10% FCS.

Figure 6.11



FBR A221 0.1 %

FBR A221 + dbcAMP

FBR A221 + 10 % FCS

FBR A221 + dbcAMP / 10 % FCS

6.9. PD98059 modulates formation of actin stress fibres in FBR

FBR A221 revertant cell lines have an increased stress fibre content. Here the effect of the MEK-1 specific inhibitor PD98059 on the actin filament distribution and the reversion of the transformed phenotype was tested.

This inhibitor has been shown to reverse of v-*ras* transformation after treatment of *ras*-transformed cells for 24 hours with 50 μM of the inhibitor (Dudley *et al.*, 1995). Incubation of v-Ki-*ras* transformed cells growing in 10% FCS with PD98059 resulted in a time-dependent reversion of morphological transformation, as shown by Rhodamine-labelled Phalloidin staining of the actin stress fibres (Figure 6.12 d and f).

Figure 6.12. Actin reorganisation in v-Ki-*ras* transformed cells by PD98059.

Cells were plated in DMEM supplemented with 10% FCS for 24 hours, and subsequently treated for 24 or 48 hours hours in the presence of 10% FCS with the MEK-1 inhibitor PD 98059 at a final concentration of 50 μM. To visualise actin stress fibres cells were fixed, permeabilized and stained with TRITC-conjugated Phalloidin by indirect immunofluorescence microscopy. This is a representative experiment of 2 independent experiments.

v-Ki-ras

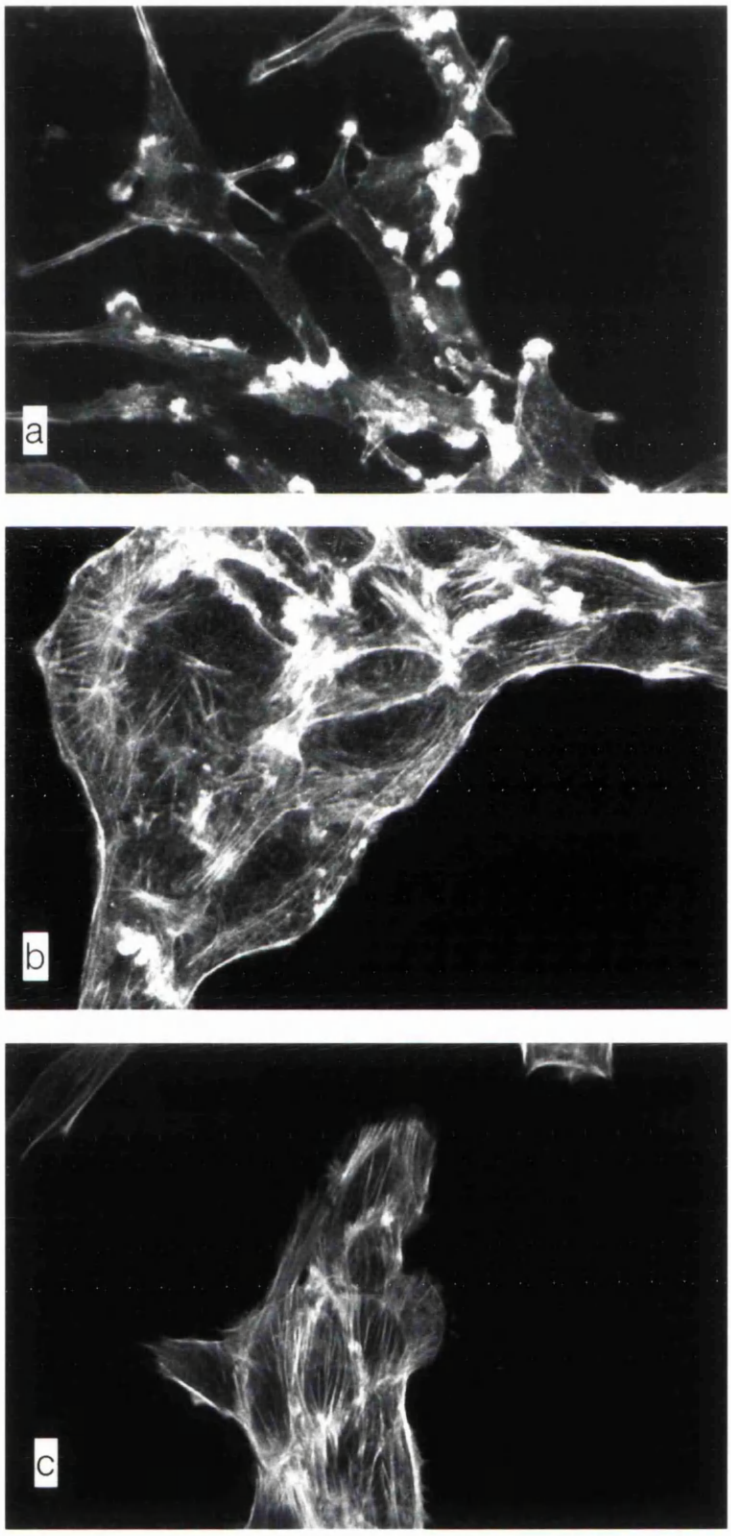


Figure 6.12

10% FCS

24 Hrs
50μM PD 98059

48 Hrs
50μM PD 98059

FBR cells growing in 10% FCS were incubated with PD98059, a partial reversion and reduction of the membrane ruffling activity in the pseudopodes of FBR was observed (Figure 6.13 c and e, compared to control FBR picture a). Use of this inhibitor at a higher concentration resulted in an increase of the F-actin content (Figure 6.14 c and d) as well as a reduction in the membrane ruffling.

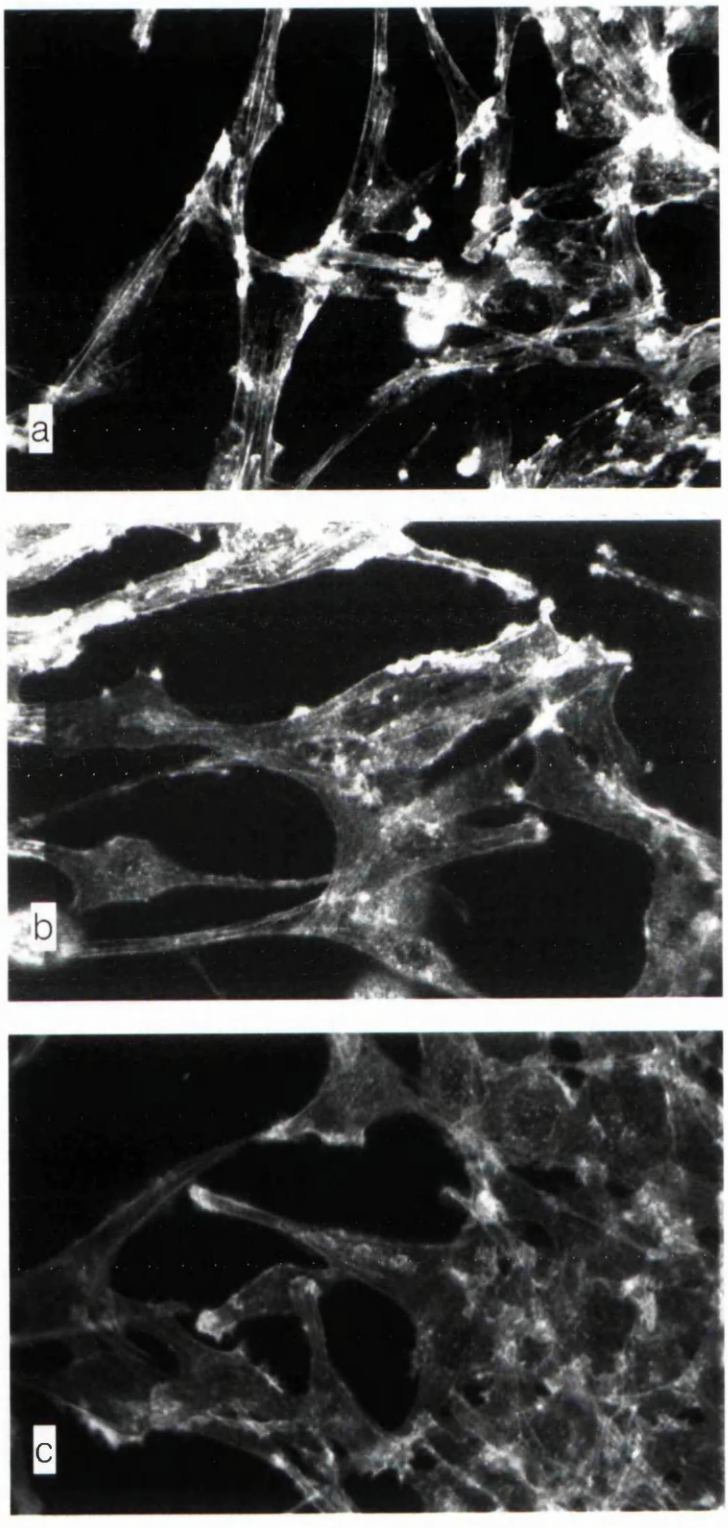
As shown previously in this chapter, FBR A221 revertant show a decreased membrane ruffling activity (Figure 6.8). This is in agreement with the results observed here, since a decrease in membrane ruffling after treatment of the FBR with the MEK-1-specific inhibitor PD98059 was seen.

Figure 6.13. Actin reorganisation in FBR by PD98059.

Cells were plated in DMEM supplemented with 10% FCS for 24 hours, and subsequently treated for 24 or 48 hours hours in the presence of 10% FCS with the MEK-1 inhibitor PD 98059 at a final concentration of 50 μM. To visualise actin stress fibres cells were fixed, permeabilized and stained with TRITC-conjugated Phalloidin by indirect immunofluorescence microscopy. This is a representative experiment of at least three independent experiments.

FBR

Figure 6.13



10% FCS

24 Hrs
50μM PD 98059

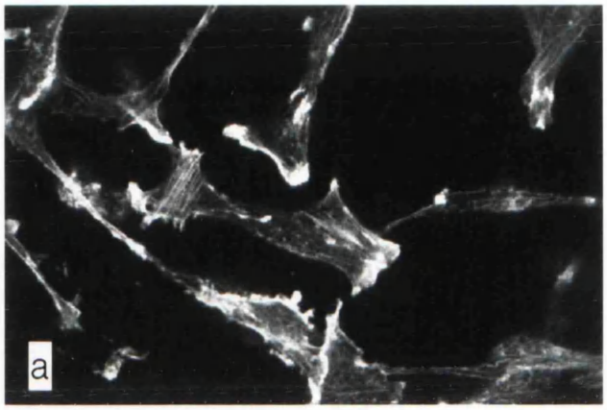
48 Hrs
50μM PD 98059

Figure 6.14. Concentration-dependent actin reorganisation in FBR by PD98059.

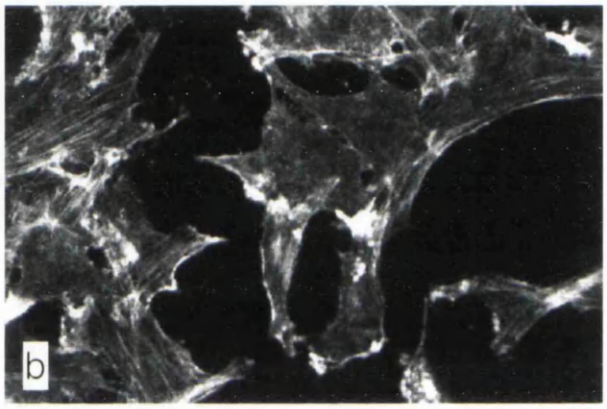
Cell lines were plated in DMEM supplemented with 10% FCS for 24 hours and treated for the indicated periods of time with the MEK-1 inhibitor PD 98059. All treatments took place in cells growing in DMEM supplemented with 10% FCS. To visualise actin stress fibers, cells were fixed, permeabilized and stained with TRITC-conjugated Phalloidin by indirect immunofluorescence microscopy . This experiment is a representative of three independent experiments.

a) FBR control in 10% FCS; **b)** FBR treated with 50 μM PD 98059 for 24 hours; **c)** FBR treated with 100 μM PD 98059 for 24 hours; **d)** FBR treated with 100 μM PD 98059 for 48 hours.

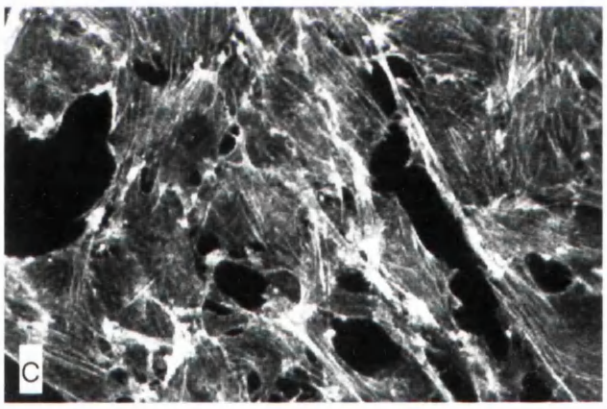
Figure 6.14



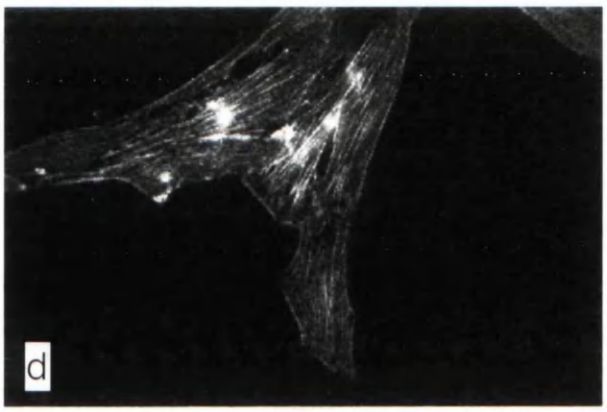
FBR 10% FCS



FBR 50 μM PD 98059, 24 hrs



FBR 100 μM PD 98059, 24 hrs



FBR 100 μM PD 98059, 48 hrs

6.10. Conclusion

Dominant negative MEK-1 abrogates v-*ras* and v-*src* induced transformation (Cowley *et al.*, 1994) and constitutively active MEK-1 transforms NIH 3T3 cells (Cowley *et al.*, 1994 ; Mansour *et al.*, 1994 ; Alessandini *et al.*, 1996 ; Greulich *et al.*, 1997). From this evidence it is apparent that the role of MEK-1 is not very well established for other types of oncogenes and its role in anchorage independence has not been investigated (Cowley *et al.*, 1994).

In this study a transfection approach was used to examine the role of a dominant negative MEK-1 in FBR v-*fos*-induced transformation. After transfection of a vector encoding a dominant negative MEK-1 with an Alanine substitution in the regulatory Serine residue 221 it was found that anchorage independence growth of FBR cells was suppressed and phosphorylation of MAP Kinases by serum was reduced.

It has been reported that MEK-1 A221 inhibits partially MAP Kinase phosphorylation by the neuropeptide bombesin in Swiss 3T3 cells and this induction could be effectively reduced by PD98059 (Seufferlein *et al.*, 1996). MEK-1 A221 and A217 had also a negative regulatory role in the induction of DNA synthesis by bombesin in Swiss 3T3 cells but not in the combination of bombesiin with an additional growth factor such as Insulin (Seufferlein *et al.*, 1996).

The MEK-1-specific inhibitor PD98059 can effectively abolish phosphorylation of MAP Kinases by serum and EGF in FBR cells, but not completely in 208F cells. This indicates the existence of another putative MEK isoform in 208F since PD98059 inhibits MEK-1 by binding to its activation site. Another possibility in the case of 208F cells is that the response to EGF-induced signalling is maintained at high levels and thus a higher amount of PD 98059 is required to abolish MAP Kinase induction.

RESULTS CHAPTER 7

GENERATION OF FBR REVERTANT CELL LINES FOLLOWING TRANSFECTION OF THE DOMINANT NEGATIVE MUTANT OF Raf-1 KINASE, NΔ Raf-1

RESULTS

CHAPTER 7. GENERATION OF FBR REVERTANT CELL LINES FOLLOWING TRANSFECTION OF THE DOMINANT NEGATIVE MUTANT OF Raf-1 KINASE, NΔ Raf-1

7.1. Raf-1 kinase is a major constituent of the Growth Factor- ras- AP-1 pathway

A major signal transduction pathway that is stimulated by growth factors involves the small GTP-binding oncoprotein $p21^{ras}$ which belongs to a family of small GTP-binding proteins. $p21^{ras}$ is also a frequently mutated target protein in different types of human cancer (Barbacid, 1987).

A requirement for $p21^{ras}$ in signalling downstream of Receptor Tyrosine Kinases and upstream of the c-*raf*-1 protooncogene was first shown in microinjection experiments (Smith *et al.,* 1986). The product of the c-*raf*-1 protooncogene, which is the cellular homologue of the v-raf oncogene (Rapp *et al.,* 1983), encodes the Serine/ Threonine Kinase Raf-1. The v-*raf* oncogene induces cellular transformation of established cell lines, and microinjection of a mutant c-Raf-1 protein results in cellular transformation of NIH 3T3 cells (Smith *et al.,* 1990).

GTP-bound $p21^{ras}$ forms complexes with Raf-1 and MEK-1, a dual specificity threonine/ tyrosine kinase MAP Kinase Kinase (Moodie *et al.,* 1993). Raf-1 kinase activates MEK-1 by phosphorylation (Kyriakis *et al.,* 1992; Dent *et al.,* 1992; Howe *et al.,* 1992). MEK-1 phosphorylates MAP Kinases resulting in activation of their catalytic activity. MAP Kinases have multiple targets such as nuclear transcription factors and cytoplasmic signalling molecules (Treisman, 1996). Raf-1 also is required for transcriptional activation of AP-1 and regulation of AP-1-dependent genes (Bruder *et al.,* 1992).

Taken together these results suggest that Raf-1 kinase might indeed be a central point of the *ras*-growth factor signalling pathway. Raf-1 kinase as mentioned already functions as a positive regulator of MAP Kinase signalling and AP-1 transcriptional activity, but also can serve other functions. These include involvement in cellular transformation (Samuels *et al.,* 1993), cell cycle (Lovric and Moelling, 1996), cell proliferation (Kerkhoff and Rapp, 1997) and cell growth (Kerkhoff and Rapp, 1998).

In this study a dominant negative mutant of Raf-1 kinase, NΔRaf-1, was used to investigate an involvement of Raf-1 in v-*fos* transformation. To approach this question FBR cells were transfected with the NΔRaf-1 and stable transfectants were generated. These cell lines have the properties of the revertant and a possible role of ΔNRaf-1 in reversion is discussed.

7.2. Transfection of NΔ Raf-1 in FBR and 208F cells

In order to continue the study of the MAP Kinase pathway in FBR cells, Raf-1 kinase activity was inhibited by transfection of the an expression vector encoding for a C-terminally truncated Raf-1 kinase NΔ Raf-1 (Schaap *et al.,* 1993). This mutant lacks the catalytic domain of Raf-1 kinase and has been shown to be a negative regulator of MAP Kinase signalling in mammalian cells by blocking activation of MAP Kinases by EGF and TPA (Schaap *et al.,* 1993).

The pEXV-NΔ Raf-1 vector was transfected together with the pSV2neo, which confers G418 resistance as a means for selection of transfectants. After selection in G418 for approximately three weeks, stable cell clones expressing NΔ Raf-1 were isolated for phenotypic and biochemical analysis.

After selection with G418 was completed the transfection dishes were stained with Giemsa to assess relative colony formation. A reduction of approximately 0.7-fold in total colony number was observed for FBR cells after transfection of NΔRaf-1 when compared with FBR cells transfected only with the pSV2neo control vector alone (Table 7.1). Therefore introduction of NΔRaf-1 did not result in inhibition of colony formation in FBR in contrast to the transfections with the dominant negative Jun TAM-67 mutant and the kinase-inactive MEK-1 A221. The NΔRaf-1 mutant was also introduced in the 208F cells. In this case a slight increase in the colony number by 1.2-fold was observed.

Table 7.1. Colony formation after transfection of NΔ Raf-1 into FBR and 208F cells.

Cells were transfected with the indicated amounts of CsCl-purified plasmid DNA using lipofection. After 3-4 weeks post-transfection and selection in DMEM/10% FCS containing 400 μg/ml G418 the colonies were fixed in 100% Methanol, washed with PBS, and stained with 10% Giemsa solution (v/v in H_2O). The approximate number of G418 resistant colonies is indicated.

Cell Line	*Transfected Plamsid*	*Number of G418-Resistant colonies*
FBR	neo 1 μg	155 colonies
FBR	NΔ Raf-1/neo 5μg/1μg	122 colonies
208F	neo 1 μg	150 colonies
208F	NΔ Raf-1/neo 5μg/1μg	200 colonies

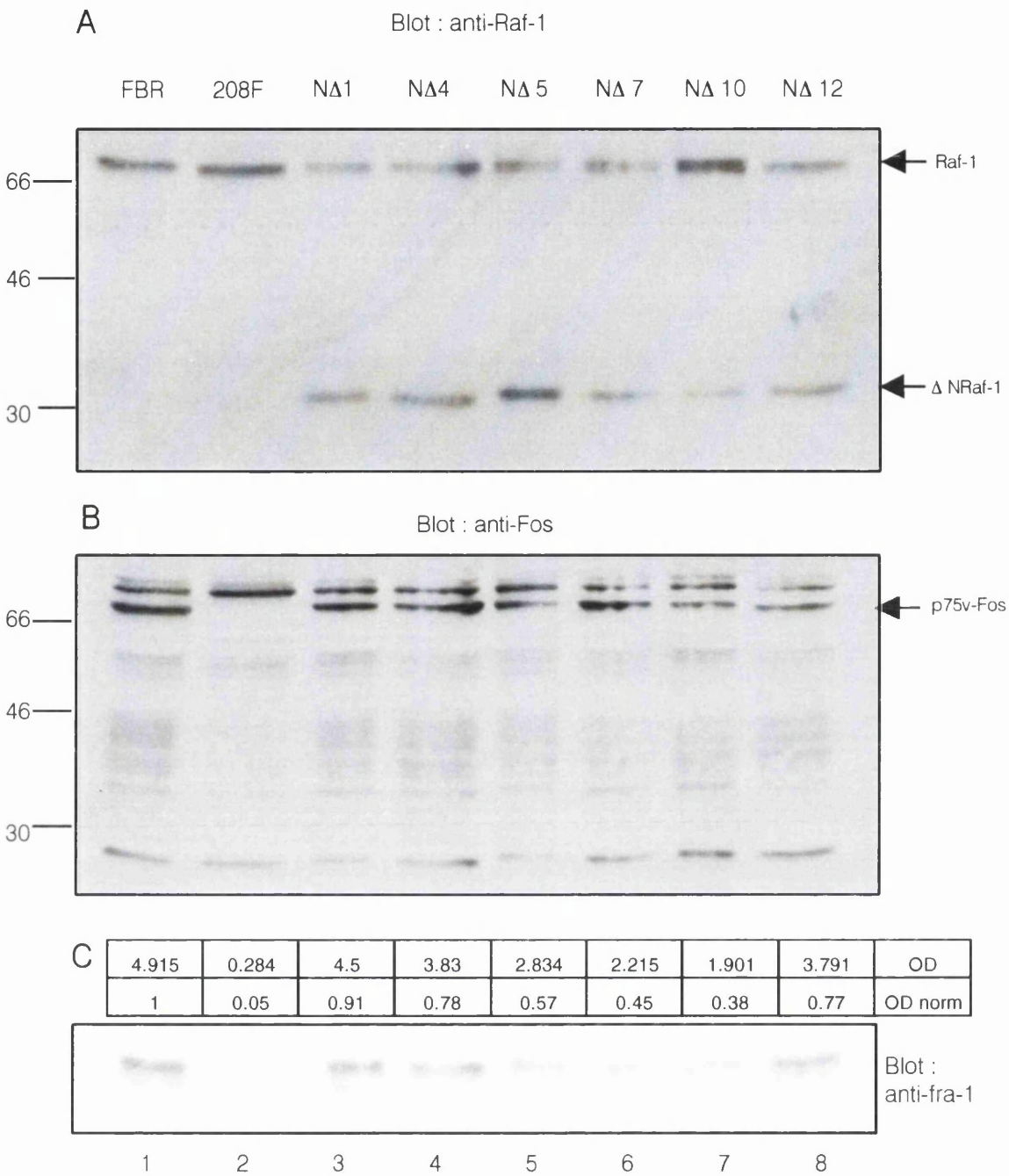
From this series of transfections revertant cells lines were isolated demonstrating a flat morphology. After the isolation of these revertants the expression of the NΔRaf-1 and the endogenous Raf-1 protein levels were analysed, to assess the efficiency of the transfection in FBR cells.

Cultures growing in DMEM supplemented with 10% FCS were washed twice with ice-cold PBS and lysed with standard Triton X-100 lysis buffer. The lysates were normalised for protein content and 100 μg of total cell lysate was separated by 10% SDS-polyacrylamide gels electrophoresis (Figure 7.1). The proteins were transferred to a PVDF membrane and blotted with a monoclonal antibody specific for the Raf-1 N-terminus. In the FBR, 208F cells and also in the 6 FBR NΔ raf-1 revertant cell lines a c-Raf-1-specific immunoreactive band of 70 kD corresponding to endogenous kinase was observed (Figure 7.1A, lanes 1, 2, 3, 4, 5, 6, 7, 8). In the FBR ΔNRaf-1 revertant 1, 4, 5, 7,10, and 12 clones a band of approximately 30kD which corresponds to the transfected NΔ Raf-, was also observed (Figure 7.1A, lanes 3, 4, 5, 6, 7 ,8) according to the previous published reports (Schaap *et al.,* 1993).

These revertant clones were also examined for expression of the p75 $^{v\text{-}Fos}$ transforming oncoprotein to ensure that it is still expressed at sufficient levels and that is not lost during the process of selection. To confirm p75 $^{v\text{-}Fos}$ expression the same membrane shown in the Figure 7.1A was stripped and probed with the K-25 antibody which identifies p75 $^{v\text{-}Fos}$ and also broadly reacts with different Fos proteins (Figure 7.1B). p75 $^{v\text{-}Fos}$ protein was seen in parental FBR cells (Figure 7.1B lane1) and in the FBR NΔRaf-1 revertant 1, 4, 5, 7, 10, and 12 cell lines (Figure 7.1B, lanes 3, 4, 5, 6, 7, 8). Therefore the transfection process did not affect significantly the expression of the transforming p75 $^{v\text{-}Fos}$ oncoprotein.

In an attempt to look for differences in *fos*-effector genes, at the levels of the Fra-1 protein, a known v-*fos*-effector which is an AP-1 component (Cohen and Curran, 1988; Bergers *et al.,* 1995). Transformation by v-*fos* and *fos*B increases the levels of *fra*-1 mRNA and protein (Bergers *et al.,* 1995; Mechta *et al.,* 1997). The filter in Figure 7.1B and was stripped and probed with the N-17 antibody specific for the Fra-1 protein. As shown in Figure 7.1C, in FBR immunoreactive bands were observed which migrated at the expected size of the Fra-1 proteins on an 10% SDS-polyacrylamide gel (Franza *et al.,* 1987). The Fra-1 protein expression appears

to be greately reduced in lysates prepared from cycling 208F fibroblasts (Figure 7.1C lane 2). Expression of Fra-1 protein was also detected in the lysates prepared from cycling FBR NΔ Raf-1 revertants (Figure 7.1C lanes 3, 4, 5, 6, 7 , 8). In four out of the six FBR NΔ Raf-1 revertant clones (Figure 7.1C lanes 5, 6, 7, 8) we found that Fra-1 protein levels were approximately 2-fold reduced compared to the parental FBR cells as determined by scanning densitometry (Figure 7.1C lane 1).



C

4.915	0.284	4.5	3.83	2.834	2.215	1.901	3.791	OD
1	0.05	0.91	0.78	0.57	0.45	0.38	0.77	OD norm

1 2 3 4 5 6 7 8

Figure 7.1. Expression patterns of $p75^{v\text{-}Fos}$, Raf-1 and NΔ Raf-1 and Fra-1 proteins in FBR, 208F cells and FBR transfected with the dominant negative C-terminal deletion mutant of Raf-1 Kinase, ΔNRaf-1.

Cell lines were grown in DMEM supplemented with 10% FCS and when sub-confluent, cell lysates were prepared. 100μg of total cell lysate was separated on 10% SDS-PAGE, transferred to PVDF membrane, and blotted with an antibody against **A)** Raf-1 (Transduction Labs, KY, USA). The same membrane was stripped and blotted with antibodies against **B)** Fos (K-25, Santa Cruz, Bio) and **C)** fra-1 (N-17, Santa Cruz, Bio.) to visualise the expression of Fos and Fra-1 proteins.
1) FBR cells **2)** 208F cells; **3)** FBR NΔ Raf-1 revertant clone 1; **4)** FBR NΔ Raf-1 revertant clone 4; **5)** FBR NΔ Raf-1 revertant clone 5; **6)** FBR NΔ Raf-1 revertant clone 7; **7)** FBR NΔ Raf-1 revertant clone 10; **8)** FBR NΔ Raf-1 revertant clone 12.
In the panel **C** the integrated and normalised OD values after scanning densitometry of the Fra-1 western are shown.

7.3. Morphological Characteristics of FBR NΔRaf-1 transfectants

FBR cells have a transformed morphology when maintained in 10% FCS (Figure 7.2 a). In contrast the cell lines that were isolated after transfection of NΔRaf-1 into FBR cells have a flat morphology (Table 7.2). As shown in the Figure 7.2 (c, d, e, e, f, d, h) transfection of FBR cells with NΔ Raf-1 reverted v-*fos*-induced transformation. The FBR NΔ Raf-1 expressing cell lines showed a flat morphology when grown in cycling conditions (Figure 7.2).

It was also observed that the morphological reversion of FBR NΔRaf-1 revertants was density dependent and the flat phenotype was more pronounced in higher cell densities. One of the clones was chosen to demonstrate the appearance of the revertant phenotype at low and a higher cell density. As illustrated in Figure 7.3, FBR NΔRaf-1 clone 4 showed an increased ability to revert at a higher density.

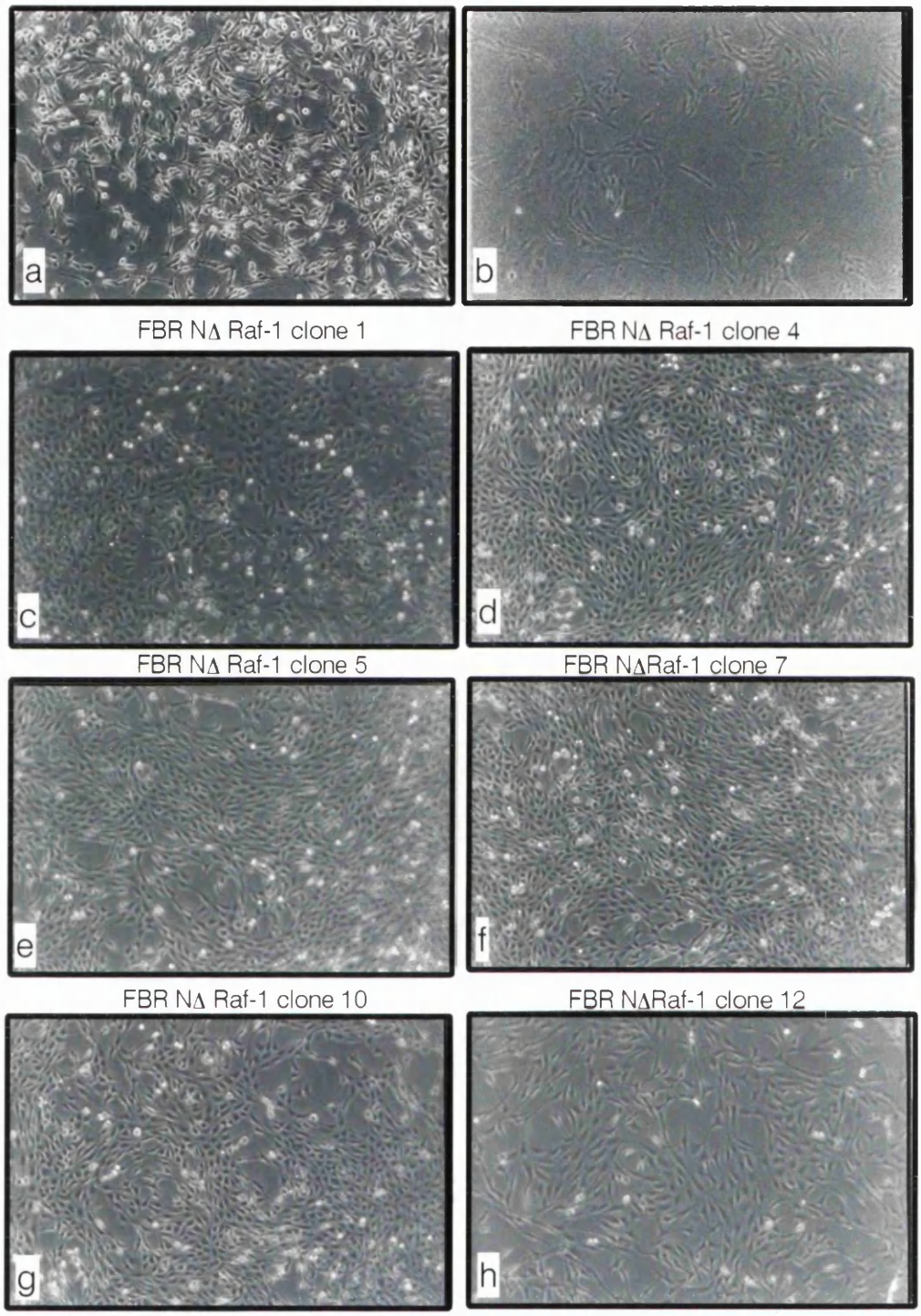


Figure 7.2. Morphology of FBR, 208F cells and FBR ΔN Raf-1 revertants.
Cell lines were grown in DMEM supplemented with 10% FCS for 24 hours and photographed with am inverted Nikon Diaphot microscope.
a) FBR cells ; **b)** 208F cells ; **c)** FBR NΔ Raf-1 revertant clone 1 ; **d)** FBR NΔ Raf-1 revertant clone 4 ;**e)** FBR NΔ Raf-1 revertant clone 5 ; **f)** FBR NΔ Raf-1 revertant clone 7 ; **g)** FBR NΔ Raf-1 revertant clone 10 ; **h)** FBR NΔ Raf-1 revertant clone 12.

Table 7.2. Morphology of FBR neo and FBR NΔ Raf-1 transfected cells lines.

Cell lines were plated in DMEM containing 10% FCS and 400 μg/ml G418 on 10cm tissue culture dishes and were allowed to attach for 48 hours to the plastic substratum to identify individual cell morphologies.

Cell line	*Transfected plasmid*	*Morphology*
FBR neo 1	neo	transformed
FBR neo 2	neo	transformed
FBR neo 3	neo	transformed
FBR neo 4	neo	transformed
FBR NΔ 1	NΔ Raf-1 / neo	flat
FBR NΔ 2	NΔ Raf-1 / neo	flat
FBR NΔ 4	NΔ Raf-1 / neo	flat
FBR NΔ 5	NΔ Raf-1 / neo	flat
FBR NΔ 7	NΔ Raf-1 / neo	flat
FBR NΔ10	NΔ Raf-1 / neo	flat
FBR NΔ 12	NΔ Raf-1 / neo	flat

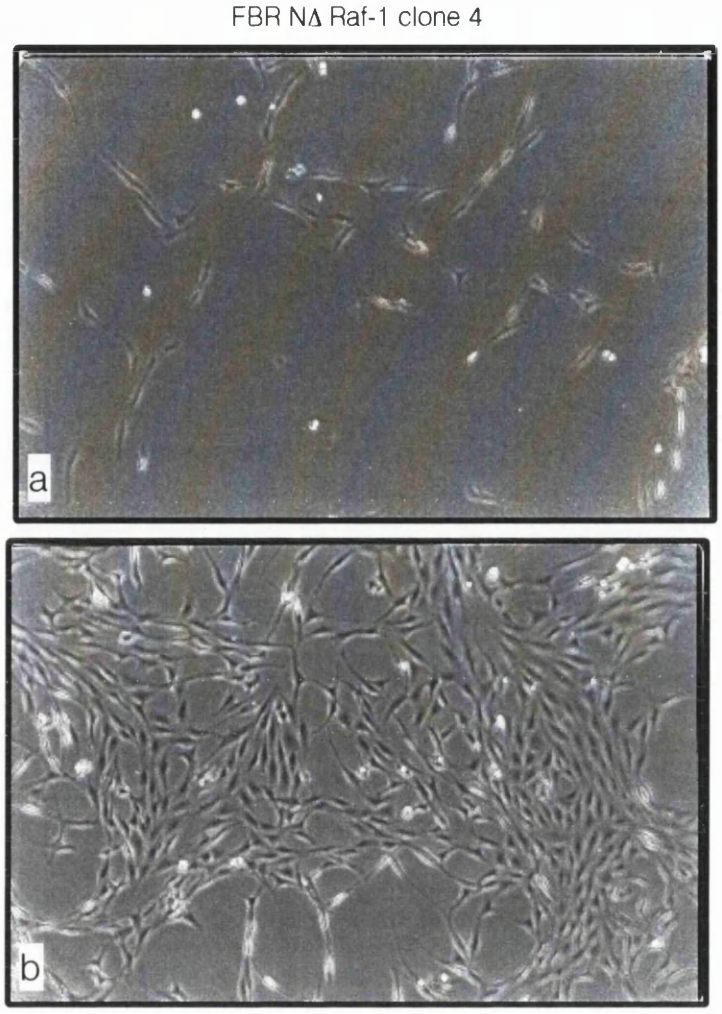


Figure 7.3. Morphological reversion at different cell densities.

Morphology of FBR NΔ Raf-1 revertant clone 4 shown at two different cell densities a) low density and b) high density.
Cell lines were grown in DMEM supplemented with 10% FCS and photographed with a Nikon inverted Diaphot microscope.

7.4. NΔ Raf-1 inhibits v-fos-induced anchorage independence

The parental FBR, 208F and the FBR NΔRaf-1 revertants were tested for their ability to grow in methylcellulose, that is under anchorage-independent conditions. Cells from the 6 revertant clones: FBR NΔ1, FBR NΔ4, FBR NΔ5, FBR NΔ7, FBR NΔ10, and FBR NΔ12, were trypsinized from cultures growing in DMEM supplemented with 10 % FCS and resuspended in serum-free media. Cell number was determined and $2x10^4$ cells plated in Ham's medium supplemented with 10% FCS containing 1,5% methylcellulose. The cells were allowed to grow for a period of 20 days. Colonies were observed using a light microscope (Figure 7.4 c, d, e, e, f, d, h) and counted to measure the approximate number present in each plate.

208F cells do not grow in anchorage-independent conditions (Figure 7.4b), however FBR cells showed a high colony-forming ability in semi-solid medium (Figure 7.4 a). A decrease in the colony formation efficiency (measured by the relative number of colonies formed per $10cm^2$ dish and this number divided by the number of cells plated initially) was seen for the clones expressing the dominant negative NΔ Raf-1 mutant. Table 7.3 summarises the results of this experiment.

These results suggest a correlation between the expression of NΔ Raf-1 and a decrease in the colony forming ability in semi-solid medium of the FBR NΔ Raf-1. These data suggest a role for the NΔ Raf-1 kinase mutant in suppression of v-*fos*-induced transformation.

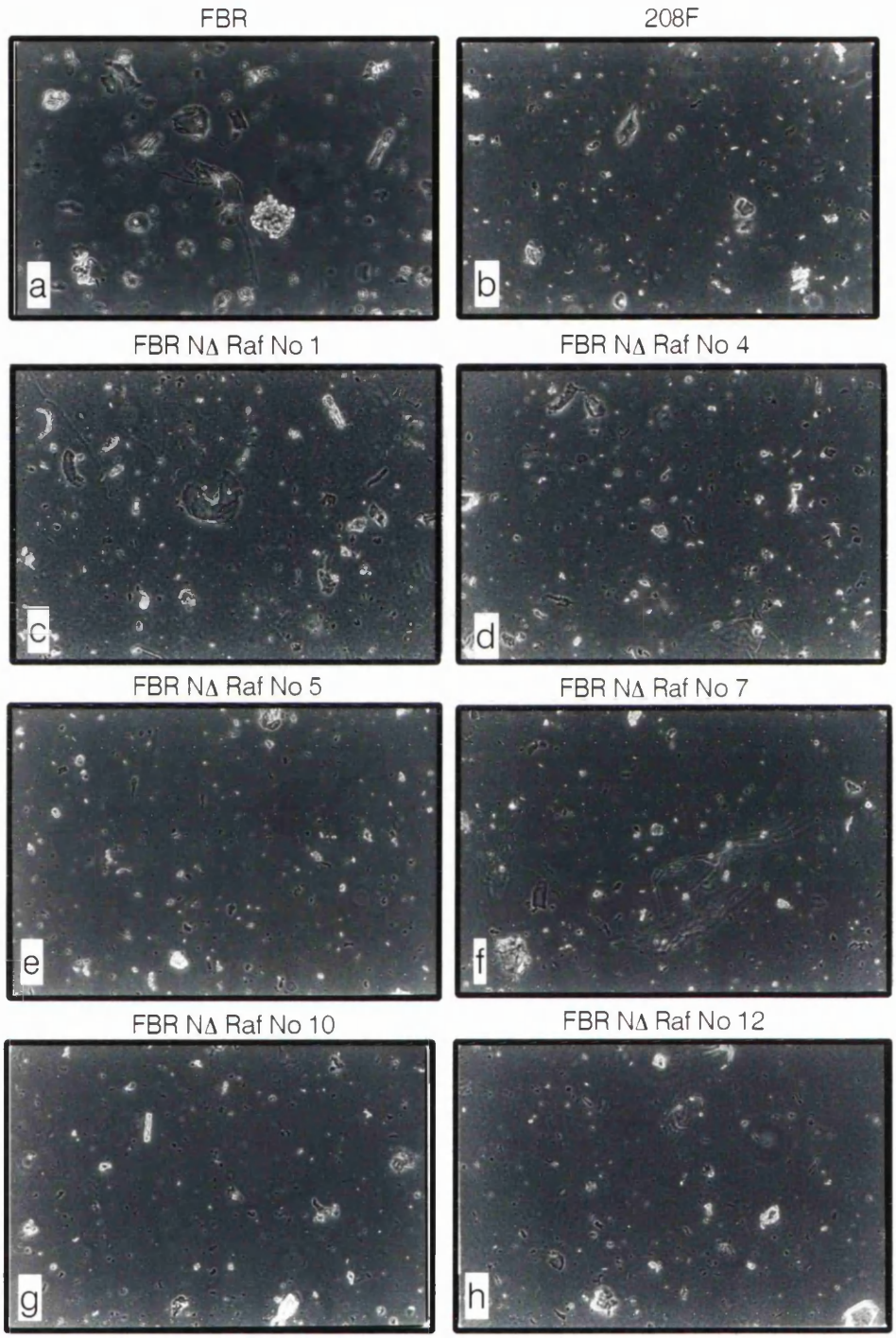


Figure 7.4. Growth in semi-solid medium of FBR, 208Fand FBR transfected with the dominant negative C-terminal deletion mutant of Raf-1 Kinase, NΔ Raf-1.

Cells were grown in methylcellulose containing serum for 20 days an photographed with a Nikon inverted Diaphot microscope. **a)** FBR cells ; **b)** 208F cells ; **c)** FBR NΔ Raf-1 revertant clone 1 ; **d)** FBR NΔ Raf-1 revertant clone 4 ; **e)** FBR NΔ Raf-1 revertant clone 5 ; **f)** FBR NΔRaf-1 revertant clone 7 ; **g)** FBR NΔ Raf-1 revertant clone 10 ; **h)** FBR NΔ Raf-1 revertant clone 12.

Table 7.3. Anchorage-Independent growth of FBR and FBR NΔ Raf-1 revertants.

$2x10^4$ cells from each cell line were plated in Ham's media containing 1.5% Methylcellulose. Colonies were counted after 3 weeks. These results represent two independent experiments, where triplicate samples were assayed.

Cell Line	*Average Colony Number*	*Mean*	*Ratio*
FBR	8064 - 8256	8160	0.4
FBR NΔ 1	806.4 - 672	739	0.03
FBR NΔ 4	144 - 380	262	0.01
FBR NΔ 5	768 - 960	864	0.04
FBR NΔ 7	921 - 816	868	0.04
FBR NΔ 10	48 - 240	144	0.007
FBR NΔ 12	144 - 96	120	0.006

7.5. The role of NΔ Raf-1 in serum-induced MAP-Kinase activation

Previously it has been shown that the NΔ Raf-1 has inhibits activation of MAP Kinases by EGF and TPA (Schaap *et al.,* 19930. Here the ability of NΔ Raf-1 to prevent MAP Kinase activation by serum in the FBR NΔ Raf-1 expressing cells was examined, compared to the activation in the parental FBR cells. To test this an antibody specific for the phosphorylated forms of MAP Kinase isoforms ERK1 and ERK2 was used for western analysis. Cell lysates were prepared from quiescent and serum-stimulated cells total using the standard Triton X-100 lysis buffer. Lysates were cleared by centrifugation, normalised for protein content, and 50 μg of lysate were separated by 10% SDS-Polyacrylamide gel electrophoresis, transferred to a PVDF membrane and blotted with the MAPK phospho-specific antibody.

Serum induces strong MAP Kinase phosphorylation in 208F as shown in Figure 7.5 lane 4 compared to FBR cells. In the FBR NΔ Raf-1 clones 1 and 4 activation of MAP Kinases ERK1 and ERK2 was 1.5-fold reduced compared in FBR cells (Figure 7.5A lanes 6 and 8, 0.29+/-0.13,n=3, $P>0.05$ in FBR cells and 0.17+/-0.005, n=3, $P>0.03$ in FBR NΔ Raf-1 clone 1 and 0.16+/-0.03, n=3 $P<0.05$ in FBR NΔ Raf-1 clone 4). These two clones express higher levels of the transfected mutant NΔ Raf-1 compared to FBR NΔ Raf-1 clone 10 (Figure 7.1C lanes 3 and 4). The relative inhibition of serum-induced MAP Kinase phosphorylation in FBR NΔ Raf-1 clone 1 was found to be reduced not very significantly compared to serum-stimulated FBR cells ($P>0.05$). In the FBR NΔ Raf-1 clones 4 and 10 the inhibition of ERK activation was found to be significant ($P<0.05$) and the activation state of MAP Kinases remained and similar to FBR as determined by scanning densitometry (Figure 7.5 lane 10). This particular clone contains reduced levels of NΔ Raf-1 mutant (Figure 7.1C lane 7). For the same experiment when ERK1 activation was measured using scanning densitometry it was found that in all three clones was reduced to approximately 2-fold compared to the FBR untransfected control cells (Figure 7.5 top panel above A, lanes 6,8 and 10). The differences in the activation status of ERK1 might reflect the potency of NΔ Raf-1 to reduce the phosphorylation of this isoform.

van Blitterswijk and co-workers (van Dijk *et al.,* 1997) have shown that the activation of MAP Kinases in Rat-1 cells transfected with the NΔ Raf-1 mutant stimulated with EGF or PDGF

is reduced by 2-fold. A similar result is seen with the FBR NΔ Raf-1 clones, suggesting a functional response to serum when the NΔ Raf-1 mutant is present in the FBR cells.

Since the inhibition of ERK1 and ERK2 phosphorylation in the FBR NΔ Raf-1 clones 1 and 4 is not striking the effect of NΔ Raf-1 in the suppression of FBR *fos*-induced transformation could be ascribed to another function of this dominant negative mutant.

It is known that the NΔ Raf-1 is an inhibitor of $p21^{ras}$ function in mammalian cells (Kolch et al., 1991). The putative inhibition of *ras* function by NΔ Raf-1 in FBR NΔ Raf-1 cells could be associated with the suppressor effect of this mutant in the MAP Kinase pathway or another pathway downstream of $p21^{ras}$. The ability of $p21^{ras}$ to control also the Phosphoinositide 3-Kinase, PI3K (Rodriguez-Viciana *et al.*, 1994, 1997; Khwaja *et al.*, 1997) and the Ral-GDS pathways (Urano *et al.*, 1996) in regulating cellular transformation is well established. Such a pathway could be involved in the transformation-suppressor function of NΔ Raf-1 in FBR cells. To addresssuch a question pharmacological inhibitors or dominant negative mutants could be used to identify a possible role of PI3K or Ral-GDS in FBR *fos*-induced transformation and anchorage independence.

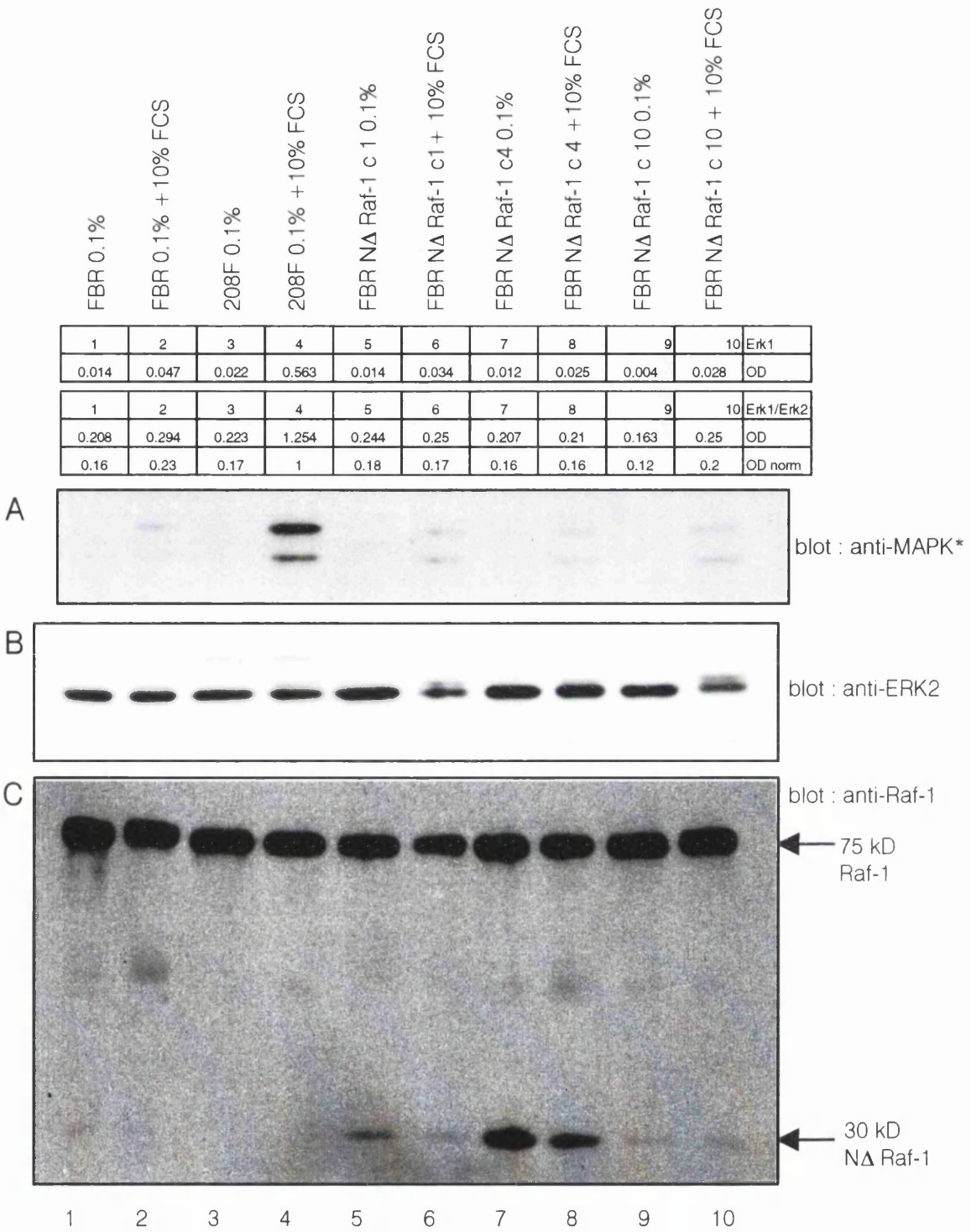
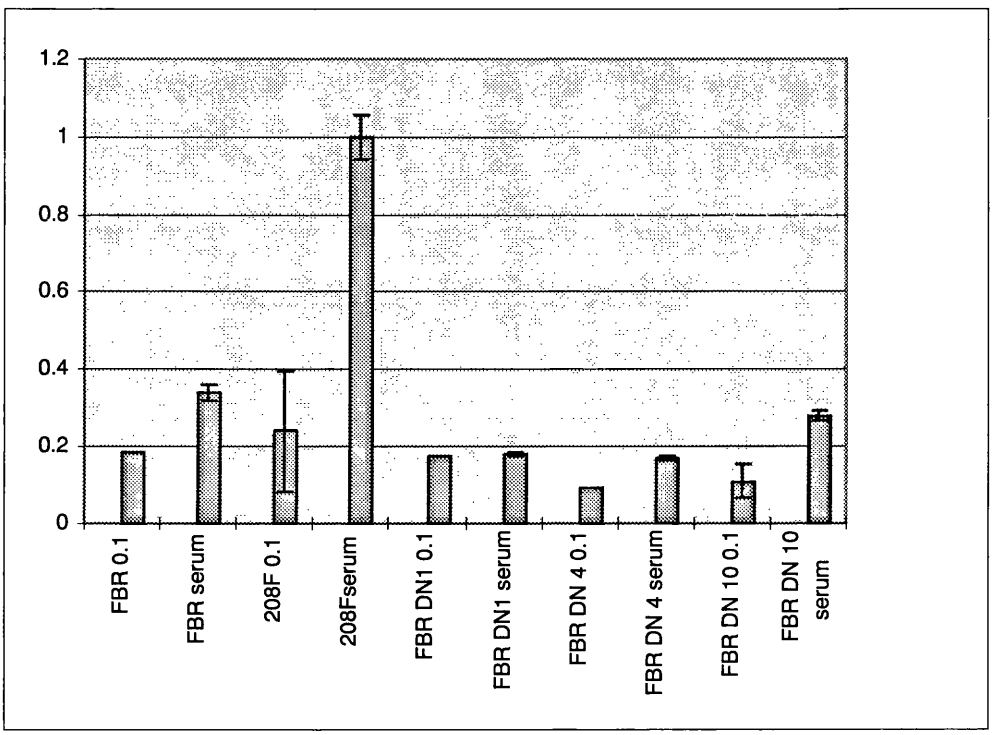


Figure 7.5. Serum-induced MAPK phosphorylation in FBR, 208F and FBR transfected with the dominant negative mutant of Raf-1 kinase, NΔ Raf-1.

Cell lines were grown to subconfluence in DMEM supplemented with 10% FCS and serum-deprived for 48 hours (0.1% FCS). Stimulation was for 5 minutes with 10% FCS. Cell lysates were prepared and 50 μg of total cell lysate was separated by 10% SDS-PAGE, transferred to PVDF membranes and probed with an antibody specific for **A)** the phosphorylated MAP Kinases ERK1 and ERK2 (MAPK*, Promega Labs, WI, USA).
Blots were stripped and probed with an antibody specific for **B)** ERK-2 as a loading control (ERK-2) (Transduction Labs, KY, USA) and for **C)** Raf-1 (Transduction, Labs, KY, USA).
Quantification was performed using scanning densitometry. In the case of quantification of the total ERK1 and ERK2 signal by densitometry the OD values presented are derived from automatic subtraction of the backround using the Pdi software. In the case of the quantification of the ERK1 signal the OD values presented are derived from subtraction of the total backround from the film.

Table 7.4 The effect of the dominant negative mutant of Raf-1, NΔ Raf-1 in the serum-stimulated MAP Kinase phosphorylation in FBR, 208F and FBR NΔ Raf-1 revertants.

Quantification of the increase in phosphorylation of ERK1 and ERK2 was performed using scanning densitometry. The band of serum-stimulated 208F cells was taken as the maximal, 100% activation. The values were normalised for control loading of ERK2 for each individual experiment. The values are the mean +/- standard deviation of three independent experiments.



7.6. FBR NΔRaf-1 revertants have increased F-Actin content

It has been shown that an inverse correlation exists between morphological transformation and the cellular content of actin stress fibres (Weber *etal.,* 1974; Pollack *et al.,* 1975; Ozanne *et al.,* 1980; Lamb *et al.,* 1997). FBR cells have a reduced number of actin stress fibres and also have a fraction of actin which is soluble and diffuse (Hennigan, 1993). In contrast, 208F cells have stress fibres when grown in the presence of serum and maintain a number of stress fibres when are serum-deprived.

The distribution of F-actin in FBR, 208F and FBR NΔRaf-1 revertant cell lines was examined by staining with Rhodamine-labelled Phalloidin and fluorescence microscopy. Cels grown in serum were fixed, permeabilised and stained with Rhodamine-Phalloidin to visualise actin.

FBR cells appear bipolar, contain a small number of actin stress fibres and have extensive membrane ruffling activity localised at the cell extensions (Figure 7.6 a). 208F cells are flat and contain actin stress fibres in large numbers extending from one side of the cell to the other.

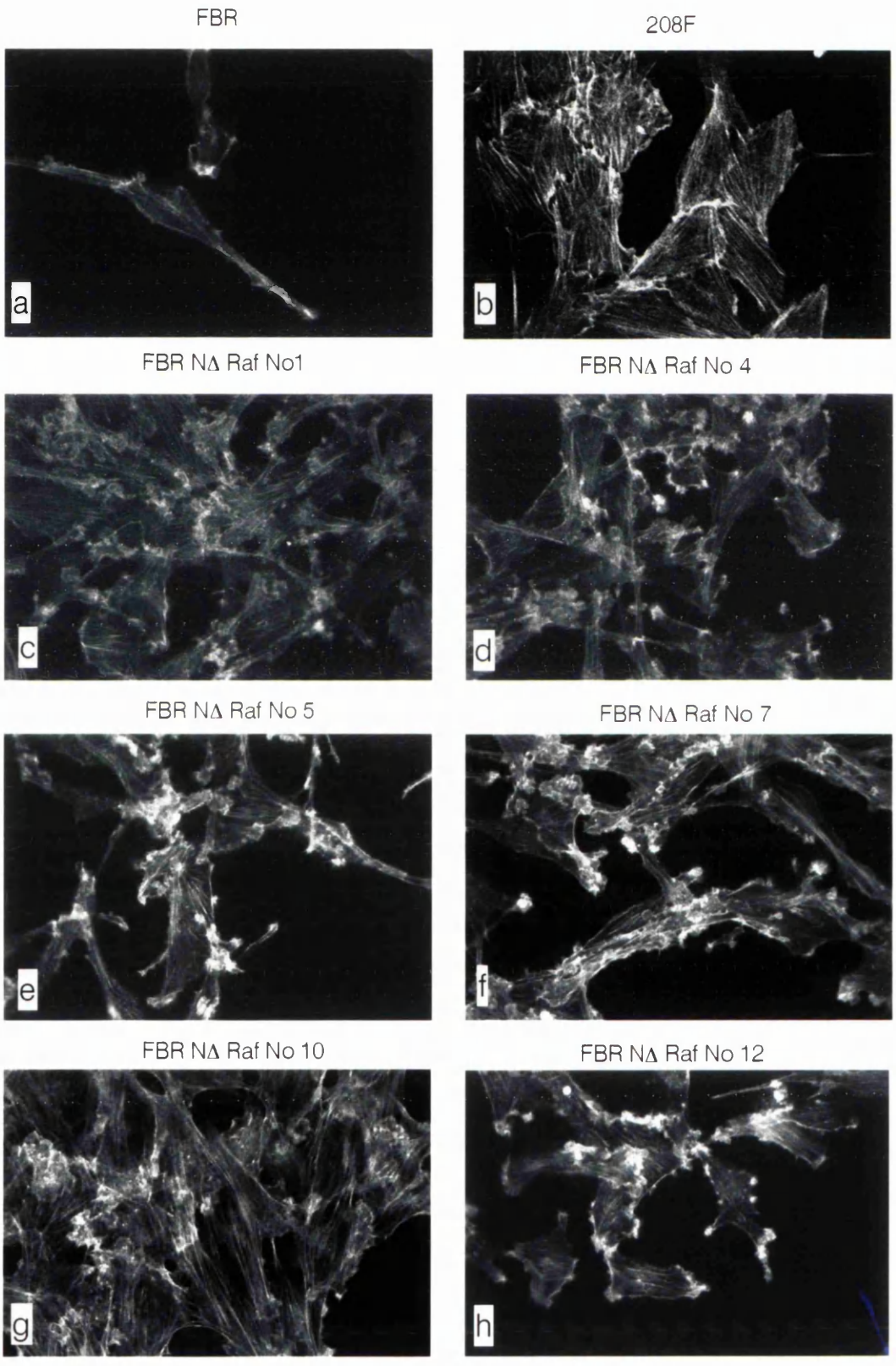
When the actin distribution was examined in the FBR NΔaf-1 revertants, the actin content was significantly increased and the cells contained stress fibres (Figure 7.6 c, d, e, e, f, g, h). Careful examination showed that the cells were morphologically reverted but a significant number of membrane ruffling activity was still present. The ability of NΔRaf-1 to revert *fos*-induced transformation and to induce stress fibre formation but not inhibit membrane ruffling is associated with the role of Raf-1 in transformation pathways (Khosravi-Far *et al.*, 1996; Joneson *et al.*, 1996).

Figure 7.6. Actin filament distribution in FBR, 208F and FBR transfected with the dominant negative mutant of Raf-1 kinase, NΔRaf-1.

Cell lines were plated on coverslips, and grown in DMEM supplemented with 10% FCS. To visulaise actin stress fibres, cells were fixed, permeabilised and stained with TRITC-conjugated Phalloidin. Images were analysed and processed using a Bio-Rad MRC 600 Confocal microscope. These figures are representative from two independent experiments.

a) FBR cells; **b)** 208F cells; **c)** FBR NΔRaf-1 revertant clone 1; **d)** FBR NΔRaf-1 revertant clone 4; **e)** FBR NΔRaf-1 revertant clone 5; **f)** FBR NΔRaf-1 revertant clone 7; **g)** FBR NΔRaf-1 revertant clone 10; **h)** FBR NΔRaf-1 revertant clone 12.

Figure 7.6


FBR
208F
a
b
FBR NΔ Raf No1
FBR NΔ Raf No 4
c
d
FBR NΔ Raf No 5
FBR NΔ Raf No 7
e
f
FBR NΔ Raf No 10
FBR NΔ Raf No 12
g
h

7.7. The effect of Raf-1 inhibitor L-779 450 in serum-induced MAPK phosphorylation

Recently, L-779 450, a novel Raf-1 Kinase-specific inhibitor has been described. This compound inhibits Raf-1 kinase activity *in vivo* at a low micromolar range. L-779 450 inhibits also *in vitro* the enzymatic activity of Raf-1 kinase.

In experiments similar to those in the previous chapter where MEK-1 inhibitor PD98059 was used, here L-779 450 was used to study serum-induced MAP Kinase phosphorylation in FBR, 208F and v-Ki-*ras* transformed cells. In serum-treated quiescent FBR there is a 2-3 fold increase in MAPK phosphorylation (Figure 7.7. lane 3). This induction can be inhibited by 2-fold with pre-treatment of the quiescent FBR with 3 μM L-779 450 for 30 minutes (Figure 7.7 lane 3 and 4). In serum treated 208F cells a strong induction of MAP Kinase phosphorylation could be reduced 3-fold by pre-treatment with L-779 450 of serum-stimulated 208F cells (Figure 7.7 lane 7 and lane 8). These data show that L-779 450 can inhibit the induction of MAP Kinases in FBR and 208F cells by serum.

In v-Ki-*ras* transformed 208F cells MAP Kinase phosphorylation basal levels were higher compared to FBR and 208F serum-starved cells (Figure 7.7 lane 9), suggesting that the pathway is efficiently activated by the presence of the oncogenic *ras* (Howe *et al.,* 1992; Kyriakis *et al.,* 1992; Dent *et al.,* 1992). Also in v-Ki-*ras* cells MAP Kinase phosphorylation did not change significantly after serum stimulation (Figure 7.7A lane 11). This correlates with evidence from others where *ras* oncogenes did not contribute to the enhancement MAP Kinase activation after serum stimulation (Samuels and McMahon, 1994; Pritchard *et al.,* 1995; Olson *et al.,* 1998). In these experiments it was found that the basal and also the serum-induced phosphorylation of MAP Kinases in *ras*- transformed cells was sensitive to inhibition by L-779 450 (Figure 7.7 lanes 10 and 12). In contrast to this the phosphorylation of MEK-1 was not inhibited in serum-stimulated v-Ki-*ras* cells (Figure 7.7B, lane 12) suggesting the existence of an alternative MEK-1 activator.

In addition to MAPK activation the activation state of MEK-1 was examined in FBR, 208F and v-Ki-*ras* cells. The blot shown in Figure 7.7A was stripped and probed with the MEK-1 phosphospecific antibody (Figure 7.7B). In this case phosphorylated MEK-1 was induced dramatically by serum in 208F (Figure 7.7B; lane 7). In the case of 208F cells as in the v-Ki-*ras* cells the phosphorylation of MEK-1 is not completely alleviated by L-779 450 (Figure 7.7B; lane 8). Serum-deprived v-Ki-*ras* cells also contain high levels of phosphorylated MEK-1 (Figure 7.7

B lane 9) which are elevated in response to serum (Figure 7.7 B 11). MEK-1 phosphorylation is sensitive to inhibition of Raf-1 activity by L-779 450 in the *ras* transformed cells (Figure 7.7 B lane 10). This shows a direct dependence of MEK-1 activation on the presence of the transforming *ras* oncogene, a potent activator of Raf-1 kinase (Howe *et al.,* 1992; Kyriakis *et al.,* 1992; Dent *et al.,* 1992). In FBR cells, the magnitude of MEK-1 activation was much weaker and inhibited 2-fold by L-779 450 (Figure 7.7 B lane 3 and 4 respectively) showing a similar pattern for MEK-1 activation to MAP Kinase activation in this cell type (Figure 7.7 panel A lanes 3 and 4).

Phosphorylation of the Raf-1 C-terminus, through a MAPK-dependent mechanism results in a decrease in its electrophoretic mobility in SDS-polyacrylamide gels (Williams *et al.,* 1992, 1993; Ferrier *et al.,* 1997). L-779 450 treatment of cells resulted in a diminished phosphorylation-dependent activation of Raf-1 kinase in FBR, 208F and v-Ki-ras cells (Figure 7.7 C, lanes 4, 8 and 12 respectively), compared to its activation by serum (Figure 7.7 C, lanes 3, 7 and 11 respectively). This shows that phosphorylation of Raf-1 detected by mobility shift is MAP Kinase-dependent Raf-1 specific. Previously MAP Kinase activation has been correlated with mobility shift assays in western immunoblotting experiments (Posada and Cooper, 1992; Leevers and Marshall, 1992) and with phosphospecific antibodies (Olson *et al.,* 1998).

The Table 7.5 summarises data from experiments with the L-779 450 and its effect in the inhibition of MAPK phosphorylation in FBR, 208F and v-Ki-*ras* cells. In the case of FBR cells the activation by serum (0.24+/-0.02, n=2, P=0.01) was inhibited by L-779 450 and this was a significant response (0.12+/-0.01, n=3, P=0.0032), although in the Figure 7.7A, lane 3 there is some ERK1 and ERK2 specific immunoreactive band. Also in 208F cells the Raf-1 inhibitor was effective and inhibited the serum-induced MAP Kinase phosphorylation (serum activation 1+/-0.00, n=3, P=0.001 and the inhibition was significant with activation reaching 0.28+/-0.02, n=3, P=0.0005). In the v-Ki-*ras* transformed 208F cells the induction by serum was not very significant (0.24+/-0.04, n=3, P>0.05), which this correlates from the results reported by Marshall and co-workers (Olson et al., 1998). The response to serum of v-Ki-*ras* cells was significant, and this was inhibited approximately by 1.5-fold by L-779 450 (activation 0.15+/-0.02, n=3, significant at P=0.02). The results presented in the Table 7.5 show that the Raf-1-specific inhibitor L-779 450 is an effective inhibitor of MAP Kinase phosphorylation in FBR and 208F cells after stimulation with serum (10% FCS).

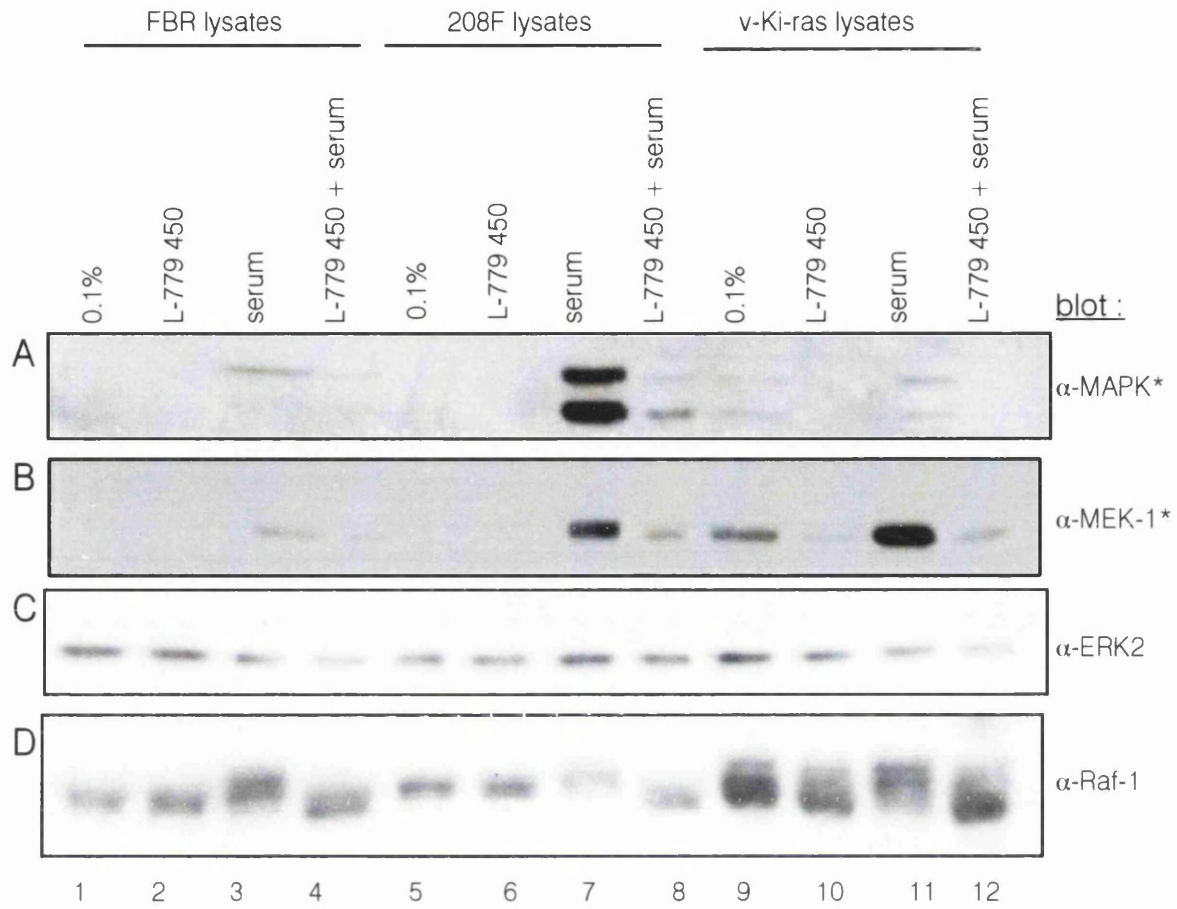
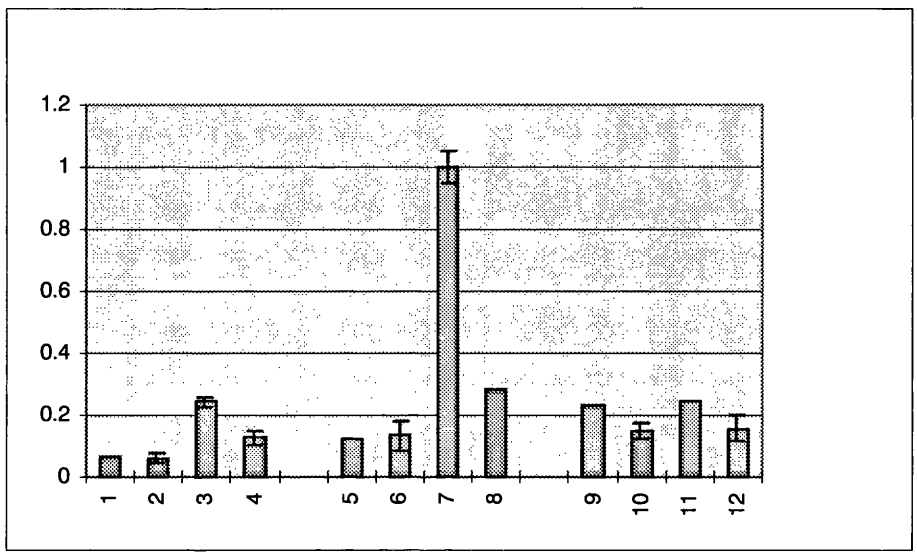


Figure 7.7. The role of the Raf-1 kinase inhibitor L-779 450 in the phosphorylation of MAPK and MEK-1 in FBR cells, 208F cells and v-Ki-ras transformed 208F cells.

Cells were grown in DMEM supplemented with 10% FCS and when sub-confluent, serum-deprived for 48 hours. Cells were stimulated with 10% FCS for 5 minutes or untreated and immediately cell lysates were prepared and 50 µg of total cell lysate was separated by 10 % SDS-PAGE, transferred to PVDF membrane and blotted with an antibody specific for **A)** activated MAPK (Promega Labs, WI, USA), **B)** activated MEK-1 (New England Biolabs, USA) **C)** ERK2 (Transduction Labs, KY, USA) .
D) 80 µg of total cell lysate separated in 7.5% SDS-PAGE, transferred to PVDF membrane and blotted with an antibody specific for Raf-1 (Transduction Labs, KY, USA).
FBR cell lysates in 1) 0.1% FCS 2) 0.1% FCS treated with L-779 450 for 30 minutes 3) treated with 10% FCS for 5 min 4) pre-treated with 3 µM L-779 450 for 30 min and with 10% FCS for 5 min
208F cell lysates in 5) 0.1% FCS 6) 0.1% FCS treated with 3 µM L-779 450 for 30 minutes 7) treated with 10% FCS for 5 min 8) pre-treated with 3 µM L-779 450 for 30 min and with 10% FCS for 5 min
v-Ki-*ras* cell lysates in 9) 0.1% FCS 10) 0.1% FCS treated with 3 µM L-779 450 for 30 minutes 11) treated with 10% FCS for 5 min 12) pre-treated with 3 µM L-779 450 for 30 min and with 10% FCS for 5 min.
The experiment shown is a representative of three experiments with very similar results.

Table 7.5. The effect of L-779 450 in serum-stimulated MAP Kinase phosphorylation in FBR, 208F and v-Ki-*ras* transformed 208F cells.

Quantification of the increase in phosphorylation of ERK1 and ERK2 was performed using scanning densitometry. The band of serum-stimulated 208F cells was taken as the maximal, 100% activation. The values were normalised for control loading of ERK2 for each individual experiment. The values are the mean +/- standard deviation of three independent experiments.



1) FBR serum-deprived cells

2) FBR cells treated with 3 μM of L-779 450

3) FBR serum-stimulated cells

4) FBR cells pre-treated with 3 μM of L-779 450 and serum-stimulated for 5 minutes

5) 208F serum-deprived cells

6) 208F cells treated with 3 μM of L-779 450

7) 208F serum-stimulated cells

8) 208F cells pre-treated with 3 μM of L-779 450 and serum-stimulated for 5 minutes

9) v-Ki-*ras* serum-deprived cells

10) v-Ki-*ras* cells treated with 3 μM of L-779 450

11) v-Ki-*ras* serum-stimulated cells

12) v-Ki-*ras* cells pre-treated with 3 μM of L-779 450 and serum-stimulated for 5 minutes

7.8. L-779 450 modulates actin filament distribution

In order to investigate the relationship of activation of Raf-1 kinase by serum and v-*fos* oncogene, the effect of L-779 450 in membrane ruffling and morphological transformation was investigated.

FBR cells growing in medium supplemented with 10% FCS display a characteristic bipolar shape with increased actin-containing structures in the leading edge of the cells when stained with Rhodamine-Phalloidin (Figure 7.8a). Since MEK-1 is downstream of Raf-1 the Raf-1 inhibitor L-779 450 was used to examine the effect on actin organisation in FBR cells.

Treatment with 3 μM L-779 450 for 24 hours resulted in partial morphological reversion of FBR and reduction of peripheral membrane ruffling. We found that the actin fibres increased compared to the untreated FBR cells and the cells appeared flatter (Figure 7.8b). When FBR cells were incubated with L-779 450 for 48 hours surprisingly the inhibitor did not induce reversion. The actin distribution of the treated cells was more similar to untreated FBR cells (Figure 7.8c). This experiment suggests that L-779 450 does not reverse very efficiently *fos*-induced morphological transformation as the PD 98059 inhibitor shown in experiments presented in the previous chapter.

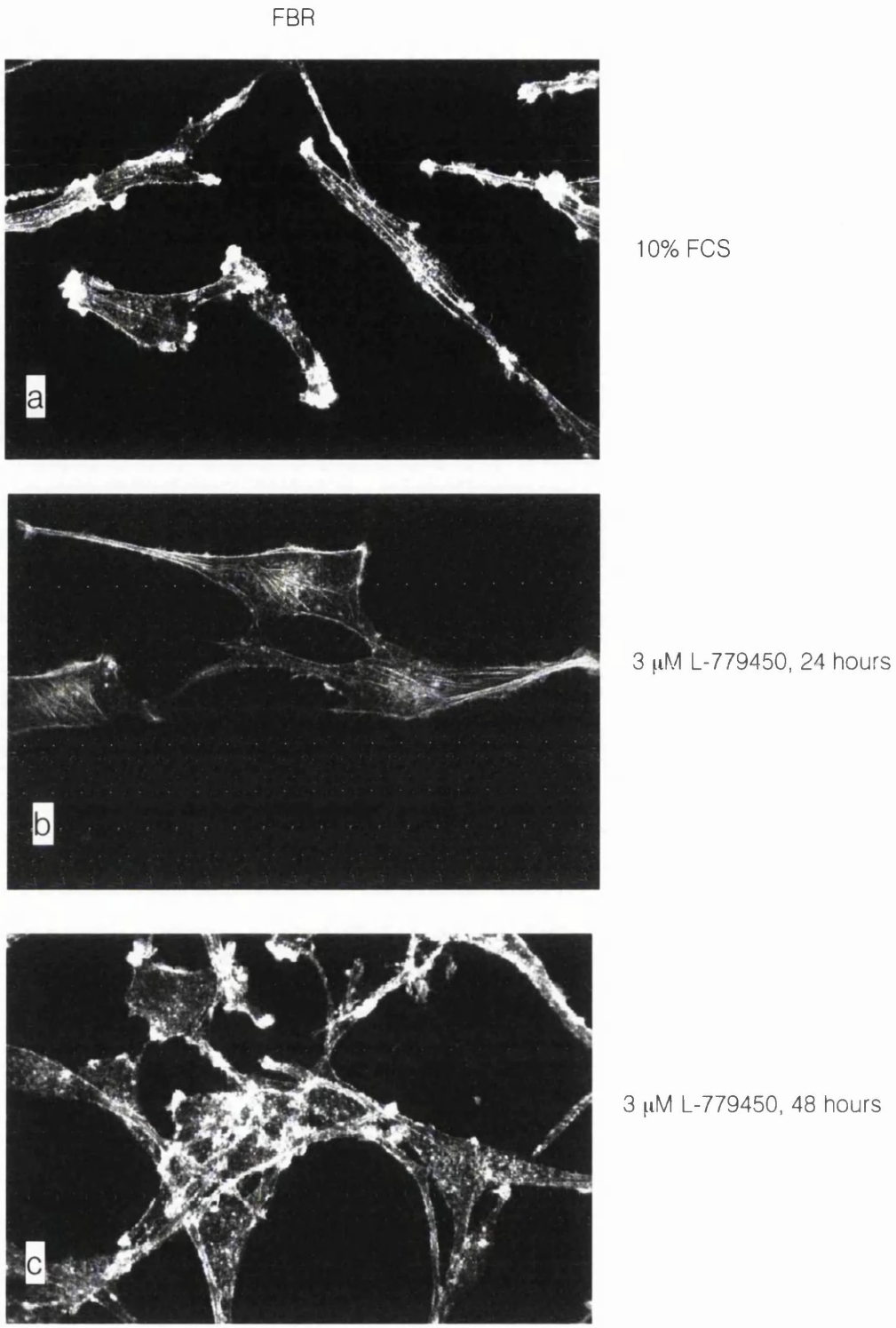


Figure 7.8 . Actin filament distribution in FBR fibroblasts treated with the inhibitor of Raf-1 kinase L-779450.

Cells were grown in DMEM supplemented with 10% FCS for 24 hours, and treated with the Raf-1-specific inhibitor L-779450 for an additional 24 or 48 hours. To visualise actin, cells were fixed, permeabilized and stained with TRITC-conjugated Phalloidin. Images were analysed and processed using a Bio-Rad MRC 600 confocal microscope.
a) FBR in 10% FCS ; **b)** FBR treated for 24 hours with 3 μM of the Raf-1 kinase-specific inhibitor L-779450 ; **c)** FBR treated for 48 hours with 3 μM of the Raf-1 kinase-specific inhibitor L-779450.

To test the specificity of L-779 450, v-Ki-*ras* 208F transformed fibroblasts were treated under the same conditions used for FBR cells (Figure 7.9). In fibroblasts transformed by *ras* Raf-1 kinase is strongly activated (Howe *et al.,* 1992). Inhibition of Raf-1 activity by L-779 450 could potentially modulate different responses of oncogenic *ras* such as actin reorganisation and membrane ruffling.

v-Ki-*ras* cells have a characteristic asteroid morphology when maintained in DMEM supplemented with 10% FCS, and are not bipolar like FBR cells when attached to plastic (Figure 7.9 a). When stained with Rhodamine-Phalloidin the v-Ki-*ras* cells show extensive membrane ruffling and have a decreased number of stress fibres (Figure 7.9 a).

v-Ki-*ras* cells responded differently from FBR after treatment with L-779 450. Incubation of v-Ki-*ras* cell with 3μM L-779 450 for 24 hours resulted in morphological reversion and the suppression of membrane ruffling activity (Figure 7.9b). L-770 450 reverts *ras* transformation even after more prolonged treatment for 48 hours (Figure 7.9 c). In this case reversion was stable and the cells had a characteristic pentagonal, flat fibroblastoid shape. However despite the morphological reversion of *ras*-transformed cells after incubation with L-779 450, reformation of stress fibres was not observed in contrast to studies with the MEK-1 inhibitor PD 98059 (Results Chapter 6, Figure 6.12 b and c).

Reversion of morphological transformation is mainly associated with the appearance of the actin and microfilament network in mammalian cells. In the case of reversion of ras transformation with L-779 450, a dissociation from this principle was revealed. This suggests that the reversion of the transformed morphology in *ras* cells by a Raf-1 kinase specific inhibitor might be independent of the formation of the actin stress fibre network and also that Raf-1 might be involved in the reversion through other mechanisms.

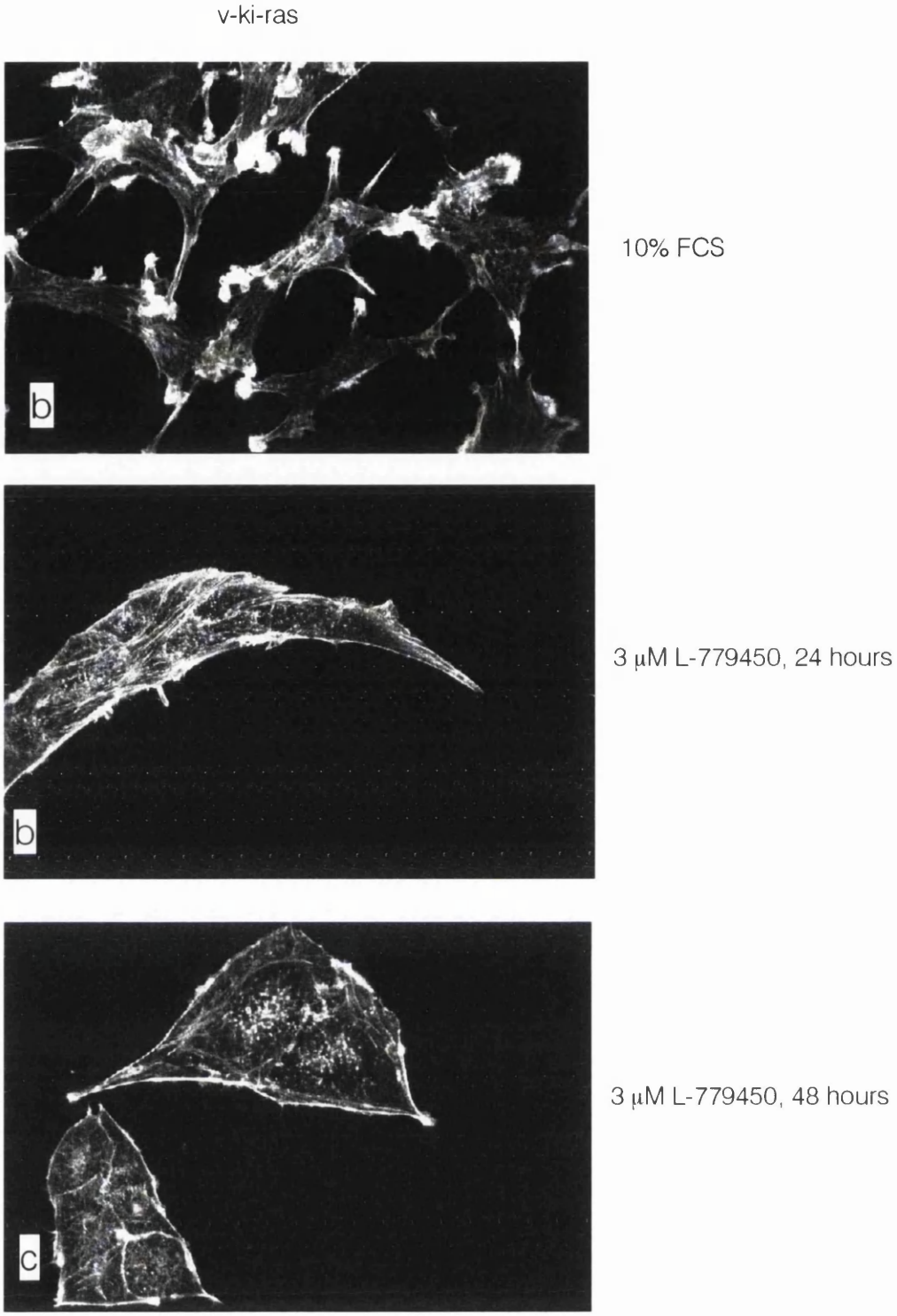


Figure 7.9. Actin filament distribution in v-Ki-*ras* 208F transformed fibroblasts treated with the Raf-1 kinase inhibitor L-779450.

Cells were grown in DMEM supplemented with 10% FCS for 24 hours, and treated with the Raf-1-specific inhibitor L-779450 for an additional 24 or 48 hours. To visualise actin, cells were fixed, permeabilized and stained with TRITC-conjugated Phalloidin. Images were analysed and processed using a Bio-Rad MRC Confocal microscope.
a) v-Ki-ras 208F transformed fibroblasts in 10% FCS ; **b)** v-Ki-ras 208F transformed fibroblasts treated for 24 hours with 3 μM of the Raf-1 kinase-specific inhibitor L-779450; **c)** v-Ki-ras 208F transformed fibroblasts treated for 48 hours with 3 μM of the Raf-1 kinase-specific inhibitor L-779450

7.9. Conclusion

In this chapter the role of Raf-1 kinase was investigated in the context of *fos*-induced transformation. Raf-1 kinase is upstream of *fos* in the growth factor - *ras* signalling pathway and this makes Raf-1 an interesting candidate for mediating signals to downstream effectors and ultimately to AP-1 (Bruder *et al.,* 1992).

Introduction of the dominant negative C-terminally truncated mutant of Raf-1 kinase ΔNRaf-1 into FBR cells resulted in morphological reversion of *fos*-induced transformation. It was also observed that the anchorage independent growth of FBR NΔRaf-1 revertant cells was also suppressed compared to parental FBR cells.

When the FBR NΔRaf-1 cells were tested for activation of MAP Kinases using phosphospecific antibodies, it was found that the ΔN Raf-1 mutant suppresses MAP Kinase activation by 2-fold. This is in agreement with transfection experiments in COS cells where the NΔRaf-1 mutant has been reported to confer a concentration-dependent reduction of MAP Kinase activation by growth factors (van Dijk *et al.,* 1997). It should also be noted that we have not been able to demonstrate activation of Raf-1 by MEK-1 substrate phosphorylation in *in vitro* kinase assays due mainly to technical obstacles.

Actin organisation is altered by NΔRaf-1 when it is stably expressed in FBR cells. The FBR NΔRaf-1 revertant cells isolated show an increase in the formation of actin stress fibres, a characteristic of normal fibroblasts, but retain membrane ruffling a characteristic of transformed fibroblasts. Thus Raf-1 could possibly regulate anchorage independent growth that is associated with actin formation (Pollack *et al.,* 1975) but not membrane ruffling which is controlled by other *ras*-dependent mechanisms (Nobes and Hall, 1995; Qiu *et al.,* 1997).

Pharmacological intervention in FBR cells using the Raf-1 inhibitor L-779 450 resulted in partial time-dependent reversion of *fos*-transformation. In contrast in the case of v-Ki-*ras* cells transformed cells reversion was evident in morphology, since these cell became very flat and lost the membrane ruffles but without any re-formation of the actin stress fibre network.

These data suggest that Raf-1 kinase activity is present in FBR cells and its inhibition by the introduction of the NΔRaf-1 reverses the transformed phenotype in these cells and inhibits the serum-induced MAP Kinase phosphorylation.

RESULTS CHAPTER 8

STUDIES IN THE FOS-RELATED ANTIGEN-1 IN FBR AND REVERTANT CELL LINES

RESULTS

CHAPTER 8. STUDIES IN THE FOS-RELATED ANTIGEN-1 IN FBR AND REVERTANT CELL LINES

8.1. Fos-related antigen-1 is positively regulated during fos-induced transformation

The induction of c-*fos* expression is associated with important biological processes such as mitogenesis (Greenberg and Ziff, 1984; Muller *et al.,* 1984) and differentiation (Muller and Wagner, 1984). Transcriptional activation of c-*fos* by growth factors is very rapid (Greenberg and Ziff, 1984) and the p55 $^{c\text{-}Fos}$ protein accumulation is maximal after 30 minutes of induction. These observations place c-*fos* in a signal transduction pathway that couples short term signals elicited on the cell surface by growth factors to the induction of gene expression in the nucleus.

p55 $^{c\text{-}Fos}$ is complexed in resting and stimulated fibroblasts with a 39 kD protein (Curran *et al.,* 1984, 1985; Franza *et al.,* 1987; Sassone-Corsi *et al.,* 1988b). In addition, p55 $^{c\text{-}Fos}$ is complexed with other polypeptides as shown by immunoprecipitation of [^{35}S]-methionine labeled lysates from quiescent and induced PC12 cells followed by two-dimensional gel electrophoresis (Franza *et al.,* 1987). These proteins appear to be recognized directly by Fos-specific antibodies (Franza *et al.,* 1987) and represent modified forms of the Fos-Related antigens Fra-1 and Fra-2. The Fra-1 protein appeared to be the major Fos-related antigen identified from PC12 cells induced by NGF or from serum-stimulated 208F fibroblasts (Cohen and Curran, 1988).

The *fra*-1 mRNA and Fra-1 protein are also up-regulated during oncogenic transformation. Rat-1 cells expressing the FBJ *fos* or *fos*B transforming genes show elevated levels of *fra*-1 mRNA (Bergers *et al.,* 1995). *fra*-1 message and Fra-1 protein is also up-regulated in *ras*-transformed NIH 3T3 cells (Mechta *et al.,* 1997), and transfection of *fra*-1 contributes positively to transformation by *ras* (Mechta *et al.,* 1987) and *jun* oncogenes (Bergers *et al.,* 1995), suggesting that *fra*-1 might act co-operatively with other nuclear oncogenes. There is evidence also in epithelial cells for *fra*-1 in malignant progression and cellular transformation. In the metastatic cell line CSML100, that represents a an *in vitro* cell line model of epithelial tumour progression, increased expression of *fra*-1 correlates with elevated AP-1 binding activity, invasiveness and metastatic properties of this cell line (Kustikova *et al.,* 1998). Moreover expression of exogenous *fra*-1 in non-malignant CSMLO cells (of this epithelial progression

model) resulted in increased metastatic potential, morphological alterations resembling fibroblastoid conversion and expression of a number of a genes associated with late stages of tumour development (Kustikova *et al.,* 1998).

The regulation of Fra-1 protein in FBR cells or 208F cells has been under analysed in this laboratory, mainly by western immunoblotting. Nuclear cell lysates prepared from exponentially growing FBR cells contain high levels of Fra-1 protein compared to 208F cells (Kim Hawker and Lynn McGarry, unpublished data).

In an attempt to look for any possible relationship between morphological reversion and the expression of Fra-1 in the revertant cell lines. Therefore the expression pattern of Fra-1 protein in FBR, 208F cells and in FBR MEK-1 A221 and FBR TAM-67 revertant cell lines was analysed by western immunoblotting.

8.2. Fra-1 protein expression in FBR and revertant cell lines

Fos-Related Antigens are up-regulated during mitogenic stimulation of fibroblasts by serum (Franza *et al.,* 1987; Cohen and Curran, 1988; Cohen *et al.,* 1989). In addition to their induction by serum and growth factors, Fra-1 and Fra-2 proteins are positively regulated at the transcriptional and post-translational level by different transforming oncogenes such as v-*ras* (Mechta *et al.,* 1997), v-*jun* (Liz Black and David Gillespie, unpublished observations) and v-*fos* (Kim Hawker and Lynn McGarry, unpublished observations). These data highlight a role for these proteins in cell proliferation and possibly transformation.

The Fra-1 protein is present in high levels in cycling FBR cells grown in 10% FCS, but at very low levels under the same conditions in non-transformed parental 208F cells. In contrast, in NIH 3T3 cells the major protein which is detected under cycling conditions is the Fra-2 protein whereas the Fra-1 and c-Fos proteins are not detected (Lallemand *et al.,* 1997)..

The levels and the variation of the Fra-1 protein in FBR and the revertant cell lines was examined by western analysis, to identify any possible relationship between the expression of Fra-1 protein and morphological reversion. To address this, lysates from exponentially growing cultures of FBR and revertant cell lines were prepared followed by western immunoblotting with Fra-1-specific antibodies. The result of such an experiment is shown in Figure 8.1. In the FBR cells multiple protein species of 46 and 32-35 kDa are recognized by commercially available anti-Fra-1 rabbit serum (Figure 8.1A lane 1). In contrast, in 208F cell lysates a Fra-1

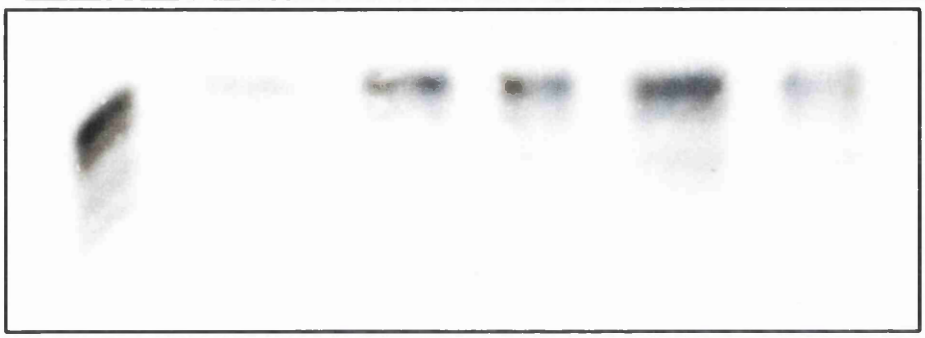
immunoreactive band which represents the Fra-1 protein is reduced 15-fold compared to Fra-1 protein from FBR lysates (Figure 8.1A lane 2). In the FBR MEK-1 A221 revertant cells (Figure 8.1A lanes 3, 4, and 5), approximately a 60% reduction in FBR A221 clone 6 and FBR A221 clone 19 in Fra-1 levels was found, but only a 20% reduction in FBR A221 clone 23. In the FBR TAM-67 lysates, a 60% reduction in the levels of Fra-1 protein was found compared to the FBR cells (Figure 8.1 lane 6 compare to lane 1).

In *ras* transformed fibroblasts Yaniv and co-workers have observed a dramatic increase in Fra-1 protein levels. This parallels the studies of Bergers et al. (1995) where it was shown that the expression of the *fra*-1 gene is controlled by an AP-1-dependent mechanism and is under positive control by AP-1 family members. In Figure 8.1B, a second experiment is shown, in which lysates from v-Ki-*ras* transformed 208F cells were also included as a positive control. Scanning densitometry revealed that the levels of Fra-1 protein in *ras* transformed 208F cells is 2-fold elevated over FBR cells.

A

blot : anti-Fra-1

FBR	208F	FA 6	FA 19	FA 23	FBR T-10	
1.583	0.12	0.52	0.676	1.325	0.522	OD
1	0.07	0.32	0.42	0.83	0.32	OD norm
1	2	3	4	5	6	

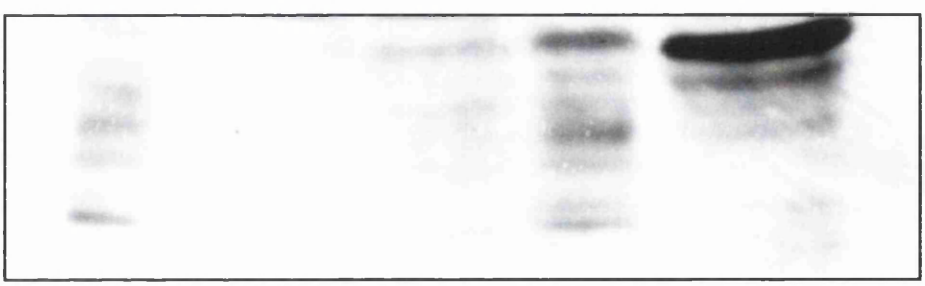


1 2 3 4 5 6

B

blot : anti-Fra-1

FBR	208F	FA 19	FA 23	208F v-Ki-ras	
1	2	3	4	5	
3.04	0.13	2.3	3.9	5.8	OD
1	0.04	0.7	1.4	2	OD norm



1 2 3 4 5

Figure 8.1. Expression of Fra-1 protein in FBR , 208F, v-Ki-*ras* transformed 208F cells and revertant cell lines.

Cells were grown in DMEM supplemented with 10% FCS to sub-confluence, cell lysates were prepared and proteins were separated by 10% SDS-PAGE, transferred to PVDF membrane and probed with an antibody specific for Fra-1 protein. (N-17 rabbit antibody, Santa Cruz Bio., CA, USA).
A) 1) FBR cells; 2) 208F cells; 3) FBR A221 clone 6; 4) FBR A221 clone 19; 5) FBR A221 clone 23; 6) FBR TAM-67 cells.
B) 1) FBR cells; 2) 208F cells; 3) FBR A221 clone 19; 4) FBR A221 clone 23; 5) v-Ki-*ras* 208F transformed cells.
The experiments are representative of three at least similar experiments

8.3. Inducibility of Fra-1 in FBR cells after extracellular stimulation

Previously Curran and co-workers have examined the patterns of Fra-1 induction in quiescent 208F cells treated with serum (Franza *et al.,* 1987 ; Cohen *et al.,* 1989) and PC12 cells treated with benzodiazepines and NGF (Franza *et al.,* 1987). The induction of Fra-1 is slower compared to $p55^{c\text{-}Fos}$ protein in fibroblasts, reaching a peak after 2-$2^1/_2$ hours after the addition of 20% FCS in 208F cells (Franza *et al.,* 1987) and in NIH 3T3 fibroblasts (Lallemand *et al.,* 1997). This lag period of Fra-1 induction suggests that it could be regulated by AP-1 components, such as Fos and FosB which are induced earlier in the cell cycle.

While the regulation of Fra-1 expression in normal cells is documented, little is known of Fra-1 inducibillity in v-*fos* transformed cells. The induction of Fra-1 protein in FBR cells and 208F cells in a short term response of 60 minutes was examined. This time point was chosen to demonstrate any rapid changes in the induction of Fra-1 that exist in FBR that will allow to distinguish this response to the previously identified $2^1/_2$ hour induction in 208F cells (Franza *et al.,* 1987).

When FBR cells are serum-deprived, Fra-1 protein levels are still detectable, suggesting that the *fra*-1 gene escapes transcriptional silencing at quiescence, during *fos*-induced transformation. Fra-1 proteins consist of multiple bands that migrate between 30 to 46 kD as shown in SDS-polyacrylamide gels and western immunoblotting (Figure 8.2A lane 1). Using scanning densitometry and normalising for ERK2 as a control a 3-fold reduction in the protein levels of Fra-1 was observed in serum-deprived 208F cells (Figure 8.2A lane 5).

During mitogenic stimulation of fibroblasts by serum the Fra-1 protein appears as a smear in SDS-polyacrylamide gels. This mainly is attributed to post-translational modifications of the protein such as phosphorylation (Cohen *et al.,* 1989; Gruda *et al.,* 1994). Stimulation of FBR cells with 10% FCS for 60 minutes induced approximately 2-fold the expression of Fra-1 (Figure 8.2A lane 3) and had no detectable effect on Fra-1 induction in 208F cells (Figure 8.2A lane 7). The patterns of *fos* protooncogene and Fos protein expression after serum stimulation, are well investigated in 208F cells and other established cell lines (Muller *et al.,* 1984; Kruijer *et al.,* 1984; Sassone-Corsi *et al.,* 1988a). c-*fos* mRNA is induced rapidly within minutes and its protein synthesis is maximal between 30 and 60 minutes after the addition of mitogens (Muller *et al.,* 1984). To identify if in this experiment the $p55^{c\text{-}Fos}$ protein is induced in 208F cells the

membrane of the Figure 8.2A was stripped and probed with the K-25 antibody which recognizes the p55 $^{c\text{-}Fos}$ protein and the p75 $^{v\text{-}Fos}$ protein also.

The result of this attempt is shown in the Figure 8.2 Panel B. There was a dramatic induction of p55 $^{c\text{-}Fos}$ protein in the 208F cells after treatment with 10% FCS (Figure 8.1B lane 7). In FBR cells the inducibility of c-Fos protein is dramatically impaired (Figure 8.1B lane 3). These data suggested that induction of Fos and Fra-1 proteins in response to mitogenic stimulation by serum in FBR and 208F cells is altered, and Fos compared to Fra-1 shows a differential pattern which allows to distinguish their post-translational modifications.

Mitogenic stimulation with serum induces post-translational modifications in Fra-1 and Fos proteins (Curran *et al.,* 1984; Cohen *et al.,* 1989). The mobility patterns by SDS-Polyacrylamide gel electrophoresis of Fra-1 and Fos after induction are easily distinguished, although was not clear whether this was the result of phosphorylation or another type of modification. Molecular Biology Grade Alkaline Phosphatase has been used previously to confirm whetther such mobility shifts are the result of phosphorylation (Barber and Verma, 1987; Lin *et al.,* 1992; Gruda *et al.,* 1994; Lallemand *et al.,* 1997). In such experiments Alkaline Phosphatase was used to investigate if phosphorylation accounts for the appearance of higher molecular weight species of Fra-1 after SDS-polyacrylamide electrophoresis (Figure 8.2A lanes 2, 4, 6 and 8).

Lysates prepared with the standard Triton X-100 lysis buffer were normalized for their protein content and treated with alkaline phosphatase and then processed as usual for SDS polyacrylamide electrophoresis. In samples from FBR cells treated with phosphatase a reduction in the formation of the higher migrating Fra-1 bands was observed, suggesting that these are phosphorylated. Similarly in lysates from FBR cells induced with serum, Fra-1 protein was found to be phosphorylated (Figure 8.2A lanes 2 and 4). After phosphatase treatment an accumulation of Fra-1 immunoreactivity in protein species of 35 kD was observed in FBR cells (Figure 8.2A lanes 2 and 4). However in 208F cells this effect was negligible (Figure 8.2A lanes 6 and 8) which was expected, as the Fra-1 protein levels are low in the 208F cells is at low levels compared to FBR cells. The effect of the phosphatase treatment in the differential modification of the p 55 $^{c\text{-}Fos}$ protein is shown in the Figure 8.2B. In this case the phosphatase altered the mobility of the induced 55 kD c-Fos protein only in 208F cells (Figure 8.2B lane 7 ; compare to lane 8) but not in the FBR cells, were is undetectable (Figure 8.2B, Lane 3) (Hawker *et al.,*

1994). These changes in mobility of Fra-1 protein in FBR cells suggest that kinase is able to modify the protein at quiescence. When FBR cells are stimulated with serum this effect is more pronounced resulting in elevation of the Fra-1 protein as well as in its post-translational modification by phosphorylation.

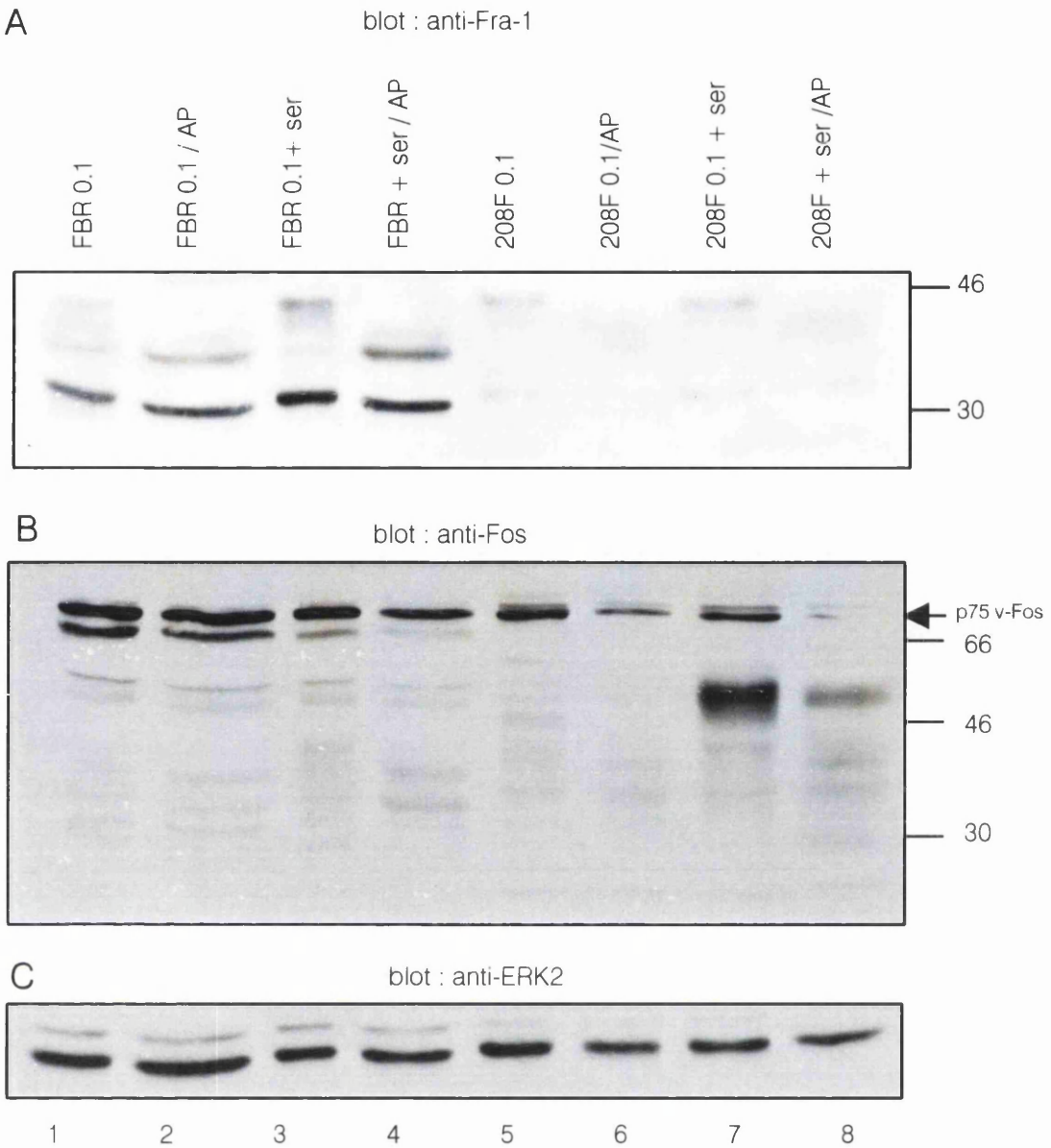


Figure 8.2. Induction of Fra-1 and Fos proteins by serum in FBR and 208F cells and the effect o in vitro incubation with Alkaline Phosphatase.

Cells were grown in DMEM supplemented with 10% FCS and when subconfluent, serum-deprived for 48 hours (0.1% FCS). Stimulation was for 60 minutes with 10% FCS. Cell lysates were prepared and incubated for 2 hours at 37^{0} C with alkaline phosphatase or remain untreated.
Proteins were separated by 10% SDS-PAGE, transferred to PVDF membrane and probed with an antibody specific for A) Fra-1 protein (N-17 rabbit antibody, Santa Cruz Bio.). Blots were stripped and probed with an antibody specific B) for FOS-proteins and FOS-related antigens (K-25 rabbit antibody, Santa Cruz Bio.). As control for protein loading, the membranes were probed with an antibody to ERK2 (Transduction Labs, KY, USA).

Lane 1) Lysate from FBR serum-deprived cells; 2) Lysate from FBR serum-deprived cells incubated *in vitro* with Alkaline Phosphatase to dephosphorylate lysate proteins; 3) Lysate from FBR serum-stimulated cells; 4) Lysate from FBR serum-stimulated cells incubated *in vitro* with Alkaline Phosphatase to dephosphorylate lysate proteins; 5) Lysate from 208F serum-deprived cells 6) Lysate from 208F serum-deprived cells incubated *in vitro* with Alkaline Phosphatase to dephosphorylate lysate proteins; 7) Lysate from 208F serum-stimulated cells; 8) Lysate from 208F serum-stimulated cells incubated *in vitro* with Alkaline Phosphatase to dephosphorylate lysate proteins.

8.4. Fra-1 is phosphorylated in FBR

The levels of Fra-1 protein in cycling FBR cells growing in 10% FCS are high compared to 208F cells (Figure 8.1). These forms of Fra-1 protein accumulate in the higher migrating forms of approximately 40-46 kD after SDS-polyacrylamide electrophoresis. These forms might represent modified forms of this protein since early studies have showed that this protein is heavily modified in cultured fibroblasts (Cohen *et al.,* 1989).In order to analyse and possibly correlate Fra-1 phosphorylation state with cells either at quiescence or after mitogenic stimulation, alkaline phosphatase was again used to identify Fra-1 phosphorylation.

Phosphatase treatment of FBR lysates prepared from cycling cells growing in 10% FCS resulted in a mobility shift of this band from 45 kD to approximately 35 kD (Figure 8.3B lane2). This demonstrates that the majority of Fra-1 in FBR cells exists in a phosphorylated form in the case that these cells are growing in the presence of 10% FCS.

Stimulation with EGF or phorbol ester for 2 hours (Figure 8.3B, lanes 5 and 7) resulted in the induction of the higher migrating forms of Fra-1 proteins, as in FBR cycling cells (Figure 8.3A lane 1 and Figure 8.3B lane 1). To demonstrate that these higher migrating forms of Fra-1 caused by EGF or TPA were the result of phosphorylation the samples were treated with phosphatase (Figure 8.3B, lanes 6 and 8). Phosphatase treatment resulted in the disappearance of the higher migrating forms of these proteins and all the Fra-1 immunoreactive bands accumulated the 35kD molecular weight species (Figure 8.3B lanes 6 and 8) (Franza *et al.,* 1987). This shows that the induction of Fra-1 proteins by agonists other than serum results in phosphorylation of Fra-1. A similar observation had been shown (Gruda *et al.,* 1994). Treatment of Swiss 3T3 cells with Insulin and TPA results in the accumulation of phosphorylated forms of Fra-1 (Gruda *et al.,* 1994).

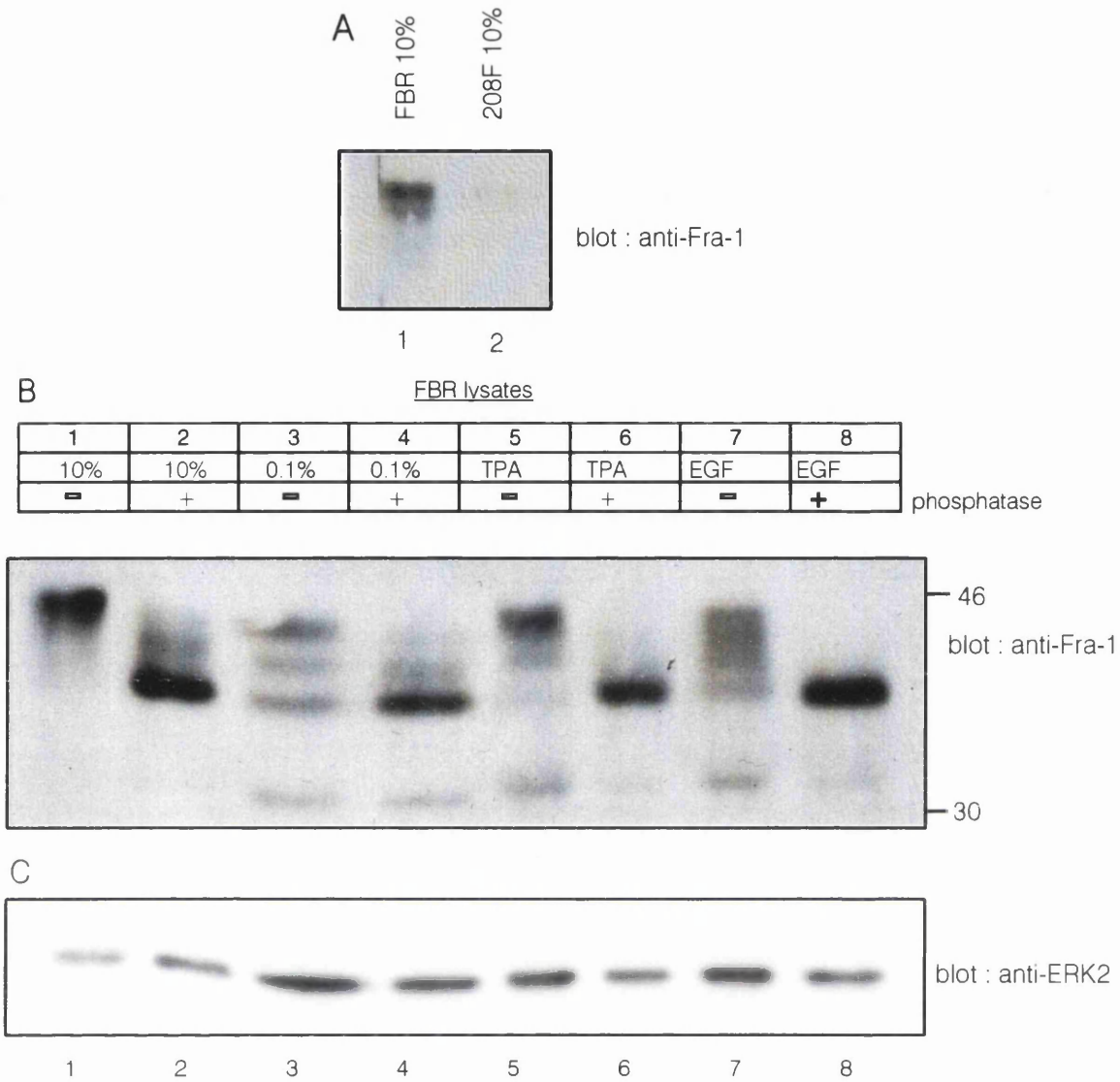


Figure 8.3. Induction of Fra-1 proteins by EGF and TPA in FBR and the effect of *in vitro* incubation with Alkaline Phopshatase.

Cells were grown in DMEM supplemented with 10% FCS and when to subconfluent serum-deprived for 48 hours (0.1% FCS). Stimulation was for 2 hours with 100 ng/ml EGF, 100 ng/ml TPA or the cells left untreated. Cell lysates were prepared and incubated for 2 hours at 37^0 C with alkaline phosphatase or left untreated.
Proteins were separated by 10% SDS-PAGE, transferred to PVDF membrane and probed with an antibody specific for **A)** Fra-1 protein (N-17 rabbit antibody, Santa Cruz Bio.). Blots were stripped and probed with an antibody specific **B)** Fra-1 protein (N-17 rabbit antibody, Santa Cruz Bio.). For a loading control the blot was probed **C)** with an antibody specific for ERK2 (Transduction Labs).
A) lane 1) FBR growing in 10% FCS ; 2) 208F cells growing in 10% FCS.
B and C) lane 1) Lysate from FBR growing in 10% FCS; 2) Lysate from FBR growing in 10% FCS incubated *in vitro* with Alkaline Phosphatase; 3) Lysate from FBR serum-deprived cells; 4) Lysate from FBR serum-deprived cells incubated *in vitro* with Alkaline Phosphatase; 5) Lysate from FBR TPA-stimulated cells 6) Lysate from FBR cells TPA-stimulated incubated *in vitro* with Alkaline Phosphatase; 7) Lysate from FBR EGF-stimulated cells; 8) Lysate from FBR EGF-stimulated cells incubated *in vitro* with Alkaline Phosphatase.

8.5. Fra-1 induction in FBR, 208F and revertant cell lines

There is evidence that the MAPK pathway is responsible for the changes in the Fra-1 phosphorylation during mitogenic stimulation. Stimulation with growth factors results in phosphorylation of Fra-1 by MAP Kinases and stimulation of its transcriptional activity (Skinner *et al.,* 1997). Here the inducibilty and phosphorylation of Fra-1 in FBR, 208F and revertant cell lines was examined, in order to correlate Fra-1 phosphorylation state with the *ras* signaling pathway in normal and transformed cells.

For these experiments subconfluent cultures were incubated in DMEM supplemented with 0.1 % FCS to induce quiescence. After 48 hours of serum deprivation, the cultures were incubated with 10% FCS for $2^1/_2$ hours and cell lysates were prepared under standard conditions. The lysates were separated by SDS-PAGE and blotted with the Fos-specific antibody K-25 (Figure 8.4A). In this case a strong induction of the $p55^{c\text{-}Fos}$ protein (Figure 8.4A, lane 4). In samples prepared from FBR and revertant cells no Fos-specific bands were identified (Figure 8.4; lanes 2, 6, 8, 10 and 12). Using the K-25 antibody in the samples prepared from stimulated FBR , 208F and revertant cells a serum-inducible band of approximately 30-45 kD was also identified. This protein could potentially represent a novel Fos-Related Antigen since it is of a similar molecular weight to Fra-1. However further investigation is required to confirm this. It is also of interest that its expression also found to be reduced in the revertant cell lines (Figure 8.4A, lanes 6, 8, 10, and 12 compare to lane 3 and 4).

To examine if the inducibility of the Fra-1 protein is affected by the introduction of dominant negative mutants, the western immunoblot in Figure 8.4A was probed with the N-17 Fra-1-specific antibody. In FBR serum-deprived cultures, low levels of Fra-1 protein are present, as previously documented (Franza *et al.,* 1987; Cohen *et al.,* 1989).

Stimulation of FBR cells with 10% FCS for $2\ ^1/_2$ hours resulted in the induction of Fra-1 protein 3-4 fold as determined by scanning densitometry (Figure 8.4B, lane 1 and 2). In the case of 208F cells the magnitude of induction of Fra-1 protein was 2-fold reduced compared to FBR cells (Figure 8.4B, lane 4). In the FBR A221 revertant clones 6 and 19, and in one clone of FBR TAM-67 cells examined here, there was a 1.5-fold reduction in the inducibility of the Fra-1 protein levels when compared to FBR (Figure 8.4B, lane 6, 8, and 12). It should be noted that in

the case of FBR A221 23 clone the induction in response to serum was similar in that of FBR cells (Figure 8.4B, lane 10).

Together these data suggest that there could be a biological correlation between reversion and the mechanism of reduction of Fra-1 protein levels in the revertant cell lines.

The MEK-1 inhibitor PD98059 was used to establish a possible molecular relationship between the dominant negative MEK-1 A221, Fra-1 induction and FBR reversion. In this experiment the MEK-1-specific inhibitor PD 98059 was used to mimic the conditions of a transfection with the dominant negative MEK-1 A221 in order to examine the role of MEK-1 in Fra-1 protein inducibility (Figure 8.5).

Serum-deprived cultures of FBR cells were incubated for 60 minutes with 50 μM of PD 98059 and then induced for $2^1/_2$ hours with 10% FCS. Cell lysates were prepared, proteins were separated by 10% SDS-PAGE and transferred to PVDF membranes. Western immunoblotting with the Fra-1-specific antibody showed that there was potent induction of Fra-1 protein (Figure 8.5A, lane 3). FBR cells were incubated with PD 98059 and treated with 10% FCS for $2^1/_2$ hours. Scanning densitometry of the western immunoblot revealed that there was a 2-fold reduction of Fra-1 protein levels suggesting a requirement of MEK-1 and MAP Kinase in the induction of Fra-1 protein expression.

To determine total Fra-1 protein content of the cells, lysates were incubated with phosphatase for a prolong period to dephosphorylate all Fra-1 protein to allow estimation of the total protein content as one 35 kD band (Figure 8.5B). In the samples from quiescent FBR cells or treated with the inhibitor there was no major difference in the amount of Fra-1 protein levels (Figure 8.5B lanes 1 and 2). In the samples treated with 10% FCS a clear induction of Fra-1 protein was observed (Figure 8.5B lane 3) which was reduced approximately 2-fold as determined by scanning densitometry (Figure 8.5B, lane 4). This experiment supports the data presented in the Figure 8.5A which suggest that a MEK-1-dependent mechanism is involved in the serum induction and post translational modification of Fra-1 protein in FBR cells.

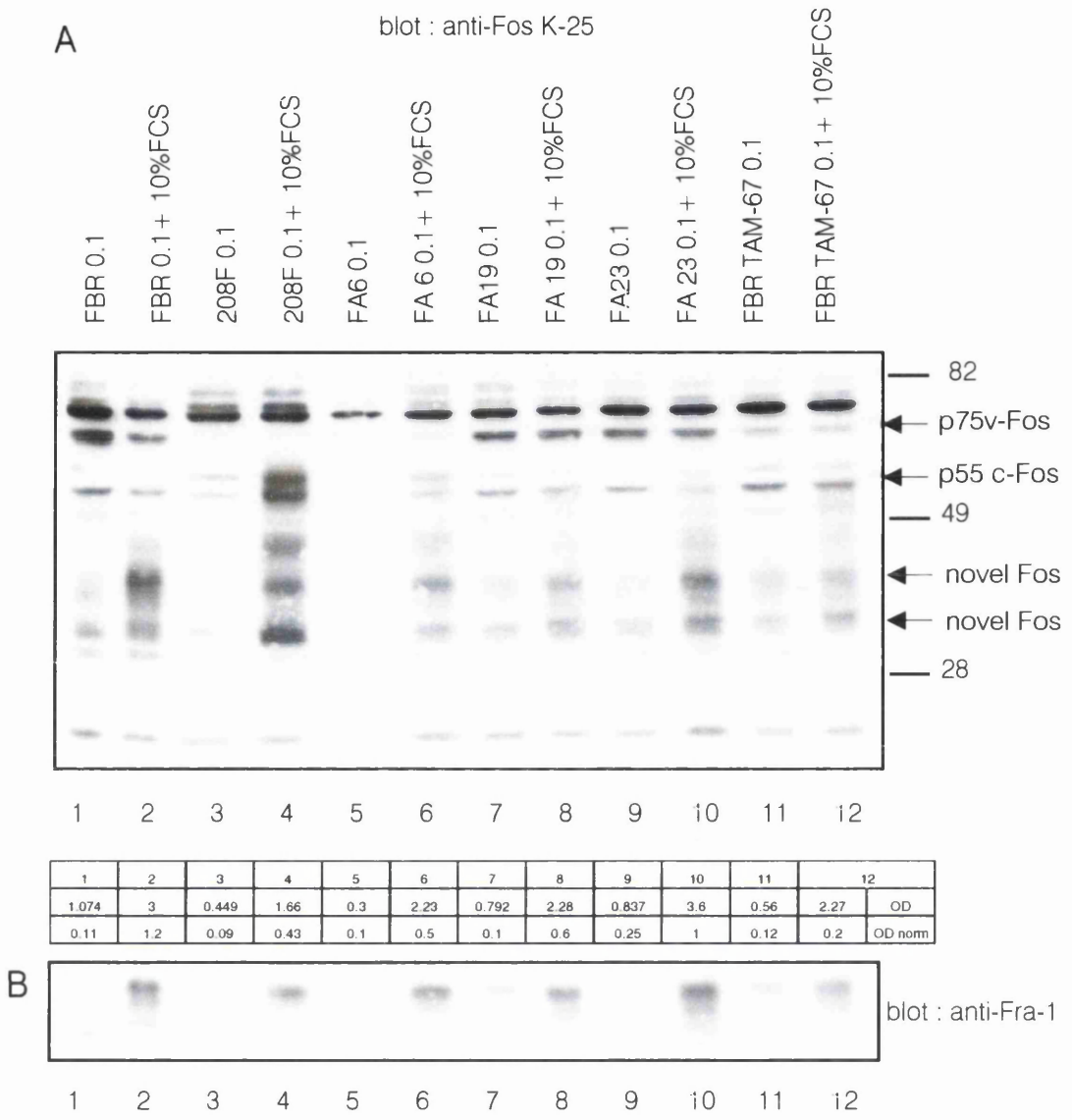


Figure 8.4. Regulation of Fra-1 and Fos proteins by serum in FBR, 208F and FBR transfected with dominant negative mutant MAPKK A221 and FBR transfected with a dominant negative deletion mutant of c-Jun TAM-67.

Cell lines were grown in DMEM supplemented with 10% FCS and when sub-confluent serum-deprived for 48 hours (0.1% FCS) Stimulation was for 2 $^{1}/_{2}$ hours with 10% FCS. Cell lysates were prepared and 50 µg of total cell lysate was separated in 10% SDS-PAGE, transferred to PVDF membrane and probed with an antibody specific for **A)** Fos protein (K-25, Santa Cruz Bio., CA, USA) stripped and probed with an antibody specific for **B)** Fra-1 proteins (N-17, Santa Cruz Bio., CA, USA)

1) serum-deprived FBR; 2) serum-stimulated FBR; 3) serum-deprived 208F cells ; 4) serum-stimulated 208F cells; 5) serum-deprived FBR A221 revertant clone 6; 6) serum-stimulated FBR A221 revertant clone 6; 7) serum-deprived FBR A221 revertant clone 19; 8) serum-stimulated FBR A221 revertant clone 19; 9) serum-deprived FBR A221 revertant clone 23; 10) serum-stimulated FBR A221 revertant clone 23; 11) serum-deprived FBR TAM-67 revertant clone 10; 12) serum-stimulated FBR TAM-67. revertant clone 23

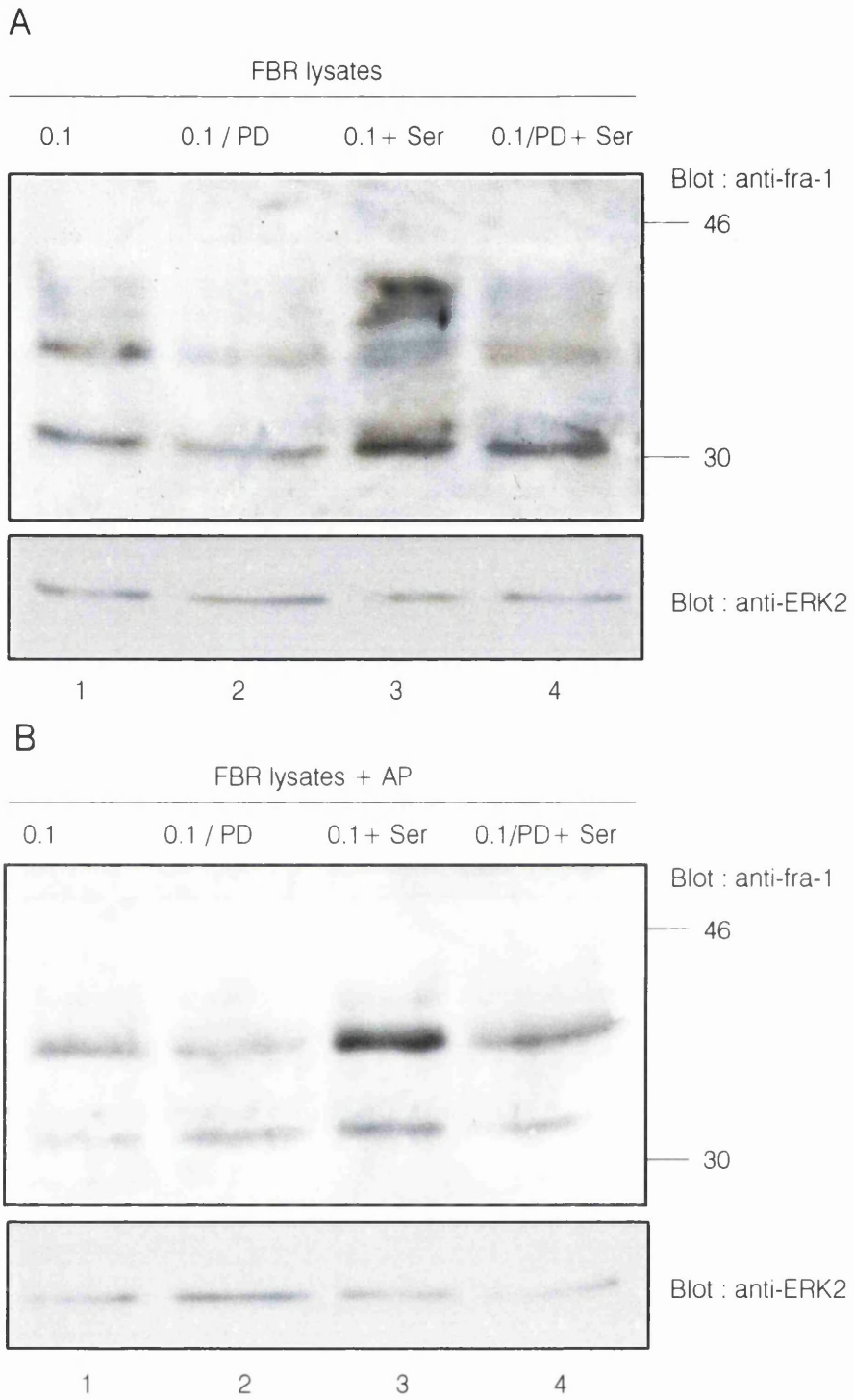


Figure 8.5 . Post-translational regulation of Fra-1 protein in FBR cells by the MEK-1 specific inhibitor PD 98059.

Cells were grown in DMEM supplemented with 10 % FCS and when sub-confluent serum-deprived for 48 hours. The pre-treatment with the MEK-1 inhibitor PD 98059 was for 60 minutes at a final concentration of 50µM. Serum stimulation with 10% FCS was for 2 ½ after the addition of the MEK-1 inhibitor. Cell lysates were prepared and incubated with alkaline phosphatase or left untreated. Proteins were separated by 10% SDS-PAGE, transferred to PVDF membrane and probed with an antibody specific for the fra-1 proteins (N17, Santa Cruz Bio., CA, USA)
To confirm equal loading the blots were stripped and probed with an antibody specific for ERK2 (Transduction Labs, KY, USA).

8.6. Conclusion

The Fos-Related Antigen 1, Fra-1 is a protein with extensive similarities in primary structure with $p55^{c\text{-}Fos}$. *fra*-1 mRNA and protein is induced by serum and mitogens in fibroblasts. This induction is AP-1 dependent due to an AP-1 responsive element in the 5' end of the *fra*-1 gene (Bergers *et al.,* 1995).

Transformation by oncogenes that activate and regulate AP-1 such as *ras* and *fos* lead to induction of *fra*-1 gene transcription (Bergers *et al.,* 1995) and to up-regulation of the Fra-1 protein (Mechta *et al.,* 1997). By western analysis Fra-1 protein levels are elevated in FBR cells during cycling conditions. In contrast, Fra-1 protein levels are dramatically reduced in 208F cells. In quiescent FBR cells expression of Fra-1 protein is induced after stimulation with 10% FCS. This suggests that the inducibility of Fra-1 protein in FBR cells has a similar pattern of induction, but the magnitude of induction is greater in FBR cells as a result of transformation with the FBR v-*fos* oncogene.

Fra-1 expression was examined in FBR MEK-1 and FBR TAM-67 revertant cell lines isolated. In most revertant cell lines there was a reduction in the levels of Fra-1 protein. This event was independent of culture conditions. Revertant cell lines cycling in serum or serum-stimulated had reduced levels of Fra-1 protein when compared to the parental FBR Fra-1 levels. This suggests that the dominant negative mutants might act in an antagonistic manner and limit the levels of AP-1 effectors such as the Fra-1 gene and protein, resulting in an inhibition of morphological transformation. It is also known that in all the revertant cell lines the $p75^{v\text{-}Fos}$ oncoprotein is expressed. This might reduce the reversion potential of the dominant negative mutants resulting in clones that are selected for the expression of the mutant by retaining morphological properties of the transformed phenotype *in vitro*.

This data and published observations from others argue for a *ras* - MEK-1 - AP-1 dependent regulation of Fra-1 expression in FBR cells. The role of the Fra-1 protein is not very well established in transformation by the *fos* oncogene. It potentially represents a downstream effector of AP-1-dependent transformation. This makes its transactivation properties important in the context of transcriptional regulation by *fos* and *jun* oncogenes. A study that could shed some light in the role of *fra*-1 gene would be to manipulate it genetically to identify its role in regulation of specific genes. The use of *fra*-1 null cells for example could be an important tool to identify AP-1 target genes in a fibroblast model of gene regulation. Such cells could be re-

transformed by *fos, ras* or other oncogenes and a differential display protocol could be carried out to search for candidate AP-1 targets.

RESULTS CHAPTER 9

PROTEIN TYROSINE PHOSPHORYLATION IN FBR AND 208F CELLS: IDENTIFICATION OF A POTENTIAL REGULATOR OF MAP KINASE SIGNALLING

RESULTS

CHAPTER 9. PROTEIN TYROSINE PHOSPHORYLATION IN FBR AND 208F CELLS: IDENTIFICATION OF A POTENTIAL REGULATOR OF MAP KINASE SIGNALLING

9.1. Tyrosine phosphorylation controls activation of the ras pathway

Protein tyrosine kinases play a major role in the transmission of extracellular signals to the cytoplasm and to the nucleus (Schlessinger and Bar-Sagi, 1994). Two types of protein kinases are involved in this process namely the receptor (RTK) and non-receptor protein tyrosine kinases (PTK). In the case of the RTK's, polypeptide growth factors such as EGF bind to the extracellular domain (Pawson, 1995) and activate the intrinsic tyrosine kinase activity of the receptor leading to receptor autophosphorylation (Schlessinger, 1988) and the generation of binding sites for other signaling proteins (Anderson *et al.,* 1990b; Matsuda *et al.,* 1990). Non-receptor tyrosine kinases, such as v-*src* (Collett *et al.,* 1980; Hunter and Sefton; 1980; Levinson *et al.,* 1980), v-*fps* (Pawson *et al.,* 1980 Neil *et al.,* 1981), and v-*fes* (Barbacid *et al.,* 1980; Beemon, 1981) have been identified as retroviral oncogenes suggesting an important role of Protein Tyrosine Kinase in transformation and cell proliferation (Jove and Hanafusa, 1987). The protooncogene *ras* and the MAP Kinase pathway are also involved in several such protein tyrosine kinase systems. Both v-*src* and the activated EGFR stimulate guanine nucleotide exchange on ras (Satoh *et al.,* 1990a; 1990b).

Many proteins that participate in mitogenic signalling are substrates for protein tyrosine kinases (Ellis *et al.,* 1990), they are regulated by protein tyrosine phosphorylation (Nishibe *et al.,* 1990; Meisenhelder *et al.,* 1990; Margolis *et al.,* 1990) or serve a binding proteins at specific phosphotyrosine-docking sites of protein tyrosine kinases (McGlade *et al.,* 1992; Rozakis-Adcock *et al.,* 1992). Several of these proteins such as rasGAP, shc and GRB2 are involved the regulation of MAP Kinase signalling.

Because changes in tyrosine phosphorylation are associated with the mitogenic response and transformation, differences in protein tyrosine phosphorylation in FBR and 208F cells were investigated which might ultimately regulate downstream mitogenic pathways such as

MAPK pathway. An antibody which recognises tyrosine phosphorylated proteins was used together with western immunoblotting and immunoprecipitation to determine whether difference exist between FBR and 208F cells.

9.2. Identifcation of Focal Adhesion Kinase as a potential regulator of MAPK signalling in FBR and 208F

Phorbol ester TPA, a tumour promoter, induces a variety of cellular responses such as protein tyrosine phosphorylation (Gilmore and Martin, 1983; Cooper *et al.,* 1984; Ferrel and Martin, 1990), MAP Kinase activation (Hoshi *et al.,* 1988) and also cytoskeletal reorganisation (Lewis *et al.,* 1996). To identify if TPA has the ability to stimulate protein tyrosine phosphorylation in FBR and 208F cells, cell lysates were prepared from each cell type and analysed for the tyrosine phosphorylated- protein content using western immunoblotting.

Serum deprived FBR and 208F cells were incubated with 20 ng/ml TPA for 10 minutes and the dishes were washed twice with ice cold PBS and lysates were prepared by lysis with the standard Triton X-100 containing buffer. The lysates were clarified by centrifugation and the 200 μg of total cell lysate proteins were separated in 7.5% SDS-PAGE gels and transferred to PVDF membranes. A monoclonal antibody against phosphotyrosine (anti-ptyr, PY20) was used to identify tyrosine phosphorylated proteins between 60 and 220-250 kilodaltons.

At quiescence FBR and 208F cells share some similarities in tyrosine phosphorylation of proteins (Figure 9.1). Phospho-proteins of approximately 50-55 kD were detected. A protein at 120kD becomes heavily phosphorylated after induction with TPA in 208F but not in FBR cells. Another single band of approximately 150 kD appears to be heavily phosphorylated in response to TPA in 208F cells but not in FBR's.

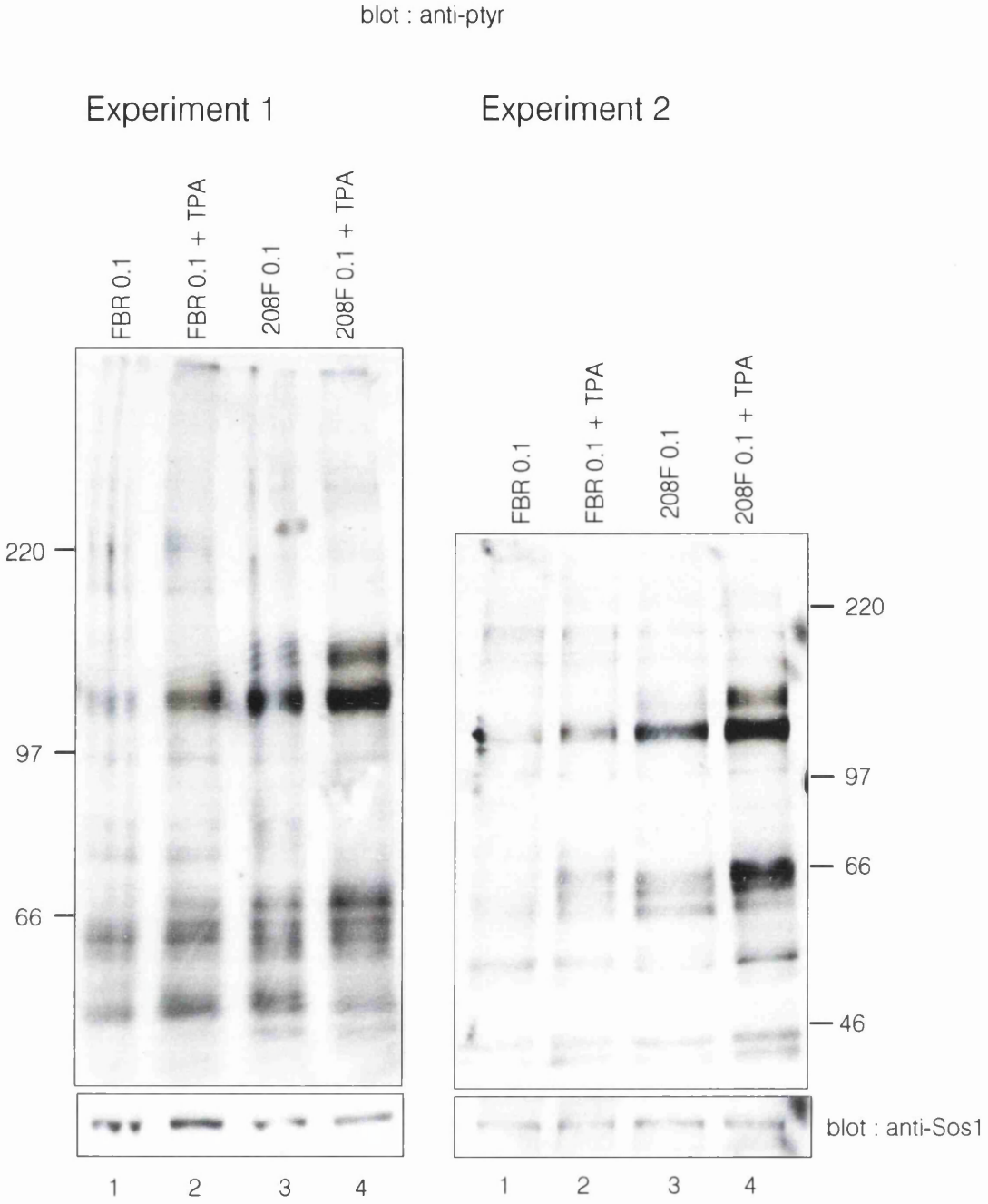


Figure 9.1. TPA-induced protein tyrosine phosphorylation in FBR and 208F cells.

Cells were grown in DMEM supplemented with 10% FCS and when subconfluent, serum-deprived for 48 hours (0.1% FCS). Stimulation was for 10 minutes with 20 ng/ml TPA.
Cell lysates were prepared and proteins were separated by 7.5% SDS-PAGE, transferred to PVDF membranes and probed with an antibody specific for phosphotyrosine (PY-20, Transduction labs, KY, USA).
To confirm equal loading the membranes were stripped, and probed with an antibody specific for Sos-1 (Transduction Labs, KY, USA).
Two independent experiments are shown.

Another mitogen, EGF was used to compare the induction of protein tyrosine phosphorylation in FBR and 208F cells. Cell lysates from EGF-treated FBR and 208F cells for 10 minutes were prepared, and examined for phosphotyrosine content using the same conditions as in the previous experiment. When quiescent 208F cells were treated with EGF a dramatic increase in tyrosine phosphorylation of proteins around 60-70 kD, and also as in the TPA-treated 208F in 120 and 150 kD (Figure 9.2). Three major tyrosine phosphorylated proteins were identified in EGF-treated 208F cells of the following molecular masses: 60-70kD, 120-150kD and 160-180kD. A protein of approximately 160-180 kD is heavily tyrosine phosphorylated in 208F cells in response to EGF (Figure 9.2). In contrast to what was found in 208F samples, no increase in the content of tyrosine phosphorylated proteins in FBR cells treated with EGF was found. One only protein of approximately 180 kD displayed an increased phosphotyrosine content in FBR cells and its was unresponsive to extracellular stimulation. This was also seen in TPA-treated FBR cells (Figure 9.1 lane 1 and 2, Experiment 1 and Experiment 2). It should be noted that the variation observed between the experiment 1 and 2 in the Figure 9.2. is due to the fact that different amounts of lysate were loaded in these gels. In the experiment 1 50 μg were used and in the experiment 2, 100 μg of total cell lysate. In both cases the membranes were incubated with identical dilution of the primary anti-phosphotyrosine antibody PY-20.

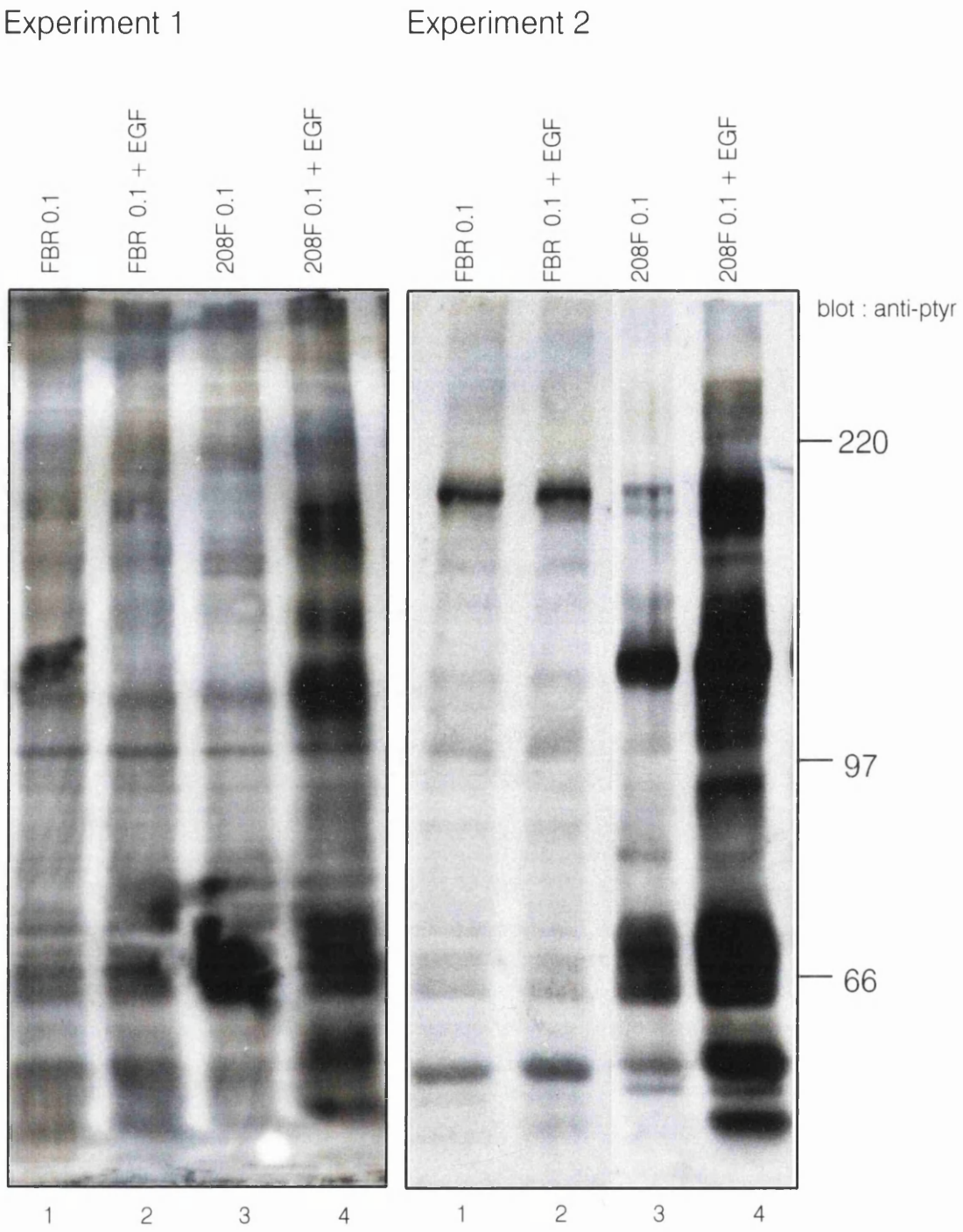


Figure 9.2. EGF-induced Protein tyrosine phosphorylation in FBR and 208F cells.

Cells were grown in DMEM supplemented with 10% FCS and when subconfluent, serum-deprived for 48 hours (0.1% FCS). Stimulation was for 10 minutes with 50 ng/ml EGF.
Cell lysates were prepared and proteins were separated by 7.5% SDS-PAGE, transferred to PVDF membrane and probed with a specific antibody for phosphotyrosine (PY-20, Transduction labs, KY, USA).
Two independent experiments are shown

Schlaepfer et al (1998) have identified a variety of different tyrosine phosphorylated proteins in lysates prepared from NIH 3T3 fibroblasts replated on fibronectin. These investigators have shown that FAK and $p130^{CAS}$ proteins are associated with a 120-140 kD protein complex, heavily tyrosine-phosphorylated upon adhesion.

Focal Adhesion Kinase is a protein tyrosine kinase localised to the focal adhesions and regulates biological functions of these cell structures. Focal adhesions serve as areas of cell attachment to the substratum and consist of many proteins that are tyrosine phosphorylated and which play a role in cell attachment and migration. It has been suggested that Focal Adhesion Kinase is tyrosine phosphorylated by *src* kinase and this phosphorylation event serves as a docking site for the binding of the GRB2 adaptor protein (Schlaepfer *et al.,* 1994; Schlaepfer and Hunter, 1996; Schlaepfer *et al.,* 1997; Schlaepfer *et al.,* 1998) providing a link to *ras* and MAP Kinase pathway (Pawson, 1992 ; Rozakis-Adcock *et al.,* 1993).

Since FBR cells are poorly adherent to the plastic substratum but 208F cells attach even in the absence of serum, it was hypothesized that FAK could be tyrosine phosphorylated in 208F cells and not in FBR cells. Thus the nature of the 120 kD protein found to be tyrosine phosphorylated in 208F cells was further investigated.

Immunoprecipitation experiments using a monoclonal antibody specific for FAK was performed using lysates from quiescent, EGF and TPA stimulated FBR and 208F cells to investigate the tyrosine phosphorylation state of FAK. Cells were stimulated with 25 ng/ml EGF, 20 ng/ml TPA or left untreated and lysates were prepared using the standard Triton X-100 containing buffer. The lysates were clarified by centrifugation and the supernatant was normalised for protein content and 1000 μg of lysate was used to immunoprecipitate FAK and its associated proteins with a monoclonal antibody against FAK. After overnight incubation of the antibody, the immunoprecipitates were washed with lysis buffer, boiled in a small volume of 2X SDS sample buffer and the complexes were separated by 7.5% SDS-PAGE gels, transferred to PVDF membranes and blotted with the monoclonal antibody against phosphotyrosine.

As shown in Figure 9.3 FAK is tyrosine phosphorylated in quiescent and stimulated 208F cells but not in the FBR treated or control samples. This correlates with the data presented in Figures 9.1 and 9.2 and with published studies of Hunter and co-workers where FAK tyrosine phosphorylation was detected in response to adhesion and attachment in rodent fibroblasts. We

were unable to identify tyrosine phosphorylation of FAK in immunoprecipitates from quiescent or stimulated FBR cells. Even after prolonged exposure, no tyrosine phosphorylation of Fak could be detected in quiescent or stimulated FBR lysates. To confirm that FAK was immunoprecipitated from all the cell lysates used, the same membrane was stripped and probed with the FAK-specific monoclonal antibody in all samples. This is shown clearly in Figure 9.3 c, where equal amount of FAK are present in each lane (Figure 9.3c).

It is well established that FAK plays a significant role in MAP Kinase signalling in fibroblasts. Oncogenic transformation associated with non-receptor tyrosine kinases such as v-*src* v-*fps* and v-*abl* or Receptor Tyrosine Kinases such as the EGFR, results in tyrosine phosphorylation of FAK and activation of MAP Kinase signalling. In FBR cells the absence of strong tyrosine phosphorylation signals derived from activated or overexpressed tyrosine kinases upstream of *fos*, together with the results presented which indicate that FAK activation might be important in the regulation of MAP Kinase signalling pathway. FAK, a membrane-associated tyrosine kinase , which is phosphorylated in FBR cells suggesting a role for FAK in the down-regulation of MAP Kinase signalling pathway

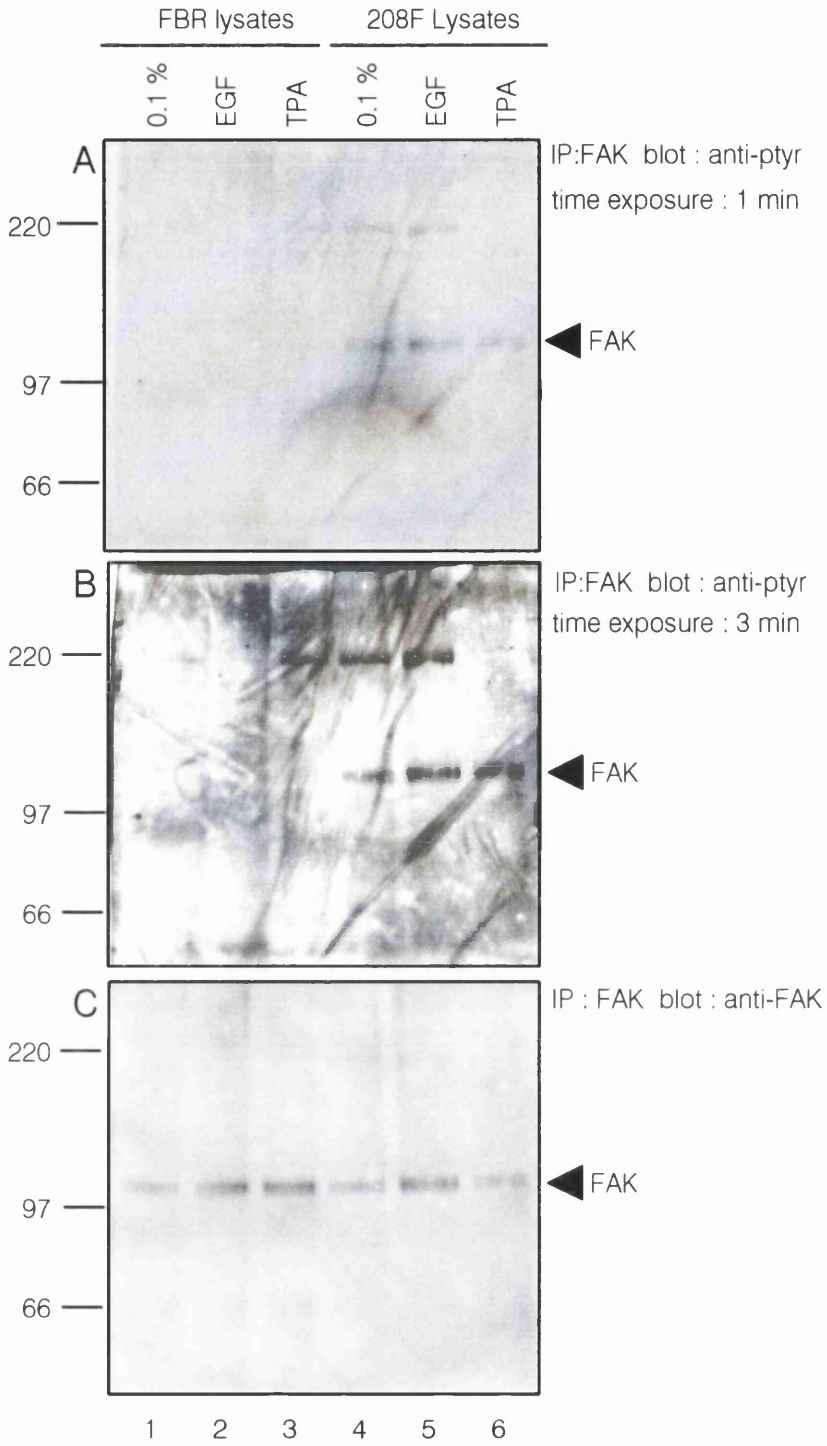


Figure 9.3. EGF and TPA-induced tyrosine phosphorylation of FAK in FBR and 208F cells.

Cells were grown in DMEM supplemented with 10% FCS to subconfluence and serum-deprived for 48 hours (0.1% FCS). Stimulation was for 10 minutes with 20 ng/ml EGF, 20ng/ml TPA or the cells remain untreated.
Cell lysates were prepared and 1000 µg of total cell lysate was immunoprecipitated with a FAK-specific antibody (Transduction Labs, KY, USA). Immunoprecipitates were incubated overnight rotating at 4^0C, washed with lysis buffer, separated by 7.5% SDS-PAGE, transferred to PVDF membrane and probed with A) and B) specific antibody for phosphotyrosine (PY-20, Transduction labs, KY, USA).
C) To verify equal loading the membrane was striped, washed, blocked and probed with the FAK-specific antibody.
Two different exposures are shown to identify tyrosine phosphorylation of FAK in 208F cells.

9.3. Conclusion

Phosphorylation of FAK by tyrosine kinases occurs in response to adhesion to extracellular matrix components and this function is implicated in the association of FAK with signalling proteins such as GRB2, PLC-γ and NCK and also in the activation of MAP Kinases. FAK also is activated in response to stimulation with extracellular signals such as phorbol esters (Woods and Couchman, 1993 Vuori and Ruoslahti, 1993 ; Lewis *et al.,* 1996), LPA (Takeda *et al.,* 1998) growth factors (Chen *et al.,* 1998; Casamassima and Rozengurt, 1998) and ultimately in the activation of MAP Kinases. These events make FAK an important mediator of extracellular signals in the activation of the MAP Kinase pathway. Recently it was also shown that serum or growth factor-dependent activation of MAP Kinases is strongly dependent on cell adhesion to the extracellular matrix (Renshaw *et al.,* 1997). These data suggest that adhesion to ECM components activates or enhances positive MAP Kinase through a mechanism that involves activation of a FAK-dependent ras-sensitive pathway.

Here the tyrosine phosphorylation state of FAK is reduced both in resting and agonist stimulated FBR cells, whereas it is tyrosine phosphorylated in 208F cells. Lack of FAK activity could significantly contribute to the loss of adhesion properties associated with FBR cells.

A differential hybridisation approach used in this lab has shown that the mRNA of a membrane-associated Protein Tyrosine Phosphatase is overexpressed in FBR compared to 208F cells (H.J. Spence, I. Johnston and B.W. Ozanne, unpublished observations). One could speculate that this phosphatase could contribute to the loss of phosphotyrosine containing proteins in FBR cells, resulting in FAK de-phosphorylation and ultimately leading to a diminished response to different signals leading to the activation of MAP Kinases.

CHAPTER 10 DISCUSSION

ISOLATION OF FBR REVERTANT CELL LINES HELPS TO UNDERSTAND THE REQUIREMENT OF MAP KINASE PATHWAY DURING ONCOGENIC TRANSFORMATION

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10.1. Properties of flat revertants derived from oncogenically transformed cells

In cancer biology the term reversion describes the conversion from the transformed, malignant state to a non-transformed one, as it appears in established cell lines (Noda, 1993). A number of flat revertant cell lines derived from transformed cells by RNA-containing sarcoma viruses have been isolated and characterised previously (Ozanne and Vogel, 1974; Deng *et al.,* 1974; Deng *et al.,* 1977; Noda *et al.,* 1983).

Kuzumaki *et al.* (1989) have classified the revertant cell lines into three different classes. The first consists of those which either retain an inactive oncogene or have lost the relevant viral oncogene (MacPherson, 1965). The second category contains revertants that bear mutations in cellular genes which interfere with morphological transformation (Zarbl *et al.,* 1987). The third class includes cells derived by activation of somatic transformation suppressor genes (Noda *et al.,* 1983, 1989 ; Kitayama *et al.,* 1989).

To generate revertant cell lines from oncogenically transformed cells, different methods have been used, for example treatment with mutagens (Kuzumaki *et al.,* 1989), or the application of tissue culture methods such as selective adhesion of sub-colonies of non-transformed cells from transformed cells and enrichment under low serum conditions (Noda *et al.,* 1989; Wisdom and Verma, 1990). Usually these stringent procedures have the disadvantage of generating cell lines with variations due to chromosomal aberrations or unidentified mutations that might lead to non-reproducible results.

Previously Wisdom and Verma (1990) and Joliqoeur and co-workers (Zarbl *et al.,* 1987) have isolated FBR and FBJ revertant cell lines respectively and have shown that these are resistant to transformation by other oncogenes.

To generate revertant cells lines of the FBR v-*fos* transformed cell line a transfection approach was used. Dominant negative mutants of the c-*jun* protooncogene, and of the MEK-1 and Raf-1 protein kinases which are upstream of AP-1 were used. The cell lines isolated after

these transfection experiments exhibit the properties of a revertant. The growth properties of these cell lines demonstrate the requirement of one at least of the three well established the *ras* pathways, during the induction of cellular transformation by the FBR-MuSV. The *ras* proto-oncogene is known that can activate except the MAP Kinase pathway the PI3K-PDK- PKB and the Ral-GDS pathway.

These cell lines appear also to be useful to study genes that are involved in morphological transformation by *fos* and *ras* oncogenes.

10.1.1. Isolation of FBR revertants following transfection of TAM-67

In order to demonstrate the requirement of Fos and Jun proteins in cellular transformation Muller and co-workers have made use of mutant Fos proteins that bear alterations in functional domains such as the NT-A, the C-terminal transactivation domain and the Basic-Leucine Zipper region (Neuberg *et al.,* 1991; Wick *et al.,* 1992) These studies showed that multiple regions in the Fos protein are important for the induction of transformation, and that the leucine zipper was necessary for these biological responses. Fos proteins have impaired transforming potential when the leucine repeat is substituted with the Jun leucine repeat (Neuberg *et al.,* 1989b). These chimeric Fos proteins, termed Ψ-Fos, are able to form dimers composed only of Ψ-Fos proteins. These dimers capable of binding DNA but do not exhibit any transforming potential, suggesting that dimerization with Jun family members is required for transformation (Neuberg *et al.,* 1991). In transfection assays the induction of transformation was maximal when Fos and Jun were combined in the assays, compared with Fos transfected alone (Neuberg *et al.,* 1991). These studies together with the observation that ES cells lacking c-*jun* are non-tumourigenic (Hilberg and Wagner, 1992), suggest that the c-*jun* protooncogene is an critical factor in AP-1-mediated transformation activity *in vitro* and *in vivo*.

Previously Iba, Curran and co-workers have used point mutants and N-terminally truncated c-*jun* genes to investigate AP-1-mediated cellular transformation (Okuno *et al.,* 1991; Suzuki *et al.,* 1994). These studies showed that AP-1 is a downstream component of signal transduction pathways, and that the mutant Jun proteins can efficiently inhibit oncogenic transformation by diverse oncogenes such as v-*src*, v-*fps*, c-Ha-*ras* and *raf* (Suzuki *et al.,*1994).

A transfection assay with the truncated c-*jun* protooncogene deletion mutant TAM-67 was used to investigate AP-1-induced transformation by v-*fos in vitro* and to inhibit its transforming potential. Dominant negative c-*jun* TAM-67 has been developed by Birrer, Karin and co-workers (Alani *et al.,* 1991; Brown *et al.,* 1993, 1994) down-regulates AP-1 activity in transfection experiments, inhibits transformation by *ras*, *myc* and SV40 and counteracts TPA-induced focus formation. More importantly it has been shown to suppress malignant conversion of epithelial cells to squamous cell carcinomas in mouse models (Domann *et al.,* 1994). These findings make it an attractive suppressor of transformation in tissue culture and animal models, although care should be taken when interpreting these data since the squamous cell expressing-TAM-67 cells can still induce tumours with a longer latency in this particular mouse model (Domann *et al.,* 1994).

Recently it was shown that TAM-67 when expressed in A431 epidermoid carcinoma cells inhibits motility, invasion and actin reorganisation by EGF, suggesting that AP-1 activity is downstream of the EGFR and that negative modulation of signalling by TAM-67 affects these biological processes (Malliri *et al.,* 1998).

TAM-67 was transfected into FBR cells and after selection with G418, FBR TAM-67 stable cell lines were isolated. In these cells TAM-67 functions in a dominant negative fashion, causing reversion of *fos*-induced morphological transformation and inhibiting cell proliferation. In this study it a reduction of the proliferative potential of the 208F TAM-67 and FBR TAM-67 expressing cell lines compared to their controls was found (not shown). This is in contrast to other studies with TAM-67 in epithelial cells (Domann *et al.,* 1994; Malliri *et al.,* 1998).

In an analogy with the dominant effects of TAM-67 previously Muller and co-workers have also shown that there is not any significant alteration in the proliferative potential of fibroblasts after transfection with dominant negative mutants Fos proteins. These mutants also reversed *ras* transformation but did not interfere with the rate of proliferation of these cells (Wick *et al.,* 1992).

These observations make it difficult to understand the role of TAM-67 in the inhibition of 208F and FBR cell proliferation and how it could be related to cellular transformation. This could distinguish the fibroblast model of transformation from that of cells of epithelial origin such as A431 cells. One could speculate that the role of TAM-67 in fibroblastic cells is associated with inhibition of proliferation by sequestering complexes involving AP-1 components, such as c-*fos*

or FBR v-*fos*. Previous studies with c-*fos* antisense vectors (Holt *et al.,* 1986; Nishikura and Murray, 1987) and microinjection experiments with *fos*-specific antibodies (Riabowol *et al.,* 1988) suggested that c-*fos* could possibly function as a positive modulator of fibroblastic cell growth.

However studies with *fos* -/- fibroblasts have shown that *fos* is not required for cell proliferation (Brusselbach *et al.,* 1995). Again it is difficult to compare the proliferative potential of knockout cells to that of TAM-67 transfected cell lines. Recent work from Greenberg and co-workers suggests that *fos* plays a role in cell cycle progression through the regulation of cyclin D1 expression levels and that its expression positively regulates cell proliferation (Brown *et al.,* 1998).

TAM-67 inhibits also anchorage independent growth of the FBR TAM-67 cells, reducing colony number and size when compared to FBR cells. Therefore TAM-67 inhibits colony formation of FBR cells but not to a 100%. This could be due to the fact that in all FBR TAM-67 revertant cell lines the p75 $^{v\text{-}Fos}$ oncoprotein is expressed at levels similar to the parental FBR cells. Therefore several of the transforming and immortalising properties that p75$^{v\text{-}Fos}$ initiates are not fully suppressed. Dong *et al.* (1994) also reported that some epithelial cell lines when grown in the presence of phorbol ester or EGF are resistant to complete suppression of anchorage independence by TAM-67. These results show that some transformation-sensitive cell lines maintain a potential for anchorage independent growth after transfection with TAM-67.

TAM-67 also has the ability to inhibit EGF-induced morphological transformation of 208F cells. EGF-induced morphological transformation is associated with anchorage-independent growth (de Larco and Todaro, 1978) and stress fibre disorganisation (Ozanne *et al.,* 1980). This biological function of EGF occurs via the EGFR signalling pathway through the GRB2 adaptor (Matuoka *et al.,* 1993) and the *ras*-GTPase Activating Protein, *ras*GAP (McGlade *et al.,* 1993). In 208F TAM-67 transfectants, TAM-67 inhibits the disorganisation of actin stress fibres upon EGF treatment for 24 hours. This phenotypic effect of TAM-67 is associated with its ability to inhibit the transforming potential of growth factors coincident with its ability to prevent oncogene-induced transformation (Brown *et al.,* 1993; Dong *et al.,* 1994; Lamb *et al.,* 1997).

10.2. Growth Factors and Transformation

The proliferation and survival of cells in culture is dependent on the presence of peptide mitogens known as growth factors (Holley, 1975). Cells transformed by RNA- or DNA-tumour viruses and some human tumour cell lines can proliferate in the absence of added growth factors in the medium (Kaplan, 1983). The v-Ki-*ras* oncogene has been reported to transform cells independent of the presence of growth factors (de Larco and Todaro, 1978; Ozanne *et al.,* 1980) and to induce growth under anchorage-independent conditions (Kaplan and Ozanne, 1983). These findings led Todaro to propose that autocrine mechanisms could be relevant to the growth of cancer cells *in vitro* as well as *in vivo* (Sporn and Todaro, 1980).

Some primary evidence suggest that v-*fos* exerts a different function in terms of the growth factor requirement *in vitro* compared to other oncogenes. Zhan and Goldfarb (1986) have found that cytoplasmic oncogenes e.g. v-*mos*, v-*sis*, v-*src* and v-*ras* can release the growth factor dependency of NIH and BALB/c 3T3 cell lines, while FBJ v-*fos* does not support such a function. In addition Vennstrom and Bravo (1987) reported that the FBR-MuSV and FBJ-MuSV do not support the growth of BALB/c 3T3 cells in semi-solid medium, in the absence of competence type growth factors such as Platelet Poor Plasma (PPP) or PDGF (Pledger *et al.,* 1977), however they are able to do so when co-infected with the MMCV retroviruses carrying the v-*myc* oncogene of the avian retrovirus OK10 (Vennstrom *et al.,* 1984).

A previous study in this laboratory has shown that incubation of FBR v-*fos* transformed fibroblasts in serum-free medium led to a marked decrease in DNA synthesis, suggesting that there was no autocrine growth factor activity from this transformed cell line which could induce DNA synthesis and proliferation (Hawker *et al.,* 1993; Hennigan *et al.,* 1994). Thus the FBR and FBJ v-*fos* transformed fibroblasts are serum-dependent for DNA synthesis and cell proliferation (Hennigan, 1993).

These observations lead to this investigation of growth factor-dependent signals of the MAP Kinase pathway in the context of cellular transformation by FBR v-*fos* oncogene.

To address this, the first question concerned whether conditioned medium collected from serum-deprived FBR could induce MAP Kinase activation. Results indicated that FBR-derived conditioned medium did not induce any activation of MAP Kinase neither FBR nor in 208F cells. Thus the activation of the MAP Kinase pathway in FBR cells is serum-dependent. The growth factor dependence of FBR cells shows that DNA synthesis and mitogenic signals

that emerge from the activation of the MAP Kinase pathway could be required for cell proliferation. DNA synthesis and ultimately proliferation are also required for transformation (McCormick, 1994 , 1995). This lead us to speculate that the growth factor dependency of MAPK signals in FBR might be also be relevant and important for morphological transformation.

10.2.1. Is MAP Kinase signalling important for cellular transformation ?

Previously other groups have reported that oncogenic transformation results in defects in activation of MAP Kinases (Kizaka-Kondoh and Okayama, 1993; Greulich *et al.,* 1996; Stofega *et al.,* 1997). In these experiments different fibroblastic cell lines have been used for experiments that demonstrate the down regulation of MAP Kinase signalling by oncogenes including NRK, NIH 3T3 and chicken embryo fibroblasts. These studies indicate that different oncogenes such as v-*src* (Stofega *et al.,* 1997), v-*raf*-1 (Kizaka-Kondoh and Okayama, 1993), v-*crk* (Greulich *et al.,* 1996) are involved in the downregulation of MAP Kinase signalling in different cell settings *in vitro*.

In human tumour cells, recent data suggest that MAP Kinase signalling might be related to tumorigenesis. ERK1 and ERK2 are over-expressed in human breast tumours compared to normal control tissue. In addition the tyrosine phosphorylation of MAP Kinases ERK1 and ERK2 is higher in tumours compared to normal controls as detected by immunoprecipitation and western immunoblotting. Unfortunately these studies do not provide any mechanistic explanation for these over-expression patterns. Two other reports also suggest a connection between MAPK activity in hepatocellular carcinomas and tumour progression of this type of cancer (Schmidt *et al.,* 1997; Ito *et al.,* 1998).

A rather intriguing report deals with the role of an activated Estrogen-dependent mutant of Raf-1 kinase : DRaf-1:ER in cell cycle entry of human lung cancer cells (Ravi *et al.,* 1997). DRaf-1:ER transfected into human lung cancer cells negatively regulates their growth by induction of the cyclin-dependent kinase inhibitor $p27^{kip}$.

Two possible reasons may account for these differences. Firstly the studies with breast tumours use material derived from tissues and not cultured cell lines that may have undergone additional alterations. Secondly, cell type specificity also contributes to observed differences in the experimental systems mentioned.

These examples show that there could be a variation in the nature of activation of MAP Kinase signalling both within naturally occurring tumours and when established cell lines are compared to oncogenically transformed cells (Gupta *et al.,* 1992; Gallego *et al.,* 1992; Greulich *et al.,* 1996; Greulich and Erikson, 1998).

It is possible that MAP Kinase activation could result in the up-regulation of mechanisms controlling cell proliferation and DNA synthesis. From experiments with fibroblasts there is evidence that a dominant negative MAP Kinase mutant can have negative effects on cell cycle entry, DNA synthesis and AP-1-dependent transcriptional activity (Pages *et al.,* 1993). Also transfection of fibroblasts with plamsids encoding antisense ERK2 and inactive ERK2 kinase resulted in impaired colony formation in a dose-dependent manner (Pages *et al.,* 1993).

In these studies stimulation of FBR and 208F cells with various stimuli resulted in differential activation of MAP Kinase signalling, with the magnitude of serum-induced activation of ERK1 and ERK2 decreased in FBR compared to the parental 208F cells. When growth factors and activators of lipid signal transduction pathways e.g. LPA, the response was similar to that found with serum: decreased levels of phosphorylated MAP Kinases in FBR cells compared to 208F controls. These observations suggest an apparent paradox: why is MAP Kinase signalling important and relevant to cell transformation, but maintained at such low levels ?

A similar paradigm is addressed by Cambell, Der and co-workers in the context of *ras* transformation, as seen in the case of various *ras* mutants which are able to induce cellular transformation without activating fully the Raf/MEK/MAPK pathway. It is interesting that the blockage of these pre-existing, low levels of MAPK activity resulted in the inhibition of *ras* transformation The answer to this question is that there is a series of events that follows tumourigenic transformation by *ras*, and that suppression of one pathway involved could significantly reverse the transformed phenotype (Cambell *et al.,* 1998).

By analogy to *ras*-induced transformation, blocking of the MAP Kinase pathway could yield insights into the regulation of morphological transformation by FBR v-*fos.* To address this question dominant negative mutants of the *ras* effectors Raf-1 and MEK-1 were transfected into FBR cells and analysis of isolated drug-resistant clones, showed that this pathway was required for *fos*-induced transformation. The FBR cell lines transfected with dominant negative MEK-1 and Raf-1 had the properties of revertant cells suggesting a putative role of the MAP Kinase pathway in *fos*-transformation. These results are discussed in more detail below.

10.2.2. Isolation of FBR revertants after transfection of of MEK-1 A221

MEK-1 is a MAP Kinase Kinase, with dual specificity kinase activity towards Threonine and Tyrosine residues (Alessandrini *et al.,* 1992; Rossomando *et al.,* 1992; Asworth *et al.,* 1992; Nakielny *et al.,* 1992; Seger *et al.,* 1992). MEK-1 phosphorylates the ERK family of MAP Kinases ERK1 and ERK2 (Extracellular signal Regulated Kinase) (Boulton *et al.,* 1991) on Threonine and Tyrosine residues *in vitro* and *in vivo.* MEK-1 is activated by phosphorylation of two serine residues Serine 217 and Serine 221 by the product of the protooncogene Raf-1, a Serine/Threonine protein kinase. This phosphorylation is required for activation of MEK-1 by Raf-1 *in vitro* and *in vivo* (Alessi *et al.,* 1994).

Experiments with mutant MEK-1 showed that substitution of either Serine 217 or Serine 221 with Alanine residues is sufficient to impair the catalytic properties of the enzyme (Alessi *et al.,* 1994). When a mutant MEK-1 bearing such mutations is microinjected into v-*ras* or v-*src* transformed NIH 3T3 fibroblasts, DNA synthesis is inhibited up to 50% (Cowley *et al.,* 1994). This result suggests that MEK-1 is downstream of the v-*ras* and v-*src* oncogenes and it can block signal transduction mechanisms downstream of Receptor Tyrosine Kinases and oncogenic *ras*. When the Serines 217 and 221 residues of MEK-1 are replaced with Glutamate or Aspartate the resulting enzyme is constitutively active with activity independent of growth factor stimulation. This gain of function mutant of MEK-1 can promote serum-independent growth of NIH 3T3 transfected cells (Alessandrini *et al.,* 1996; Greulich and Erikson, 1998) and promote serum and growth factor-independent growth in tissue culture (Cowley *et al.,* 1994). Additionally it induces tumours when injected into nude mice (Cowley *et al.,* 1994).

This evidence highlight a role for MEK-1 in cellular transformation (Cowley *et al.,* 1994) and cell cycle progression by the *ras* oncogene (Greulich and Erikson, 1998). Since the *ras* oncogene is upstream of *fos* oncogenes the role of the dominant negative MEK-1 in cellular transformation by the FBR *fos* was examined. Introduction of dominant negative MEK-1 with the Alanine 221 substitution into FBR cells inhibited anchorage-independent growth in serum-containing semi-solid medium.

MAP Kinase activation also seems to be related to AP-1 activation in tissue culture cells. Microinjection experiments with ERK-specific substrate peptides inhibits AP-1 activation by serum and phorbol esters (Frost *et al.,* 1994) and over-expression of ERK1 and ERK2 kinase-

inactive mutants blocks activation of AP-1 reporters by *ras*, serum and phorbol esters (Frost *et al.,* 1994).

All these data argue for an important role of MAP Kinases in signal transduction and activation of AP-1. Here the role of MEK-1, one of the constituents of the *ras* pathway in AP-1/*fos*-induced transformation was investigated.

Marshall and co-workers who pioneered these studies have examined the DNA synthesis patterns of *ras* and *src*-transformed fibroblasts microinjected with MEK-1 A217 and MEK-1 A221 mutants. These experiments resulted in a significant reduction of the ability of *ras* and *src* oncogenes to induce DNA synthesis after microinjection of the MEK-1 interfering mutants. Microinjected *ras*-transformed cells with MEK-1 A221 or MEK-1 A217 vectors resulted in are morphological reversion with concomitant restoration of the actin stress fibres. In contrast, the activated mutant of MEK-1 E217/E221 induces morphological transformation and promotes serum-free growth of NIH 3T3 cells (Cowley *et al.,* 1994). This indicates that MEK-1-dependent transformation of NIH 3T3 cells is related to transformation by oncogenic *ras*, and that MEK-1 is necessary for abnormal growth control and morphological transformation. In FBR A221 revertant cells lines similar effects were observed: restoration of the actin cytoskeleton and the stress fibres present in contrast to FBR where the cytoskeleton is dissolved with very few actin stress fibres present. FBR A221 revertant cells share another property with the parental 208F cells in that the adhesion components, possibly focal adhesions, are restored.

It is known that serum-, EGF- or TPA-induced MAP Kinase activation can be inhibited by the introduction of MEK-1 A221 and MEK-1 A217 mutants (Cowley *et al.,* 1994; Seger *et al.,* 1994). This can be measured by several ways such as the activation of MAP Kinases using an *in vitro* kinase assay or by supershifts of ERK1 and ERK2 in SDS-polyacrylamide electrophoresis. Quiescent FBR A221 revertant cell lines when stimulated with serum have decreased phosphorylation of ERK1 and ERK2, as shown by western immunoblotting with phosphospecfic antibodies. Orthovanadate, a broad inhibitor of protein-tyrosine phosphatases was also tested in the activation of ERK1 and ERK2 in FBR and FBR A221 revertant cells. There response to activation of ERK1 and ERK2 by orthovanadate in the FBR A221 cells was similar to the effects of serum: there was not any significant elevation of ERK phosphorylation compared to 208F cells were stimulation with orthovanadate induces ERK activation.

When these cells were tested for a high concentration of EGF, there was no inhibition but there was a potent response to this mutant, suggesting that the dominant negative MEK-1 A221 could be activated under these conditions as previously shown by others (Cowley *et al.,* 1994; Seger *et al.,* 1994). It should be noted that since there is no evidence for the expression of this mutant in FBR A221, it is not known if the observed differences are due to the presence of the transfected gene or are a result of transfection selection.

10.2.3. Isolation of FBR revertants after transfection of NΔRaf-1

The role of Raf-1 kinase in cellular transformation has been established since the identification of the v-*raf* oncogene as the component of the transforming retrovirus 3611 (Rapp *et al.,* 1983). Like most retroviral oncogenes v-*raf*-1 has the ability to transform established cells lines and to induce them to grow under anchorage independent conditions.

Raf-1 also can transform cells in co-operation with *ras* oncogenes in cell culture experiments. A truncated c-Raf-1, BXB Raf-1 kinase transforms NIH 3T3 cells in a co-operative fashion with *ras* (Kolch *et al.,* 1991) and also in co-operation with ERK1 and ERK-2 MAP Kinases (Troppmair *et al.,* 1994). A chimeric protein consisting of the Raf-1 kinase domain fused to the ligand binding domain of the Estrogen Receptor (ER) can transform established cell lines *in vitro* and induce them to grow under anchorage-independent conditions (Samuels *et al.,* 1993). This transformation function of v-*raf* is not independent of functional *ras* as shown by microinjection of neutralising *ras* antibodies (Smith *et al.,* 1986) and by expression of the dominant negative allele N17*ras* (Feig and Cooper, 1988). Conversely *ras*-induced transformation can be suppressed by the expression of a kinase-inactive Raf-1 mutant (Kolch *et al.,* 1991).

ras also plays an important role in the activation of Raf-1 by extracellular signals. The dominant negative allele N17*ras* (Feig and Cooper, 1988) is required for ligand-induced phosphorylation and activation of Raf-1 in PC12 and NIH 3T3 cells (Wood *et al.,* 1992 ; Troppmair *et al.,* 1992). Expression of oncogenic *ras* in fibroblasts results in Raf-1 phosphorylation (Morrison *et al.,* 1988; Wood *et al.,* 1992). Activation of downstream effectors of *ras* signalling such as the MAP Kinases is independent of *ras* in v-*raf* transformed Swiss 3T3

cells (Howe *et al.,* 1992). These studies reveal a potentially important role of Raf-1 in signalling mechanisms induced by growth factors and intracellular transformation by *ras* oncogenes.

In most cases nuclear oncogenes do not enhance activation of the *ras* pathway and ultimately MAP Kinase phosphorylation and activity. Cuadrado *et al.* (1994) have shown that FBR *fos* does not induce hyper-phosphorylation of Raf-1 kinase nor activate MAP Kinases and also v-*myc* behaves similarly (Howe *et al.,* 1992). These data suggest that activation of MAP Kinase signalling in transformed cell lines is not a consequence of transformation by nuclear oncogenes. Activation of this pathway depends on cytoplasmic oncogenes such as v-*ras* and v-*src* (Howe *et al.,* 1992). Although this appears to be true in the case of this study, it does not necessarily preclude the lack of importance of this pathway in morphological transformation by *fos*.

As shown in the case of MEK-1, a dominant negative MEK-1 A221 could reverse the *fos*-induced transformed phenotype in transfection studies. Since the activator of MEK-1 is Raf-1 in many cell types a similar approach was taken to study the role of the dominant negative Raf-1 by expressing NΔRaf-1 in the FBR background. This truncated mutant has an intact N-terminus regulatory domain containing a cysteine-rich domain that is the putative binding site for a modulator of Raf-1 activity (Bruder *et al.,* 1992). Also certain amino acids in this domain serve as interaction sites with $p21^{ras}$ (Vojtek *et al.,* 1993) suggesting that when over-expressed in tissue culture cells, this regulatory domain will interfere with early activation events of the MAP Kinase pathway since Raf-1 acts as a MAP Kinase Kinase Kinase.

Transfection of NΔRaf-1 into COS cells inhibits MAP Kinase activation by EGF, TPA and LPA (Schaap *et al.,* 1993; Howe and Marshall, 1993). Recently these results have been confirmed by an analysis of this inhibitory function of NΔRaf-1 on the MAP Kinase pathway also in Rat-1 cells (van Dijk *et al.,* 1997). These studies have shown that Raf-1 is central to the activation of the MAPK pathway by diverse mehanisms such as growth factors and activators of lipid pathways, as the Phosphatidyl Choline-specific phospholipase C (PC-PLC) and atypical protein kinase C-ζ (PKC-ζ). Taken together these data support the role of Raf-1 as a mediator of *ras* and growth factor-induced activation of MAP Kinases.

The biological effect of transfected NΔRaf-1 was tested in the context of *fos*-induced transformation. FBR cells were transfected with NΔRaf-1 and G418-resistant stable cell lines

were isolated . The stable cell lines designated FBR NΔRaf-1 expressed sufficient levels of the mutant as shown by western immunoblotting. NΔRaf-1 reversed *fos*-induced transformation and the all the FBR NΔRaf-1 expressing cells displayed a flat phenotype. NΔRaf-1 also reduced the ability of FBR to grow in semi-solid medium, that is in anchorage-independent conditions. These data show that NΔRaf-1 reverses transformation and that the FBR NΔRaf-1 expressing cells have properties of revertant cells.

Serum-induced activation of MAP Kinases ERK1 and ERK2 in FBR NΔRaf-1 expressing cells was examined by western immnuoblotting with phospho-specific antibodies. When serum-starved FBR NΔ Raf-1 revertants are stimulated with serum there is a 2-fold reduction in ERK activation compared to FBR cells suggesting that dominant negative NΔRaf-1 has a functional role and blocks serum-induced MAP Kinase phosphorylation. Apart from growth factors and phorbol esters (Schaap *et al.,* 1993; van Dijk *et al.,* 1997) other activators of MAP Kinases are sensitive to inhibition by NΔRaf-1. Howe and Marshall (1993) showed a 60-80% inhibition of MAP Kinase activity in response to LPA stimulation of COS cells transfected with NΔRaf-1. Therefore NΔ Raf-1 is an effective inhibitor of MAP Kinase induction by multiple stimuli as measured by different assays in Rat-1 and COS cells. Although these data are consistent others (van Dijk *et al.,* 1997), other reports have provided contrasting evidence regarding the role of dominant negative Raf-1 mutants (Chao *et al.,* 1994). In this latter report the Raf-1 mutant 301, was employed to investigate the requirement of Raf-1 in the activation of MAP Kinases by EGF, IGF-I, calcium and phorbol ester. This particular mutant of Raf-1, has a Lysine to Tryptophan substitution in the kinase domain which renders it inactive (Kolch *et al.,* 1991). In these experiments a stable transfected Balb/c3T3 cell line with Raf-1 301 was tested for MAP Kinase activation using in-gel kinase assays. Stimulation with IGF-I and Thapsigargin, a calcium mobilising agent, found to be sensitive to this mutant, whereas Raf-301 did not block activation of MAP Kinases by EGF and phorbol ester in these cells (Chao *et al.,* 1994).

There is the possibility that cell type specificity determines some parameters of signal transduction mechanisms of Raf-1 activation and the suppression of MAPK pathway by dominant acting forms of the Raf-1 kinase. Most groups have similar data obtained from transient transfection assays, and this suggests that dominant negative Raf-1 kinase mutants are able to inhibit growth factor activation of MAP Kinases as measured by various methods.

It seems that in most cases activation of the MAPK pathway is associated with Raf-1 kinase activation by mitogenic signals. In experiments where DNA synthesis is induced by activators of other pathways, such as cAMP activation of PKA, a dissociation between MAPK activation and DNA synthesis is observed (Withers *et al.,* 1995).

FBR cells are spindle shaped, refractile and exhibit a loss of actin stress fibres when plated onto plastic in the repsence of serum (Hennigan, 1993 ; Lamb *et al.,* 1997). FBR NΔRaf-1 revertants are flat and have an increased number of stress fibres as shown by staining with Rhodamine-labelled Phalloidin. This is an interesting correlation between suppression of anchorage independence by NΔRaf-1 in FBR cells and morphological reversion (Weber *et al.,* 1974). FBR cells also have increased membrane ruffling activity in the area of the pseudopods. Despite the morphological reversion in FBR NΔRaf-1 revertants, no decrease in ruffling activity was observed but instead this remained persistent in cells actively growing in 10% FCS. This suggests that the introduction of NΔRaf-1 into FBR cells, unlike FBR MEK-1 A221 revertants, did not interfere with membrane ruffling but only with the formation of microfilaments. Therefore in the case of FBR NΔRaf-1 revertants the re-formation of the microfilament network might lead to anchorage-dependence, although the membrane ruffling activity remains unchanged. Previous microinjection studies with the activated mutants of Ras and Raf suggested that Raf-1 is not sufficient to induce potent membrane ruffling activity in fibroblasts. Microinjection of an activated form of *ras*, V12 Ras to Swiss 3T3 and REF-52 fibroblasts resulted in the induction of membrane ruffling (Ridely *et al.,* 1992; Joneson *et al.,* 1996). Conversely, microinjection of REF-52 fibroblasts with an activated, membrane-localised RAF-CAAX is not sufficient to induce membrane ruffling. The Ras mutant Ras(V12C40) efficiently transforms NIH3T3 cells and also co-operates with Raf in fibroblast transformation but itself does not induce loss of actin stress fibre formation, suggesting a Rho-depedent transforming function of this mutant. This is in contrast to other studies where the introduction of a constitutively active V12 GTPase *cdc42* into NIH 3T3 fibroblasts resulted in tumourigenicity, increased membrane ruffling but not full morphological transformation (Qiu *et al.,* 1997). It seems that the signals elicited during FBR transformation are different when compared to the signals after introduction of the V12 mutant of Rac-1 and RhoA activated GTPases. The mechanisms of transformation in a Raf-independent manner are believed to be dependent on Rho-like GTPases such as Rac1, Rho

and cdc42. The reversion mode that exists in the FBR NΔRaf-1 revertants is likely to be associated with the function of Raf-1 in the context of FBR transformation, leaving a window for other mechanisms to occur as those mentioned above.

The Raf-1 kinase inhibitor L-779 450 was also tested in its response to serum-induced MAPK phosphorylation in FBR, 208F and v-Ki-*ras* transformed 208F cells. These experiments also confirmed previous results. A dramatic decrease in the response to serum-induced MAP Kinase activity was observed in the FBR cells compared to 208F cells. The serum-induced phosphorylation of MAP Kinases in FBR cells was inhibited approximately 1.5-fold by L-779 450. This result suggests that the function of L-779 450 is similar to that by the dominant negative mutant of Raf-1 kinase, NΔRaf-1. In 208F cells, L-779 450 exerted a more potent inhibitory function since it inhibited MAPK phosphorylation approximately 7-fold but it did not significantly reduce the levels of activated MEK-1 suggesting the existence of different MEK-1 activators alternative to Raf-1. Serum-starved v-Ki-*ras* transformed cells displayed low levels of phosphorylated MAP Kinases. Similarly also to what others had observed this activity was not induced upon serum stimulation (Olson *et al.,* 1998). In v-Ki-*ras* cells L-779 450 inhibited the activity of MAP kinases by 1.5-fold and reduced the levels of phosphorylated MEK-1, suggesting that the v-Ki-*ras* oncogene is the major activator of Raf-1 kinase in these cells.

The distribution and organisation of actin in FBR and v-Ki-*ras* transformed cells was also examined, to determine if L-779 450 could reverse *fos*-induced transformation of FBR cells, as in the case of the the MEK-1-specific inhibitor PD98059 where reversion appeared to correlate in a time- and concentration-dependent manner. Treatment of FBR with L-779 450 resulted in partial morphological reversion of *fos*-transformation. In the case of v-Ki-*ras* cells the reversion was evident but different from the mode of reversion after treatment with PD98059: the cells lost the membrane ruffling activity and became flat but reversion was not accompanied with the reformation of stress fibres.

Considering the biochemical and the biological data from the three different cell types examined, it is obvious that Raf-1 activity leading to MAP Kinase stimulation is reduced in FBR cells and sensitive to inhibition by L-779 450, and in general its levels are low in this cell type. Despite the fact that the Raf-1 activity is possibly low in FBR cells its inhibition could possibly still be sufficient for the reversion of morphological transformation as shown by transfection

experiments with the NΔRaf-1. In this case it is not clear why pharmacological inhibition with L-779 450 did not result in the reversion of *fos*-induced transformation.

In terms of Raf-1 kinase activation, evidence is limited since various technical obstacles were encountered in kinase assays with bacterially produced substrates of GST-MEK-1 and GST-ERK2. The lack of evidence does not allow us to determine the activity of did not allow determination of Raf-1 kinase activity from lysates of stimulated FBR cells compared to stimulated 208F cells. This could have been potentially interesting since it has been proposed that alternative activators of MEK-1 exist (Zheng *et al.,* 1994). Previously Raf-1 activation as shown by Roberts and co-workers by the relatively straightforward method of measuring activation in lysates of tissue culture cells. These investigators have shown by western immmunoblotting, *in vitro* auto-kinase assays and *in vivo* [^{32}P] labelling that Raf-1 could become autophosphorylated and activated (Morrison *et al.,* 1988; Williams *et al.,* 1992 ; 1993). This was shown by the mobility shift of the protein to a slower migrating electrophoretic species by western immunoblotting. The electrophoretic mobility of Raf-1 is similar between FBR and 208F cells and in case of the FBR samples there is a multiple pattern of Raf-1 specific-immunoreactivity which resembles the situation seen in v-Ki-*ras* transformed cells were Raf-1 is dramatically activated (see Figure 7.7). Raf-1 activation however does not correlate with MEK-1 activation in FBR cells following mitogenic stimulation, suggesting that this way of measuring Raf-1 activation is perhaps not a reliable assay to determine the activity of Raf-1. Previously a similar mechanism that involves the activation of Raf-1 phosphorylation by MAPK has been suggested (Ueki *et al.,* 1994). Lowy and co-workers have found that this type of activation of the Raf-1 C-terminus is due to the modification of a Serine residue by a MAPK-dependent mechanism, since activated MEK-1 induces this shift *in vivo* (Ferrier *et al.,* 1997). In our case, the multiple immunoreactive Raf-1 bands in serum-induced lysates from FBR cells are not explained by down-modulation of the MAPK pathway in these cells. It is therefore possible that this mode of Raf-1 activation in FBR cells results from another, as yet unidentified mechanism, most probably dissociated from the MAP Kinase signals that are relatively low in FBR. The existence of other activators of MEK-1 apart from Raf-1 suggests that there could be alternative ways to activate MAP Kinases (Okazaki and Sagata, 1995 ; Withers *et al.,* 1995 ; Seufferlein *et al.,* 1996). There is some evidence that MEK-1 could be phosphorylated by another activator in

PC12 (Pang *et al.,* 1995) and Swiss 3T3 cells (Zheng *et al.,* 1994) not yet molecularly cloned. One possibility to be excluded however is the activation of MEK-1 by B-Raf (Papin *et al.,* 1995). In Rat-1 cells B-Raf levels are not detectable by western immunoblotting (Erhardt *et al.,* 1995) and this makes the possibility of activation of Raf-1 in 208F and FBR cells very small, unless a genetic event has occurred that might lead to selection, and over-expression of B-Raf mRNA and protein in FBR cells. A lack of appropriate reagents has prevented further investigation of this possibility.

10.3. Identification of high molecular weight tyrosine phosphorylated proteins in FBR and 208F : A putative relationship to MAP Kinase pathway down-regulation

Ligand activation of growth factor Receptor Tyrosine Kinases (RTK's) results in their autophosphorylation, dimerisation (Schlessinger, 1988; Schlessinger and Bar-Sagi, 1994), and phosphorylation of downstream target proteins involved in signal transduction (Kazlauskas and Cooper, 1989). These targets serve as components of mitogenic pathways and include proteins of high molecular weight such as Phospholipase Cγ-1 (Meisenhelder *et al.,* 1990; Nishibe *et al.,* 1990), Phosphoinositide 3-Kinase (Valius and Kazlauskas, 1993), *ras*GTPase activating Protein (Gibbs *et al.,* 1990; Molloy *et al.,* 1989), the tyrosine phosphatase SHP-2 (Feng *et al.,* 1993), the family of the IRS proteins (Lavan *et al.,* 1992), and Focal Adhesion Kinase (FAK) (Chen *et al.,* 1998). Proteins of small molecular weight such as the Shc proteins are also regulated by tyrosine phosphorylation (Pronk *et al.,* 1993). These signal transducing proteins regulate and are required for downstream signal transduction to MAP Kinase activation (Margolis *et al.,* 1990; Medema *et al.,* 1991; Medema *et al.,* 1992; Lowenstein *et al.,* 1992; Stephens *et al.,* 1994; Roche *et al.,* 1996).

These mitogenic components are subject to regulation by retroviral oncogenes which contain a tyrosine kinase activity associated with their catalytic domain (Ellis *et al.,* 1990). Such oncogenes as v-*src*, v-*abl*, v-*fps*, v-*yes*, v-*fes* are members of the non-receptor Protein Tyrosine Kinases (PTK's) as are their cellular homologues. This family of oncogenes is relatively large suggesting that they might exert an important role in cell proliferation and oncogenic transformation of different vertebrate species. Most studies in this field rely on the over-expression of oncogene products such as v-*src* and v-*abl* or the over-expression of growth

factor Receptor Tyrosine Kinases (Di Fiore *et al.,* 1987) in order to facilitate biological responses, to potentiate substrate recognition and to understand phosphorylation mechanisms. However in some cases this appears naturally, as in the case of EGFR over-expression in the A431 carcinoma cell line (Ullrich *et al.,* 1984).

From these studies it is apparent that the components of tyrosine phosphorylation-related signal transduction are modulators of early mitogenic signals occurring in the plasma membrane resulting in activation of pleiotropic cellular responses and gene expression.

The patterns of tyrosine phosphorylation in the cell lines used throughout this thesis were examined in an attempt to identify any differences that could account for the down-regulation of MAP Kinase pathway. The 208F cell line, a derivative of Rat-1 cells has been used to generate FBR transformants. The 208F cells are attached well to the substratum and one would expect that mitogenic activation would involve some adhesion components. In contrast FBR cells are refractile, rounded and poorly adherent, features common in many oncogenically transformed cell lines. To investigate this, western immunoblotting with anti-Phosphotyrosine antibodies seemed to be a relatively straightforward approach to such a complicated issue.

This approach suggested that a reproducible differential pattern of tyrosine phosphorylation existed between FBR and 208F under relatively low concentrations of extracellular stimuli used. This event could be relevant to transformation since a subtractive hybridisation approach yielded a variety of gene expression alterations, a phenomenon that was expected since FBR v-*fos* is a trans-regulator of gene expression (Johnston *et al.,* submitted). Some of the transactivated genes identified are possibly involved in the regulation of mitogenic signalling including a Protein Tyrosine Phosphatase and a Raf-1 kinase-binding protein.

During these experiments the 120 kDa Focal Adhesion Kinase was identified as a possible modulator of MAP Kinase pathway in 208F but not FBR cells. Increased tyrosine phosphorylation of FAK content was detected in 208F but not in FBR cells. Previous reports are consistent with a role for FAK in potentiation and regulation of MAP Kinase signalling. It was first identified by replating NIH 3T3 cells on ECM proteins (Schlaepfer *et al.,* 1994). FAK was shown to be activated and linked to the *ras* pathway through GRB2 binding and Sos-1 association with the plasma membrane and also by induction of its tyrosine phosphorylation status by extracellular ligands or activation of PKC. The finding that FBR cells contain less tyrosine

phosphorylated FAK compared to 208F could shed some light on the mechanisms of MAP Kinase activation in this cell type.

The reduced tyrosine phosphoprotein content in FBR cells is somewhat surprising considering the high mitogenic rate of these cells (Hennigan, 1993 ; Hawker *et al.,* 1994). If tyrosine phosphorylation is involved in the generation of positive mitogenic signals across the membrane, then possibly another factor could contribute to the effects seen in FBR cells. A 180-190 kDa protein that is tyrosine phosphorylated in FBR cells to a greater extent was also identified, which is independent of ligand stimulation. Whether or not this could account for the observed differences in the mitogenic potential of FBR cells is not known. It would be interesting in the future to identify the nature of this putative signalling molecule and determine its role in the activation of trans-membrane signals in FBR cells.

10.4. Multiple effectors determine activation of the MAP Kinase pathway

MAP Kinases are stimulated by various agonists and serve as transducers of multiple intracellular signals (Ray and Sturgill, 1987, 1988). MAP Kinases have cytosolic and nuclear substrates with multiple functions. In the cytosol and the plasma membrane MAP Kinases phosphorylate enzymes such as the EGFR (Alvarez *et al.,* 1991) and phospholipase A2 (Lin *et al.,* 1993). As a result of mitogenic stimulation a fraction of MAP Kinases translocates to the nucleus and regulates gene transcription by phosphorylating transcriptional activators (Chen *et al.,* 1992, 1993), such as Elk-1 (Gille *et al.,* 1992; 1995; Marais *et al.,* 1993; Janknecht *et al.,* 1993).

MAP Kinases are stimulated by growth factors, such as NGF and EGF (Boulton *et al.,* 1991) and lipids, such as LPA (Cook *et al.,* 1993; Howe and Marshall, 1993; Hordijk *et al.,* 1994). The main elements that compose the MAP Kinase pathway are the small GTP-binding protein $p21^{ras}$ and its target the Raf-1 kinase (Howe et al., 1992) which phosphorylates and activates the major MAP Kinase activator, MEK-1 (Gomez and Cohen, 1991; Ahn et al., 1992; Seger et al., 1992;).

Fibroblasts, such as Rat-1 cells stimulated by multiple agonists show an increase in the phosphorylation and activation of MAP Kinases ERK1 and ERK2 (Boulton et al., 1991; Ahn et al., 1990; Ahn and Krebs, 1990; Haystead et al., 1990). This action is a sequential event that requires growth factor Receptor Tyrosine Kinase stimulation of guanine nucleotide exchange on

$p21^{ras}$ (Satoh *et al.,* 1990a, 1990b) and Raf-1 kinase activation (Troppmair *et al.,* 1992; Wood *et al.,* 1992; Howe *et al.,* 1992). Activation of the MAP Kinases is regulated also by dual specificity protein Tyrosine/Threonine phosphatases products of immediate early genes that reduce MAP Kinase activity (Keyse and Emslie , 1992; Alessi *et al.,* 1993).

Cytoplasmic oncogenes are also known to activate the MAP Kinase pathway. Membrane-associated oncogenes such as v-*src* and *gip2*/G_{i2} activate MAP Kinases in a constitutive manner (Gupta *et al.,* 1992; Gallego *et al.,* 1992) and also cytoplasmic oncogenes associated with protein kinase activity such as v-*raf*, v-*abl* and v-*mos* (Howe *et al.,* 1992; Dent *et al.,* 1992; Troppmair *et al.,* 1994; Okazaki and Sagata, 1995).

The specificity of the interaction of MEK-1 with Raf-1 and $p21^{ras}$ is high and it seems likely that $p21^{ras}$ is one of the major upstream activators of this signalling complexe. However recent evidence suggests also that the MAP Kinase pathway can be more complex than previously thought and not linear (Meier and Evan, 1998; Sternberg and Alberola-Ila, 1998). The regulation of early events leading to the activation of a MAP Kinase Kinase Kinase such as Raf-1 is dependent on $p21^{ras}$ activity and also a Tyrosine Kinase signal is necessary (Morrison *et al.,* 1993; Fabian *et al.,* 1993; Dent *et al.,* 1995; Marais *et al.,* 1995). However recently the identification of new proteins that participate in *ras* signalling in *Drosophila* and *C. Elegans* show that this activation could be the result of the participation of a protein network leading to the activation of Raf-1 (Sternberg and Alberola-Ila, 1998). The KSR protein: Kinase Suppressor of Ras identified in *drosophila* is a putative alternative regulator of Raf-1 kinase that is activated by other mechanisms (Therrien et al., 1996). Other mechanisms reported to result in a MEK-1-dependent Raf-1-independent mechanisms of MAP Kinase pathway is the recent demonstration of the role of Protein Kinase C (PKC) atypical isoforms (Schonwasser et al., 1998).

There is also evidence that oncogenic transformation is not necessarily associated with the MAP Kinase activation. Stimulation of quiescent FBR cells with serum or growth factors does not result in dramatic activation of MAP Kinase phosphorylation, as in the case of 208F cells. This novel finding might be associated with the up-regulation of a regulatory molecule which affects the intensity of MAP Kinase signals. For example a Protein Tyrosine Phosphatase (PTP) that will reduce the intensity of extracellular stimuli could be a possible candidate in FBR cells. Such an enzyme could interfere with the de-phosphorylation of Protein Tyrosine Kinases

such as Focal Adhesion Kinase (FAK). A schematic representation of such alterations is depicted in Figure 10.1.

The MAP Kinase pathway has been shown to be activated by adhesion to Integrins and this event can be augmented by growth factors such as EGF (Miyamoto *et al.,* 1996). Recently it was shown that the Protein Tyrosine Phosphatase PTEN, down-regulates the MAP Kinase pathway by dephosphrylation of the adaptor protein Shc, without interfering with activation and tyrosine phosphorylation of the EGFR (Gu et al., 1998). Over-expression of such an enzyme could potently reduce the signals downstream of RTK's and also from FAK and other integrin-mediated pathways (Danen *et al.,* 1998; Gu *et al.,* 1998) making the hypothesis of the presence of a putative Protein Tyrosine Phosphatase in FBR more realistic (Figure 10.1). A transient transfection approach can be employed in order to identify if this phosphatase could dephosphorylate proteins in FBR cells that are not tyrosine phosphorylated to the same extent into FBR cells compared to 208F cells.

In Figure 10.1 a schematical representation of multiple activators of MAP Kinase pathway is shown.Raf-1 kinase can be activated by a mechanism that involves $p21^{ras}$ and a protein kinase such as the recently identified Pak3 (King et al., 1998) and v-*src* (Marais et al., 1995). The existence of other activators of MEK-1 is also presented. It has been reported that NEK-1 might have alternative activators except the Raf-1 kinase (Zheng et al., 1994; Pang et al., 1995). A candidate approach to identify a different activator of MEK-1 except Raf-1, in FBR cells is to use a bacterially expressed recombinant activated form of MEK-1 immobilised in a column and pass through the column FBR cytoplasmic lysates to identify any proteins that might associate with MEK-1. These proteins could be subsequently identified by sequence determination and molecular cloning.

Finally in the Figure 10.1a a possible biological role for the different dominant negative mutants in the FBR v-*fos* transformation system is presented, derived mainly from the experimental results presented in this thesis. The function of NΔ Raf-1 is to suppress anchorage independence although its role in MAP Kinase phosphorylation is less apparent (see Figures 7.4 and 7.5). MEK-1 A221 has a significant effect in cell morphology and also suppresses serum-induced MAP Kinase phosphorylation (see Figures 6.3 and 6.8). The dominant negative deletion mutant of c-Jun TAM-67 can effectively inhibit FBR v-*fos* transformation (see also Figure 4.4 for anchorage independent growth of FBR TAM-67 revertants).

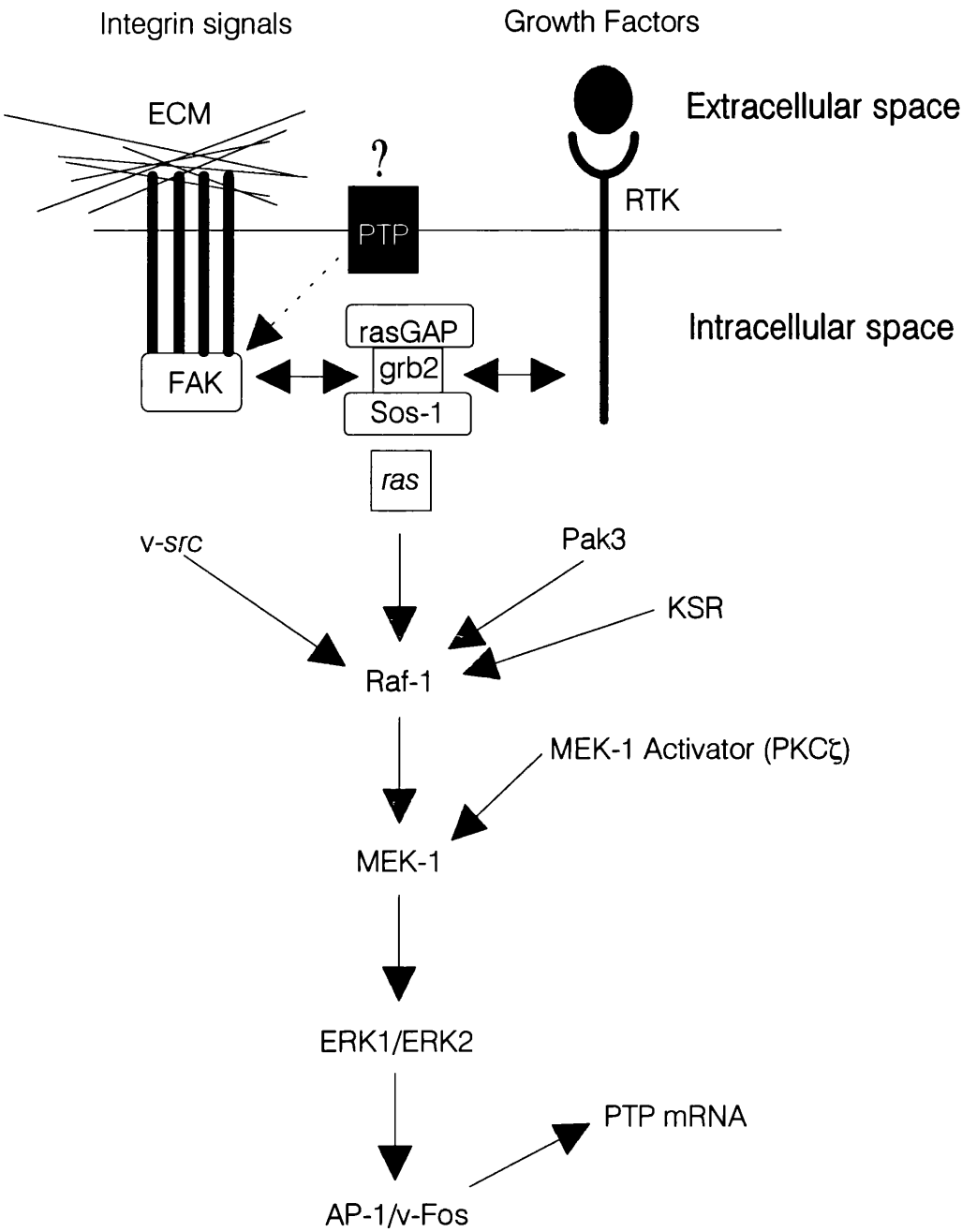

Figure 10.1. Activation of MAP Kinase Pathway in FBR cells by extracellular signals.

MAP kinase activation can be elicited by extracellular signals. These include soluble growth factors, such as EGF and PDGF that act through Receptor Tyrosine Kinases (RTK's). Signals through the Extracellular Matrix (ECM) which is composed from insoluble ligands such as Fibronectin act by binding to Integrins which in turn activate the Focal Adhesion Kinase (FAK). Both signalling pathways activate *ras* signalling and MAP Kinase pathway through the formation of complexes containing the adaptor protein GRB2 that links ras to the exchange factor Sos-1 and the GTPase activating protein *ras*GAP. This process can be attenuated in FBR cells by the over-expression of putative Protein Tyrosine Phosphatase (PTP) that might dephosphorylate FAK or components of Receptor signalling pathways.
Downstream of RTK's Raf-1 kinase can be activated by *ras* and by another signals emerging from a Serine Kinase such as Pak3 or by Tyrosine Kinases such as v-*src*. The MAP Kinase activator MEK-1 can be also activated by other mechanisms except Raf-1. For example MEK-1 can be inhibited by the mammalian version of the Kinase Suppressor of Ras (KSR) a protein recently identified in *Drosophila*. Alternative activators of MEK-1 have been reported in mammalian cells although their identity is not fully established. A candidate for such a function is the atypical Protein Kinase C ζ.

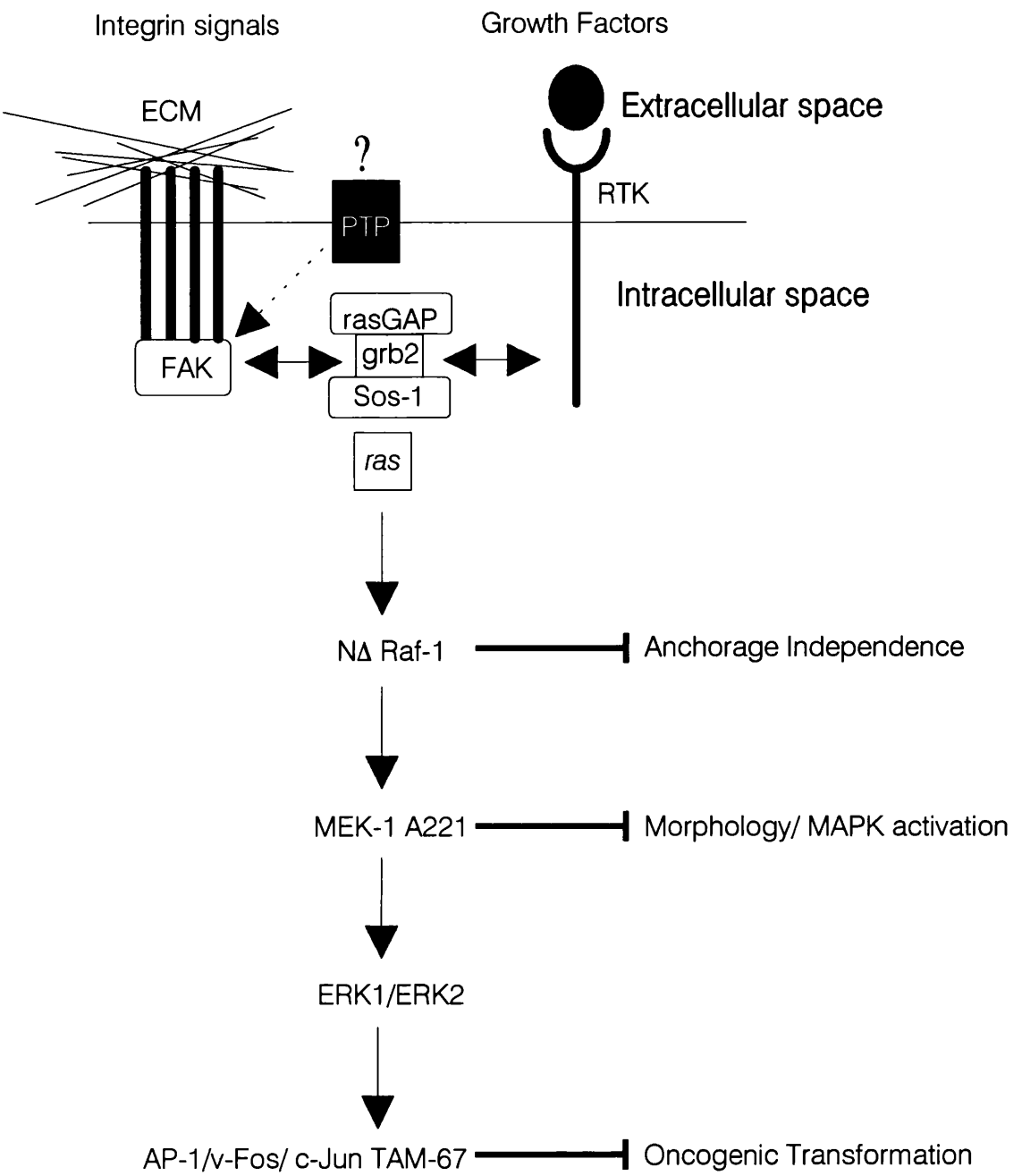

Figure 10.1a. Putative biological function of dominant negative mutants in fos-induced transformation.

MAP kinase activation can be elicited by extracellular signals. These include soluble growth factors, such as EGF and PDGF that act through Receptor Tyrosine Kinases (RTK's). Signals through the Extracellular Matrix (ECM) which is composed from insoluble ligands such as Fibronectin act by binding to Integrins which in turn activate the Focal Adhesion Kinase (FAK). Both signalling pathways activate *ras* signalling and MAP Kinase pathway through the formation of complexes containing the adaptor protein GRB2 that links ras to the exchange factor Sos-1 and the GTPase activating protein *ras*GAP.
The dominant negative mutants of Raf-1 ND Raf-1, MEK-1 A221 and c-Jun TAM-67 could have multiple roles in regulating MAP Kinase signalling, morphology and transformation. For example the truncated dominant negative mutant of Raf-1. ND Raf-1 prevents growth under anchorage independent conditions. The mutant MEK-1 A221 inhibits serum-induced MAP Kinase phosphorylaytion and reverses morphological changes in FBR cells. Finally TAM-67 is an inhibitor of oncogenic transformation, proliferation and induces effectively reversion of the transformed phenotype..

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