A novel non-spreading variant of transformed hamster fibroblasts

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Summary

Using repeated plating on fibronectin-coated surfaces as a selection procedure, I isolated three different mutants unresponsive to fibronectin, from thioguanine resistant Py-BHK (TG) cells in a simple assay of cell spreading. All three were recloned on soft agar. Two had morphologies similar to lines which had been selected previously, F1 and F2. The mutant F3, then a unique isolate, had a different morphology in culture. Whereas F1 colonies contain exclusively rounded cells, and F2 have a few partially spread cells, more scattered than F1, F3 has a spread epithelial-like morphology in culture.

I selected F3 from TG cells, on the basis that this mutant is non-responsive to fibronectin. However, in spreading assays, I found that F3 spreads on fibronectin, when the simple saline in the assay (HEPES-buffered Hanks') is supplemented with foetal bovine serum, or replaced by Ham's F10. Unexpectedly, I found that the active component stimulating spreading of F3 was pyruvate. To explain the pyruvate requirement, I searched for an abnormality of glucose utilisation in F3. Glucose uptake, studied using ^{14}C-labelled glucose, suggested there could be some such abnormality. However, separating metabolites from TG and F3 by one and two dimensional paper chromatography gave inconclusive results.

The activity of mitochondrial dehydrogenases of TG and F3, measured using MTT, responded similarly to glucose, suggesting that pyruvate in F3 is more likely needed to supply metabolites than as an energy source. The possible identity of such metabolites and explanation for how F3 came to be selected, are discussed.

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CHAPTER 1. INTRODUCTION

List of abbreviations

ABPs	Actin-binding proteins
A-CAM	Adherence junction-specific cell adhesion molecule
AIDS	Acquired immune deficiency syndrome
ADP	Adenosine di-phosphate
cAMP	Cyclic adenosine mono-phosphate
ATP	Adenosine tri-phosphate
BD	Bradykinin
BO	Bombesin
CAS	Cadherin-associated substrate
CNS	Central nervous neurons
CRT	Calreticulin
cDNA	Complementary deoxy-ribonucleic acid
ECM	Extracellular matrix
EGF	Epidermal growth factor
ERK	Extracellular regulated kinase
FAK	Focal adhesion kinase
F-actin	Filamentous actin
FAP	Focal adhesion plaque
FMDV	Foot and mouth disease virus
Fn	Fibronectin
GAPs	GTP-ase-activating proteins
GDI	Guanine nucleotide dissociation inhibitor
GDP	Guanosine di-phosphate
GEFs	GDP-GTP exchange factors

GF	Growth factor
GFR	Growth factor receptor
GMP	Guanosine mono-phosphate
GTP	Guanosine tri-phosphate
Grb2	Growth factor receptor bound
HIV	Human immuno-deficiency virus
IAP	Integrin-associated protein
I-CAM	Intercellular adhesion molecule
IFs	Intermediate filaments
ILK	Integrin-linked kinase
IRM	Interference reflection microscopy
JNK	Jun kinase
LPA	Lysophosphatidic acid
MAPs	Microtubule-associated proteins
MAPK	Mitogen-activated protein kinase
MEK	MAPK/ ERK kinase
MTOCs	Microtubule-organising centres
MTT	[3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide
PDGF	Platelet-derived growth factor
PI 5K	Phosphatidylinositide 4-phosphate-5-kinase
PI 3K	Phosphatidylinositol 3'kinase
PIP	Phosphatidylinositol 4-phosphate
PKC	Protein kinase C
PKN	Protein kinase N
PMA	Phorbol myristate acetate
Rac-EF	Rac exchange factor
RGD	Arg-Gly-Asp

ROCK	Rho-associated coiled-coil containing protein kinase
RSV	Rous sarcoma virus
SDS	Sodium dodecyl sulphate
SH	Src homology domain
Tβ4	Thymosin β4
Tβ10	Thymosin β10
TGF-β	Transforming growth factor β
TG	6-thioguanine
Vn	Vitronectin
V-CAM	Vascular cellular adhesion molecule

Acknowledgements

This work is dedicated to my late father Naji Abu-Zayyad who died on April 4th 1979 at the age of 45. It was my father's dream that I grow up to be a scientist. I hope that I have lived up to his expectations.

A. N. A. Z

April 4th 1979

INTRODUCTION

Introduction

1. Cell spreading

When animal tissue cells are allowed to come into contact with a solid substrate coated with a suitable molecule of the extracellular matrix (ECM), they respond as follows: they attach, spread, and may locomote. Attachment involves specialised receptors (integrins) interacting with the substrate and reorganisation of the cytoskeleton.

For a cell to spread, its edges must move outwards. This movement seems to involve rearrangements of cytoskeleton and membrane similar to those shown by locomoting cells. The integrins, through interaction with the ECM, provide a stimulus: the cell responds by spreading. Some signal transduction pathways presumably link the stimulus to the response.

1.1 Integrin-Extracellular matrix interactions

1.1.1 Extracellular matrix and its components

The extracellular matrix (ECM) consists of the cell-free 'ground substance' which is considered to be the filler that surrounds cells and provides the strength to soft tissues and the rigidity to skeletal tissues (Bernfield, 1989). The ECM consists of very large, multi-domain molecules that are linked together by covalent and non-covalent bonds into an insoluble compound. Initially, the most common components were isolated and characterised, particularly type I collagen, and the cartilage chondroitin sulphate proteoglycan. The discovery of fibronectin and laminin, the adhesive glycoproteins of the interstitial matrix and basal lamina,

respectively, stimulated cell biologists to search for more of these proteins, to characterise their molecular interactions and to learn how they affected cells (Engel, 1991; Flaumenhaft *et al.*, 1991). Each of the two major types of matrices, the interstitial matrix and the basal lamina, contains as major components a type of collagen, other cell-binding adhesive glycoproteins and proteoglycans. The interstitial matrix is produced by mesenchymal cells which migrate through it. It contains fibrillar collagens (types I, III, V and others), fibronectin, hyaluronic acid and fibril-associated proteoglycans (e.g. decorin). The interstitial matrix exists in various forms, such as tendon, bone, and dermis. The basal lamina is produced by parenchymal cells. The cells are polarised to it, especially epithelia whose basal surfaces abut on it. Basal laminae contain a mesh-like collagen framework (type IV), laminin and a large heparan sulphate proteoglycan (filtrican). The basal lamina shows structural order, but the precise arrangement of its components is unclear.

I will give a very brief introduction to some of the components of the ECM: vitronectin, laminin, collagens and proteoglycans, discuss briefly their interactions with cells, and then focus on fibronectin in more detail, since this protein was central to my work.

1.1.1.1 Vitronectin

Vitronectin (Vn) is a 70 kDa RGD-containing multifunctional serum protein, produced primarily in the liver, also known as S-protein or serum spreading factor (Riberio *et al.*, 1995). It has also been known as epibolin (Underwood *et al.*, 1989). Vn was first identified as a cell attachment factor with high activity on glass surfaces (Yamada 1991). It is found in serum at a concentration of 200-400 μg/ml. However, it also appears in specific tissues. For example, Vn was found to localise within the stroma of wound tissue and elastin fibers of the skin. Vn affects the humoral immune systems (Felding-Habermann

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et al., 1993). It also serves as a major matrix-associated regulator of blood coagulation on the basis of its ability to bind heparin (Ribeiro *et al.*, 1995), plasminogen, plasminogen activator inhibitors and thrombin-antithrombin III complexes. Vn promotes cellular attachment, spreading and migration of a wide variety of cell types (Underwood *et al.*, 1989).

1.1.1.2 Laminin

Laminin is the major glycoprotein found in basement membranes, the thin extracellular matrix which underlies all epithelia and surrounds muscle, peripheral nerve and fat cells (Kleinman *et al.*, 1989; Mercurio, 1990; Yamada, 1991; Tryggvason, 1993; Yurchenco *et al.*, 1994). Laminin also binds to other components in the matrix (i.e. type IV collagen, heparan sulphate proteoglycan and entactin) and to itself. Laminin is a complex and interesting extracellular matrix component (Tryggvason 1993) with multiple biological activities, including promotion of cell adhesion, growth, migration, maintenance of cellular phenotype, control of development, differentiation, collagenase IV activity, neurite outgrowth, tyrosine hydroxylase activity and tumour metastasis, cellular proliferation and gene expression, in addition to being a true structural component of the basement membrane meshwork.

Laminin is a large glycoprotein (M_r = 800,000) which consists of A (M_r = 400,000), B1 (M_r = 210,000) and B2 (M_r = 200,00) chains which are held together in a cross-like structure by disulphide bonds. The centre of the cross was found to have cell adhesion activity while the end of the long arm had both cell attachment and neurite outgrowth activity. A unique sequence of five amino acids, YIGSR, from one of the EGF-like repeats in the B1 chain was identified as promoting cell adhesion and migration. A nearby sequence (RYVVLPRPVCFEKGMNYTVR), termed F9, on the internal globular domain was also found to have cell attachment activity (Fig. 1), which bound specifically

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to heparin. An RGD sequence is located on laminin A chain in the central EGF repeat (Kleinman *et al.*, 1989).

A chain
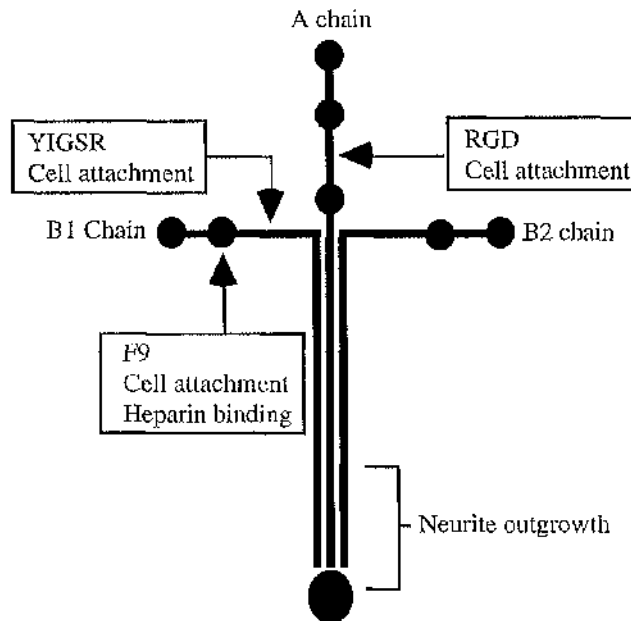
Fig. 1.1 Schematic model of laminin showing approximate locations of biologically active sites. For more details see text. (adapted from Kleinman H. K. *et al.*, 1989).

The complete structure has been determined through cDNA cloning and sequencing (Yamada *et al.*, 1992). The chains previously designated A, B1 and B2 chains comprising a tri-molecular monomer are renamed α, β and γ, the corresponding isoforms for each are numbered in order of discovery, as are the complete laminins. The genes for these chains use the letters A, B and C to correspond to the three chains (Yurchenco *et al.*, 1994; Brown *et al.*, 1994).

1.1.1.3 Collagens

Collagens are the major class of insoluble fibrous proteins in the ECM and in connective tissues (Bornstein *et al.*, 1980; Dedhar *et al.*, 1987a; Engvall *et al.*, 1986; Tanzer, 1989). In fact they are the most abundant components of the ECM.

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There are at least nineteen different types of collagens plus numerous other non-structural proteins that contain at least one collagen triple helix as a structural motif. It was recently proposed that all proteins containing a collagen triple helix be regarded as members of a collagen superfamily (Mayne et al., 1993). Types I, II, and III are the most abundant and form fibrils of similar structure. Type IV, a main component of the basal lamina, in contrast, forms a two-dimensional reticulum: a covalently- stabilised polymer network. The chains most widely distributed and present in largest amount are the $\alpha 1(IV)$ and $\alpha 2$ (IV) chains [$\alpha 1(IV)_2\alpha 2(IV)$] (Yurchenco et al., 1994). The type IX-like family of collagens (Mayne et al., 1993) was discovered a decade ago in several different cartilages and recently a number of additional collagens were described in other connective tissues and contain one or more domains in common with domains of type IX collagen. These include type XII, type XIV and type XVI collagens together with an additional collagen chain, $\alpha 1(Y)$. Recently, several cDNA clones were described that contain short stretches of collagenous sequence interspersed by non-collagenous sequences but are not members of the type IX collagen-like family. Three of these cloned cDNAs are designated as collagen types XIII, XV, and XVII (Mayne et al., 1993). All collagens contain a three-stranded helical structure and globular domains. The fundamental structural unit is 300 nm long and 1.5 nm in diameter, and consists of three coiled subunits: Two $\alpha 1$ chains and $\alpha 2$. Each chain contains exactly 1050 amino acids wound around each other in a characteristic right-handed triple helix. Many regions of collagen chains are composed of the repeating motif Gly-Pro-X, where X can be any amino acid. Hydrogen bonds linking the peptide bond NH of a glycine residue with a peptide carbonyl (C=O) group in an adjacent polypeptide help hold the three chains together. It appears that most collagen exons encode six Gly-X-Y sequences, and other exons encode two or three of these primordial units, where Y is a hydroxyproline.

The role of collagens is mainly structural, as an example, type I collagen fibers are used as the reinforcing construction of bone, but various collagen types have been shown to promote the adhesion and migration of a variety of cell types (Dedhar *et al.*, 1987a). Cells have integrins such as $\alpha_1\beta_1$ and $\alpha_2\beta_1$ which recognise motifs on collagens (e.g. type I collagen) and which have been shown to promote directional motility of normal and transformed cells (Ruoslahti, 1991; Faassen *et al.*, 1992).

1.1.1.4 Fibronectin

Fibronectin (Fn) is a multifunctional extracellular glycoprotein that exists in a soluble form in body fluids and in an insoluble form (cellular Fn) in the ECM. Insoluble Fn has been found on the surface of most untransformed cells and in basement membranes (Vartio *et al.*, 1987; Zerlauth *et al.*, 1988). Both forms of Fn are encoded by the same single gene. Fn plays a central role in cell adhesion. It interacts with cells to promote attachment and spreading of cells (Hynes, 1990). It plays a major role in many important physiological processes, such as embryogenesis, wound healing, haemostasis and thrombosis, cell differentiation, maintenance of normal cell morphology, cytoskeletal organisation, and oncogenic transformation, (Vartio *et al.*, 1987; Yamada 1989; Hynes, 1989; Hynes, 1990; Schwarzbauer *et al*, 1991; Potts *et al*, 1994; Watt *et al.*, 1994). Intensive research on Fn has made it an ideal protein for investigations of cell adhesion and spreading (Ruoslahti *et al.*, 1988)

Fibronectin structure and its functional domains

The complete amino acid sequence of the Fn polypeptide has been determined from cloned cDNA and protein for several species. There is a high degree of sequence homology between fibronectins from the various species.

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All forms of Fn are large glycoproteins containing around 5% carbohydrate (Hynes, 1990). The carbohydrates protect the Fn polypeptide against proteolysis, and this may be the general significance of Fn glycosylation (Jones *et al.*, 1986; Ruoslahti *et al.*, 1988). Glycosylation may also modulate fibroblast adhesion and spreading. Fn is secreted as a dimmer, with a monomer molecular weight of approximately 220-250 kDa; the monomers are joined by two disulphide bonds near the carboxyl terminus of the protein (Fig. 2). Like many proteins of the ECM, including tenascin, laminin and thrombospondin, Fn is a mosaic protein, composed of modular protein units that often correspond to the exon structure of the gene. These modular units are of three types: module I, II, and III which contain ~40, 60, and 90 amino acid residues, respectively (Baron *et al.*, 1990; Potts *et al.*, 1994). The modules are folded into functional domains that are resistant to proteolysis and contain binding sites for ECM proteins such as collagen and thrombospondin, cell-surface receptors such as integrins, circulating blood proteins such as fibrin, and glycosaminoglycans such as heparin and chondroitin sulphate. It has been demonstrated that circulating Fn and Fn obtained from fibroblast cultures, both showed specific binding to collagen and gelatin (Ruoslahti *et al.*, 1977). Different isoforms of Fn result from alternative splicing of the ED-A and ED-B modules and of the type III connecting segment (IIICS; Fig. 2) (Gutman *et al.*; 1986, Carnemolla *et al.*, 1989; Potts *et al.*, 1994). Fibronectin expression is controlled at several levels: transcription, splicing, and protein secretion. Expression is stimulated by serum, cAMP, glucocorticoids and by transforming growth factor β (TGF-β), and suppressed by oncogenic transformation and cell density (Dean *et al.*, 1988; Schwarzbauer, 1991).
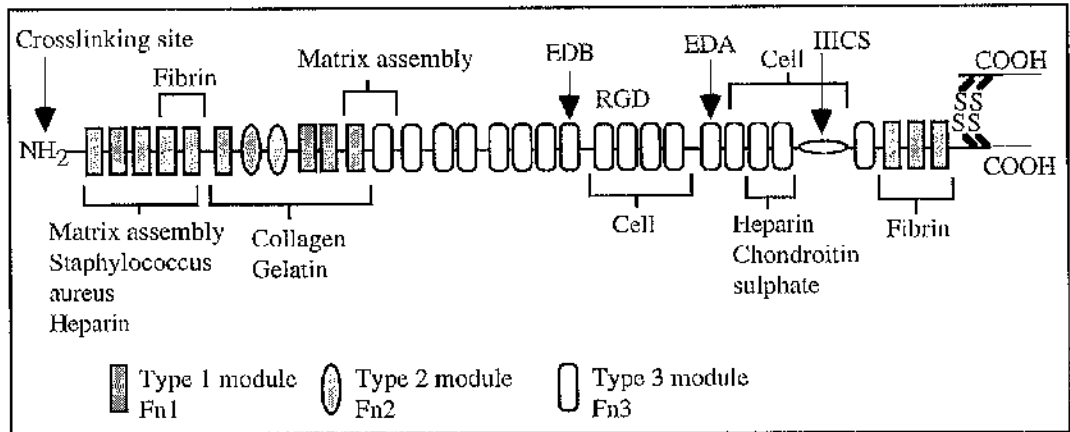
Fig. 1.2 The modular structure of Fn, showing the position of the three types of modules; Fn1, Fn2 and Fn3. EDA, EDB and IIICS are alternatively spliced regions. [10]Fn3 contains the RGD sequence involved in integrin binding. (adapted Potts *et al.*, 1994)

Splicing of the IIICS region can be combined with splices of the ED-A and ED-B regions (EIIIA and EIIIB) to form the 20 variants constituting the human Fn family (Schwarzbauer *et al.*, 1987; Yamada, 1989; Schwarzbauer, 1991).

Cell adhesion sequences in fibronectin

Most cells can adhere to fibronectin, at least in part by binding to the centrally located "cell-binding" domain. A crucial sequence in this domain is Arg-Gly-Asp-Ser (RGDS). The first three amino acids are particularly important, the "RGD" recognition site, and they are the essential structure recognised by most cells in fibronectin (Dedhar *et al.*, 1987b; Ruoslahti *et al.*, 1987; Yamada, 1991). Mutagenesis experiments and monoclonal antibody inhibition studies appear to confirm that this region contains a second binding region located about 14-28 kDa toward the amino terminus of the protein, that functions in synergy with the RGD sequence in cell adhesion, migration, and fibronectin matrix assembly.

The alternatively spliced sequences of the IIICS or V region are unique in that they encode cell-type-specific adhesion sequences. Sites involved in cell

adhesion and spreading reside between repeat III_6 and III_{15}. A synergistic region located at least 20 kDa of polypeptide sequence away from the RGD site is important for complete cell attachment and spreading, and for transmembrane effects of Fn on organising actin-containing microfilament bundles (Schwarzbauer, 1991; Yamada, 1989). Cell adhesion and spreading is essential for normal cellular behaviour and growth. This cell-type-specific region undergoes complex alternative splicing of precursor Fn mRNA to form up to five different human Fn variants. Two of these sites, termed the LDVP (Humphries *et al.*, 1987) and Arg-Gly-Asp-Val "RGDV" (Humphries *et al.*, 1986) sequences, are recognised by a variety of neural crest derivatives, including melanoma cells, sensory and sympathetic ganglion cells, and crest cells themselves. Certain lymphocytes also recognise the LDV site (Wayner *et al.*, 1989; Wayner *et al.*, 1992).

The collagen-binding domain, which was the first functional domain to be identified, seems to account for all the capacity of fibronectin to bind to collagen/gelatin.

Fn contains two heparin-binding domains, which are thought to interact most often with heparan sulphate proteoglycans.

Fn contains at least two fibrin-binding domains; a third is detectable after proteolysis of the protein. The major binding domains bind to fibrin or fibrinogen, but binding tends to be relatively weak.

1.1.2 Integrins

Integrins are a large group of transmembrane glycoproteins that bind to adhesive macromolecules in extracellular matrices and on cell surfaces. They are the major class of adhesion receptor and make a dominant contribution to the process of cell-cell and cell-substratum adhesion (Turner *et al.*, 1991; Tuckwell *et*

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al., 1993). The term "integrins" was originally coined to reflect the role of these receptors in integrating the intracellular cytoskeleton (actin microfilaments) with the extracellular matrix (fibronectin) (Hynes, 1987; Mosher, 1991; Haas, 1994). It has been reported that the cytoplasmic domain is essential for the accumulation of integrin into focal adhesions, and that the entire cytoplamic domain is essential for complete integrin function (Mueller *et al.*, 1989; Turner *et al.*, 1991; Reszka *et al.*, 1992; Sastry *et al.*, 1993; Miyamoto *et al.*, 1995a). The β_1 and β_3 classes predominantly mediate cell-matrix adhesion, while the β_2 class are cell-cell adhesion receptors (Humphries, 1990). In terms of ligand-binding specificity, β_1 integrins are generally involved in adhesion to connective tissue macromolecules such as fibronectin, laminin and collagens and they are expressed on many haematopoietic and leukocyte cell types (Springer 1990), β_3 receptors bind to vascular ligands such as fibrinogen, entactin, tenascin, von Willebrand factor, thrombospondin and vitronectin (Ruoslahti *et al.*, 1987; Wayner *et al.*, 1988; Ruoslahti, 1991; Hynes, 1992). β_1 and β_2 integrins have a widespread occurrence and are expressed by most cells (reviewed Humphries, 1990).

Integrin research links such diverse fields as haematology, neurobiology, thrombosis, cancer biology, inflammation, AIDS and developmental biology (Hynes, 1992; Churchill *et al.*, 1993; Roche *et al.*, 1993; Smith *et al.*, 1993; Pilewski *et al.*, 1993; Haas *et al.*, 1994).

1.1.2.1 Integrin structure

All integrins are dimmers consisting of unrelated α and β subunits. The α subunits vary in size between 120 and 180 kDa. β subunits are 90-110 kDa (Ruoslahti *et al.*, 1987; Hynes, 1992). Both α and β subunits have a large extracellular domain, a transmembrane segment, and with the exception of β_4, a small cytoplasmic domain (Hynes *et al.*, 1989; Hynes, 1992; Reszka *et al.*, 1992; DiPersio *et al.*, 1995). The structure and binding properties of integrins suggest

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the presence of domains controlling at least three major functions: subunit association, ligand binding, and cytoskeletal interactions (Solowska et al., 1991). The extracellular domains of the α subunits contain several calmodulin-type divalent cation-binding sites (EF-hand, Fig. 3), while the β subunits may have one such site (Loftus et al., 1990; Ruoslahti, 1991; review Humphries et al., 1996). The divalent cation dependency of integrin function is likely to derive from the presence of these structures. Certain α subunits, $\alpha 1$, $\alpha 2$, αL, αM and αX, contain a distinct domain between EF-hand domains 2 and 3, known as the inserted- or I-domain (Larson et al., 1989). $\alpha 3$, $\alpha 5$, $\alpha 6$, $\alpha 7$, $\alpha 8$, αIIb and αV subunits are subject to a post-translational cleavage at a conserved site within the EF-hand domains and the transmembrane region, the protease cleavage site, which does not contain any homology to known structural domains. It has been proposed that the region between the EF-hands and the cleavage site is composed of a short α helix-rich region followed by 12 β strands ((Nermut et al., 1988). The extracellular, N-terminal region in β subunits contains a segment of about 200 residues which is highly conserved between subunits (Moyle et al., 1991). The other well characterised section of the integrin β subunit is a cysteine-rich region in the C-terminal part of the extracellular domain, which is made up of four EGF-like domains (Nermut et al., 1988). The α and β subunits are noncovalently bound to one another, and this association is promoted by divalent cations. It has been established that divalent cations, such as Ca^{2+} and Mg^{2+} are crucial to integrin structural stability and that cations can alter the specificity and affinity of integrins for their ligands (Grinnell, 1984; Edwards et al., 1987; Edwards et al., 1988; Argraves et al., 1987; Elices et al., 1991; Grezesiak et al., 1992; Tuckwell et al., 1993; Hass et al., 1994; Hogg et al., 1994; review Humphries et al., 1996).
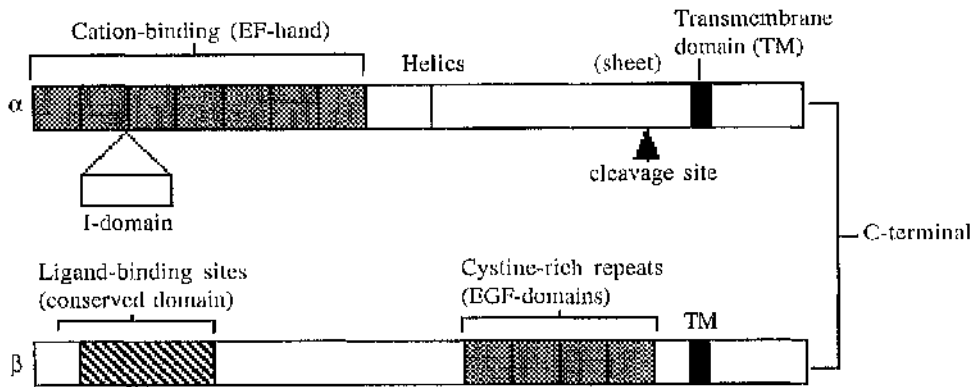
Fig. 1.3 Diagrammatic structure of integrins, showing the domains comprising α and β subunits. The N-terminal portion of a subunits is made up of 7 repeating units (EF-hand domains). I-domain: certain α subunits, α1, α2, αL, αM and αX, contain a distinct domain between EF-hand domains 2 and 3, known as inserted- or I-domain, for more details see text. adapted Tuckwell *et al.*, 1993)

The twenty two or so known integrins offer the possibility of great flexibility in cell adhesion, and this flexibility is probably further increased by alternative splicing (Hynes 1992).

1.1.2.2 Integrin diversity

Most integrins are expressed on a wide variety of cells, and most express several integrins. There are at least 15 α subunits (α1-8, αM, αL, αX, αV, αIIβ, and αIEL) and 8 β subunits (β1-8), which have been identified in vertebrates (Vogel *et al.*, 1990; Elices *et al.*, 1991; Hynes *et al.*, 1992; Tuckwell *et al.*; 1993, Sheppard, 1993; Smith *et al.*, 1993; Hogg *et al.*, 1993; Diamond *et al.*, 1994; Venstrom *et al.*, 1995). These subunits are capable of assembling into at least 22 functional heterodimeric receptors. The family is still growing but slowly. Integrins bind to diverse array of ligands, including ECM proteins, plasma proteins, that are deposited at sites of injury in haemostasis (fibrin) and complement activation (iC3b), and integral membrane proteins. Many integrins bind to more than one of these ligands, and some ligands bind to more than one

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integrin, using either the same or distinct recognition sites. For example, at least eight integrins have been shown to bind to fibronectin (Hynes *et al.*; 1992; Sheppard, 1993). Integrin ligands now include bacterial and viral proteins, coagulation and fibrinolytic factors, complement proteins and cellular counter-receptors, in addition to epithelial and vascular matrix components (Sheppard, 1993, Haas *et al.*, 1994, Diamond *et al.*, 1994; McDonald *et al.*, 1995; Schnapp *et al.*, 1995). Table 1 summarises the diversity of vertebrate integrins as presently understood.

Ligand	Comments	Integrin
ECM proteins		
Cytotactin/tenascin	Large ECM protein composed of multiple distinct domains, including 6-15 homologous repeats of the Fn type III module. The third type III repeat in cytotactin contains an RGD sequence and supports cell attachment and spreading mediated by α_v integrin (Prieto *et al.*, 1993)	$\alpha_v\beta_3$, $\alpha_v\beta_6$, $\alpha\beta_1$
Epiligrin	Epithelial basement membrane component synthesised by basal keratinocytes (Wayner *et al.*, 1993; Symington *et al.*, 1995)	$\alpha_3\beta_1$
Collagens	see 1.1.1.3	$\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_{IIb}\beta_3$
Denatured collagens		$\alpha_5\beta_1$, $\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$
Fibronectin	see 1.1.1.4	$\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_4\beta_7$, $\alpha_5\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_6$, $\alpha_{IIb}\beta_3$
Laminin	see 1.1.1.2	$\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$
Vitronectin	see 1.1.1.1	$\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_{IIb}\beta_3$
Thrombospondin	A large glycoprotein that is released from platelets, also present in various extracellular matrices due to its interactions with heparin, fibronectin and collagen. It has been found to promote cell adhesion and other biological events, including neurite outgrowth and inhibition of tumour metastasis	$\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$

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Pathogenic ligands

HIV Tat protein	The Tat protein of human HIV type 1, a growth factor for AIDs Kaposi sarcoma and cytokine-activated vascular cells induces adhesion of the same cell types by using integrins recognising the RGD sequence (Barillari et al., 1993).	$\alpha_v\beta_5$, $\alpha_v\beta_3$
FMDV	RGD-containing capsid protein of foot-and-mouth disease virus (Logan et al., 1993)	?
Echovirus 1	$\alpha_2\beta_1$-echovirus 1 interaction is independent of both $\alpha_2\beta_1$ activation state and divalent cations, therefore contrasting with collagen and laminin interactions with $\alpha_2\beta_1$ (Bergelson et al., 1993).	$\alpha_2\beta_1$
Borrelia burgdorferi	Causes Lyme disease, *Borrelia burgdorferi*, binds to platelets through $\alpha_{IIb}\beta_3$ in an interaction inhibited by RGD and fibrinogen chain peptides (Coburn et al., 1993)	$\alpha_{IIb}\beta_3$

Disintegrins

e.g. Kistrin, Echistatin	Originally discovered in snake venoms	$\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$
Decorsin Cyritestin, etc...	as potent inhibitors of platelet aggregation by $\alpha_{IIb}\beta_3$ (Blobel et al., 1992; Weskamp et al., 1994)	

Haematopoieses and thrombosis

Factor X		$\alpha_M\beta_2$
Fibrinogen	Non-activated platelet integrin $\alpha_{IIb}\beta_3$ binds to immobilised fibrinogen, but not soluble. Certain sites in fibrinogen, including the RGD at residues 72-74, become accessible to site-specific monoclonal antibodies when the fibrinogen is immobilised on a surface. Thus, the physical state of the ligand may influence its recognition specificity (Ugarova et al., 1993).	$\alpha_M\beta_2$, $\alpha_v\beta_3$, $\alpha_x\beta_2$, $\alpha_{IIb}\beta_3$
von Willebrand factor		$\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$

Others

iC3b	A major opsonic form of complement C3 (Rabb et al., 1993)	$\alpha_M\beta_2$, $\alpha_x\beta_2$
ICAMs	Immunoglobulin-like counter-receptors. Certain integrins on leukocytes mediate adhesion to endothelial cells by binding to counter-receptors, which consist of a series of immunoglobulin-like domains (De Fougerolles et al., 1992)	$\alpha_L\beta_2$, $\alpha_M\beta_2$

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Invasin	$\alpha_4\beta_1$
Mucosal ACAM-1	$\alpha_4\beta_7$
Osteopontin	$\alpha_v\beta_3$
RGD sequence	$\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_{IIb}\beta_3$
VCAMs	$\alpha_4\beta_1$, $\alpha_4\beta_7$
?	$\alpha_8\beta_1$, $\alpha_9\beta_1$,
$\alpha_v\beta_8$	

Table 1.1 Extracellular ligands of the integrin family (adapted from Haas *et al.*, 1994).

1.1.2.4 Fibronectin receptors

Fibronectin receptors that interact with the RGD cell attachment site have been identified in cultured cells and tissues by affinity chromatography on Fn cell attachment fragments, or in a less direct way by means of antibodies that inhibit the attachment of cells to Fn (Ruoslahti *et al.*, 1987; Ruoslahti 1988; Gailit *et al.*, 1988; Wayner *et al.*, 1988; Dalton *et al.*, 1995). There is no single "Fn receptor", the various Fn receptors are members of a homologous receptor family that also includes receptors for other ligands (reviewed by Hynes, 1990; Vogel *et al.*, 1990). It has been proposed that a 140 kDa Fn receptor complex may be part of a cell surface linkage between Fn and cytoskeleton, which anchors cells to their substrata and maintains normal morphology (Pytela *et al.*, 1985; Chen *et al.*, 1986).

Fibronectin is recognized by at least six different cell surface receptors. The integrin $\alpha_5\beta_1$ was the first one isolated and characterised, and is often referred to as the "classical" Fn receptor. The $\alpha_5\beta_1$ integrin binds to the RGD cell attachment site of Fn. The other integrins that can bind Fn are $\alpha_{IIb}\beta_3$, $\alpha_v\beta_x$, perhaps $\alpha_3\beta_1$ and a novel neutrophil integrin. Recent studies indicate functions of the $\alpha_4\beta_1$ receptor in lymphocyte and melanoma adherence to the cell-type-specific, alternatively spliced CS1 sequence (Wayner *et al.*, 1989; Wayner *et al.*, 1992; Dean *et al.*, 1993; Kassner *et al.*, 1995), and there is evidence that $\alpha_8\beta_1$ is a Fn receptor that recognises the RGD sequence (Müller *et al.*, 1995; Venstrom *et*

al., 1995). Because of its abundance and comparatively high affinity, most work has focused on the $\alpha_5\beta_1$ Fn receptor. Like other integrin receptors, the receptor is composed of two subunits: an α subunit of 1,008 amino acids which is processed into two polypeptides disulphide bonded to one another, and a β subunit of 778 amino acids. Each subunit has near its COOH terminus a hydrophobic segment, which serves as a transmembrane domain anchoring each subunit in the membrane and dividing each into a large extracellular domain and a short cytoplasmic domain. It has been claimed that ECM recognition by the $\alpha_5\beta_1$ integrin plays a role in the control of cell proliferation (Grinnell, 1992). It has been suggested that a reduction of this Fn-receptor may contribute to morphological changes in transformed cells: reduced adhesion and rounded morphology (Chen *et al.*, 1986; Roman *et al.*, 1989; Giancotti *et al.*, 1990). The cytoplasmic domain of the β_1 subunit has been found to be phosphorylated in cells transformed by tyrosine kinase oncogenes (Yamada, 1989; Giancotti *et al.*, 1990).

Studies on Fn-receptors have shown that the Fn-receptor affinity is regulated by divalent cations, such as Mg^{2+} or Ca^{2+} for binding to fibronectin (Ruoslahti, 1988; Gailit *et al.*, 1988; Marks *et al.*, 1991). Mn^{2+} was also found to increase the binding of the receptor liposomes to Fn 2-3 fold over their binding in buffers containing Ca^{2+} and Mg^{2+}. The increased Fn-receptor activity in the presence of micromolar Mn^{2+} appeared to be due to an increase in the affinity of the receptor for the RGD sequence (Gailit *et al.*, 1988). In mM Mn^{2+} Cells can adhere and spread on proteins lacking the RGD sequence (Edwards *et al.*, 1988).

1.1.2.5 Vitronectin receptors

There are at least four known integrin receptors capable of recognizing Vn: including $\alpha_v\beta_3$, $\alpha_v\beta_5$ (Wayner *et al.*, 1991; Orlando *et al.*, 1991), $\alpha_{II}\beta_3$, and $\alpha_v\beta_1$ (Vogel *et al.*, 1990; Marshall *et al.*, 1995). Integrin $\alpha_v\beta_3$ appears to be

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highly restricted to vitronectin and is probably the best known Vn receptor. It mediates a broad spectrum of cell-cell and cell-substrate interactions, including attachment to Vn, von Willebrand factor, fibrinogen, and thrombospondin (Charo et al., 1990 ; Orlando et al., 1991). $\alpha_v\beta_3$ was reported to cooperate with $\alpha_5\beta_1$ in Chinese Hamster Ovary cell migration on fibronectin and vitronectin (Felding-Habermann et al., 1993).

1.1.2.6 Laminin receptors

Given the diversity of laminin and its multiple active sites, several members of the integrin superfamily interact with it, such as $\alpha_1\beta_1$, α_2b_1, $\alpha_3\beta_1$, $\alpha_7\beta_1$ (Hynes 1987; Hynes 1992; Goodman et al., 1991; Solowska et al., 1991; DiPersio et al., 1995; Gu et al., 1994). Inhibition studies using α_6-specific antibodies have shown that $\alpha_6\beta_1$ functions as a specific receptor for laminin (Sonnenberg et al., 1991; Marshall et al., 1995; Borradori et al., 1996).

1.1.2.7 Collagen receptors

The collagen-binding integrins are $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$ (Grzesiak et al., 1992; Tuckwell et al., 1995). There are many RGD sequences in collagens, but much of the collagen-mediated cell attachment is not RGD dependent (Dedhar et al., 1987a; Ruoslahti, 1991).

1.1.3 Structural aspects of adhesion to ECM

Cell adhesion of normal anchorage-dependent cells to extracellular matrix progresses through three stages: attachment, spreading, and the formation of specialised contact zones known as focal adhesions or focal contacts (Singer et

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al., 1988; Bloch, 1992). After the adhesion event takes place, cells typically spread and organise ligand-bound integrins into focal contacts that colocalise with the ends of actin filaments. Another type of adhesion site termed the extracellular matrix contact was described (Chen *et al.*, 1982). It is composed of co-linear transmembrane associations of actin microfilaments, a fibronectin receptor, and fibronectin-containing extracellular matrix fibers closely applied to the substratum (Chen *et al.*, 1985; Singer *et al.*, 1988). Electron microscopy studies have shown that extracellular matrix contacts contain fibronexuses, which are close transmembrane associations of fibronectin-containing fibers and bundles of 5 nm actin microfilaments in hamster and human fibroblasts (Singer *et al.*, 1979; Singer *et al.*, 1981; Singer *et al.*, 1982).

1.1.3.1 Focal adhesions

Many cells grown in tissue culture adhere tightly to the underlying substrate through distinct regions of the plasma membrane, referred to as focal adhesion sites or focal contacts or adhesion plaques. Using interference reflection microscopy (IRM), three types of regions on the ventral surface of cultured cells were classified (Izzard *et al.*, 1976; Izzard et al., 1980; Burridge *et al.*, 1988):
a) Focal contacts, the closest regions, which appear black by IRM, had a separation of 10-15 nm. Fully developed (mature) focal contacts have elongated shapes. Their length can reach 5-10 μm (Vasiliev, 1985).
b) Close contacts "gray in IRM", corresponding to a separation of about 30 nm.
c) Faint gray or white, corresponding to a separation of 100 nm or more from the substrate.

In focal contacts, now known as focal adhesions, the surface of the cell comes closest to the substrate and the plasma membrane is specialised at its cytoplasmic face for anchoring stress fibers (Grinnell, 1986; Burridge *et al.*, 1988). They are not essential for cell attachment and spreading, but their

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presence correlates with increased strength of cell attachment and restricted cell

motility. Focal adhesions contain different components (Geiger *et al.*, 1984;

Grinnell, 1986; Izzard, 1988; Turner *et al.*, 1991). These components are located

in distinct molecular domains (Fig. 4): one is associated with the peripheral ends

of microfilament bundles, which consist, at least, of F-actin, filamin, and α-

actinin (myosin, tropomyosin, and spectrin are excluded); and closest to the

plasma membrane structural proteins, such as vinculin, talin, paxillin (Geiger *et*

al., 1984; Bershadsky *et al.*, 1985; Sastry *et al.*, 1993), regulatory proteins

including tyrosine kinases (e.g. $pp125^{FAK}$, Schaller *et al.*, 1992a) and pp60v-src,

protein kinase C, a calcium-dependent proteolytic enzyme (calpain II), and

several phosphoproteins (reviewed Sastry *et al.*, 1993, Gingell, 1993).
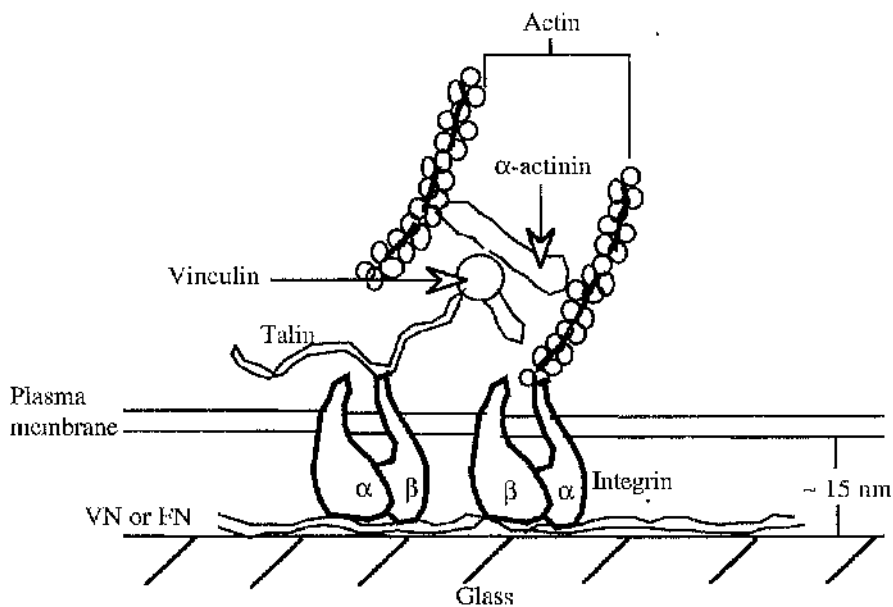
Fig. 1.4 a diagram showing some of the interactions that have been determined in vitro for proteins in focal adhesions. Vitronectin (VN) or fibronectin (FN) are shown adsorbed to he glass (Adapted Burridge *et al.*, 1988).

A number of other proteins that are targets for tyrosine phosphorylation by

pp60v-src in RSV-transformed cells also localise to focal adhesions (Hirst *et al.*,

1986; Turner *et al.*, 1991). Paxillin, which shares many of the characteristics of

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the other cytoskeletal components of focal adhesions is one of these proteins. Zyxin, an 82 kDa protein has also been localised to focal adhesions. Among the other interesting focal adhesion proteins, is the actin-binding protein tensin, which contains a src homology 2 (SH2) domain. It has been suggested that the interaction of tenascin with other focal adhesion proteins may be regulated through their state of phosphorylation (Turner *et al.*, 1991; Craig *et al.*, 1996). Inhibition of protein kinase C (PKC) but not of cyclic AMP- or cyclic GMP-dependent kinases, can prevent focal adhesion formation (Woods *et al.*, 1992). These experiments indicate that PKC-mediated phosphorylation changes, and may be an important signalling mechanism in focal adhesion and stress fibre formation.

1.2 Role of motility in cell spreading

For a cell to spread, its edges must move outward. This movement, involves rearrangements of filaments and membrane similar to those shown by locomoting cells: ruffles, microspikes and filopodia. Surface attached particles and groups of receptors linked to the cytoskeleton also move centripetally along the spreading axes.

1.2.1 Locomotion and cell motility

Abercrombie and his colleagues were first to draw close attention to the phenomenon of substrate-associated cell movement of metazoan cells (Abercrombie *et al.*, 1970).

Locomotion and motility of individual cells is critically important to many basic biological processes, such as embryonic development, wound healing and inflammation (review, Lauffenburger et al., 1996). Many different types of cells,

including amoebae, leukocytes, fibroblasts, epithelial cells and neurite growth

cones, move by crawling across solid substrates in a similar process. Animal

tissue cells move by cycles of polar extension of a leading lamella, an actively

motile anterior membrane protrusion (leading edge), establishment of contact

with the underlying substrate, and retraction of the posterior trailing tail into the

advancing cell body (reviewed Geiger *et al.*, 1984, Rinnerthaler *et al.*, 1988;

Small *et al.*, 1993).

1.2.2 Structure of lamellipodium

Lamellipodia are the most visible and therefore the most familiar of the

locomotory organelles of crawling cells, and with few exceptions, all lamellipodia

have a similar structure. For example, the lamellipodia of fibroblasts, which

appear to have a structural counterpart in a variety of cells, including nerve

growth cones, the advancing edge of leukocytes, blood platelets and small

amoebae are thin (around 0.1 μm -0.2 μm, reviewed Small *et al.*, 1993, Gingell

1993) , veil-like processes that commonly extend from the margins of the lamella,

but may occur from less active regions of the cell and from the centre.

Lamellipodia lack cytoplasmic inclusions and are devoid of microtubules and

intermediate filaments, with the exception of lamellipodia of keratocytes, which

contain microtubules and intermediate filaments, and myosin-II as well (Lee *et*

al., 1993a). Fluorescent-phalloidin images of the fibroblast lamellipodium (Heath

et al., 1993) showed a characteristic criss-cross pattern of F-actin filaments, this

pattern is broken up by thicker bundles of F-actin called ribs or microspikes.

These F-actin ribs arise suddenly within the criss-cross network and then can

display a complicated pattern of extension and retraction, lateral motion and

fusion of adjacent ribs. Lamellipodia also contain the single headed myosin-1

(Zot *et al.*, 1992; Gingell, 1993; Cramer *et al.*, 1994; Williams *et al.*, 1994) which

can associate with membranes. The membranes of lamellipodia have adhesive

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proteins and, in the case of the nerve growth cone, abundant G-proteins which suggest signalling pathways (Gingell, 1993). Lamellipodia also contain α-actinin, filamin, vinculin and talin (Geiger *et al.*, 1984; Rinnerthaler *et al.*, 1988; Heath *et al.*, 1993; Lee *et al.*, 1993b).

1.2.3 Dynamic behaviour of actin filaments

In fibroblasts lamellipodia continually extend, lift up, and then fall back and dissolve into the dorsal surface of the lamella in a familiar process called ruffling (Bray *et al.*, 1988; Stossel, 1993). This process has been shown by many different types of cells. An interesting feature of lamellipodia is the prominent rearward flux of the filament network (Heath *et al.*, 1993). Rearward transport of continuous filament network was supported by cytochalasin B pre-treatment of neuronal growth cones. The drug caused a rapid withdrawal of the whole F-actin network from the cell margin. It has been proposed that withdrawal of the F-actin away from the cell margin is due to a centripetal pull on the filaments by a myosin motor. Immuno-fluorescence microscopy studies (Fukui *et al.*, 1989; Gingell *et al.*, 1992) showed that non-filamentous myosin I occurs at the leading edges of the lamellipodial projections of migrating *Dictyostelium* amoebae, which are devoid of myosin II, whereas filamentous myosin II is concentrated in the posterior of the cells, and it was suggested that actomyosin I might contribute to the forces that cause extension of the leading edge of a motile cell, while the contraction of actomyosin II at the rear squeezes the cell mass. Other possibilities are that the motor is located either on some stable elements within the lamellipodium, such as F-actin ribs, or, more favoured by Heath *et al.*, 1993, that the motor, in part at least, resides in the more central regions of the cytoskeleton in particular within the dorsal cortical F-actin sheath of the lamella, where myosin-II is found.

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Polymerisation of actin filaments is believed to be particularly important in cell locomotion. The mechanisms of force transduction and movement are still subject to speculation. Models for the dynamic behaviour of actin in different cells have been proposed, such as the original treadmilling model for fibroblasts (Wang 1985) and the nucleation-release model for keratocytes (Small *et al.*, 1993).

The treadmilling model

Electron microscopic studies on the leading edge of living fibroblasts demonstrated a presence of actin meshwork of clearly continuous actin filaments, as well as numerous actin filament bundles referred to as microspikes, extending from the edge of the cell toward the centre. In addition, most actin filaments in this region have a uniform polarity, with the fast extending "barbed" ends associated with the membrane. Myosin, is either absent or present in only a very small amount (reviewed Wang, 1985). Wang (1985) studied the rate and the pattern of actin recovery in live gerbil fibroblasts and he proposed a treadmilling model. In this model the actin filaments are oriented with their fast growing, active "barbed" ends towards the cell margin, and must be long enough to span the entire breadth of the lamellipodium. All polymerisation is expected to occur at the cell margin, and all depolymerisation at the rear of the lamellipodium (Theriot *et al.*, 1991; Small *et al.*, 1993; Small, 1995).

The nucleation-release model

This model has been proposed in conjunction with studies on fish keratocyte cells. Epidermal fish and amphibian keratocytes are among the most rapidly moving metazoan cells. These cells have recently attracted increased attention from investigators of cell motility. Their striking feature is their shape,

which has been likened to a "canoe" (Small *et al.*, 1993), and the term "fan cells" has also been given to them. Theriot *et al.*, 1991 proposed that the lamellipodium of keratocytes is filled by large numbers of short actin filaments that are continuously treadmilling subunits. The filaments are nucleated at the cell margin and then released to fill the lamellipodium. This model, the nucleation-release model, would lead to a uniform density of short actin filaments in the lamellipodium in fish keratocytes. Such a model has been also proposed for fibroblasts. This model does not require any particular orientation of actin filaments. A coherent flux of actin subunits through the lamellipodium occurs because of the movement of a meshwork of short, cross-linked actin filaments as a unit, with new filaments being created primarily at or near the leading edge and subsequently released.

1.2.4. Motility requiring force from myosin-1 activity

Myosin-1 is a class of monomeric myosins that probably function at the membrane to mediate motility (Zot *et al.*, 1992; Williams *et al.*, 1994). This class of myosins has a single head domain and at least one light chain, that cannot polymerise into thick filaments (Gingell *et al.*, 1992). Like muscle myosins, the head contains binding sites for ATP and actin and transduces the energy from the hydrolysis of ATP into mechanical work (Zot *et al.*, 1992; Cramer *et al.*, 1994; Ruppel *et al.*, 1995). Actin stimulates ATP hydrolysis by myosin-1, and particles containing myosin-1 move along actin. Phosphorylation of the head domain by myosin-1 heavy chain kinase is required to activate myosin-1. This phosphorylation results in enhanced binding to phospholipid vesicles that depends on calcium in conjunction with the dissociation of calmodulin. The phosphorylation of the tail region may provide an alternative means of regulating myosin-1 through the phosphoinositide pathway and may be another means of controlling myosin-1 activity (Titus, 1993). A study by (Zot *et al.*, 1992)

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demonstrated that myosin-1 bound to pure lipid bilayers moves actin filaments. They also showed that there is a preferential binding of myosin-1 to membranes containing 5-40 % phosphatidylserine, a composition consistent with that of cellular membranes.

1.2.5 Function of myosin-II in cell motility

It has been proposed that one specific mechanical contribution of myosin-II to locomotion is to provide a contractile force that detaches the cell from its posterior adhesion sites, and its importance for locomotion increases with the adhesivity of the substratum (Jay *et al.*, 1995; Weber *et al.*, 1995). Myosin-II is required for cortical movements such as capping of cell-surface receptors (reviewed Cramer *et al.*, 1994). Mutant *Dictyostelium* cells that lack myosin-II crawl more slowly (about half as fast) as their parental wild-type (Jay et al., 1995). Therefore, myosin-II is not essential for locomotion, but it does contribute.

1.2.6 Role of integrins in cell motility

Members of the integrin family of cell-surface proteins are perhaps the best characterised receptors involved in ECM-mediated motility (McDonald, 1989; Zetter *et al.*, 1990; Palecek *et al.*, 1997). Monoclonal antibody studies suggest that β_1 integrins are involved in the migration of a variety of cell lines on laminin, fibronectin and collagen. In particular, the $\alpha_5\beta_1$ integrins and $\alpha_2\beta_1$ integrins appear to be important in cell migration on fibronectin and collagen, respectively (Yamada *et al.*, 1990). It has been claimed by Yamada and others that β_1 integrins are also ECM-molecule specific as well as cell-type specific (Akiyama *et al.*, 1989; Yamada *et al.*, 1990). $\alpha_4\beta_1$ integrin mediates cell motility in the absence of $\alpha_5\beta_1$ and also promotes cell motility in response to VCAM-1

(Wu *et al.*, 1995a). It has been shown that a large fraction of the Fn receptors in locomoting cells is mobile, whereas in stationary cells the receptors are concentrated in focal contacts and fibrillar streaks where they are immobile (Duband *et al.*, 1988; Yamada, 1989, Fig. 5).
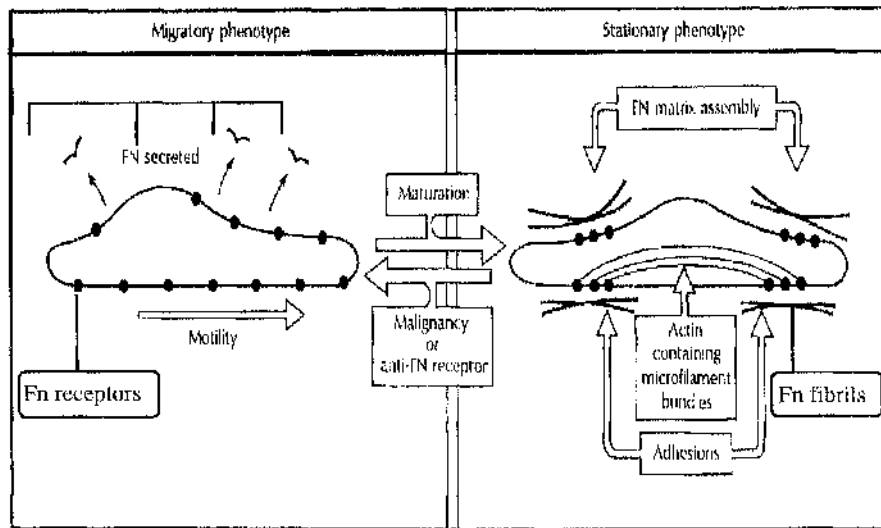
Fig. 1.5 Fn receptor and cell motility. This model represents two states of cells: the migratory one which displayed by rapidly migrating embryonic cells, and the stationary one expressed after by most cells which is characterised by slower rates of migration and changes in ECM, membrane receptor, and cytoskeleton. (adapted Yamada, 1989).

The organisation of integrins in transformed cells was found to be altered to a pattern similar to that found in highly motile cells, a diffuse surface distribution (Chen *et al.*, 1986; Mueller *et al.*, 1989; Roman *et al.*, 1989; Mueller *et al.*, 1991). Highly motile cells bind to immobilised Fn and other ECM components by localisation of β_1 integrins and talin at sites of membrane-ECM contacts, which might account for the motility-driven adhesion necessary for cell migration and invasion.

1.3 Structural aspects of membrane and filaments in spread cells

The cytoskeleton is the system of fibrillar structures in the cytoplasm of eukaryotic cells. There are three main types of filaments forming these structures,

which are: microtubules, actin filaments, and intermediate filaments. Many cytoskeletal structures are highly dynamic. The cytoskeleton can be regarded as a dynamic cytoplasmic matrix, surrounding and embedding other intracellular structures. This matrix may determine the position and movements of other cellular structures as well as the shape of the whole cell. This matrix is actively involved in cell spreading (section 1.1), and may be actively involved in the control of metabolic activity of other organelles and of the whole cell.

1.3.1 Microtubules

Microtubules are highly dynamic, universal components of all eukaryotic cells, which have the largest diameter of all cytoskeletal filaments, usually about 25 nm. The wall of microtubules is about 5 nm wide. Microtubules are tubular polymers assembled from a cytosolic pool by the addition of noncovalently linked 100 kDa tubulin heterodimers to protofilaments which associate laterally to form microtubules. Microtubules, and often bundles of microtubules, are important in the development and maintenance of cell shape and in a number of forms of cell motility (Redenbach et al., 1994). However, studies on some epithelial cell types have shown that they do not require microtubules either to adopt or to maintain a polarised morphology (Middleton et al., 1988; Middleton et al., 1989). Microtubules facilitate the transport of proteins from the Golgi complex to the apical domain, and they also play roles in the delivery of transport vesicles between different plasma membrane domains (Mays et al., 1994).

Microtubules act as tracks for organelle transport powered by the motor proteins kinesin (toward the plus-end) and cytoplasmic dynein (toward the minus-end) (Hoyt et al., 1994; Schroer et al., 1994). Polymerisation and the stabilisation of microtubules, as well as associations between these organelles, have been shown to be promoted by a variety microtubule-associated proteins (MAPs) (Ludueña et al., 1992; Lee, 1993b; Marya et al., 1994; Hirokawa, 1994; Amos et

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al., 1997). MAPs are attached to microtubules *in vivo* and copurify with tubulin through cycles of assembly and disassembly. In vitro, MAPs stimulate microtubule assembly. MAPs can control the stability of microtubules. MAPs also act as the targets of intracellular regulatory signals, such as altered concentration of cAMP or of calcium.

In interphase cells, microtubules participate in the maintenance of cell shape and the organisation and transport of organelles within the cytoplasm. The assembly of tubulin heterodimers confers a structural and kinetic polarity with plus (fast-growing) and minus (slow-growing) ends. One or both ends of cellular microtubules are often associated with special structures called microtubule-organising centres "MTOCs" (Joshi, 1994).

Effect of microtubule-destroying drugs

The role of microtubules in the development and maintenance of the shape of tissue cells at interphase was studied in detail in experiments with elongated polarised cells, such as fibroblasts or neurons. It has been found that depolymerisation of microtubules by specific drugs prevents or reverses polarisation of these cells. In particular, fibroblasts become unable to achieve and to maintain elongated shapes; differentiation of their edges into pseudopodially active and stable zones disappears. Elongated cytoplasmic processes of neuronal cells also disappear (Domnina *et al.*, 1985).

1.3.2 Actin

Actin filaments, also called microfilaments or F-actin (filamentous actin) are the major components of the cytoskeleton and appear to be essential for cell spreading and locomotion. Actin filaments are polymerised from globular actin monomers, G-actin; a 42-kDa actin monomer, which has a bound nucleotide

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(Bremer *et al.*, 1992; Yu *et al.*, 1994). Most actins consist of 375 amino acid residues, are acetylated at their amino terminus and are methylated at His68. The structure of the actin molecule in complex with DNAase I has been solved to atomic resolution (Bremer *et al.*, 1992; Reisler, 1993). The actin molecule consists of two domains (Fig. 6), called 'large' and 'small', although they are similar in size. These two domains can be subdivided further into two subdomains each, the small domain being composed of subdomains 1 and 2, and the large domain of sub-domains 3 and 4. The four subdomains are held together and stabilised mainly by salt bridges and hydrogen bonds to the phosphate groups of the bound nucleotide and to its associated divalent cation localised in the centre of the molecule.
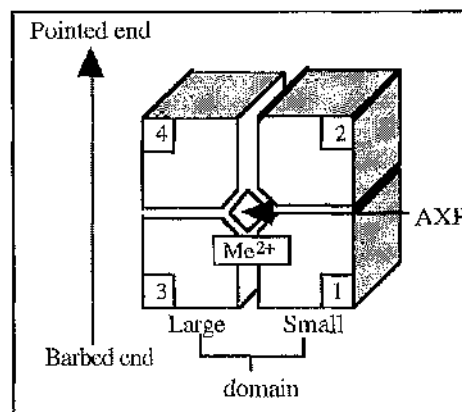
Fig. 1.6 Schematic view of the actin molecule.
(adapted Bremer *et al.*, 1992)

Actin-filaments are grouped together within cells and form many types of structures. Four large groups of these structures can be distinguished: bundles of parallel filaments with uniform polarity (usually do not contain myosin II, for example microvilli), bundles of filaments with alternate polarities (usually contain myosin, for example, stress fibers), three-dimensional networks, and the spectrin-actin submembranous network, which is the only cytoskeletal structure present within mammalian erythrocyte. These actin structures are attached to the plasma membrane at sites where cells adhere to substrates or to each other. At these

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regions a cell establishes a transmembrane linkage between components of the ECM and the actin-rich cytoskeleton. Actin interacts with a large variety of actin-binding proteins to play a central role in various forms of cell motility, including muscle contraction, and cell spreading.

Polymerised and nonpolymerised actin can interact with many proteins, called actin-binding proteins (ABPs). These proteins regulate the degree of polymerisation of actin and the stability, length, and distribution of actin filaments. A large number of actin monomer binding proteins have been identified. Among these, profilin and two major mammalian isoforms, thymosin $\beta4$ (T$\beta4$) and thymosin $\beta10$ (T$\beta10$) are identified as significant actin monomer sequestering proteins, which may be involved in regulating actin filament assembly in living cells (Vandekerckhove *et al.*, 1992; Nachmias, 1993; Yu *et al.*, 1994). The second group of ABPs either induce or inhibit early events in actin polymerisation (e.g. gelsolin and actobindin). Third, those that bind to one of the ends of the actin filaments and that determine the direction of filament growth or that anchor the filament ends to other proteins or structures (e.g. capZ36/32 and gcap39 and acumentin). Fourth, proteins that bind laterally to the actin filaments, these proteins interconnect filaments, connect filaments with other proteins or regulate the interaction of other ABPs with filamentous actin (F-actin), examples are α-actinin, villin, synapsin and tropomyosin. Finally, a set of proteins are able to sever/cap actin filaments by distributing the interaction between adjacent F-actin protomers (e.g. gelsolin, Weeds *et al.*, 1993; Borovikov *et al.*, 1995).

Myosins are a large family of actin-based motor proteins, of special importance. Motor proteins are functionally defined by their ability to generate directional movement in a nucleotide-dependent manner. All myosins contain conserved ~ 80 kDa 'head' domains which are responsible for motor activity. Interactions of actin with myosin are accompanied by the consumption of ATP and can lead to the movements of actin microfilaments (Svitkina *et al.*, 1986;

Cheney *et al.*, 1992; Titus, 1993). These interactions provide the molecular basis for many types of motility.

Each actin molecule contains one molecule of bound nucleotide (ATP or ADP) and one bound ion (Ca^{2+} or Mg^{2+}). The living cell usually contains more Mg^{2+} than Ca^{2+} and more ATP than ADP (Bremer, 1992).

Proteins attaching actin filament to the membrane

Attachment of actin structures to the outer cell membranes is likely to be mediated by a group of special proteins. For example, ankyrin in erythrocytes binds both to spectrin and to the integral membrane protein, called band 3 protein or anion transporter. Protein 4.1, can link spectrin and another membrane protein, glycophorin. The 110 K-calmodulin complex of brush-border microvilli binds both to actin filaments and to plasma membrane in an ATP-sensitive manner (Rosok *et al.*, 1983). Two integral membrane glycoproteins have been found to associate directly with actin. These are ponticulin, and the epidermal growth factor receptor (Hitt *et al.*, 1994). Several characteristic proteins were found in the submembranous termini of the filament bundles of cultured fibroblasts, which are attached to the focal cell substrate contacts. These contacts contain vinculin and talin in close proximity to the membrane. Talin binds vinculin in vitro and also binds transmembrane 140-kDa, integrin. Vinculin is also accumulated in the submembranous areas of cell-cell contacts associated with microfilament bundles (Rosok *et al.*, 1983).

1.3.3 Intermediate filaments

Besides tubulin-containing microtubules and actin-containing microfilaments, intermediate filaments (IFs) are smooth, long dynamic cellular filaments, approximately 10-nm in diameter, usually assembled into cytoskeletal

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networks extending from the nucleus to the cell membrane (Vikstrom *et al.*, 1989; Stewart, 1993; Eriksson *et al.*, 1992; Fuchs *et al.*, 1994), and represent the third filament system involved in forming the complex cytoskeleton of eukaryotic cells.

1.4. Integrin-mediated cell-signalling

Integrins, as receptors for constituents of ECM, play a much greater role than simply serving as mediators of ECM-cytoskeleton linkage. A major function of integrins is to mediate a bi-directional transfer of signals : from ECM to the cell interior, "outside-in signalling", and from the interior of the cell to ECM "inside-out signalling". Outside-in signalling modulates a broad spectrum of cellular responses including cytoskeletal re-organisation and cell spreading, gene expression, and cell proliferation, while inside-out signalling regulates the conformation, and thus affinity, of the receptor from inside the cell.

1.4.1 Outside-in signalling

It appears that many of the well known signal transduction pathways identified previously for growth factors and cytokines are also activated by integrins (Damsky *et al.*, 1992; Gingell, 1993; Sastry *et al.*, 1993; Richardson *et al.*, 1995). Among these are pathways controlling activation of both protein tyrosine kinases and members of the rho family of small GTP-binding proteins (Dedhar *et al.*, 1996; Parsons *et al.*, 1996).

It is not clear which (if any) if these pathways signal the events of initial spreading. Vuori (1993), suggested that a protein tyrosine kinase C may be important, and in some cells a phospholipid kinase, PI 3-kinase has been

implicated, by use the inhibitor wortmannin (Shimizu *et al.*, 1995; Reif *et al.*, 1996; Parker *et al.*, 1995).

Focal adhesions (sec 1.1.3.1) are regarded as centres of signal transduction. Activation of a tyrosine kinase associated with focal adhesions (pp125^{FAK}) may be an important early step in intracellular signal transduction pathways triggered in response to cell interactions with the ECM (Hanks *et al.*, 1992). After integrin occupancy and clustering, focal adhesion kinase (FAK) becomes hyperphosphorylated, by an as yet poorly understood mechanism. Phosphorylated FAK can then associate with other signalling molecules via their SH2 domains (e.g. Fig. 7).
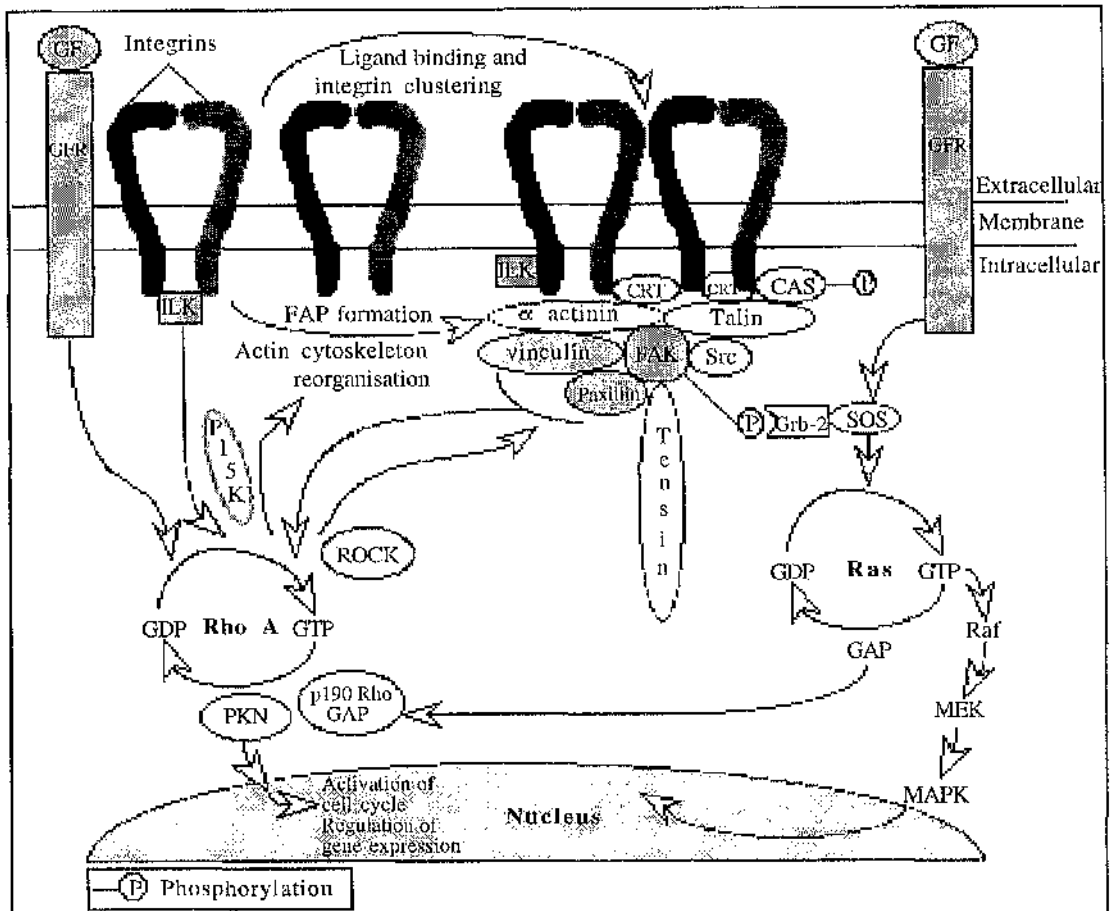
Fig. 1.7 Molecular events involved in integrin-mediated 'outside-in' signalling (see text). FAP, focal adhesion plaque; CRT, calreticulin; CAS, cadherin-associated substrate; GF, growth factor; GFR, growth factor receptor; PKN, protein kinase N; PI5K, phosphatidylinositide 4-phosphate-5-kinase; MAPK, mitogen-activated protein kinase; MEK, ERK kinase; ILK, integrin-linked kinase; ROCK, Rho-associated coiled-coil containing protein kinase; GAP, GTPase-activating protein. (adapted Dedhar *et al.*, 1996)

The phenomenon of anchorage-dependent cell growth may depend on signalling by integrins (Guan *et al.*, 1992; Ruoslahti *et al.*, 1994; Hannigan *et al.*, 1996)S. Like growth factors, integrins can also inhibit apoptosis (Ruoslahti *et al.*, 1994; Aharoni *et al.*, 1996). Cells denied anchorage can undergo apoptosis and integrin-mediated signalling may be the controlling factor in this phenomeonon. (Meredith *et al.*, 1993; Frisch *et al.*, 1994). Since anchorage-independent cell growth is a hallmark of oncogenically transformed cells, the mechanism of signal transduction at focal contacts is clearly relevant to both normal and neoplastic

growth processes. Signals believed to be induced by integrins are summarised in Table 2.

Type of signal transduction	Comments	reference
Ca^{2+} influx	Certain α_v integrins, inhibited by anti-IAP antibodies	(Schwartz et al., 1993)
H^+ exchange (alkalinization)	Can be triggered by ligand-coated beads	(Schwartz et al., 1992; Schwartz et al., 1994)
Tyrosine phosphorylation of FAK	Induced by a variety of ECM ligands, but not polylysine	(Richardson et al., 1994; Schaller et al., 1994
Activation of Src and related tyrosine kinases	—	(Shattil et al., 1994)
Accumulation of signal transduction adapters and enzymes	Rapidly increasing numbers, including Grb2-Sos and many others	(Yamada et al., 1995; Miyamoto et al., 1995; Schlaepfer et al., 1994)
Monocyte activation , (tyrosine kinases, transcription factors)	May also be induced by adhesion to plastic	(TH et al., 1995)
Phosphoinositide mediators	In endothelial cells, via activation of PIP-5 kinase and Rho	(McNamee et al., 1993; Chong et al., 1994)
Activation of protein kinase C and arachidonic acid pathway	—	(Clark et al., 1995)
Growth regulation (e.g. via cyclins and retinoblastoma protein	—	(Schwartz et al., 1992; Varner et al., 1995)
ERK activation	—	(Schlaepfer et al., 1994; Chen et al., 1994)
JNK activation	—	(Miyamoto et al., 1995)
Enhanced gene expression	Time course differs from ERK type of MAP kinase	(Roskelley et al., 1994)
Prevention or stimulation of apoptosis	—	(Ruoslahti et al., 1994; Meredith et al., 1993)

Table 1.2. Integrin-mediated signal transduction. ERK, extracellular regulated kinase; FAK, focal adhesion kinase; IAP, integrin-associated protein; JNK, Jun kinase; MAP, mitogen-activated protein; PIP, phosphatidylinositol 4-phosphate. (adapted Yamada et al., 1995).

1.4.2 Inside-out signalling

The affinity of integrins for their extracellular ligands can change in response to cytoplasmic signals initiated via the activation of other cellular receptors (Ginsberg et al., 1992; Hynes et al., 1992; Schwartz et al., 1995; Yamada et al., 1995; Dedhar et al., 1996; Humphries et al., 1996). The activities of both protein kinases and protein phosphatases have been implicated in integrin signalling regulation. Integrins of the β_1, β_2 and β_3 integrin families have all been shown to undergo 'activation'. Inside-out signalling has been proposed to involve the propagation of conformational changes from the cytoplasmic domains to the extracellular binding site in response to intracellular signalling (Williams et al., 1994; O'Toole et al., 1994). Although the molecular nature of the conformational changes is not yet understood, recent advances have been made in the identification of the regions within integrin cytoplasmic domains which may be involved in regulating inside-out signalling (Humphries et al., 1996).

1.5 Signalling through small G proteins

The rho-family proteins have recently contributed exciting insights into how cells regulate their shape and motility through modulation of the actin cytoskeleton (Machesky et al., 1994; Nobes et al., 1995b; Hall et al., 1990; Ridley et al., 1996; Zigmond et al., 1996). The mammalian ras-related GTPases consist of rho (A, B, and C), rac (1 and 2), Cdc42 (Cdc42Hs and G25K), rho G, and TC10, each having 50-55% homology with each other and around 30% homology to ras.

Rho-family proteins as members of the ras GTPase superfamily act as molecular switches and are regulated by GTPase-activating proteins (GAPs), GDP-GTP exchange factors (GEFs) and a GDP dissociation inhibitor (GDI).

They are active when binding GTP, inactive when this is converted to GDP by their intrinsic GTPase activity.(Hall *et al.*, 1992; Hall *et al.*, 1990; Hall *et al.*, 1994; Lamarche *et al.*, 1994; Diekmann *et al.*, 1994; Quilliam *et al.*, 1995; Tapon *et al.*, 1997).

1.5.1 Signalling through rho

Rho is an essential component of a signal transduction pathway linking growth factor receptors to the assembly of focal adhesions and the polymerisation of actin into stress fibers (Nobes *et al.*, 1995b). This has been investigated by using an enzyme from *Clostridium botulinum* (C3 transferase) which inactivates rho, and by microinjection of recombinant rho proteins.

When microinjected into serum-starved Swiss 3T3 cells, rho rapidly stimulated stress fibre and focal adhesion formation. Re-addition of serum produced a similar response. This activity was due to lysophosphatidic acid (LPA), bound to serum albumin (Ridley *et al.*, 1992a). Other growth factors including PDGF induced actin reorganisation initially to form membrane ruffles, and later stress fibres. For all growth factors tested, the stimulation of focal adhesion and stress fibre assembly was inhibited when endogenous rho function was blocked, whereas membrane ruffling was unaffected (Ridley *et al.*, 1994). It has also been demonstrated that stimulation of fibroblasts with LPA induces myosin light chain phosphorylation. This precedes the formation of stress fibres and focal adhesions and is accompanied by increased contractility. When contractility is inhibited, integrins disperse from focal adhesions as stress fibres and focal adhesions disassemble (Chrznowska *et al.*, 1994; Chrznowska *et al.*, 1996). How rho stimulates these cytoskeletal events has not been resolved.

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1.5.2 Signalling through rac

Rac proteins are approximately 60% identical to rho, suggesting that rac and rho may have related functions. Like rho, rac is an essential component of the signal transduction pathways linking growth factors to the organisation of actin cytoskeleton. It has been shown that rac can act as signal transducer for distinct PDGF-induced responses, and proteins that can regulate rac activity, such as Bcr, are likely to act on this signalling pathway (Ridley *et al.*, 1992b; Ridley *et al.*, 1994). PDGF (Hawkins *et al.*, 1995; Wennström *et al.*, 1994) and EGF with insulin, initially activates rac, which stimulates membrane ruffling, and also leads to rho activation, accounting for an observed delay in stress fibre formation. Microinjection of recombinant rac 1 protein into serum-starved Swiss 3T3 cells has a dramatic effect on the actin cytoskeleton, that is quite distinct from rho-induced effects; rac has been observed to stimulate the rapid polymerisation of actin at the plasma membrane to produce lamellipodia and membrane ruffles (Ridley *et al.*, 1992b; Hall *et al.*, 1994; Nobes *et al.*, 1995b). Some of the events that underlie cell spreading might depend on signalling via rac.

Recent studies have concluded that activation of rac by PDGF and insulin receptors is mediated by PI 3-kinase (Nobes *et al.*, 1995a; Parker *et al.*, 1995; Reif *et al.*, 1996). The role of rac, as a key regulator of membrane ruffling in fibroblasts is shown in (Fig. 8).
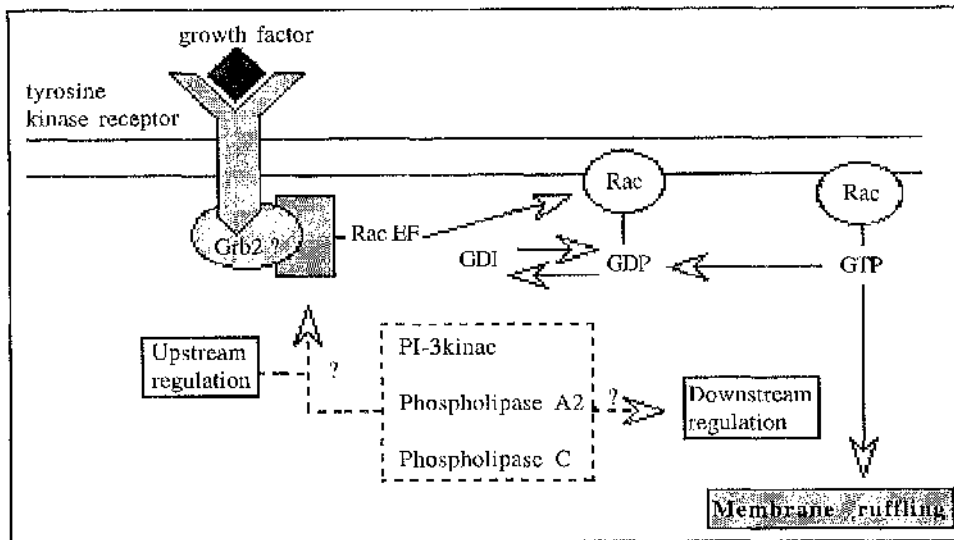
Fig. 1.8 A possible model for rac activation leading to membrane ruffling. In resting cells, rac is normally complexed in the cytosol with a GDI (guanine nucleotide dissociation inhibitor), preventing membrane binding, nucleotide exchange or interaction with GAPs or presumably with target proteins. Upon activation of cells by an extracellular factor, this complex dissociates and rac is able to bind to the membrane. In the case of tyrosine kinase receptors, a Grb-2-like protein brings a rac exchange factor (Rac EF) to the plasma membrane by binding via a SH2 domain to an activated receptor. This leads to activation of rac and stimulation of downstream events leading to membrane ruffling. Alterations in phospholipid metabolism mediated by PI-3-kinase (PI3K), phospholipase A2 and phospholipase C may also be required for membrane ruffling, but whether they act upstream or downstream of rac is not known. Rac can be inactivated by the action of GAPs (adapted Ridley *et al.*, 1994)

1.5.3 Signalling through Cdc42

Cdc42 was originally detected in the yeast *Saccharomyces cerevisiae* as a mutation that caused defects in budding and cell polarity (Adams *et al.*, 1990; Johnson *et al.*, 1990; Zheng *et al.*, 1994). However, recent studies have shown, that there are at least two activities associated with homologous Cdc42 in mammalian cells: first an action on the cytoskeleton, and second on rac. When Cdc42 was microinjected into confluent Swiss 3T3 cells, it led to the formation of lamellipodia and actin stress fibres. By coinjecting inhibitors of rac and rho, it could be shown that those responses were due to the activation of endogenous rac and rho by Cdc42 (Ridley *et al.*, 1992b). Time-lapse video recordings of subconfluent cells in culture, revealed that in the absence of endogenous rac activity, around 20-30 filopodia were induced on each cell within 5 minutes after

injecting Cdc42, which could grow up to around 10-25 μm in length over 30 minutes (Kozma et al., 1995).

In conclusion, figure 9 summarises the rho, rac, and Cdc42 signal transduction pathways in Swiss 3T3 cells. These authors have concluded that there is a hierarchical relationship between the members of the rho family (Ridley et al., 1992; Nobes et al., 1995a; Nobes et al., 1995b: Tampon et al., 1997).
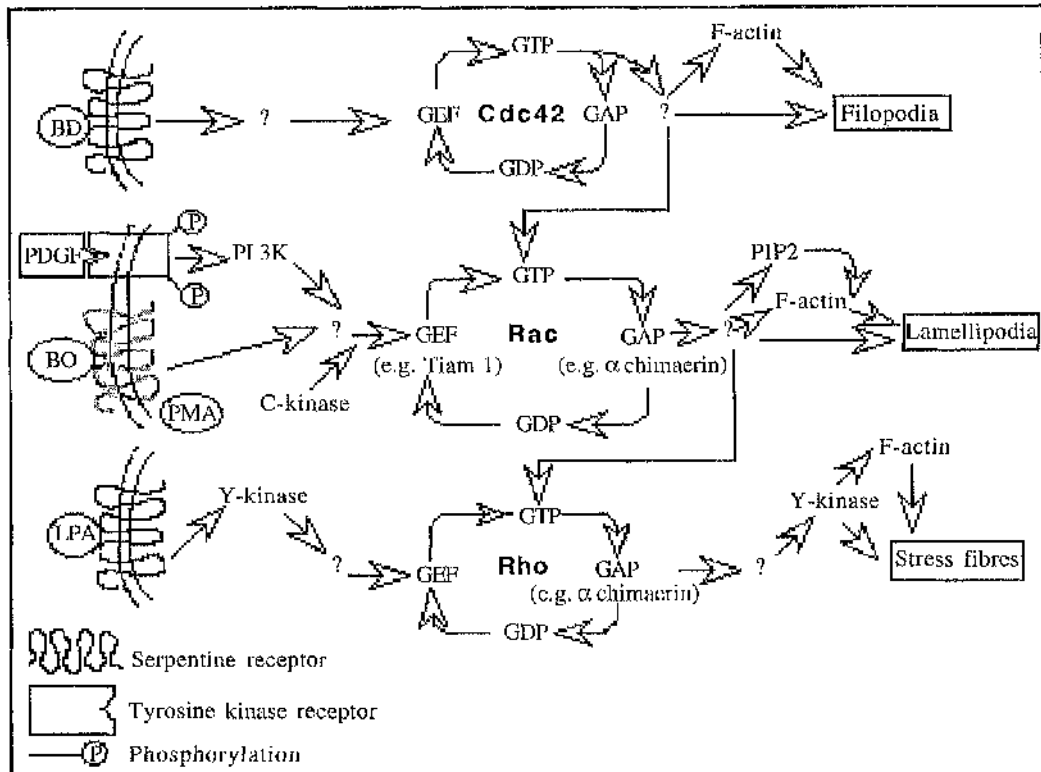
Fig. 1.9 Rho, Rac, and Cdc42. Signal transduction pathways in Swiss 3T3 fibroblasts. This diagram illustrates proposed relationships between receptors, Rho family members, and the cytoskeleton. The extracellular ligands are bradykinen (BD), bombesin (BO), PDGF, PMA, and LPA. The receptors for the extracellular ligands are serpentine receptors linked to heterotrimeric G proteins, tyrosine kinase receptors, or protein kinase C (C kinase). The kinases PI3K, which was identified by sensitivity to wortmannin, C kinase (the protein kinase C target of PMA), and Y kinase (tyrosine kinases, which were identified by sensitivity to tyrphostin. GTP and GDP are the nucleotides bound by the Rho family G proteins. (adapted Zigmond et al., 1996)

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2. Selection of mutants of altered adherence properties

One approach to investigate the relation between cell-substratum adhesion and cell-spreading is the selection of low adherence mutants unresponsive to fibronectin or other ECM constituents.

A number of mutants selected for poor adhesion to various substrata have been described. Two mutants of Balb/c 3T3 cells AD6 and AD8 defective in adhesion to tissue culture polystyrene in the presence of serum were isolated. Both mutants had decreased adhesion to their substrate (Pouyssegur $et\ al.$, 1976). Subsequent studies on AD6 showed that only 10% of AD6 cells formed focal contacts, which were very small (Norton $et\ al.$, 1982). A mutant of Chinese Hamster Ovary cells termed CHO^{att-}, was isolated which was altered in its response to "cell attachment protein", probably fibronectin. These cells were defective in their attachment to collagen and were also altered in their ability to attach to synthetic substrata, such as glass and plastic surfaces (Klebe $et\ al.$, 1977). Another adhesion variant (AD^v) of Chinese Hamster Ovary cells was selected for reduced adhesion to serum-coated tissue culture plates and reduced adhesion to substrata composed of collagen layers coated with bovine serum or with fibronectin (Harper $et\ al.$, 1980). The adhesion defect in some AD variants could be corrected by raising intracellular c-AMP levels (Cheung $et\ al.$, 1985). AD^v appeared to have an altered type I c-AMP dependent protein kinase with lower affinity for c-AMP (Cheung $et\ al.$, 1987). A variant of human lymphoblastoid cells, termed adhesion-negative clone (Ad$^-$) was isolated that was incapable of cell spreading (Sorrentino $et\ al.$, 1983). Edwards $et\ al.$, 1985 reported selection from polyoma transformed hamster fibroblasts (Py-BHK21) of cells poorly adhesive to fibronectin and serum coated surfaces. These cells showed a dramatically altered morphology, and spread very poorly on any protein-coated surfaces tested. Subsequently similar mutant cells resistant to 6-thioguanine (TG), and two different mutants of low adherence (F1 and F2) were

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selected from Py-BHK21 (Hameed, 1988). These mutants were found not to spread on fibronectin or on serum coated surfaces. F1 colonies contained exclusively rounded cells and F2 colonies had a few partially spread cells and were more scattered than F1. Adhesion mutants of the mouse L929 line were selected for low critical sheer of detachment (Sarwar *et al.*, 1990).

Mutants for increased adhesiveness were also selected. Mutants of SV40-transformed mouse fibroblasts with greatly increased cell-substratum adherence were isolated. These mutants yielded 2.5-10 fold more substratum-attachment material than the parental cell lines (Bennoni *et al.*, 1986). MG-63 human osteosarcoma cells with higher adhesion to fibronectin-related peptide were selected for attachment and growth in the presence of increasing concentrations of a synthetic peptide containing the cell attachment-promoting Arg-Gly-Asp (RGD) sequence derived from the cell- binding region of fibronectin. Cells capable of attachment and growth in 5 mM concentrations of a peptide having the sequence Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) overproduce the cell surface receptor for fibronectin but not vitronectin receptors and were morphologically different from the parental MG-63 cells (Dedhar *et al.*, 1987b). Two variant lines, SK-N-SH and IMR-32 of neuroblastoma cells were selected for resistance to detachment by an RGD-containing synthetic GRGDSP peptide. These variant cell lines exhibited weaker attachment to type 1 collagen and laminin, but a similar level of attachment to fibronectin as compared to the parental cells. They stably overproduced up to 20-fold the $\alpha 1$ subunit (VLA-1); and in the IMR-32 variant cells, the common $\beta 1$ subunit was also overproduced. The level of expression of $\alpha 2$ and $\alpha 3$ subunits was considerably reduced and that of the $\alpha 5$ subunit unchanged relative to the parental cells (Dedhar *et al.*, 1989). Recently a variant of the K562 erythroleukemia cell line, FA-K562 was isolated by selection for strong adhesion to human plasma fibronectin. FA-K562 expressed fourfold more cell-surface $\alpha 5 \beta 1$ than parental K562, displayed slower growth under standard culture conditions and failed to grow in soft agar or nude mice. Both GRGDS and

anti-$\alpha_5\beta_1$ antibodies specifically stimulated the anchorage-independent growth of FA-562 in a dose-dependent fashion. Morphologically, this variant had become fibroblastoid in appearance, assembled filamentous actin, and differed from K562 in vimentin staining intensity and pattern.

3. Pyruvate requirements in cell culture

Pyruvate is frequently included as a supplement in culture media for mammalian cells. It has been reported that addition of pyruvate is often essential for the utilisation of some carbohydrates such as galactose, mannose and fructose (Eagle *et al.*, 1958; Burns *et al.*, 1976). Pyruvate is also known to enhance the proliferation of certain types of mammalian cells in glucose-containing media, especially when cell densities are low (Eagle *et al.*, 1962). The requirement for pyruvate was found to be stringent in cultures of tracheal epithelial cells in which it promotes their growth by inhibiting terminal differentiation (Wasilenko *et al.*, 1984). It is required for the preimplantation and development of early embryos in cultures during human *in vitro* fertilisation (Conaghan *et al.*, 1993). During maturation of mouse oocytes, pyruvate appears to be needed for nuclear membrane assembly and maintenance (Kim *et al.*, 1991). Pyruvate was also required, together with lactate, to support normal development of embryos of certain inbred mouse strains, and their F1 hybrids to the morula stage *in vitro* . Glucose was an essential component of the culture medium for development to the blastocyst stage (Brown *et al.*, 1991). Pyruvate was required for growth of a line of transformed hamster embryo fibroblasts (Sens *et al.*, 1982), and for the growth and survival of human malignant melanoma cells (Ellem *et al.*, 1983).

So what is the role of pyruvate in supporting the survival and growth of many types of cells in culture? Do cells need pyruvate as an energy source, when there is an inadequate supply of endogenous substrates for ATP synthesis via the

citric acid cycle, or does it supply metabolites? These could include amino acids and acetyl-CoA. The latter in turn feeds into various biosynthetic pathways including the endogenous synthesis of amino acids, or biosynthesis of fatty acids.

It is known that pyruvate is essential for the survival of prenatal neurons in culture, and it has been suggested that *in vivo* the glial cell population is the most likely external source of pyruvate and other citric acid cycle intermediates such as α-oxoglutarate, oxaloacetate, or amino acids that transaminate to them (Selak *et al.*, 1985), and it has been found that pyruvate (plus malate) increased the respiration rate while ATP levels were unchanged (Villalba *et al.*, 1994). Survival of chick embryo central nervous neurons (CNS), cultured as monolayers at low density need exogenous pyruvate. Other α-oxo acids support cell survival only in the presence of α-amino acids that transaminate to α-oxoglutarate, oxaloacetate, or pyruvate (Facci *et al.*, 1985a; Facci *et al.*, 1985b; Facci *et al.*, 1986). It has been concluded that pyruvate is needed as an energy source as well as a source of amino acids by transamination reactions in the CNS. It has been found that although pyruvate carboxylase is found primarily in astroglial cells, both neurons and astroglia require constant replenishment of oxaloacetate or other products derived from it. Without replenishment, the capacity of the citric cycle to oxidise pyruvate to $CO_2 + H_2O$ would be diminished in both neurons and astroglia, and the level of intermediates available for the biosynthesis of the excitatory neurotransmitters, glutamate and aspartate, and of γ-aminobutyric acid (GABA) , would fall (Kaufman *et al.*, 1992). Indeed, in patients with pyruvate carboxylase deficiency, due to genetic disease, this is exactly what is observed (De-vivo *et al.*, 1977).

It is known that other compounds rather than pyruvate itself can enter the citric acid cycle via acetyl-CoA (e.g. fatty acids, ketone bodies), and that glutamine is oxidised in part by the citric acid cycle and it has been assumed that pyruvate oxidation may play a minor but significant role in the provision of ATP (Newsholme *et al.*, 1987; Curi *et al.*, 1988).

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Recent studies have shown that hepatocytes cultured with pyruvate had a much higher ATP level than those without pyruvate, and it has been suggested that pyruvate enhances lipogenesis in cultures of rat hepatocytes cell culture, as well as producing enough energy for their maintenance (Tomita et al., 1993; Tomita et al., 1995).

In conclusion it seems that the role of pyruvate may be sought in a variety of pathways, since it holds such a central position in intermediary metabolism.

MATERIALS
&
METHODS

1. Materials

1.1 Cell line

TG cells, the parental wild type, were selected from Py3 cells (a sub-clone of PyY) (Stoker *et al.*, 1964) for resistance to 6-thioguanine (Hameed, 1988).

1.2 Media

Water for culture medium and solutions was purified by reverse osmosis and ion exchange, using Milli RO cartridges and Milli Q.

HEPES Buffered Water

For 1 litre

HEPES   5.25 g

pH adjusted to 7.5, and stored at 4° C.

HEPES is N-[2 hydroxethyl] piperazine-N'[2-ethane sulphonic acid] (Cambridge Research Biochemicals).

7.5 % Sodium hydrogen carbonate

Sodium bicarbonate 75 g in 1 litre water, dispensed in 20 ml aliquots, and stored at room temperature.

Tryptose Phosphate Broth (TPB) (Gibco)

29.5 g powder in 1 litre water, pH adjusted to 7.5, dispensed in 20 ml aliquots, autoclaved, and stored at 4° C.

GPSA

GPSA is a mixture containing the amino acid glutamine, the antibiotics Penicillin and Streptomycin, and the antimycotic Fungizone (Amphotericin B).

Glutamine	114 mM
Penicillin	1905 units/ml
Streptomycin	1905 μg/ml
Fungizone	11.9 μg/ml

All the ingredients except glutamine (Flow laboratories) were from Gibco, ordered in the form:

Penicillin (5,000 units/ml), Streptomycin (5,000 μg/ml) in normal saline solution. Fungizone (Amphotericin B, 250 μg/ml) prepared in water. GPSA was dispensed in 20 ml aliquots, and stored at - 20° C.

Ham's F10

For 1 litre

20 mM HEPES water pH 7.5	180 ml
X10 concentrated Ham's F10 (Flow)	20 ml
7.5 % NaHCO$_3$	1 ml
GPSA	5 ml
Foetal calf serum (FCS, Gibco)	20 ml
Tryptose Phosphate Broth (TPB)	20 ml

HEPES Saline buffer (HS)

For 1 litre

Sodium chloride	8 g
Potassium chloride	0.4 g

D-Glucose	1 g
HEPES	2.38 g
Phenol Red	0.5 %

pH adjusted to 7.5 with 5 M NaOH.

Hanks HEPES buffer (HH)

For 1 litre

Sodium chloride	8 g
Potassium chloride	0.4 g
Calcium chloride ($2H_2O$)	0.19 g
Magnesium chloride ($6H_2O$)	0.2 g
D-Glucose	1 g
HEPES	2.38 g
Phenol Red (0.5%)	2.0 ml

pH adjusted to 7.5.

Phosphate Buffered Saline (PBS)

For 1 litre

NaCl	9.86 g
KCl	0.25 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.25

pH adjusted to 7.2.

Buffered Formalin (Formol Saline)

For 1 litre

100 mls of formaldehyde (40%) were added to 900 ml of phosphate buffered saline.

1.3 Tissue Culture Vessels

Tissue culture petri dishes were obtained from Corning Glass Works or Sterilin (U.K), tissue culture flasks from Greiner (Germany) or Sterilin (U.K.). Glass bottles for roller culture had an area of about 800 cm^2.

1.4 Solutions for detaching cells from culture surface

Versene

For 1 litre

Sodium chloride (NaCl)	8 g
Potassium chloride (KCl)	0.2 g
Di-sodium orthophosphate (Na$_2$HPO$_4$)	1.15 g
Potassium dihydrogen phosphate (KH$_2$PO$_4$)	0.2 g
EDTA (ethylene diamine tetra-acetic acid)	0.2 g
Phenol Red (0.5%)	2.0 ml

Trypsin

Sterile 0.25 %w/v trypsin (Gibco 1:250) in normal saline. pH 7.5, dispensed in 2 ml aliquots, and stored at - 20° C. 0.5 ml of this solution was added to 20 ml versene.

1.5 Medium for freezing cells for storage

FCS (Gibco)   9 ml

Glycerine (BDH)   1 ml

1.6 Buffers for the Affinity Chromatography of Calf Serum on Gelatin- Sepharose

1 mM Sodium chloride

For 1 litre

1 ml of 1 M HCl, was added to 1 litre distilled water.

Coupling Buffer

For 1 litre

Sodium chloride   29.24 g

Sodium bicarbonate (NaHCO$_3$)   8.4 g

pH adjusted to 8.0.

Tris Buffer

For 1 litre

Tris-base (Trizma) 12.1 g made up with 1 litre deionised distilled water, pH adjusted to 8.

Acetate buffer

For 1 litre

Sodium acetate (NaCH$_3$COO.3H$_2$O)   13.6 g

NaCl   29.23 g

pH adjusted to 4.0

Citrate buffer

For 1 litre

Na$_2$HPO$_4$ 1.8 g

KH$_2$PO$_4$ 0.2 g

NaCl   9.0 g

Sodium citrate   2.49 g

pH adjusted to 7.2

8 M UREA

Fot 1 litre

Tris base   6.05 g

Urea   480 g

pH adjusted to 7.5

These buffers were dispensed in 100 ml aliquots, autoclaved, and stored at 4º C.

1.7 Polyacrylamide Gel Electrophoresis (PAGE)

Reagents

All reagents were electrophoresis grade, obtained from the following sources:
Acrylamide and bis acrylamide from BDH Chemicals Ltd, Poole, England.
Sodium dodecyl sulphate (SDS), Bromo-phenol Blue, Kenacid Blue, and glycine
from Sigma. N, N, N', N', tetra methyl ethylene diamine (TEMED), and ß-
mercaptoethanol from Koch-Light Ltd. Ammonium persulphate from May and
Baker Ltd. Tris, (hydroxymethyl) aminoethane, from Boehringer Mannheim
GmbH Ltd.

Stock solutions

30% acrylamide

Acrylamide	28.5 g
Bis acrylamide	1.5 g
Distilled water	100 ml

Running gel buffer

Tris	18.5 g
SDS	0.4 g

dissolved in 50 ml water, pH adjusted to 8.9 with HCl, and made up to 100 ml
with water.

TEMED (N, N, N', N' , tetra methylethylene diamine).

Ammonium persulphate

10% w/v in water freshly made up before use.

Stacking gel buffer

Tris	5.9 g
SDS	0.4 g

dissolved in 50 ml water, pH adjusted to 6.7 with HCl, and made up to 100 ml with water.

Upper tank buffer

Prepared as a 5x concentrate, diluted for use in water.

Tris	31.6 g
Glycine	20 g
SDS	5 g

In one litre of water.

Lower tank buffer

Prepared as 5x concentrate, diluted for use in water.

Tris	60.5 g
SDS	5 g

pH adjusted to 8.1 with HCl and made up to a final volume of one litre with water.

Protein solubilising medium (boiling mixture)

Stacking gel buffer	2 ml
ß-mercaptoethanol	2.5 ml
SDS	1 g
Glycerol	2 g
Bromophenol blue (1%)	10 mg

Made up to 10 ml water.

After the addition of boiling mixture, used in a ratio of 5 parts sample (fibronectin) to 1 part boiling mixture, samples were placed in a boiling water bath for 3 minutes, used or stored at - 20° C.

Molecular weight standards

The following proteins were mixed to a final concentration of 1 mg/ml.

Fibronectin (bovine serum) (Gibco)	220 kD
ß-galactosidase (Sigma)	130 kD
Phosphorylase A (Sigma)	125 kD
Catalase (Sigma)	60 kD
Ovalbumin (Sigma)	45 kD
Concanavalin A (Sigma)	22 kD

Gel staining

Gels were stained with Kenacid blue.

For 0.1% Kenacid blue:

Methanol	250 ml
Distilled water	250 ml
Glacial acetic acid	35 ml
Kenacid blue	0.5 g

Gel destainer

Methanol	250 ml
Distilled water	250 ml
Glacial acetic acid	35 ml

The gel stain was also used for staining in the spreading assays (see methods).

1.8 Solutions for paper chromatography

All preparations were carried out in the fume cupboard, and were prepared according to (Veneziale *et al.*, 1969).

Solvent A

Solvent A consists of equal parts of aqueous propionic acid (BDH) [(620 ml propionic acid and 790 ml water), and aqueous n-butanol (Pro Labo) (1246 ml n-butanol + 84 ml water)].

Both solutions were mixed immediately before use.

Solvent B

Consisted of 75 parts 100% ethanol and 30 parts 1.0 M ammonium acetate.

Silver staining

5 grams of silver nitrate were boiled to 100° C in 4 ml distilled water, then cooled
to room temperature.

Saturated AgNO$_3$	0.5 ml
Acetone	500 ml

Developer (0.5% NaOH in ethanol)

For 1 litre

NaOH	5 g
Ethanol (100%)	1000 ml

1.9 Radioactive materials

[U-^{14}C] glucose

[U-^{14}C] glucose high specific activity 56.2 MBq/mg (1.52 mCi/mg), was supplied
by Amersham International plc. in aqueous solution containing 3% ethanol. It was
diluted in glucose-free Hanks HEPES (GHHH) to a concentration of 10 μCi/ml.

1.10 Solutions for mitochondrial dehydrogenases study

MTT solution

MTT (Sigma), was 3-[4,5-Dimethyl-thiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; (thiazoyl blue). Stock solution was prepared in Hanks HEPE (5 mg/ml) and stored at - 20° C.

Acidic isopropanol (Solubilization solution)

0.1 N HCl in absolute isopropanol (Scott the chemist, Byres road, Glasgow).

1.11 Glucose inhibitor solution

2-deoxyglucose from Sigma, was prepared in GFHH at a concentration 100 mM at the beginning, and then it was prepared in HH at concentration 100 mM for comparison with those experiments in which GFHH was used.

HH, HS, haemoglobin, and fibronectin were as the solutions for the standard spreading assays (see methods).

2. Methods

2.1 Cell culture

TG cells and F3 (0.5×10^{6}) were grown for 2-3 days in 75 cm^2 tissue culture flasks in Ham's F10 supplemented with 10% foetal calf serum and 10% tryptose phosphate broth at 37$^{\text{o}}$ C. Attached cells were subcultured at 2/3 confluency. The medium was poured off and the cell monolayer washed twice with HEPES saline. 5 ml trypsin-versene mixture was added for 1 minute. This was poured off and the cells were left for 4 minutes. The trypsin activity was stopped by adding 5 ml fresh medium. The cell suspension was transferred to a universal container, aspirated and counted in a haemocytometer.

2.2 Selection and cloning

TG wild type cells, selected and recloned (Edwards *et al* ., 1988), were used to select variants unresponsive to fibronectin. For selection, cells were used in experiments for 5 weeks then discarded. Cells were grown for 2-3 days in Ham's F10 medium. Then the medium was changed and grown in two 200 cm^2 tissue culture flasks in Ham's F10 medium for a further 2-3 days. About 30-50 million cells from this stage were cultured overnight in each of two 800 cm^2 roller glass bottles. Unattached cells were subcultured by shaking the culture bottle, aspirating the cells to disperse small clumps and replacing directly for the next culture.

On the second day, cells which had not attached during roller culture or which could be released by a gentle rinse with growth medium, were recovered by centrifugation and resuspended in 20 ml growth medium. Cells were distributed between two 75 cm^2 plastic culture flasks, the growth surface of

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which had been previously coated with 25 μg/ml bovine plasma fibronectin. After 5-6 hours the unattached cells were transferred in 30 ml growth medium to another single 75 cm^2 flask with or without fibronectin (in different experiments) and incubated at 37° C overnight.

The same procedure was repeated on day 3 and 4. The cells unattached after 5-6 hours on day 4 were plated on dishes for colony counts and inspection of morphology. At each stage, cells were counted and plated on 90 cm^2 tissue culture plastic dishes. After one week, the unattached cells were recovered and recloned by dilution in soft agar.

An agar underlay was prepared in 90 mm bacterial grade plastic petri dishes with Ham's medium containing 10% foetal calf serum, 10% TPB and 0.5% Noble agar. This was overlaid with 1.5 ml of Ham's containing 0.3% Noble agar and cells in a range of 50-500 per dish were plated. The dishes were incubated at 37° C until colonies were developed.

2.2.1 Selection and recovery of clones

After 10-14 days, healthy clones, well separated from other clones, were selected. The medium was removed from the dish with a pipette and the dishes rinsed twice with about 10 ml HEPES-saline (HS). For each type of clone, a cloning ring was positioned over a marked colony. 5 drops of versene was added to each cloning ring, and incubated at room temperature for about 10 minutes. Detached cells were well aspirated by a Pasteur pipette, and transferred to universals containing 10 mls Ham's F10. Some drops of this medium were added to the cloning rings, to recover remaining detached cells, then the cell suspensions from each type were transferred to 25 cm^2 culture flasks (3 flasks from each).

2.3 Fluorochrome staining for mycoplasma

Contamination by mycoplasma can subtly alter phenotypes of cells in culture, so TG, F1, F2, and F3 cells were grown overnight on 13 mm coverslips in 24-well culture plates containing Ham's medium. Next day, cells were fixed for 2 minutes with (methanol 3: glacial acetic acid 1). The fixative was removed and the cells were incubated for a further 5-10 minutes with more fixative. The coverslips were rinsed twice in distilled water. 2 ml of bis-benzimide fluorochrome "Hoechst 33258" (5×10^{-5} mg/ml) stain was added and incubated at 37° C for 30 minutes. The coverslips were rinsed thoroughly in distilled water, mounted on a glass slide with PBS/glycerol 1:1 and examined using the fluorescence microscope.

2.4 Coupling of Gelatine to Sepharose 4B

5 gms of cyanogen bromide-activated Sepharose 4B (CNBr-activated Sepharose 4B) were rehydrated in 30 ml of 1 mM HCl for 10 minutes. 100 mgs of gelatin were dissolved in 100 ml of coupling buffer by stirring at 100° C. The gel was washed five times with 30 ml aliquots of 1 mM HCl, using a sintered funnel assembly and then with 30 ml coupling buffer. A 1 mg/ml solution of gelatin in coupling buffer (see materials) was used to rinse and scrape the gel slurry into a 100 ml cylinder and more of this solution was used to make the volume to 60 ml. The gel was distributed between six universals and agitated gently using the end-over-end Emscope mixer for 1.5 hours. The gel was then washed with 30 mls coupling buffer on a sintered funnel, resuspended in 30 mls Tris buffer and agitated for a further 1.5 hours. The gel was washed with 30 mls coupling buffer, followed by acetate buffer, followed by coupling buffer.

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2.5 Affinity chromatography of calf serum on Gelatin-Sepharose 4B

2.5.1 Affinity purified Fibronectin

Fibronectin was isolated from bovine serum on gelatin-Sepharose columns using the method of Engvall and Ruoslahti (1977).

Using a peristaltic pump, a column containing gelatin-Sepharose (Sepharose to which gelatine has been covalently coupled), was washed with 20 mls of PBS/citrate buffer (0.9% NaCl, 0.01 M Sodium citrate). 40 mls of new born calf serum was run on via the pump.

The column was washed with PBS/citrate until the UV absorbance trace on the chart recorder returned close to that of the PBS baseline. Fibronectin was then eluted from the column with 8 M Urea (8 M urea in 0.05 M Tris, pH 7.5). The optical density of the eluted protein (fibronectin) was read at 280 nm, using a spectrophotometer (Shimadzu, UV-160), and made up to a concentration of 1 mg/ml in 8 M Urea.

The gel was washed with approximately 20 mls of isotonic saline containing sodium azide as preservative and stored at 4° C for future use.

2.6 Spreading assay

2.6.1 Preparation of coverslips

22 mm glass coverslips were cleaned in 1:20 diluted Decon by boiling for 5 minutes, rinsed thoroughly with tap water for 15 minutes then rinsed 3 times in distilled water for 15 minutes. Glass coverslips to be coated from solutions of fibronectin or other proteins were placed in 35 mm diameter culture dishes and 2 ml of the protein solution at the desired concentrations added (for fibronectin, 25

μg/ml in HH). After a standard time at room temperature (about 30-60 minutes) the coating solution was removed and the dishes were rinsed twice with HH. 2 ml HH, containing 0.5 mg/ml haemoglobin (Hb) was added to the coverslips and incubated at room temperature for 15 minutes. The function of Hb is to block with an inactive protein any free adsorption sites not occupied by fibronectin. Hb was chosen for its homogeneity and inactivity in spreading (Edwards *et al.*, 1987). After 15 minutes Hb was replaced with HH and coverslips were then rinsed twice in HH.

Some spreading assays were carried out in 35 mm diameter plastic tissue culture dishes without coverslips.

2.6.2 Cell suspension

After trypsin-versene, the cells were pipetted in 5 ml of HH containing 10% calf serum. The cell suspension was pelleted twice in a bench centrifuge at 1000 rpm for about 5 minutes each. The cells were resuspended in 5 ml appropriate buffer (HH).

2.6.3 Incubation for spreading

Immediately before use, the HH was removed from the coverslips, the dishes were taken to the 37° C room and 2 ml of cell suspension was added to each dish. (The dishes were always in duplicate). After 45-60 minutes (in some experiments, 60 or 180 minutes), the cells were fixed in formol-saline (1 ml each) for 15 minutes.

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2.6.4 Staining and mounting

The fixative and medium were removed and cells stained for 15 minutes with 0.1% Kenacid (= Coomassie) Blue. The stain was removed and the coverslips were washed twice with 2 ml distilled water. Then the coverslips were removed from the second 2 ml water and placed on a sheet of tissue, cells uppermost, in labelled positions, blotted off dry and carried to the hot room to dry. The coverslips were mounted in pairs on slides, using clearmount or Depex, cells facing downwards in the mountant.

2.6.5 Measurement of area and shapes

Mean spread area (MSA) was measured as described by (Edwards *et al.*, 1993). To determine the extent of cell spreading, I measured the projected area of digitised images of fixed and evenly distributed cells. Images were obtained with a x50 objective on a Leitz Ortholux microscope equipped with a Hamamatsu Vidicon C1000 camera and Archimedes digitiser. A routine specially written in Acorn Risc Machine assembler for an Acorn Archimedes 310 microcomputer, was used for analysis. This substracts background shading, identifies cell outlines and rejects objects smaller than unspread cells. It then calculates area (1 mm2= 6.4 pixels), dispersion and elongation, as described by Dunn and Brown (1986).

2.7 Measurement of mitochondrial dehydrogenases

MTT (3-[4,5-Dimethyl-thiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; thiazoyl blue) was used for measuring the mitochondrial dehydrogenases of TG and F3 cell lines. The procedure was adopted from the supplier's description (Sigma). (Carmichael *et al.*, 1987). Cells were grown up in Ham's F10 in 35 mm

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in diameter tissue culture dishes at 37º C until confluent. Cells were washed twice in GFHH, then incubated for 30 minutes with 2 ml of GFHH + L-Glutamine, HH, GFHH + sodium pyruvate, or GFHH. After 30 minutes MTT was added from a 5 mg/ml stock solution, 0.2 mls per 2 mls culture volume and incubated for 2-3 hours at 37º C. The culture solutions were discarded and 2 ml of 0.04-0.1 N HCl in isopropanol was added. (All the steps of this experiment were carried out in the hot room, at 37º C). The absorbance of the extracted dye was measured at a wavelength of 570 nm with background subtraction at 630-690 nm.

2.8 Separation and identification of ^{14}C-labelled metabolites of glucose in TG and F3 by paper chromatography

Cells were grown in 35 mm diameter tissue culture dishes. The number of cells used for labelling was approximately 300,000. Cells were washed twice in GFHH. 5 μCi ^{14}C-labelled glucose (20 μCi in some experiments) in 4 ml GFHH was added to the dishes of TG and F3 cells (2 mls each). The cells were incubated for 45 minutes, then washed twice (thoroughly) in prewarmed HH. 0.4 ml 10% TCA was added, incubated for two minutes and 0.1 ml of the solution transferred to a vial for scintillation counting.

TCA samples, and 5 μl glucose labelled with ^{14}C-labelled glucose as a standard, were applied to Whatman SG 81 paper chromatography 46x57 cm, using different volumes from each sample (10, 20, 30, and 40 μl). Chromatograms were developed with solvent A in a chromatography tank. The tank was first saturated with solvent A then the spots were dried and then the separation took place in a chromatography tank, by using solvent A [Veneziale and Gabrielli (1969)], see materials. After approximately 30 hours, the paper was dried, placed against X-OMAT film and incubated at -70o C for 5-10 weeks, before developing the autoradiograph.

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For two dimensional paper chromatography, 40 μls of the TCA extracts were applied to the chromatography paper, 46x57 cm. 5 μl labelled glucose with ^{14}C-labelled glucose was applied to another paper of the same size as a standard. Separation took place using solvent A, for about 30 hours, followed by solvent B for about 16 hours.

The tank was saturated always for at least 30 minutes with the appropriate solvent. The autoradiographs were exposed for about 10 weeks at - 70° C. The sensitivity of the above method was studied by drying down on paper known amounts (cpm) of labelled ^{14}C-glucose. Serial dilutions of ^{14}C-labelled glucose in GFHH were made, and were run on paper (10 μl each) in an order of 1/1, 1/100,..., up to $1/1x10^7$. 100 μl from each was transferred to a vial for scintillation counting, autoradiographed for two weeks at -70° C.

2.8.1 Silver staining for paper chromatograms

The chromatogram was placed in a tray with silver stain for 5-10 minutes under constant agitation. A brown precipitate was formed but disappeared on shaking. The paper was dried, and was developed in 0.5% sodium hydroxide in ethanol. The spots of sugars became visible 30-90 seconds after the developer was added.

Aims of my research

The original aim of my project was to investigate the hypothesis that post-translational modification of integrins, such as phosphorylation, might underlie the low adherent phenotypes of certain mutant mammalian cells previously selected for lack of response to fibronectin. However, repeating the selection procedure led to isolation of a mutant with a novel phenotype, and a different aim emerged: to characterise this mutant and identify its abnormality.

RESULTS
&
# DISCUSSION

Results and discussion

1 Selection of non-adherent mutant cells

1.2 Isolation of F3, a new phenotype

Edwards *et al.* (1985) reported the isolation of variants unresponsive to fibronectin from polyoma transformed cells. To see if further mutants with similar properties could be selected, the original selection procedure was repeated using TG cells, the parental wild type, selected by Hameed (1988). Three different variants were isolated successfully. Two of the clones had morphologies (F1, F2) similar to the clones previously isolated from Py3 and TG cells. F1 colonies contained exclusively rounded cells and F2 colonies had a few partially spread cells and were more scattered than F1 (Fig. 1 a, b, c, d). A new variant F3, had a morphology not previously found. F3 cells had a spread epithelial-like morphology (Fig. 1d) which was clearly recognisable, because they lacked the elongated processes associated with most TG cells. The cells were mycoplasma-negative as shown by staining with Hoechst 33258. A line of similar phenotype was isolated several months later in repeating the selection.
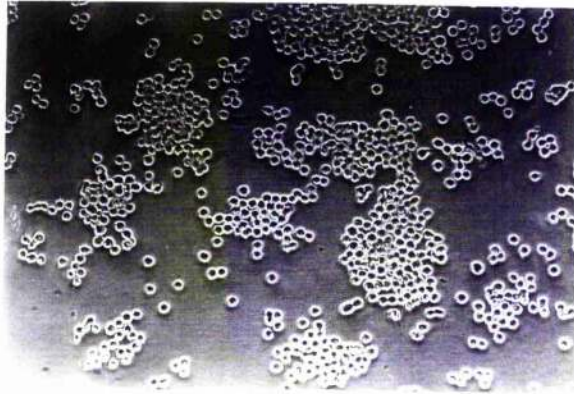
a) F1
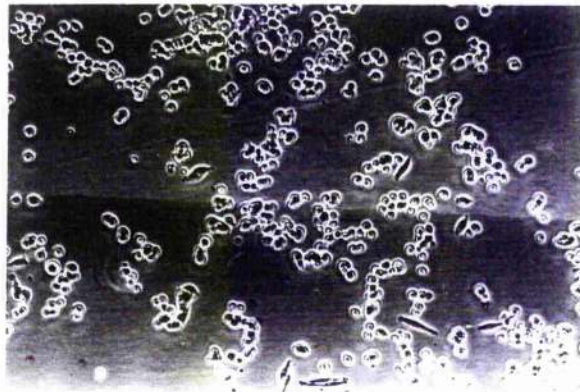
b) F2

Fig. 1 (1a, 1b)F1 colonies contained exlusively rounded cells and F2 colonies had a few partially spread cells and were more scattered than F1 (Objective x-10)
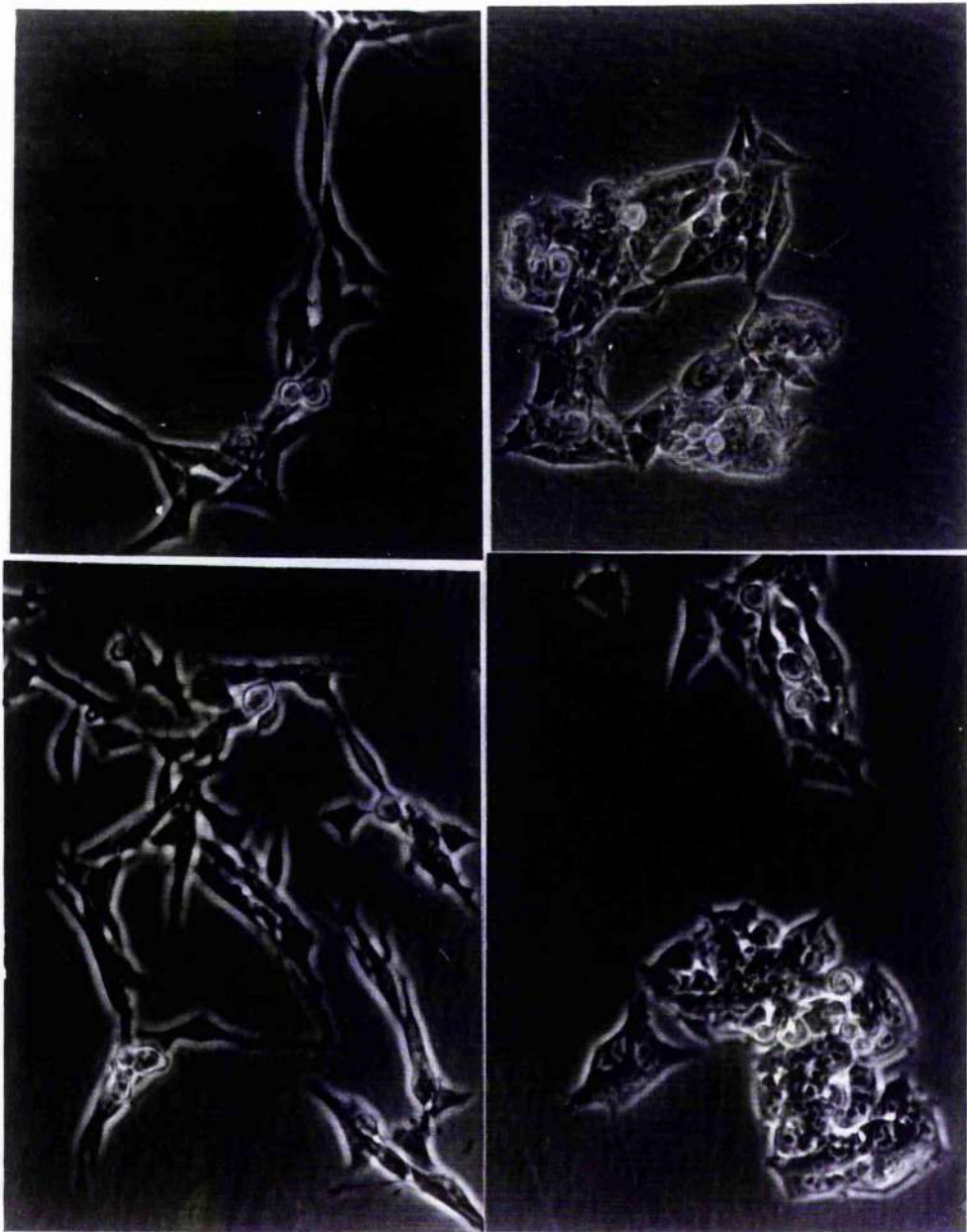
c) TG   d) F3

Fig. 1c, 1d TG, the parental wild type and F3 cells, isolated from TG which had different morphology from the isolated mutants F1 and F2 (Objective x-32)

1.1. 2 Phenotype of F3

1.1.2.1 Time-lapse video-tape recording of TG and F3

Superficially the phenotype of F3 appeared similar to that of TG when they were in confluent culture, so I studied the behaviour of the two lines, using time-lapse video-tape and a x 32 phase contrast objective. Low number of cells (0.1 x 10^5/ml) were cultured in 25 cm^2 tissue culture flasks. The recording suggested that there were differences between the motility of TG and F3. TG cells more often showed elongated processes. These seemed to be "tails" formed as cells move (Fig. 2a, a, b, c, and d, sequence d, 1, 2, 3, 4, 5, p. 73) which shows this most clearly. These processes were not shown in F3, which appeared to be less motile (Fig. 2b, e, f. g, and h).
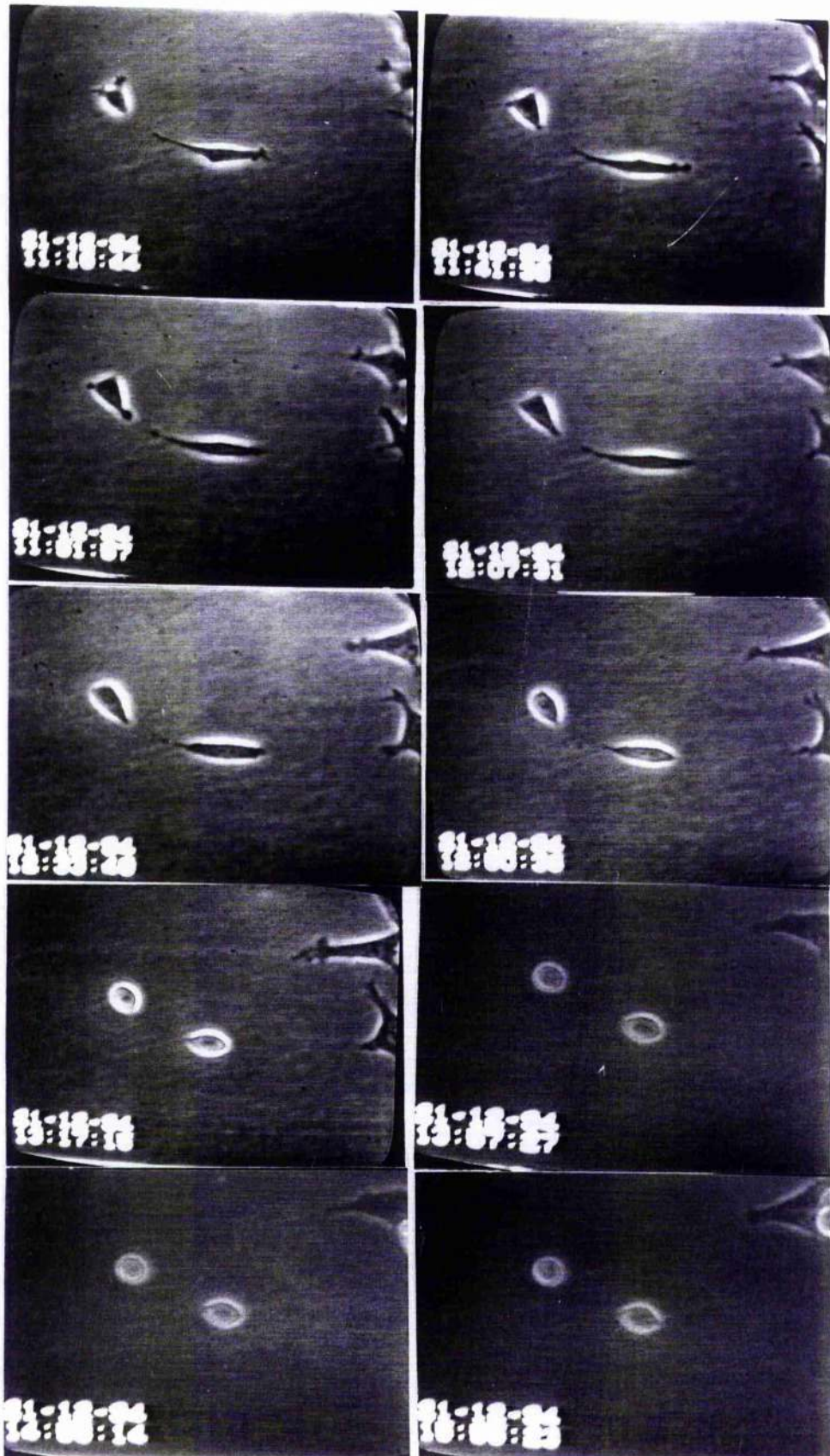
**Fig. 2a** Time-lapse video recording of TG cells in culture

(1)

(2)

(3)

(4)

(5)
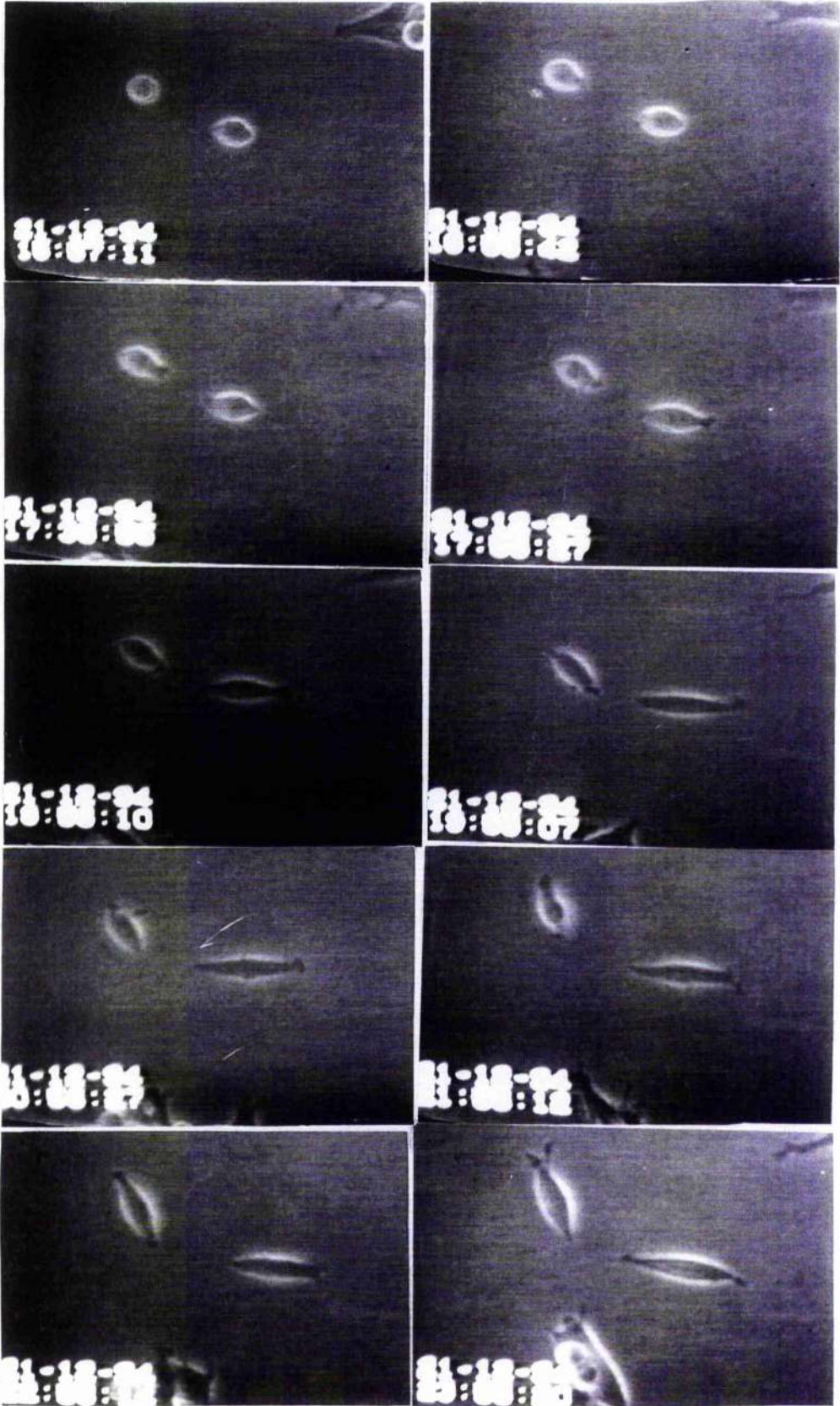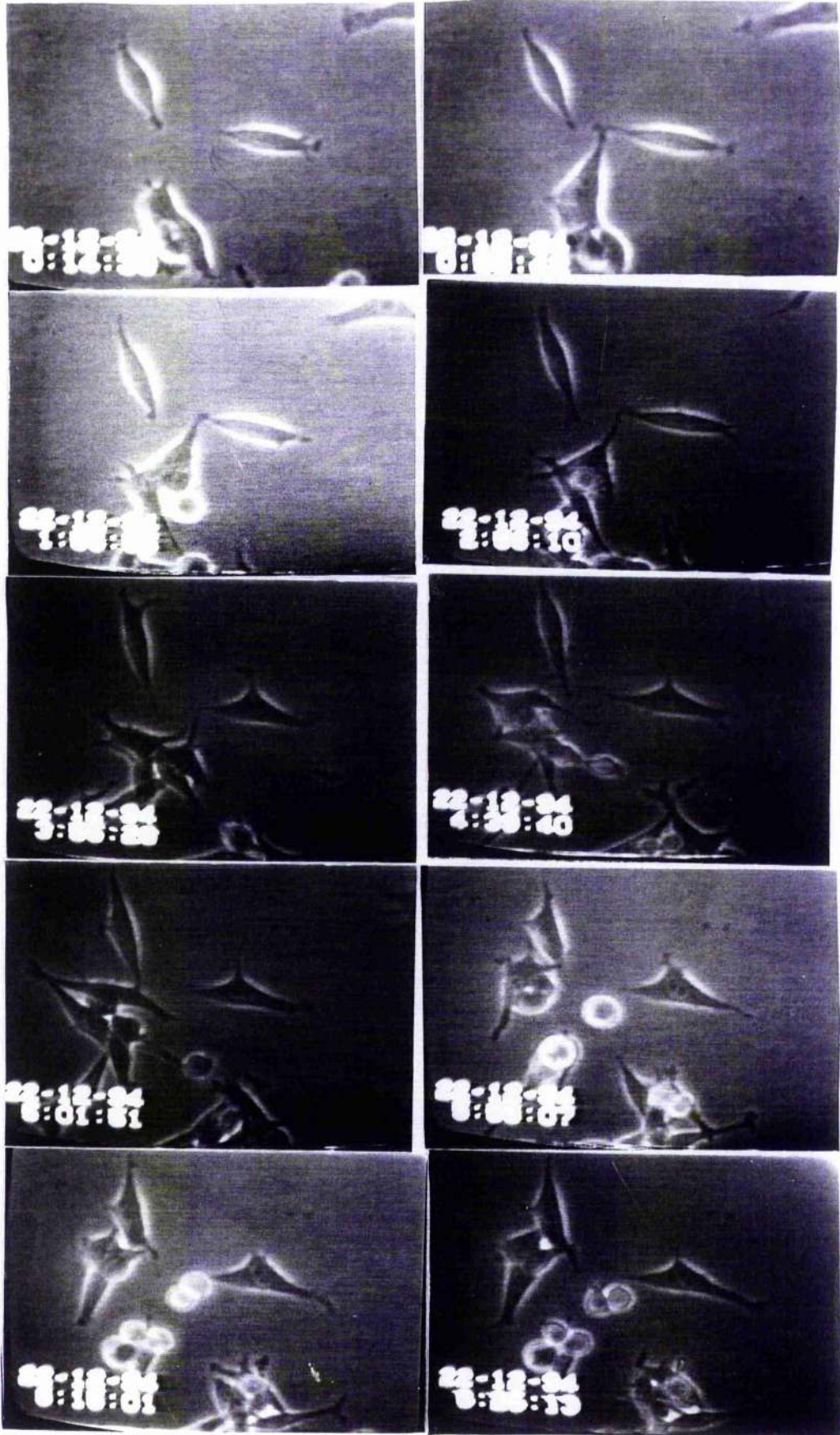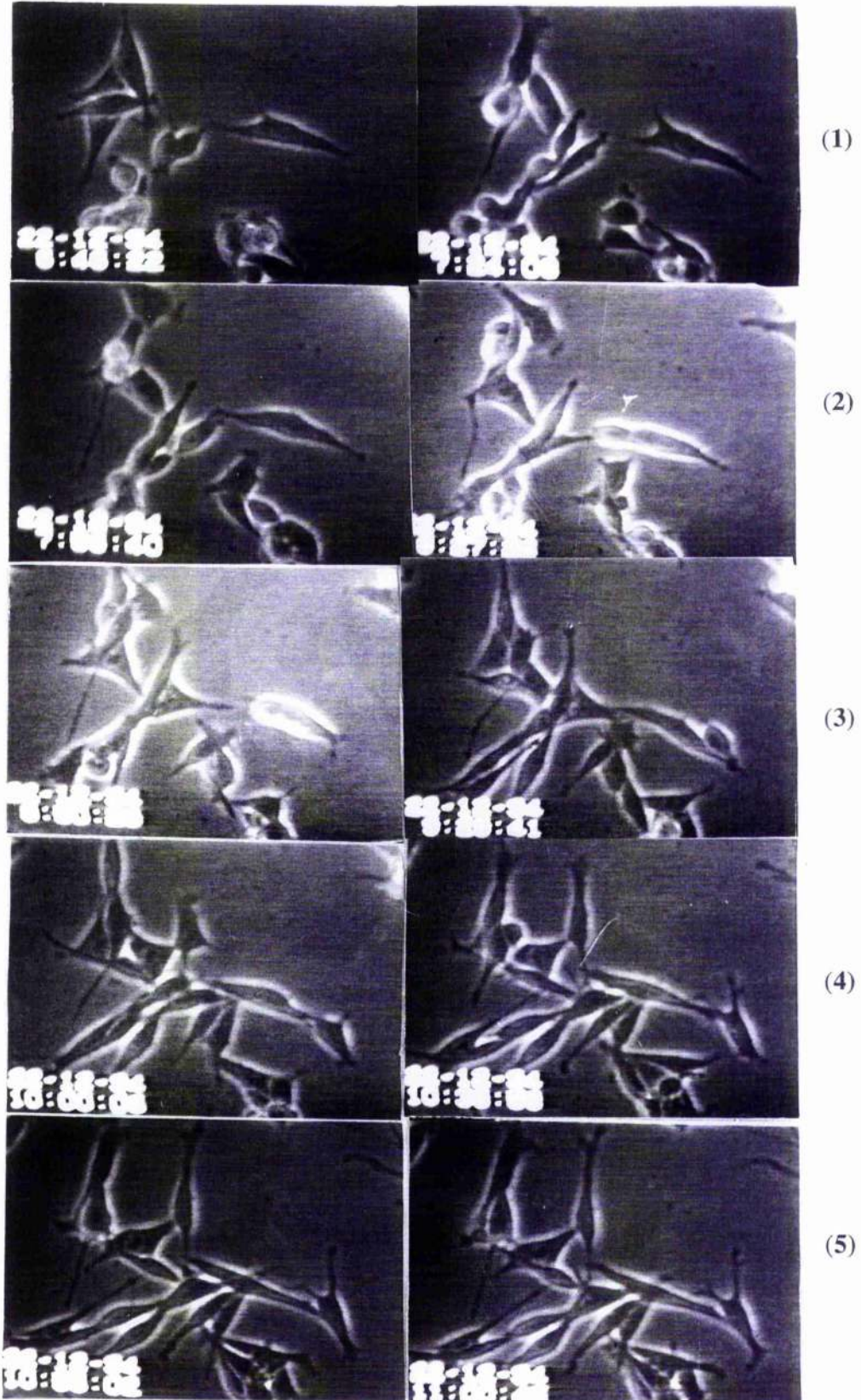
e)
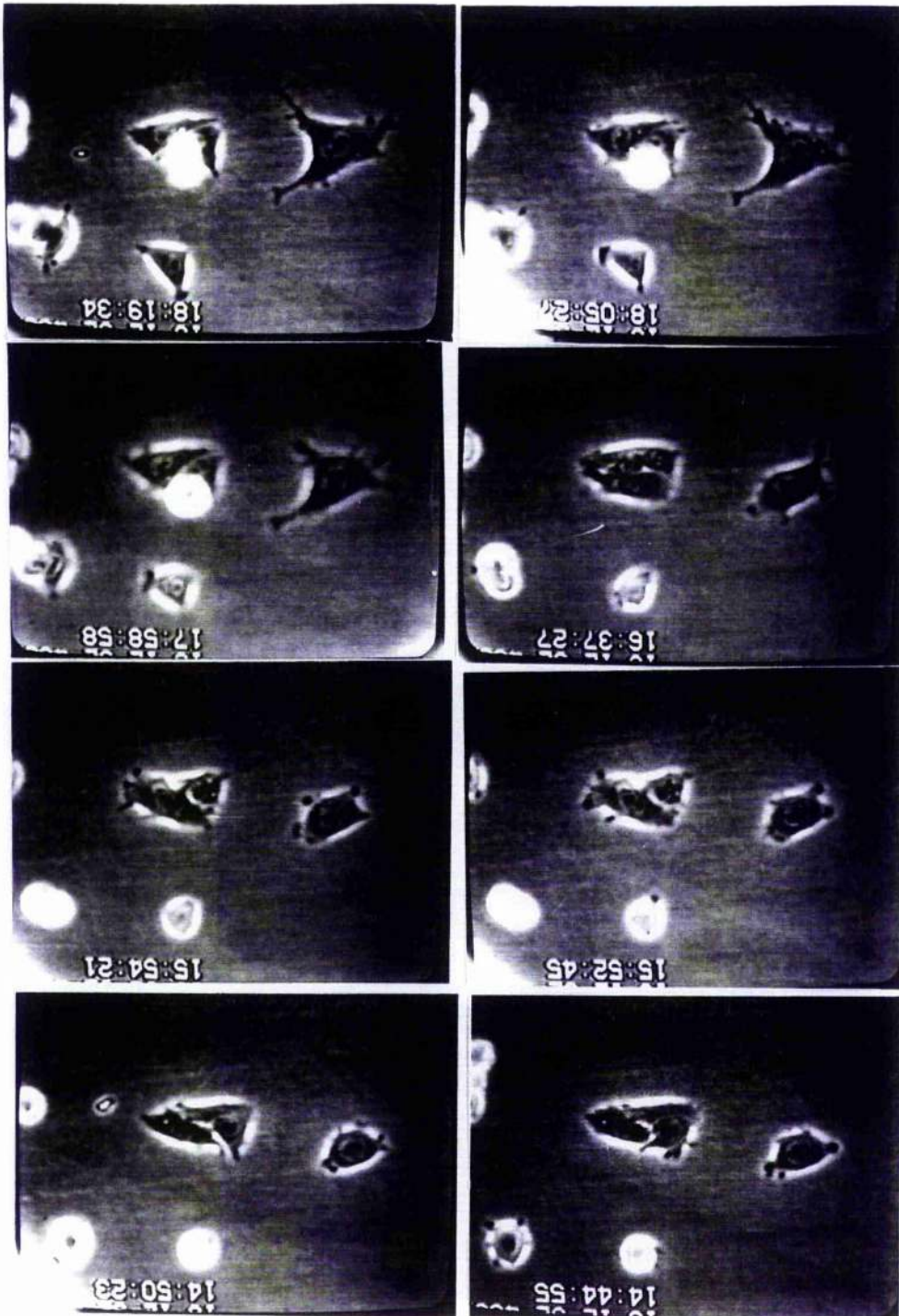
**Fig. 2b** Time-lapse video recording of F3 cells in culture

75

78
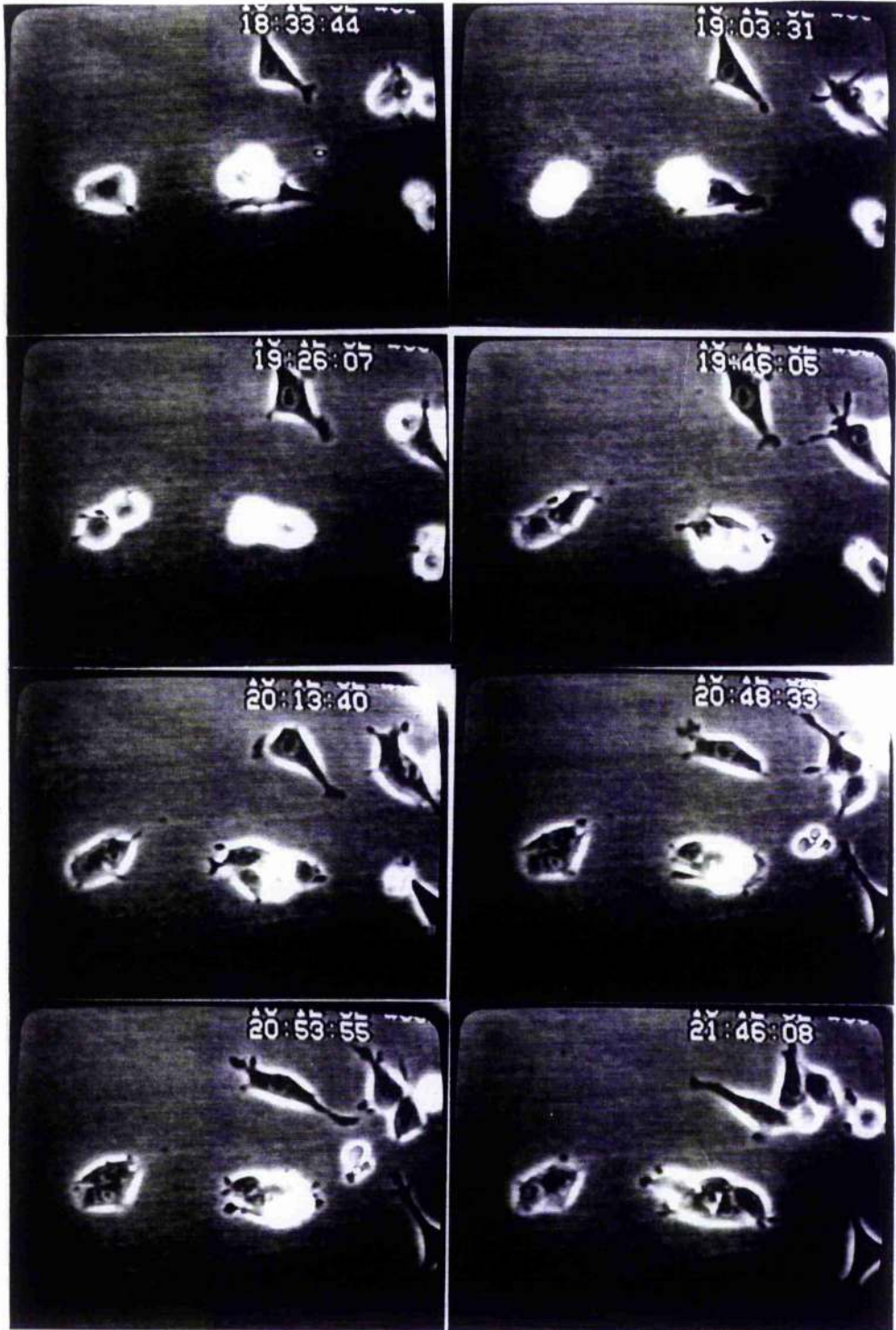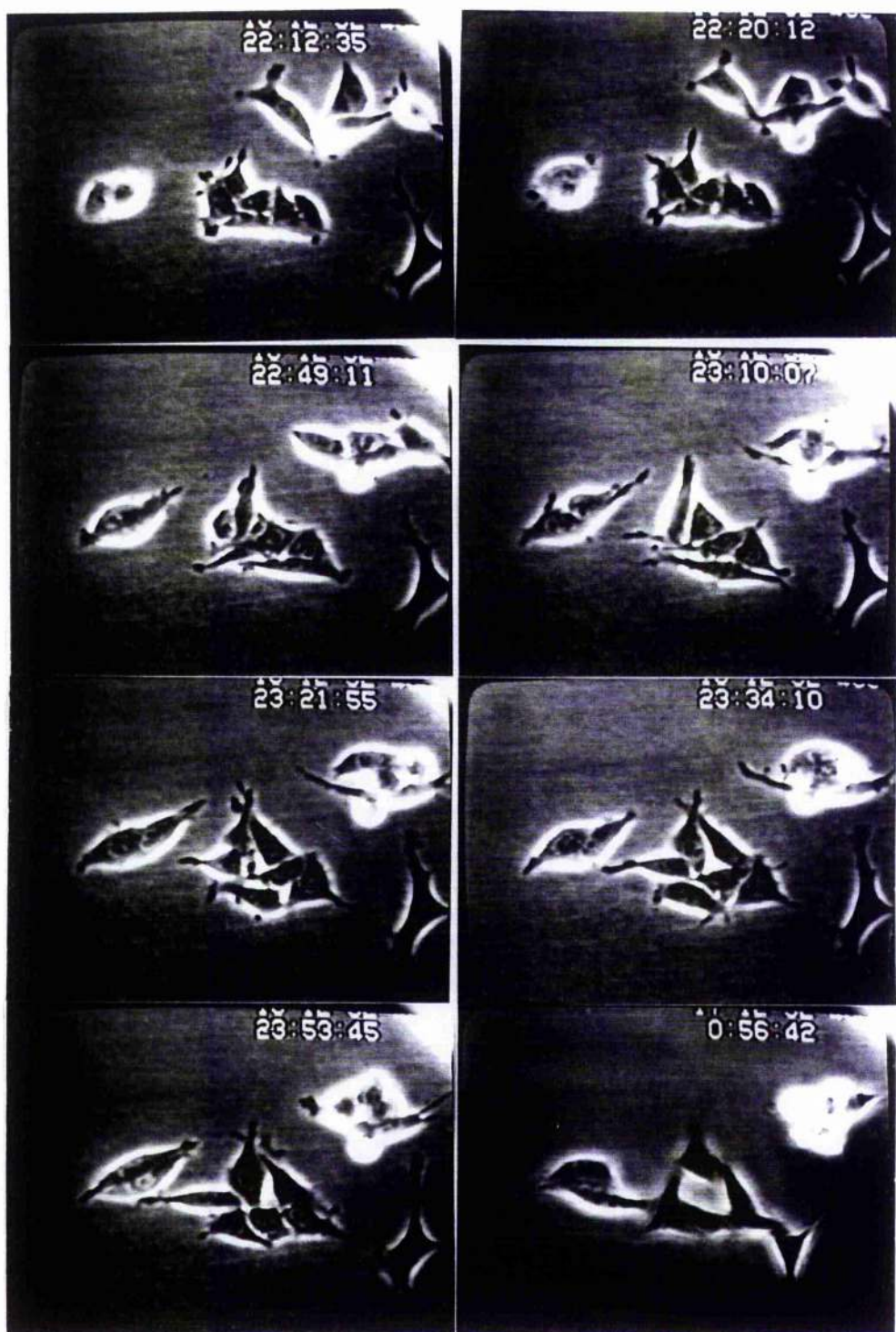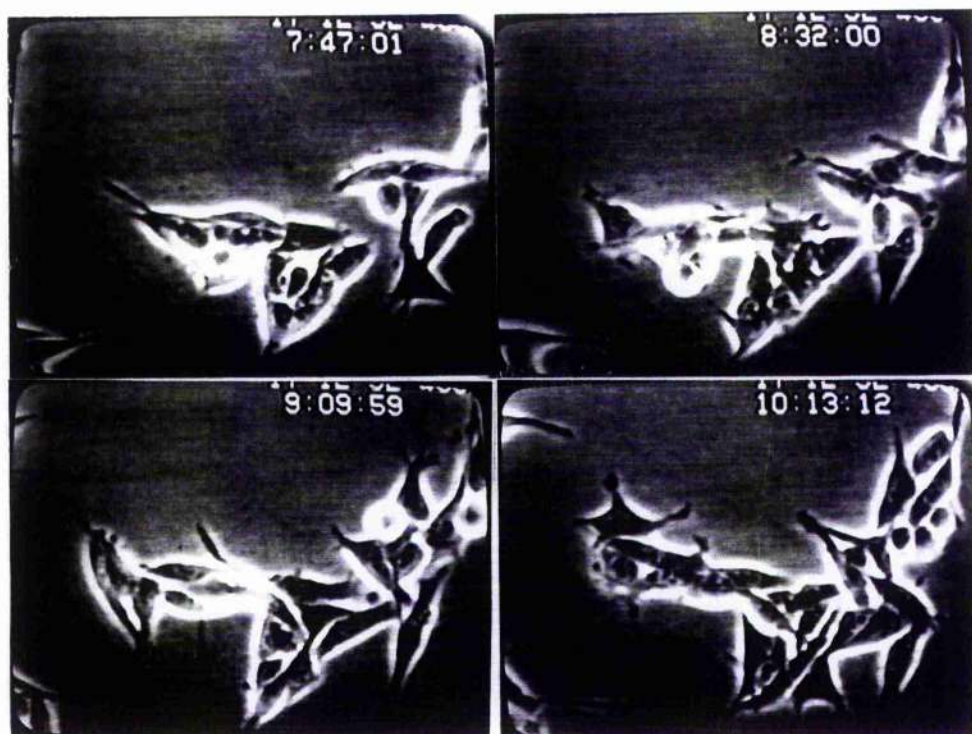
1.1.2.2 Spreading of F3 on Fn-coated glass coverslips

The spreading of F1, F2, F3 and TG cells was measured on Fn using the standard spreading assay and the mean spread area (MSA) was measured as described by Edwards (1987). The results showed that F1, F2, and F3 did not spread on fibronectin under these conditions (Fig 3).
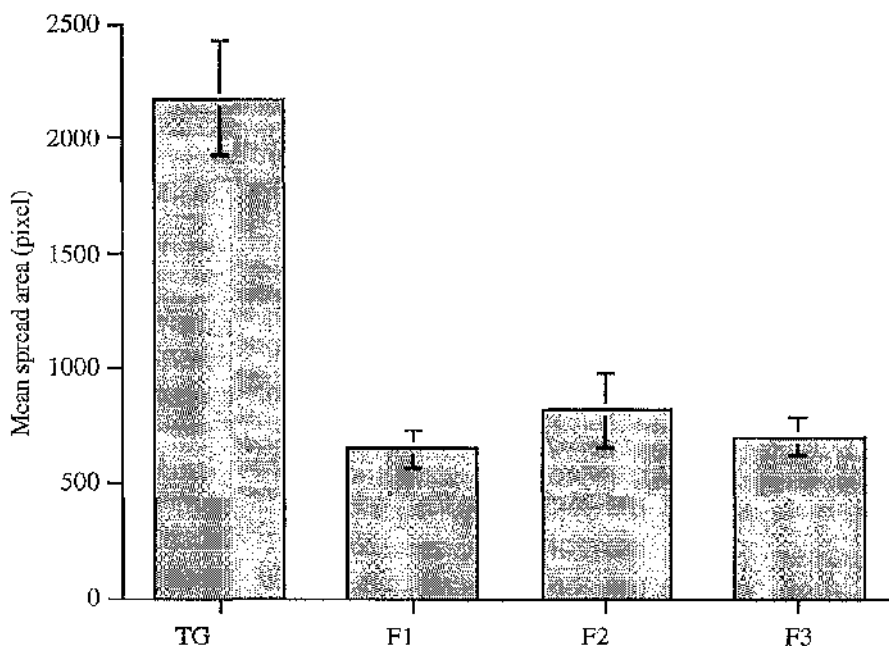
Fig.3 Spreading of TG, F1, F2 and F3 on Fn-coated coverslips.

Glass coverslips with 25 µg/ml fibronectin for 30 minutes, then were blocked with 0.5 mg/ml haemoglobin for 15 minutes. Each cell suspension was prepared in 5 ml Hanks' HEPES, with 1 x 10^5/ml cells of each type. 2 mls of cell suspensions were added to each coverslip, and cells were incubated for 60 minutes in the hot room, and processed as described in methods. The results are means of 8 experiments (16 samples), error bars = standard deviations.

The mean spread area for F3 was not significantly greater than the completely unspread F1 cells that had a value of about 650 pixels. In the histograms of spreading assays which follow, the value 650 pixels is substracted from the ordinate, which therefore shows unspread cells at zero increase in pixels.

F3 was consistently found not to spread on fibronectin in the standard spreading assay. This left a puzzle: why does F3 not spread in these assays while it does spread in culture? One possibility was that F3 responds to other components of the culture medium such as serum. Alternatively the difference might reflect the changed substrate.

2 Effect of medium components on the spreading of F3

2.1 Growth medium

Cell suspensions in growth medium (Ham's F10) with or without 10% foetal serum and cell suspensions in Hanks HEPES with or without serum for comparison, were tested for spreading, both on Fn-coated glass coverslips and plastic dishes. Mean spread area was measured. The results showed that on glass F3 spreads a little in the presence of Ham's with or without serum. The addition of serum in Hanks HEPES had little or no effect . On plastic F3 spreads much more in the presence of Ham's or with addition of serum to Hanks HEPES, than in Hanks HEPES alone (Fig 4).
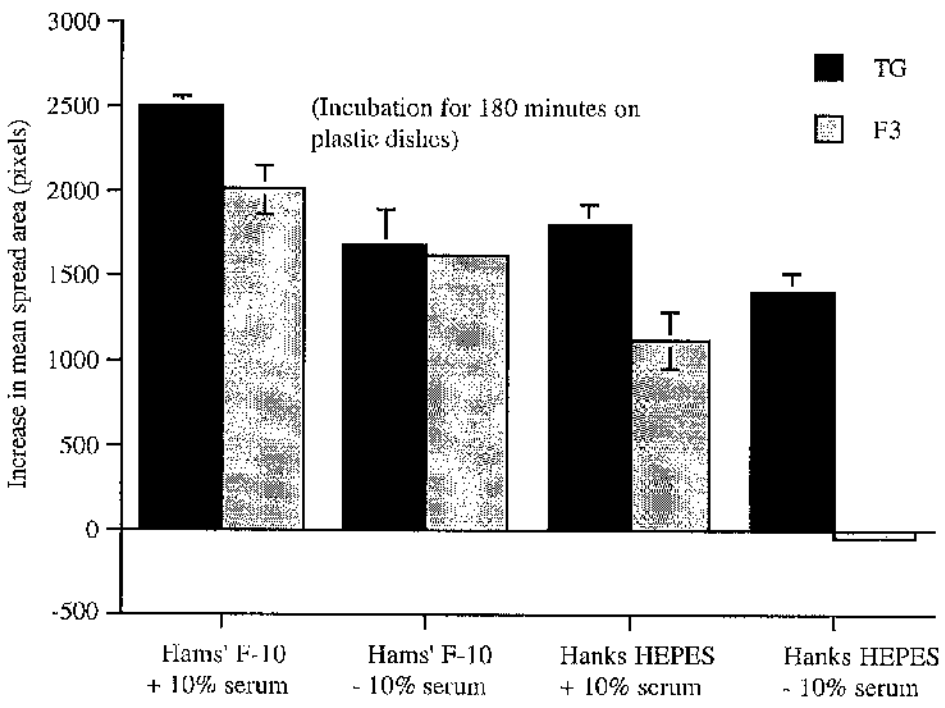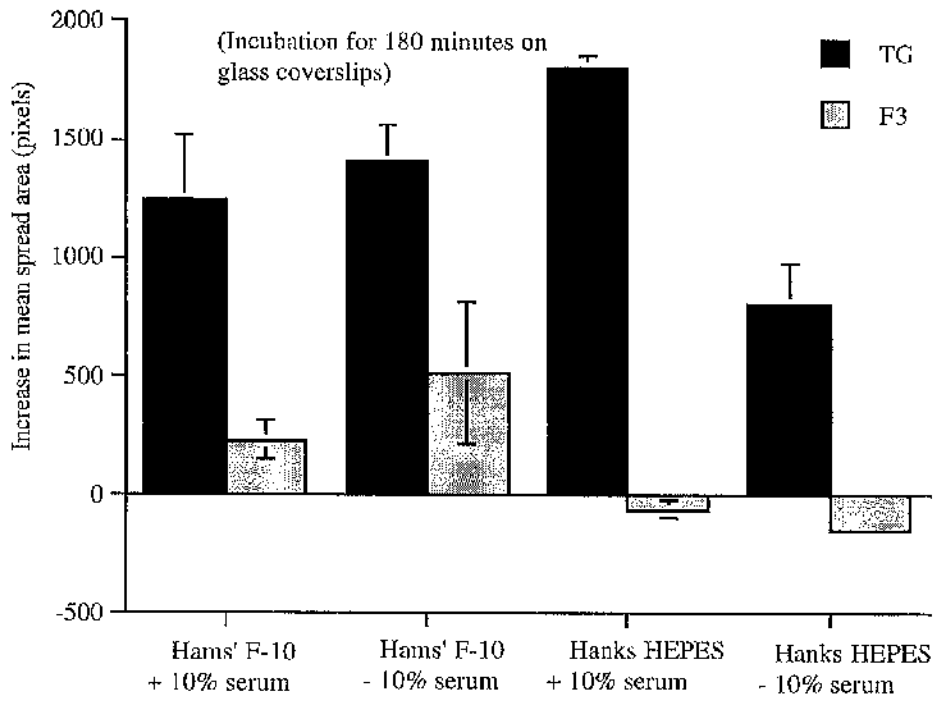
Fig. 4 Effect of different medium with or without serum on the spreading of TG and F3 cells, on plastic and glass coated with fibronectin.

The results are means of 8 experiments (16 samples), error bars = standard deviation. Error bars not shown where too small.

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2.2 Dialysed serum

Serum could contain macromolecules or low molecular substances that cause the spreading of F3 cells in culture.

To investigate this, 10% foetal calf serum was dialysed against Hanks HEPES. Cell suspensions with or without dialysed serum, were tested for spreading, both on Fn-coated glass coverslips and Fn-coated plastic dishes. The results showed there was untypical partial spreading of F3 in this group of experiments, however addition of dialysed serum inhibited rather than stimulated on both glass and plastic. Leaving out the haemoglobin block had no effect (Fig. 5).

Dialysed serum was ineffective but Hams' F-10 stimulated spreading. I therefore investigated the effect of various low molecular components of Hams' which are missing from Hanks HEPES.
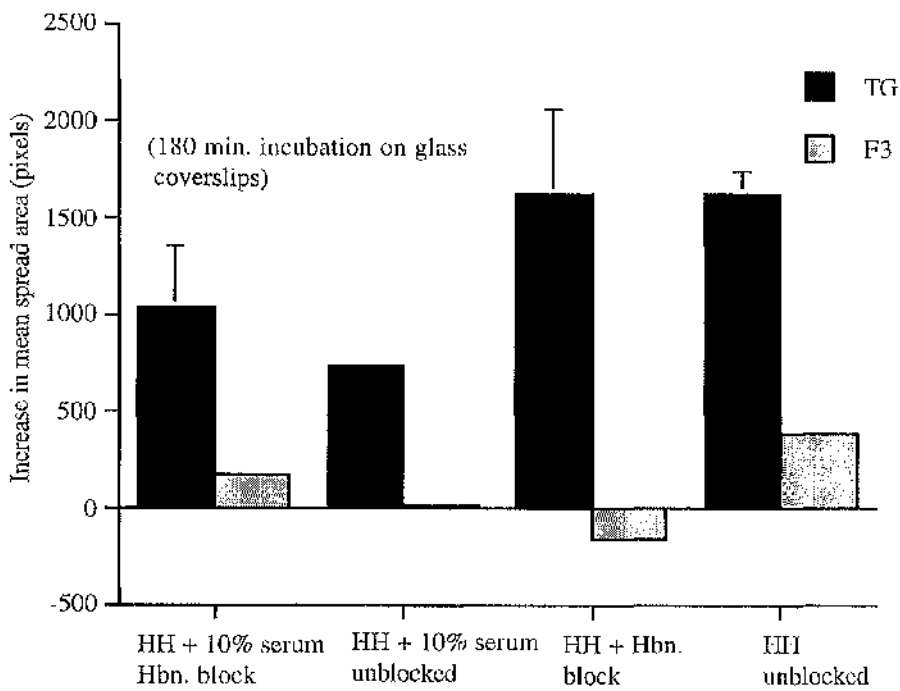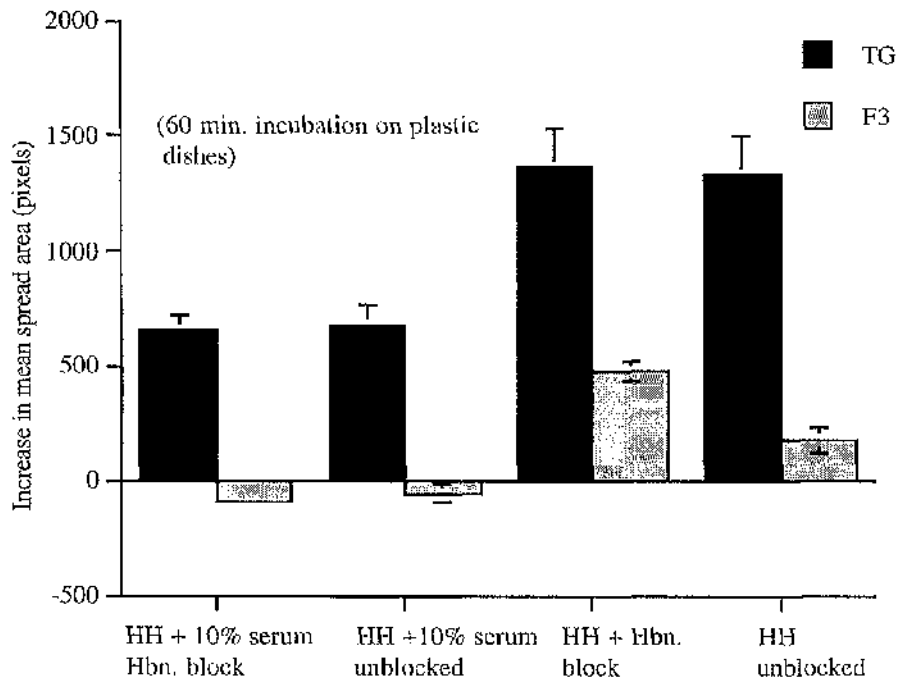
Fig. 5 Effect of adding dialysed serum to HH in the assay on spreading of TG and F3 cells on Fn-coated surfaces.

(3 experiments, error bars = standard deviation)
Error bars not shown where too small

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2.3 Pyruvate, L-glutamine, and succinate

I found that F3 spreads very well in HANKS HEPES provided that 1 mM pyruvate is added (Fig. 6a, 6b and 6c), and a little in 1 mM L-glutamine, but 1 mM succinate had no effect, particularly on plastic.

Omission of glucose had no effect on the wild type (TG) spreading (Fig. 6b, Fig. 6c).

<div align="center">

TG (HH)   F3 (HH)

TG (HH + Pyruvate)   F3 (HH + pyruvate)

</div>
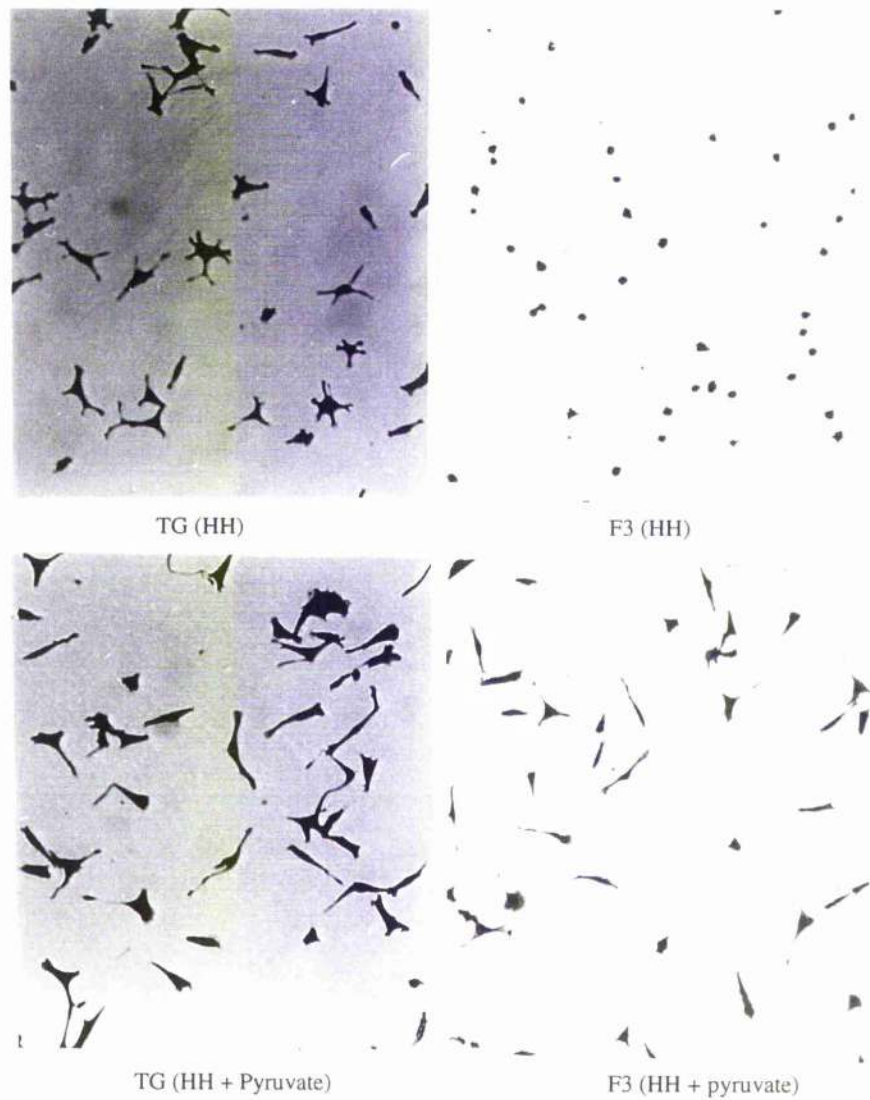
Fig. 6a. Effect of pyruvate on the spreading of TG and F3 on Fn-coated coverslips.
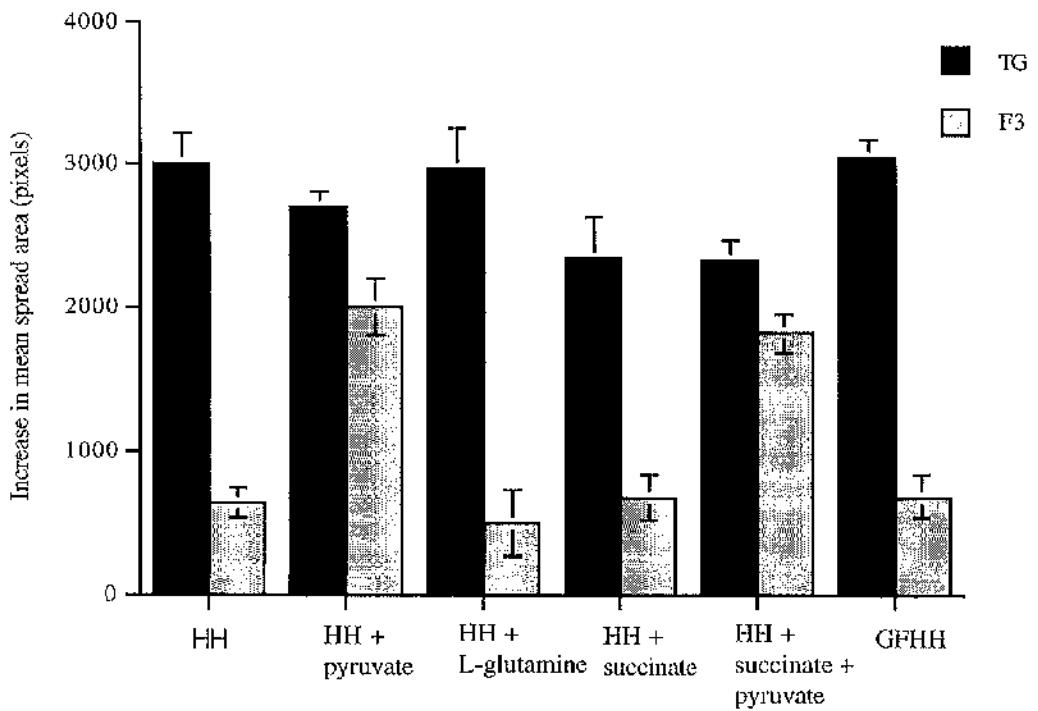
Fig. 6b Effeect of 1 mM pyruvate, L-glutamine, succinate, and 5 mM glucose on the spreading of TG, and F3 cells on Fn-coated plastic petri dishes.

(8 experiments, error bars= standard deviation)
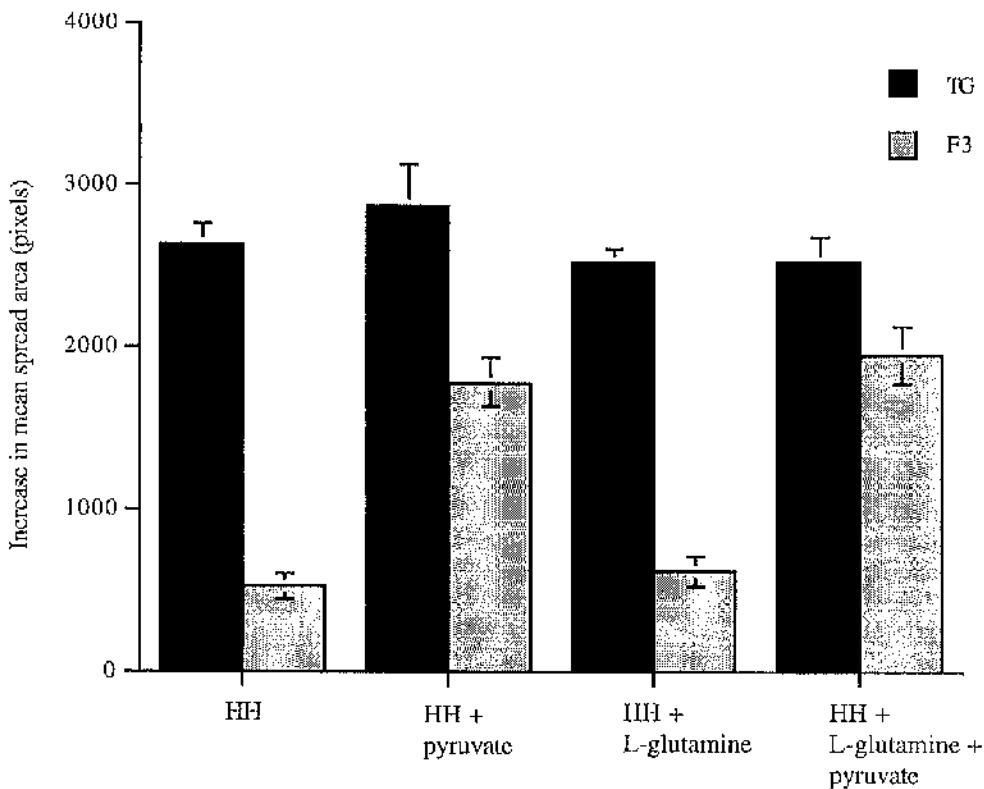
Fig. 6c Effeect of 1 mM pyruvate, L-glutamine, and 5 mM glucose on the spreading of TG, and F3 cells on Fn-coated glass coverslips.

(3 experiments, error bars= standard errors)

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2.4 Effect of pyruvate concentration on the spreading of F3 on fibronectin

The effect of sodium pyruvate concentration was studied. Different concentrations were added and spreading was compared with the cell suspension in HH alone.

Spreading increased with concentrations to about 0.5 mM, but increased little at higher concentration (Fig. 7).
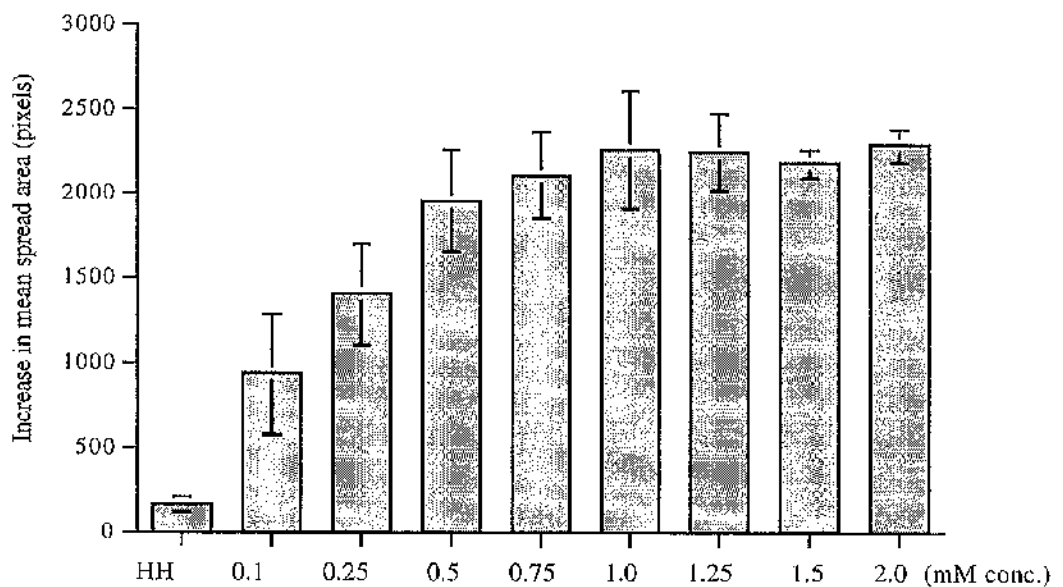
Fig. 7 Effect of increased pyruvate concentration on the spreading of F3 on glass coverslips.

Cell suspensions were prepared in HH and different concentrations of pyruvate were added.
The results are means of 3 experiments, and the error bars = standard errors.

2.5 Time course of spreading of TG and F3, in the presence of pyruvate

Cell suspensions were prepared in 5 ml HH, with 1 mM sodium pyruvate. The effect of different incubation times on the spreading of TG and F3 in pyruvate was investigated.

Data showed the mean spread area (MSA) of F3 in HH was hardly increased with the increase of the incubation time. In pyruvate, MSA was increased through the 90 minutes incubation (Fig. 8). In the experiments it is noteworthy that pyruvate also increased the MSA of TG. This was not noticed in the photographs (Fig. 6a).
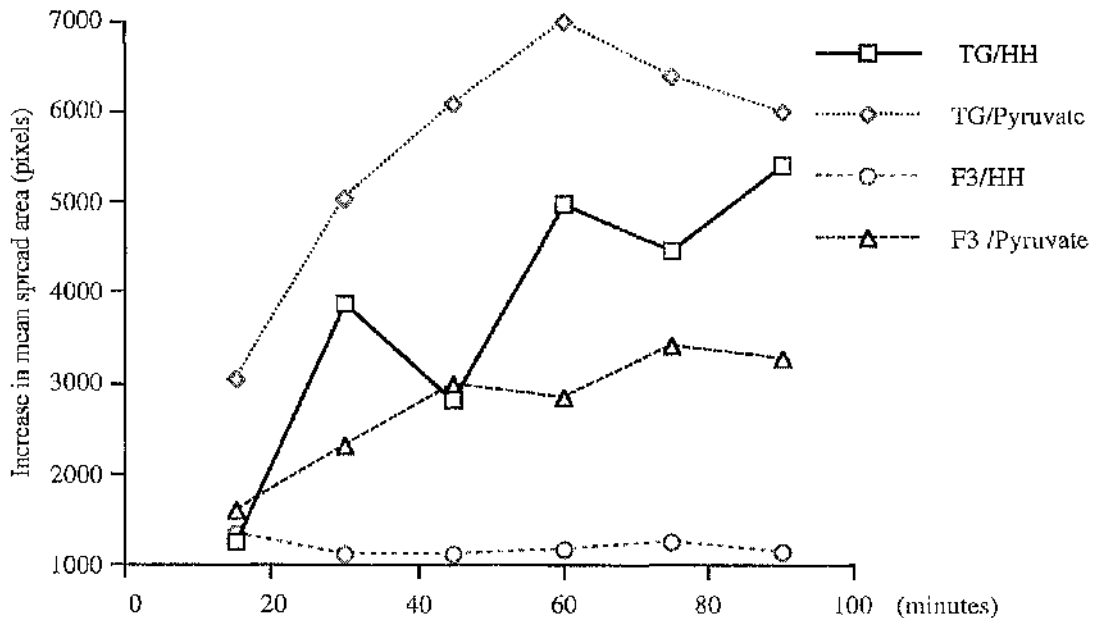
Fig. 8 Spreading of TG and F3 on Fn-coated coverslips at different incubation times.

Cell suspensions of TG and F3 were prepared in HH, and 1 mM pyruvate in HH. Following the standard spreading assay, cells were allowed to spread on 25 µg/ml fibronectin, and cells were fixed with formol saline at different times. The results are means of 3 experiments (Objective x-50).

2.6 Effect of EDTA on spreading

A simple interpretation of the result of Fig. 6 could be that pyruvate acts as an energy source. One unlikely possibility was that F3 cells are more sensitive than TG cells to inhibitory heavy metal ions which might contaminate the glucose in HH. However pyruvate could then act by chelating these inhibitory ions. To eliminate this possibility I tested the effect of both adding 10^{-5} M EDTA in the spreading assay and omitting the glucose source. This should work because Ca^{2+} and Mg^{2+} in Hanks HEPES are millimolar and although they bind to EDTA, ions such as Cu^{2+} and Fe^{3+} bind much more strongly.
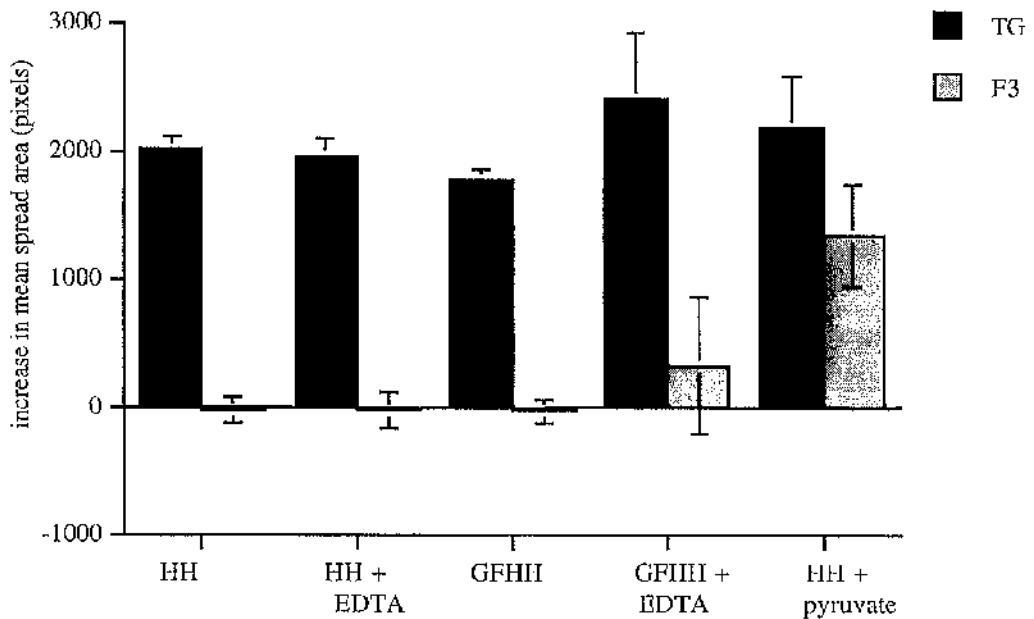
Fig. 9 Effect of EDTA on the spreading of TG and F3 cells

Plastic tissue culture dishes (35 mm diameter) were coated with Fn for 30 minutes. Cell suspensions were applied to unblocked dishes and were incubated for 180 minutes in the hot room. The results are means of 5 experiments, the error bars = standard deviations.

The results showed there was no significant effect on the mean spread area for TG or F3, so this possibility was discounted (Fig. 9).

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2.7. The effect of removing pyruvate on spread F3 cells

Previous results showed that F3 require pyruvate to spread. The question arose if pyruvate is required to maintain the spread state. Cell suspensions were prepared in HH with or without 1.0 mM pyruvate and allowed to spread on Fn-coated glass coverslips for 90 minutes. Half the samples were fixed and processed. For the remainder, the medium was removed using a filter pump and washed thoroughly with prewarmed HH to avoid losing cells, and 2 mls of prewarmed HH with or without pyruvate was added. The samples were incubated for a further 90 minutes.

Addition of pyruvate after 90 minutes to cells preincubated in HH without pyruvate, showed that the cells could still respond to pyruvate although not as well as when pyruvate was added initially (Fig. 10). Removal of pyruvate after 90 minutes resulted in decrease of the mean spread area. This result shows that cells do indeed require pyruvate to remain spread in the period 90-180 minutes.
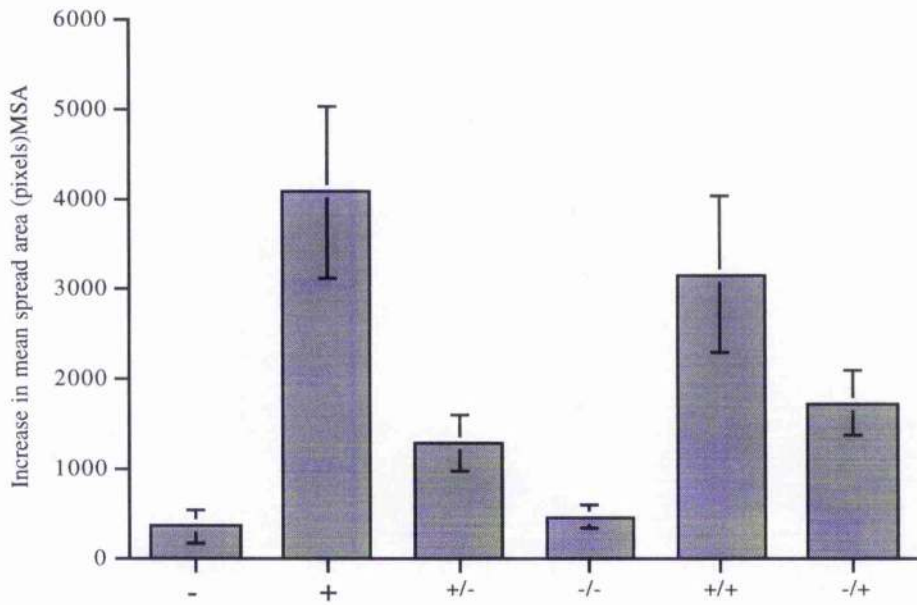
Fig. 10 The effect of removing pyruvate on the spreading of F3

- HH
+ HH + pyruvate/ incubation for 90 minutes
+/- pyruvate removed at 90 minutes of incubation
-/+ pyruvate added to HH after 90 minutes of incubation
+/+ incubation with pyruvate for 180 minutes
-/- incubation with HH for 180 minutes

The results are means of 5 experiments (10 samples each), error bars = standard deviations

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3. Effect of an inhibitor of the glycolytic pathway (2-deoxyglucose) on the spreading of TG

The previous results showed that F3 requires pyruvate (perhaps), both for spreading and to maintain the spread state (Fig. 10).

One possible explanation is that pyruvate acts as an energy source. However TG does not require exogenous glucose for spreading (see Fig. 6a, 6b). Presumably cells must require metabolic energy to spread, and TG might derives energy via glycolysis from endogenous glucose, (e.g from glycogen).

To investigate if TG derives energy for spreading from endogenous glucose, I used 2-deoxyglucose, which is an inhibitor of the glycolytic pathway. 2-deoxy-D-glucose is rapidly phosphorylated by cells via the hexokinase reaction. The resulting 2-deoxy-D-glucose-6-phosphate is a powerful inhibitor of the enzyme phospho-glucose isomerase, which catalyse the conversion of glucose-6-phosphate to fructose-6-phosphate. As a result, glucose cannot enter the glycolytic pathway in the presence of this inhibitor.

Following the standard spreading assay, different concentrations of 2-deoxyglucose were added to GFHH with or without 1 mM pyruvate.

The results showed that the spreading of TG cells was not inhibited at 10 mM and only partially at 100 mM to about a half of that in GFHH control. 2-deoxyglucose inhibition of TG was evident in presence of pyruvate. With F3, there was slight inhibition of pyruvate- induced spreading even at the lower concentrations of 2-deoxyglucose (Fig. 11). The concentrations of 2-deoxyglucose used were very high, raising the possibility of non-specific inhibition.
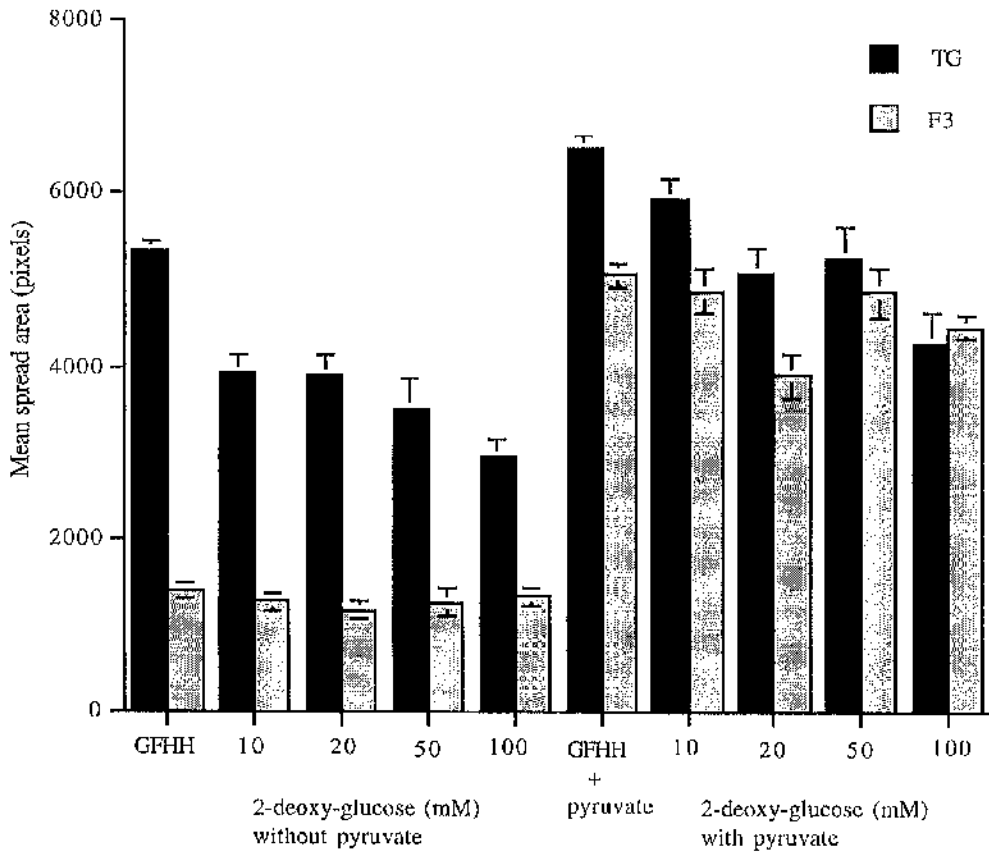
Fig. 11 Effect of 2-deoxy-glucose on the spreading of TG and F3

Cell suspensions were prepared in GFHH with or without 1 mM
pyruvate, and different concentrations of 2-deoxy-glucose were added, and were allowed
to spread on Fn-coated coverslips for 180 minutes, then processed as described in methods.
The results are means of 6 experiments (objective 50x). Error bars = standard deviations

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4. Measurement of the activity of mitochondrial dehydrogenases

Spreading assays showed that F3 requires pyruvate to spread and to maintain the spread state. Pyruvate could act as an energy source. TG (the wild type which gave rise to F3) spreads in both Hanks HEPES which contains glucose and in glucose free Hanks HEPES. TG could derive energy for spreading via glycolysis from endogenous glucose (glycogen), as suggested by the 2-deoxyglucose inhibition assay (Fig. 11).

The end product of glycolysis in the presence of oxygen is pyruvate. Pyruvate can then be completely oxidised to CO_2 and H_2O by enzymes present in the inner membrane of the mitochondria. The question arose whether F3 is unable to derive energy from glucose, but instead maintains its ATP from the oxidation of pyruvate.

To test this hypothesis the activity of the mitochondrial dehydrogenases was measured using MTT in which the absorbance of the converted dye was measured at a wavelength of 570 nm with a background subtraction at 630 nm. Three different conditions were investigated

1) TG and F3 cells were grown to confluence in 35 mm diameter tissue culture dishes, washed twice in GFHH, and incubated for 30 minutes in HH, or GFHH, to which L-glutamine or pyruvate were added. After 30 minutes, 0.1 ml of 0.5 mg/ml stock MTT was added and the cells were incubated for two hours in the hot room. At the end of the incubation, the medium was removed and the converted dye was solubilized with acidic isopropanol. The absorbance of the converted dye was measured at a wavelength of 570 nm with a background subtraction at 630 nm . Cell numbers were approximately equal for each treatment (0.32 and 0.3 x 10^6/ml for TG and F3 (Fig.12).
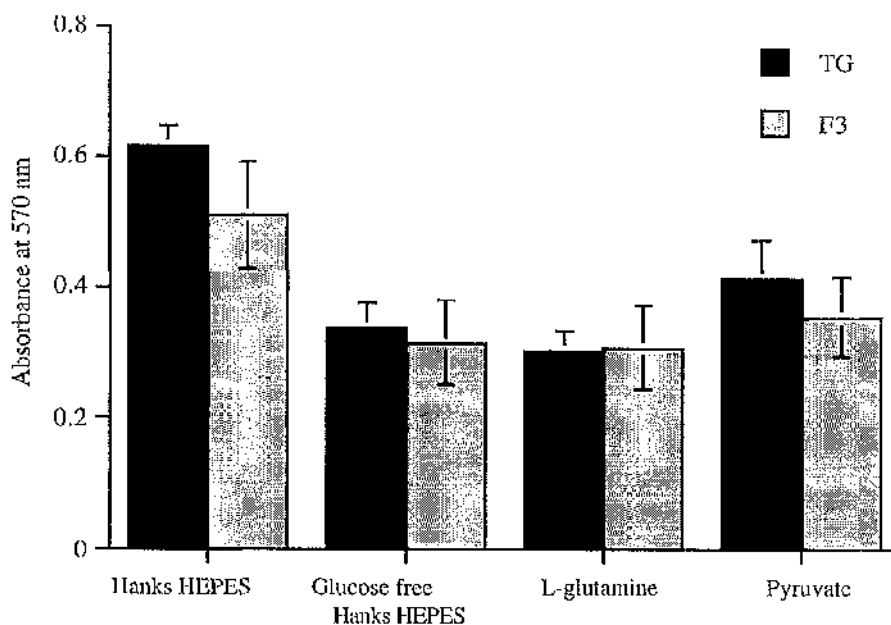
Fig. 12 Measurement of the mitochondrial dehydrogenases of TG and F3 using MTT (condition 1)

The results are means of 3 experiments (6 samples each), and the bars = standard errors

2) Cells were incubated with GFHH for 45 minutes, washed twice in GFHH, then incubated for a further 45 minutes in HH, or GFHH, to which L-glutamine or pyruvate, then 0.2 ml MTT was added and incubated for 3 hours, then later stages were carried out as described above (Fig. 13).
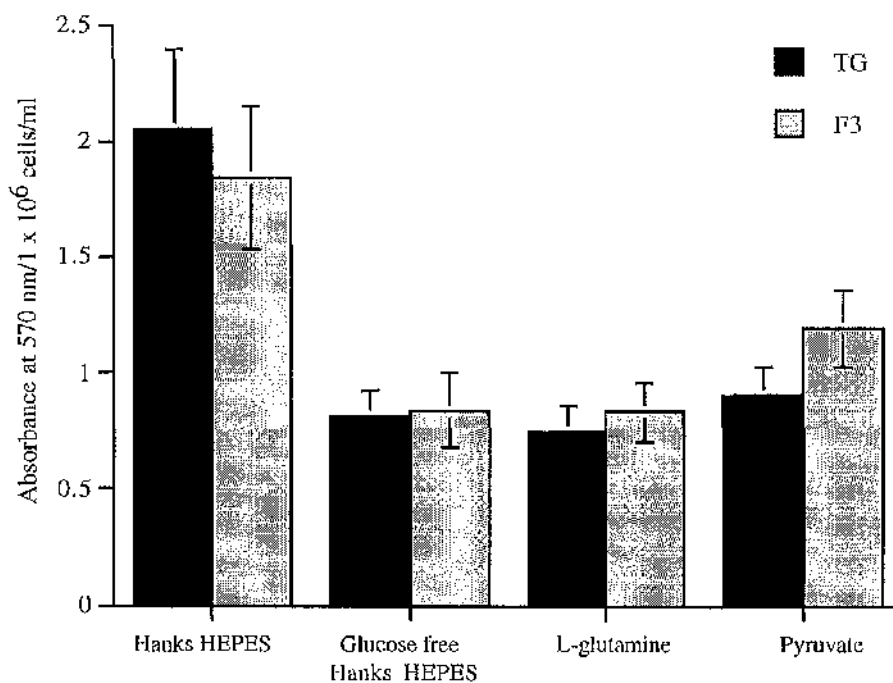
Fig. 13 Measurement of the mitochondrial dehydrogenases of TG and F3 using MTT (condition 2)

Results were corrected according to cell number (1×10^6). The results are means of 4 experiments (8 samples each), and the bars = standard errors

3) As in 2, but MTT was added together with HH, or GFHH, and incubated for 3

hours to which L-glutamine or pyruvate was added, and processed as described in

1 (Fig. 13).
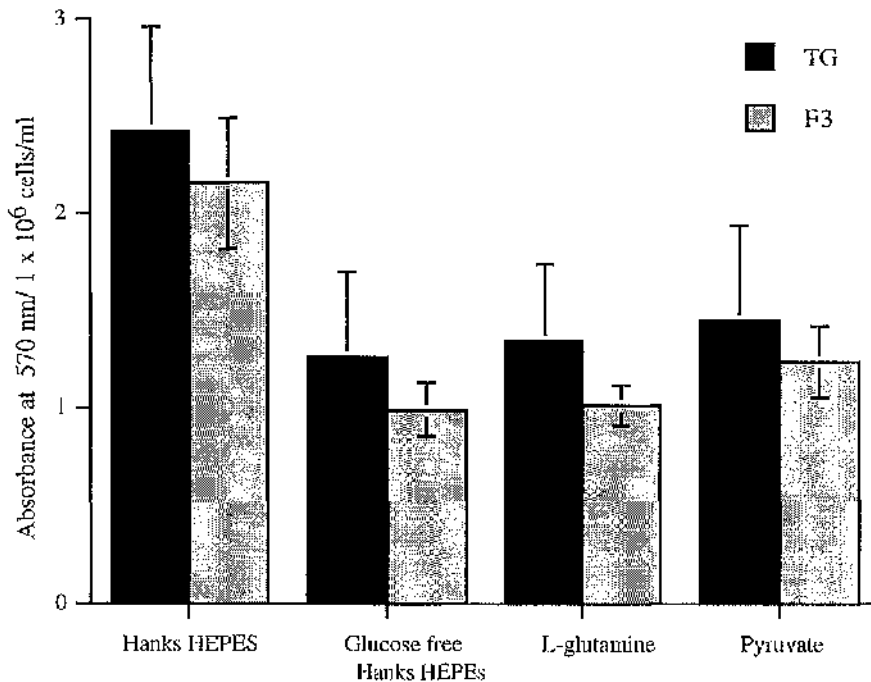
Fig. 13 Measurement of the mitochondrial dehydrogenases of TG and F3 using MTT (condition 3)

Cells were grown to confluence in 35 mm diameter tissue culture dishes, incubated with GFHH for 45 minutes, washed twice in GFHH and incubated for further 45 minutes in HH, or GFHH, or L-glutamine or pyruvate, then 0.2 ml MTT was added and were incubated for 3 hours. The cell cultures were then processed as described in condition 1. The results were corrected according to cell number (1×10^6). The results are means of 4 experiments (8 samples each), and the bars are standard errors

The results in (Fig. 12, Fig. 13 and Fig. 14) showed that TG and F3

respond similarly to glucose (as a component of HH) and GFHH. Neither showed

much increase in MTT in L-glutamine or pyruvate. These results appear to be

inconsistent with the hypothesis that F3 is deficient in energy generation from

glucose. The phenotype of F3 was confirmed after these experiments, by

repeating the spreading assay for TG and F3 in the presence of pyruvate, the

results showed results consistent with earlier experiments, that F3 spread when pyruvate was added.

5. Analysis of ^{14}C-labelled glucose metabolites of TG and F3

5.1 Uptake of ^{14}C-labelled glucose by TG and F3

5.1.1 Time course

The pyruvate requirement of F3 for spreading in culture and on Fn- coated surfaces suggested that this cell line might be defective somewhere in the utilisation of glucose. To investigate this I began by examining the uptake of ^{14}C-labelled glucose, and then analysed glucose metabolites in TG and F3 by one and two dimensional paper chromatography.

Uptake of ^{14}C-labelled glucose by TG and F3 was studied, using 1% Triton X-100 to extract the radioactive metabolites from the cells. Cells were grown in 35 mm diameter tissue culture dishes. The number of cells used for labelling, for both TG and F3 was about 300,000/ml. Cells were washed twice with GFHH and 1 μCi ^{14}C-labelled glucose in 2 ml GFHH was added to each dish. The cells were incubated for zero, 15, 30 and 45 minutes, (60 minutes in some later experiments), washed twice in prewarmed HH and 0.4 ml 1% Triton X-100 in HH was added. Cells were then incubated for two minutes and 0.1 ml of extract was transferred to vials for scintillation counting.
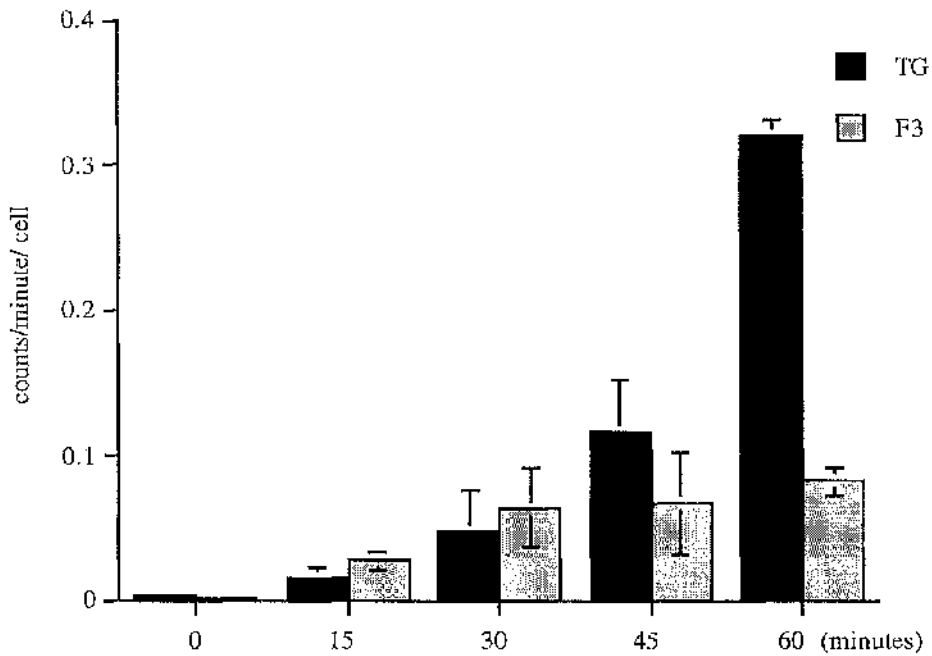
Fig. 15. Uptake of ^{14}C-labelled glucose by TG and F3 after different incubation times.

The results are means of 7 experiments (one sample/ experiment) except that the only three experiments were continued for 60 minutes incubation. The bars are standard errors.

The results showed there was no significant difference between the glucose uptake by TG and F3 during the incubation times zero, 15, 30 and 45 minutes (Fig. 15), which argues against a defect in F3 in the early stages of glucose utilisation, such as entry of glucose through the cell membrane.

However, the figure showed a dramatic difference in glucose uptake between TG and F3 by 60 minutes, which suggesting F3 might be defective in slower processes requiring glucose uptake.

5.2 Analysis of ^{14}C-labelled glucose metabolites of TG and F3 by paper chromatography

5.2.1 Sensitivity of the method

The sensitivity of detection was studied by drying on paper known amounts of ^{14}C-labelled glucose. 10 fold serial dilutions 1/1, 1/10, up to 1/1x107 of ^{14}C-labelled glucose in GFHH were prepared and 10 μl from each dried on chromatography paper. 100 μl from each was transferred to vials for scintillation counting. The paper was placed against X-OMAT film and incubated for 10 days at -70° C.

After 10 days the films was developed. Three distinct spots could be seen, the lightest spot corresponded to 1/100 which was around 1497 cpm (Fig. 16). The results showed that higher activity of ^{14}C-labelled glucose would be needed than used for the time course. Even using 10-20 μCi/ml/ each cell line, very long exposure of the X-OMAT films was needed (4-5 months).
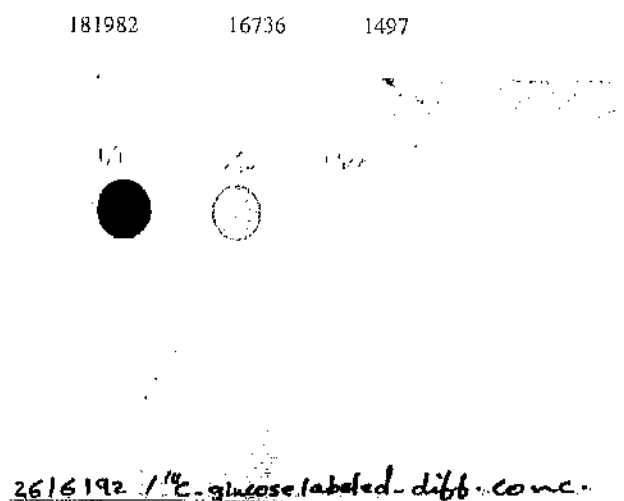
Fig. 16 Sensitivity of the method

5.2.2 One dimensional paper chromatography

Two different batches of silica gel-loaded chromatography paper were used in the one dimensional experiments. Details of the first (older batch) were not available and the second was 3 MM Whatman SG 81 paper.

Cells were incubated with 5 μCi ^{14}C-labelled glucose for 45 minutes and one dimensional chromatography paper was run, using solvent A (Veneziale and Gabrielli, 1969). In some experiments samples from TG and F3 were run each on two papers, to allow one to be cut for scintillation counting. The first paper was dried, placed against X-OMAT film and incubated at -70° C for 5-10 weeks, depending on how much ^{14}C-labelled glucose was used, and the X-OMAT films were developed. The other paper was dried, cut into small strips and then transferred to vials for scintillation counting. Samples were run in duplicate and total counts applied on the first paper were 7530.87 and 7563.00 for TG and 12397.8 and 12477.5 for F3.
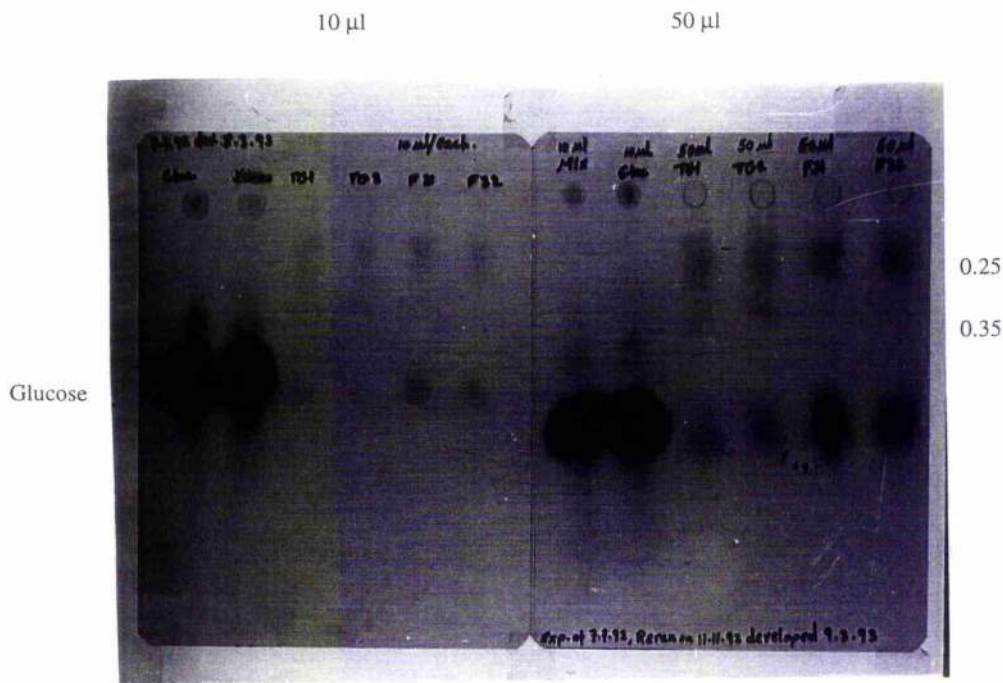
Fig. 17 One dimensional paper chromatography
5 μCi ^{14}C-labelled glucose was used for labelling and two different volumes of
TC extracts (10 μl, 50 μl) were applied to the paper.

All samples showed a glucose spot, and a spot of motility relative to

glucose 0.25 in TG and F3, but a spot of motility 0.35 in TG, was missing in F3

(Fig. 17). This suggested there might be a block in the glycolytic pathway in F3.

To confirm this result, the one dimensional paper chromatography was re-run

using the same samples from this experiment. The results (Fig. 18) confirmed the

missing spot in (Fig. 17), which supported the idea of a defect in a metabolic

pathway in F3.

40 µl

Glue.   Mix   40 µl   40 µl   40 µl   40 µl
                TG1   TG2   F31   F32

Glucose

7/7, Result. on 11.1.92 develop. on 8/3/93.
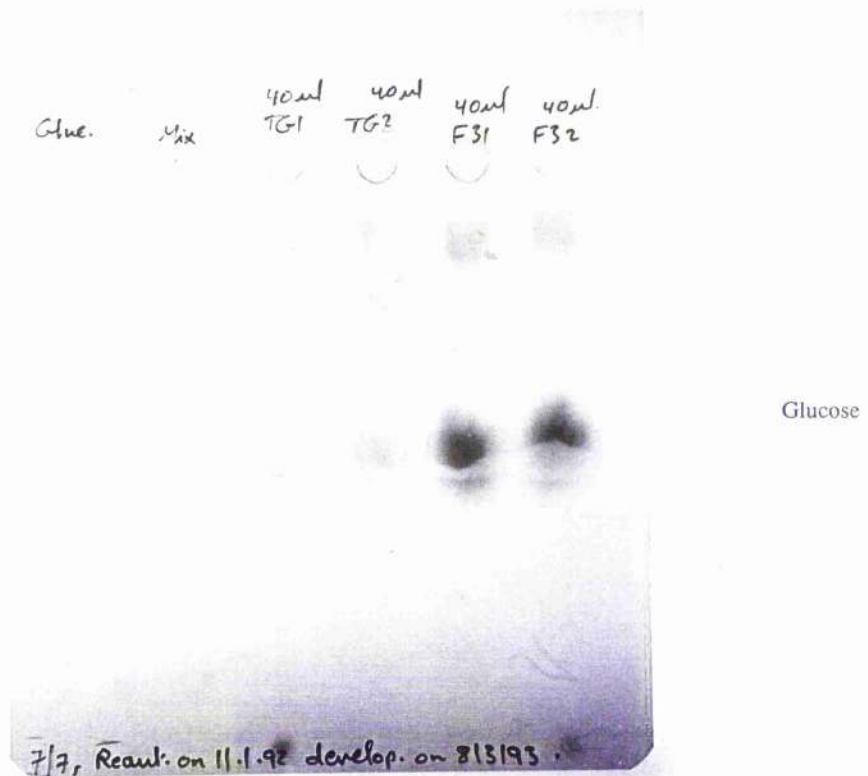
Fig. 18 One dimensional paper chromatography
(Second separation of the extracts analysed in Fig. 17)

In further experiments, the TG specific component (motility relative to glucose 0.35) was not seen. Two papers were run and 5 µCi ^{14}C-labelled glucose was used for TCA extracts for each cell lines. Samples were in duplicate and the total amounts loaded on the first paper was 10349 and 8343 for TG and 2334 and 2166 for F3.
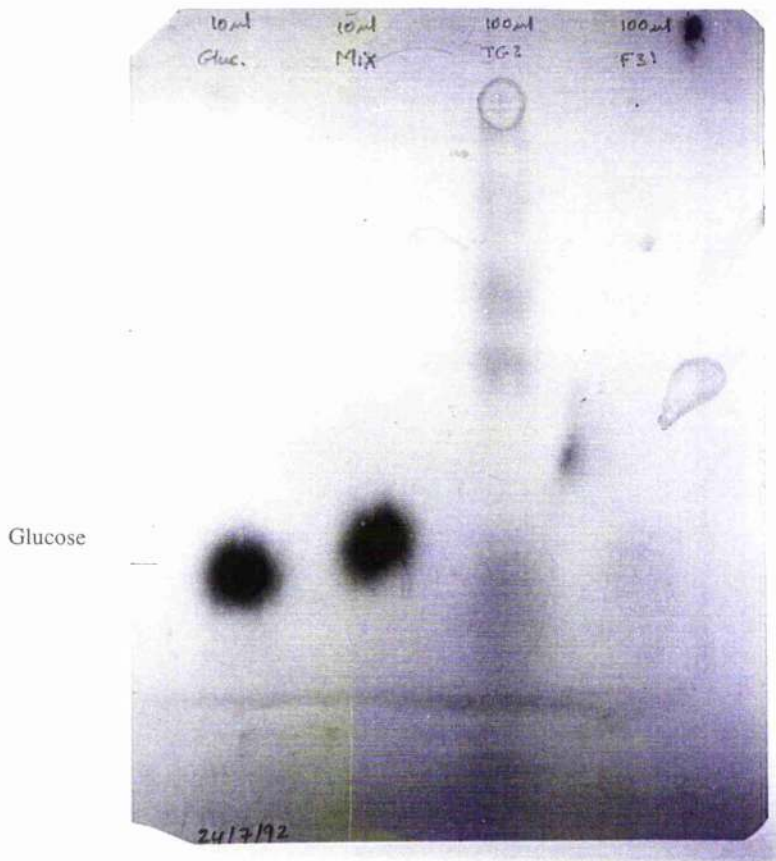
Fig. 19 One dimensional paper chromatography
5 µCi 14C-labelled glucose was used for labelling, and 100 µl TCA extracts were
applied to the paper.

The results showed three distinct spots for TG as seen in Fig. 17 and 18
(Fig. 19), while nothing for F3, which might be due to the lower amount of the
radioactivity loaded on the paper. The second paper (loaded with less extract, the
remaining 25 µl) was dried and cut into strips for scintillation counting. Total
amounts loaded on the second paper was 1252 for TG and 326 for F3.

I attempted to quantitate this result as follows: the paper was dried, cut into small strips, and the strips transferred to vials for scintillation counting (Fig. 20).
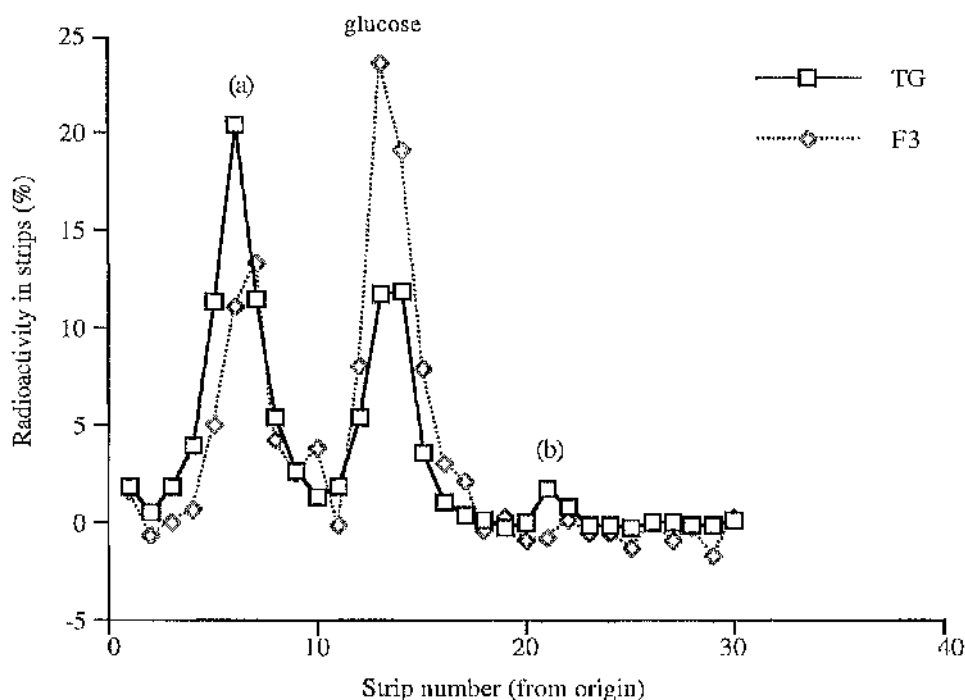
Fig. 20 One dimensional paper chromatography

25 µl sample was loaded on paper, the paper was dried and cut into strips for scintillation counting

Although the amounts loaded on paper were very low and for F3 were not seen on the autoradiograph (Fig. 19), the result again showed an increase in the percentage of the radioactivity recovered as glucose in F3 (summarised below in table 1). A trace of fast moving radioactivity (1.77% of the total recovered, motility relative to glucose 1.6) was detected in TG and not in F3 (strip 21=b).

In a further experiment, 10 µCi ^{14}C-labelled glucose was used for TCA extracts for each cell line. Samples were in duplicate and the total amounts loaded on paper were 12041 and 13439 for TG and 10511 and 13508 for F3.
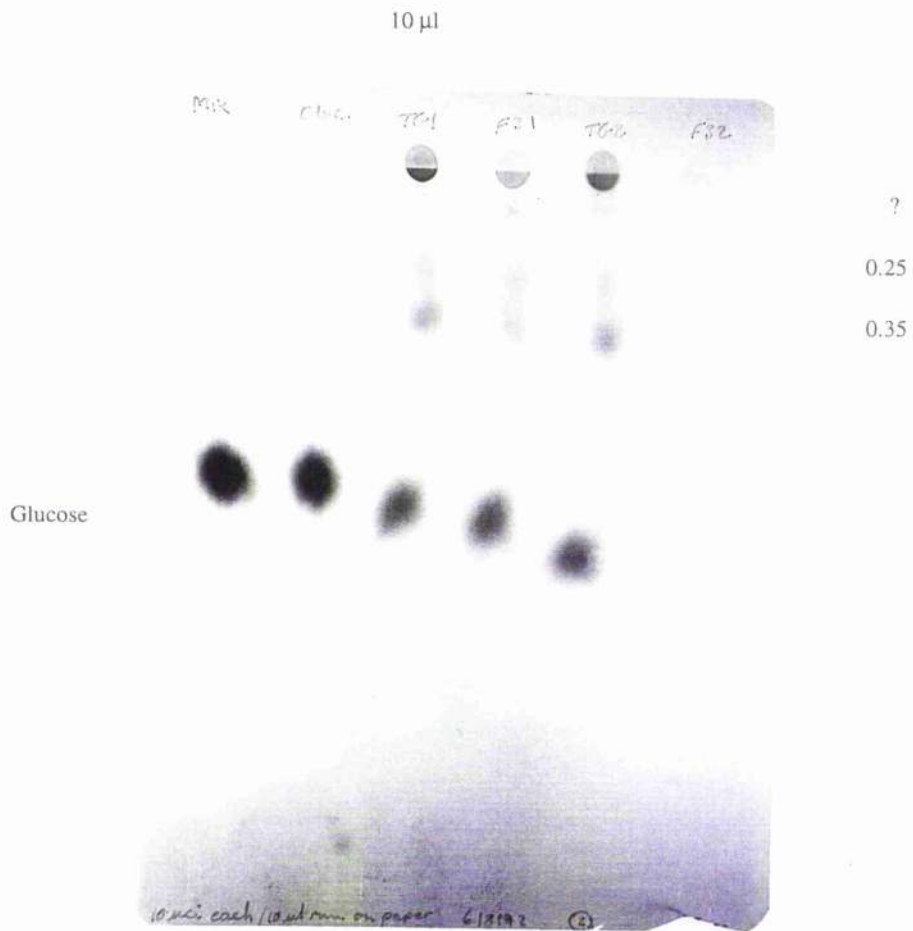
Fig. 21 One dimensional paper chromatography

10 µCi ^{14}C-labelled glucose was uses for labelling, and 10 µl TCA
extracts were applied to the paper.

The results showed two distinct spots for TG as seen before (Fig. 17 and Fig. 19) and a suggestion of a third, running slower. This slower material was absent for F3.

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As before a second paper was run and cut into strips for scintillation counting.
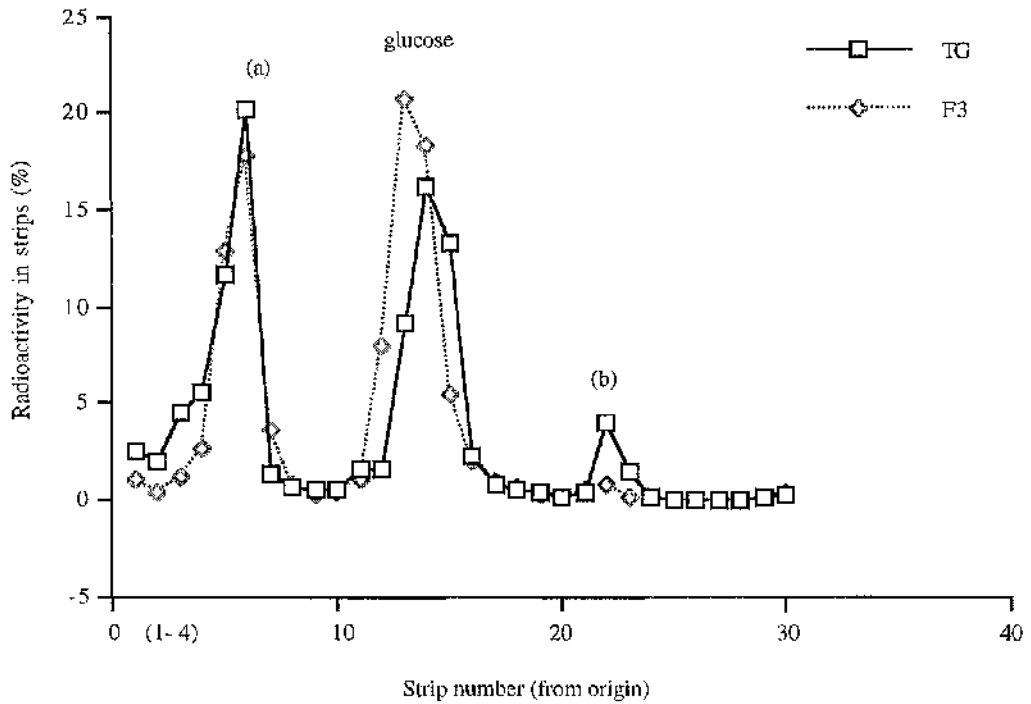
Fig. 22 One dimensional paper chromatography

25 µls extracts from TG and F3 were loaded on paper, the paper was dried and cut into strips for scintillation counting

The results again showed an increase in the percentage of the radioactivity recovered as glucose in F3 (Fig. 22) and an increase in percentage recovered glucose in TG in which was 3.95% in TG (motility relative to glucose 1.57).and very low in F3 (0.73%)

(strip 22=b). Strips 1-4 confirmed the presence of slower running material in TG but not F3.

In a repeat of this experiment, in which percentage of total amounts loaded were 26947 for TG and 7147 for F3, the results again showed an increase percentage of radioactivity recovered as glucose in F3 0.6% and 0.89% (motility relative to glucose 1.57) in TG, (Fig. 23, strip 22=b). However the difference in the slower material was not observed.
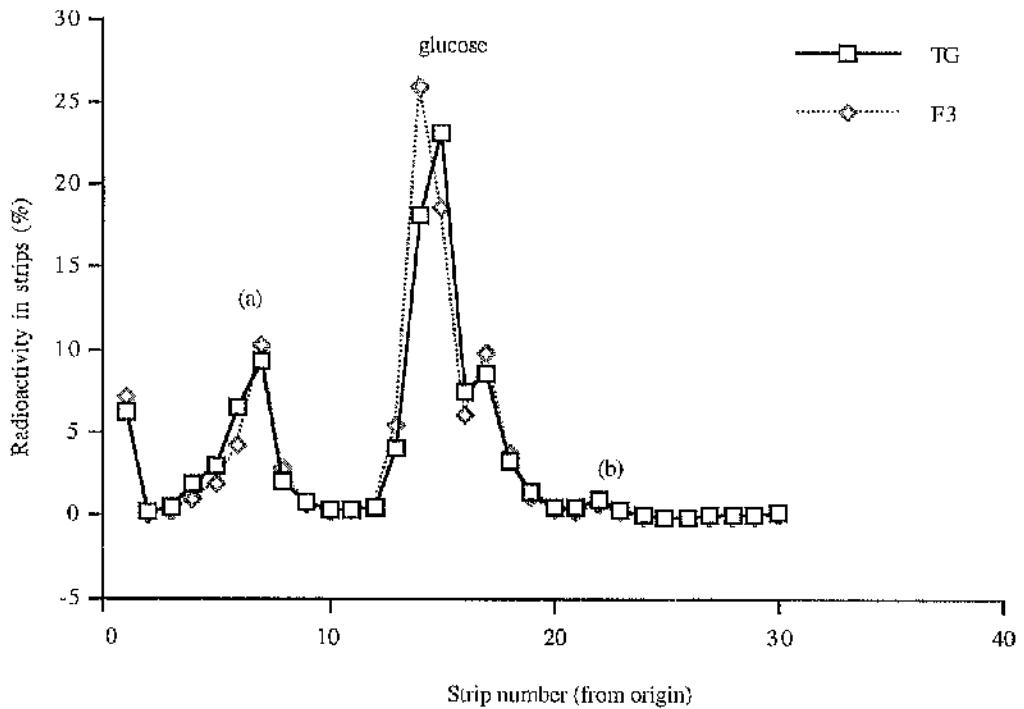
Fig. 23 One dimensional paper chromatography

10 µCi ^{14}C-labelled glucose was used for TCA extracts for TG and F3 and 10 µl extract was loaded on paper, and the paper was cut into strips for scintillation counting

The results of all the one dimensional chromatography analysis are summarised in the table below:

	Components in TG not in F3		% total cpm in glucose		
	0.35	1.6	TG	F3	F3/TG
1) a.	+	-	45.7	58.7	1.28
b.	+	-			
2)	+	+	27.19	50.7	1.86
3)	-	+	38.5	44.37	1.15
4)	+	+	48.7	50.5	1.04

Table 3.1 Summary of the results of the one dimensional paper chromatography

From the summary in table 1, the results in some of the experiments suggested there might be a defect in a metabolic pathway glucose utilisation in F3. However the components detected were inconsistent between different experiments.

In order to investigate these glucose metabolites further, I decided to analyse extracts using two dimensional paper chromatography on 3 MM Whatman SG 81 paper. In this technique the second dimension used was solvent B (see materials and methods).

In the experiments for two dimensional separations I used bigger tissue culture dishes (60 mm diameter), higher radioactivity (40 µCi and 50 µCi), and lower volumes of 10% TCA to extract the cells (0.5 ml).

(Two components in TG not seen in F3)

(More components in TG, right)

(TG)   (F3)
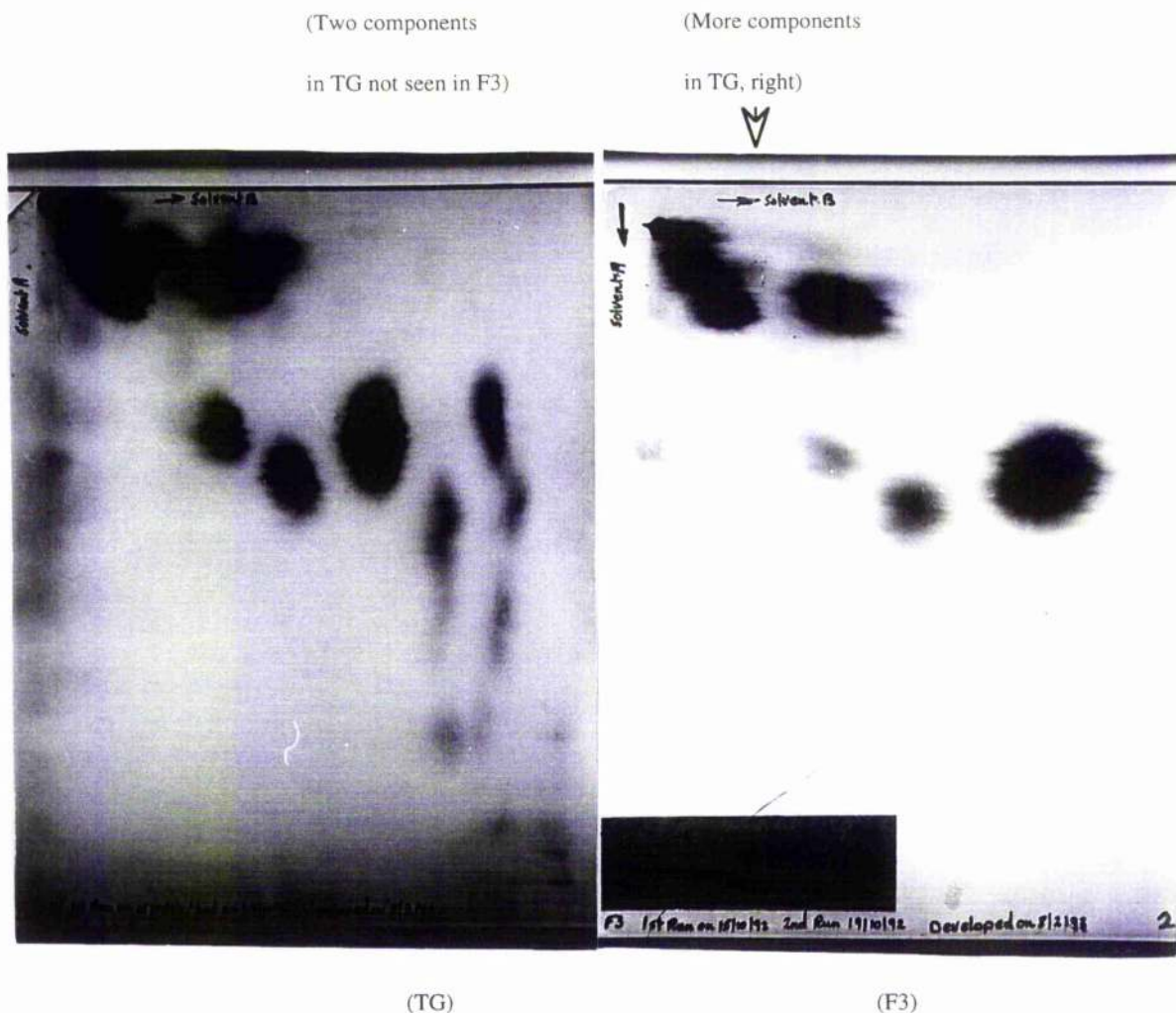
Fig. 24 Two dimensional paper chromatography
Cells were grown to confluence in 60 mm diameter tissue culture dishes
(TG 0.75 x 106/ml and F3 0.57 x 106/ml), washed twice in GFHH, and 0.5 ml containing
40 µCi ^{14}C-labelled glucose was added. Cells were incubated for 45 minutes at 37°C,
then washed twice in prewarmed HH and 0.5 ml of 10% TCA was added to the cell
cultures and incubated for further 2 minutes in the hot room. The autoradiograph was
exposed 15 weeks.

The results showed a clear difference between TG and F3 (Fig. 24). There were two components in TG not seen in F3 as well as more components "amino acids ?" in the far right on the paper (TG).

In an attempt to confirm this result the experiment was repeated, using 50 μCi ^{14}C-labelled glucose.

| TG | F3 |
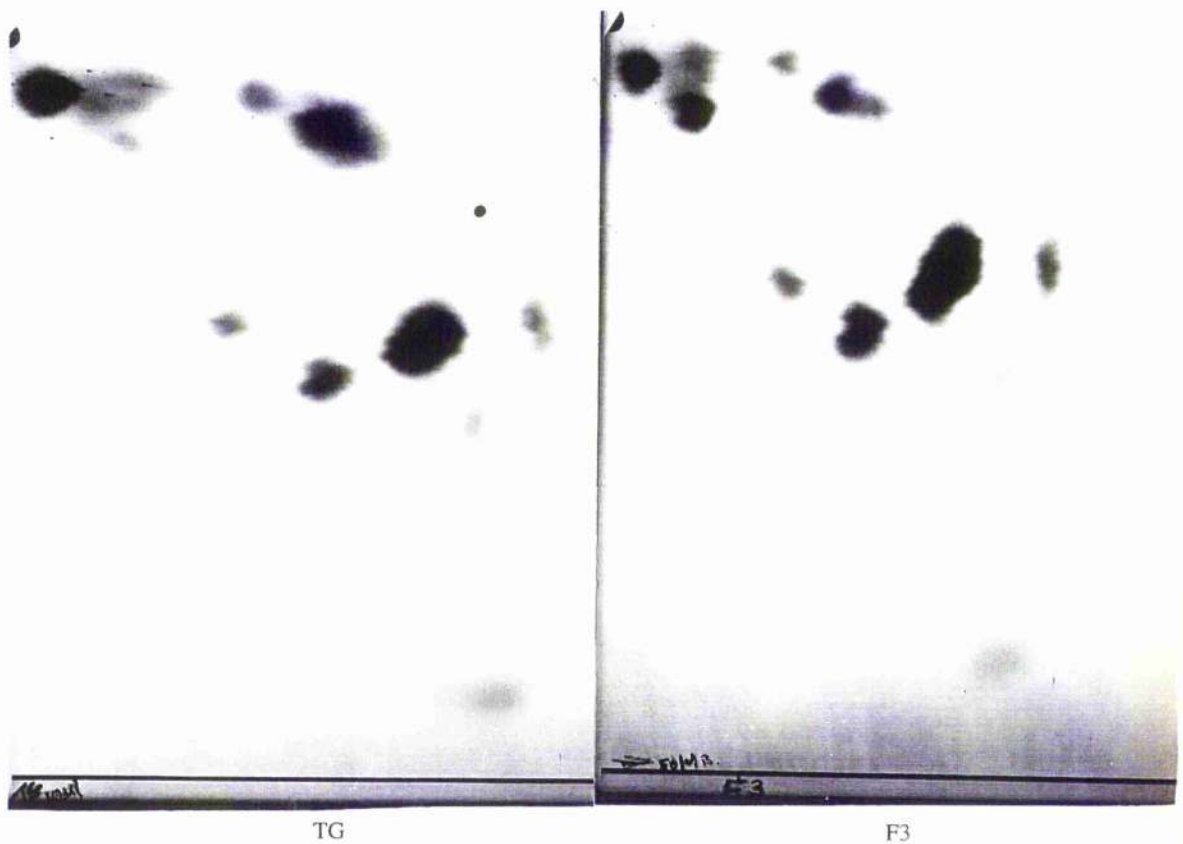
Fig 25 Two dimensional paper chromatography

Cells were grown to confluence in 60 mm diameter tissue culture dishes (TG 1.45 x 106/ml), washed twice in GFHH and incubated in 0.5 ml containing 50 μCi ^{14}C-labelled glucose for 45 minutes at 37°C, the cell cultures were then washed twice in prewarmed HH and incubated with 0.5 ml 10% TCA for further 2 minutes in the hot room. The autoradiograph was exposed for 13 weeks.

The results (Fig. 25) did not reproduce the clear difference seen in the previous experiment, Fig. 24. The variation in the results of the one and two dimensional chromatography did not allow a definite conclusion about a defect in glucose utilisation in F3.

CHAPTER 4

GENERAL DISCUSSION

General discussion

The F3 line consistently required addition of pyruvate to Hanks' HEPES to spread, and maintain spreading, on fibronectin. Although pyruvate has been found necessary for growth and maintenance of various cell types in culture, a specific requirement for spreading has not been previously reported. The pyruvate could be needed either as a source of ATP, or to supply one or more metabolites. In some spreading assays, pyruvate also stimulated spreading of the TG "wild type" line. Two questions need to be answered: first, why would pyruvate need to be added to F3 and not the wild type; second, why is pyruvate needed for spreading?

It is possible that in F3 less pyruvate is generated from glycolysis, which means there is some deficiency in this pathway. Or, less likely, that more pyruvate is needed by F3 than TG. Glucose uptake studies suggested that there could be a deficiency in glucose metabolism in F3. However, two-dimensional paper chromatography results were inconsistent. This is clearly worth further investigation, but the achievement of results is very slow.

The activity of mitochondrial dehydrogenases showed that both lines respond similarly to glucose with reduction of the dye, suggesting that both lines can probably generate enough ATP. This was supported by the observation that when glutamine was added to HH, it did not stimulate spreading of F3, in comparison with pyruvate. Glutamine is normally needed as a secondary source of ATP generation in animal cells. These two observations favour the idea that F3 does not need pyruvate for production of ATP, but rather for supply of some metabolites. These metabolites could be amino acids or fatty acids (formed via acetyl co-enzyme A). Amino acids seem unlikely, because cell spreading in short term assays is not dependent on protein synthesis (Grenz et al., 1993).

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One situation is known where a fatty acid appears to be required for a process similar to cell spreading. Patterson *et al.*, 1994 found that tunicamycin inhibits neurite outgrowth from PC12 cells, and showed by using long chain homologues that the inhibitor was acting by blocking palmitoylation, rather than glycosylation. Palmitate added to medium was able to reverse the block. The target for palmitoylation was believed to be the protein GAP43 (Jochen *et al.*, 1995). The idea that pyruvate is needed to generate palmitate could be explored in various ways. For example, palmitate added to medium might increase spreading, and cerulenin, an inhibitor of fatty acid synthesis, should inhibit. (Cerulenin was found to block palmitoylation of selected proteins, and internalisation of insulin in rat adipocytes (Jochen *et al.*, 1995).

Since pyruvate (which is present in Hams' F10) was present during selection, it is not clear why F3 should have been selected by the procedure used. Did F3 perhaps consume much of the pyruvate during the 6-8 hour selection? Or are these cells less spread than the wild type and therefore more easily detected?

In conclusion, my selection of a mutant with a specific requirement for spreading (which is related to various other important processes in animal cells) shows the usefulness of this approach..

REFERENCES

References

Abercrombie, M., Heaysman, J. E. M., and Pegrum, S. M. (1970). The locomotion of fibroblasts in culture. *Exp. Cell Res.* **62**: 389-398.

Adams, A. E. M., Johnson, D. I., Longnecker, R. M., Sloat, B. F., and Pringle, J. R. (1990). Cdc42 and Cdc43, two additional genes involved in budding and establishment of cell polarity in the yeast *Saccharomyces cervisiae*. *J. Cell Biol.* **111**: 131-142.

Aharoni, D., Meiri, I., Atzmon, R., Vlodavsky, I., and Amsterdam, A. (1996). Differential effect of components of the extracellular matrix on differentiation and apoptosis. *Curr. Biol.* **7**: 43-51.

Akiyama, S. K., Yamada, S. S., Chen, W.-T., and Yamada, K. M. (1989). Analysis of receptor function with monoclonal antibodies: roles in cell adhesion, migration, matrix assembly, and cytoskeletal organisation. *J. Cell Biol.* **109**: 863-875.

Amos, L. A. (1997). The structure of microtubule-motor complexes. *Curr. Opin. Cell Biol.* **9**: 4-11.

Argraves, W. S., Suzuki, S., Arai, H., Thompson, k., Pierschbacher, M. D., and Ruoslahti, E. (1987). Amino acid sequence of the human fibronectin receptor. *J. Cell Biol.* **105**: 1183-1190.

Barillari, G., Gendelman, R., Gallo, R. C., And Ensoli, B. (1993). The Tat protein of human immunodeficiency virus type 1, a growth factor for AIDS

Kaposi Sarcoma and cytokine-activated vascular cells, induces adhesion of the same cell types by using integrin receptors recognising the RGD amino acid sequence. *Proc. Natl. Acad. Sci. USA.* **90**: 7941-7945.

Baron, M., Norman, D., Willis, A., and Campbell, I. D. (1990). Structure of the fibronectin type I module. *Nature* **345**: 642-646.

Bergelson, J. M., Chan, B. M., Finberg, R. W., and Hemler, M. E. (1993). The integrin VLA2 binds Echovirus 1 and extracellular matrix ligands by different mechanisms. *J. Clin Invest.* **92**: 232-239.

Bernfield, M. (1989). Extracellular matrix. *Curr. Opin. Cell Biol.* **1**: 953-955.

Bennoni, D., and Bradley, W. E. C. (1986). Highly adherent mutants of SV40-transformed mouse fibroblasts: genetic and biochemical characterisation. *J. Cell Physiol.* **126**: 174-180.

Bershadsky, A. D., Tint, I. S., Neyfakh, Jr. and Vasiliev, J. M. (1985). Focal contacts of normal and RSV-transformed Quail cells. *Exp. Cell Res.* **158**: 433-444.

Blobel, C. P., and White, J. M. (1992). Structure, function and evolutionary relationship of proteins containing a disintegrin domain. *Curr. Opin. Cell Biol.* **4**: 760-765.

Bloch, R. J. (1992). Clusters of neural cell adhesion molecule at sites of cell-cell contact. *J. Cell Biol.* **116** (**No. 2**): 449-463.

Bloom, G. S. 1992. Motor proteins for cytoplasmic microtubules. *Curr. Opin. Cell Biol.* **4**: 66-73.

Borradori, L., Sonnenberg, A. (1996). Hemidesmosomes: roles in adhesion, signalling and human diseases. *Curr. Opin. Cell Biol.* **8**: 647-656.

Bornstein, P., Sage, H. (1980). Structurally distinct collagen types. *Ann. Rev. Biochem.* **49**: 957-1003.

Borovikov, Y. S., Norman, J. C., Price, L. S., Weeds, A., and Koffer, A. (1995). Secretion from permeabilised mast cells is enhanced by addition of gelsolin: contrasting effects of endogenous gelsolin. *J. Cell Sci.* **108**: 657-666.

Bray, D., and White, J. G. (1988). Cortical flow in animal cells. *Science.* **239**: 883-888.

Bremer, A., and Aebi, U. (1992). The structure of the F-actin and the actin molecule. *Curr. Opin. Cell Biol.* **4**: 20-26.

Brown, J. J. G., and Whittingham, D. G. (1991). The roles of pyruvate, lactate and glucose during preimplantation development of embryos from F_1 hybrid mice *in vitro. Development.* **112**: 99-105.

Brown, J. C., Weidemann, H., and Timpl, R. (1994). Protein binding and cell adhesion properties of two laminin isoforms (AmB1eB2e, AmB1sB2e) from human placenta. *J. Cell Sci.* **107**: 329-338.

Burridge, K., Fath, K., Kelly, T., Nuckolls, G., and Turner, C. (1988). Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. *Annu. Rev. Cell Biol.* **4**,: 487-525.

Burns, R. L., Rosenberger, P. G., and Klebe, R. J. (1976). Carbohydrate preferences of mammalian cells. *J. Cell Physiol.* **88**: 307-316.

Carmichael, J., DeGraff, W. G., Gazdar, A. F., Minna, J. D., and Mitchell, J. B. (1987). Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res.* **47**: 936-942.

Carnemolla, B., Balza, E., Siri, A., Zardi, L., Nicotra, M. R., Bigotti, A., and Natali, P. G. (1989). A tumour-associated fibronectin isoform generated by alternative splicing of messenger RNA precursors. *J. Cell Biol.* **108**: 1139-1148.

Charo, I. F., Nannizzi, L., Smith, J. W., and Cheresh, D. A. (1990). The vitronectin receptor $\alpha_v\beta_3$ binds fibronectin and acts in concert with $\alpha_5\beta_1$ in promoting cellular attachment and spreading on fibronectin. *J. Cell Biol.* **111 (No. 6, Pt. 1)**: 2795-2800.

Chen, W.-T., Greve, J. M., Gottlieb, D., and Singer, S. J. (1985). Immunocytochemical localisation of 140 kd cell adhesion molecules in cultured chicken fibroblasts, and chicken smooth muscle and intestinal epithelial tissue. *J. Histochem. Cytochem.* **33**: 576-586.

Chen, H. C., and Guan, J. L. (1994). Association of focal adhesion kinase with its potential substrate phosphatidylinositol 3-kinase. *Proc. Natl. Acad. Sci. USA.* **91**: 10148-10152.

Chen, Q., Kinch, M. S., TH, L., Burridge, K., and Juliano, R. L. (1994). Integrin-mediated cell adhesion activates mitogen-activated protein kinases. *J. Biol. Chem.* **269**: 26602-26605.

Chen, Y. P., O'Toole, T. E., Shipley, T., Forsyth, J., LaFlamme, S. E., Yamada, K. M., Shattil, S. J., and Ginsberg, M. H. (1994). Inside-out signal transduction inhibited by isolated cytoplasmic domains. *J. Biol. Chem.* **269**: 18307-18310.

Chen, W.-T., and Singer, S. J. (1982). Immunoelectron microscopic studies of the sites of cell-substratum and cell-cell contacts in cultured fibroblasts. *J. Cell Biol.* **95**: 205-222.

Chen, C. S., Thiagarajan, P., Schwartz, S. M., Harlan, J. M., and Heimark, R. L. (1987). The platelet glycoprotein Ib/ IIIa-like protein in human endothelial cells promotes adhesion but not initial attachment to extracellular matrix. *J. Cell Biol.* **105**: 1885-1892.

Chen, W.-T., Wang, J., Hasegawa, T., Yamada, S. S., and Yamada K,. M. (1986). Regulation of fibronectin receptor distribution by transformation, exogenous fibronectin, and synthetic peptides. *J. Cell Biol.* **103**: 1649-1661.

Cheney, R. E., and Mooseker, M. S. (1992). Unconventional myosins. *Curr. Opin. Cell Biol.* **4**: 27-35.

Cheung, E., Brown, P. J., and Juliano, R. L. (1987). Altered type 1 protein kinase in adhesive defective CHO cell variants. *J. Cell Physiol.* **130**: 118-124.

Cheung, E., and Juliano, R. L. (1985). cAMP induced phenotypic reversion of adhesion defective CHO cell variant. *J. Cell Physiol.* **124**: 337-343.

Chong, L. D., Traynor-Kaplan, A., Bokoch, G. M., and Schwartz, M. A. (1994). The small GTP-binding protein Rho regulates a phosphatidylinositol 4-phosphate 5-kinase in mammalian cells. *Cell.* **79**: 507-513.

Chrzanowska-Wodnicka, M., and Burridge, K. (1994). Tyrosine phosphorylation is involved in reorganisation of the actin cytoskeleton in response to serum or LPA stimulation. *J. Cell Sci.* **107**: 3643-3654.

Chrzanowska-Wodnicka, M., and Burridge, K. (1996). Rho-stimulated contractility drives the formation of stress fibres and focal adhesions. *J. Cell. Biol.* **133 (No. 6)**: 1403-1415.

Churchill, L., Gundel, R. H., Letts, L. G., and Wegner, C. D. (1993). Contribution of specific cell-adhesive glycoproteins to airway and alveolar inflammation and dysfunction. *Am. Rev. Respir Dis.* **148**: S83-S87.

Clark, E. A., Burgge, J. S. (1995). Integrins and signal transduction pathways: the road taken. *Science.* **268**: 233-238.

Coburn, J., Leong, J. M., and Erban, J. K. (1993). Integrin $\alpha_{IIb}\beta_3$ mediates binding of the lyme disease agent *Borrelia burgdorferi* to human platelets. *Proc. Natl. Acad. Sci.* USA. **90**: 7059-7063.

Conaghan, J., Handyside, A. H., Winston, R. M. L., and Leese, H. J. (1993). Effects of pyruvate and glucose on the development of human preimplantation embryos in vitro. *J. Reproduction and Fertility.* **99**: 87-95.

Cramer, L. P., Mitchison, T. J., and Theriot, J. A. (1994). Actin-dependent motile forces and cell motility. *Curr. Opin. Cell Biol.* **6**, 82-86.

Curi, R., Newsholme, P., Newsholme, E. A. (1988). Metabolism of pyruvate by isolated rat mesenteric lymphocytes, lymphocyte mitochondria and isolated mouse macrophages. *Biochem J.* **250**: 383-388.

Craig,. S. W., and Johnson, R. P. (1996). Assembly of focal adhesions: progress, paradigms, and portents. *Curr. Opin. Cell Biol.* **8**: 74-85.

Dalton, B. A., McFarland, C. D., Underwood, P. A., and Steele, J. G. (1995). Role of the heparin binding domain of fibronectin in attachment and spreading of human bone-derived cells. *J. Cell Sci.* **108**: 2083-2092.

Damsky, C. H., and Werb, Z. (1992). Signal transduction by integrin receptors for extracellular matrix: co-operative processing of extracellular information. *Curr. Opin. Cell Biol.* **4**: 772-781.

Dean, D. C., Iademarco, M. F., Rosen, G. D., and Sheppard, A. M. (1993). The integrin $\alpha 4\beta 1$ and its counter VCAM-1 in development and immune function. *Am. Rev. Respir. Dis.* **148**: S43-S46.

Dedhar, S. Argraves, W. S., Suzuki, S., Ruoslahti, E., and Pierschbacher, M. D. (1987b). Human osteosarcoma cells resistant to detachment by an Arg-Gly-Asp-containing peptide overproduce the fibronectin receptor. *J. cell Biol.* **105**: 1175-1182.

Dedhar, S., and Hannigan, G. E. (1996). Integrin cytoplasmic interactions and bi-directional transmembrane signalling. *Curr. Opin. Cell Biol.* **8**: 657-669.

Dedhar, S., Ruoslahti, E., and Pierschbacher, M. D. (1987a). A cell surface receptor complex for collagen type I recognises the Arg-Gly-Asp sequence. *J. Cell Biol.* **104**: 585-593.

De Fougerolles, A. R., and Springer, T. A. (1992). Intercellular adhesion molecule 3, a third adhesion counter-receptor for lymphocyte function-associated molecule 1 on resting lymphocytes. *J. Exp. Med.* **175**: 185-190.

DeVivo, D. C., Haymond, M. W., Lackie, M. P., Busssmann, Y. L., McDougal, D. B., Jr., and Pagliara, A. S. (1977). The clinical and biochemical implications of pyruvate carboxylase deficiency. *J. Clin. Endocrinol. Metab.* **45**: 1281-1296.

Diamond, M. S., and Springer, T. A. (1994). The dynamic regulation of integrin adhesiveness. *Curr. Biol.* **4 (No. 6)**: 506-517.

Diekmann, D., Abo, A., Johnston, C., Segal, A. W., and Hall, A. (1994). Interaction of rac with p67^{phox} and regulation of phagocytic NADPH oxidase activity. *Science.* **265**: 531-533.

DiPersio, C. M., Shah, S., and Hynes, R. O. (1995). $\alpha 3A\beta 1$ integrin localises to focal contacts in responses to diverse extracellular matrix proteins. *J. Cell Sci.* **108**: 2321-2336.

Domnina, L. V., Rovensky, J. A., Vasiliev, J. M., and Gelfand, I. M. (1985). Effect of microtubule-destroying drugs on the spreading and shape of cultured epithelial cells. *J. Cell Sci.* **74**: 267-282.

Duband, J. L., Nuckolls, G. H., Ishihara, A., Hasegawa, T., Yamada, K. M., Thiery, J. P., and Jacobson, K. (1988). Fibronectin receptor exhibits high lateral

mobility in embryonic locomoting cells but is immobile in focal contacts and fibrillar streaks in stationary cells. *J. Cell Biol.* **107**: 1385-1396.

Dunn, G. A. and Brown, F. (1986). Alignment of fibroblasts on grooved surfaces described by a simple geometric transformation. *J. Cell Sci.* **83**: 313-340.

Eagle, H., Barban, S., Levy, M., and Schulze, H. O. (1958). The utilisation of carbohydrates by human cell cultures. *J. Biol. Chem.* **233 (No. 3)**: 551-558.

Eagle, H., and Piez, K. (1962). The population-dependent requirement by cultured mammalian cells for metabolites which they can synthesise. *J. Exp. Med.* **116**: 29-42.

Edwards, J. G., Campbell, G., and Salama, N. (1985). Variants of Polyoma-transformed BHK21 cells unresponsive to fibronectin. *Cell Biol. Int. Reports.* **9 (No. 8)**: 737-745.

Edwards, J. G., Robson, R. T., and Campbell, G. (1987). A major difference between serum and fibronectin in the divalent cation requirement for adhesion and spreading of BHK21 cells. *J. Cell Sci.* **87**: 657-665.

Edwards, J. G., Hameed, H., and Campbell, G. (1988). Induction of fibroblast spreading by Mn^{2+}: a possible role for unusual binding sites for divalent cations in receptors for proteins containing Arg-Gly-Asp. *J. Cell Sci.* **89**: 507-513.

Elices, M. J., Urry, L. A., and Hemler, M. E. (1991). Receptor functions for the integrin VLA-3: fibronectin, collagen, and laminin binding are differentially

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influenced by Arg-Gly-Asp peptide and by divalent cations. *J. Cell Biol.* **112** (**No. 1**): 169-181.

Ellem, K. A. O., Kay, G. F. (1983). The nature of conditioning nutrients for human malignant melanoma cultures. *J. Cell Sci.* **62**: 249-266.

Engel, J. (1991). Common structural motifs in proteins of the extracellular matrix. *Curr. Opin. Cell Biol.* **3**: 779-785.

Engvall, E., Hessle, H., and Klier, G. (1986). Molecular assembly, secretion, and matrix deposition of type VI collagen. *J. Cell Biol.* **102**: 703-710.

Engvall, E., Ruoslahti, E. (1977). Binding of soluble form of fibroblast surface protein, fibronectin, to collagen. *Int. J. Cancer.* **20**: 1-5.

Eriksson, J. E., Opal, P., and Goldman, R. D. (1992). Intermediate filament dynamics. *Curr. Opin. Cell Biol.* **4**: 99-104.

Faassen, A. E., Schrager, J. A., Klein, D. J., Oegema, T. R., Couchman, J. R., and McCarthy, J. (1992). A cell surface chondroitin sulphate protoglycan, immunologically related to CD44, is involved in type I collagen-mediated melanoma cell motility and invasion. *J. Cell Biol.* **116** (**No. 2**): 521-531.

Facci, L., Skaper, S. D., and Varon, S. (1985a). Specific replacements of pyruvate for trophic support of central and peripheral nervous system neurons. *J. Neurochem.* **45** (**No. 3**): 926-934.

Facci, L., Skaper S. D., and Varon S. (1985b). Selected nutrients reduce the pyruvate requirement for survival *in vitro* of Chick central nervous system neurons. J. Neurosci Res., 14: 293-302.

Facci L., Skaper, S. D., and Varon, S. (1986). Transamination of glutamate to tricarboxylic acid-cycle intermediates in cultured neurons correlates with the ability of oxo-acids to support neuronal survival *in vitro* . *Biochem.* **234**: 605-610.

Felding-Habermann, B., and Cheresh, D. A. (1993). Vitronectin and its receptors. Curr Opin Cell Biol. **5 (No 5)**: 864-868.

Flaumenhsft, R., and Rifkin, D. B. (1991). Extracellular matrix regulation of growth factor and protease activity. *Curr. Opin. Cell Biol.* **3**: 817-823.

Frisch, S. M., and Francis, H. (1994). Disruption of epithelial cell-matrix interaction induces apoptosis. *J. Cell Biol.* **124**: 619-626.

Fuchs, E. (1994). Intermediate filaments: Structure, dynamics, function, and disease. *Annu. Rev. Biochem.* **63**: 345-382.

Fukui, Y., Lynch, T. J., Brzeska, H., and Korn, E. D. (1989). Myosin I is located at the leading edge of locomoting *Dictyostelium* amoebae. Nature **341**: 328-331.

Gailit, J., and Clark, A. F. R. (1994). Wound repair in the context of extracellular matrix. *Curr. Opin. Cell Biol.* **6**: 717-725.

Gailit, J., and Ruoslahti, E. (1988). Regulation of the fibronectin receptor affinity by divalent cations. *J. Biol. Chem.* **263 (No. 26)**: 12927-12932.

Geiger, B. (1991). What the papers say: Long-range morphogenetic signals and cell adhesion. *BioEssays.* **13 (No. 12)**: 665-666.

Geiger, B., Avnur, Z., Rinnerthaler, G., Hinssen, H., and Small, V. J. (1984). Microfilament-organising centres in areas of cell contact: cytoskeletal interactions during cell attachment and locomotion. *J. Cell Biol.* 99 **(No. 1 Pt. 2)**: 83s-91s.

Giancotti, F. G, and Ruoslahti, E. (1990). Elevated levels of the $\alpha_5\beta_1$ fibronectin receptor suppress the transformed phenotype of Chinese Hamster Ovary cells. *Cell.* **60**: 849-859.

Gingell, D. (1993). Contact signalling and cell motility. *SEB symposium.* **No. 47**: 1-33.

Goodman, S., Aumailley, M., and von der Mark, H. (1991). Multiple cell surface receptors for the short arms of laminin: $\alpha_1\beta_1$ integrin and RGD-dependent proteins mediate cell attachment only to domains III in murine tumour laminin. *J. Cell Biol.*, **113 (No. 4)**: 931-941.

Gingell, D., and Owens, N. (1992). How do cells sense and respond to adhesive contacts? Diffusion-trapping of laterally mobile membrane proteins at maturing adhesions may initiate signals leading to local cytoskeletal assembly response and lamella formation. *J. Cell Sci.* **101**: 255-266.

Ginsberg, M. H., Du, X., and Plow, E. F. (1992). Inside-out integrin signalling. *Curr. Opin. Cell Biol.* **4**: 766-771.

Grenez, H., Carbonetto, S., and Goodman, S. L. (1993). $\alpha_3\beta_1$ integrin is moved into focal contacts in kidney mesangial cells. *J. Cell Sci.* **105**: 739-751.

Grinnell, F. (1984). Manganese-dependent cell-substratum adhesion. *J. Cell Sci.* **65**: 61-72.

Grinnell, F. (1986). Focal adhesion sites and the removal of substratum-bound fibronectin. *J. Cell Biol.* **103 (No. 6, Pt. 2)**: 2697-2706.

Grinnell, F. (1992). Wound repair, keratinocyte activation and integrin modulation. *J. Cell Sci.* **101**: 1-5.

Grzesiak, J. J., Davis, G. E., Kirchhofer, D., and Pierschbacher, M. D. (1992). Regulation of $\alpha_2\beta_1$-mediated fibroblast migration on type I collagen by shifts in the concentrations of extracellular Mg^{2+} and Ca^{2+}. *J. Cell Biol.* **117 (No. 5)**: 1109-1117.

Guan, J. L., and Shalloway, D. (1992). Regulation of focal adhesion associated protein tyrosine kinase by both cellular adhesion and oncogenic transformation. *Nature.* **358**: 690-692.

Gu, M., Wang, W., Song, W. K., Cooper, D. N. W., and Kaufman, S. (1994). Selective modulation of the interaction of $\alpha_7\beta_1$ integrin with fibronectin and laminin by L-14 lectin during skeletal muscle differentiation. *J. Cell Sci.* **107**: 175-181.

Gutman, A., Yamada, K. M., and Kornblihtt, A. (1986). Human fibronectin is synthesised as a pre-polypeptide. *FEBS.* **207 (No. 1)**: 145-148.

Hanks, S. K., Calaib, M. B., Harper, M. C., and Patel, S. K. (1992). Focal adhesion protein-tyrosine kinase phosphorylated in response to cell spreading on fibronectin. *Proc. Natl. Acad. Sci. USA.* **89**: 8481-8491.

Hall, A. (1990). The cellular functions of small GTP-binding proteins. *Science.* **249**: 635-638.

Hall, A. (1992). Signal transduction through small GTPases: a tale of two GAPs. *Cell.* **69**: 389-391.

Hall, A. (1994). Small GTP-binding proteins and the regulation of the actin cytoskeleton. *Annu. rev. Cell Biol.* **10**: 31-54.

Hameed, A. H. M. (1988). Selection and characterisation of transformed BHK21 cells altered in responses to fibronectin. *Thesis.* University of Glasgow.

Hannigan, G. E., Leung-Hagestcijn, C., Fitz-Gibbon, L., Coppolino, M. G., Radeva, G., Filmus, J., Bell, J. C., and Dedhar, S. (1996). Regulation of cell adhesion and anchorage-dependent growth by a new β_1-integrin-linked protein kinase. *Nature.* **379**: 91-96.

Harper, P. A., and Juliano, R. L. (1980). Isolation and characterisation of Chinese Hamster Ovary cell variants defective in adhesion to fibronectin-coated collagen. *J. Cell Biol.* **87**: 755-763.

Hass, T. A., and Plow E. F. (1994). Integrin-ligand interactions: a year in review. *Curr. Opin. Cell Biol.* **6**: 656-662.

Hawkins, P. T., Eguinoa, A., Qiu, R. G., Stokoe, D., Cooke, F. T., Walters, R., Wennström, S., Claesson-Welsh, L., Evans, T., Symons, M., and Stephens, L. (1995). PDGF stimulates an increase in GTP-rac via activation of phosphoinositide 3-kinase. *Curr. Biol.* **5 (No. 4)**: 393-403.

Heath, J. P., and Holifield, B. F. (1993). On the mechanisms of cortical actin flow and its role in cytoskeletal organisation of fibroblasts. *SEB symposium.* **No. 47**: 35-71.

Hirokawa, N. (1994). Microtubule organisation and dynamics dependent on microtubule-associated proteins. *Curr. Opin. Cell Biol.* **6**: 74-81.

Hirst, R., Horwitz, A., Buck, C., and Rohrschneider, L. (1986). Phosphorylation of the fibronectin receptor complex in cells transformed by oncogenes that encode tyrosine kinases. *Proc. Natl. Acad. Sci. USA.* **83**: 6470-6474.

Hitt, A. L., and Luna, E. J. (1994). Membrane interactions with the actin cytoskeleton. *Curr. Opin. Cell Biol.* **6**: 120-130.

Hogg, N., Harvey, J., Cabanas, C., and Landis, R. C. (1993). Control of leukocyte integrin activation. *Am. Rev. Respir. Dis.* **148**: S55-S59.

Hogg, N., Landis, R. C., Bates, P. A., Stanley, P., and Randi, A. M. (1994). The sticking point: how integrins bind to their ligands. *Trends Cell Biol.* **4**: 379-382.

Hoyt, M. A. (1994). Cellular roles of Kinesin and related proteins. *Curr. Opin. Cell Biol.* **6**: 63-68.

Humphries, M. J. (1990). The molecular basis and specificity of integrin-ligand interactions. *J. Cell Sci.* **97**: 585-592.

Humphries, M. J. (1996). Integrin activation: the link between ligand binding and signal transduction. *Curr. Opin. Cell. Biol.* **8**: 632-640.

Humphries, M. J. , Akiyama, S. K., Komoriya, A., Olden, K., and Yamada, K. (1986). Identification of an alternatively spliced site in human plasma fibronectin that mediates cell type-specific adhesion. *J. Cell Biol.* **103**: 2637-2647.

Humphries, M. J. , Komoriya, A., Akiyama, S. K., Olden, K., and Yamada, K. (1987). Identification of two distinct regions of the type III connecting segment of human plasma fibronectin that promote cell type-specific adhesion. J. Biol. Chem. **262**: 6886-6892.

Hynes, R. O. (1990). Fibronectins, Springer series in molecular biology.

Hynes, R. O. (1992). Integrins: versatility, modulation, and signalling in cell adhesion. *Cell.* **69**: 11-25.

Hynes, R. O., Marcantonio, E. E., Stepp, M. A., Urry, L. A., and Yee, G. H. (1989). Integrin heterodimer and receptor complexity in avian and mammalian cells. J. Cell Biol. **109**: 409-420.

Izzard, C. S. (1988). A precursor of the focal contact in cultured fibroblasts. *Cell Motil. Cytosk.* **10**: 137-142.

Izzard, C. S., and Lochner, L. R. (1976). Cell to substrate contacts in living fibroblasts: an interference reflection study with an evaluation of the technique.

J. Cell Sci. **21**: 129-160.

Izzard, C. S., and Lochner, L. R. (1980): Formation of cell-to-substrate contacts during fibroblast motility: an interference reflection study. J. Cell Sci. **42**: 81-116.

Jay, P. Y., Pham, P. A., Wong, S. A., and Elson, E. L. (1995). A mechanical function of myosin II in cell motility. *J. Cell Sci.* **108**: 387-393.

Jochen, A. L., Hays, A., Mick, G. (1995). Inhibitory effects of cerulenin on protein palmitoylation and insulin internalisation in rat adipocytes. *Biochimica et Biophysica Acta.* **1259**: 65-72.

Jones, G. E., Arumugham, R. G., and Tanzer, M. L. (1986). Fibronectin glycosylation modulates fibroblast adhesion and spreading. *J. Cell Biol.* **103**: 1663-1670.

Joshi, H. C. (1994). Microtubule organising centres and γ-tubulin. *Curr. Opin. Cell Biol.* **6**: 55-62.

Kassner, P. D., Alon, R., Springer, T. A., and Hemler, M. E. (1995). Specialised functional properties of the integrin $\alpha 4$ cytoplasmic domain. *Mol. Cell Biol.* **6**: 661-674.

Kaufman, E., and Driscoll, B. F. (1992). Carbon dioxide fixation in neuronal and astroglial cells in culture. *J. Neurochem.* **58 (No. 1)**: 258-262.

Kim, H., and Schuetz, A. (1991). Regulation of nuclear membrane assembly and maintenance during in vitro maturation of mouse oocytes: role of pyruvate and protein synthesis. *Cell Tissue Res.* **265**: 105-112.

Klebe, R. J, Rosenburger, P. G., Naylor, S. L., Burns, R. L., Novok, R., and Kleinman, H. (1977). Cell attachment to collagen: isolation of a cell attachment mutant. *Exp. Cell Res.* **104**: 119-125.

Kleinman, H. K., and Weeks, B. S. (1989). Laminin: structure, functions and receptors. *Curr. Opin. Cell Biol.* **1**: 964-967.

Klymkowsky, M. W. (1995). Intermediate filaments: new proteins, some answers, more questions. *Curr. Opin. Cell Biol.* **7**: 46-54.

Kozma, R., Ahmed, S., Best, A., and Lim, L. (1995). The Ras-related protein Cdc42 and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts. *Mol. Cell. Biol.* **15 (No. 4)**: 1942-1952.

Lamarche, N., and Hall, A. (1994). GAPs for rho-related GTPases. *Trends in Genetics.* **10 (No. 12)**: 436-440.

Larson, R. S., Corbi, A. L., Berman, L., and Springer, T. (1989). Primary structure of the leukocyte function-associated molecule-1 α subunit: an integrin with an embedded domain defining a protein superfamily. *J. Cell Biol.* **108**: 703-712.

Lauffenburger, D. A., and Horwitz, A. F. (1996). Cell migration: a physically integrated molecular process. *Cell* **84**: 359-369.

Lee, G. (1993b). Non-motor microtubule-associated proteins. *Curr. Opin. Cell Biol.* **5**: 88-94.

Lee, J., Ishihara, A., and Jacobson, K. (1993a). The fish epidermal keratocyte as a model system for the study of cell locomotion. *SEB symposium.* **No. 47**: 73-89.

Loftus J. C., Plow, E. F., O'Toole, T. E., Glass, A., Frelinger, A. L., and Ginsberg, M. H. (1990). A $\beta 3$ integrin mutation abolishes ligand binding and alters divalent cation-dependent conformation. *Science.* **249**: 915-918.

Logan, D., Abu-Ghazaleh, R., Blakemore, W., Curry, S., Jackson, T., king, A., Lea, S., Lewis, R., Newman, J., and Parry, N. (1993). Structure of a major immunogenic site on Foot-and-Mouth disease virus. *Nature.* **362**: 566-568.

Ludueña, R. F., Banerjee, A., and Khan, I. A. (1992). Tubulin structure and biochemistry. *Curr. Opin. Cell Biol.* **4**: 53-57.

Machesky, L. M., and Hall, A. (1996). Rho: a connection between membrane receptor signalling and the cytoskeleton. *Trends in Cell Biol.* **6**: 304-308.

Marshall, J. F., Rutherford, D. C., McCartney, A. C. E., Mitjans, F., Goodman, J. F., and Hart, I. R. (1995). $\alpha_v \beta_1$ is a receptor for vitronectin and fibrinogen, and acts with $\alpha_5 \beta_1$ to mediate spreading on fibronectin. *J. Cell Sci.* **108**: 1227-1238.

Marya, P. K., Syed, Z., Fraylich, P. E., and Eagles, A.M. (1994). Kinesin and tau bind to distinct sites on microtubules. *J. Cell Sci.* **107**: 339-344.

Mayne, R., and Brewton, R. G. (1993). New members of the collagen superfamily. *Curr. Cell Biol.* **5**: 883-890.

Mays, R. W., Beck, K. A., and Nelson, W.J. (1994). Organisation and function of the cytoskeleton in polarised epithelial cells: a component of the protein sorting machinery. *Curr. Opin. Cell Biol.* **6**: 16-24.

McDonald, J. A. (1989). Matrix regulation of cell shape and gene expression. *Curr. Opin. Cell Biol.* **1**: 995-999.

McDonald, K. A., Lakonishok, M., and Horwitz, A. F. (1995). α_V and α_3 integrin subunits are associated with myofibrils during myofibrillogenesis. *J. Cell Sci.* **108**: 975-983.

McNamee, H. P., Ingber, D. E., and Schwartz, M. A. (1993). Adhesion to fibronectin stimulates inositol lipid synthesis and enhances PDGF-induced inositol lipid breakdown. *J. Cell Biol.* **121 (No. 3)**: 673-678.

Mercurio, A. M. (1990). Laminin: multiple forms, multiple receptors. *Curr. Opin. Cell Biol.* **2**: 845-849.

Meredith, J. E., Jr., Fazeli, B., and Schwartz, M. A. (1993). The extracellular matrix as a cell survival factor. *Mol. Biol. Cell.* **4**: 953-961.

Mosher, D. F. (1991). How do integrins integrate. *Curr. Biol.* **1 (No. 6)**: 394-396.

Moyle, M., Napier, M. A., and McLean, J. W. (1991). Cloning and expression of a divergent integrin subunit β_8. *J. Biol. Chem.* **266 (No. 29)**: 19650-19658.

Middleton, C.A., Brown, A. F., Brown, R. M., Karavanova, I. D., Roberts, D. J. H. and Vasiliev, J. M. (1989). The polarisation of fibroblasts in early primary cultures is independent of microtubule integrity. *J. Cell Sci.* **94**: 25-32.

Middleton, C.A., Brown, A. F., Brown, R. M., and Roberts, D. J. H. (1988). The shape of cultured epithelial cells does not depend on the integrity of their microtubules. *J. Cell Sci.* **91**: 337-345.

Miyamoto, S., Akiyama, S K., and Yamada, K. M. (1995). Synergestic roles for receptor occupancy and aggregation in integrin transmembrane function. *Science.* **267**: 883-885.

Miyamoto, S., Teramoto, H., Coso, O. A., Gutkind, J. S., Burbelo, P. D., Akiyama, S K., and Yamada, K. M. (1995). Integrin function: molecular hierarchies of cytoskeletal and signalling molecules. *J. Cell. Biol.* **131 (No. 3)**: 791-805.

Mueller, S. C., Kelly, T., Dai, M., and Chen, W. T. (1989). Dynamic cytoskeleton-integrin associations induced by cell binding to immobilised fibronectin. *J. Cell Biol.* **109 (No. 6, Pt. 2)**: 3455-3464.

Mueller, S. C., and Chen, W.-T. (1991). Cellular invasion into matrix beads: localisation of β_1 integrins and fibronectin to the invadopodia. *J. Cell Sci.* **99**: 213-225.

Müller, U., Bossy, B., Venstrom, K., and Reichardt, L. F. (1995). Integrin $\alpha_8\beta_1$ promotes attachment, cell spreading, and neurite outgrowth on fibronectin. *Mol. Biol. Cell.* **6**: 433-448.

Nachmias, V. T. (1993). Small actin-binding proteins: the β-thymosin family. *Curr. Opin. Cell biol.* **5**: 56-62.

Nermut, M. V., Green, N. M., Eason, P., Yamada, S. S., and Yamada, k. M. (1988). Electron microscopy and structural model of human fibronectin receptor. *EMBO J.* **7**: 4093-4099.

Newsholme, P., Gordon, S., and Newsholme, E. A. (1987). Rates of utilisation and fates of glucose, glutamine, pyruvate, fatty acids and ketone bodies by mouse macrophages. *Biochem J.* **242**: 631-636.

Nobes, C. D., Hawkins, P., Stephens, L., and Hall, A. (1995a). Activation of the small GTP-binding proteins rho and rac by growth factor receptors. *J. Cell Sci.* **108**: 225-233.

Nobes, C. D., and Hall, A. (1995b). Rho, Rac, and Cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibres, lamellipodia, and filopodia. *Cell.* **81**: 53-62.

Norton, E. K., and Izzard, C. S. (1982). Fibronectin promotes formation of the close cell-to-substrate contact in cultured cells. *Exp Cell Res.* **139**: 463-467.

Orlando, R. A., and Cheresh, D. A. (1991). Arginine-Glycine-Aspartic acid binding leading to molecular stabilisation between integrin $\alpha_v\beta_3$ and its ligand. *J. Biol. Chem.* **266 (No. 29)**: 19543-19550.

Otto, J. J. (1994). Actin-bundling proteins. *Curr. Opin. Cell Biol.* **6**: 105-109.

O'Toole, T. E., katagiri, Y., Faull, R. J., Peter, K., Tamura, R., Quaranta, V., Loftus, J. C., Shattil, S. J., and Ginsberg, M. H. (1994). Integrin cytoplasmic domains mediate inside-out signal transduction. *J. Cell Biol.* **124**: 1047-1059.

Palecek, S. P., Loftus, J. C., Ginsberg, M. H., Lauffenburger, D. A., and Horwitz, A. F. (1997). Integrin-ligand binding properties govern cell migration speed through cell substratum adhesiveness. *Nature*. **385**: 537-540.

Parker, P. J. (1995). PI 3-kinase puts GTP on the Rac. *Curr. Biol.* **5**: 577-579.

Parry, D. A. D., and Steinert, P. M. (1992). Intermediate filament structure. *Curr. Opin. Cell Biol.* **4**: 94-98.

Parsons, J. T. (1996). Integrin-mediated signalling: regulation by protein tyrosine kinases and small GTP-binding proteins. *Curr. Opin. Cell Biol.* **8**: 146-152.

Patterson, S. I., Skene, J. H. P. (1994). Novel inhibitory action of tunicamycin homologues suggests a role for dynamic protein fatty acylation in growth cone-mediated neurite extension. *J. Cell Biol.* **124**: 521-536.

Pilewski, J. M., and Albelda, S. M. (1993). Adhesion molecules in the lung. *Am. Rev. Respir. Dis.* **148**: S31-S37.

Potts, J. R., and Campbell, I. D. (1994). Fibronectin structure and assembly. *Curr. Opin. Cell Biol.* **6**: 648-655.

Pouyssegur, J. M., and Pastan, I. (1976). Mutant of mouse fibroblasts altered in the synthesis of cell surface glycoproteins. *J. Biol. Chem.* **252**: 1639-1646.

Prieto, A. L., Edelman, G. M., and Crossin, K. L. (1993). Multiple integrins mediate cell attachment to cytotactin / tenascin. *Proc. Natl. Acad. Sci. USA.* **90:** 10154-10158.

Pytela, R., Pierschbacher, M. D., and Ruoslahti, E. (1985). Identification and isolation of a 140 Kd cell surface glycoprotein with properties expected of a fibronectin receptor. *Cell.* **40:** 191-198.

Quilliam, L. A., Khosravi-Far, R., Huff, S. Y., and Der, C. J. (1995). Guanine nucleotide exchange factors: activators of the Ras superfamily of proteins. *BioEssays.* **17 (No. 5):** 395-404.

Rabb, H., Michishita, M., Sharma, C. P., Brown, D., and Arnout, M. A. (1993). Cytoplasmic tails of human complement receptor type 3(CR3, CD11b/CD18) regulate ligand avidity and internalisation of occupied receptors. *J. Immun.* **151:** 990-1002.

Redenbach, D. M., Richburg, J. H., and Boekelheide, K. (1994). Microtubules with altered assembly kinetics have a decreased rate of kinesin-based transport. *Cell Motil. Cytosk.* **27:** 79-87.

Reif, K., Nobes, C. D., Thomas, G., Hall, A., and Cantrell, D. A. (1996). Phosphatidylinositol 3-kinase signals activate a selective subset of Rac/Rho-dependent effector pathways. *Curr. Biol.* **6 (No. 11):** 1445-1455.

Reisler, E. (1993). Actin molecular structure and function. *Curr. Opin. Cell Biol.* **5:** 41-47.

Reszka, A. A., Hayashi, Y., and Horwitz, A. F. (1992). Identification of amino acid sequences in the integrin β1 cytoplasmic domain implicated in cytoskeletal association. *J. Cell Biol.* **117 (No. 6)**: 1321-1330.

Riberio, S. M. F., Schultz-Cherry, S., and Murphy-Ullrich, J. E. (1995). Heparin-binding vitronectin up-regulates TGF-β production by bovine aortic endothelial cells. *J. Cell Sci.* **108**: 1553-1561.

Richardson, A., and Parsons, J. T. (1995). Signal transduction through integrins: A central role for focal adhesion kinase ? *BioEssays.* **17 (No. 3)**: 229-236.

Ridley, A. J. (1994). Membrane ruffling and signal transduction. *BioEssays.* **16 (No. 5)**: 321-327.

Ridley, A. J. (1996). Rho: theme and variations. *Curr. Biol.* 6 **(No. 10)**: 1256-1264.

Ridley, A. J., and Hall, A. (1992a). The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibres in response to growth factors. *Cell.* **70**: 389-399.

Ridley, A. J., and Hall, A. (1992b). The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell.* **70**: 401-410.

Ridley, A. J., and Hall, A. (1994). Signal transduction pathways regulating Rho-mediated stress fibre formation: requirement for a tyrosine kinase. *EMBO J.* **13 (No. 11)**: 2600-2610.

Rinnerthaler, G., Geiger, B., and Small, J. V. (1988). Contact formation during fibroblast locomotion: involvement of membrane ruffles and microtubules. *J. Cell Biol.* **106**: 747-760.

Roche, W. R., Montefort, S., Baker, J., and Holgate, S. T. (1993). Cell adhesion molecules and the bronchial epithelium. *Am. Rev. Respir. Dis.* **148**: S79-S82.

Roman, J., LaChance, R. M., Broekelmann, T. J., Kennedy, C. J. R., Wayner, E. A., Carter, W. G., and McDonald, J. (1989). The fibronectin receptor is organised by extracellular matrix fibronectin: Implications for oncogenic transformation and for cell recognition of fibronectin matrices. *J. Cell Biol.* **108**: 2529-2543.

Roskelley, C. D., Desprez, P. Y., and Bissell, M. J. (1994). Extracellular matrix dependent tissue-specific gene expression in mammary epithelial cells requires both physical and biochemical signal transduction. *Proc. Natl. Acad. Sci. USA.* **91**: 12378-12382.

Rosok, M. J., and Rohrschneider, L. R. (1983). Increased phosphorylation of vinculin on tyrosine does not occur during the release of stress fibers before mitosis in normal cells. *Mol. Cell Biol.* 3 (**No. 3**): 475-479.

Ruoslahti, E. (1988). Fibronectin and its receptors. *Ann. Rev. Biochem.* **57**: 375-413.

Ruoslahti, E. (1991). Integrins as receptors for extracellular matrix. Second edition, edited by Elizabeth D. *Hay Plenum press.* New York.

Ruoslahti, E., and Pierschbacher M. D. (1987). New perspective in cell adhesion. *Science.* (Wash. DC), **238**: 491-497.

Ruoslahti, E., and Reed, J. C. (1994). Anchorage dependence, integrins, and apoptosis. *Cell.* **77**: 477-478.

Ruppel, K. M., and Spudich, J. A. (1995). Myosin motor function: structural and mutagenic approaches. *Curr. Opin. Cell Biol.* **7**: 89-93.

Sarwar, M., and Whish, W. J. D. (1990). Mammalian cell adhesion on protein-modified surfaces and selection of adhesion mutants. *Biochemical Society Transactions.* **18 (No. 5)**: 1015.

Sastry, S. K., and Horwitz, A. F. (1993). Integrin cytoplasmic domains: mediators of cytoskeletal linkages and extra- and intracellular initiated transmembrane signalling. *Curr. Opin. Cell Biol.* **5**: 819-831.

Schaller, M. D., Borgman, C. A., Cobb, B. S., and Vines, R. R. (1992a). pp125^{FAK}, a structurally distinctive protein-tyrosine kinase associated with focal adhesions. *Proc. Natl. Acad. Sci. USA.* **89**: 5192-5196.

Schaller, M. D., and Parsons, J. Th. (1994). Focal adhesion kinase and associated proteins. *Curr. Opin. Cell Biol.* **6**: 705-710.

Schlaepfer, D. D., Hanks, S. K., Hunter, T., and Geer, P. V. D. (1994). Integrin-mediated signal transduction linked to Ras pathway by Grb2 binding to focal adhesion kinase. *Nature.* **372**: 786-791.

Schnapp, L. M., Breuss, J. M., Ramos, D. M., Sheppard, D., and Pytela, R. (1995). Sequence and tissue distribution of the human integrin α_8 subunit: a β_1-associated α subunit expressed in smooth muscle cells. *J. Cell Sci.* **108**: 537-544.

Schroer, T. A. (1994). Structure, function and regulation of cytoplasmic dynein. *Curr. Opin. Cell Biol.* **6**: 69-73.

Schwartz, M. A., Brown, E. J., and Fazeil, B. (1993). A 50 kDa integrin-associated protein is required for integrin-regulated calcium entry in endothelial cells. *J. Biol. Chem.* **268**: 19931-19934.

Schwartz, M. A., Ingber, D. E. (1994). Integrating with integrins. *Mol. biol. Cell.* **5**: 389-393.

Schwartz, M. A, and Lechene, C., and Ingber, D. E. (1991). Insoluble fibronectin activates the Na/H antiporter by clustering and immobilising integrin α5β1, independent of cell shape. *Proc. Natl. Acad. Sci. USA.* **88**: 7849-7853.

Schwartz, M. A, and Lechene, C. (1992). Adhesion is required for protein kinase C-dependent activation of the Na^+ /H^+ antiporter by platelet-derived growth factor. *Proc. Natl. Acad. Sci. USA.* **89**: 6138-6141.

Schwartz, M. A., Schaller, M. D., and Ginsberg, M. H. (1995). Integrins: emerging paradigms of signal transduction. *Annu. Rev. Cell Biol.* **11**: 549-600.

Schwarzbauer, J. E. (1991). Fibronectin: from gene to protein. *Curr. Opin. Cell Biol.* **3**: 786-791.

Schwarzbauer, J. E., Mulligan, R. C., and Hynes, R. O. (1987). Efficient and stable expression of recombinant fibronectin polypeptides. *Proc. Natl. Acad. Sci. USA.* **84**: 754-758.

Selak, V., Skaper, S. D., and Varon, S. (1985). Pyruvate participation in the low molecular weight trophic activity for central nervous system neurons in glia-conditioned media. *J Neuroscience.* **5 (No. 1)**: 23-28.

Shattil, S. J., Ginsberg, M. H., Brugge, J. S. (1994). Adhesive signalling platelets. *Curr. Opin. Cell Biol.* **6**: 695-704.

Sheppard, D. (1993). Identification and characterisation of novel airway epithelial integrins. *Am. Rev. Respir. Dis.* **148**: S38-S42.

Shimizu, Y., Mobley, J. L., Finkelstein, L. d., and Chan, S. H. (1995). A role for phosphatidylinositol 3-kinase in the regulation of β_1 integrin activity by the CD2 antigen. *J. Cell Biol.* **131 (No. 2)**: 1867-1880.

Sens, D. A., Hochstadt, B., and Amos, H. (1982). Effects of pyruvate on the growth of normal and transformed Hamster embryo fibroblasts. *J. Cellular Physiol.* **110**: 329-335.

Singer, I. I. (1979). The fibronexus: a transmembrane association of fibronectin containing fibers and bundles of 5 nm microfilaments in hamster and human fibroblasts. *Cell.* **16**: 675-685.

Singer, I. I. (1982). Association of fibronectin and vinculin with focal contacts and stress fibers in stationary hamster fibroblasts. *J. Cell Biol.* **92**: 398-408.

Singer, I. I., and Paradiso, P. R. (1981). A transmembrane relationship between fibronectin and vinculin (130 kD protein): serum modulation in normal and transformed hamster fibroblasts. *Cell.* **24**: 481-492.

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Singer, I. I., Scott, S., Kawka, D. W., Kazazis, D. M., Gailit, J., and Ruoslahti, E. (1988). Cell surface distribution of fibronectin and vitronectin receptors depends on substrate composition and extracellular matrix accumulation. *J. Cell Biol.* **106**: 2171-2182.

Small, J. V. (1995). Getting the actin filaments straight: nucleation-release or treadmilling. *Trend. Cell Biol.* **5**: 52-55.

Small, J. V., Rohlfs, A., and Herzog, M. (1993). Actin and cell movement 1993. *SEB symposium* **No. 47**: 57-71.

Smith, C. H., Barker, J. N. W. N., and Lee, T. H. (1993). Adhesion molecules in allergic inflammation. *Am. Rev. Respir. Dis.* **148**: S75-S78.

Solowska, J., Edelman, J. M., Albeda, S. M., and Buck, C. A. (1991). Cytoplasmic and transmembrane domains of integrin β_1 and β_3 subunits are functionally interchangeable. *J. Cell Biol.* **114**: 1079-1088.

Sonnenberg, A., Calafat, J., Janssen, H., Daams, H., van der Raaij-Helmer, L. M. H., Falcioni, R., Kennel, S., Aplin, J. D., Baker, J., Loizidou, M., and Garrod, D. (1991). Integrin α_6/β_4 complex is located in hemidesmosomes, suggesting a major role in epidermal cell-basement membrane adhesion. *J. Cell Biol.* **113**: 907-917.

Sorrentino, J. A., Millis, A. J. T, Whipple, A. P., and Colquhoun, W. R. (1983). Lymphoblastoid cell variant in contact-mediated cell spreading. *Exp. Cell Res.* **145**: 369-379.

Springer, T. A. (1990). Adhesion receptors of the immune system. *Nature*. **346**: 425-434.

Stewart, M. (1993). Intermediate filament structure and assembly. *Curr. Opin. Cell Biol.* **5**: 3-11.

Stoker, M. O., and Macpherson, I. (1964). Syrian hamster fibroblast cell line BHK21 and its derivatives. *Nature*. **203**: 1355-1357.

Stossel, T. P. (1993). On the crawling of animal cells. *Science*. **260**: 1086-1094.

Strohmeier, R., and Bereiter-Hahn, J. (1984). Control of cell shape and locomotion by external calcium. *Exp. Cell Res.* **154**: 412-420.

Symington, B. E. (1990). Fibronectin receptor overexpression and loss of transformed phenotype in a stable variant of the K562 cell line. *Cell Regulation*. **1**: 637-648.

Symington, B. E., and Carter, W. G. (1995). Modulation of epidermal differentiation by epiligrin and integrin $\alpha_3\beta_1$. *J. Cell Sci.* **108**: 831-838.

Svitkina, T. M., Neyfakh, A. A., and JR, (1986). Actin cytoskeleton of spread fibroblasts appears to assemble at the cell edges. *J. Cell Sci.* **82**: 235-248.

Tanaka, J., Watanabe, T., Nakamura, N., and Sobue, K. (1993). Morphological and biochemical analysis of contractile proteins actin, myosin, caldesmon and tropomyosin in normal and transformed cells. *J. Cell Sci.* **104**: 595-606.

Tanzer, M. L. (1989). Collagens and elastin: structure and interactions. *Curr. Opin. Cell Biol.* **1**: 968-973.

Tapon, N., and Hall, A. (1997). Rho, Rac and Cdc42 GTPases regulate the organisation of the actin cytoskeleton. *Curr. Opin. Cell Biol.* **9**: 86-92.

Theriot, J. A., and Mitchison, T. J. (1991). Actin microfilament dynamics in locomoting cells. *Nature.* **352**: 126-131.

TII, L., Rosales, C., Mondal, K., Bolen, J. B., Haskill, S., and Juliano, R. L. (1995). Integrin-mediated tyrosine phosphorylation and cytokine message induction in monocyte cells. *J. Biol. Chem.* **270**: 16189-16197.

Titus, M. A. (1993). Myosins. *Curr. Opin. Cell Biol.* **5**: 77-81.

Tomita, Y., Abraham, S., Noda, C., and Ichihara, A. (1993). Pyruvate stimulates hormonal induction of lipogenic enzymes in primary cultured rat hepatocytes. *Biochemica et Biophysica Acta.* **1170**: 253-257.

Tomita, Y., Yuasa, C., Ni R., Ishimura, K., and Ichihara, A. (1995). Long-term maintenance of functional rat hepatocytes in primary culture by additions of pyruvate and various hormones. *Biochemica et Biophysica Acta.* **1243**: 329-335.

Tryggvason, K. (1993). The laminin family. *Curr Opin. Cell Biol.* **5**: 877-882.

Tuckwell, D. S, Calderwood, D. A., Green, L. J., and Humphries, M. J. (1995). Integrin α_2 I-domain is a binding site for collagens. *J. Cell Sci.* **108**: 1629-1637.

Tuckwell, D. S, Weston, S. A., and Humphries, M. J. (1993). Integrins: a review of their structure and mechanisms of ligand binding. *SEBsymposium*. **No. 47**: 107-136.

Turner, C. E. and Burridge, K. (1991). Transmembrane molecular assemblies in cell-extracellular matrix interactions. *Curr. Opin. Cell Biol.* **3**: 849-853.

Ugarova, T. P., Budzynski, A. Z., Shattil, S. J., Ruggeri, Z. M., Ginsberg, M. H., and Plow, E. F. (1993). Conformational changes in fibrinogen elicited by its interaction with platelet membrane glcoprotein GPIIb-IIIa. *J. Biol. Chem.* **268 (No. 28)**: 21080-21087.

Underwood, P. A., and Bennett, F. A. (1989). A comparison of the biological activities of the cell-adhesive proteins vitronectin and fibronectin. *J. Cell Sci.* **93**: 641-649.

Vandekerckhove, J., and Vancompernolle, K. (1992). Structural relationships of actin-binding proteins. *Curr. Opin. Cell Biol.* **4**: 36-42.

Varner, J. A., Emerson, D. A., and Juliano, R. L. (1995). Integrin $\alpha_5\beta_1$ expression negatively regulates cell growth: reversal by attachment to fibronectin. *Mol. Biol. Cell.* **6**: 725-740.

Vartio, T., Laitinen, L., Närvänen, O., Cutolo, M., Thornell, L. E., Zardi, L., and Virtanen, I. (1987). Differential expression of the ED sequence-containing form of cellular fibronectin in embryonic and adult human tissues. *J. Cell Sci.* **88**: 419-430.

Vasiliev, J. M. (1985). Spreading of non-transformed and transformed cells. *Biochimica et Biophysica Acta*. **780**: 21-65.

Veneziale, C. M., and Gabrielli, F. (1969). Separation and identification of ^{14}C-labelled glucogenic compounds by paper chromatography. *Anal. Biochem.* **28**: 206-215.

Venstrom, K., and Reichardt, L. (1995). β_8 integrins mediate interactions of Chick sensory neurons with laminin-1, collagen IV, and fibronectin. *Mol. Biol. Cell.* **6**: 419-431.

Vikstrom, K. L., Borisy, G. G., and Goldman, R. D. (1989). Dynamic aspects of intermediate filament networks in BHK-21 cells. *Proc. Natl. Acad. Sci. USA.* **86**: 549-553.

Villaba, M., Martinez-Serrano, A., Gomez-Puertas, P., Blanco, P., Börner, C., Villa, A., Casado, M., Gimenez, C., Pereira, R., Bogonez, E., Pozzan, T., and Satrustegui, J. (1994). The role of pyruvate in neuronal calcium homeostasis. *J. Biol. Chem.* **269** (**No. 4**): 2468-2476.

Vogel, B. E., Tarone, G., Giancotti, F. G., Gailit, J., and Ruoslahti, E. (1990). A novel fibronectin receptor with unexpected subunit composition ($\alpha_v\beta_1$). *J. Biol. Chem.* **265** (**No. 11**): 5934-5937.

Vuori, K., and Ruoslahti, E. (1993). Activation of protein kinase C precedes $\alpha_5\beta_1$ integrin-mediated cell spreading on fibronectin. *J. Biol. Chem.* **268** (**No. 29**): 21459-21462.

Wang, Y. L. (1985). Exchange of actin subunits at the leading edge of living fibroblasts: possible role of treadmilling. *J. Cell Biol.* **101**: 597-602.

Watt, F. M., and Hodivala, K. J. (1994). Fibronectin and integrin knockouts come unstuck. *Curr. Biol.* **4 (No. 3)**: 270-272.

Wasilenko, W. J., and Marchok, A. C. (1984). Pyruvate regulation of growth and differentiation in primary cultures of rat tracheal epithelial cells. *Exp. Cell Res.* **155**: 507-517.

Wayner, E. A., Carter, W., Piotrowicz, R. S., and Kunicki, T. (1988). The function of multiple extracellular matrix receptors in mediating cell adhesion to extracellular matrix: preparation of monoclonal antibodies to the fibronectin receptor that specifically inhibit cell adhesion to fibronectin and react with platelet glycoproteins Ic-IIa. *J. Cell Biol.* **107**: 1881-1891.

Wayner, E. A., Garcia-Pardo, A., Humphries, M. J., McDonald, J. A., and Carter, W. G. (1989). Identification and characterisation of the T lymphocyte adhesion receptor for an alternative cell attachment domain (CS-1) in plasma fibronectin. *J. Cell Biol.* **109**: 1321-1330.

Wayner, E. A., Gil, S. G., Murphy, G. F., Wilke, M. S., and Carter, W. G. (1993). Epiligrin, a component of epithelial basement membranes, is an adhesive ligand for $\alpha 3 \beta 1$ positive T lymphocytes. *J. Cell Biol.* **121**: 1141-1152.

Wayner, E. A., Orlando, R. A., Cheresh, D. A. (1991). Integrins $\alpha_v \beta 3$ and $\alpha_v \beta 5$ contribute to cell attachment to vitronectin but differentially distribute on the cell surface. *J. Cell Biol.* **113**: 919-929.

Wayner, E. A., and Kovach, N. L. (1992). Activation-dependent recognition by haematopoietic cells of LDV sequence in the V region of fibronectin. *J. Cell Biol.* **116**: 489-497.

Weber, I., Wallraff, E., Albrecht, R., and Gerisch, G. (1995). Motility and substratum adhesion of *Dictyostelium* wild-type and cytoskeletal mutant cells: a study by RICM/ bright-field double-view image analysis. *J. Cell Sci.* **108**: 1519-1530.

Weeds, A., and Maciver, S. (1993). F-actin capping proteins. *Curr. Opin. Cell Biol.* **5**: 63-69.

Welch, M. D., Holtzman, D. A., and Drubin, D. G. 1994. The yeast actin cytoskeleton. *Curr. Opin. Cell Biol.* **6**: 110-119.

Wennström, S., Hawkins, P., Cooke, F., Hara, K., Yonezawa, K., Kasuga, M., Jackson, T., Claesson-Welsh, L., and Stephens, L. (1994). Activation of phosphoinositide 3-kinase is required for PDGF-stimulated membrane ruffling. *Curr. Biol.* **4** (**No. 5**): 385-393.

Weskamp, G., and Blobel, C. P. (1994). A family of cellular proteins related to snake venom disintegrins. *Proc. Natl. Acad. Sci. USA.* **91**: 2748-2751.

Williams R., and Coluccio, L. M. (1994). Novel 130-kDa rat liver myosin-1 will translocate actin filaments. *Cell Motil. Cytosk.* **27**: 41-48.

Woods, A., and Couchman, J. R. (1992). Protein kinase C involvement in focal adhesion formation. *J. Cell Sc.* **101**: 277-290.

Wu, C., Fields, A. J., Kapteijn, B. A. E., and J. A. McDonald, (1995). The role of $\alpha_4\beta_1$ in integrin in cell motility and fibronectin matrix assembly. *J. Cell Sci.* **108**: 821-829.

Wu, C., Chung, A. E., and McDonald, J. A. (1995). The role of $\alpha_3\beta_1$ integrins in extracellular matrix assembly. *J. Cell Sci.* **108**: 2511-2523.

Yamada, K. M. (1989). Fibronectins: structure, functions and receptors. *Curr. Opin Cell Biol.* **1**: 956-963.

Yamada, K. M. (1991). Cell biology of extracellular matrix, second edition, edited by Elizabeth D. Hay, *Plenum Press*. New York.

Yamada, K. M., Kennedy, D. W., Yamada, S. S., Gralnick, H., Chen, W.-T, and Akiyama, S. K. (1990). Monoclonal antibody and synthetic peptide inhibitors of human tumour cell migration. *Cancer Res.* **50**: 4485-4496.

Yamada, Y., and Kleinmann, H. K. (1992). Functional domains of cell adhesion molecules. *Curr. Opin. Cell Biol.* **4**: 819-823.

Yamada, K. M., and Miyamoto, S. (1995). Integrin transmembrane signalling and cytoskeletal control. *Curr. Opin. Cell. Biol.* **7**: 681-689.

Yu, F. X., Lin, S. C., Morrison-Bogorad, M., and Yin, II. L. (1994). Effects of thymosin β_4 and thymosin β_{10} on actin structures in living cells. *Cell Motil Cytsk.* **27**: 13-25.

Yurchenco, P. D., and O'Rear, J. (1994). Basal lamina assembly. *Curr. Opin. Cell Biol.* **6**: 674-681.

Zerlauth, G., Wesierska-Gadek, J., and Sauermann, G. (1988). Fibronectin observed in the nuclear matrix of HeLa tumour cells. *J. Cell Sci.* **89**: 415-421.

Zetter, B. R., and Brightman, S. E. (1990). Cell motility and the extracellular matrix. *Curr. Opin. Cell Biol.* **2**: 850-856.

Zheng, Y., Cerione, R., and Bender A. (1994). Control of the yeast bud-site assembly GTPase Cdc42. *J. Biol. Chem.* **269 (No. 4)**: 2369-2372.

Zigmond, S. H. (1996). Signal transduction and actin filament organisation. *Curr. Opin. Cell Biol.* **8**: 66-73.

Zot, H. G., Doberstein, S. K., and Pollard, T. D. (1992). Myosin-I moves actin filaments on a phospholipid substrate: Implications for membrane targeting. *J. Cell Biol.* **116**: 367-376.