A Proteomic Analysis of Raf-1 signalling Pathways

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Abstract

Raf-1 is an integral part of the Ras/Raf/MEK/ERK signalling pathway that relays mitogenic and survival signals to the nucleus, leading to suppression of apoptosis, growth and proliferation. The ability of Raf-1 to fulfil these diverse functions is likely to require its ability to form multiple complexes which are regulated by phosphorylation and sub-cellular localisation. The aim of the work described in this thesis was to develop proteomic approaches to study dynamic changes in Raf-1 signalling complexes in vivo.

Recent studies have indicated that Raf-1 not only plays a crucial role in relaying growth signals through the MAPK pathway but it also has a kinase independent function in protecting cells from apoptosis. To characterise this anti-apoptotic role of Raf-1, DIGE (different gel electrophoresis) was used to determine changes in protein expression resulting from the reconstitution of Raf-$1^{-/-}$ fibroblasts with kinase-dead or wild-type Raf-1. Numerous pro- and anti-apoptotic proteins were differentially expressed between these three cell lines. Furthermore levels of several potential Raf-1 binding proteins and proteins involved in actin dynamics and motility were altered. The changes in protein expression identified by proteomic analysis are consistent with the hypersensitivity of Raf-$1^{-/-}$ fibroblasts to apoptotic stimuli and the alterations in their actin cytoskeleton and motility.

In addition to examining global protein changes by DIGE, we developed a gel-less, mass spectrometry based two-dimensional liquid chromatography approach to analyse protein samples of medium complexity and successfully adapted it to identify Raf-1 protein complexes. We identified over 100 potential Raf-1 interacting partners, some of which required the presence of S259 site to bind to Raf-1 whereas others interacted only when cells were induced with mitogens or apoptotic insults. Two of these interactions (protein phosphatase 5 and SIP1) were verified by co-immunoprecipitations and functional analysis was performed on one of these proteins. Furthermore we developed a highly sensitive and

robust method for phosphorylation site mapping and demonstrated its potential by identifying phosphorylation sites of proteins present in Raf-1 complexes.

Raf-1 activation is a complex process in which phosphorylation plays a key role. While several kinases have been shown to phosphorylate Raf-1, only one phosphatase, PP2A, has been identified to regulate Raf-1 activity in vivo by dephosphorylating serine 259 upon activation with mitogens. We identified protein phosphatase 5 (PP5) as a potential Raf-1 interacting protein. This interaction was validated by co-immunoprecipitation of endogenous Raf-1 with PP5 in COS-1 cells. PP5 dissociated from Raf-1 upon stimulation with EGF and specifically dephosphorylated a crucial activation site, S338, in-vivo and in-vitro. Furthermore we provide evidence that cross talk between the MAPK pathway and the Gα12 pathway is regulated by PP5.

The results presented here show that we have developed robust proteomic methods enabling us to analyse protein complexes as well as changes in protein expression and phosphorylation with high sensitivity and accuracy.

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Declaration: *The work presented in this thesis is my own work unless otherwise stated.*

To my parents

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List of Abbreviations

2D-LC:	Two dimensional liquid chromatography
2D-PAGE:	Two dimensional PAGE
ALG2:	apoptosis-linked gene 2
ALIX:	ALG2 interacting protein X
APS:	Ammonium Persulphate
ASK1:	Apoptosis signal-regulating kinase 1
BAD:	BCL2 Antagonist of cell Death
BSA:	Bovine Serum Albumin
cAMP:	cyclic Adenosine Mono Phosphate
CNK:	Connector enhancer of ksr
CRD:	Cysteine Rich Domain
DAPI:	4'6-Diamidino-2-phenylindole dihydrochloride
DIGE:	Different image gel electrophoresis
DMEM:	Dulbecco's Modified Eagle's Medium
DNA:	DeoxyriboNucleic Acid
DTT:	DiThioThreitol
ECL:	Enhanced ChemiLuminescence
E.coli:	Escherichia coli
EDTA:	Diaminoethanetetra-acetic acid
EGF:	Epidermal Growth Factor
ESI:	Electrospray ionisation
ERK:	Extracellular signal-Regulated Kinase/MAPK
FACS:	Fluorescence-Assisted Cell Sorting
FCS:	Foetal Calf Serum
GAP:	GTPase Activating Protein
GDP:	Guanosine DiPhosphate
GEF:	GTP Exchange Factor
G-Protein:	GTP-binding protein
GRB2:	Growth factor Receptor-Bound protein2
GST:	Glutathione S-Transferase
GTP:	Guanosine Tri Phosphate
HSP90:	Heat Shock Protein 90
IDA:	Iminodiacetic acid
IP:	ImmunoPrecipitate
kD:	Kilodalton
KSR:	Kinase Suppressor of Ras
$MgCl_2$:	Magnesium Chloride
MALDI	Matrix assisted laser desorption ionisation
MAPK:	Mitogen Activated Protein Kinase/ERK
MAPKK:	MAPK Kinase/MEK
MAPKKK:	MAPK Kinase Kinase/Raf
MBP:	Myelin Basic Protein
MEF:	Mouse embryonic fibroblast
MEK:	MAPK Kinase/ERK Kinase
mg:	milligram
min(s):	minute(s)

ml:	millilitre
mM:	millimolar (millimoles per litre)
mRNA:	messenger RNA
MST2:	Mammalian Sterile20-like kinase 2
n:	nano
ng:	Nanogram
NF-κB:	Nuclear Factor-kappa B
NTA:	Nitrilotriacetic acid
PAGE:	PolyAcrylamide Gel Electrophoresis
PAK:	p21 Activated Kinase
PBS:	Phosphate Buffered Saline
PD:	Phosphatase dead
PDGF:	Platelet-Derived Growth Factor
PI3-kinase:	Phosphatidylinositol triphosphate kinase
PKA:	Protein Kinase A
PKB:	Protein Kinase B/Akt
PKC:	Protein Kinase C
PP2A:	Protein Phosphatase 2A
PP5:	Protein phosphatase 5
PVDF:	PolyVinylidene DiFluoride
RACK1:	Receptor of active PKC
Rb:	Retinoblastoma protein
RBD:	Ras Binding Domain
RKIP:	Raf Kinase Inhibitor Protein
RNA:	RiboNucleic Acid
RTK:	Receptor Tyrosine Kinase
rpm:	revolutions per minute
SDS:	Sodium Dodecyl Sulphate
sec(s):	seconds
Sf9:	Spodoptera frugiperda 9
SIP1:	SIAH interacting protein
SoS:	Son Of Sevenless
TBS:	Tris Buffered Saline
TCA:	Trichloricacetic acidμ
TCF:	T-cell Factor
TEMED:	N,N,N',N'-Tetraamethylethylenediamine
TOF:	Time of flight
Tris:	Tris (hydroxymethyl) aminomethane
Tween 20:	Polyoxyethylene sorbitan monolaurate
TPA:	O-Tetradecanoylphorbol 13-acetate
VDAC:	Voltage-dependent anion channel
WT:	Wild Type

μg:	microgram
μl:	microlitre
°C:	degrees centigrade

Single letter amino acid code

Alanine:	Ala (A)
Arginine:	Arg (R)
Asparagine:	Asn (N)
Aspartic Acid:	Asp (D)
Cysteine:	Cys (C)
Glutamic Acid:	Glu (E)
Glutamine:	Gln (Q)
Glycine:	Gly (G)
Histidine:	His (H)
Isoleucine:	Ile (I)
Leucine:	Lue (L)
Lysine:	Lys (K)
Methionine:	Met (M)
Phenylalanine:	Phe (F)
Proline:	Pro (P)
Serine:	Ser (S)
Threonine:	Thr (T)
Tryptophan:	Trp (W)
Tyrosine:	Tyr (Y)
Valine:	Val (V)

Chapter 1:

Introduction

1.1 Raf isoforms

Mitogen activated protein kinase (MAPK) pathways play an integral part in relaying extracellular signals to the nucleus and other intracellular destinations. MAPK pathways can be found in all eukaryotic organisms and consist of kinase modules organised in a three-tiered cascade (reviewed in (Garrington and Johnson, 1999; Schaeffer and Weber, 1999; Widmann et al., 1999). This setup not only ensures the amplification of the signal but additionally facilitates regulation and fine-tuning of the signal outcome, by providing interfaces for additional regulation of signal duration, amplitude and kinetics. The basic module consists of a small G-protein located at the membrane that upon activation recruits and induces activation of a MAP kinase kinase kinase (MAPKKK) which then itself binds to a MAP kinase kinase (MAPKK) and activates it through phosphorylation. MAPKK then phosphorylates MAP kinase (MAPK). Raf is a MAPKKK and functions downstream of Ras as the G-protein and upstream of MEK as MAPKK and ERK as MAPK. The Ras/Raf/MEK/ERK pathway mainly regulates proliferation and differentiation pathways through activation of ERK which itself has the ability to phosphorylate either cytoplasmic substrates, such as RSK, or can translocate to the nucleus where it can activate transcription factors. Not surprisingly hyperactivation of this pathway leads to unchecked proliferation and differentiation, and occurs in approximately 30% of human tumours (reviewed in (Bos, 1989; Katz and McCormick, 1997; Lowy and Willumsen, 1993) Consistent with this observation mutational activation of members of this cascade can induce transformation of cells in tissue culture.

Evolution provided mammalian organisms with three distinctive Raf isoforms compared with only one in Drosophila and C. elegans. All three isoforms A-Raf, B-Raf and Raf-1 share a common structure and large parts of the proteins are conserved throughout the isoforms; see (Kolch, 2000; Wellbrock et al., 2004) for reviews. The conserved structure

consists of the conserved regions, CR1, CR2 and CR3. CR1 and CR2 lie in the regulatory domain and CR3 encodes the C-terminal kinase domain. The regulatory domain also includes the Ras binding domain (RBD) and a cysteine rich domain (CRD) both of which are essential for Raf/Ras binding. The CRD has been shown to bind to a multitude of proteins in a large scale yeast-two-hybrid screen, making this domain an important hub for protein-protein interactions (Yuryev and Wennogle, 2003). Remarkably, despite the high degree of conservation in this domain several isoform-specific interactions were found.

Raf proteins have been found to be strongly regulated by multisite phosphorylation. Several of these sites are conserved throughout the family, suggesting a common regulatory mechanism, while others are not conserved suggesting at least a degree of differential regulation. B-Raf is subject to multiple splicing resulting in the expression of isoforms with a mass range of 70 to 95 kDa (Barnier et al., 1995), whereas no alternative splice variants of A-Raf and Raf-1 have been reported. A-Raf has a molecular weight of 68kDa and Raf-1 of 75kDa. mRNA studies suggested that Raf-1 is ubiquitously expressed, while A-Raf and B-Raf showed are more restricted expression pattern (Storm et al., 1990). B-Raf has been found expressed at high levels in neuronal cells but lower levels of B-Raf are expressed in wide range of other tissues. A-Raf mRNA is especially high in urogenital organs, although protein expression studies have shown that it is fairly ubiquitous in all tissues (Luckett et al., 2000).

All three isoforms share Ras as a common activator and all three can activate MEK, although with varying efficiencies. B-Raf is by far the best MEK kinase followed by Raf-1 and A-Raf (Papin et al., 1996). Despite having a common activator and substrate all three isoforms are essential for mouse development. A-Raf deficient mice are born but, depending on the background, die because of intestinal and neurological defects (Pritchard et al., 1996). Raf-1 knock-out mice die in midgestation due to massive apoptosis in the liver and placenta (Huser et al., 2001; Mikula et al., 2001) and B-Raf knock-outs die of

vascular defects in midgestation (Wojnowski et al., 1997). Interestingly MEK and ERK activation in response to mitogens in not impaired in either the A-Raf or the Raf-1 knock-out, whereas MEK activation is severely impaired in the B-Raf knock-out mice. This finding indicates that B-Raf might be the main activator of the MEK/ERK pathway whereas Raf-1 and A-Raf may perform other tasks. The phenotype of the Raf-1 knock-out mice and newly discovered anti-apoptotic functions of Raf indicate that Raf-1's main function may not be MEK activation, but protection from apoptosis (see Raf and apoptosis) (Baccarini, 2002).

1.2 Activation of the Raf-1 pathway

Raf proteins are activated by numerous mitogens and growth factors such as EGF, serum, PDGF and NGF. Activation of the pathway occurs upon binding of the mitogens to specific cell surface receptors. In the case of receptor tyrosine kinases this induces oligomerisation that results in activation of their tyrosine kinase activity through inter molecular tyrosine phosphorylation. These tyrosine phosphorylations sites create protein-protein docking sites for adaptor proteins containing SH2 and SH3 domains (Li et al., 1993; Rozakis-Adcock et al., 1993). These newly attached adaptor proteins, such as Grb-2, have the ability to recruit RasGEFs (Guanosine nucleotide Exchange Factors) such as SOS, Cdc25 and RasGRF (Guanosine nucleotide Release Factor) to the membrane, which stimulate the release of bound GDP on Ras, thus creating a binding pocket for either GTP or GDP (Egan et al., 1993). Due to the much higher concentrations of GTP in the cell, GTP binding is favoured. It induces a conformational change in the effector binding region of Ras facilitating the interaction with effectors such as Raf-1 and so initiating a signalling cascade resulting in phosphorylation of ERK (Wittinghofer and Nassar, 1996). Active ERK can then in turn terminate Ras activation by phosphorylating the GEF SOS resulting in disassembly of the Grb-2/GEF complex and release of SOS back into the cytosol, thus terminating the activation of Ras (Buday et al., 1995; Cherniack et al., 1995; Waters et al.,

1995a; Waters et al., 1995b) . Beside ERK other kinases that are regulated by MAPK-signalling have also been identified to exert a similar negative feed-back effect on SOS. RSK-2, a kinase downstream of ERK, has been reported to phosphorylate SOS in PC12 cells in response to EGF activation (Douville and Downward, 1997).

Raf-1 recruitment to the membrane by RasGTP is initiated by the binding of the RBD and the CRD to Ras. Point mutation in either one of the RBD and CRD disrupt the membrane localisation of Raf, suggesting that both domains are needed for the successful translocation and Ras binding in fibroblasts (Bondeva et al., 2002). RBD binding to Ras is dependent on the GTP-induced conformational change in Ras, whereas the CRD binds either conformation. Membrane localisation of Raf-1 is only the initial event that leads to full activation. In order to achieve full activation a complex set of steps that involves sequential dephosphorylation, phosphorylation and conformational changes is needed to fully activate Raf-1. Targeting Raf-1 to the plasma membrane by fusing the farnesylation sequence of Ras to the C-terminus of Raf-1 (Raf-CAAX) is sufficient to activate Raf-1 constitutively and seemingly independent of Ras (Stokoe et al., 1994). However Raf-CAAX is not fully active and its kinase activity can be further increased by mitogens and RasGTP. Recent reports even suggested that mutationally activated Ras is able to enhance Raf-CAAX activity by direct binding (Mineo et al., 1997), possibly by initiating Raf dimerisation or by forcing the release of the kinase from the regulatory domain, indicating that Ras does more than merely recruiting Raf-1 to the membrane. Although most reports suggest that Ras/Raf binding is essential for Raf-1, activation studies using a Raf-1 mutant that is deficient in Ras binding showed that this mutant could still be activated by EGF, TPA and during mitosis, suggesting that at least part of the activation process might not be dependent on the Ras/Raf interaction (Mischak et al., 1996; Ziogas et al., 1998).

The activation of A-Raf is less well studied, but seems to be similar to Raf-1. In contrast, B-Raf activation is simpler, as Ras binding alone suffices to activate B-Raf. This seems to

be due to the fact that B-Raf exists in an already pre-activated form. In B-raf two crucial tyrosines (Y447 and Y448, equivalent to Y340 and Y341 in Raf-1) are replaced by phosphomimetic aspartic acid residues, and the adjacent S445 and S446 (equivalent to S338 and S339 in Raf-1), whose phosphorylation is required for activation, are already constitutively phosphorylated (Kolch, 2000; Wellbrock et al., 2004).

1.3 Regulation of Raf-1 by phosphorylation

Phosphorylation of Raf-1 plays a key role in regulating its activity, subcellular localisation, and its interaction with other proteins. In resting cells Raf-1 is phosphorylated on S43, S259 and S621 (Morrison et al., 1993). Upon stimulation with growth factors S338 and possibly Y341 phosphorylation is induced as well as phosphorylations of T491 and S494 in the activation loop (Kolch, 2000; Wellbrock et al., 2004). The basal S259 phosphorylation site is assumed to be mainly an inhibitory site, whereas the latter sites are phosphorylated in conjunction with increasing Raf-1 activity, therefore assumed to be activating (Figure 1.1).

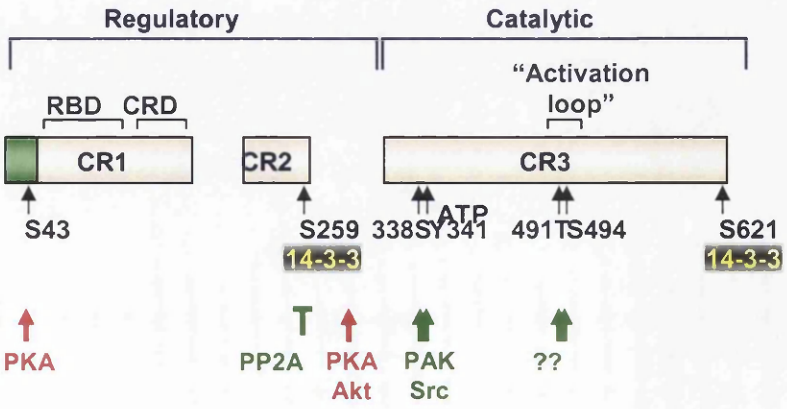

Figure 1.1: Cartoon of Raf-1 domains and phosphorylation sites

Raf-1 is phosphorylated on both S621 and S259 in its inactive cytoplasmic confirmation although the stochiometry of these phosphorylations is not known (Dhillon et al., 2002a; Michaud et al., 1995; Tzivion et al., 1998). These phosphorylations create two 14-3-3

interaction sites. This interaction can stabilise Raf-1 in its inactive conformation, where the regulatory domain shields the kinase domain, preventing access to substrates and activating kinases. Upon stimulation with mitogens the dephosphorylation of pS259 is one of the early events necessary for full activation of Raf-1 and the inhibition of this crucial dephosphorylation step inhibits the subsequent activation. It has been reported that Raf-1 binding to Ras-GTP destabilises the 14-3-3/pS259 interaction, thus releasing 14-3-3 and making this site accessible to phosphatases localised at the plasma membrane (Rommel et al., 1996). Treatment with okadaic acid and other phosphatase inhibitors have suggested that PP2A and PP1 both can dephosphorylate pS259 (Abraham et al., 2000; Jaumot and Hancock, 2001; Mitsuhashi et al., 2003). Inhibition of these phosphatases increases pS259 and 14-3-3 bound to Raf-1 at the membrane, thus increasing the proportion of inactive Raf at the membrane. Consequently the dephosphorylation of pS259 impedes the reassembly of the 14-3-3 dimer with the Ras/Raf complex, thus releasing the kinase domain form the regulatory domain. 14-3-3 is thought to remain bound to pS621 and disruption of this interaction leads to the loss of kinase activity. Confirmation of the proposed inhibitory role of S259 phosphorylation was obtained by mutational analysis as Raf-1 S259 mutants have an increased basal kinase activity that can be further increased by growth factor stimulation. This indicates that pS259 dephosphorylation is essential for activation, but itself is not sufficient, suggesting that these mutants resemble an intermediate conformation. The kinases Akt and PKA have been identified as the enzymes that can phosphorylate S259, although the kinase which is responsible for the basal S259 phosphorylation remains elusive. In some cell types, including muscle cells and breast cancer cells, the activation of Akt via PI3-kinase is sufficient to block Raf-1 activity and to impair signalling through the MAPK pathway (Moelling et al., 2002; Rommel et al., 1999; Zimmermann and Moelling, 1999). This cross-talk between the Akt and Raf-1 signalling pathway can change the outcome of the stimulation such as shifting the cellular response from cell cycle arrest caused by Raf-1 hyperactivity to proliferation in breast

cancer cell lines. The occurrence of the Raf/Akt cross-talk seems to be highly dependent on the cell type and differentiation stage, suggesting that Akt might not be the sole kinase responsible for the S259 phosphorylation or that accessory proteins are involved.

PKA has also been shown to phosphorylate S259. Activation of PKA via cAMP was reported to inhibit MAPK activation induced by EGF and this has been correlated with increases in phosphorylation on S43,S233, S259 and S621 (Chuang et al., 1994; Wu et al., 1993). Mutation of these sites suggested that the S259 is the major site involved in PKA mediated Raf-1 inhibition (Dhillon et al., 2002b), although another report suggested that all sites involved have to be mutated to abolish cAMP initiated inhibition of Raf-1 signalling (Dumaz et al., 2002).

Upon activation and translocation to the membrane several inducible, activating phosphorylations events take place. Two crucial phosphorylation sites, T491 and S494, lay both within the activation loop and are essential for Raf-1 activation. Mutation of both sites to alanines blocks Raf-1 activation by mitogens and by active Ras. The corresponding mutation to phosphomimetic amino acids on the other hand does not activate Raf-1, quite in contrast to B-Raf, and additional mitogen stimulation is essential for Raf-1 activation (Chong et al., 2001; Zhang and Guan, 2000). This confirms that the phosphorylations in the activation loop are essential, but not sufficient for full Raf-1 activation. So far, the kinases responsible for phosphorylating these two sites have not been identified.

Finally, phosphorylation of two more sites is essential for Raf-1 activation. S338 and Y341 both are located in the CR3 region and have been shown to be induced by mitogens. S338 is conserved throughout the Raf isoforms, but it has been suggested that it is constitutively phosphorylated in B-Raf, whereas it is strongly induced in Raf-1 (Diaz et al., 1997; Mason et al., 1999). Mutation of S338 and the adjacent S339 to alanines severely impedes the transformation ability of membrane targeted Raf-CAAX and severely reduces Ras

dependent activation. Mutation to acidic amino acids on the other hand only increases the kinase activity mildly, once again suggesting that this sites are essential for activation but not sufficient. Initial studies identified Pak3 as the kinase that is responsible for phosphorylating S338, but subsequent studies indicated that Pak2 is probably the kinase involved (Chaudhary et al., 2000; King et al., 1998; Sun et al., 2000). The Pak family kinases are cytosolic serine/threonine kinases that are regulated by the Rho family of small GTPases, specifically CDC42 and Rac. Ras can activate Rac via a PI3-kinase dependent mechanism possibly explaining how active Ras can induce pS338 phosphorylation on Raf-1. pS338 appears to be important not only for Raf-1 activity, but may also serve as a platform for integrating signals from CDC42 and Rac into ERK signalling. More recently the involvement of Paks in phosphorylating S338 residue has been disputed (Chiloeches et al., 2001), as inhibition of the PI3-kinase pathway with chemical inhibitors did not influence pS338 phosphorylation levels and Pak3 kinase activity did not correlate with S338 phosphorylation. This contradicted earlier publications suggesting that chemical inhibitors of PI3-kinase signalling were able to inhibit pS338 phosphorylation (Sun et al., 2000). A possible explanation for this discrepancy is that the inhibitors can inhibit Ras at high unspecific concentrations and therefore can reduce pS338 phosphorylation by inhibiting Ras instead of reducing Pak activity. However, studies on the physical interactions between Raf-1 and Pak1 have supported the role of this Pak isoform as a S338 kinase (Zang et al., 2002). Thus, the role of Pak as the physiological regulator of S338 remains unresolved.

Tyrosine 341 is the fourth site essential for Raf-1 activation. It is conserved in A-Raf and Raf-1 but is replaced by a phosphomimetic acid amino acid in B-Raf. The exchange in B-Raf in conjunction with the constitutive phosphorylation S445, the equivalent of S338 in Raf-1, explains why B-Raf has a higher activity than Raf-1 and is more readily stimulated by mitogens, as it only requires membrane translocation and two phosphorylations in the activation loop. Tyrosine 340/341 site on Raf on the other hand is strongly phosphorylated

by active Src (Marais et al., 1997), which may explain why active Src and Ras synergise in activating Raf-1.

Although co-expression assays have established that active Src is capable of phosphorylating tyrosines 340 and 341 (Fabian et al., 1993; Jelinek et al., 1996; Marais et al., 1995), the importance of tyrosine phosphorylation in the physiological activation of Raf-1 remains disputed, mainly because tyrosine phosphorylation of Raf-1 by mitogen stimulation occurs on a low level and is difficult to detect both by phospho-peptide mapping of in vivo labelled Raf-1 or by western blotting with phosphospecific antibodies. More recently a report suggested that distinctive growth factor stimulations could not only phosphorylate the Y341 and S338 residues, but were selective for either one of these residues. In human endothelial cells vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) induced either tyrosine phosphorylation on 340/341 or serine phosphorylation on S338/339 (Alavi et al., 2003). More interestingly, pY340/341 was linked to Raf-1 mediated protection from extrinsic apoptotic stimuli, whereas serine 338 phosphorylation protected the cells from intrinsically induced apoptosis (Alavi et al., 2003) (also see Raf and apoptosis).

Although the processes that lead to Raf-1 activation have been extensively studied, little is known how Raf-1 is deactivated. Taking into account that only a small fraction of the Raf pool is active at any given time and is localised at the membrane very little is known how Raf relocalises from the membrane back to the cytosol. Recent studies suggest binding of Raf-1 to 14-3-3 may play an important role in this process (Roy et al., 1998; Dhillon et al., 2002; Light et al., 2002). In addition, dephosphorylation of activating Raf-1 sites is likely to be a crucial step in the inactivation of Raf-1. To date no phosphatases have been identified that dephosphorylate either of the activating sites and identifying the players in this game will certainly help to clarify the mechanisms involved in inactivating Raf-1.

1.4 Regulation of Raf-1 signalling by protein-protein interactions

In addition to Ras, 14-3-3 and MEK, Raf-1 has been reported to interact with several other proteins. The core of the Raf-1 complex is formed by an assembly of chaperones and 14-3-3 proteins that keep Raf-1 in conformations required for its functions. HSP90, HSP70, CDC37, FKBP65 and Bag-1 all are chaperones that have been reported to bind to Raf-1 (Coss et al., 1998; Grammatikakis et al., 1999; Silverstein et al., 1998; Wang et al., 1996b). Disruption of the interaction between Raf-1 and the chaperone complex renders Raf-1 insoluble and prone to degradation. This is exemplified by treating cells with geldanamycin or radicicol, both drugs which interfere with HSP90 function. Upon treatment with these drugs Raf-1 protein levels drop and Raf-1 becomes undetectable within a few hours, demonstrating their crucial role in stabilising Raf-1 (Schulte et al., 1997; Schulte et al., 1995; Soga et al., 1998). Apart from merely stabilising and correctly folding Raf, chaperones have been shown to be crucial for regulating Raf membrane localisation and its activity. For example, HSP70 and Bag-1 have been found to regulate Raf-1 activity and the subsequent activation of ERK in concert (Song et al., 2001). Upon heat-shock, HSP70 levels increase and displace Bag-1 from Raf-1. This exchange antagonizes Raf-1 activity thus reducing signalling through the MAPK pathway and halting DNA synthesis. Bag-1 mutants deficient in HSP70 binding constitutively activate Raf-1, whereas HSP70 mutants that cannot bind Bag-1 do not inhibit Raf-1 activity.

As chaperones ensure correct folding of Raf-1, scaffolding proteins ensure that individual signalling modules maintain their specificity and fidelity of signalling by facilitating the physical interaction of the proteins involved in the cascade in a spatially and temporally restricted manner.

Sur-8 is a scaffold proposed to act at the Ras/Raf level. Sur-8 was identified in a *Caenorhabditis elegans* loss of function screen as a protein that when mutated could suppress an active Ras phenotype. Sur-8 was shown to function upstream of Raf, at the

level of Ras (Sieburth et al., 1998). Sur-8 interacted with Ras, but did not bind to a dominant negative mutant. Human Sur-8 was found to have similar functions. Overexpression of Sur-8 did not activate the MAPK pathway per se, but enhanced ERK and Raf-1 activity induced by mitogens and active Ras. Sur-8 was found to bind not only to Ras and active Ras mutants, but also Raf-1 and was present in ternary complex consisting of Ras/Sur-8/Raf-1. The enhancement of Ras signalling seems to be Raf specific as Sur-8 was not able to enhance signalling through PI3-kinase/Akt or JNK (Li et al., 2000), suggesting that Sur-8 is indeed a Ras/Raf specific scaffold that organises and enhances signalling through the MAPK pathway.

Connector enhancer of KSR (CNK or MAGUIN) in another scaffold regulating the Ras/Raf interaction and was identified in a *Drosophila melanogaster* genetic screen as a gene which interfered with the rough-eye phenotype caused by expression of dominant negative KSR (Therrien et al., 1998). Drosophila CNK is a multidomain protein with no apparent enzymatic function but comprises several protein-protein interaction domains, indicating that its main purpose is to assemble protein complexes. Genetic studies suggested that CNK functioned in the Ras pathway either upstream or in parallel of Raf. A subsequent biochemical analysis in Schneider insect cells revealed that the interaction with Raf was mediated by two N-terminal domains and a short C-terminal domain, whereas the C-terminus binds to Ras. When Ras is inactive the interaction of the C-terminus with Raf inhibited its catalytic function, whereas it changes into a potent activator upon Ras activation by interacting with the N-terminal domains (Douziech et al., 2003). The functions of CNK in mammalian system remains sketchy. Human CNK2 interacted with Raf-1 in a manner not dependent of Raf-1 activity and its overexpression inhibited MAPK activation by mitogens (Lanigan et al., 2003). The other human homologue CNK1 has been recently found to be involved in Rho mediated transcription but did not affect Rho-dependent stress fibre formation (Jaffe et al., 2004). Moreover it was found to augment proapoptotic Ras signalling through RASSF1A/C and MST1/2 (Rabizadeh et al., 2004).

Clearly the functions of CNK in the mammalian system are more complex than the studies in Drosophila suggested and more work is needed to fully characterise the functions of the mammalian homologues of CNK.

Kinase suppressor of Ras (KSR) is another scaffold involved in regulating MAPK signalling. KSR was also identified in genetic screens in worms and flies (Kornfeld et al., 1995; Sundaram and Han, 1995; Therrien et al., 1995) as a protein that was able to reverse the phenotype of mutationally activated Ras. KSR is a multidomain protein containing five conserved domains and has a high degree of similarity to Raf kinases. The CA1 region is a 40 amino acid stretch unique to KSR proteins, CA2 is a proline rich region, CA3 is a cysteine rich domain, CA4 is a serine/threonine rich region and CA5 is a putative kinase domain. Although the name, the similarity to Raf and the presence of kinase domain suggested an enzymatic function, so far only few reports have found the kinase domain to be active (Wang and Studzinski, 2001; Xing et al., 2004; Xing and Kolesnick, 2001; Xing et al., 2000; Zhang et al., 1997) and identified Raf-1 as a potential substrate. Other studies challenged the proposed kinase function as they failed to detect kinase activity (Michaud et al., 1997; Stewart et al., 1999). Furthermore, the essential lysine involved it the phospho-transfer reaction in the kinase domain of KSR is exchanged for an arginine, indicating that this protein might not be able to utilise ATP. Moreover the expression of a truncation mutant of KSR that contains the C-terminal kinase domain inhibited MAPK signalling (Joneson et al., 1998; Yu et al., 1998), in contrast to Raf, where the equivalent deletion produces a constitutively active kinase.

It is now widely accepted that KSR functions as a scaffold in MAPK signalling independently of its proposed kinase function. The idea of KSR being a scaffold emerged when it was found to bind to several proteins involved in MAPK signalling. It was found to bind to Raf-1, MEK and ERK (Denouel-Galy et al., 1998; Yu et al., 1998) and mutations in protein-protein binding domains of KSR render it functionally inactive (Roy

et al., 2002). KSR's interaction with MEK is constitutive and mutations that disrupt this interaction disrupt KSR function (Muller et al., 2000; Stewart et al., 1999). Raf-1 and ERK binding on the other hand is not constitutive and is induced by Ras activation (Jacobs et al., 1999). Raf-1/KSR binding seems to be limited to the membrane and does not require either MEK or Ras to be present in the complex (Roy et al., 2002; Therrien et al., 1996; Xing et al., 1997). Interestingly forcing the interaction between Raf and MEK is sufficient for Raf-mediated MEK phosphorylation (Roy et al., 2002). The proposed function of KSR as a molecular scaffold coordinating the Raf/MEK interaction was further confirmed by mouse knock-out studies. Although the mice were born alive and no phenotypical deficiencies were detectable, ERK activation was impaired upon stimulation by mitogens, indicating that KSR potentates MAPK signalling (Nguyen et al., 2002). Gel filtration of mouse lysates showed that MEK, ERK and KSR are present in high molecular protein complexes, whose size was reduced in the knock-out mice, further supporting the scaffolding function of KSR.

The similarity between KSR and Raf not only occurs at the structural level but also at the level of regulation. Like Raf, 14-3-3, CDC37 and HSP90 bind to KSR and control its conformation and solubility. One particularly intriguing similarity between Raf and KSR is their regulation by 14-3-3 binding. KSR is phosphorylated in resting cells on S297 and S392 which enables binding to 14-3-3 proteins (Cacace et al., 1999). Upon activation, these sites are dephosphorylated by PP2A, enabling membrane translocation of KSR. The inhibition of this dephosphorylation step prevents the membrane localisation of KSR, thus preventing MAPK activation (Ory et al., 2003). Thus the similarity of Raf and KSR regulation by PP2A and 14-3-3 is remarkable.

As scaffolding proteins organise and facilitate Raf interactions, other proteins inhibit the formation of the active signalling complex. Raf kinase inhibitory protein (RKIP) was initially identified in a yeast-two-hybrid screen for proteins interacting with the C-terminal

kinase domain of Raf-1 (Yeung et al., 1999). RKIP interacts not only with Raf-1 but also with MEK and ERK, but fails to interact with Ras. This is similar to KSR but the function of RKIP is quite the opposite. RKIP inhibits Raf dependent phosphorylation of MEK in-vitro and in-vivo, but does not affect ERK phosphorylation by constitutively active MEK mutants in-vitro or in-vivo, suggesting that RKIP functions at the Raf/MEK interface. Furthermore the inhibitory function of RKIP is very specific to MEK phosphorylation by Raf as it does not reduce the kinase activity of Raf-1 per se, and RKIP does not inhibit MEK phosphorylation by MEKK1. RKIP acts by specifically disrupting the physical interaction between Raf-1 and MEK, thus behaving like a competitive inhibitor. Interestingly, the Raf-1 domains needed for MEK and RKIP binding and the MEK domains needed for Raf and RKIP interaction do not overlap, suggesting that RKIP does not function as a straightforward competitor but reduces the affinity of Raf towards MEK by either inducing a conformational change or by steric hindrance (Yeung et al., 2000). In accordance with these findings, RKIP-bound Raf-1 is unable to bind to MEK and vice versa, whereas MEK and ERK are able to bind to RKIP simultaneously. Upon activation with mitogens, RKIP can be phosphorylated by protein kinase C (PKC) which causes it to dissociate from Raf-1 (Corbit et al., 2003; Lorenz et al., 2003). RKIP is phosphorylated by PKC on serine 153, which reduces its affinity for Raf-1, but enables it to bind and inhibit G-protein-coupled receptor kinase 2 (GRK-2). This inhibits receptor internalisation and enhances signalling through both G-protein-coupled receptors and the ERK pathway (Lorenz et al., 2003). PKC phosphorylation of RKIP explains the widely observed RKIP dissociation from Raf-1 upon stimulation with mitogens, although it cannot be excluded that other kinases or mechanisms, such as direct modifications of Raf, contribute to this dissociation. More recently RKIP has been identified as a metastasis suppressor gene in prostate cancer (Fu et al., 2003) and malignant melanomas (Schuierer et al., 2004). RKIP expression and mRNA levels were widely reduced in the metastases in comparison to the

primary tumours. The decrease in RKIP levels correlated with increased phospho-ERK levels, confirming the pivotal role of RKIP in regulating MAPK signalling.

1.5 Raf-1 and apoptosis

In mice, one essential role of Raf-1 that could not be compensated by other Raf family members is its ability to inhibit apoptosis. Raf-1 is essential for mouse development and knocking out the gene resulted in a lethal phenotype (Huser et al., 2001; Mikula et al., 2001). The mouse embryos died in mid-gestation due to massive apoptosis in the placenta and the liver. Apoptosis is the cellular mechanism of programmed cell death and can be induced by several stimuli. These stimuli can be divided into intrinsic and extrinsic insults, and Raf-1 has been reported to protect against both (Alavi et al., 2003). Replacement of wild-type Raf-1 with a mutant, Raf-1 YY340/341FF, which has impaired kinase activity and is not efficiently activated by Ras signalling, was sufficient to revert the apoptotic phenotype completely (Huser et al., 2001; Mikula et al., 2001). These results confirmed previous observations that Raf-1 plays a crucial role in cell survival and that, at least part of this function, is seemingly independent of kinase activity and signalling through the MEK/ERK module. Several mechanisms have been so far proposed to explain the function of Raf-1 in preventing apoptosis, some of which require MEK/ERK signalling, while some require Raf-1 kinase activity while others require neither.

One of the first indications of an anti-apoptotic function of Raf-1 came from the observation that the anti-apoptotic Bcl-2 protein was able to bind to Raf-1 and recruit it to the mitochondria. Raf-1 was unable to phosphorylate Bcl-2 but it was suggested that it could phosphorylate and inactivate the pro-apoptotic protein BAD (von Gise et al., 2001; Wang et al., 1996a). However, no Raf-1 phosphorylation site has been detected in BAD and it is only a very poor in vitro substrate for Raf-1 (von Gise et al., 2001; Wang et al., 1996a). Non-phosphorylated BAD can bind to Bcl-2 and prevent it from interacting with other proapoptotic BH3-domain proteins such as Bax and Bak. Phosphorylation of BAD

causes it to bind 14-3-3, which sequesters it to the cytosol. This increases the level of free Bcl-2, therefore increasing Bcl-2 and Bax/Bak heterodimerisation and thus preventing destabilisation of the mitochondrial membrane potential and release of cytochrome c. Mitochondrial targeting of activated Raf-1 by fusing it to a p70 Mas targeting sequence protected the cells to the same extent as over-expression of Bcl-2 and Raf-1, demonstrating that mitochondrial translocation and kinase activity was sufficient to inhibit apoptosis (von Gise et al., 2001). As kinase activity seems to be required for protection from apoptosis it has still to be determined how Raf-1 can be activated at the mitochondrial membrane as no conclusive mechanism has been found. There is data suggesting that Bag-1, a protein that binds to Bcl-2 and heat shock proteins, could play a role in the activation of Raf-1 at the mitochondria as incubation of Raf-1 with recombinant Bag-1 was sufficient to increase kinase activity (Wang et al., 1996b), possibly resolving this mystery.

A further potential anchor protein for mitochondrial Raf-1 has been identified as the voltage-dependent anion channel (VDAC), a protein crucial for mitochondrial membrane trafficking. VDAC was reported to bind to both Raf-1 and Bcl-2 (Le Mellay et al., 2002), although the interaction of Raf-1 with VDAC seemed to be Bcl-2 independent. Binding of kinase active Raf-1 to VDAC limited the in-vitro reconstitution of VDAC in the outer mitochondrial membrane thus reducing the conductivity of the ion-channel and cytochrome C release, but had no effect on already established VDAC pores. These reports indicate that the anti-apoptotic function of Raf-1 at the mitochondria requires kinase activity which contradicts the results obtained in the mouse knock-in studies. The reasons for this discrepancy are not presently clear. One possibility is that active Raf-1 at the mitochondria protects cells from a different set of stimuli compared to the kinase-independent protection mechanism.

It has been shown that Raf-1 can interact with apoptosis signal-regulating kinase 1 (ASK1) and prevent apoptosis induced by overexpression of ASK1 (Chen et al., 2001). ASK1 is an

important mediator of apoptosis as it can relay pro-apoptotic signals induced by numerous insults, such as Fas, TNF-α, DNA-damage and oxidative stress. ASK-1 overexpression has been shown to induce mitochondrial dependent cell death. The antiapoptotic function of Raf-1 in these studies was MEK independent as inhibiting MEK/ERK signalling did not decrease the protective function of Raf-1 and more intriguingly Raf-1 kinase activity was not needed either. The direct interaction of Raf-1 and ASK1 was essential as apoptosis induced by an ASK1 truncation mutant that was unable to bind to Raf-1 was not inhibited by Raf-1. Additional confirmation of the physiological relevance of this interaction emerged from cross-breeding of ASK1 and Raf-1 deficient mice. Mice with a heart-specific deletion of Raf-1 showed heart dilatation caused by an increase in cardiomyocyte apoptosis . Cross-breeding these mice with ASK1 knock-out reverted this phenotype suggesting that, at least in the heart, this interaction does prevent ASK1 mediated apoptosis (Yamaguchi et al., 2004). Perhaps a whole-animal ASK-1/Raf-1 knock-out would have generated more insights and possibly confirmed if this mechanism is more general.

Another, possibly more general, mechanism how Raf-1 can inhibit apoptosis has been identified. MST2, a proapoptotic Ste20-like kinase was found to directly bind to Raf-1. In Raf-1 deficient cells MST2 kinase activity was increased and could be further increased through Fas signalling. The apparent inhibitory effect of Raf-1 was not dependent on kinase activity as kinase dead Raf-1 inhibited MST2 as efficiently as wild type Raf-1. Further confirmation of the necessity of this interaction was the observation that a simultaneous knock-down of Raf-1 and MST2 in several cell lines reverted the increased apoptotic sensitivity of Raf-1 knock-downs. This indicates that at least a large proportion of Raf-1 anti-apoptotic function is dependent on this interaction (O'Neill et al. 2004 in press).

Apart from these three anti-apoptotic functions of Raf-1 that are not dependent MAPK-signalling, Raf-1 is also involved in survival signalling relayed through MEK and ERK.

Both MEK and ERK have been shown to have antiapoptotic functions as expression of active MEK or ERK is sufficient to repress cell death in several situations. Expression of oncogenic v-Raf did also suppress apoptosis through a MEK dependent pathway as this effect was nullified by co-expression of dominant negative MEK. Furthermore it was shown that Akt was necessary for Raf/MEK dependent cell survival (von Gise et al., 2001). This suggests that Raf/MEK signalling activates PI 3-kinase and subsequently Akt. Active Akt then has the ability to inhibit apoptosis by phosphorylating several down-stream targets. It has seen shown that phosphorylation of BAD is Akt and ERK dependent (Cross et al., 1995; Hayakawa et al., 2000; Scheid and Duronio, 1998) as well as dependent on phosphorylation and inactivation of proapoptotic GSK-3β (Cross et al., 1995).

In another study using human endothelial cells, the pathways through which Raf-1 could protect from apoptosis were separated (Alavi et al., 2003). The authors showed that extrinsic apoptotic insults, such as death receptor signalling, were inhibited if Src dependent tyrosine phosphorylation of the 340/341 sites was induced by vascular endothelial growth factor (VEGF). On the other hand signalling through the S338 kinase (possibly Pak), stimulated by bFGF, resulted in phosphorylation of serine 338 and could protect from intrinsic apoptotic insults, such as starvation and DNA damage. The induction of phosphorylation at serine 338 resulted in mitochondrial translocation of Raf-1 and quite intriguingly mutation of the serine at the 338/339 position to alanines inhibited the sub-cellular redistribution indicating that serine 338 phosphorylation might be essential for mitochondrial translocation and protection form apoptosis.

Taken together, these studies provide strong evidence for a role of Raf-1 in apoptosis protection. Clearly, much more work is needed to clarify the physiological relevance of these different mechanisms by which Raf-1 is proposed to act and to determine if they coexist in cells or whether they are cell-type or species specific. In addition, the

relationship, if any, between Raf-1 dependent apoptosis protection and other anti-apoptotic pathways (e.g. Akt) requires further investigation.

1.6 Proteomic analysis of differentially expressed proteins

1.6.1 Two-dimensional gel electrophoresis

Proteomic analysis of proteins generally requires the initial separation of the proteins in the sample. In less complex samples conventional one dimensional gel electrophoresis may be sufficient to separate the proteins but the resolution limit of this method is quickly reached in biological samples. Therefore more powerful methods have to be used especially if the samples contain hundreds to thousands of individual proteins, a complexity easily reached in cell lysates as an example. If the resolution of one dimensional separation is not sufficient a second separation that is perpendicular to the first can improve the overall capacity of the method. Thus, two-dimensional gel electrophoresis has been used for samples of medium to high complexity. The most widely used first dimension in protein analysis is the separation according to the isoelectric point using immobilised pH gradients (reviewed in (Gorg et al., 2000). Typically the sample is denatured with strong chaotropic reagents and solubilised using non-ionic detergents, as these do not affect the overall charge and hence migration of the protein. The protein mixture is then separated in an electric field and the proteins migrate in the pH gradient to the point where they do not carry any net charge, the isoelectric point. This point is not only dependent on the primary

□Ā□□□□□□□□

f the protein, i.e. the amino acid composition, but is also strongly affected by posttranslational modifications. In particular, modifications that alter or introduce acidic or basic groups change the isoelectric point noticeably, therefore enabling the separation of differentially modified protein isoforms. It is therefore possible to detect changes in the phosphorylation status, as this modification introduces a strongly acidic group onto a less acidic amino acid, thus decreasing the isoelectric point of the overall protein. The second dimension is normally a denaturing sodium dodecylsulfate polyacrylamide gel (SDS-

PAGE) that separates the proteins according to size resulting in a gel separated by the isoelectric point on the x and molecular weight on the y axis. These gels can resolve several thousand individual proteins into single protein spots enabling the simultaneous comparison of a large number of proteins in one sample.

The proteins separated by 2D gel electrophoresis are then stained by either conventional methods such as silver or Coomassie staining or by fluorescent dyes that either bind to the proteins or the SDS molecules surrounding the protein in the gel (Steinberg et al., 1996). A comparison between individual samples separated by 2D gels can reveal differentially expressed and differentially modified proteins on a large scale. The final stage of analysis is the identification of the differentially expressed or modified proteins, which can be either performed by Edman sequencing or by mass spectrometric methods (Shevchenko et al., 1996).

1.6.2 Soft ionisation methods and mass spectrometric analysers

The mass spectrometric analysis of proteins and peptides began in the 1960s with the introduction of electron impact and chemical ionisation mass spectrometry. These methods are limited with respect to the maximal weight of the molecules that can be analysed as they have to be transferred into the gas phase before ionisation can occur. This limited the methods to thermically stable and evaporable substances and chemical modification were necessary in order to analyse peptides. The introduction of ionisation methods in a condensed phase was able to alleviate the problems encountered in the gas-phase ionisation methods. Electrospray (Whitehouse et al., 1985) and matrix assisted laser desorption ionisation (MALDI) (Karas and Hillenkamp, 1988) are the two most widely used "soft" ionisation techniques used to date and have replaced other methods (Figure 1.2), such as fast atom bombardment and plasma desorption (Macfarlane and Torgerson, 1976; Morris et al., 1981). These soft methods enable ionisation of peptides and proteins directly form the condensed phase, so that no derivatisation is needed for thermolabile and non-volatile

molecules, such as proteins and peptides. MALDI was introduced in the late 1980s and represents an ionisation technique suitable for peptides and proteins. The ionisation is performed in solid phase. The sample is mixed with an UV absorbing mixture and co-crystallised on a metal target. The ions are generated by irradiating the sample/matrix crystals with a pulse of light generated by a UV laser. This leads to desorption of matrix and sample molecules and ions. The ions are then accelerated in an electric field in a vacuum and their flight time is measured at the end of a flight tube (time-of-flight analyser/TOF). The flight time is directly proportional to their mass over charge (m/z), therefore it can be translated into mass units with the appropriate calibration. TOF analysers combine speed, wide mass range and extreme sensitivity as all ions are detected (Figure 1.3B).

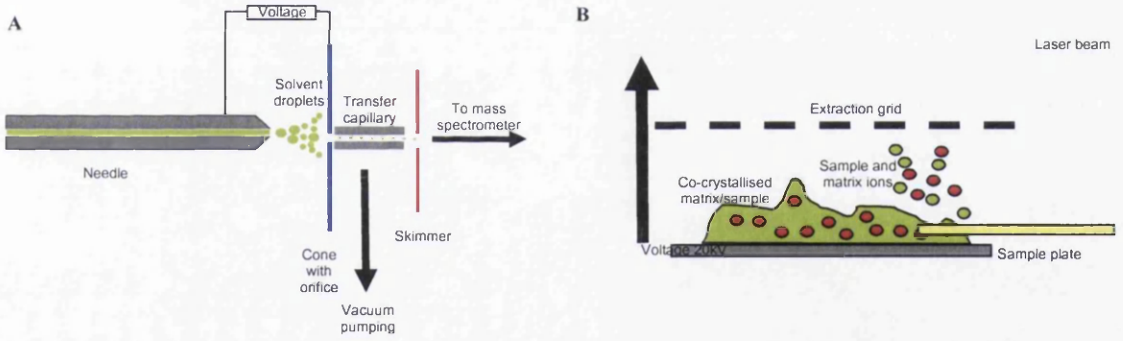

Figure 1.2: Schematic drawings of soft ionisation methods. A) Electrospray ionisation: The analyte solution flow passes through the electrospray needle that has a high potential difference applied to it (typically in the range from 1-2.5kV). This forces the spraying of charged droplets from the needle with a surface charge of the same polarity to the charge on the needle. The droplets are repelled from the needle towards the source sampling cone on the counter electrode (shown in blue). As the droplets traverse the space solvent evaporation occurs until the ions are left without solvent. **B)** Matrix assisted laser desorption ionisation: The solid analyte/matrix solution is irradiated with a UV laser beam. The matrix absorbs at that wavelength and transfers the energy to the analyte resulting in the evaporation of the matrix and analyte. The matrix molecules stabilise in the gas phase by protonating the analyte molecules, leading to matrix and sample ions.

In contrast to MALDI the sample ionisation in ESI occurs in solution. The analyte is introduced to the source either loaded into a nanospray needle (nano-flow ES) (Wilm and Mann, 1996) or with a constant flow generated from a HPLC pump (LC-MS). The solubilised sample is sprayed from a needle that carries a voltage potential. Small charged droplets are formed and repelled from the needle that carries the same charge. The solvent starts to evaporate as the droplets travel through the space towards the sample cone

attached to the counter electrode and are finally sucked into the mass spectrometer. The solvent in the droplets surrounding the sample ions is completely evaporated whilst the droplet proceeds through several stages of pressure reduction until the ion is left "naked". The ion is then transported to the analyser. Contrary to MALDI, ESI generated ions tend to carry multiple charges and peptides commonly carry more than one charge. The introduction of nanospray ionisation (nano ESI) decreased the sample consumption of electrospray ionisation, thereby increasing the sensitivity by several orders of magnitude, as signal intensity of electrospray ionisation is dependent on the concentration of the sample.

As ESI produces a steady stream of ions, quite in contrast to the pulse generated in MALDI sources by the laser beam, TOF analysers are not the preferred method of separating these ions, since TOF analysers use pulsed accelerations and are not compatible with constant ion currents. Most widely used in conjunction with ESI are quadrupole analysers because of their compactness, robustness and compatibility with a steady ion stream. The quadrupole consists of four metal rods that are assembled in parallel. A varying voltage is applied to the metal rods that results in a fluctuating electric field. At a certain voltage and voltage to frequency ratio (radio frequency (RF)/DC is constant) only ions with a specific m/z value can pass through the electric field and reach the detector. By varying the voltage and RF the nature of the electric field can be changed, allowing ions with different m/z to pass, thereby enabling scanning through the whole mass range (Figure 1.3A).

Figure 1.3

Other analysers used for ESI are ion traps. The ions, produced in the source of the instrument, enter into the trap through the inlet and are trapped through action of the three electrodes: the ring electrode and the entrance and exit endcap electrodes. The ions are forced into a stable trajectory by varying the potentials of these electrodes. For detection of the ions the potentials are varied hence destabilising the trajectories of ions with a certain m/z ratio. Thus these ions are released from the trap and can be detected in order of increasing m/z. More recently linear ion traps have been constructed (LIT), the advantage of these analysers is their ability to function either as a quadruple or as an ion trap, hence combining the versatility of the quadrupole analyser with the enhanced sensitivity of an ion-trap.

1.6..3 Mass spectrometric protein identification methods

The identification of a protein in a database by using a set of molecular masses generated by proteolytic digest is called peptide mass fingerprinting. This method emerged over 10 years ago as the first mass spectrometric method to identify proteins isolated from 2-dimensional gels. Before that, proteins had to be either identified by Edman sequencing, a time consuming and insensitive method whereas MALDI based peptide mass fingerprinting enabled rapid and sensitive identification of gel separated proteins in an automated fashion with high accuracy (Henzel et al., 1993; Mann et al., 1993; Pappin et al., 1993; Yates et al., 1993). Soon afterwards other methods were developed that used short sequence data obtained by fragmenting peptides in the mass spectrometer, so called peptide sequence tags (Mann and Wilm, 1994), for searching against a protein or expressed sequence tag (EST) database (Neubauer et al., 1998). This method, especially if combined with peptide mass fingerprinting, increased the accuracy of the protein identification and also made it feasible to de-novo sequence at least small parts of proteins not present in the database. In contrast to peptide mass fingerprinting the generation of peptide sequence tags is more laborious and not robustly automatable. The operator has to identify possible

sequence fragments by hand and by experience, making large scale analyses of fragmentation data a daunting task. This was resolved with the advent of fully automated search programs that did not use amino acid sequences generated by the operator, but used the raw MS/MS data and by comparing the fragmentation to the theoretical fragmentation of peptides derived from theoretical digests of proteins present in databases (Perkins et al., 1999; Yates et al., 1995).

The increasing size of the databases following large scale sequencing projects increases the chance of the protein identification as the protein is more likely to be present in the database. However, the expansion of the databases presented the field with new problems as it also increased the chances of a false positive identification, creating the need for more accurate and reliable methods. This need was met by the development of more advanced mass spectrometers with increased resolution, mass accuracy and sensitivity. Delayed extraction (Brown and Lennon, 1995) combined with reflectrons (Brown and Lennon, 1995) increased the mass accuracy and resolution of MALDI-TOF machines to such an extent that mass accuracy below 20ppm is routinely achievable with in-gel digested biological samples, facilitating protein identification by peptide mass fingerprinting. More recently new hybrid mass spectrometers have become widely available that combine high sensitivity and mass accuracy with the possibility to obtain fragmentation data (Brown and Lennon, 1995). Triple quadrupole and ion-trap mass spectrometers were the first generation of machines used routinely for peptide fragmentation (see Figure 1.4A for an example of a triple quadrupole/linear-ion trap mass spectrometer). Later on, quadrupole-TOF machines became available with the benefit of a high resolving, fast and sensitive second analyser (Figure 1.4B). More recently TOF-TOF mass spectrometers have been developed (Medzihradszky et al., 2000). This specific setup enables the fast generation of sequence data form MALDI-ionised samples, combining the speed and sensitivity of MALDI-TOF data acquisition with the generation of fragmentation data that usually required ES ionisation techniques.

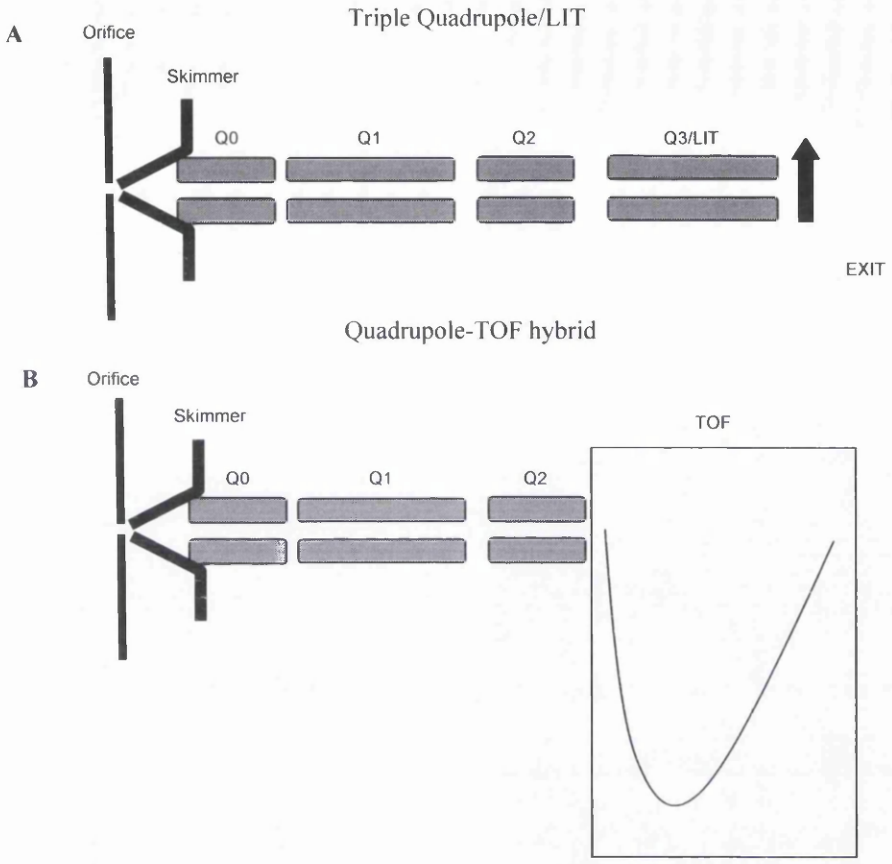

Figure 1.4: Schematic drawings a quadrupole/TOF and a quadrupole/linear ion-trap hybrid mass spectrometer. Q0 is a RF-only quadrupole used to cool the ion beam, Q1 can be used in selective RF/DC mode for product/precursor/neutral loss or a RF-only for standard MS. Q2 is an enclosed pressurised quadrupole used for CID. **A)** Q3 can be used as a standard RF/DC quadrupole for precursor/neutral loss scans or as a linear ion trap (LIT) for MS and MS/MS scans. **B)** The second analyser is a time of flight that can be used for MS, MS/MS and for precursor ion scans.

1.7 Analysis of Protein-Protein interactions

Signal transduction, the cell cycle, targeted proteolysis and gene expression are just a few examples of cellular events that are regulated by protein-protein interactions. These interactions, often regulated by interaction domains, organise enzymes, scaffolds and adaptors into functional multiprotein signalling complexes that execute distinctive functions (reviewed in (Pawson and Nash, 2003). The regulation of these interactions is essential for the understanding of cellular regulation, as interacting proteins often link signalling modules from the receptors to their ultimate substrate. Typically, protein-protein interactions require distinctive interaction domains that regulate the assembly of the complexes. Some of these domains, for instance SH2 and PTB domains, require modified amino acids for the interaction. These domains are present in a multitude of cytoplasmic proteins and they directly recognise phosphorylated tyrosines, which are typically found on activated tyrosine kinase receptors. The phospho-tyrosine dependent assembly of signalling complexes at activated tyrosine kinase receptors enables the transmission of receptor signals to their cytoplasmic targets. Phosphoserine and threonine on the other hand can be recognised by WD40 repeats, 14-3-3 and FHA domains driving the assembly of other protein complexes as the result of reversible phosphorylation.

Because of the multitude of regulations exerted by protein-protein interactions, it is not surprising that a multitude of methods has been developed that address the detection of potential interacting proteins. Some of these are based on yeast genetics, while others are based on *in-vitro* protein interactions and on affinity purifications of complexes that are formed *in-vivo*.

1.7.1 Yeast two hybrid screens

The two hybrid system is a molecular biological tool that enables the screening for protein-protein interactions. Developed fifteen years ago it is probably the most widely used

screening method for these interactions (Fields and Song, 1989). It is based on the fact that the DNA binding and transcriptional activation domains of transcription factors can function as independent modules that will form a functional transcription factor even when artificially linked. Initially the system used the Gal4 transcription factor to assay for interacting proteins. One gene sequence is fused to the Gal4 binding domain and the other is fused to the Gal4 activating domain to produce chimeric proteins that are co-expressed in a yeast cell. If the two test proteins interact they reconstitute a functional transcription factor that can initiate Gal4 driven transcription. This transcription can then initiate the transcription of an essential marker gene, which usually encodes a metabolic enzyme that enables the colony to grow in selection medium. Refined systems usually have more than one marker gene with different copies of the Gal4 DNA binding motif in their promoters. Promoters with more binding sites are more sensitive and respond to weaker interactions than promoters with only one binding site, thus permitting a semiquantitative evaluation of the strength of the interaction. By using the protein or protein domain of interest as bait a library of chimeric proteins can be screened for interaction partners. The surviving colonies are then isolated and the chimeric proteins that interacted with the "bait" are identified by reisolating the expression plasmids and sequencing the gene inserts. This simple and powerful assay enables large library screens, and screens of complete genomes have been produced (Ito et al., 2001; Uetz et al., 2000), identifying a myriad of protein-protein interactions. Several variations of the original two-hybrid system have been developed more recently. A general shortcoming of the yeast two hybrid system is that it only detects binary interactions, and that the interactions have to take place in the nucleus of a yeast cell, which is a rather artificial environment for most proteins, e.g. membrane proteins. In order to remedy the latter shortcoming, Ras and SOS recruitment systems have been developed (Aronheim et al., 1997; Broder et al., 1998; Hubsman et al., 2001). Both are based on a different readout than the original yeast two hybrid screen and these methods can overcome some shortcomings of the transcriptional based selection. The

selection is based on the activation of Ras at the membrane by bringing Ras to the vicinity of the SOS exchange factor through interacting proteins. In the Ras recruitment screen active Ras, lacking the membrane localising CAAX-box is fused to the bait proteins and the "prey" protein is fused with a membrane targeting sequence. If "bait" and "prey" interact physically, active Ras is localised at the membrane and enables the survival of the cell. These assays are suitable for hydrophobic membrane proteins as the bait is localised at the membrane and does not require nuclear localisation and solubility. Furthermore some basic interaction studies can be performed in mammalian systems (Maroun and Aronheim, 1999).

Other variants of this screen include reverse two-hybrid screens that screen for mutations and molecules that disrupt a pre-existing protein-protein interaction (Leanna and Hannink, 1996) and yeast-three-hybrid screens that screen for protein/protein/ RNA interactions (SenGupta et al., 1996; Zhang and Lautar, 1996).

1.7.2 Protein arrays

Several recent reports have used purified recombinant proteins immobilised to a matrix to study protein-protein interactions (Ge, 2000; MacBeath and Schreiber, 2000; Ramachandran et al., 2004; Zhu et al., 2001). Zhu et al. produced protein chips containing purified proteins derived from 5800 yeast open reading frames, enabling a genome wide screen for interacting proteins. The purified proteins are printed at high density onto glass slides and are fixed to the surface with reactive cross-linkers or by affinity interactions. These chips can then be used to screen for protein-protein and protein-phospholipid interactions. The protein-protein or protein-phospholipid interactions are formed by incubating the molecule that is screened with the protein-chip. The interactions are formed and after intensive wash steps, specifically binding molecules can be detected on the chip by a multitude of techniques, such as chemoluminiscence, fluorescence and radioactivity. So far protein microarrays have been used in "proof of principle" studies in yeast and on a

small scale in mammalian systems. Large scale mammalian protein microarrays have still to be produced, but clearly the application of these chips will contribute to our knowledge of protein-protein interactions as well as other interactions mediated by protein domains, such as drug and DNA binding.

1.7.3 Protein-protein interaction screening using mass spectrometric methods

Mass spectrometric methods have both been used to screen for interacting proteins as well as for determining possible interaction domains, such as antibody epitope screening. The analysis of interacting sequences relies on limited proteolysis of two interacting proteins and assaying for peptide sequences that have not been cleaved as an indication of domains that are not exposed in a protein complex (Suckau et al., 1990), or on the ability of the interacting protein to specifically affinity purify the peptides (Zhao et al., 1996). Other methods to detect the interacting sequences involve deuterium-hydrogen exchange reactions as these reactions occur more quickly on exposed amino acids (Anand et al., 2002; Hughes et al., 2001; Mandell et al., 2001; Mandell et al., 1998). Upon formation of a complex the interacting sequences are not exposed to the solvent anymore, therefore the exchange is slower. A direct comparison of peptides derived from the digested isolated and the complexed protein can reveal the sequences that are involved in the interaction.

The methods involved in screening for interacting proteins are more straightforward and utilise the ability of mass spectrometric methods to identify proteins. Protein pull-downs and co-precipitations are two of the most widely used methods for proteomic interaction screening. Both methods have been used successfully and have distinctive advantages and produce complementary information when used in parallel (compare (Jin et al., 2004; Rubio et al., 2004) (reviewed in (Aebersold and Mann, 2003).

Protein pull-downs involve the binding of a purified recombinant protein to a matrix, which is then exposed to a protein lysate. Interacting proteins that bind to the bait protein

can be identified by mass spectrometric methods after being eluted from the bait. The formation of complexes in pull-down assays takes place in-vitro, quite in contrast to co-precipitation experiments. In these experiments the complex is formed in the cell and the intact complexes are then precipitated by binding the bait protein to an affinity matrix. As the success of this purification relies on enriching intact complexes it is crucial that the complex is not disrupted by the purification process, hence mild, non-denaturing conditions and speed are two crucial factors for a successful analysis.

The most common affinity purification for co-precipitations studies is antibody based, and some problems can arise by using this purification method. Certain protein complexes might not be purified because an interacting protein is masking the binding epitope of the antibody. Moreover certain complexes can be disrupted by the interaction of the antibody with the epitope, thus limiting the identification of interacting proteins.

Although immunoprecipitation of endogenous, untagged proteins is feasible, most purifications rely on the use of overexpressed, tagged proteins. The introduction of a tag into the bait protein has several advantages over protein specific immunoprecipitations. A specific tag can allow a very high degree of purification in a single step, as highly specific antibodies are commercially available. Moreover, elution can often be performed under mild conditions by using immunospecific peptides that preserve the complex and its biological activity. Importantly, the conditions for purification are generally more dependent on the affinity tag than on the protein to which it is attached. Purification methods can be optimised and standardized for a particular tag, allowing many different proteins or complexes to be purified by essentially similar methods.

As mentioned before the ease and reproducibility of the purification is essential for successful large scale screen. A single tag is often insufficient for homogeneity so several systems have been developed that fuse two more tags (Honey et al., 2001; Rigaut et al.,

1999) to the bait protein. The introduction of several tags allows the consecutive affinity purification resulting in a higher purity of the purified protein complex. These tandem affinity purification tags (TAP tags) have been developed in yeast but more recently tags compatible with mammalian expression systems have been developed. However, one drawback of this approach is that the two-step method increases the purification time, which might eliminate the identification of transient or weak interactions.

Subsequently the eluted complexes can be separated by denaturing, native or by 2D gel electrophoresis and then identified by MALDI or ESI. Successful identification of the molecular components of these complexes requires highly sensitive approaches. Most commonly the proteins separated by PAGE are digested in-gel and identified by MALDI mass fingerprinting (Mann et al., 1993; Pappin et al., 1993), although the identification rate can be increased if tandem mass spectrometry methods are used. The first large scale analyses of this type used a single FLAG-tag or a TAP-tag to generate tagged proteins of 10% of the predicted yeast proteome followed by immunopurification, gel electrophoresis and mass spectrometric identification of the protein complexes (Gavin et al., 2002; Ho et al., 2002). The comparison between this approach and large scale yeast two hybrid screens showed discrepancies. The immunopurification approach identified three times more interacting proteins, which is not surprising given that the proteomics approach is not confined to the detection of binary interactions, but can identify all the proteins in a complex regardless whether their interaction with the bait is direct or indirect. However, the proteomics approaches failed to identify about 50% of the proteins found in the yeast-two-hybrid approaches, indicating that both methods are complementary (Ito et al., 2002; von Mering et al., 2002).

More recently gel-less approaches have been used for complex analysis. Multidimensional Protein Identification Technology (MudPIT) (Link et al., 1999), a form of 2D liquid chromatography, uses a strong ion-exchange matrix as first and reverse phase LC as

second dimension in order to separate tryptic peptides that then can be online injected into a mass spectrometer for identification. MudPIT has been used in combination with the TAP-tag technology in yeast to analyse protein complexes involved in mitosis, identifying over 300 potential interactions (Graumann et al., 2004).

1.8 Analysis of phosphopeptides by mass spectrometry

Post-translational modifications are processing events that include either cleavage of the protein by proteases to change its properties or the covalent binding of modifying groups to one or several amino acids. These modifications can change the protein's activity, cellular location and stability, as well as inducing or disrupting protein-protein interactions. In signalling processes, reversible phosphorylation is probably a major determining factor, as kinases can be turned on and of by these modifications. Knowledge of the particular residues that are phosphorylated can provide insights into the working of a signalling cascade, specifically how and when the kinases are activated and which kinases are responsible for this action. So it is not surprising that protein phosphorylation is possibly the most studied post-translational modification. Protein phosphorylation is one of the most ubiquitous modifications of proteins, with approximately 2% of the human genome encoding for kinases and possibly up to a third of all proteins being phosphorylatable. However, despite the large prevalence of phosphorylation events the identification of the specific amino acid which is modified is labour intensive and not always successful (Cohen, 2000; Mann et al., 2002).

1.8.1 Radioactive ^{32}P-labelling

The typical procedure used to identify phosphorylation sites involves labelling of a substrate protein with radioactive ^{32}P using an appropriate kinase in-vitro. The labelled protein in then digested with a protease to obtain peptides that are more amenable to further analysis than the full-length proteins. The peptides are then separated by either thin-

layer-chromatography (TLC) or more recently by high-performance liquid chromatography (HPLC) (Boyle et al., 1991; van der Geer and Hunter, 1994). The collected radioactively labelled purified peptides are then generally sequenced by Edman degradation using a sequencer. The amino acid composition and the position of the radioactive aminoacid can be determined as the loss of radioactivity is monitored after each cycle and the nature of the amino acid is determined by their characteristic HPLC retention times. However this method can be insensitive, and successful identification requires large amounts of phosphopeptides that are challenging to obtain. The detection of the labelled amino acid on the other hand requires little material, therefore the sensitivity of the method can be improved vastly by omitting the sequencing step and relying on mass spectrometric methods to determine either the mass or the sequence of the phosphorylated peptide (Ben-Levy et al., 1995). Instead of submitting the entire HPLC fraction containing the labelled peptide to the Edman sequencer, the Edman degradation is only used to determine the position of the phosphorylated amino acid by monitoring at which degradation cycle the ^{32}P labelled phosphoamino acid is released. The majority of the fraction is analysed by mass spectrometry in order to determine the identity of the peptide. The masses of the peptides are generally determined by MALDI-TOF mass spectrometry and the presence of a phosphorylation site can be further confirmed by post-source decay (PSD) and a neutral loss of 90 Da. The Edman sequencing reveals the position of the labelled amino acid and the mass spectrum reveals the mass of the peptide. Comparing these findings with a theoretical digest of the proteins then reveals the exact position of the phosphorylation site in the protein. This method is probably the most successful method used to date and thousands of phosphorylation sites have been determined using this set-up.

1.8.2 Affinity purification of phosphopeptides

Other methods to determine phosphorylation sites have been used that do not rely on in-vitro or in vivo radioactive phosphorylation. These methods attempt to detect

phosphorylation sites by enriching phosphorylated peptides by affinity purification, by modifying phosphorylated amino acids through chemical or biological reactions or by specifically detecting phosphorylation sites by "marker ions" with mass spectrometric ions.

To date the preferred enrichment method for phosphopeptides is by immobilised metal ion affinity chromatography (IMAC). In this technique, metal ions such as Fe^{3+} and Ga^{3+} are bound to a chelating support. Agarose or silica gel usually forms the matrix and nitrilotriacetic acid (NTA) or iminodiacetic acid (IDA) are the chelating groups (Ficarro et al., 2002; Muszynska et al., 1992; Neville et al., 1997; Scanff et al., 1991). Phosphopeptides are selectively bound to the chelated metal ion because of the phosphoric acid segment. The phosphopeptides are bound at a low pH and can be released by either high pH or phosphate buffer. The latter requires further desalting prior to mass spectrometric analysis. Limitations to this approach are the possible loss of phosphopeptides unable to bind to the matrix, unspecific binding of unphosphorylated peptides and difficulty in eluting multiply phosphorylated peptides. Attempts have been made to address all three problems, albeit not completely satisfactorily. Some studies have suggested that IDA is less specific than NTA resins while other reported little difference (Neville et al., 1997; Nuhse et al., 2003). Moreover Ga^{3+} ions were reported to be more specific than Fe^{3+} ions (Cleverley et al., 1998). Seemingly the specificity of the method is highly dependent on the specific binding and elution conditions, but to date no uniquely successful method has been suggested and confirmed. Methyl esterification of peptide mixtures with methanolic HCl has been reported to enhance selectivity in some systems and a high degree of enrichment was obtained in a large scale analysis of the complete yeast proteome (Ficarro et al., 2002). The benefit of this modification has been disputed more recently as no added benefit was detected. Seemingly the benefit of masking carboxyl groups through esterification is dependent on the stringency of the subsequent IMAC purification and on the nature of the metal ion and the chelator. Prefractionation by strong ion exchange chromatography prior to IMAC was recently reported to enhance the

enrichment of phosphopeptides and also to increase the proportion of singly phosphorylated peptides in the purification (Nuhse et al., 2003). A large scale study looked at overall binding and recovery of radiolabelled phosphopeptides derived from in-vivo labelled plasma membranes and found that prefractionation and optimisation of elution and binding conditions increased the overall level of mono-phosphorylated peptides that were detectable. This finding can be explained by the possible out-competition of weakly binding phosphopeptides by stronger binding multiply phosphorylated peptides. Overall IMAC methodology has improved dramatically over the past years but additional method development is still required. Several reports have been published where IMAC prefractionation enabled the authors to identify several hundred phosphopeptides in one analysis, which although impressive, amounts to only a small fraction of the total phosphorylation sites in a whole cell system (Ficarro et al., 2002). It also has to be taken into account that these large scale analyses have been performed using large amount of material that would not always be available.

In addition to IMAC, immobilised anti-phospho-amino acid antibodies have been used to purify phosphopeptides and phosphoproteins. The use of phosphoserine or phosphothreonine antibodies for immunoprecipitations has been reported but is not as widely used as the corresponding phosphotyrosine based purification. Phosphoserine/threonine antibodies have been shown to perform reasonably well in immunoprecipitation of whole proteins (Gronborg et al., 2002), but so far have not been used as a tool to purify phosphopeptides in order to facilitate phosphorylation site mapping. In contrast, phosphotyrosine immunoprecipitations have been used widely in phosphorylation mapping approaches, both on protein and peptide level. In some approaches phosphotyrosine immunoprecipitations were performed as an initial purification of either the complete phosphotyrosine proteome or of a specific protein. The enrichment of the phosphoprotein then facilitated the subsequent mapping of the phosphorylation site greatly. Normally the first purification step is followed by other

purifications, either IMAC or PAGE and the analysis of the phosphorylation site is then performed by mass spectrometric methods. Anti-phosphotyrosine antibodies have also shown to be able to enrich phosphopeptides that were derived from purified tyrosine phosphorylated proteins (De Corte et al., 1999). The phosphopeptide was then detected by mass spectrometry, either by directly mixing the matrix with the antibody/phosphopeptide beads and MALDI analysis, or by eluting the peptide and then analysing it. So far this method, although looking promising, has not been exploited to map global phosphorylation changes in whole cell lysates.

1.8.3 Modification of phosphorylation sites

Recently chemical and biological modifications of phosphorylation sites have been utilised to map phosphorylation sites in proteins. Three distinctive methods have been utilised to convert phosphoserine/threonine amino acids into moieties that are more amenable for enrichment and subsequent analysis. All three methods exploit the tendency of alkyl phosphate esters to undergo β-elimination under basic conditions, converting serine into dehydroalanine and threonine into 2-amino-2,3-dehydrobutyric acid. The unsaturated sidechain can then undergo Michael addition, normally performed by a sulfhydryl moiety. This reaction then allows conversion of the former phosphoaminoacid into either a "hook", such as biotin or a sulfhydryl group that can be affinity purified, or a residue similar to lysine (Goshe et al., 2001; Jaffe et al., 1998; Knight et al., 2003; Oda et al., 2001). The inclusion of a sidechain similar to lysine changes the tryptic digest pattern and enables the detection of the former phosphorylated aminoacid without the need for sequencing, i.e. peptide mass fingerprinting. Although these methods have been shown to work with purified protein standards, such as casein, they have yet to be proven to be feasible for detecting phosphorylation sites involved in signalling pathways. It has to be noted that only very simple and efficient chemical derivatisation techniques can be used. If any heterogeneity is introduced through these reactions the task of identifying modified amino

acids becomes more challenging, and only abundant phosphorylation sites might be detected. A further drawback of this method is the inability to detect phosphotyrosine residues, as these do not undergo β-elimination.

Dephosphorylation by alkaline phosphatase is one of the most established methods used for detecting phosphorylation sites of biological samples (Larsen et al., 2001; Stensballe et al., 2001; Zhang et al., 1998). The general procedure is that the sample set is divided into two aliquots, one is treated with the phosphatase and the other one is left untreated. The mass spectra are then compared to each other. If a shift of 80Da (or multiples of 80Da) is detected in the phosphatase treated sample this is regarded as proof of a phosphorylation site. This method enables laboratories without MS/MS capabilities to perform at least rudimentary phosphopeptide mapping. This general method has been more recently combined with IMAC enrichment to obtain a purified phosphopeptide sample. The phosphopeptides are then dephosphorylated either prior to or after IMAC. Comparison of the non-treated samples with the dephosphorylated ones then reveals the phosphorylation sites (Stensballe et al., 2001). This method was recently reported to have been miniaturised and performed on a compact disc, where all the steps, from IMAC to MALDI are performed on the same surface (Hirschberg et al., 2004). Although method development has improved the performance and reliability of this method, the major drawback of this method is that without MS/MS data from the phosphorylated peptide, it is impossible to detect the exact phosphorylation site if several potential phosphorylation sites are present in the peptide. Further problems arise if isobaric peptides are present and if the exact sequence of the protein to be analysed is unknown.

1.8.4 Precursor ion and neutral loss scanning

Phosphopeptides behave atypically after being ionised in the mass spectrometer. Peptides containing unmodified amino acids fragment under low energy collision induced dissociation (CID) conditions normally around the peptide bond of the peptide backbone.

Side-chain fragmentations occur only at very high collision energies and are not normally observed under these conditions. Quite the opposite occurs with peptides that carry a phosphorylated amino acid. The phosphate group is quite unstable and the phosphoester group is more likely to fragment upon CID, rendering it possible to scan for the phosphospecific marker ions.

The hallmark of phosphorylated aliphatic amino acids is the tendency to β-eliminate phosphoric acid upon CID and under basic conditions. This lability is not only exploited for chemical modifications, but is also utilised to produce a marker of phosphorylation in mass spectrometry. The ionised peptide undergoes CID and phosphoric acid is eliminated resulting in loss of 98Da. The charge of the ion does not change upon dissociation of this fragment, therefore the loss is called neutral. The analysis is normally performed using mass spectrometers that contain two analysers. The first analyser, typically a quadrupole, scans through the mass range, while the second analyser scans at an offset of -98Da, the mass shift resulting from the loss of phosphoric acid (Figure 1.5B). The ions undergo CID between the analysers and therefore only peptides that produce a product ion with a mass difference of 98Da, equalling the loss of phosphoric acid, are recorded. Ions identified can then be scanned to obtain complete MS/MS data that then ideally reveal the sequence and the site of the phosphorylated amino acid. This method has been widely used for phosphopeptide mapping, initially using triple quadrupole mass spectrometers (Schlosser et al., 2001), but more recently using Q-TOFs (Bateman et al., 2002). The enhanced resolution and mass accuracy of the TOF analyser has been reported to enhance this scanning method by reducing false positive identifications. As the scan is performed in positive-ion mode and no switching is required for a subsequent MS/MS scan, which is also performed in positive-ion mode, this facilitates automated method development. Thus this method has been widely used in conjunction with HPLC separation of the peptide mixtures (Hunter and Games, 1994). Problems arise from the fact that the value of the neutral loss depends on the charge state of the precursor, i.e. 49 for doubly charged and

32.33 for triply charged ions, thus both losses have to be scanned. This not only increases the scan time, thus limiting the sensitivity, but also increases the likelihood of false positive identifications.

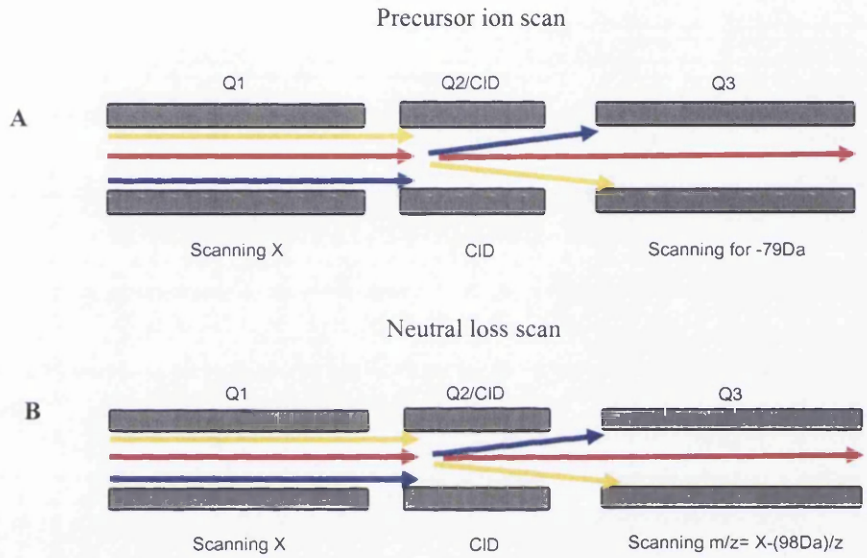

Figure 1.5: Schematic drawings of precursor-ion and neutral loss scans used for phosphopeptide analysis using a triple quadrupole mass spectrometer. A) Q1 is in RF/DC mode and scans the whole mass range, Q2 induces CID of the ions selected by Q1, Q3 scans in RF/DC mode for m/z 79. **B)** Q1 is in RF/DC mode and scans the whole mass range, Q2 induces CID of the ions selected by Q1, Q3 scans in RF/DC with an offset to Q1 equalling the mass of phosphoric acid.

Neutral loss is not the only mass spectrometric method used to scan for phosphopeptides. A precursor ion scan is a mass spectrometric method that is used to identify the mass of the precursor of a fragment. Two distinctive precursor ion scans have been used for phosphopeptide mapping. The first and more widely used method scans for the precursor of PO_3^- and the second scans for the precursor of the phosphotyrosine-immonium ion.

The PO_3^- precursor ion scan has been widely used for several years and is probably one of the most successful mass spectrometry based methods for phosphorylation site mapping (Carr et al., 1996; Neubauer and Mann, 1999; Wilm et al., 1996) (reviewed in (Neubauer

and Mann, 1999). The method requires a mass spectrometer with two analysers, similar to neutral loss scanning, and is in performed in negative-ion mode. The first analyser scans through the whole mass range and the second analyser screens for a fragment of -79Da, the mass of PO_3^-. If the -79Da fragment is detected after the second analyser this indicates that the precursor ion contained a phosphorylated amino acid (Figure 1.5A). As there are few CID induced negatively charged peptide fragments that are isobaric, this method is highly specific and only few false positive identifications are expected. However this type of scan is not without difficulties. The pH of the spray is required to be basic to obtain optimal sensitivity, a condition unsuitable for HPLC separations. Thus, prefractionation is normally performed off-line. Furthermore it is quite challenging to obtain a stable spray in negative ion mode as this is frequently hampered by discharges from the tip of the capillary, further complicating matters. In addition, peptide sequencing in negative ion mode is not feasible and an additional positive ion MS/MS spectrum has to be acquired. This involves rebuffering the solvent to lower the pH or the use of another aliquot, further increasing sample handling time. Initially this method was not used in conjunction with HPLC because of the mentioned limitations, but recent publications have shown that using a multidimensional approach LC separation is feasible and enables to analyse more complex samples (Annan et al., 2001; Zappacosta et al., 2004; Zappacosta et al., 2002).

With the appearance of Q-TOF mass spectrometers another precursor ion scan method has been reported that takes advantage of the superior resolution of the TOF analyser. Unlike aliphatic phospho-amino acids, the phosphorylation residue of the tyrosine side chain is stable under positive-ion CID conditions and the intact phosphotyrosine immonium ion, with a mass of 216.04Da, is detectable in product ion scans. Unfortunately several theoretical fragments possess a similar mass to the immonium ion and would so increase false positive identifications. With the advent of hybrid mass spectrometers and the high resolution TOF analyser this did not represent a challenge anymore as the resolution is high enough to distinguish between the different ions (Steen et al., 2001a). The feasibility

of the method was shown in studies of EGF induced tyrosine phosphorylation (Steen et al., 2002) and to map Abl phosphorylation sites (Steen et al., 2003). The main advantage of this method in comparison to the -79 precursor ion scan is that it is performed at low pH in positive ion mode, therefore sequencing can be done in the same experiment.

1.9 Concluding remarks

A relatively small number of signalling pathways in the cells enables the cell to react appropriately and with a high degree of specificity toward a multitude of extracellular signals. This high degree of flexibility and fidelity requires a high degree of spatial and temporal control of the kinase cascade and it is becoming increasingly accepted that the interplay of protein-protein interactions in combination with phosphorylation events substantially determine the final outcome of the signal, would it be differentiation, proliferation or cell death. To fully understand signalling pathways it is therefore not sufficient to determine the outcome of the signal but also to analyse the individual complexes and the regulation of their formation and turnover. As mentioned beforehand phosphorylation events do not only effect enzymatic activities but play also a crucial role in assembling these multiprotein signalling entities, such as linking receptors to their substrates.

We are interested in Raf-1 mediated signalling and therefore decided to use proteomic techniques to gain a deeper understanding of Raf-1 regulated signalling. We decided not only to determine the effects Raf-1 has on a whole cell system, but wanted also to detect and identify changes in Raf-1 protein complexes that are dependent on specific extracellular signals. Furthermore, as phosphorylation plays a pivotal role in signalling events, we wanted to analyse the phosphorylation status in these complexes and determine their effects.

Chapter 2

Materials & Experimental Procedures

2.1 Materials

2.1.1 Antiserum:

All antiserum was used at a concentration of 1:1000 unless otherwise stated

BD Transduction Labs

Anti Raf-1 (R19120)

Anti PP5; used at 1/500

Calbiochem

Anti pS621 Raf-1

Cell signalling

Anti pS338 Raf-1

New England Biolabs

Anti phospho-ERK (9101)

Anti MEK1/2 (9122)

Anti phosphoMEK1/2 (9121)

Anti pS259 Raf-1

Santa Cruz

Anti GST: used at 1:5000

Anti-Raf-1 C12

Anti Gα12

Sigma

Anti ERK1/2 (M5670)

Anti phosphoERK1/2

2.1.2 General reagents

All reagents were purchased from Sigma, unless listed here.

Amersham Pharmacia: ^{32}P-ATP, GST-Sepharose, protein A-Sepharose, protein G-Sepharose, Cy2, Cy3, Cy5 DIGE dyes, PVDF blotting membrane, SDS gel caster, SDS gel running tank, Western blotting tank, Ettan 2D gel equipment, Typhoon laser scanner, IPG-strips, Pharmalytes

Becton Dickinson, Collaborative Research: 10cm tissue culture dishes

Biorad: agarose tanks and agarose gel casters

Calbiochem: Nonidet P-40

Cell Signalling/New England Biolabs: mouse secondary antibody (HRP conjugate), rabbit secondary antibody (HRP conjugate)

Costar: plates for tissue culture

Fisher Scientific: glycerol, glycine, KCl, $MgCl_2$, $MgSO_4$, NaCl, SDS

Invitrogen: LipofectAMINE and LipofectAMINE 2000 transfection reagent

Kodak: Ammonium Persulphate

Life Technologies/Gibco: BL21 (DE3) competent cells, DH5α competent cells, DMEM, Hepes, L-glutamine, MBP, trypsin

Melford Laboratories: IPTG, Tris base

Nunc: tissue culture flasks

Qiagen: Effectene transfection reagent, plasmid purification kits (mini and maxi),

Roche diagnostics/Boehringer Mannheim: blocking reagent, ECL (enhanced chemiluminescence) system, EDTA, EGTA, leupeptin, Tris-HCl ,

Stratagene: Mutagenic PCR kit

Severn Biotech Ltd: 30% Acrylamide (37.5:1 bis-acrylamide)

Sterilin: dishes for agar plates

2.1.3 Plasmids

FLAG-Raf-1; obtained from D. Morrison; vector: pCDNA3 purchased from Invitrogen; promoter: CMV (all Raf-1 mutants were produced by site specific mutagenesis using this plasmid as a template)

HA-PP5; obtained form T. Cohen; vector CMV; promoter CMV

Gα12 and Gα12QL; a gift from Silvio Gutkind

2.1.4 Primers

All primers were made by technology services staff at the Beatson Institute. All primers are written 5' to 3'

PP5 mutation of 304 H to A- forward
CCT TCG AGG CAA CGC CGA GAC AGA CAA CAT G

PP5 mutation of 304 H to A

CAT GTT GTC TGT CTC GGC GTT GCC TCG AAG G

Raf-1 SS338/339DE – forward
GGC CTC GTG GAC AGA GAG ATG ACA GCT ATT ATT GGG AAA TAG

Raf-1 SS338/339DE – reverse
CTA TTT CCC AAT AAT AGC TGT CAT CTC TCT GTC CAC GAG GCC

Raf-1 K375M – forward
GGA GAT GTT GCA GTA ATG ATC CTA AAG GTT GTC

Raf-1 K375M – reverse
GAC AAC CTT TAG GAT CAT TAC TGC AAC ATC TCC

2.2 Cell culture and transfections

2.2.1 Maintenance of 1COS-1 cells and 3T3 fibroblasts

Cos-1 were maintained in complete media (DMEM supplemented with 1:100 L-Glutamine and 10% FCS). Cells were grown in a 37°C/5% CO_2 incubator. Upon reaching 90% confluency, cells were washed in 5mls PBS and trypsinised in 1ml Trypsin (1:10). Cells were incubated with trypsin for no longer than 5 min. until cells appeared rounded and detached from the plastic tissue culture dish. 1ml of suspended cells was diluted into 10mls fresh complete media. 1ml of the diluted cell suspension was placed into a new tissue culture flask with 10mls fresh complete media. Cells were "split" in this way 2-3 times per week.

2.2.2 Transfection of COS-1 cells

Cos-1 cells were transfected using Effectene transfection reagent (Gibco) according to manufacturer's instructions. Briefly $2x10^5$ cells were seeded per well of a 6-well plate. The following day 0.3-1.0µg K-Ras/EXV or 50ng-0.3µg H-Ras/EXV was mixed with 100µl Buffer EC. Vector only was used to equalise total DNA content. 4.0µl enhancer was added to each mix, mixed thoroughly and incubated for 2mins at room temperature. 6.0µl of Effectene reagent was then added, pipetted up and down 4-5X and incubated at room temperature for 15-30mins. Meanwhile cells were washed 1x PBS and over laid with 1.5ml serum free DMEM. Complexes were over-laid onto the cells and incubated for 5-6 hours at 37◦C/5%CO_2. Cells where then washed 1x PBS and fresh 10% FCS/DMEM added. 30 hours post transfection cells washed 1x PBS and starved overnight in 0.2% FCS/DMEM. The following day cells were washed 3 times ice cold PBS and either store at –70 ◦C or lysed directly.

2.3 DIGE preparation

2.3.1 Mammalian cell lysate preparation for DIGE

Cells were seeded in 10% FCS/DMEM at 800.000 per 10cm dish. Cells were let to attach for 4h and then either lysed or washed with PBS and overlaid with 0.1% FCS supplemented DMEM. Cells were starved for 16-20hours. Cells were lysed in 20mM Tris-HCl pH8.0, 2mM EDTA, 1% Triton, supplemented with protease inhibitors and phosphatase inhibitors (1mM PMSF, 2mM NaF, 1mM Na vanadate, 5ug/ml leupeptin and 2.2ug/ml aprotinin). Plates were scraped using a disposable cell scraper and incubated on ice for 5-10mins. Lysates were collected in pre-cooled 1.5ml eppendorf tubes and clarified by centrifugation at 13000rpm for 15mins at 4°C. Supernatants where transferred to fresh pre-cooled eppendorfs and assayed for protein concentration using the BCA protein concentration assay KIT (Pierce). Proteins where then precipitated by TCA.

2.3.2 TCA precipitation

Lysate or proteins eluted from FLAG-beads were incubated for 30 min on ice with 0.002% sodium deoxycholate in dH_20. 22% of 100% TCA solution was added and rested on ice for 15 min. Precipitated proteins were pelleted by centrifugation at 13000rpm for 15 min at 4°C. The supernatant was removed and the pellet was washed twice with ice-cold acetone for 30 min. Pellet was allowed to air-dry for 5 min and either digested or dissolved in SDS-loading buffer or DIGE labelling buffer.

2.3.3 DIGE labelling

Labelling of the protein samples was performed according to the manufacturer' protocols. Briefly the protein samples were dissolved in 20mM Tris-HCl, pH 8.5, 2M thiourea. 6M urea and 4% CHAPS in dH_20 to a concentration of 10mg/ml. The pH was checked by spotting 0.5µl onto pH-paper. If the sample was too acidic the pH was adjusted with 2M Tris-HCl pH 8.9. Per 50µg of protein 1µl of diluted Cy2, Cy3 or Cy5 NHS activated ester (Amersham Pharmacia) was added. Samples were placed in an ice-bucket for 30min. The remaining unreacted esters were blocked by adding 1µl of a 10mM lysine solution and incubated on ice for 30min. The labelled samples were then mixed and diluted to a total volume of 450µl with 2M thiourea, 6M urea and 4% CHAPS in dH_20. DTT and Pharmalytes 3-10 (Amersham Pharmacia) were then added to a final concentration of 0.5% each.

2.3.4 Casting large gels for 2D-gel electrophoresis

Gels were cast in an EttanDALT6 gel caster and low fluorescence Ettan DALT gel glass plates were used (Amersham Pharmacia). Gels were bound to the backplate of the gel by treating it with bind silane solution made out of 10µl Bind Silane (Amersham Pharmacia), 8ml ethanol, 200µl acetic acid, 1.8ml dH_20. The prepared solution was applied onto the backplate with a lint-free cloth at let to air-dry for two hours. Then two reference markers (Amersham Pharmacia) were stuck to the backplate and the gels were assembled in the gel

caster. The gel solution consisting of 10% acrylamide (Acrylamide/Bis-acrylamide 37.5), 0.1% SDS, 350mM Tris-HCl pH 8.9 was filtered through a 0.2um filter.). 0.05% TEMED and 0.05% APS was added and the solution was poured into the gel caster. 2ml water saturated n-butanol was placed on top of the gel solution and the gels were let to polymerise for 2h. The gels were stored in 0.1% SDS, 350mM Tris-HCl pH 8.9 at 4°C.

2.3.5 Running 2D gels

For the first dimension a 24cm strip 3-10 immobilised pH gradients (IPG) strips were used (Amersham Pharmacia). A total sample volume of 450µl containing 150µg (DIGE gels) or 500µg (preparative gels) were transferred into IPGPhor (Amersham Pharmacia) coffins. The protective covering was removed from the IPG-strip and the strip was placed gel-side down on the sample. It was ensured that the sample was spread evenly and that no air bubbles were trapped underneath the strip. The strip was covered with 2ml of IPG cover fluid (Amersham Pharmacia) and placed on an IPGPhor unit Amersham Pharmacia). The following settings were used for DIGE gels: after 12h of rehydration a maximum voltage of 500V was applied for 1h, followed by an additional hour at 1000V and further 9h at 8000V. To prevent burning of the strip a maximum current of 60µA was allowed per strip. After the program was finished the strips were removed from the coffins the excess fluid was drained and the strips were rinsed briefly under a stream of dH_20. The strips were incubated with 100mM Tris-HCl, 6M urea, 30%glycerol 2% SDS,0.5% DTT in dH_20 under shaking for 15min. The strips were removed, briefly rinsed with dH_20 and incubated with 100mM Tris-HCl, 6M urea, 30%glycerol 2% SDS, 4.5% iodacetamide in dH_20 under shaking for 15min. Meanwhile the PAGE gels were stacked in an upright position and excess fluid was blotted away using Whatman 3MM paper. 0.5% Agarose in running buffer (see PAGE) with 30% glycerol was melted in a microwave. The strips were removed from the alkylating buffer, briefly rinsed with dH_20 and placed on top of the gel. Precautions were taken not to damage the strip and not to trap air bubbles between the gel

and the strip. The strip was sealed in place with the agarose solution. The gel was placed in an Ettan12 gel-tank (Amersham Pharmacia) and run at fixed 1.5W (no limit of V or A) per gel over night until the dye-front just had run off the gel.

2.3.6 Scanning of DIGE and Sypro-Orange gels

The gels were scanned using a Typhoon 9400 (Amersham Pharmacia) laser scanner with an external blue laser source. Following settings were used:

Dyes	Excitation	Filter	Laser energy
Cy2	Blue; 488nm	520nm width 40nm	500-550
Cy3	Green; 532nm	580nm width 30nm	500-550
Cy5	Red; 633nm	670nm width 30nm	500-550
SyproOrange	Blue, 488nm	580nm width 30nm	480-530

First the settings for the optimal laser energy were determined by using a quick scan at a low resolution (1000 micron pixel size). The laser energy was adjusted so that the most intensive spot was below saturation at around 80000 when visualised using ImageQuant (Molecular Dynamics). The final scan was performed at high resolution (100microns pixel size). Gel images were then cropped using the ImageQuant software before being analysed.

2.3.7 Analysis of DIGE gels

Gels were analysed using the DeCyder software package (Amersham Pharmacia). The spot number was set to 2500, artefacts were deleted by filtering using following settings: Area<200; Peak height<200; Volume<10000. The settings were determined using one gel as a template. The number was set to a value where spots were neither split nor merged. Area, peak height, volume were set to values which excluded most artefacts without eliminating true protein spots. The slope was not used as an exclusion parameter as it eliminated true protein spots that colocalised with dust particles

The master gel was selected as the Cy2 gel with the highest number of identified spots after filtering. All gels were matched to the master by landmarking an average of 50 spots manually, matching was then performed by the software. Spots matched erroneously by the software were corrected manually. Protein spots were picked that showed a variation of >1.7 fold between either set.

2.3.8 *Spot-picking, tryptic digest and MALDI-plate spotting by robotic workstation*

The robotic workstation was used in conjunction with 2D-gels. The spot picking was controlled by a "pick-list", in essence a file containing X,Y coordinates for the spots to be picked. The spot-picker was calibrated using two reference marker on the gel. Spot picking, digest and MALDI spotting were performed according to the manufacturer's recommendations. Briefly the spots were excised and destained by washing with 50% methanol 50mM NH_4CO_3H in dH_20 and 75% acetonitrile in dH_20. The digestion used modified porcine trypsin (Promega) at a concentration of 20ng/µl in 50nM Ammoniumbicarbonate in water and was conducted for 4h at 37°C. Peptides were extracted, dried and resolubilised in 3µl of 50% acetonitrile, 0.1% TFA in HPLC grade H_20 and 0.5µl were spotted on a MALDI plate and mixed on target with a 90% saturated solution of recrystallised α-cyano-4- hydroxycinnamic acid in 50% acetonitrile, 0.1% TFA in HPLC grade H_20.

2.4 Tryptic digests

2.4.1 In-gel digest

The coomassie stained bands were cut out using a scalpel and placed in eppendorfs. The gel slices were 2x washed with 50% methanol 50mM NH_4CO_3H in dH_20 for 5min. 10mM DTT, 50mM NH_4CO_3H in dH_20 was added and heated to 60°C for 15min. The supernatant was removed and replaced by 50mM iodacetamide, 50mM NH_4CO_3H in dH_20 and incubated for 30min in the dark. The supernatant was removed and the gel slices were

washed 2x with 50% methanol 50mM NH_4CO_3H in dH_20 and 1x with 75% acetonitrile in dH_20 for 5min at each step. Gel slices were dried by speedvac and were either kept at -20°C or directly digested.

Ice-cold modified porcine trypsin (Promega) was at a concentration of 12.5ng/µl was added to the gel slices until covered. Eppendorfs were placed in ice for 15min until the gel slices were rehydrated. Excess buffer was removed and replaced by 50mM NH_4CO_3H in dH_20 until gel slices were covered. The gel slices were incubated overnight at 37°C. The supernatant was replaced into a fresh eppendorf and peptides were extracted from the gel by shaking it for 5min with 50% acetonitrile 5% formic acid in dH_20. The supernatant was removed, pooled with the previous one and lyophilised by speedvac.

2.4.2 In-solution digest

The washed pellet was solubilised in 4µl of 8M Urea, 0.1% n-octyl-glucoside, 200mM NH_4CO_3H in dH_20 by shaking for 30 min. 0.5µl of 1M DTT was added and shaken for 30 min at 37°C. 32µl of dH_20 and 2µl of 125ng/µl porcine trypsin (Promega) were added and incubated over night at 37°C. The reaction was stopped by adding 2µl of formic acid.

2.5 Analysis of Raf-1 complexes

2.5.1 FLAG-Raf-1 immunoprecipitation for mass spectrometric analysis

3 x 10cm plates of COS-1 cell were transfected with the Raf-1 constructs. After 24h the cells were washed with PBS and either overlaid with 0.1%, 10% FCS/DMEM or taxol 10% FCS/DMEM. After 24h the cells were washed 3 times with PBS and lysed with 3 times 500µl Cells were lysed in 20mM HEPES pH8.0, 2mM EDTA, 0.5% NP-40, supplemented with protease inhibitors and phosphatase inhibitors (1mM PMSF, 2mM NaF, 1mM Na vanadate, 5ug/ml leupeptin and 2.2ug/ml aprotinin). Plates were scraped using a disposable

cell scraper and incubated on ice for 5-10mins. Lysates were collected in 3 pre-cooled 1.5ml eppendorf tubes and clarified by centrifugation at 13000rpm for 15mins at 4°C. 60µl of FLAG-Agarose and ProteinA-agarose was washed in the lysis buffer, then 0.1M glycine pH2.5 in dH_20 and then lysis buffer. The pooled supernatants were incubated with 60µl of washed ProteinA-agarose for 15min. The agarose was pelleted by centrifugation and the supernatant removed and incubated with 60µl of washed FLAG-agarose for 2-4h. The beads were pelleted by centrifugation and washed 3 times with 20mM HEPES pH8.0, 2mM EDTA, 0.1% NP-40. The proteins were then eluted form the beads by incubating them 3 times for 5min with 0.1M glycine pH2.5 in dH_20. The combined eluates were then neutralised with 2M pH 8.9 Tris-HCl buffer. TCA precipitated as described and in-solution digested as described.

2.5.2 2D-LC MS/MS

The peptides were separated using a UltiMate Nano LC System (LC Packings) using a strong cation exchange (SCX) column in the first dimension and nano reversed phase in the second dimension and detected using a Q-Star mass spectrometer (Applied Biosystems). 40µl of the digest were injected onto the column in two injections of 20 µl. The digest was firstly run over an strong cation exchange column (BioX-SCX, 0.5mm ID x 15mm, LC Packings) with a flow rate of 30µl/min. Peptides that did not bind to the first column were run over a C18 reverse phase (RP) cartridge (PepMap, 300µm ID x 5mm, LC Packings) functioning as a trap. The peptides bound to the C18 RP cartridge were then eluted from the trap and separated using a 75µm ID C18 RP column (PepMap, 15cm, LC Packings) with a 60min gradient from 0-40% acetonitrile 0.1% formic acid with a flow rate of 200nl/min. The peptides bound to the SCX column were eluted with increasing concentrations of KCl and run over the C18 RP cartridge at 30µl/min. The peptides bound to the RP cartridge were then eluted from the trap and separated using the 75um ID C18 RP column. The elution form the SCX and subsequent RP separation was repeated 8 times

up to a concentration of 1000mM KCl (50, 100, 150, 200, 250 400, 600, 1000mM). The eluted peptides were sprayed through a nano-LC needle (Pico emitter, 20μm ID, 10 μm orifice, New Objectives) and analysed using a data-dependent acquisition program on the Q-Star. The four most intensive multiply charged ions with an ion-count of >50 were selected by the software for further MS/MS analysis. Ions were excluded from MS/MS analysis for 120s after analysis, collision energy was set automatically by the software. The scan time was set to 3s in MS mode and 10s in MS/MS mode.

2.6 Peptide purification

2.6.1 C18 ZipTip peptide purification

If necessary peptide samples were desalted using ZipTips (Millipore) according to the manufacturer. Briefly the tip was washed with 50% Acetonitrile in HPLC grade H_20 by pipetting 10μl up and down. The matrix was then equilibrated with 1% formic acid in HPLC grade H_20 by pippetting 10μl up and down. The peptides were then loaded on the matrix by pipetting the solution slowly up and down for 10 times and then washed 3 times with 1% formic acid in HPLC grade H_20 by pipetting up and down. The peptides bound to the matrix were then eluted by pipetting 3μl of 30% Acetonitrile, 1% formic acid in HPLC grade H_20 up and down into a clean eppendorf. The peptide mixture was then analysed by mass spectrometric methods.

2.6.2 IMAC purification of peptides

IMAC purification of the casein digest and of the synthetic peptides was performed using $ZipTip_{MC}$ (Millipore) according to the manufacturer's instructions. Briefly the tips were washed 3 times with 0.1% acetic acid with 50% acetonitrile in HPLC grade H_20. Then the matrix was charged with iron(III) ions by 10 aspirate and dispense cycles with 10 μL 200

mM of Ferric Chloride in HPLC grade H_20 with 10 mM HCl. The tip was washed 3 times with HPLC grade H_20 and 3 times with 1.0% acetic acid with 10% acetonitrile in HPLC grade H_20. The tip was then equilibrated by washing 5x with 0.1% acetic acid with 10% acetonitrile in HPLC grade H_20. The lyophilised sample was dissolved in 0.1% acetic acid with 10% acetonitrile in HPLC grade H_20 and loaded onto the matrix by 10 aspirate and dispense cycles. And then washed 3 times with 0.1% acetic acid with 50% acetonitrile in HPLC grade H_20 and 3 times with HPLC grade H_20. Bound peptides were eluted with 0.3 N ammonium hydroxide solution in HPLC grade H_20 into an eppendorf and lyophilised by speedvac.

2.6.3 Crosslinking of 14-3-3 and phosphospecific antibodies

We used cyanogen bromide activated agarose (Sigma) to cross link proteins to be able to perform the peptide-pull-down experiments. We crosslinked the proteins according to the manufacturers instructions. Briefly we dialysed the proteins against 0.1 M $NaHCO_3$ buffer containing 0.5 M NaCl, pH 8.3 to ensure that no free amino groups were present in the buffer. Then we washed the cyanogen-bromide activated resin in cold 1 mM HCl for 30 min. The resin was washed with distilled water and then with the $NaHCO_3$ /NaCl coupling buffer and afterwards the protein solution was added immediately. The slurry was mixed for 2 hours at room using a rotation mixer. The reactive groups were then blocked with 0.2 M glycine, pH 8.0 for 2 hours at room temperature. The slurry was then washed extensively to remove the blocking solution, first with 0.1 M $NaHCO_3$ buffer containing 0.5 M NaCl, pH 8.3 and then with acetic acid 0.1 M, NaCl 0.5 M, pH 4. This wash cycle was repeated 5 times. The resin was then washed with PBS containing 0.02% sodium azide and stored at 4°C.

2.6.4 Phosphopeptide pull-downs

The phosphopeptides were dissolved in PBS, 0.05% Tween-20 and incubated with the either 14-3-3 or anti-phospho Ser/Thr agarose overnight. The slurry was then loaded into a home-made spin column and washed 3 times with PBS, 0.05% Tween-20 and 3 times with PBS. The peptides were eluted by incubating the beads 3 times for 5min with 5% acetic acid in H_20. The eluates were then lyophilised and desalted by ZipTips prior to MALDI analysis.

2.7 Mass spectrometric methods

2.7.1 MALDI mass spectrometry

All MALDI mass spectrometric analysis was performed using a Voyager-DE mass spectrometer (Applied Biosystems). Peptide samples were either previously de-salted with ZipTips or solubilised in 50% acetonitrile, 0.1% TFA in HPLC grade H_20. A saturated solution of recrystallised α-cyano-4- hydroxycinnamic acid was freshly prepared in 50% acetonitrile, 0.1% TFA in HPLC grade H_20 and mixed on target with the peptide solution. Typically 0.5µl was spotted. Positive-ion mode was performed using the reflector to enhance resolution, in negative-ion mode the reflector was switched off and the mass spectrometer was used in linear mode. Acceleration voltage was 20kV and laser intensity was set at around 2300. The data was calibrated either internally by using the trypsin auto-digest peaks or by near-point calibration using a peptide mixture.

If the samples were spotted by the robotic workstation (Amersham Pharmacia) the acquisition was performed automatically controlled by the Voyager software.

2.7.2 ESI mass spectrometry, product-ion scan

Electrospray ionisation (ESI) was done on either a Q-STAR or QTRAP (Applied Biosystems). Ions were scanned in a mass range of 400-1200Da. Scan time was 3s. Multiply charged peptides were selected by the software in an information dependent acquisition mode (IDA) and subjected to further MS/MS analysis. Scan time was 10s. If an LC interface was used the ions were excluded for 120s after analysis, otherwise for the whole length of the analysis. Collision energy settings were set by the software in accordance of mass and charge state. MS/MS spectra were analysed by the Analyst software package (Applied Biosystems) and Mascot (Matrix science)

2.7.3 -79 precursor ion scan single-shot Q-Star

3µl of the desalted casein digest was pipetted into a nano-spray needle (Protana). The needle was centrifuged to ensure that the sample was at the sharp end of the needle. Backpressure was applied using a glass syringe and if needed the needle was opened by tapping it gently against the metal plate surrounding the orifice. When a droplet appeared the backpressure was reduced and the needle was brought into position in line with the orifice. The appropriate voltage was applied that resulted in a steady spray without plasma formation at the tip. Typically the voltage was set between -600 and -1000V. The ion spray was checked in MS mode and if ions were visible the mass spectrometer was switched to precursor ion mode for the -79Da ion with the pulsing function active. The mass window on the TOF analyser was set to 0.5Da. Collision energy was set at 50eV. If precursors were identified the charge was determined by analysing the previously recorded MS spectrum. The mass spectrometer was then switched to positive ion mode and the appropriate MS/MS spectrum was recorded. The collision energy was adjusted to generate high and low molecular weight fragments.

2.7.4 -79 precursor ion scan Q-Trap

Needle was prepared as in 2.7.3. The ion spray was checked in MS mode and if ions were visible the mass spectrometer was switched to precursor ion mode for the -79Da ion. The resolution of the first and third quadrupole was set to "low" and the step-size was set to 1Da. The collision energy was set to 70eV. If precursors were identified the mass spectrometer was switched to positive-ion mode and the charge and mass of the precursor was determined by an "enhanced resolution" scan at a scan-rate of 250Da/s. If the ion identified was multiply charged it was subjected to an MS/MS scan. The collision energy was adjusted to generate high and low molecular weight fragments and the mass range was scanned at a scan-rate of 4000Da/s.

2.7.5 216.04 precursor ion scan Q-Star

The mass spectrometer was calibrated using GluFib peptide fragmentation. The mass spectrometer had to be calibrated just before the analysis was performed because to the high mass accuracy needed for this specific experiment. After the calibration the needle was prepared similar to 2.7.3 Typically the voltage was set at 1000V. The spray was checked first in MS mode, if ions were visible then the machine was switched into precursor-ion scan with the pulsing function active and a step-size of 0.5Da. The mass window was set to 216.04Da with a width of 0.04Da. The collision energy was either varied between 40 and 80eV. Identified precursor were then further analysed by MS/MS. The collision energy was adjusted to generate high and low molecular weight fragments.

2.7.6 -79 precursor ion scan LC MS/MS

The peptides were separated using a UltiMate Nano LC System (LC Packings) and detected using a Q-Trap mass spectrometer (Applied Biosystems). A 30min gradient from 0-40% acetonitrile and 0.1% formic acid was used as reverse-phase separation condition. A data-dependent acquisition program was used. The precursor ion scan was set at the same

values as used in the single-shot analysis. If the precursor scan detected an ion of >1000 counts an enhanced resolution scan was performed on the peak-area. Settings for this scan were equivalent to the values set in the single shot analysis. The charge state of the most abundant peak was determined automatically and the ion was then selected for MS/MS analysis. The optimal collision energy was calculated by the software and the scan rate was set at 4000Da/s.

2.7.7 Neutral loss scan LC MS/MS

The peptides were separated using a UltiMate Nano LC System (LC Packings) and detected using a Q-Trap mass spectrometer (Applied Biosystems). A 30min gradient from 0-40% acetonitrile and 0.1% formic acid was used as reverse-phase separation condition. A data dependent acquisition program was used. The first scan was a neutral-loss scan in positive-ion mode for loss of 49 and 33Da, the equivalent of loss of a phosphate for doubly and triply charged ions. The collision energy was rolled and the step-size was set to 1Da. The resolution of the first and third quadrupole was set to "low". If a neutral loss was detected the mass and charge-state of the ion was determined by an enhanced resolution scan at a scan-rate of 250Da/s. The most abundant peak was then selected for MS/MS analysis. The optimal collision energy was then calculated by the software by taking m/z and the charge state into account.

2.8 Database searching

2.8.1 MASCOT based protein identification by peptide mass fingerprinting

The MALDI spectra were processed by the MASCOT Demon (Matrix science) software in conjunction with the Voyager Dataexplorer (Applied Biosystems) software. The internally calibrated spectra were smoothed, the base-line was corrected and the peaks were de-isotoped. The masses over 1000 were selected for peptide mass fingerprinting searches. Cysteins were selected as modified by carbamylation and methionines were selected as

variably oxidised. Mass accuracy was set at 50ppm and searches were conducted using an in-house version of MASCOT searching against the rodent subsection of the NCBI database.

2.8.2 MASCOT based protein identification of MS/MS data

The MS/MS data was processed using a MASCOT script downloaded form http://www.matrixscience.com/help/instruments_analyst.html. The spectra were smoothed and centroided. Distanced below 0.1Da were merged. The mass accuracy was set according to the accuracy of the calibration. Typically the settings were 200ppm in MS mode and 0.4Da in MS/MS mode. If the calibration was performed on the same day the values were lower. The resulting compressed data files were searched against the NCBI database using an in-house MASCOT server.

2.9 3T3 cell line characterisation

2.9.1 Phalloidin staining

Cells were seeded onto 15mm glass coverslips in 12-well plates at a density of 1x 10^4 cells per well. 3T3 fibroblasts were fixed for 30mins in 4% paraformaldehyde (final concentration) in phosphate buffered saline (PBS), washed 3 x in PBS and permeabilised with 0.05% Triton X-100, 50mM glycine for 20mins. Cells were washed 3 times in PBS cells and blocked (10% FCS, 0.5% BSA in PBS) for 20mins. Cells were then incubated for 45mins with FITC-conjugated Phalloidin (0.11g/ml in blocking solution) in the dark. Cells were then washed 2 times in blocking solution, with a final two washes in PBS. Coverslips were mounted onto glass slides, overlaid with a drop of Vectashield and glass rectangular 30mm coverslip. Slides were sealed to protect from drying out using clear nail varnish.

2.9.2 Wound Assays

Cells were seeded into 35mm/6-well tissue culture dishes. Cells were plated at a suitable density so that in two days the cells would be 100% confluent. Using a sterile cell scraper a straight wound was pressed down the middle of the layer of cells. Fresh media was added to the cells. Wounds were then examined and suitable areas, marked using a cloning ring and photographed using a digital camera. Wound assays were incubated overnight at photographed again the next morning.

2.9.3 Cell-cycle profiling

After induction of cell death the cell culture medium was pipetted of the plates and placed into 15ml falcon tubes. The cell were then washed, trypsinised and pooled with the medium in the falcon tubes. The cells were pelleted by centrifugation and washed 2x with PBS. The cells were resuspended in 100µl PBS and fixed with 900µl of ice-cold 70% ethanol in H_20 for 1h on ice. The cells were sedimented by centrifugation and incubated in 300µl PBS containing 250ug/ml RNAse and 10ug/ml propidium iodide for 30min at room temperature. Fluorescent assisted cell sorting (FACS) analysis was performed on a Becton Dickinson FACScan. The FACS data was analysed using the CellQuest (Becton Dickinson) software package.

2.9.4 Annexin stain

After induction of cell death the cell culture medium was pipetted of the plates and placed into 15ml falcon tubes. The cell were then washed, trypsinised and pooled with the medium in the falcon tubes. The cells were pelleted by centrifugation and washed 2x with PBS. Annexin-V-Alexa 568 (Roche) was mixed 1/50 with 10mM Hepes pH 7.4, 140mM NaCl, 5mM $CaCl_2$ in H_20. The cell pellet was resuspended in this solution and incubated for 15min. The suspension was diluted with PBS to 300µl and analysed using a FACScan

machine (Becton Dickinson). The FACS data was analysed using the CellQuest (Becton Dickinson) software package.

2.10 Polyacrylamide gel analysis

2.10.1 PAGE

Lysates were resolved on SDS-polyacrylamide gels by SDS-PAGE gel electrophoresis. Resolving gels of an 10-12% acrylamide concentration were cast using Amersham Pharmacia mini-gel gel caster. Prior to pouring of either resolving (acrylamide 10-12%, 2mM EDTA, 0.35mM Tris-HCl pH 8.9) or stacking gels (acrylamide 4.5%, 125mM Tris-HCl pH6.8, 1%SDS, 4mM EDTA), ammonium persulphate and TEMED were added. For resolving gels, 500µl APS and 50µl TEMED were added per 30ml solution, mixed by inverting 3-4 times, poured and overlaid with butan-2-ol. Resolving gels were allowed to set at room temperature for 1-2 hours. Once set, butan-2-ol was removed and gels were rinsed with dH_20. Excess water was blotted away using Whatman 3MM paper. Appropriate comb was inserted into the gel apparatus and stacking gels prepared. 15µl of APS and 4µl TEMED were added per ml of gel solution, mixed by pipetting up and down, poured and allowed to set for 10mins at room temperature. Both reservoirs were then filled with 1x SDS-PAGE running buffer (10x SDS-PAGE running buffer: 24.2g/L Tris, 144.1g/L glycine, 1% (w/v) SDS), the comb was carefully removed and wells rinsed with running buffer prior to loading samples. Proteins were denatured prior to loading by heating to 100°C for 5 min. in 5x Laemmli (Laemmli, 1970) sample buffer (62mM Tris-HCl pH 6.8, 2% SDS, 10% Glycerol, 0.1% Bromophenol blue, 4% β-mercaptoethanol) and cooled on ice. Protein extracts where resolved together with the appropriate molecular weight markers (Gibco).

2.10.2 Coomassie staining

SDS-gels were stained with colloidal coomassie stain (Sigma) according to the manufacturer for 2 hours – overnight. Gels were destained (50% methanol, 10% acetic acid in dH_20 and 50% methanol in dH_20) until proteins bound to coomassie appeared blue against a clear background. Gels were stored in 5% acetic acid at 4°C.

2.10.3 Western Blotting

Following electrophoresis, resolved proteins were transferred to PVDF membranes (Hybond-P, Amersham Pharmacia biotech UK) by wet blotting method. Membranes where cut to the size of the gel and soaked in methanol. Membranes where briefly soaked in water and the gel was then transferred onto the membrane. Two pieces of Whatmann 3MM paper were used to sandwich the membrane and gel. This sandwich was then placed in a blotting cassette (Bio-rad) and wet blotted (25mM Tris, 130mM glycine in dH_20) in a Transblot wet blotting apparatus (Bio-rad) for overnight at 8V.

2.10.4 Western analysis

Western blots where rinsed in PBS-T (0.05% Tween) and blocked for a minimum of 1 hour in blocking buffer (5% BSA, PBS-T supplemented with 0.2% sodium azide). Blots where probed with primary antibodies diluted in blocking solution generally at a dilution of 1:1000 unless otherwise stated. Membranes where washed 3 x 10 min. in PBS-T and incubated for 45min at room temperature with the appropriate peroxidase-conjugated secondary antibody (1:5000). After 3 x 10 min washes in PBS-T, bands where visualised by exposure to Fuji Medical X-Ray film. Immunoblots were stripped (0.2M Glycine pH 2.5, 1% SDS) for between 10-30min at room temperature with agitation. Membranes were subsequently washed with water and re-blocked prior to incubating with primary antibody.

2.11 Enzymatic activity assays

2.11.1 Immunoprecipitation for western blot analysis.

Protein A (for rabbit antibodies) or Protein G beads (for mouse antibodies) were washed 2x with lysis buffer. The cleared lysates were incubated over night with the beads and the antibody. The beads were washed 3 times with lysis buffer and either denatured with SDS-loading buffer or washed with kinase dilution buffer (50mM Tris-HCl pH7.5, 5mM EGTA, 75mM NaCl, 5mM $MgCl_2$) and frozen for kinase assays.

2.11.2 Raf-1 radioactive2-step kinase assay

The Raf-1 immunoprecipitation was adjusted to 10µl with kinase dilution buffer (50mM Tris-HCl pH7.5, 5mM EGTA, 75mM NaCl, 5mM $MgCl_2$) and incubated with 0.2µg of purified bacterial expressed GST-MEK-1 and with 0.4µg of bacterial expressed GST-ERK-2 and dilution buffer to a total volume of 20ul. 5µl of start buffer was added (50mM $MgCl_2$, 0.8mM ATP). The mixture was incubated for 20min at 32°C with agitation,. Afterwards the reaction was placed in ice and 100µl of assay buffer (1mM DTT, 1mM Na_3VO_4 in dilution buffer) was added. 25µl of the dilution was placed in a fresh tube with 15µg of MBP in 25µl dilution buffer. 2.2µl of radioactive reaction start mix was added (37.5mM $MgCl_2$, 1.25mM ATP and 0.2µl ^{32}P-ATP in dilution buffer). The mixture was incubated for 10min at 32°C with agitation and stopped by addition of SDS sample buffer. Each sample was electrophoresed by SDS-PAGE on a 12.5% gel, western blotted for Raf-1 and exposed to a phosphoimager.

2.11.3 Raf-1 "cold" kinase assay

The Raf-1 immunoprecipitation was adjusted to 10µl with kinase dilution buffer (50mM Tris-HCl pH7.5, 5mM EGTA, 75mM NaCl, 5mM $MgCl_2$) and incubated with 0.2µg of purified Sf9 expressed GST-MEK-1. 5µl of start buffer was added (50mM $MgCl_2$, 0.8mM ATP). The mixture was incubated for 30min at 32°C with agitation. The reaction was

stopped by adding SDS sample buffer. Each sample was electrophoresed by SDS-PAGE on a 10% gel and western blotted for pMEK and Raf-1.

2.11.4 In-vitro phosphatase assay

GST proteins for full-length PP5 (GST–PP5) and the truncation mutant lacking the phosphatase domain(GST–ΔPP5) were purified as described using glutathione–Sepharose beads. COS-1 cells transfected with Flag-Raf-1 and RasV12 were lysed. The lysates were immunoprecipitated with anti-Flag antibodies as described. The beads containing Flag-Raf-1 washed in a final step with phosphatase assay buffer (50 mM Tris–HCl pH 8.0, 2 mM $MnCl_2$, 10mM DTT) and then incubated with GST–PP5 or GST–ΔPP5 in the presence of 100 mM arachidonic acid, and reaction mixtures were incubated at 30°C for 30 min. Phosphatase reaction was stopped by adding SDS sample buffer. The beads were then resolved on SDS–PAGE followed by electroblotting onto PVDF membranes and analyzed by immunoblotting with ECL.

2.12 Recombinant protein expression

2.12.1 Purification of GST-Fusion proteins from E.coli

BL21 cells were transfected with GST-PP5 or GST-PP5Δ construct, resultant clones were used to prepare 50ml overnight culture supplemented with 1001g ampicillin. Overnight culture was diluted to 500mls LB-broth and left to grow to OD at 600nm 0.4-0.6. Protein expression was induced (0.2mM IPTG, 4h 37°C). Cells were re-suspended in 20ml GST-lysis buffer (PBS, 1% Triton, 1mM DTT, 11g/ml leupeptin, 1mM aprotinin, 1mM Na vanadate, 1mM PMSF) and lysed by sonication (3 times 5 sec). Lysates were incubated with Glutathione Sepharose beads (overnight/41C) to purify the fusion proteins. Beads were washed 5 times with PBS and eluted by incubation with glutathione. Eluates were

dialysed over against 30% glycerol PBS with 1mM PMSF over night. Purified proteins were stored at -20°C.

2.12.2 Sf9 (Spodoptera frugiperda 9 cells) expression of GST tagged proteins (GST-MEK)

Sf9 cells were grown in suspension at 27°C in TC100 media (Gibco) that was supplemented with 50ml of heat treated FCS, 0.5ml pluronic 1%solution (Sigma) and 0.5ml Amphotericin B solution (Sigma). Cells were centrifuged and the medium was replaced prior to been seeded. The cells were seeded at 20Mio/14cm dish and let to set down for 1h. 2ml of GST-MEK virus were pipetted into each dish. The cells were kept at 27°C for 48h and then lysed in 2ml/14cm dish with GST-lysis buffer (PBS, 1% Triton, 1mM DTT, 11g/ml leupeptin, 1mM aprotinin, 1mM Na vanadate, 1mM PMSF). Plates were scraped using a disposable cell scraper and incubated on ice for 5-10mins. The lysates were cleared by centrifugation and the supernatant was incubated with Glutathione Sepharose beads (overnight/41C) to purify the fusion proteins. The beads were washed 3 times with the lysis buffer. The proteins were eluted by incubation with glutathione. Eluates were dialysed over against 30% glycerol PBS with 1mM PMSF over night. Purified proteins were stored at -20°C.

2.13 Mutagenesis and plasmid DNA isolation

2.13.1 Mutagenic PCR

Generation of 338/339 FLAG-Raf-1 and HA-PP5 (H304A) mutant

Mutagenic PCR kit (Stratagene) was used according to the manufacturer's instructions. The primers stated in section: 2.1E, were used to generate the mutations:

PCR reaction samples were digested with Dpn1 enzyme for 1hour at 37 ◦C and transformed into DH5α (section 2.2H.2). All constructs were checked by complete sequencing of the insert (Agowa sequencing service)

2.13.2 Bacterial Transformation

Following addition of DNA, cells were incubated on ice for 30 min., Cells were heat shocked for 45secs at 42 °C and incubated on ice for 5 min. 0.5ml of room temp LB was added and cells were left for 1 hour, shaking at 37 °C to allow expression of ampicillin resistance gene. 100µl were then plated onto solid agar (+antibiotic) and left overnight at 37 °C. Colonies were picked the next day and overnight cultures set up. 1.5ml of overnight culture was minipreped (Qiagen) to isolated plasmid DNA.

Results and Discussions

Chapter 3

Proteomic Analysis of Raf-1 knock-out Fibroblasts and Complexes

Chapter 3: Introduction

Growing evidence suggests that Raf-1 has cellular functions that are distinct from its well documented role in MAPK signalling. Studies using knockout mice have indicated that the three Raf isoforms play different roles in vivo and that some of their functions are non-redundant (Huser et al., 2001; Mikula et al., 2001) (Baccarini, 2002). Raf-1 is essential during mouse development and Raf-$1^{-/-}$ mice die in midgestation due to massive apoptosis in the liver and placenta. As MAPK signalling was not impaired cells derived from these mice, these studies concluded that Raf-1 was not required for activation of MEK–ERK signalling during development. However, when the wild-type Raf-1 gene was replaced by a Raf-1 mutant, RafYY340/1FF, which possesses basal activity but cannot be activated by growth factors, the mice were born with no apparent phenotype indicating that kinase activity is not needed for the unique survival role of Raf-1. Previous studies have also suggested that Raf-1 has an anti-apoptotic function, which may be mediated partly by its ability to translocate to the mitochondria and increasing phosphorylation of the pro-apoptotic BH3 protein Bad (Wang et al., 1996a; Wang and Reed, 1998). More recent studies have shown that Raf-1 can prevent apoptosis through inhibiting the activity of the pro-apoptotic kinases, ASK1 (Chen et al., 2001; Yamaguchi et al., 2004) and MST2 (O'Neill et al., in press). From these studies it becomes clear that Raf-1 may use several different signalling pathways and mechanisms to regulate apoptosis. Therefore, we want to use an unbiased proteomic approach to discover such pathways.

The aim of the studies described in this chapter was to characterise the anti-apoptotic function of Raf-1. To do this we compared the protein expression profiles and post-translational modifications in immortalised Raf-1 knock-out fibroblasts with cells that were reconstituted with either wild-type Raf-1 or a kinase-dead mutant of Raf-1. Using this approach we hoped to be able to distinguish between changes that were related to kinase activity and those that occurred because of kinase independent functions. As the method of analysis we chose two-dimensional gel electrophoresis combined with mass spectrometry.

The method has the unique ability to detect post-translational modifications such as phosphorylations and truncations which play a pivotal role in the induction and execution of apoptotic pathways.

One of the most challenging aspects of detecting differences in protein patterns using 2D gel electrophoresis is the necessity to obtain reproducible spot patterns to enable cross gel analysis. Furthermore, standard methods to visualise protein spots such as Coomassie and silver staining have very limited ranges of linearity between staining intensity and protein content, making an accurate assessment of changes very demanding, if not impossible. Therefore we decided to utilise a novel method that involves covalently labelling the proteins prior to separation with fluorescent dyes, typically Cy3 and Cy5 (Unlu et al., 1997). The differentially stained proteins are then mixed and separated using one gel thereby avoiding any ambiguities arising from the necessity to compare samples run on different gels. Because these fluorescent dyes retain the sensitivity of silver staining protocols but have a linear relationship between fluorescence and concentration across a wide range, they enable the detection of even small changes in protein expression. Additionally an internal standard, a mixture of equal amounts of both samples, is labelled with a third dye, Cy2, and included into the mixture, further benefiting the experimental design. As each spot contains its own internal standard changes in protein spot concentration can be identified with even higher accuracy and reproducibility (Alban et al., 2003; Friedman et al., 2004; Knowles et al., 2003).

Chapter 3: Results

3.1 Phenotypic characterisation of Raf$^{-/-}$ and reconstituted cells

3.1.1 Wild type Raf-1 and kinase-dead Raf-1 protect from starvation- induced cell death

Primary and immortalised fibroblasts derived from Raf-1 knock-out mice have been shown to be hypersensitive to cell death inducing stimuli (Huser et al., 2001; Kolbus et al., 2002; Mikula et al., 2001). Since only a subset of the phenotypic characteristics of the Raf-1$^{-/-}$ fibroblasts was dependent on its kinase function, we used Raf-1$^{-/-}$ fibroblast cell lines stably reconstituted with either vector alone (control), wild-type or kinase dead Raf-1 for our experiments (Fig. 3.1A). We induced cell death by starving the cells in medium containing 0.1% FCS. Afterwards we analysed the cell cycle distribution and identified the percentage of cells in the sub-G1 phase, an indication of DNA fragmentation occurring in apoptotic cells. Whereas cell lines reconstituted with wild-type or kinase-dead Raf-1 were unaffected by starvation for 24 hours, cells reconstituted with the vector alone showed increased cell death (Figure 3.1B), confirming that these vector-containing cells are hypersensitive to starvation-induced cell death in comparison to the other two cell lines used.

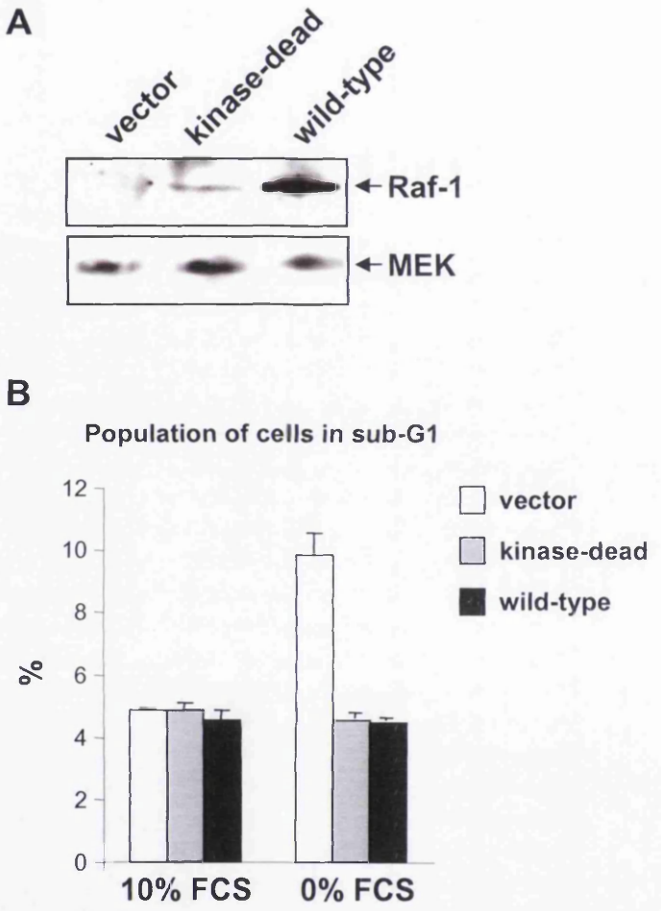

Figure 3.1: Wild-type and kinase dead Raf-1 protect from starvation induced cell death. A) Raf-1 proteins were stably expressed in Raf-1$^{-/-}$ MEFs. The expression levels were determined by immunoblotting of the cell lysates. **B)** MEFs reconstituted with Raf-1^{WT} and Raf-1^{K375M} are less sensitive to starvation induced cell death. MEFs were seeded at sub-confluent densities and either grown in medium containing 0.1% or 10% serum overnight. Cell cycle analysis was performed using FACS. The proportion of cell in sub-G1 indicates the level of cell death.

3.1.2 Raf-1 $^{-/-}$ cells have less actin stress fibres and are less motile

In addition their hypersensitivity to apoptosis, we noticed that Raf-1$^{-/-}$ cell were also morphologically different from their parental counterparts, indicating that cytoskeletal rearrangements had taken place. To visualise if these changes were due to reorganisation of the actin cytoskeleton, we stained cells with FITC-phalloidin, a toxin that binds specifically to F-actin. The cells exhibited remarkable differences. Wild-type cells seemed to be more polarised and showed pronounced actin stress fibres. The knock-out cells on the other hand were less polarised, more rounded and contained less stress fibres (Figure 3.2A). To test if these changes in actin cytoskeleton affected the motility of the cells, wound assays were performed. To do this a wound was scratched in confluent monolayers of the two cell types and the extent to which the wound had closed was measured after 16 hours later measured. During this period, the wild-type cells closed the wound almost completely whereas the knock-out cells were much slower (Figure 3.2B). This result shows that deletion of Raf-1 impairs the motility of cells.

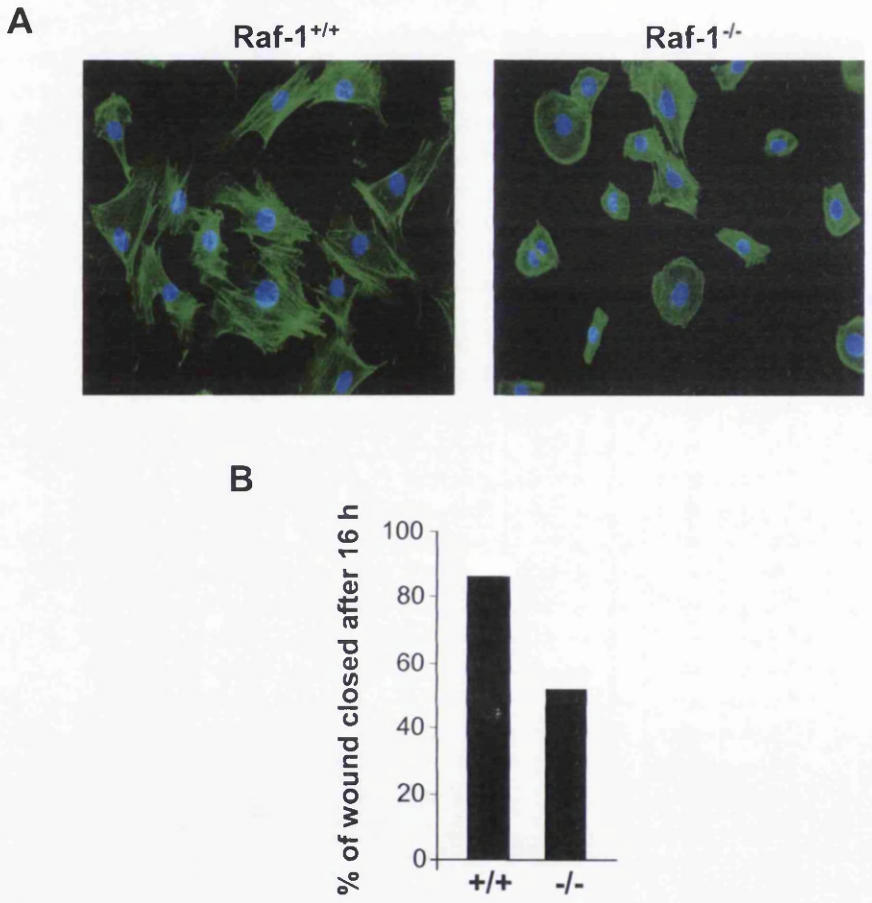

Figure 3.2: Raf-1$^{-/-}$ cells have less stress fibres and are less motile than Raf-1$^{+/+}$ cells. A) Raf-1 knock-out MEFs have less pronounced stress fibres and are more rounded than wild type cells. Growing cells were fixed and stained with FITC-phalloidin for F-actin, DAPI for nuclear DNA and imaged by fluorescent microscopy. **B)** Wild-type MEFs are more motile in a wound assay. Cells were plated and after reaching confluence the cell monolayer was scratched inflicting a "wound". The scratch was photographed and the cells were left in medium containing 10% serum for 16h and photographed again. The width of the wound at 16h was compared to the initial wound width.

3.2: DIGE method development

3.2.1 Minimal labelling of TCA precipitated cell lysate

The three fluorescent labelling dyes that were used in DIGE experiments (Cy2, Cy3 and Cy5) have an activated ester as the reactive group. This NHS-ester reacts with primary amines to form an amide bond. This reaction is used to link the fluorophors to the primary amine of the lysine side chain and therefore to the proteins in the lysate. The efficiency of the labelling reaction depends on several parameters. Firstly the pH of the lysate has to be basic between 8 and 9. Lower pH hinders the labelling reaction because primary amines tend to be protonated and therefore lose the nucleophilic character needed for the formation of the amide bond. However, at higher pH values hydrolysis of the ester is the predominant reaction. Secondly the protein concentration has to be between 5 and 10 mg/ml to ensure that enough lysines are labelled before the NHS-ester hydrolyses. Thirdly, the ratio between dye and protein has to be such that on average only one lysine per protein is labelled, otherwise quantitative comparisons between different proteins would be compromised as they would depend on the number of lysines labelled. Thus, under typical conditions only 3-5% of total lysines are labelled reducing the amount of multiply labelled proteins to a minimum (Marouga et al., 2005). In addition to the changes of electrophoretic mobility, especially for low-molecular proteins by multiple labelling,, "overlabelling" can negatively affect protein solubility (Shaw et al., 2003; Unlu et al., 1997). The general scheme of the experimental design of our DIGE experiments is shown in Figure 3.3. The total study included three individual clones of Raf-1 knockout fibroblasts reconstituted with an empty vector, kinase dead and wild type Raf-1. The cell lysates were harvested form growing cells and one biological repeat was run, resulting in a total of 6 DiGE gels containing 4 gel-images per clone. The internal standard was an equal mixture of all 6 samples.

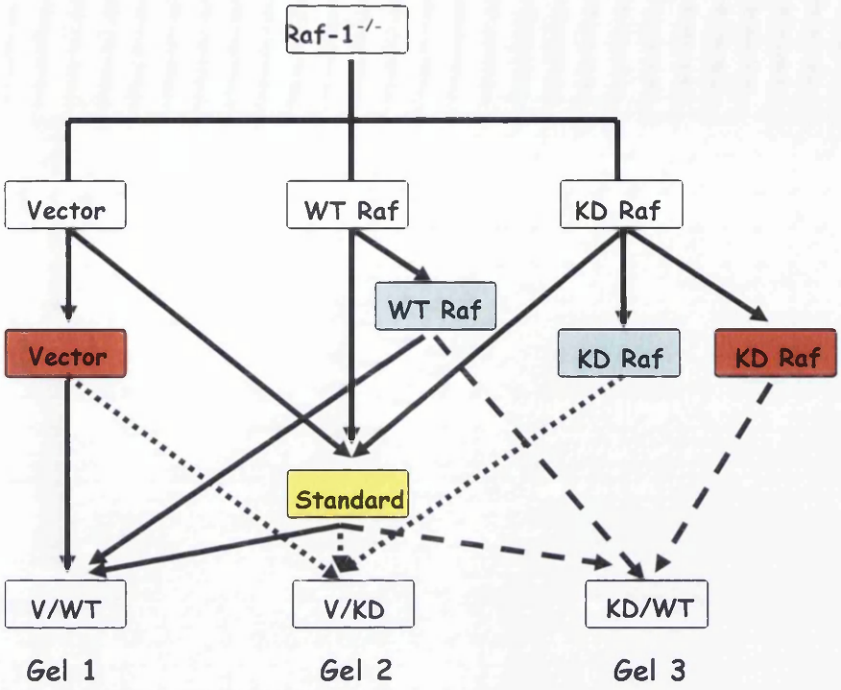

Figure 3.3: Schematic display of the DIGE experiments. Raf-1 knock-out MEFs were stably transfected with an empty vector, wild-type Raf-1 or kinase-dead Raf-1, Raf-1^{K375M}. The lysates were labelled in the following way: Vector with Cy3, Raf-1^{WT} with Cy5 and Raf-1^{K375M} with Cy3 and Cy5. A mixture of all three lysates was labelled with Cy2 to form the internal standard. Labelled vector, wild-type and standard lysates were mixed and separated by 2D-gel electrophoresis (Gel 1). Gel 2 and 3 consisted of either vector/Raf-1^{K375M}/standard or Raf-1^{K375M}/Raf-1^{WT}/standard. Two biological repeats were performed.

In initial studies, we trypsinised cells prior to lysis to achieve the desired protein concentration. However, we later abandoned this method because of concerns that detaching the cells from the plates would alter their properties including protein abundance, modifications and processing. It was also difficult to determine the exact protein concentration using protein assays because of interference from detergents and thiourea. We thus decided to use non-denaturing lysis conditions and to lyse cells on the plates without detaching them. Afterwards the protein concentration of the cleared lysates was determined using a protein assay before precipitation of the proteins with TCA, assuming total recovery of the material. A certain degree of protein loss will occur, especially for proteins with molecular weights <20kDa but the loss of proteins should have been equal between all the samples and the fluorescent labelling reaction is robust enough to cope with a slightly reduced protein concentration. After extensive washes with acetone to remove residual acid, we solubilised the pellet in the labelling buffer. We then

determined the pH and if necessary increased the pH by adding Tris-base to the buffer. The samples were then labelled and run on a standard 1D-SDS gel to determine if the labelling reaction was satisfactory. All samples appeared to be correctly labelled as no apparent double bands were visible in the low molecular region of the gel and the intensity of the fluorescent signal corresponded to the values reported in the manufacturer's protocols. All three samples showed similar patterns on the one dimensional gel (see Figure 3.4). The Cy2 samples generally needed a higher laser intensity to achieve similar signal strength and hence resulted in higher background fluorescence. This could indicate reduced labelling efficiency but was probably associated with different quantum efficiency of the fluorophor.

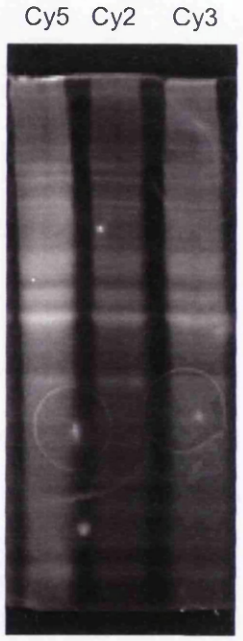

Figure 3.4: Minimal labelling of the MEF lysates: Flurorecent scan of 5μg of DiGE labelled lysate; Cy5 is wild type reconstitued MEF lysate, Cy2 is the standard mixture; Cy3 is the vector reconstituted MEF lysate

3.2.2 Identification of differences

As we wanted to use fluorescence as means of detection we had to cast gels using low fluorescent glass plates. We were not able to use pre-cast gradient gels as these gels are bound to a polymer film that itself is fluorescent and would increase the background. Since we were not able to cast highly reproducible gradient gels ourselves we decided instead to determine an optimal acrylamide concentration which would enable the best separation for the cell lysates for the purposes of our studies. A high gel reproducibility is paramount in 2D-gel analysis as this facilitates inter-gel matching of protein spots, permitting to compare multiple sets of samples. Our initial studies were performed using 12.5% acrylamide gels which showed an optimal separation for small proteins. Unfortunately the majority of protein spots were present in the top quarter of the gel and the separation for the bulk of spots was unsatisfactory. Therefore we decided to use 10% gels although they compromised on the detection of small proteins. These gels improved the overall separation and increased the number of protein spots that were detected. Figure 3.5 shows representative examples of 2D-gels that we obtained. The proteins had molecular masses of between 20 to over 200 kDa. The majority of protein spots were present between pI 4-7 and 40-70kDa in a region of the gel that was also the most reproducible. The basic pI and the high molecular weight region of the gels showed the highest variability. The pI region between 7-11 was the least resolved, a problem commonly encountered in 2D gel electrophoresis as the cysteines contained in the proteins tend to reform S-bonds, leading to "streaking" of proteins spots in the basic pH region. High molecular weight proteins sometimes did not focus into single spots and some proteins with a very high molecular weight were only present in a subset of gels indicating that these proteins did not readily enter the gel and poorly resolved in the second dimension. Additional variation occurred because some gels, although cast at the same time and in the same caster, had regions of lower density resulting in vertical distortions.

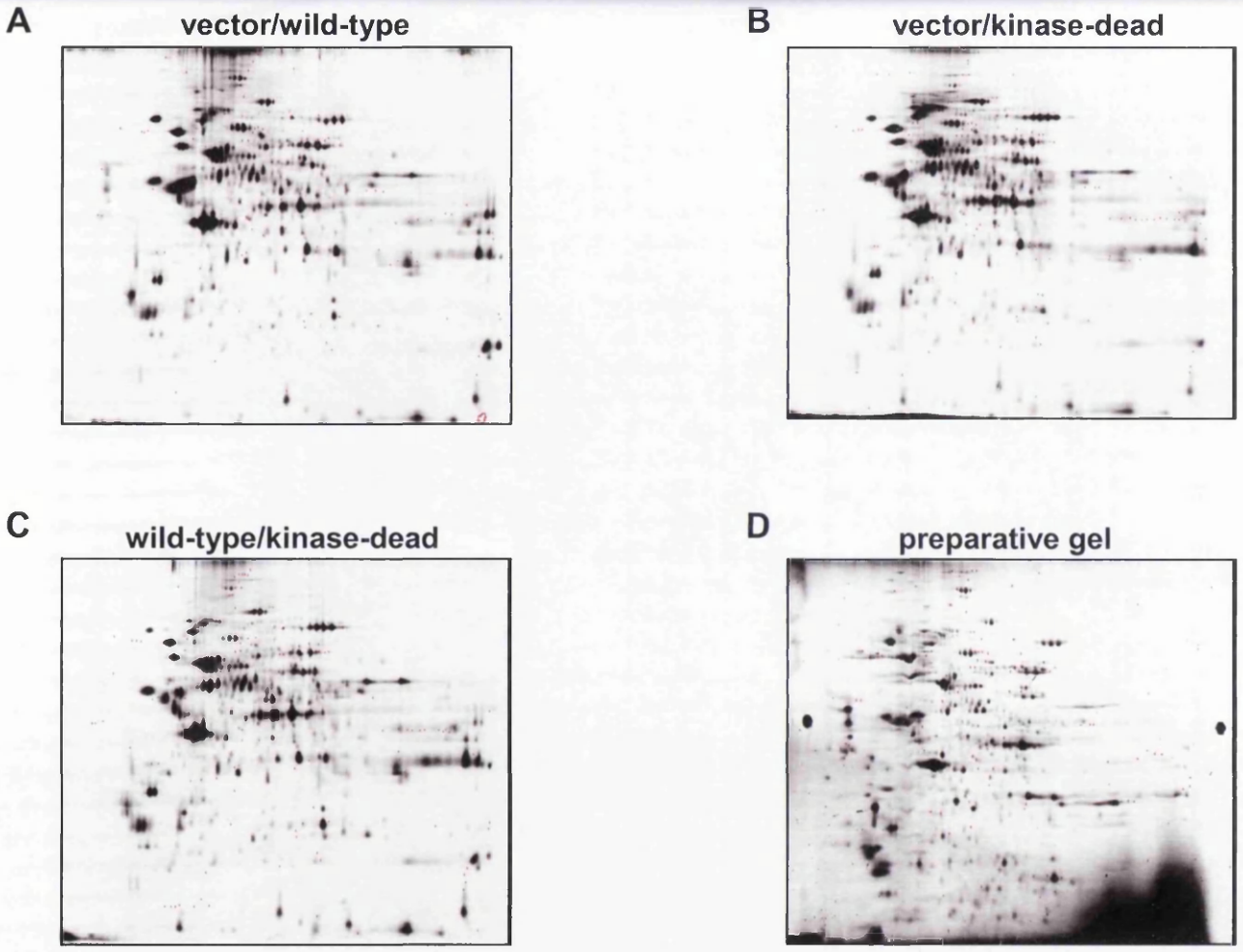

Figure 3.5: Reproducibility of DIGE gels. Cy2 images of 3 of the 12 3-10 pI, 10% acrylamide, 2D gels used in the DIGE analysis plus the preparative gel used for mass spectrometric identification of protein spots (see Figure 3.3 for sample loading). **A)** Gel 1, **B)** Gel 2, **C)** Gel 3 and **D)** "preparative" gel for mass spectrometric analysis (stained with SyproOrange).

After the gels were imaged spot detection was performed using the Decyder software package. An average of 1800 individual protein spots was detected after artefacts were deleted. The status of master gel was then assigned to the image of the standard gel with the most detected protein spots. Thereafter all gel images, resulting form 6 individual gels containing sets of comparisons between all 3 clones (see Figure 3.3) and one biological repeat, were matched to the master. Automatic matching using the software was erroneous, especially in the border regions of the gels. Consequently we matched an average of 50 protein spots manually and assigned these matches as landmarks. The succeeding matching of the remaining spots was done by the software and the accuracy was satisfactory. We routinely matched between 900 and 1700 spots, averaging at around 1100. In a preceding study we assessed the influence of biological variation on protein changes in mouse fibroblasts. Two biological repeats of Raf-1 knock-out fibroblasts were analysed using 6 DiGE gels. Using a t-test value of 0.05, indicating a 95% chance of the mean difference being significant, no protein spots showed a change in the mean spot intensity with a fold change of >1.7 (see Figure 3.7). We therefore decided to use these thresholds for our studies, thus eliminating variances that are likely to arise from technical and systematical errors as well as from biological variances. Hence protein spots showing a >1.7 fold increase or decrease within at least one of the three sets (i.e. vector/wt; vector/kinase dead, kinase dead/wt) with a t-test value of <0.05 were picked for further analysis. To increase the chances of identifying proteins by mass spectroscopy we ran an additional preparative gel with a high protein load. The higher loading negatively affects resolution, but improves the chance of protein identification by mass spectrometry. We were able to match 877 spots from the preparative gel to the master gel. The protein spots were then cut out and processed by a robotic workstation, spotted on a MALDI plate and analysed. Only a limited number of spots could be identified using peptide mass fingerprinting. Some samples contained only the trypsin autodigestion peptides but all samples where additional masses were detected and mass fingerprinting failed to return a significant identification

with a confidence level of >95% were further analysed using LC-MS/MS. In total 63 differentially expressed protein spots were positively identified, containing 55 individual proteins. In 13 spots we identified mixtures containing up to four different proteins. All proteins identified were identified with a confidence of 95% or greater. In case of multiple proteins within one spot there is no certainty which protein changes, therefore an additional quantification has to occur, possibly by immunoblotting.

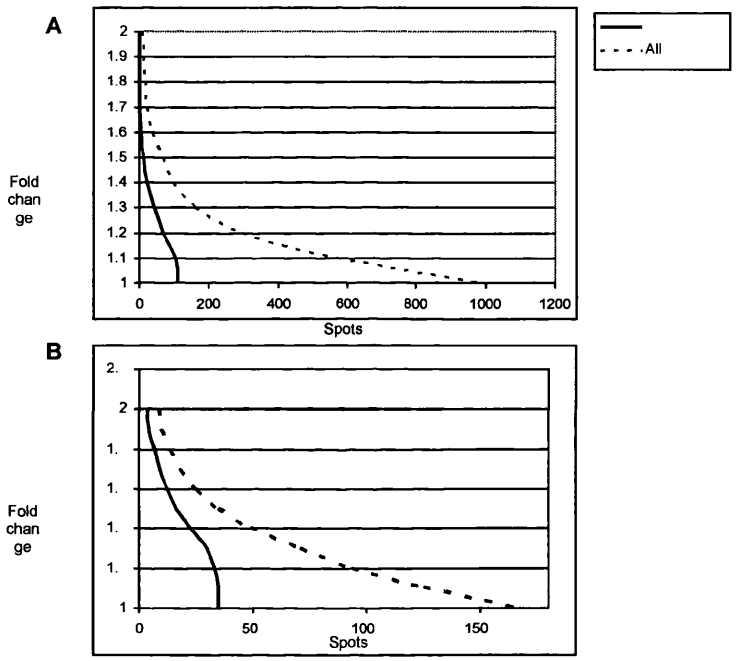

Figure 3.6: Number of protein spot changes above certain thresholds: The x-axis represents the number of protein spots that change, the y-axis represent the fold changes. A) analysis of biological variation of 2 biological repeats of -/- Raf-1 MEFs. If p is set to <0.05 all protein spots that change, change <1.7 fold B) analysis of changes of two biological repeats between Vector and Wild type Raf-1 reconstituted MEFs.

3.3: Protein expression profiles of $Raf^{-/-}$ and reconstituted cells

A list of the proteins identified using DIGE is shown is Tables 3.1 and 3.2. Table 3.1 contains the protein names, means and confidence of the identification, molecular weights and isoelectric points. The proteins are listed in sequenced according to the master gel number (see Figure 3.7). If a mixture of proteins was identified then all proteins are listed according to the Mascot score. The protein accession numbers relate to the protein entries in the NCBI non-redundant database (*http://www.ncbi.nlm.nih.gov/*). The theoretical molecular weight and pI were calculated using a web tool (*http://us.expasy.org/tools/pi_tool.html*), the peptide number indicate how many peptides were identified and if peptide mass fingerprinting was used to identify the protein, the sequence coverage is given. The Mascot score indicates the confidence in the identifications, the threshold was set to >95%.

Table 3.2 lists the properties of the individual protein spots. All spots listed in the table have a increased or decrease in volume >1.7 between at least two of the three sample sets. If the spots were detected and matched in several gels a p value of <0.05 in at least one sample pair indicating a 95% confidence of a change in the spot volume was used as an additional threshold.

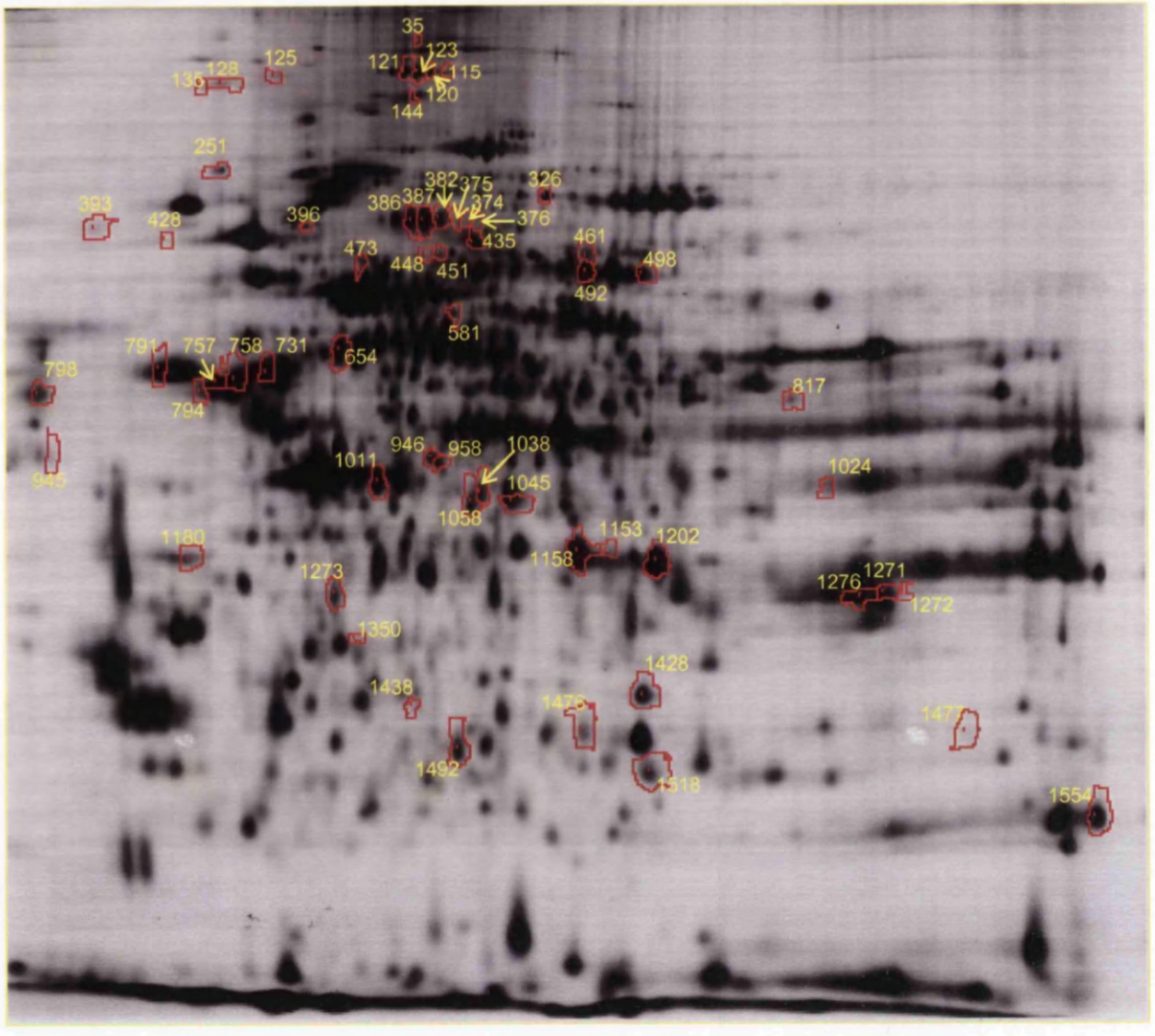

Figure 3.7: Master gel with master spot numbers. Cy2 images of the master gel 12 3-10 pI, 10% acrylamide. Number represent the master spot number (see Table 3.1 and 3.2) of proteins identified that change >1.7 fold.

Table 3.1:

Master No.	Name	Protein AC	pI	Mw	peptides# (coverage)	mascot score	identificatic
115	procollagen, type I, alpha 1	34328108	5.65	138032	20	366	MS/MS
120	Procollagen, type I, alpha 1	29476768	5.65	138032	27(33%)	157	MALDI
123	Procollagen, type I, alpha 1	29476768	5.65	138032	31(34%)	210	MALDI
125	tubulin alpha-3 chain	90217	4.93	50095	2	51	MS/MS
128	beta tubulin	49481	4.85	49741	1	44	MS/MS
134	thrombospondin	511869	4.72	129647	1	52	MS/MS
144	Endosome-associated protein p162	38090688	5.59	160914	15(14%)	48	MALDI
326	ALG-2 interacting protein AIP1	4633515	6.27	96151	16 (21%) (34)	165 (1149)	MALDI (MS/MS)
374	Gsn protein	38014369	5.52	80762	7	198	MS/MS
	palladin	38089265	5.87	152131	7	191	
375	Gsn protein	38014369	5.52	80762	8	196	MS/MS
	palladin	38089265	5.87	152131	5	91	
	alpha-macroglobulin	2144118	6.32	163614	1	56	
	Alpha-1-inhibitor III	112893	5.70	163773	1	45	
376	Gsn protein	38014369	5.52	80762	10 (21%)	48	MALDI
382	Gsn protein	38014369	5.52	80762	10 (21%)	63	MALDI
386	Gsn protein	38014369	5.52	80762	10 (21%)	43	MALDI
387	Gsn protein	38014369	5.52	80762	12 (22%)	114	MALDI
396	heat shock protein 1, beta	194027	4.95	83232	3	31	MS/MS
428	heat shock protein 1, beta	194027	4.95	83232	10	328	MS/MS
435	zyxin	1430883	6.47	60790	5	102	MS/MS
448	thimet oligopeptidase	11385994	5.77	77994	5	110	MS/MS
451	Thimet oligopeptidase 1	21619359	5.67	78026	10	184	MS/MS
461	caldesmon 1	21704156	6.98	60452	10	338	MS/MS
	moesin	6754750	5.94	66481	9	276	
492	moesin	6754750	5.94	66481	13 (18%)	55	MALDI
498	moesin	6754750	5.94	66481	11 (16%)	76	MALDI
581	Asparaginyl-tRNA synthetase	31127138	5.62	63190	4	93	MS/MS
635	Calreticulin	13097432		48136	11 (31%)	113	MALDI
654	Heat shock protein 1 (chaperonin)	16741093	5.91	60955	7 (19%)	54	MALDI
719	Prolyl 4-hydroxylase, beta polypeptide	14250251	4.77	57058	8 (20%)	98	MALDI
731	tubulin alpha	223556	4.94	50241	10 (37%)	60	MALDI
757	tubulin, beta	46237656	4.78	49670	17 (36%)	181	MALDI
758	beta tubulin	49481	4.85	49741	10	210	MS/MS
794	beta tubulin	49481	4.85	49741	7	152	MS/MS
798	Calreticulin	13097432	4.33	47994	10 (21%)	70	MALDI
817	ATP synthase, H+ transporting, mitochondrial F1 complex	6680748	9.22	59752	6	69	MS/MS
945	calumenin isoform 1	6680840	4.49	37063	5	133	MS/MS
946	annexin A7	6753062	5.91	49939	10 (23%)	84	MALDI
958	Adenosine kinase	16307144	5.84	40148	6 (14%)	67	MALDI
1011	Serpinb6a protein [Mus musculus]	34784412	5.53	42598	8 (23%)	68	MALDI
1024	phosphoglycerate kinase	987048	8.02	44562	8 (21%)	45	MALDI
1038	serine (or cysteine) proteinase inhibitor, clade B,	15826842	6.28	42733	3	76	MS/MS
1045	Septin 2	6754816	6.1	41525	8 (19)	81(625)	MALDI
	serine (or cysteine) proteinase inhibitor clade B	15826842	6.28	42733	15	485	MS/MS
	Hsp40	13278364	5.92	40555	9	371	
	mCBP	495128	8.48	35016	3	70	
1058	Serpinb1a protein	15029834	5.85	42574	17	515	MS/MS
1153	annexin A1	6754570	6.57	38691	13 (45%)	207	MALDI
1158	annexin A1	6754570	6.57	38691	7 (23%)	84	MALDI
1180	eukaryotic translation elongation factor 1 delta	26336931	4.96	31292	10	297	MS/MS
	TPR containing protein	21313588	4.99	34322	5	120	
	vacuolar adenosine triphosphatase subunit D	3955100	4.89	40319	3	89	
1202	annexin A1	6754570	6.57	38691	12 (42%)	212	MALDI
1271	Annexin A2	13097099	7.55	38676	9 (34%)	114	MALDI

1272	Annexin A2	13097099	7.55	38676	8 (27%)	78	MALDI
1273	G-protein beta subunit	51116	5.6	37376	2	56	MS/MS
	pyrophosphatase	27754065	5.37	32667	12	321	
1276	Annexin A2	13097099	7.55	38676	10 (38%)	151	MALDI
1428	glutathione S-transferase omega 1	6754090	6.91	27497	9	315	MS/MS
	methylthioadenosine phosphorylase	45544618	6.71	31061	4	104	
1438	Platelet-activating factor acetylhydrolase IB beta	3024348	5.78	25492	5	176	MS/MS
	translation initiation factor eIF-4E	110568	5.79	25053	1	68	
	death domain protein FADD	1167559	5.77	23004	1	43	
1476	Pgam1 protein	12805529	6.67	28831	8	193	MS/MS
	platelet-activating factor acetylhydrolase, isoform 1b, alpha1	6679201	6.42	25853	4	119	
1492	peroxiredoxin 4	7948999	6.67	31052	7	262	MS/MS
	1-Cys peroxiredoxin protein	4139186	5.98	24840	6	193	
	Smfn protein	13097411	5.84	23613	1	40	
1518	triosephosphate isomerase	1864018	5.62	22505	10	412	MS/MS
	glutathione S-transferase, mu 2	6680121	6.91	25716	3	93	
1554	smooth muscle protein SM22 homolog	321246	8.53	22490	9 (46%)	47	MALDI

Table3.1: Proteins differentially expressed in Raf-1 knock-outs and reconstituted fibroblasts. Lane 1: Master gel number (compare Figure 3.7) Lane 2: Protein name from NCBI database, Lane 3: Accession number of the identified proteins, Lane 3 and 4: theoretical pI and molecular weight of proteins, Lane 5: peptides identified and sequence coverage in brackets and %, Lane 6: Mascot score, Lane 7: method of identification, MS/MS indicates identification using LC-MS/MS analysis with QSTAR, MALDI indicates an identification based on peptide mass fingerprinting.

Table 3.2:

Master #	T-test ev/wt	Av. Ratio ev/wt	T-test ev/kd	Av.Ratio ev/kd	T-test kd/wt	Av. Ratio kd/wt
115		1.18		1.77	0.039	-1.5
20	0.24	-1.4	0.24	1.27	0.027	-1.77
123	0.039	-1.96	0.93	-1.01	0.053	-1.94
125	0.028	-1.8	0.19	-1.36	0.018	-1.32
128	0.015	-1.85	0.51	1.09	0.0012	-2.01
134	0.048	-2.27	0.38	-1.25	0.089	-1.82
144	0.011	-1.45	0.027	-1.39	0.74	-1.04
326	0.00031	-1.73		-1.58		-1.1
374	0.052	-1.85	0.22	-1.54	0.26	-1.2
375	0.0085	-2.32	0.27	-1.42	0.047	-1.63
376	0.089	-1.77	0.17	-1.42	0.083	-1.25
382	0.0052	-1.86	0.08	-1.52	0.4	-1.23
386	6.00E-05	-1.77	0.0075	-1.43	0.082	-1.24
387	4.40E-06	-2.01	0.00083	-1.58	0.019	-1.27
396	0.038	-3.08	0.15	-1.72	0.11	-1.79
428	0.017	2.14	0.075	2.11	0.83	1.01
435	0.0048	-1.95	0.0073	-1.64	0.027	-1.19
448	0.00066	1.72	0.018	1.46	0.15	1.18
451	0.0003	1.81	0.012	1.37	0.045	1.32
461	0.0036	-1.77	0.016	-1.61	0.23	-1.1
492	0.00029	-2.53	5.50E-05	-2.43	0.52	-1.04
498	1.00E-05	-2.19	0.00013	-2.04	0.45	-1.07
581	0.0041	1.8	0.23	1.39	0.22	1.29
635	0.0098	-2.24	0.82	-1.1	0.0035	-2.03
654	0.00032	2.12	0.0098	1.64	0.16	1.29
719	0.0083	1.61	0.0051	1.91	0.1	-1.19
731	0.012	-1.75	0.58	-1.13	0.001	-1.55
757	0.07	-1.75	0.95	-1.05	0.0084	-1.68
758	0.015	-1.89	0.16	-1.4	0.11	-1.35
794	0.056	-1.82	0.9	-1.02	6.50E-05	-1.77

798	0.016	-2.67	0.81	-1.09	0.0074	-2.44
817	0.0049	2.02	0.0076	2.11	0.83	-1.04
945	0.022	-2.64	0.031	-1.71	0.1	-1.55
946	0.00011	-1.81	0.0006	-1.48	0.045	-1.22
958	4.70E-05	-2.07	0.00013	-1.8	0.0098	-1.15
1011	0.00068	-1.8	0.00045	-1.71	0.48	-1.05
1024	0.0012	-1.52	0.00013	-2.16	0.0027	1.43
1038	0.0083	-2.72	0.0041	-3.13	0.69	1.15
1045	0.065	-1.99	0.061	-2.15	0.69	1.08
1058	0.26	-1.43	0.024	-2.55	0.12	1.78
1153		1.07	0.046	-1.6		1.71
1158	0.95	1.03	0.011	-1.78	0.058	1.84
1180	0.014	1.43	0.004	1.71	0.15	-1.19
1202	0.061	-1.8	0.0023	-2.73	0.18	1.51
1271	0.81	-1.05	0.057	-1.72	0.044	1.65
1272	0.053	-1.55	0.0087	-2.1	0.19	1.35
1273	0.00031	-2.02	0.0001	-1.74	0.15	-1.16
1276	0.0082	-2.19	0.0081	-2.53	0.53	1.15
1428	0.37	-1.1	0.00013	-2	0.0097	1.82
1438	0.059	1.69	0.042	1.77	0.76	-1.05
1476	0.00062	1.89	0.22	1.13	0.0013	1.67
1492	0.0003	2.1	0.013	1.73	0.073	1.22
1518	0.0023	1.9	0.11	1.25	0.019	1.53
1554	0.00032	-2.07	0.0017	-2.03	0.81	-1.02

Table3.2: Differences between protein spots volumes and confidence in these changes. Lane 1: master number, Lane 2: p values from T-test between empty vector and Raf-1 wild type fibroblasts, Lane 3: Changes in volume between empty vector and Raf-1 wild type fibroblasts, Lane 4: : p values from T-test between empty vector and Raf-1 kinase dead fibroblasts, Lane 5: Changes in volume between empty vector and Raf-1 kinase dead fibroblasts, Lane 6: : p values from T-test between kinase dead and Raf-1 wild type fibroblasts, Lane 7: Changes in volume between kinase dead and Raf-1 wild type fibroblasts.

3.3.1 Proteins involved in apoptosis

We identified several anti-apoptotic proteins, a large proportion of which were cysteine-protease inhibitors, which can inhibit caspases. In vector-reconstituted cells, three apoptosis inhibitory proteins were identified as being expressed at elevated levels. These were the serine (or cysteine) proteinase inhibitor, clade B (Figure 3.8C), Serpin B1a and Serpin|B6a (Ekert et al., 1999). Amongst the other proteins in this group, Alix/Programmed cell death 6 interacting protein (Figure 3.8A), an ALG-2 binding protein

that has been reported to both inhibit and promote apoptosis and cell proliferation was found at elevated levels only the vector-reconstituted cells (Chatellard-Causse et al., 2002; Missotten et al., 1999). The expression of FADD, the FAS-associated death domain-containing protein, was specifically reduced in the vector-containing cells. FADD is an adaptor that recruits caspase-8 or caspase-10 to the activated Fas receptor (Wajant, 2002). The resulting complex is called the death-inducing signalling complex (DISC). This complex when assembled on the activated Fas receptor induces the activation of caspase-8 through proteolytic cleavage. Activated caspase 8 then mediates apoptosis by cleaving and activating Bid or directly activating other caspases, such as caspase 3 (Wajant, 2002). It is uncertain though if this change was accurate as it was only identified in a protein spot containing a mixture of four proteins. We attempted to validate the differentially expressed proteins by quantification via western blots, unfortunately the antibodies we used did not recognise mouse-FADD, nor could we quantify proteins downstream of the Fas receptor, such as cleaved Caspase 8 and truncated Bid.

Proteins involved in cell motility, adhesion and actin dynamics

Zyxin, moesin (Figure 3.8C) and gelsolin were all identified in protein spots that were unregulated in the vector-reconstituted cells. All four proteins are involved in motility, adhesion or actin dynamics. Zyxin (Franco et al., 2004; Wang and Gilmore, 2003) is present in focal adhesions whereas moesin is a linker between the actin cytoskeleton and the plasma membrane, a function that is possibly regulated by RhoGTPases (McClatchey, 2003). Gelsolin on the other hand is involved in capping actin filaments and so preventing monomer exchange (McGough et al., 2003). The changes in these proteins are consistent with the altered actin cytoskeleton and motility in cells lacking Raf-1.

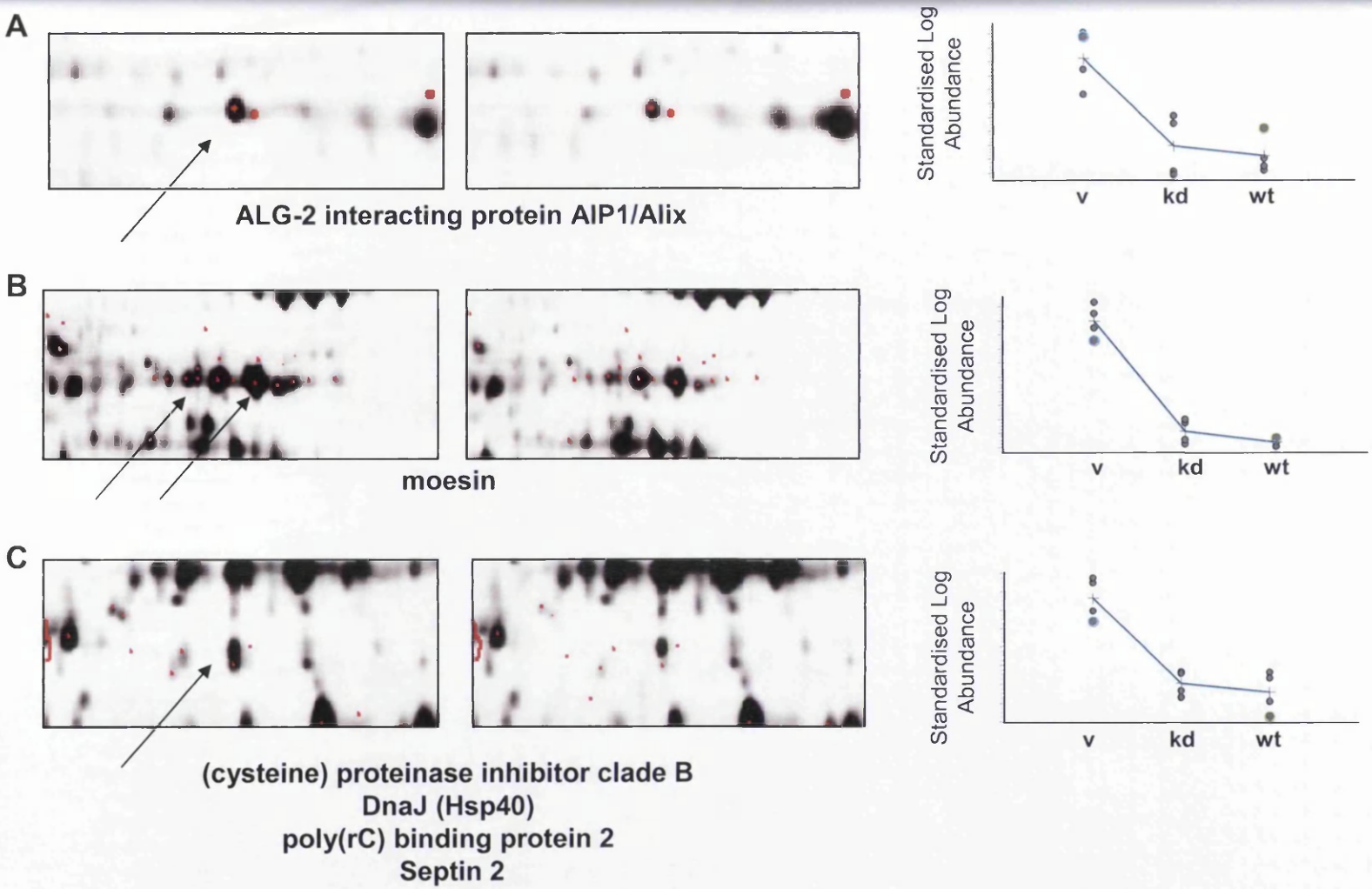

Figure 3.8: Proteins downregulated by reconstitution of wild-type or kinase-dead Raf-1 in Raf-1$^{-/-}$ cells. The left panel shows the Cy3-labelled spot (vector), the middle panel shows the Cy5 labelled spot (Raf-1^{wt}) of the "master" gel. The right panel shows the spot intensity distribution in relation to the standard for vector (v), kinase dead (kd) and wild-type Raf-1 (wt) reconstituted Raf-1$^{-/-}$ cells. **A)** down-regulation of AIP1/Alix, a pro-apoptotic protein; **B)** down-regulation of several moesin isoforms; **C)** a protein spot containing a mixture of four proteins was reduced two-fold in Raf-1 reconstituted cells.

3.3.2 Calcium binding proteins

Several annexin isoforms were detected as differentially distributed proteins. We identified annexin A1, A2, A4 and A7 in several protein spots. In general, these differences were not detected in all annexin containing spots, therefore it is likely that they are related to post-translational modifications. Other calcium binding proteins that were down-regulated included calreticulin (Figure 3.9B) and calumenin. Little is known about the function of calumenin, but we have also identified it as a potential Raf-1 interacting protein in immunoprecipitations experiments. Calreticulin has been proposed to be an upstream regulator of calcineurin, a protein that is involved in ER-dependent apoptosis (Groenendyk et al., 2004).

3.3.3 Changes of proteins involved in reducing peroxides

Two enzymes involved in deactivating peroxides were detected at reduced levels in the vector cells. The 1-Cys peroxiredoxin protein was present in a spot mixed with peroxiredoxin 4 (Figure 3.10A). It has been shown that 1-Cys peroxiredoxin requires glutathione to reduce and recycle the oxidised cysteine (Manevich et al., 2004). This reaction is mediated by glutathione S-transferase (GST) that forms a hetereodimer with peroxiredoxin. We additionally found one GST isoform (Figure 3.10C) at elevated levels in the vector and another isoform was elevated in the kinase dead cells indicating a possible connection between the identified changes.

3.3.4 Changes in potential Raf-1 binding proteins

In addition to the decrease in calumenin expression, we identified a G-protein beta subunit as a protein whose expression was altered. We had previously identified this protein as a potential Raf-1 interaction partner in serum-stimulated cells using our 2D-LC-MS/MS

approach. The expression of G-protein beta seemed to be strongly increased in the vector cells whereas no difference could be detected in the kinase-dead cells. Once again we could not be certain if the amount of this G-protein really had changed as it was detected in a mixture, further validation was impeded by non-availability of an appropriate antibody. In vector-reconstituted cells we also found a two-fold reduction in the expression of chaperonin, a protein previously reported to bind to the N-terminus of Raf-1 in a yeast-two-hybrid screen (Yuryev and Wennogle, 2003).

3.3.5 Changes in protein expression not rescued by kinase dead Raf-1

We identified four protein spots whose expression was altered in a manner dependent on Raf-1 kinase activity. These changes included spots containing collagen type I, beta tubulin, thrombospondin and calreticulin (Figure 3.9). The changes in the spot intensity of both the collagen and the tubulin spot only occurred in one specific spot suggesting that the change is specific to one isoform.

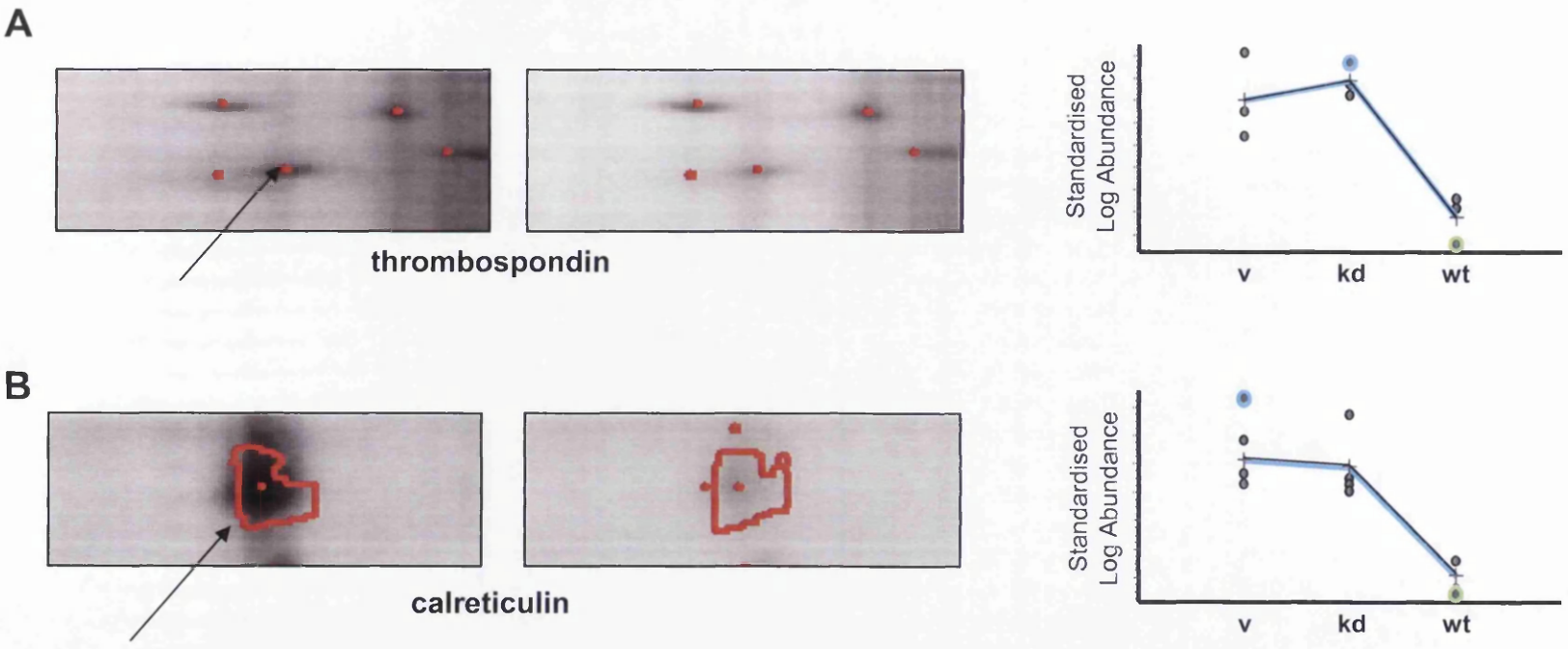

Figure 3.9: Proteins whose expression was dependent on Raf-1 kinase activity. The left panel shows the Cy3 labelled spot (vector), the middle panel shows the Cy5 labelled spot (Raf-1^{wt}) of the "master" gel. The right panel shows the spot intensity distribution in relation to the standard for vector (v), kinase-dead (kd) and wild-type Raf-1 (wt) reconstituted Raf-$1^{-/-}$ cells. Two examples of proteins whose expression was downregulated specifically when Raf-1-/- cells were reconstituted with wild-type Raf-1. **A)** thrombospondin; **B)** calreticulin.

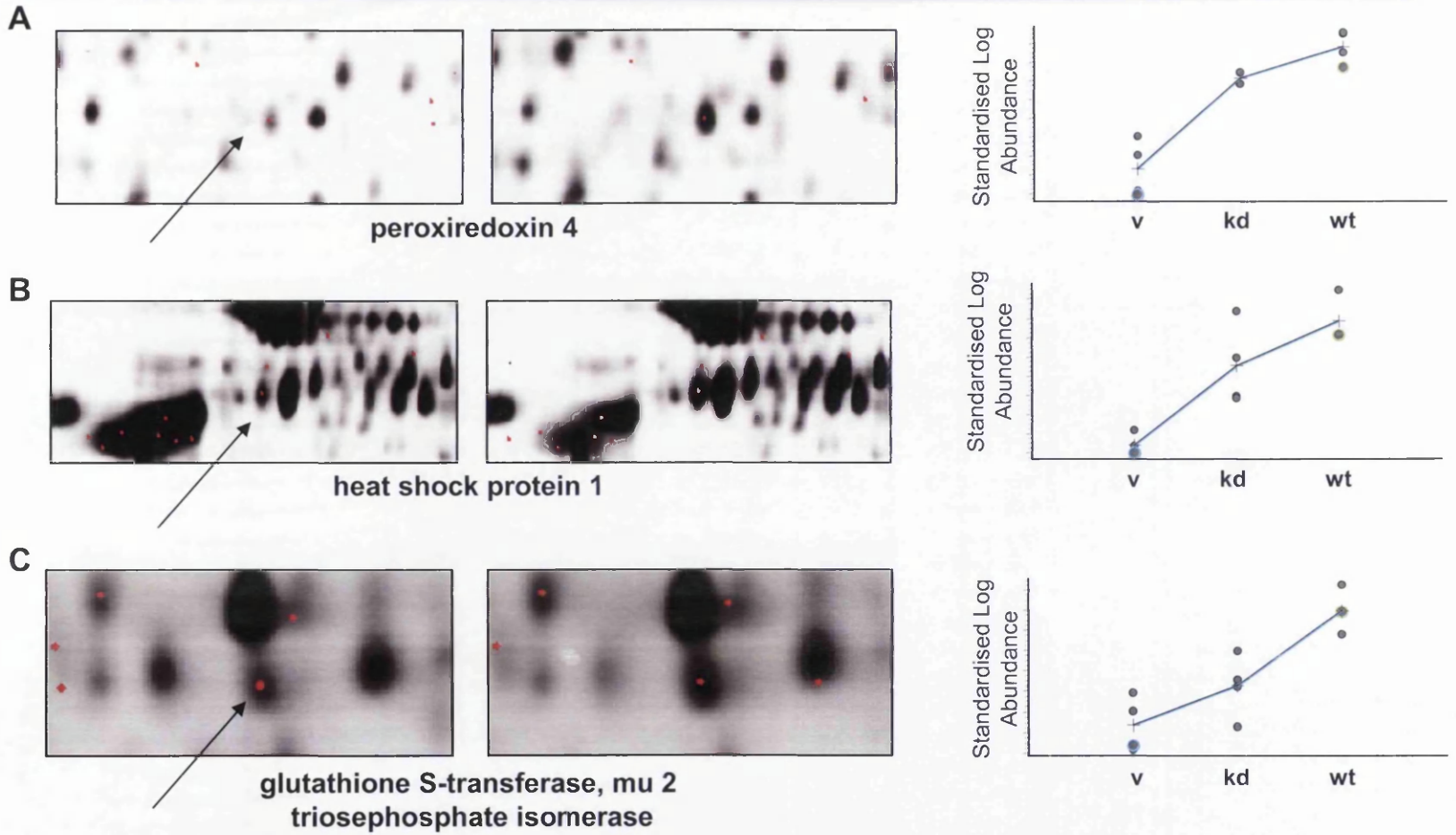

Figure 3.10: Proteins up regulated by reconstitution of Raf-1$^{-/-}$ cells with wild-type or kinase-dead Raf-1. The left panel shows the Cy3 labelled spot (vector), the middle panel shows the Cy5 labelled spot (Raf-1^{wt}) of the "master" gel. The right panel shows the spot intensity distribution in relation to the standard for vector (v), kinase-dead (kd) and wild-type Raf-1 (wt) reconstituted Raf-1$^{-/-}$ cells. **A)** upregulation of Heat shock protein 1; **B)** upregulation of peroxiredoxin 4; **C)** a spot containing a mixture of two proteins was two fold upregulated in the Raf-1 reconstituted cells.

Chapter 3: Discussion

Initially we desired to use pools of reconstituted cells, unfortunately we were unable to detect stably reconstituted Raf-1 in our pools and therefore had to resort to use single clones, where Raf-1 expression was detectable. Because of these limitations some of the differences we detected might be due to clonal variation. It is quite encouraging though that the majority of the identified differentially expressed proteins were either elevated or reduced in both clones that were reconstituted with either kinase dead of wild type Raf-1, therefore indicating that these alterations might not be due to previously mentioned clonal variation..

We successfully analysed changes in protein expression induced by reintroducing Raf-1 or kinase-dead Raf-1 into immortalised Raf-$1^{-/-}$ fibroblasts. Use of DIGE methodology enabled us to detect numerous differentially expressed proteins in these cell lines. Unsurprisingly the biggest differences were detected between the vector and the wild-type Raf-1 reconstituted cells. The protein expression profile of cells reconstituted with kinase-dead Raf-1 generally resembled those of the wild-type Raf-1 reconstituted cells indicating that Raf-1 kinase activity plays only a minor role in inducing the identified changes. In some cases the kinase-dead Raf-1 reconstituted cells showed changes in protein expression that were intermediate between the vector and wild-type reconstituted cells. One explanation of this observation is that Raf-1 protein levels were higher in the wild type cells and the effects could be dependent on the total amount of Raf-1 present in the cells. The exceptions from these general trends were annexin A1, one GST isoform, tubulin, calreticulin, thrombospondin and collagen. The latter two proteins are involved in cell-cell and cell-matrix adhesion (Sid et al., 2004). These results suggest that the kinase activity of Raf-1 is required for some aspects of adhesion but further experiments should be performed to confirm this hypothesis. The changes observed in annexin A1 and the GST

isoform were not matched by the vector reconstituted cells, suggesting that these differences were due to clonal variations. Some of the biggest differences detected were in proteins that inhibit caspases and this is probably due to cells adapting to higher basal levels of protease activity, a likely consequence of the proapoptotic phenotype. The differential expression of Alix is possible another protection mechanism as overexpression of the C-terminus of this protein protects neuronal cell from apoptosis by blocking caspases, whereas overexpression of the full length is sufficient to induce apoptosis in the same cells (Trioulier et al., 2004). Further experiments, especially a siRNA knock-down experiment, are needed to elucidate the effect Alix has in Raf-$1^{-/-}$ fibroblasts.

The detection of the differences using DIGE increases the understanding of some aspects of the phenotype of the Raf-1 knock-out cells such as hypersensitivity to apoptosis, impaired mobility and a defective actin cytoskeleton. On the other hand we were not able to elucidate how Raf-1 protects from apoptosis. Though we identified numerous proteins that are involved in apoptosis such as FADD and the caspase inhibitors, it is likely that these responses are adaptive features of cells that are hypersensitive toward apoptotic stimuli and therefore are selected to contain higher levels of anti-apoptotic proteins. Thus the cells have to adopt mechanisms to survive such as overexpressing potent inhibitors of caspases or by reducing Fas receptor signalling. Thus the DIGE analysis seems to have identified the consequences of cells adapting to the loss of Raf-1 rather than direct targets of Raf-1 involved in protecting cells from apoptosis. The possible exception is Alix but further experiments will be required confirm this.

From a technical point of view, use of the novel DIGE approach was highly satisfactory. The method was robust and spot patterns on gels were reproducible and it was possible to identify differences between samples with ease. This is a great improvement over other 2D gel staining techniques as it is comparatively quick and easy to identify intensity changes and provide accurate quantification. To achieve a similar comparison using other modern

fluorescent staining methods such as SyproRuby staining would have involved running a larger set of gels, as it is possible to run three samples on one gel using DIGE. Furthermore, the workload would have increased because matching all gels to one master gel is essential for cross-gel comparisons. It is far easier to match 6 gels, as we used in each of our conditions, than to match 18 gels. The use of an internal standard further reduced the need to run additional gels as it was possible to compare samples from different sets to each other. In the case of our experiments, we were able to compare the vector samples against vector/wild type samples and to a kinase-dead sample from a kinase dead/wild type gel as the standards in all the samples were identical. Therefore running 6 gels with three different cell lines resulted in a quadruple set of cross-comparisons. By using DIGE we managed to circumvent several drawbacks that the 2D gel approach inherently possesses. Unfortunately, we still did encounter problems that are the hallmark of this type of approach. Though we reproducibly were able to match more than 1000 protein spots to the master gel we were still looking at only a small subset of the total proteome.

Taking into account that several proteins are present in multiple spots we were only comparing a small set of proteins, possibly derived from as few as 400 genes. To further increase the uncertainties a large proportion of the spots contained a mixture of up to four proteins, frequently making it impossible to assign the detected changes to one protein. The problem of resolution can be partially overcome by using narrow pI range IEF strips and gradient gels, but on the other hand this increases the workload and is costly as the dyes cost £100 per gel. Furthermore, even though MALDI mass fingerprinting was able to identify a substantial number of protein spots, it would be desirable to analyse all peptide samples with a mass spectrometer capable of acquiring MS/MS data to be more confident about the identifications. Because we were only able to acquire MS/MS data using an LC-system coupled to a Q-TOF the throughput was limited to approximately 30 samples per 24 hours. We therefore decided to analyse only the samples that failed in the MALDI but

still contained ions that were not of trypsin origin. The limiting factor of this type of approach is no longer the sensitivity of the mass spectrometer, as we were able to identify proteins in spots that were hardly visible, but the overall resolution of the 2D gels resulting in multiple identifications of proteins in individual spots.

Because of these disadvantages it would be preferable to conduct similar comparisons using methods that do not rely on 2D gel electrophoresis. Several methods have been developed recently that enable comparisons between samples using isotope tags. Most notably SILAC (Ong et al., 2002) and ICAT (Gygi et al., 1999; Zhou et al., 2002) are two methods that could be used for these comparisons, especially when combined with high resolution LC methods such as 2D-LC or gel-assisted LC. These methods are capable of analysing several thousand peptides in one run and have been shown to be able to detect several hundred individual proteins or even over 1000 if pre-fractionation is used (Link et al., 1999; Washburn et al., 2001). 2D gels still retain one advantage over these methods, it the most practical method to detect post translational modifications, but especially when changes in phosphorylations are involved (Hinsby et al., 2004), new gel-less approaches are emerging and will probably replace 2D gel electrophoresis as the preferred method of choice.

Chapter 4

Analysis of Raf-1 complexes

Chapter 4: Introduction

Proteins involved in signal transduction act mostly as part of multiprotein complexes (Kolch, 2000; Pawson and Scott, 1997). These complexes can contain adaptors, chaperones, linkers, effectors and regulators of the core proteins. Raf-1 has been shown to exist as a complex of ~500kDa (Wartmann and Davis, 1994). The core complex seems to consist of several heat shock proteins and 14-3-3, while adaptors such as KSR and CNK enhance the interaction of Raf-1 with regulators, effectors and substrates in response to extracellular signals (Kolch, 2000). In addition to these, proteins such as RKIP can bind Raf-1 and disrupt its interaction with its substrate, MEK (Yeung et al., 1999). The net effect of this multitude of protein-protein interactions is to control the signal flux through the pathway, regulate crosstalk with other pathways, and ensure signalling specificity and fidelity.

The analysis of the components of protein complexes is crucial to the understanding of how signalling processes are regulated. Conventional approaches for identifying protein-protein interaction such as yeast-two-hybrid screens focus on binary and direct interactions in the nucleus of a yeast cell, which is a rather artificial environment for mammalian proteins (Fields and Song, 1989). Proteins that do not bind or may only bind weakly without scaffolding molecules may not be identified using these methods. More recently, several methods based on the ability of mass spectrometric analysis to identify proteins have been used to analyse protein complexes on a large scale (Ho et al., 2002; Rubio et al., 2004). Firstly, interacting proteins can be purified by firstly cross-linking the "bait" to a matrix and then incubating it with a protein lysate. Proteins that bind to the bait are then eluted and identified using mass spectrometry. Secondly, intact multi-protein complexes can be isolated from cell lysates by immunoprecipitating the bait and identifying the bound proteins by mass spectroscopy. We decided to use the latter method to purify complexes

because it presented several advantages over other methods. Immunoprecipitating intact protein-complexes ensures that these have formed in their "natural" environment and not in-vitro on a column or inside a yeast cell nucleus. Secondly it possible to analyse changes in the composition of the complexes in response to different stimuli. Thirdly it enables us to study the entire complex and not only direct one to one interactions between the bait and the protein. Standard analysis of proteins involves separating them using PAGE, cutting out the stained bands and using peptide mass fingerprinting to identify them. Though powerful, peptide mass fingerprinting is problematic when the proteins are not isolated with high resolution as it is virtually impossible to identify mixtures as are often present in 1-dimensional gels. Additional problems arise from separating proteins by PAGE. Several amino acids can be modified by the running conditions. For example, methionines are oxidised and cysteines can be modified by unpolymerised acrylamide. Moreover, the proteins have to be digested in gel, adding several wash and elution steps. This potentially decreases the total amount of material available to be injected into the mass spectrometer, as especially hydrophobic peptides tend to be retained in the gel matrix and a proportion of eluted peptides will adhere to the tubes used. Finally, contamination from keratins and polymers can arise more readily because more manual handling is needed. We therefore decided to use a gel-less method to study protein complexes, combining in-solution tryptic digests with a 2-dimensional LC approach coupled to a QTOF mass spectrometer able to generate MS/MS data. We used FLAG-tagged human Raf-1 overexpressed in COS-1 cells as a model.

As the interactions between proteins can be regulated by their phosphorylation status, we also wanted to develop new methods that would enable us to identify phosphorylated peptides in the immunoprecipitated Raf-1 complexes. Reversible protein phosphorylation is a major mechanism of regulating protein function (Cohen, 2002; Manning et al., 2002). For example, the biological functions of key signalling molecules such as Raf-1, are regulated through multisite phosphorylation at activating and inhibitory sites (Dhillon and

Kolch, 2002). Mass spectrometry is a powerful technique to identify and sequence phosphorylation sites. Even with the introduction of new and more sensitive mass spectrometers, sequencing of phosphopeptides still remains a demanding task due to the nature of the modification. The acidity of phosphorylations impedes efficient ionisation in positive ion mode. Therefore the signal intensity of the modified peptide in comparison with the unmodified one is greatly reduced. In complex mixtures, the ion of the phosphorylated peptide is quite frequently not detectable in the positive ion mode, but identification of modified peptides can be greatly improved through prior purification and fractionation of the peptide pool. Standard phospho-specific purification methods involve immobilised metal affinity chromatography (IMAC) and chemical modification to transform the phosphor group into a modification that would facilitate the purification (Mann et al., 2002). In this chapter, we attempted a different approach by utilising phosphorylation-dependent binding proteins and antibodies to purify serine- and threonine-phosphorylated peptides from complex mixtures. Another method that was chosen to tackle the problem was to perform precursor ion scans on either the 216Da phospho-tyrosine immonium-ion or the 79Da phosphoric acid ion (Steen et al., 2001a; Steen et al., 2001b). These results were also compared with a neutral loss scan of 98Da to find the most sensitive and practical method to identify in vivo phosphorylation sites of Raf-1 and its binding partners.

Chapter 4: Results

4.1 Identification of proteins which co-immunoprecipitate with Raf-1

4.1.1 MALDI peptide mass fingerprinting of SDS-PAGE separated Raf-1 multiprotein complexes

To assess if our method of identifying proteins was an improvement over standard methods, we initially used the well established gel electrophoresis protocol to separate proteins. FLAG-Raf-1 was expressed in COS-1 and immunoprecipitated by incubating the cell lysate with anti-FLAG-antibody coupled to agarose beads (Ho et al., 2002). The antibody used was covalently linked to the agarose to minimise contamination of the sample by co-elution of immunoglobulin. We decided to use a pH drop (glycine pH 2.5) to elute FLAG-Raf-1 and interacting proteins instead of using a more specific elution by incubating the beads with an immunoreactive peptide. We were concerned that large amounts of the peptide in the eluate might impede subsequent analysis, particularly when the proteins would be digested in solution prior to separation by 2D-LC. The eluted proteins were TCA precipitated, washed extensively with acetone and then resolubilised with SDS-PAGE loading buffer. The proteins were then separated by gel electrophoresis and stained with Coomassie. The complete lane was cut into 40 strips and after extensive destaining and washing we performed in-gel digestion with trypsin. The digests were subsequently analysed by MALDI-TOF peptide mass fingerprinting using the MASCOT search engine to identify the proteins.

Although most samples contained multiple ions that did not correspond to tryptic auto digested peptides of contaminants such as keratins, we were only able to identify 9 individual proteins. We assumed that the majority of the samples contained multiple proteins and therefore rendered it impossible to identify the proteins without obtaining MS/MS data. Most proteins identified were well known Raf-1 interacting proteins, such as

HSP90, HSP70 and 14-3-3 isoforms. Three of the identified proteins, a G-protein, a hypothetical protein and a histone had not been reported to bind to Raf-1 (see Figure 4.1).

4.1.2 2D-LC MS/MS of whole cell lysates

It has been reported that 2D-LC MS/MS coupled to a mass spectrometer can identify up to 500 individual proteins with one run. These studies were performed using 120µg of a yeast lysate (Link et al., 1999). As we did not expect to be able to generate an immunoprecipitated sample of such high a concentration of protein, we wanted to test the performance of our 2D-LC MS/MS setup (Figure 4.2) and our in-solution digest protocol using 50µg of a mammalian cell lysate. We precipitated the lysate with TCA to mimic the protocol used for our eluates and resolubilised the pellet in urea and n-octylglucoside. By adding the detergent we hoped to facilitate the resolubilisation of the proteins. Additionally, it has been reported that adding suitable detergents enhances the tryptic digest and reduces sample loss (Erdjument-Bromage et al., 1998). As this is a non-ionic detergent it should not bind to the ion-exchange column and, and as it elutes at a higher acetonitrile concentration than most peptides from a C18 reverse phase column, will not affect peptide identification on the first HPLC run. The proteins were then incubated with DTT at 37°C to reduce the cysteine bonds. Increasing the temperature further was avoided to minimise carbamylation of side chains. Trypsin has a low reactivity at high urea concentrations, therefore we diluted the urea to under 0.5M before adding trypsin. We incubated the solution at 37°C overnight. To avoid sample loss through concentration steps the digest was only acidified and subsequently injected into the LC. We used 8 salt step-gradient elutions and a 90 minute acetonitrile gradient to separate the peptides. The LC was coupled to a Q-TOF (Q-STAR Pulsar) mass spectrometer that acquired MS/MS spectra of all multiply charged ions, as peptides tend to carry 2 or more charges when electrospray is used as means of ionisation. The mass spectra were then analysed using MASCOT.

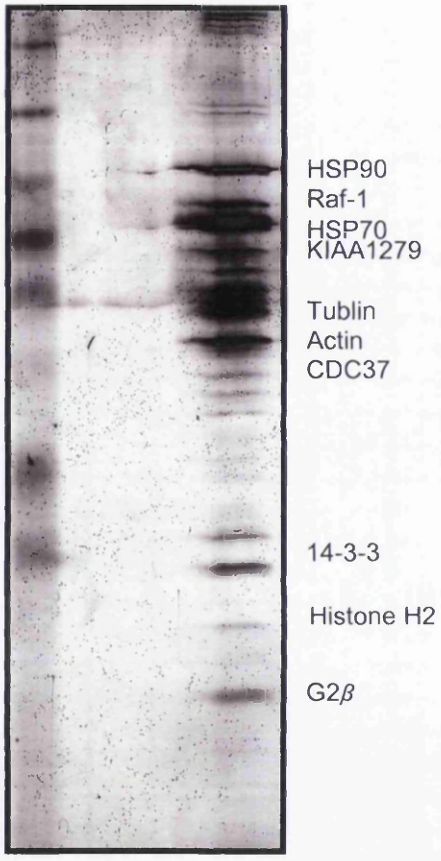

Figure 4.1: Silver stain of FLAG-Raf-1 and co-immunoprecipitating proteins. FLAG-Raf-1 was immunoprecipitated on α-FLAG beads and eluted with a pH drop. The proteins were TCA precipitated and individual protein bands were resolved by SDS-PAGE. The whole lane was cut into ca. 1mm slices, in-gel digested with trypsin. Eluted peptides were then analysed by MALDI-MS and proteins were identified by peptide mass fingerprinting. Proteins were often identified in multiple lanes. The index on the left indicates the region of the where the proteins were identified

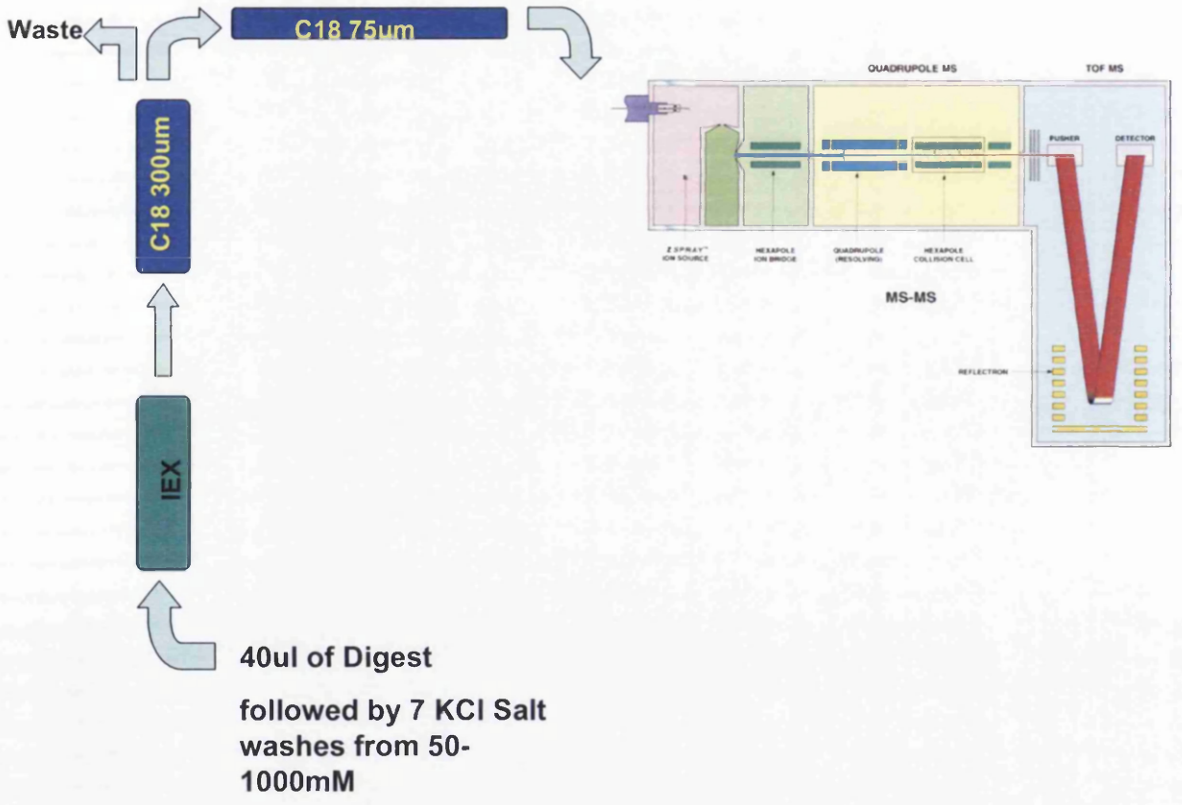

Figure 4.2: Schematic design of the 2D-LC MS/MS setup. A tryptic digest is injected onto a strong ion exchange column (IEX) and peptides that do not bind to it are trapped on the first C18 reverse phase column. These peptides are then separated using a second C18 column before being ionised and analysed by the Q-TOF mass spectrometer. Peptides bound to the IEX column are eluted stepwise and then separated by reverse phase chromatography. This cycle was repeated 7 times.

We were able to identify 70 individual proteins. No oxidised methionine was found and only one peptide was carbamylated. The tryptic digest was successful as no increase in the number of missed cleavages in comparison to typical in-gel digests was found. These results confirmed that our in-solution digest protocol worked and that our 2D-LC setup would be powerful enough to analyse samples of medium complexity, such as immunoprecipitated protein complexes..

4.1.3 2D-LC analysis of wild-type FLAG-RAf-1 and FLAG-Raf-1^{S259A}

Although we expected to increase the sensitivity of the analysis using our gel-less protocol we were concerned that we would not be able to generate information that can be derived from visualising proteins bands on gels before analysing them by mass spectrometry. We expected to lose apparent molecular weight information and also information about changes in absolute protein amounts. To test the ability of our set-up to detect known changes in composition of protein complexes, we compared wild-type Raf-1 against Raf-1^{S259A} immunoprecipitates. Unlike the wild-type protein, Raf-1^{S259A} is known to bind 14-3-3 protein poorly. This difference in 14-3-3 binding can be easily detected when wild-type and Raf-1^{S259A} immunoprecipitates are run in parallel on a 1D gel. Additionally the Raf-1^{S259A} mutant has other interesting characteristics. Despite its ability to constitutively activate MEK to a level comparable to the oncogenic v-Raf protein, this mutant is unable to transform cells. One possible explanation for this observation is that the mutant impedes signalling pathways important for transformation, possibly by interacting with other proteins (Dhillon et al., 2003; Dhillon et al., 2002a). Thus, comparing complexes formed by Raf-1 and Raf-1^{S259A} is an ideal test for the method, as some changes in complex partner proteins are known while further changes are expected.

We immunoprecipitated FLAG-wild-type and FLAG-Raf-1^{S259A} and identified the proteins in the complexes by 2D-LC MS/MS. A comparison of the proteins identified showed that several were unique to either the wild-type or mutant Raf-1 (Figure 4.3). A more detailed functional study of one of the associated proteins, protein phosphatase 5 (PP5), will be reported in a subsequent chapter. Most notable of these was the presence of five 14-3-3 isoforms in the wild-type sample but only one in the Raf-1^{S259A} sample, confirming the reduced binding of 14-3-3 proteins to the mutant. The ability to detect differences in 14-3-3 binding to Raf-1 suggests that our approach was sensitive enough to identify absolute changes in the composition of protein complexes.

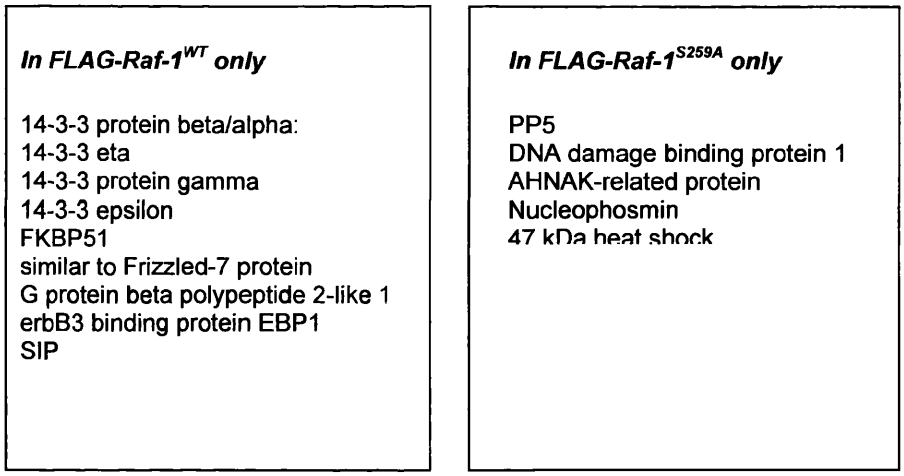

Figure 4.3: Proteins identified only in FLAG-Raf-1^{WT} or FLAG-Raf-1^{S259A} immunoprecipitates by 2D-LC MS/MS analysis.

4.1.4 2D-LC MS/MS analysis of Raf-1 complexes isolated from starved, serum-stimulated and taxol-treated cells

Based on the results described above we next wanted to test if we could identify changes in the composition of Raf-1 complexes under different conditions. We decided to use the Raf-1^{K375M} mutant as bait. This mutation of the essential lysine in the ATP binding pocket of

Raf-1 prevents ATP binding, hence destroys kinase activity. This kinase-dead Raf-1 may bind more strongly to prospective substrates by functioning as a "substrate trap". To compare dynamic changes in Raf-1 complexes, we compared the composition of complexes formed after serum-starvation, serum-activation (a mitogenic stimulus) and taxol-treatment (an apoptotic stimulus). We transfected Cos-1 cells with FLAG-Raf-1^{K375M}, and after 24 h starved the cells in medium containing 0.1% FCS or treated them with taxol overnight. One set of serum-starved cells was also stimulated for 15 minutes with 10% FCS. The Raf-1 complexes were immunoprecipitated and analysed using 2D-LC MS/MS as described previously. We identified 98 proteins in immunoprecipitates from the serum-stimulated cells, 82 in serum-starved cells and 63 in taxol-treated cells. Most of the proteins were chaperones, adaptors and ribosomal proteins, but we also identified low abundance proteins, such as kinases, phosphatases, transcription factors and G proteins. Several proteins were uniquely present in Raf-1^{K375M} complexes from either serum-activated or serum-starved cells, and two proteins were exclusively present in the taxol-treated samples (Figure 4.4).

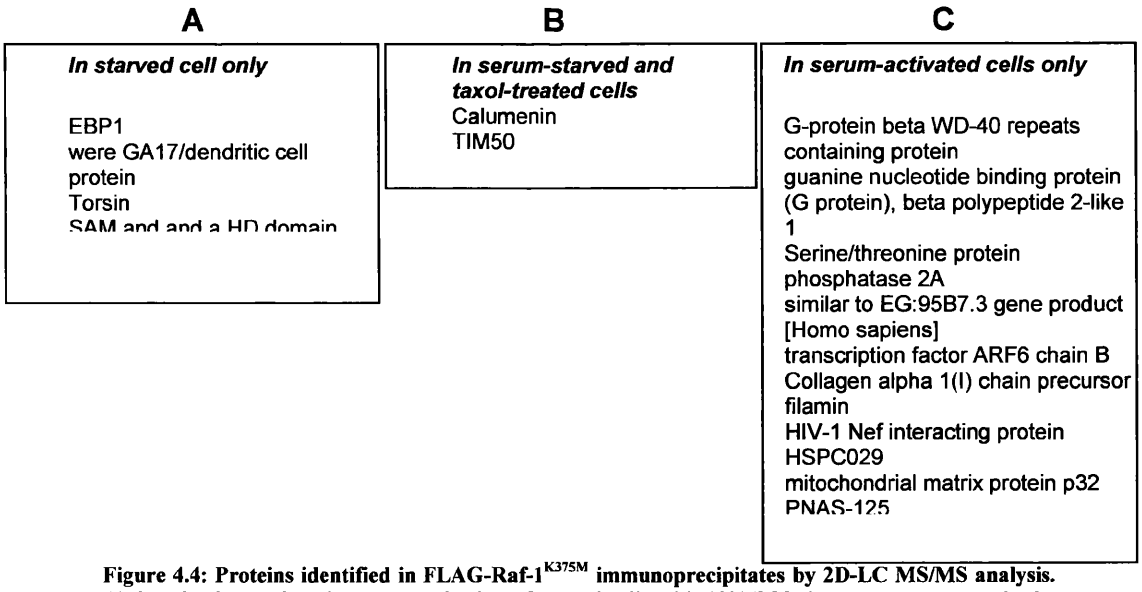

Figure 4.4: Proteins identified in FLAG-Raf-1^{K375M} immunoprecipitates by 2D-LC MS/MS analysis. A) Proteins interactions lost upon activation of starved cells with 10% FCS that were not present in the taxol treated sample B) Protein interactions induced by treating cells with taxol overnight and present in starved cells; C) Proteins interactions induced by treating cells with 10% FCS for 15min.

We evaluated the validity of our approach by comparing the strength of one individual ions belonging TIMM50, a protein identified in the serum starved and taxol treated Raf-1 immunoprecipitation. A doubly charged ion with m/z 559.9 was identified by the Mascot search engine as being a tryptic peptide derived from TIMM50. The ion was present at a strength of 70 and 55 counts at its maximum in the 50mM salt step of the serum starved and taxol treated immunoprecipitation but only with a count of 20 in the serum activated Raf-1 immunoprecipitation. A direct comparison with the co-eluting ions additionally confirms that the ion is relatively reduced (Figure 4.5).

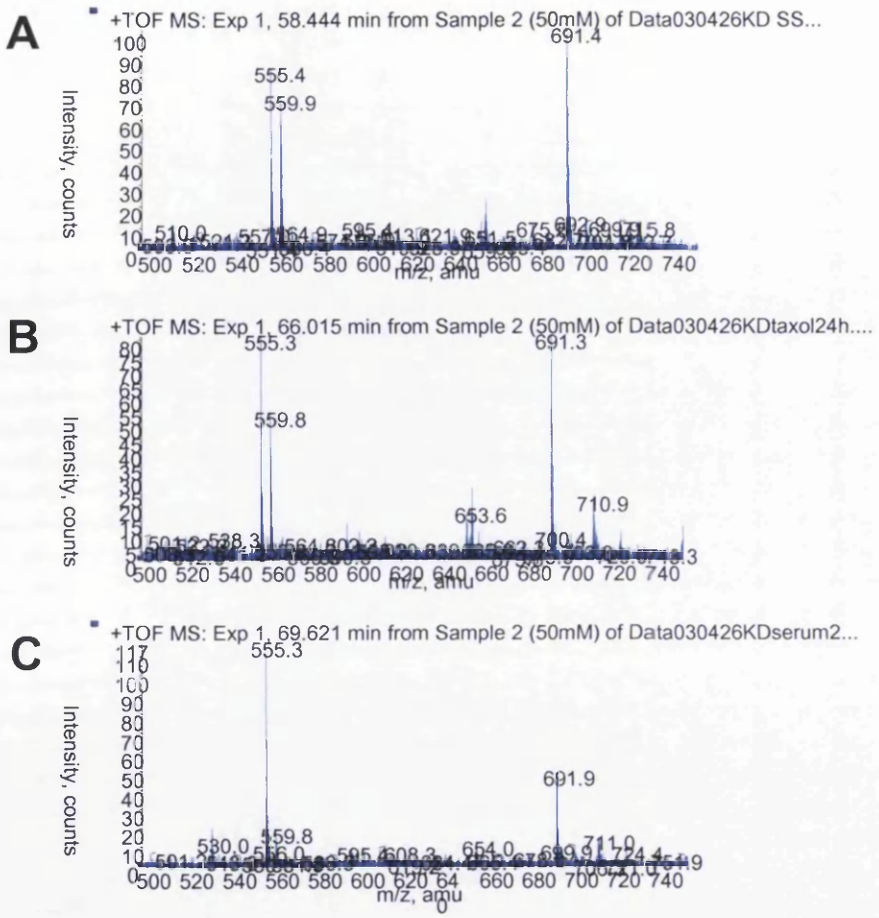

Figure 4.5: Comparison of ion-counts of TIM50 peptide: 559.8 represents a doubly charged tryptic peptide (VLLDLSAFLK) all three MS spectra were taken from the 50mM salt elution step. The time point shows the highest recorded ion intensity of the 559.8 peptide A) MS of the serum starved IP at 58.44 min B) MS of the taxol treated IP at 66.02 minutes B) MS of the serum activated IP at 69.62 minutes

Table 4.1 gives a list of all proteins identified from the Raf-1 interaction studies. The first column contains proteins that have been previously described to be either binding to Raf-1 or a core members of the HSP90/70 complex, therefore likely to be contained in the Raf-1 core complex that contains Raf-1/HSP90/HSP70/CDC37/14-3-3/BAG-1. The second column contains proteins that have not been described to be binding to Raf-1 previously but are likely to be specific interaction partners as these proteins were not identified in immunoprecipitations form unrelated proteins. Finally column three contains likely contaminants, as these proteins are regularly found in immunoprecipitations and seem to be precipitating regardless of the nature of the bait. Additionally to the listed proteins keratin, BSA and trypsin were identified.

Table 4.1: Proteins identified in FLAG-Raf-1 co-immunoprecipitations

Raf-1 and HSP90 interacting proteins	Likely interacting proteins	Likely contaminants
14-3-3 epsilon	52-kD SS-A/Ro autoantigen	26S proteasome regulatory chain 12 - human
14-3-3 eta	ADP,ATP carrier protein T2	40S RIBOSOMAL PROTEIN P40
14-3-3 protein beta/alpha	annexin A2	40S ribosomal protein S10
14-3-3 protein tau	calumenin	40S ribosomal protein S12
14-3-3 zeta	CBP-50 protein	40S ribosomal protein S13
14-3-3gamma	erbB3 binding protein EBP1	40S ribosomal protein S14
BAG-family molecular chaperone regulator-2	filamin	40S ribosomal protein S15a
calmodulin	fission yeast Skb1 protein homolog - human	40S ribosomal protein S16
CDC37 homolog	GA17 protein	40S ribosomal protein S17
chaperonin containing t-complex polypeptide 1, delta subunit	gi|12857398	40S RIBOSOMAL PROTEIN S19
chaperonin containing TCP1, subunit 2	G-protein beta WD-40 repeats containing protein	40S ribosomal protein S2
chaperonin containing TCP1, subunit 3	guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1	40S ribosomal protein S20
chaperonin containing TCP1, subunit 4	H+-transporting ATP synthase	40S ribosomal protein S21
chaperonin containing TCP1, subunit 8	H1 histone family, member 3; histone H1c	40S ribosomal protein S24
DnaJ (Hsp40) homolog, subfamily A,	H3 histone family, member T	40S ribosomal protein S25
DnaJ (Hsp40) homolog, subfamily B, member 4	Heterogeneous nuclear ribonucleoprotein K (hnRNP K)	40S ribosomal protein S28
DnaJ subfamily A member 2	histone H1.c - human	40S ribosomal protein S3
dnaK-type molecular chaperone grp78	histone H2B, testis -	40S ribosomal protein S30
dnaK-type molecular chaperone HSPA5 precursor	histone H4 [Homo sapiens	40S ribosomal protein S3a
FKBP51	HIV-1 Nef interacting protein	40S RIBOSOMAL PROTEIN S4
FKBP54	HSPC029	40S ribosomal protein S7
heat shock 27kD protein 1	KIAA1279 protein	40S ribosomal protein S9
Heat shock 70 kDa protein 1	mitochondrial matrix protein p32	40S RIBOSOMAL PROTEIN SA (P40)
heat shock 70kD protein 9B (mortalin-2)	PNAS-125	60S ACIDIC RIBOSOMAL PROTEIN P1

Heat shock cognate 71 kDa protein	ribosomal protein S18	60S ACIDIC RIBOSOMAL PROTEIN P2
heat shock protein 27	RuvB (E coli homolog)-like 1 [Homo sapiens]	60S RIBOSOMAL PROTEIN L12
heat shock protein 70	RuvB-like protein 1; Pontin 52	60S ribosomal protein L23
heat shock protein 90-beta - human	SAM domain and HD domain 1; DKFZP564A032 protein; SAM domain and HD domain	actin beta
Heat shock protein HSP 90-alpha	serine/threonine kinase 38	actin, skeletal muscle
mortalin mot-2=hsp70 homolog perinuclear form	similar to dendritic cell protein	Alpha-S1 casein precursor
Serine/threonine protein phosphatase 2A	similar to EG:95B7.3 gene product [Homo sapiens]	Alpha-S2 casein precursor
stress-induced-phosphoprotein 1 (Hsp70/Hsp90-organizing protein	similar to Frizzled-7 protein - human	beta-actin
t-complex 1; T-complex locus TCP-1; t-complex 1	similar to T-complex protein 1, epsilon subunit (TCP-1-epsilon)	beta-lactoglobulin
T-complex protein 1, epsilon subunit	SKB1 homolog; SKB1 (S. cerevisiae) homolog	Collagen alpha 1(I) chain precursor
telomerase binding protein p23	spindlin	elongation factor 1-alpha
TIM50	transcription factor ARF6 chain B	Elongation factor 1-alpha 1 (EF-1-alpha-1)
v-raf-1 murine leukemia viral oncogene homolog 1; Oncogene RAF1	SIP1	Eukaryotic translation initiation factor 3 subunit 9
PP5	DNA damage binding protein 1	eukaryotic translation initiation factor 3, subunit 10 theta
	AHNAK-related protein	eukaryotic translation initiation factor 3, subunit 2 beta
	Nucleophosmin	eukaryotic translation initiation factor 3, subunit 3 gamma
		eukaryotic translation initiation factor 3, subunit 4
		eukaryotic translation initiation factor 3, subunit 4 delta
		eukaryotic translation initiation factor 3, subunit 5 epsilon,
		eukaryotic translation initiation factor 3, subunit 6
		eukaryotic translation initiation factor 3, subunit 6 interacting protein
		eukaryotic translation initiation factor 3, subunit 8
		Eukaryotic translation initiation factor 4 gamma
		eukaryotic translation initiation factor 4B
		phosphoribosyl pyrophosphate synthetase 1
		phosphoribosyl pyrophosphate synthetase-associated protein 2
		phosphoribosylphosphate synthetase (PRPS2)
		small nuclear ribonucleoprotein D3 polypeptide 18kDa
		translation initiation factor eIF-4A
		Tubulin alpha-2 chain
		tubulin alpha-3 chain
		tubulin beta 5
		tubulin beta chain
		tubulin beta chain 15
		tubulin, alpha 4; tubulin alpha 4 [Mus musculus]
		tubulin, beta, 2

Table 4.1: Proteins identified by 2D-LC-MS/MS of FLAG-Raf-1 immunoprecipitations: All proteins listed were identified with >95% certainty using the Mascot search engine and comprise the cumulative number of proteins identified from FLAG-Wild type, FLAG-S259A and FLAG-K375M(serum starved/taxol/serum activated)_Raf-1 co-immunoprecipitations.

4.1.5 Proteins unique to Raf-1 complexes from serum-starved or taxol-treated cells

As Raf-1 protects cells from cell death induced by both serum-starvation and by taxol treatment we hypothesized that Raf-1 would bind to proteins under these conditions that could explain its anti-apoptotic function. Two proteins were specifically found in FLAG-Raf-1^{K375M} complexes under these conditions (Figure 4.4B). One of these was calumenin, a calcium-binding protein that we also identified by DIGE as one of the proteins whose expression differed in vector/kinase-dead Raf-1 reconstituted fibroblasts in comparison to wild-type Raf-1 reconstituted cells The second protein was TIM50, a mitochondrial membrane protein that, at least in yeast, links the outer membrane translocator complex to that of the inner membrane (Yamamoto et al., 2002). Recent studies in human cell lines have shown that human TIM50 is also an integral part of the mitochondrial membrane. Down-regulation of TIM50 was found to increase the sensitivity of the cells towards apoptotic stimuli by accelerating cytochrome c release from the mitochondria (Guo et al., 2004). Interestingly, TIM50 was also identified in a yeast-two-hybrid screen using the N-terminus of Raf-1 as bait, indicating that TIM50 and Raf-1 bind directly (Yuryev and Wennogle, 2003). Taken together, the results from the published literature and our 2D-LC data suggest that the TIM50 could play a role in Raf-1-dependent protection from apoptosis, possibly by acting as a mitochondrial anchor for Raf-1.

4.1.6 Proteins unique to Raf-1 complexes from serum-starved cells

Four proteins were exclusively present in the Raf-1 complexes isolated from serum-starved cells (Figure 4.4A). One is EBP1, originally found as an erbB3 binding protein (Yoo et al., 2000). It binds to erbB3 in starved cells and was also reported to bind to Rb and suppress E2F1-mediated transcription (Xia et al., 2001). Interestingly, Raf-1 has also been reported to bind to Rb but this binding correlated with an enhancement of E2F-mediated

transcription, contrary to EBP1's effect (Wang et al., 1998). Further studies are required to determine whether the binding of Raf-1 and EBP1 to Rb is mutually exclusive or whether the three proteins form a ternary complex. The remaining proteins specifically identified in complexes from starved cells were GA17/dendritic cell protein, Torsin and a protein containing a SAM and HD domain. Unfortunately no further information is currently available on these proteins.

4.1.7 Proteins unique to Raf-1 complexes stimulated with serum

Several proteins were identified specifically in complexes from cells stimulated with serum. A few of these appeared to be contaminants, mostly from serum proteins. However, PP2A was one of the proteins identified unique to these samples. PP2A has been shown to dephosphorylate pS259 on Raf-1 and was reported to bind to Raf-1 upon activation (Abraham et al., 2000). Our results confirm these observations and show that we are able to detect dynamic changes in Raf-1 complexes using the 2D-LC/MS approach. Lastly, we identified two G proteins in these complexes, G-protein beta WD-40 repeats containing protein and G-protein beta polypeptide 2-like 1 (aka RACK1), suggesting that membrane localised Raf-1 might interact with to these two proteins. RACK1 is particularly interesting because it was reported to anchor active PKC and Src to the cells membrane (Chang et al., 1998; Ron et al., 1994). In addition several other proteins were unique to serum activated Raf-1 complexes (Figure 4.4 C)

4.1.8 PP5 and SIP co-immunoprecipitate with Raf-1

Having identified over 100 proteins as potential components of Raf-1 signalling complexes, we decided to further validate two of these, PP5 and SIP1. We chose these proteins for further analysis as they are known to be expressed at moderate and low levels, respectively, and had not previously been reported to interact with Raf-1.

PP5, PP2A and PP1 are members of a family of serine/threonine phosphatases (Chen et al., 1994). Both PP2A and PP1 have been implicated in dephosphorylating Raf-1 at S259 and S621. However, it is still unknown which phosphatases dephosphorylate the other regulatory sites on Raf-1. The identification of PP5 in Raf-1 complexes suggested that PP5 may be one such phosphatase. Therefore we decided to verify the interaction between Raf-1 and PP5 by co-expressing them in cells and testing their ability to form a complex. Both wild-type Raf-1 and the Raf-1^{S259A} specifically co-immunoprecipitated PP5. Conversely PP5 was able to precipitate both wild-type Raf-1 and the mutant, confirming that Raf-1 and PP5 form a complex in-vivo (Figure 4.6 A & B).

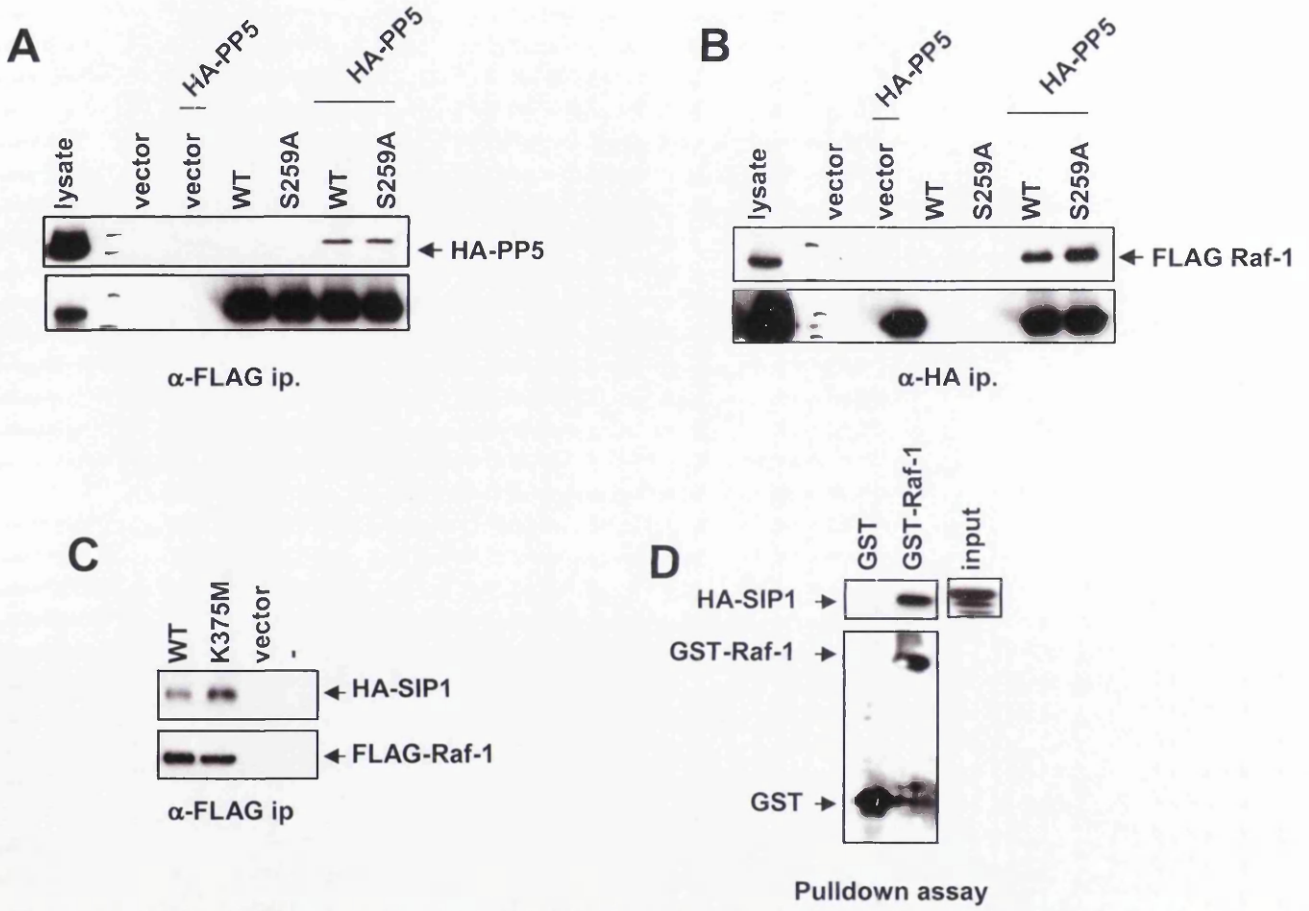

Figure 4.6: Characterisation of two proteins identified in Raf-1 complexes. Cos-1 cells were transfected with plasmids as indicated, 48h post transfection the tagged proteins were immunoprecipitated. Co-precipitating proteins were determined by immunoblotting A) HA-PP5 co-precipitates with wild-type and FLAG-Raf-1^{S259A} when co-expressed in Cos-1 cells; B) Wild-type and FLAG-Raf-1^{S259A} co-precipitated with HA-PP5 when co-expressed in Cos-1 cells; C) HA-SIP co-precipitates with wild-type and kinase-dead FLAG-Raf-1 when expressed in Cos-1 cells; D) Sf9-purified Raf-1 is able to "pull-down" HA-SIP1 in Cos-1 cell lysates (experiments C/D were done by AS Dhillon).

SIP1 is an adaptor protein involved in a novel pathway regulating β-catenin degradation, independently of its phosphorylation by GSK3. In this pathway, SIP1 links β-catenin to Siah, a ubiquitin ligase whose expression is induced by p53 (Matsuzawa and Reed, 2001). Our identification of SIP1 as a potential Raf-1-interacting protein raised the exciting possibility that SIP1 may be a link between Raf-1 signalling and this novel pathway of β-catenin degradation. Once again by co-immunoprecipitation experiments, we found that both kinase-dead and wild-type Raf-1 formed a complex with SIP1 in cells. Curiously, kinase-dead Raf-1 consistently demonstrated an enhanced ability to interact with SIP1 in these experiments. We also found that GST-Raf-1 bound to glutathione-Sepharose beads was able to "pull-down" SIP1 expressed in Cos-1 cells suggesting that the interaction between these two proteins was a direct one (Figure 4.6 C & D).

4.2 Purification of phosphopeptide standards with antibodies and 14-3-3: a comparison with established methods

4.2.1 Anti phospho-serine/threonine antibodies only purify a subset of synthetic phosphopeptides

The use of anti-phosphotyrosine antibodies to immunoprecipitate proteins has been very successful due to the high affinity and specificity of these antibodies and their ability to bind phosphotyrosine with little dependence on the surrounding amino acid sequence. Unfortunately the antibodies that recognise phosphorylated serine and/or threonine are of poorer quality. The surrounding amino acids influence the affinity of the antibody for its target, presumably because of the smaller size of serine and threonine side chains in comparison to the bulkier aromatic ring of tyrosine. To test if phospho-specific antibodies could be used to enhance the identification of phosphopeptides prior to MALDI-MS, a mixture of anti-phosphoserine/threonine antibodies was first cross-linked to agarose beads to cover the largest possible variation of phosphopeptides. A mixture of synthetic peptides,

comprising 3 phosphorylated peptides from Raf-1 containing the pS621, pS259 and pS338 sites and an excess of a non-phosphorylated peptide, Raf-1 S43, were incubated with the antibody beads overnight, washed and eluted using a pH drop. The eluted peptides were then identified by MALDI mass spectrometry. In most cases the eluted peptides had to be further purified with C18 Zip-Tips in order to be detected by mass spectrometry. Very hydrophilic peptides might have been lost in this purification step. Using this approach, we were able to enrich the pS338 phosphopeptide from the mixture but the purification was not completely compatible with subsequent mass spectrometric analysis, even using a MALDI source with its high tolerance towards contaminants such as salts or detergents (Figure 4.7A & B). Thus the eluted peptides had to be further purified in order to be ionised and visible in the mass spectrum.

4.2.2 14-3-3β agarose beads enrich the Raf-1 pS621-containing synthetic phosphopeptide

The ability of 14-3-3 proteins to bind to serine/threonine phosphorylated proteins was exploited in this approach. 14-3-3 proteins show a strong affinity to a specific recognition motif (R-S-X-pS/T-X-P or R-X-X-X-pS/T-X-P) in which binding occurs only when the central serine or threonine are phosphorylated. Several approaches have been published that utilise this phosphospecific protein-protein interaction domain to isolate and identify 14-3-3 binding proteins (Basu et al., 2003; Milne et al., 2002; Rubio et al., 2004). We wanted to test if 14-3-3 bound to agarose would be a suitable affinity purification matrix for phosphopeptides containing 14-3-3 recognition sites. Raf-1 contains two phosphorylation-dependent 14-3-3 binding sites, one at serine 259 and the other at serine 621. Both phosphorylated peptides were incubated with agarose-14-3-3 beads, alongside the non-phosphorylated control peptides (S43) and one phosphorylated peptide that is not able to bind 14-3-3 proteins (pS338).

The binding peptides were eluted by dropping the pH of the buffer. As was the case with immunoprecipitations using the phosphospecific antibodies, the eluted peptides had to be

further purified with Zip-Tips for them to be detected by MALDI mass spectroscopy. This additional purification step is a major disadvantage in comparison to IMAC based purifications, as these can be eluted in a mass spectrometry compatible solvent and the IMAC resin does not seems to retain less contaminants than the agarose matrix. The 14-3-3 β protein beads were able to enrich, but by no means purify, only the phosphopeptide containing the pS621 site. The other peptide containing the pS259 14-3-3 binding site was not found to bind to the beads specifically (Figure 4.7C & D).

4.2.3 IMAC purification of synthetic phosphopeptides and β-casein derived tryptic peptides

We wanted to compare our "biological" affinity purifications to well established phosphopeptide enrichment methods. Therefore we chose to use IMAC affinity purification (Flotow et al., 1990; Muszynska et al., 1992; Neville et al., 1997; Scanff et al., 1991) using a β-casein digest and our mixture of phosphopeptides. Phosphorylated amino acids, their peptides and even whole proteins can be purified with IMAC matrices. This affinity purification relies on the affinity of the strongly acidic nature of the phosphor ester group. The advantage of this "chemical" affinity is that the influence of the side chains of the surrounding amino acids is smaller than in the other methods described above. The binding of some peptides might be inhibited through formation of hydrogen bridges and ionic interactions with amino acids containing nucleophilic groups which can lead to masking of the phosphor ester group. The biggest disadvantage of this approach is the poor specificity of the interaction since acidic peptides tend to be co-purified and can make up the bulk of the isolated peptides when a complex mixture of peptides, such as a digest of a whole cell lysate, is used as starting material.

Using a β-casein tryptic digest with Fe^{3+}IMAC we were able to purify the mono-phosphorylated 2060Da casein peptide with little contamination of the non-phosphorylated peptide. In negative ion mode even the quadruply phosphorylated peptide at 3186Da was visible (Figure 4.8). Using the Raf-1 phosphopeptide mixture IMAC was only able to

enrich pS338 (Figure 4.7E & F). The method was fully compatible with subsequent MALDI analysis and no additional de-salting step had to be performed.

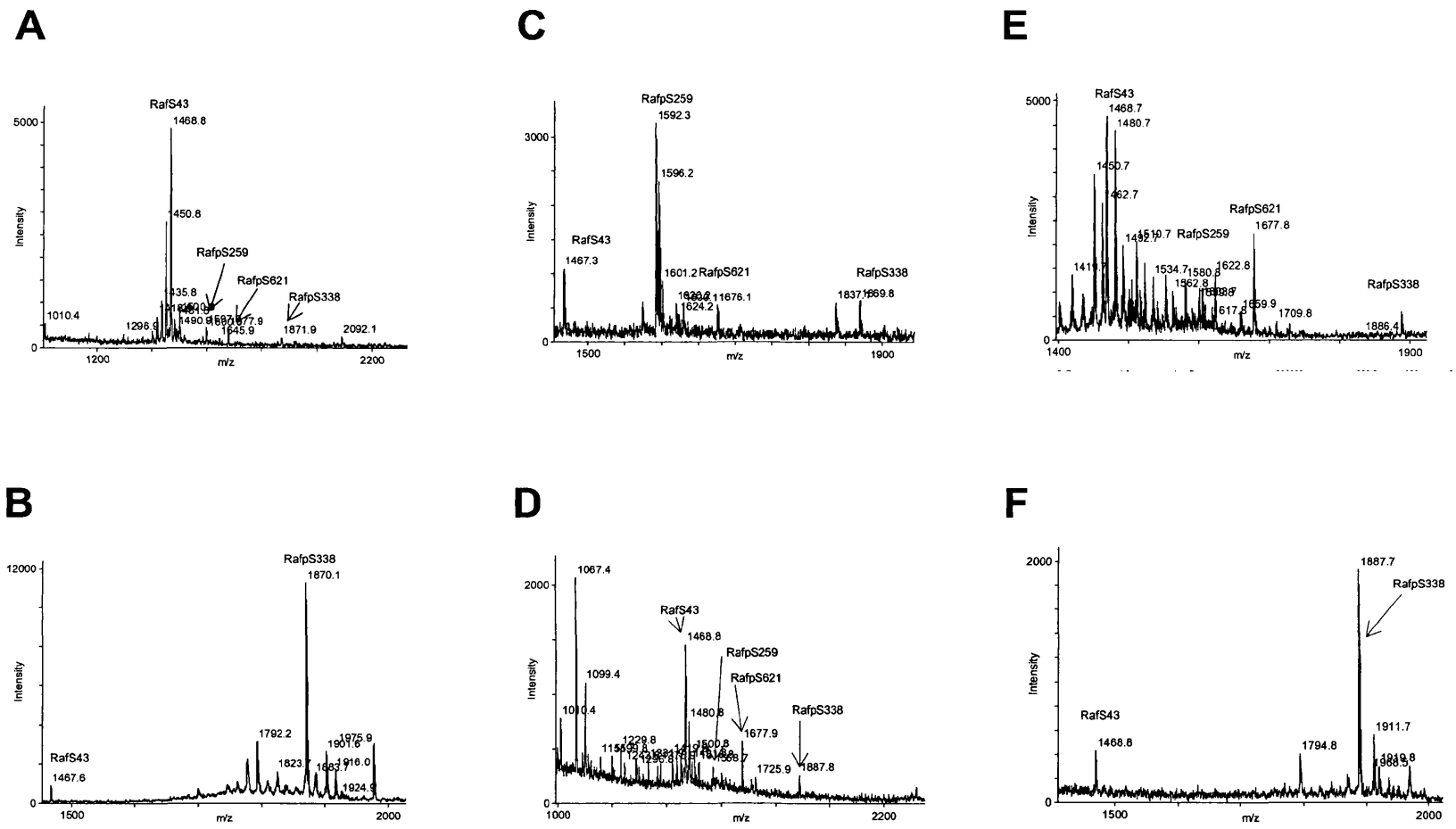

Figure 4.7: Affinity purification of synthetic Raf-1 phosphopeptides. MALDI-TOF spectra of the synthetic peptide mixture A) Peptide mixture before incubation with antibody-coupled beads; B) Peptides purified from antibody-coupled beads - only the pS338 peptide was purified using phosphospecific antibodies; C) Peptide mixture before incubation with 14-3-3 β beads; D) Peptides eluted from 14-3-3β beads - no phosphorylated peptide was purified, but the pS621 peptide was enriched E) Peptide mixture before IMAC; F) Peptides eluted from IMAC - only pS338 was purified using the IMAC resin.

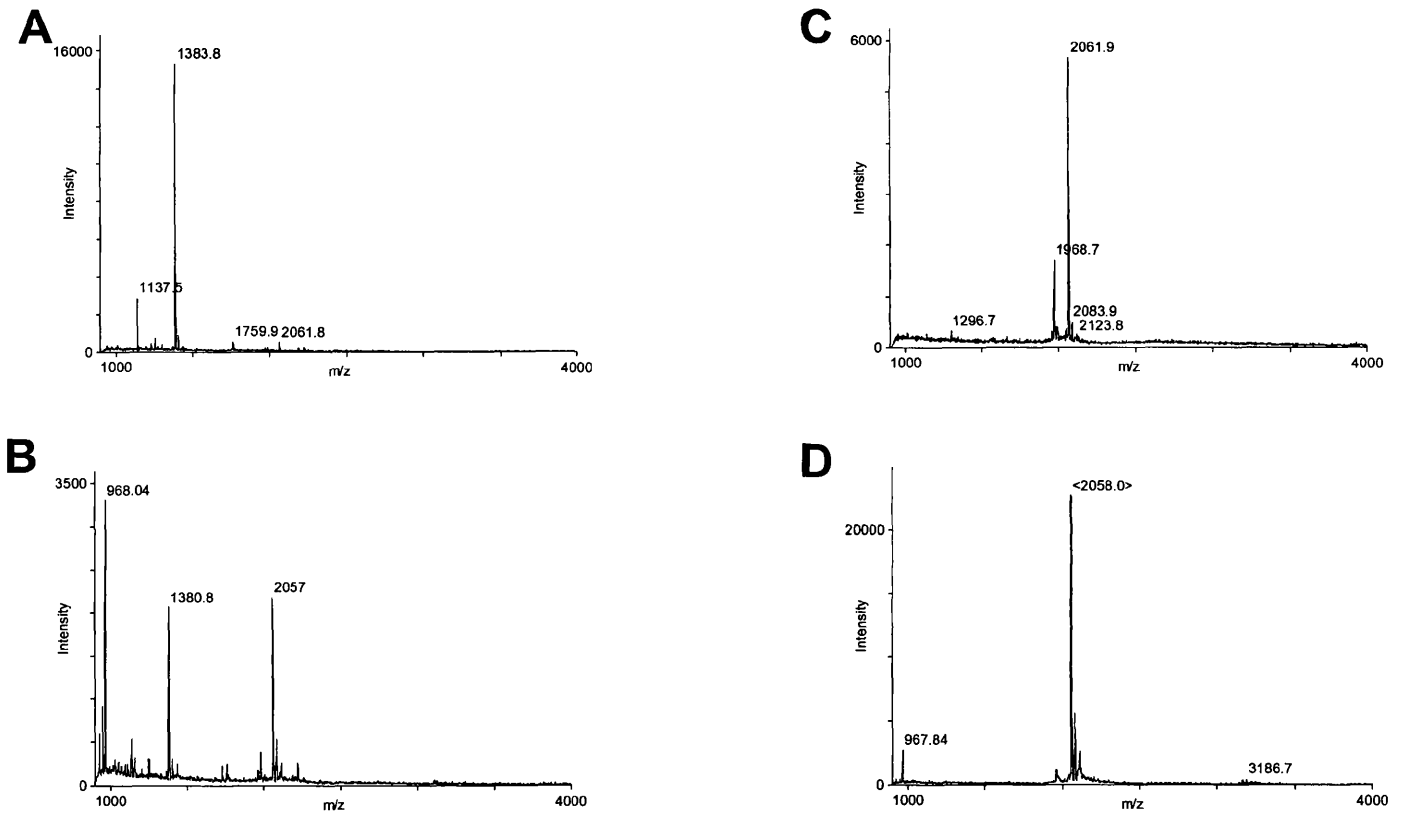

Figure 4.8: IMAC purifies phosphopeptides derived from tryptic β-casein digests. MALDI-TOF spectra of 5pM of a tryptic casein digests A) casein digest analysed in positive ion mode; B) casein digest analysed in negative ion mode; C) IMAC elution of casein digest analysed in positive ion mode; D) IMAC elution of casein digest analysed in negative ion mode.

4.3 Identification of phosphopeptides from mixtures

The first method tested was a precursor ion scan for a phosphite ion that can be observed after fragmentation of a phosphate-containing peptide in negative ion mode. This method is able to identify modifications present on serine, threonine and tyrosine. The first quadrupole screens in negative ion mode through the mass range, the second quadrupole, or TOF analyser, is set at 79Da to screen for the phosphite ion. If an ion of a mass of 79Da was detected in the fragmentation spectrum the precursor of this ion was then further analysed in positive ion mode. The voltage was switched and the charge state and exact m/z of the precursor was determined. The ion was then further analysed by MS/MS.

A second method was used to specifically identify phosphotyrosine containing peptides. The ion scanned is the immonium ion of phosphotyrosine with a mass of 216.04Da. The scan is performed like the 79Da scan but in positive ion mode

Additionally a neutral loss scan for loss of 49 and 33Da was performed on the Q-Trap spectrometer. These masses represent the often observed β-elimination of the phosphate-ester from the side chain of serine or threonine. Phosphotyrosine does not undergo β-elimination and cannot be detected using this method. The first quadrupole screens through the mass range set, the second screens at an off-set of 33Da for triply charged peptides, and for 49Da, for doubly charged peptides. If ions were detected at the specified off-set mass the ions from the mass range of origin were further analysed to determine charge state and the exact mass of the precursor. The precursor was then subjected to a MS/MS analysis to determine the amino acid sequence and location of the phosphorylation site.

4.3.1 Precursor ion scans for 79 and 216.04Da are able to identify phosphopeptides

The ability of the Q-Star and the Q-Trap spectrometers was first tested to identify phosphorylated peptides using β casein as a standard for the 79Da and a synthetic phosphotyrosine-containing peptide for the 216.04Da precursor ion scan. The 79Da precursor ion scan was performed using both the Q-Star and Q-Trap, whereas only the Q-Star was used for the 216.04Da scan because of the higher mass accuracy and resolution of the second analyser.

10pmol of a tryptic digested β-casein peptide mixture was desalted using a C18 Zip-Tip and eluted within 3μl of 50% Acetonitrile/Water, filled into a nano tip and analysed in negative ion mode. Both mass spectrometers were able to identify the singly phosphorylated β casein peptide (Figure 4.8A). The Q-Trap identified the phosphorylated peptide within seconds whereas the Q-Star needed more than a minute to identify the peptide. Therefore all further experiments involving the 79Da scan were performed on the Q-Trap due to its higher sensitivity in this application.

We used 10pmol of a pY340 Raf-1 synthetic peptide to test the phosphotyrosine immonium-ion scan. The width of the precursor scan was set at 0.04 mass units to minimise false identifications. As the precursor ion scan was able to detect the peptide with ease (Figure 4.8C), we further wanted to test the selectivity of the method using a trypsin digested PDGF tyrosine kinase receptor purified from Sf9 cells. The precursor ion scan identified several ions whose fragmentation showed presence of a mass equivalent to the phosphotyrosine immonium ion (Figure 4.9A). Further MS/MS analysis of these ions was not able to identify the sequence of the precursors. Most of the peptides showed the same fragmentation pattern, indicating that the ions did not represent a peptide but originated from contaminants (Figure 4.8B-F). Due to the poor specificity and lack of sensitivity, the phosphotyrosine immonium-ion scan method was abandoned.

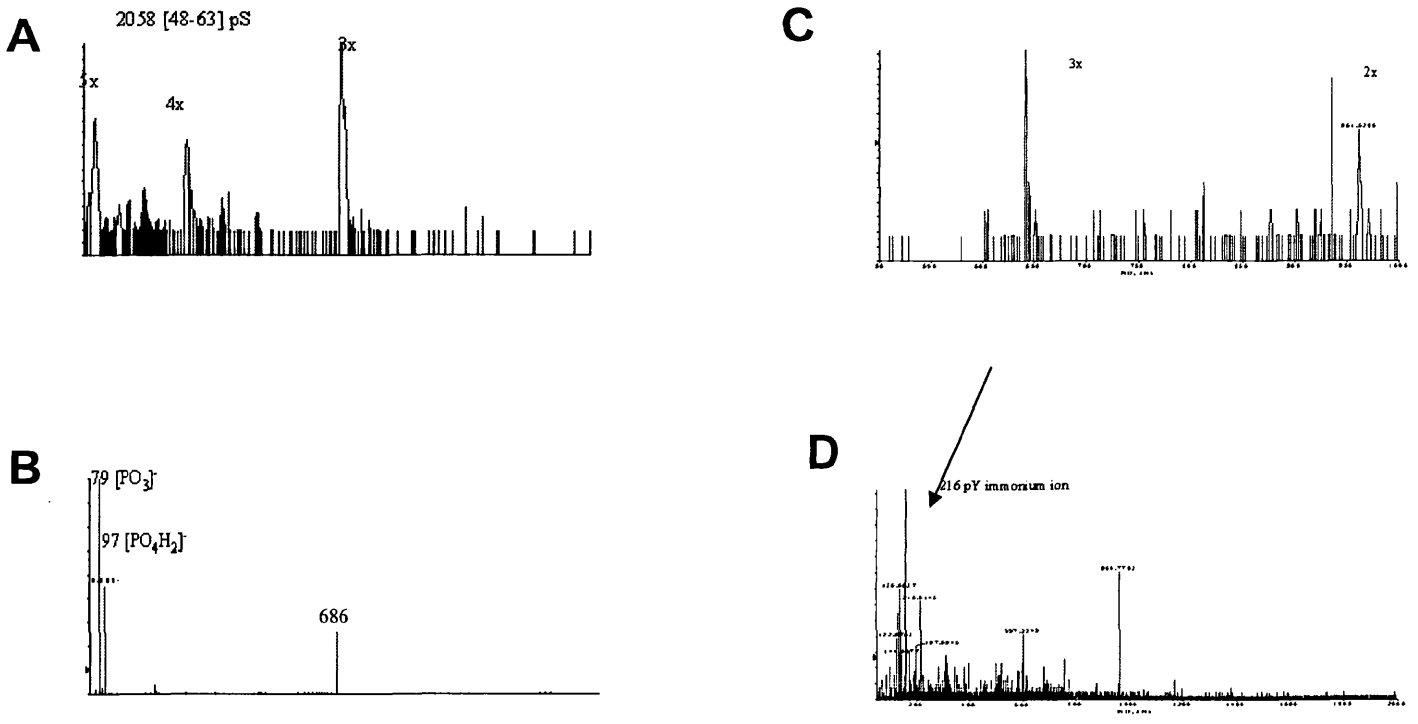

Figure 4.9: 79 and 216Da precursor ion scan of phosphorylated peptides. A) 79 precursor ion scan of beta casein digest; B) MS/MS of 686m/z (2058Da) ion in negative ion mode; C) 216.04 precursor ion scan of pY containing synthetic peptide using the Q-Star; D) MS/MS of pY peptide identified in C.

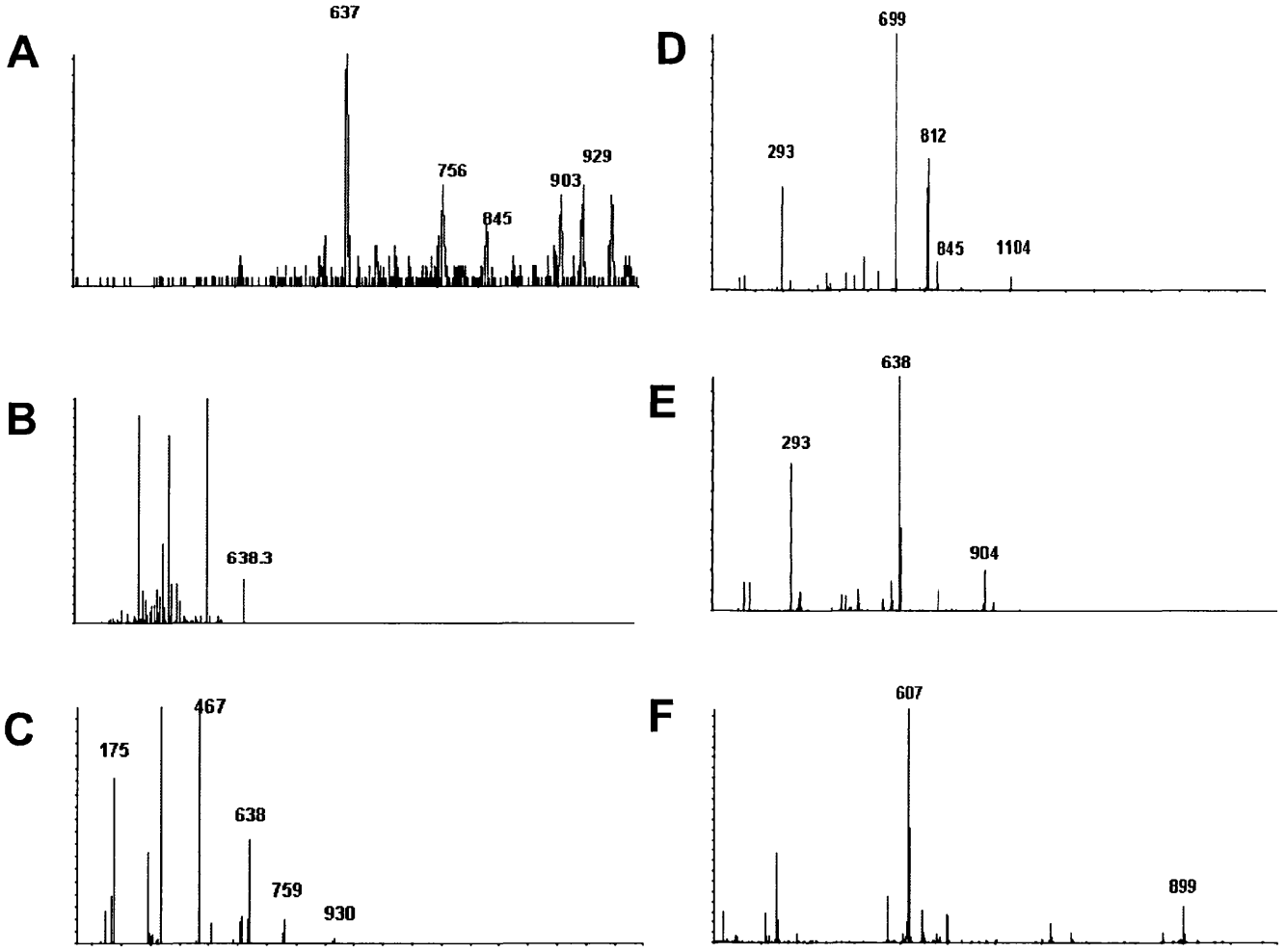

Figure 4.10: 216.04 scan of PDGF receptor and MS/MS of the precursors. Sf9 expressed PDGF receptor was purified and digested in-solution with trypsin. The 216.04Da precursor ion scan was performed using a Q-Star mass spectrometer A) 216.04Da precursor ion scan; B) MS/MS of the 638 ion; C) MS/MS of the 756 ion; D) MS/MS of the 845 ion; E) MS/MS of the 904 ion; F) MS/MS of the 929 ion.

4.3.2 LC-MS/MS -79Da precursor ion scan is more sensitive and specific than neutral loss

We wanted to combine our precursor ion scan with a LC to increase the robustness and performance of the analysis. The mass spectrometric analysis of peptides using an ESI source is very sensitive towards contaminating molecules. In particular, detergents such as SDS, salts or polymers can sometime render it impossible to detect peptides even when they are present at a relatively high concentration. Therefore all samples have to be purified prior to analysis, typically using C18 reverse phase resin. If this purification is performed using a one step elution contaminating salts are generally removed successfully. Contaminating polymers, such as poly-ethylene glycols (PEG), that are frequently present in samples derived from in-gel digests, can still be present and hinder the subsequent analysis. Another disadvantage of eluting all the peptides in one fraction is that the complexity of the sample is not reduced and co-eluting peptides will suppress the ionisation of the phosphopeptides. Furthermore the fraction volume typically used in a one-step elution is around 1-3µL whereas the volume at which peptides elute from a nano LC column is between 100 and 200nL, thus increasing the concentration of the eluting peptide around 10-fold and increasing detectability. Using in solution digested casein two different LC-MS/MS approaches to identify phosphorylation sites were tested.

100pmol of peptides derived from a casein digest were separated on a nano-LC and directly analysed by mass spectrometry. Firstly we used a neutral loss scan of 98Da and secondly a -79Da precursor ion scan to detect phosphorylated peptides. Upon detection of a phosphorylated peptide the mass spectrometer switched automatically to an enhanced resolution scan to determine the peptide mass and charge and then selected the ion to be further analysed by MS/MS. Both the neutral loss scan and the 79Da precursor ion scan were able to detect phosphopeptides, although the neutral loss scan detected only one phosphopeptide and numerous non-phosphorylated peptides. The precursor ion scan on the

other hand not only identified both phosphorylation sites of β casein but also identified 4 other sites contained in two α casein proteins that were evidently present as a contaminant in the β casein sample (Table 5.1). The direct comparison between neutral loss scanning and precursor ion scanning showed a higher specificity and higher sensitivity for the latter method, therefore the precursor ion scan was chosen as the method to be used for further analyses.

4.3.3 LC-MS/MS precursor ion scan detects 500fmol of phosphorylated β-casein

To determine the sensitivity of our approach, four additional LC runs were performed using 10pmol, 1pmol, 500fmol and 100fmol of β-casein. One phosphorylation site was still detectable in the 500fmol injection but no phosphorylation site was detected in the 100fMmol injection, setting the sensitivity of the method between 100 and 500fmol of injected phosphopeptide (Table 5.2).

Table 4.2: Phosphopeptides identified from a β-Casein digest (90% purity) by LC-MS/MS using different concentrations and different detection methods

	P-peptides identified	100pmol precursor scan	10pmol precursor scan	1pmolpre cursor scan	500fmol precursor scan	100pmol neutral loss
casein alpha-S2	NMAINPpSKENLC	x	-	-	-	-
	TVDMEpSTEVFTK	x	x	-	-	-
beta casein	FQpSEEQQQTEDELQDK	x	x	x	x	x
	RELEELNVPGEIVEpSLpSpSpSEESITR	x	-	-	-	-
alpha-S1-casein	DIGpSEpSTEDQAMEDIK	x	x	x	-	-
	YKVPQLEIVPNpSAEER	x	x	x	-	-

4.3.4 Identification of four phosphopeptides derived from Raf-1 complexes

Having established the sensitivity and specificity of our method, we then wanted to identify phosphorylation sites in Raf-1 complexes. Therefore a FLAG-Raf-1 co-immunoprecipitate was digested with trypsin in solution and the resulting peptides were analysed by a LC coupled to a Q-Trap in precursor ion scan mode. The resulting MS/MS spectra were processed by the MASCOT search engine and five peptides were identified over a significant threshold. Four peptides contained a phosphorylation site and in each peptide the position of the phosphorylated aminoacid was identified. Only one peptide originated from Raf-1, confirming the phosphorylation on the serine at the 259 position (Figure 4.11A). The other phosphopeptides identified belong to proteins previously not identified to be interacting with Raf-1. ABP-7 has similarities to the translation initiation factor IF2. We identified translation initiation factors with our previous immunoprecipitations but no further analysis of the specificity of the interaction was performed. These proteins are among the most abundant proteins in the cell, therefore it cannot be ruled out that the interaction is unspecific.

Methylosome subunit pICln and Ac2-269 (similar to methylosome protein 50, MEP50) both play a role in arginine methylation and have been found previously in the same complex (Friesen et al., 2002). MEP50 is a protein containing WD repeat domains which function as protein-protein interaction domains, indicating that the function of this protein is to act as a scaffold for the complex. The identification of two proteins from the same complex strengthens the possibility that the interaction with Raf-1 is functional although non-specificity cannot be ruled out without further investigations. Although these two proteins were not identified in our previous immunoprecipitations we were able to identify one additional protein involved in the same methyliosome complex (JBP1/SKB or Jak1 interacting protein)(Friesen et al., 2002).

The other peptides were from proteins that were present in the immunoprecipitate and could have been Raf-1 interacting proteins. The location of the phosphorylation carrying amino acid was identified by MS/MS in all peptides that were above the significant threshold (Figure 4.11). Several other peptides that could not be identified following the MASCOT search showed a neutral loss of 98 mass units, typical for phosphopeptides. However, the quality of the fragmentation data was not good enough to positively identify these peptides.

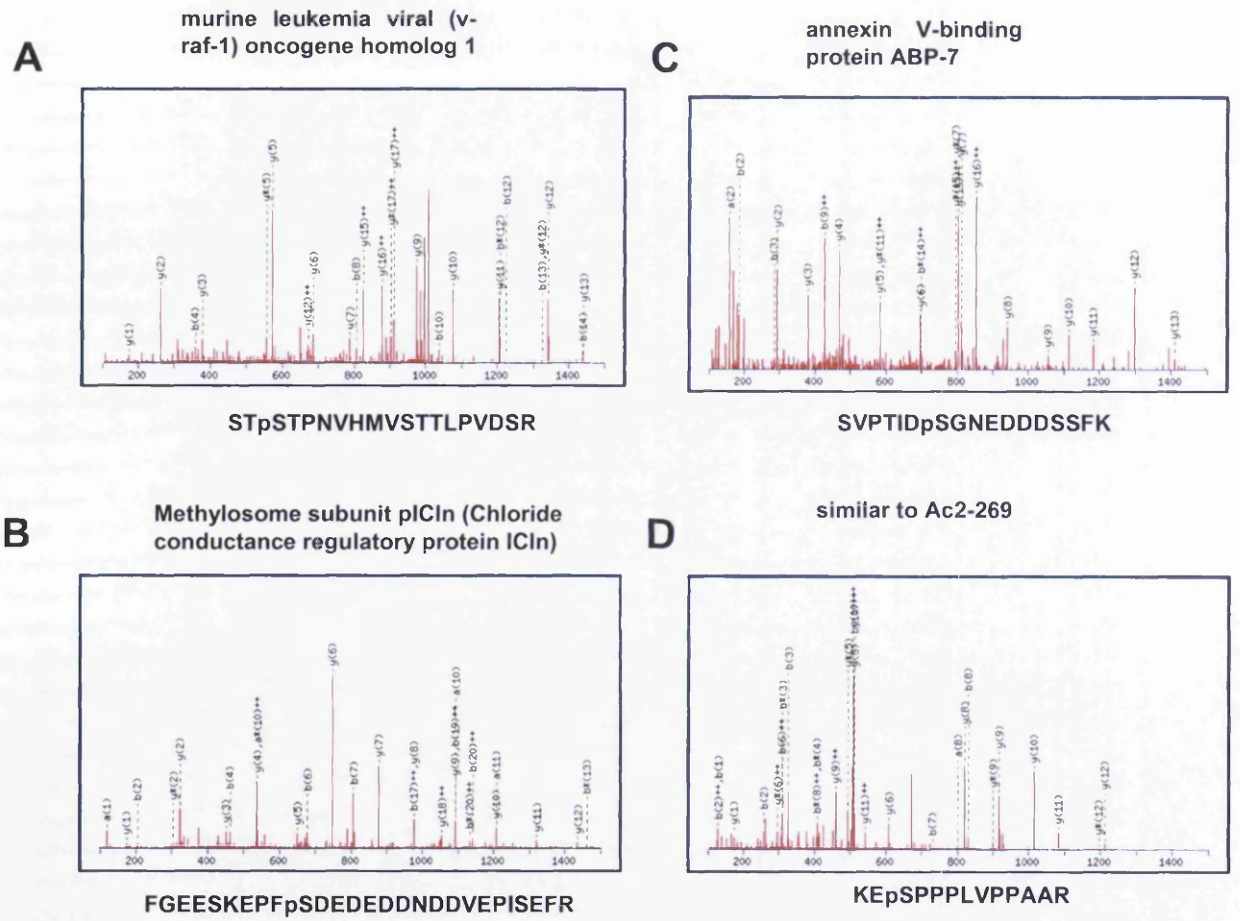

Figure 4.11: Phosphopeptides detected in digests of Raf-1 protein complexes by LC-MS/MS 79 parent ion scan: FLAG-Raf-1 was immunoprecipitated from stable expressing Rat1 cells and digested in solution peptides were analysed by LC-precursor ion-MS/MS using a Q-Trap mass spectrometer, phosphopeptides were identified using the MASCOT search engine A) pS259 Raf-1 B) pS115 ABP-7 C) pS95 Chloride conductance regulatory protein ICln D) pS5 similar to Ac2-269

Chapter 4: Discussion

We set out to improve methods of analysing multi-protein complexes by using more accurate and sensitive mass spectrometers combined with new separation techniques. By using two-dimensional liquid chromatography we were able to separate peptides generated from immunoprecipitated samples and identify them by an online mass spectrometer. By using this approach we increased the number of identified proteins by more than 10-fold when compared to our initial MALDI approach, demonstrating the increased sensitivity and accuracy of our improved methodology. We now have a powerful and robust tool that enables us to analyse protein complexes and have confirmed the validity of our approach by verifying two interactions with alternative methods.

Although this method is very useful, further improvements are needed to unlock the full potential of mass spectrometry-based approaches to study protein-protein interactions. One of the most surprising findings was that some well documented Raf-1 binding proteins evaded detection by our approach despite our increasing the sensitivity of the methodology. One possible explanation for these results is the lysis conditions used and the presence of detergent in the buffer. It is plausible that some proteins will detach from Raf-1 complexes upon contact with detergents. Other proteins might detach from Raf-1 in a time-dependent manner For example, Ras would detach from Raf-1 if GTP hydrolysis occurred after prolonged buffer exposure. Other problems arise from non-specific binding proteins. Although they do not seem to hamper the detection of genuine interacting proteins, the presence of these non-specific binders can make it time consuming to sort out the wheat from the chaff. Tandem affinity purification tags have been proposed to solve the latter problem (Rigaut et al., 1999). Although using these tags will increase specificity these methods will inevitably increase sample handling times as two affinity purification steps and incubation with a specific protease are needed to cleave the tag and purify the protein

complex. These extra steps could result in the loss of loosely and transiently bound proteins, rendering this method unsuitable for analysing dynamic changes in the composition of protein complexes. There is no easy solution to these problems, but using detergent free lysis buffer whenever possible and using highly specific antibodies preferably against endogenous proteins will most certainly improve the accuracy and validity of this approach.

Further improvements have arisen in recent years. In particular, the use of stable isotope labelling is set to revolutionise gel-less proteomics. We used a crude method to estimate changes in protein binding, an all or nothing approach. If we would have used methods like SILAC we would have been able to detect even slight changes in protein binding, making this method most suitable to detect transient interactions. SILAC (Stable Isotope Labelling in Cell culture) uses ^{13}C and ^{15}N substituted amino acids in cell culture. Cell grown in this medium will incorporate the modified aminoacids into newly synthesised proteins. Lysates from cells grown in "heavy" and "light" medium are mixed and further purification steps, such as immunoprecipitation, can be performed on the mixture. The protein mixture can is then analysed by mass spectrometric means and peptides derived from these proteins will elute at the same time from an LC and will have identical ionisation properties, despite having different masses. The comparison of the signal strength of the individual ions can therefore be used for relative quantitation. As recent publications have shown, use of these methods enables the identification of novel insights into well studied cellular systems (Blagoev et al., 2003; de Hoog et al., 2004).

In the second part of this chapter we attempted to improve phosphorylation site mapping of protein complexes by using specific purification and detection methods. None of the methods that were tried to enrich phosphopeptides was completely successful. Several reasons might explain this. IMAC, as shown several times before, is an efficient method to enrich tryptic phosphopeptides that originate form casein digests (Stensballe et al., 2001).

We were able to highly enrich both the singly and the quadruply phosphorylated peptide. However, the purification was not satisfactory when even a simple mixture of synthetic phosphopeptides was used. Only one of the synthetic phosphopeptides was enriched using this affinity purification. The results were similar to the results achieved with the affinity matrices generated from either phosphospecific antibodies or 14-3-3. In recent years IMAC methods have been improved by introduction of new chelating matrices, the use of different ions and the modification of acid amino acids. Future improvements may render it possible for IMAC to be used more widely and initial attempts using yeast lysates have been reported (Ficarro et al., 2002).

Because the interaction between the peptides and their interacting proteins relies on a biological affinity all the incubations were performed in PBS. The peptide pull-down could only be efficient in a physiological salt-containing buffer that does not affect the tertiary structure of the proteins. The salt contained in the sample had to be removed using ZipTips, possibly resulting in sample loss. Additionally the elution with acetic acid might not have been complete, especially in case of the 14-3-3 affinity purification. This approach has been very successful in purifying whole proteins (Basu et al., 2003; Rubio et al., 2004). In these experiments the proteins were competed off the beads by using phosphopeptides containing the 14-3-3 recognition site. Therefore there is no reason why both the pS259 and pS621 peptide should not have been able to bind to the bait proteins. The most conceivable explanation is that we failed to elute the peptides from the 14-3-3 beads. Instead of trying a pH drop maybe the use of a chaotropic elution buffer would have been more efficient. The use of these reagents is on the other hand is not compatible with subsequent mass spectrometry and extensive washes, with the possibility of sample loss, would have had to follow the elution.

Our antibody-based enrichment method failed presumably because of weak interactions between some of the peptides and the antibodies. More recent studies have shown that

certain Ser/Thr phosphospecific antibodies perform quite well in immunoprecipitating phosphorylated proteins (Gronborg et al., 2002). Thus, with a broadening arsenal of antibodies recognising multiple phosphorylation sites, a more successful enrichment of phosphopeptides from a complex sample might become possible.

Specific detection of phosphopeptides by precursor ion scans has been more fruitful for the task of sequencing phosphorylated peptides from biological samples. Our initial method development showed that both machines, the Q-Star and the Q-Trap, were able to detect phosphopeptides by precursor ion scanning with a high sensitivity and high accuracy. In negative ion mode the phosphopeptides seem to fragment nearly exclusively into a fragment of 79 and 97Da mass units (Figure 4.7B), hence the high sensitivity of the method. Overall the linear iontrap had a higher sensitivity. The probable reason for the better performance is the design of the machine, having a linear iontrap as a second analyser that functions as a third quadrupole when used in precursor ion mode, as this analyser is built for a continuous ion stream as originating from the electrospray. The TOF analyser in the Q-Star could obviously not perform as well; one reason might be the pulsing nature of the TOF design.

The performance and the practicality of the phosphorylation mapping increased dramatically by use of an LC system coupled to the mass spectrometer. The sensitivity achieved with 500fmol of peptide is reasonable and comparable to methods published that utilised multidimensional LC analysis (Zappacosta et al., 2004; Zappacosta et al., 2002) and non LC-based precursor ion scanning (Neubauer and Mann, 1999). In contrast to other published methods the method is amenable to automation and the complete scan of a sample and sequencing of the detected peptides is achieved with one LC run of 40 minutes. Full automation though could not be achieved. Unfortunately the electrospray is unstable in the negative ion mode and plasma formation at the tip of the needle is quite frequent, so that the operator has to be present at all times to be able to adjust the needle.

In total four phosphorylated peptides were identified in Raf-1 immunoprecipitates, quite a small number considering the large number of proteins identified in the complexes. This small number can be explained by the fully automated selection of ions designated to be further analysed by MS/MS. Firstly it is possible that the correct precursor ion might not be chosen accurately. The enhanced resolution scan that is executed in the mass range that contained the precursor of the 79Da fragment has a width of 10Da and selects the ion with the highest intensity for analysis. This selection might not be accurate at all times, especially if the sample is complex and other peptides or contaminants elute in the same mass range. Additionally the analysis assumes that a peptide ion that carries a given amount of charges in negative ion mode will carry the same charge in positive ion mode, as only that mass range is selected for further analysis in positive ion mode. This assumption will not be correct for all peptides, especially since phosphopeptides tend to carry a lesser charge in positive ion mode due to the strong negative charge carried by the phosphorester. Confirming this assumption was the observation that the trace of the 79Da precursor ion scan showed several peaks that did not result in a positive identification of a phosphorylated peptide. Secondly co-eluting peptides could have suppressed the ion corresponding to the phosphopeptide rendering it invisible to the mass spectrometer. The feasible combination of precursor ion scan and 2D-LC might cure some of these problems and so possibly change the detection of phosphorylation sites in multiprotein complexes to a robust and easy technique.

Chapter 5

Characterisation of the Raf-1/PP5 interaction

Chapter 5: Introduction

Numerous studies have shown that phosphorylation plays a key role in regulating the activity and signalling by Raf-1 (Dhillon and Kolch, 2002; Kolch, 2000). While several kinases have been reported to phosphorylate some of the major regulatory sites in Raf-1, much less is known about how theses sites are dephosphorylated. Protein phosphatases PP1 and PP2A have both been shown to dephosphorylate pS259 upon activation by mitogens (Abraham et al., 2000; Jaumot and Hancock, 2001; Mitsuhashi et al., 2003). Dephosphorylation of this inhibitory site is crucial for translocation of Raf-1 to the membrane and binding to active Ras, its phosphorylation on activating sites such as S338 and its ability to activate MEK (King et al., 1998; Mason et al., 1999). While these studies have shown that dephosphorylation plays an important role in Raf-1 activation, the role and the identity of phosphatases involved in deactivating Raf-1 is poorly understood. We identified PP5 as a novel component of Raf-1 complexes and verified the interaction between PP5 and Raf-1 by co-immunoprecipitation experiments in COS-1 cells (see Figure 5.4). The aim of the experiments described in this chapter was to characterise the role of PP5 in Raf-1 signalling. In particular, we wanted to test if PP5 directly dephosphorylated site(s) on Raf-1.

PP5 is a member of the PPP family of serine/threonine specific protein phosphatases which also includes PP1, PP2A and PP2B. PP5 was initially reported to be a predominantly nuclear protein, but more recent studies have confirmed that it is also cytoplasmic and can translocate to the cell membrane (Chen et al., 1994). PP5 comprises a catalytic domain which shows approximately 40% identity to the other PPP family members. PP5 can be inhibited by okadaic acid, but at higher concentrations to those required to inhibit PP1 and PP2 (Brewis et al., 1993; Chen et al., 1994). A distinctive feature of PP5 is that it possesses an N-terminal tetratrico-peptide repeat (TPR) domain, which is found in numerous signalling proteins. As it is believed that the TPR domain is crucial for protein-protein interactions it is possible that it targets PP5 to its substrates. The process that leads to

activation of PP5 is controversial. In contrast to PP1 and PP2, PP5 has a very low basal activity and it seems that, whereas PP1 and PP2 are regulated primarily by targeting to their substrates, PP5 can be activated by removing the N-terminus. PP5 can also be activated by long-chain polyunsaturated fatty acids, such as arachidonic acid, that interact with the TPR domain and protein-protein interactions mediated via the TPR domain increase PP5 activity, at least in-vitro (Chen and Cohen, 1997; Yamaguchi et al., 2002; Zeke et al., 2004; Zeke et al., 2005). These studies indicate that the N-terminal TPR domain shields the C-terminal active site from interacting with substrates and so regulates its activity. Although numerous proteins have been shown to interact with PP5, only a few have been shown to be genuine substrates. Thus far ASK1 and DNA-PKcs are the only two bona fide substrates that have been identified (Morita et al., 2001; Wechsler et al., 2004). These studies reveal a remarkable specificity for PP5 in choosing its targets, quite in contrast to PP1 and PP2.

Recent studies have shown that subcellular localisation of PP5 plays an important role in regulating its activity. Nuclear localisation appears to be dependent on the C-terminal region, whereas localisation to the membrane can be induced by activating the heterotrimeric G-proteins, Gα12 or Gα13 (Yamaguchi et al., 2002). These results indicate that changes in PP5 localisation induced by extracellular signals may play an important role in regulating its signalling properties.

Chapter 5: Results

5.1 Characterisation of the interaction between Raf-1 and PP5

5.1.1 Endogenous Raf-1 and PP5 form a complex in serum starved COS-1 cells

PP5 was identified as a potential Raf-1 interacting partner in a proteomic screen using Raf-1^{S259A} as bait. This interaction was validated using co-immunoprecipitation analysis (Figure 4.6). Having verified the interaction using overexpressed proteins we then wanted to test if both proteins form a complex when expressed at endogenous levels. Therefore, we immunoprecipitated endogenous Raf-1 from COS-1 cells with an antibody that recognised the 12 C-terminal residues of Raf-1. We found that PP5 interacted with Raf-1 specifically in serum-starved cells as less endogenous PP5 was found to co-precipitate with Raf-1 in growing cells (Figure 5.1A). This result indicated that PP5 and Raf-1 form an endogenous complex which is regulated by extracellular signals.

5.1.2 PP5 binds specifically to the kinase domain of Raf-1

Next we wanted to determine the region of Raf-1 that interacted with PP5. We coexpressed HA-PP5 with Flag-tagged versions of full-length Raf-1, the N-terminal regulatory domain and the C-terminal kinase domain in COS-1 cells. Analysis of Flag immunoprecipitates prepared from these cells showed that both full-length Raf-1 and its kinase domain coprecipitated PP5 (Figure 5.1B). These results show that PP5 interacts specifically with the kinase domain of Raf-1. They also raised the possibility that PP5 may dephosphorylate sites within this region of Raf-1.

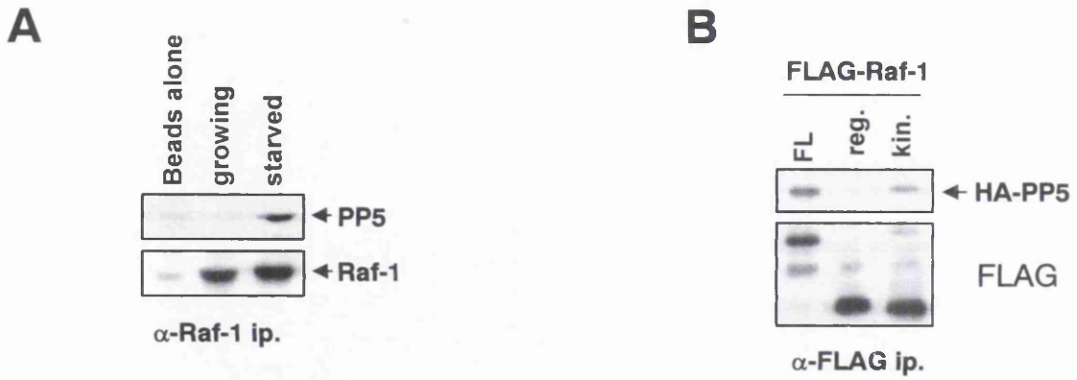

Figure 5.1: Characterisation of the interaction of PP5 with Raf-1: A) Endogenous PP5 co-precipitates with endogenous Raf-1 in serum starved cells. Cos-1 cells were either starved overnight or left growing in 10% FCS before Raf-1 was immunoprecipitated; B) PP5 binds to full length and to the kinase domain of Raf-1. Full-length (FL), the regulatory domain and the kinase domain of FLAG-tagged Raf-1 were co-expressed with HA-PP5 in Cos-1 cells. 24h post transfection the cells were starved and the Raf-1 proteins were immunoprecipitated and analysed by immunoblotting.

5.1.3 PP5 dissociates from Raf-1 upon stimulation with EGF

The poor binding of PP5 to Raf-1 in cells growing in medium containing 10% FCS prompted us to ask if the interaction between the two proteins was regulated by extracellular growth factor signals. Therefore we compared the interaction of PP5 with Raf-1 in serum-starved COS-1 cells with cells stimulated with EGF, a strong activator of the Raf-1-MEK-ERK pathway. EGF treatment led to the rapid dissociation of PP5/Raf-1 complex, with little PP5 coprecipitating with Raf-1 after just 5 minutes (Figure 5.2). Thus, the interaction between PP5 and Raf-1 is disrupted by growth factor signalling.

5.2 Effect of PP5 on signalling via the Raf-1-MEK-ERK pathway

5.2.1 PP5 impairs serum-stimulated activation of MEK

To explore the effect of PP5 on Raf-1 signalling, wild-type and catalytically-inactive (phosphatase-dead) PP5 were coexpressed in COS-1 cells with Raf-1 and HA-MEK. After serum-starvation overnight the cells were stimulated with 10% FCS for 5 minutes. HA-MEK was immunoprecipitated and immunoblotted with an antibody recognising the Raf-mediated phosphorylation sites on MEK. Phosphorylation of these sites is required and sufficient for MEK activation. Thus, MEK activation can be conveniently measured by determining the phosphorylation at theses sites. Active PP5 reduced MEK phosphorylation whereas phosphatase-dead PP5 had no effect on pMEK levels (Figure 5.3). These results show that PP5 negatively regulates activation of the Raf-MEK pathway.

5.2.2 PP5 reduces Raf-1 kinase activity of wild type and activated Raf-1 mutants

To test if PP5 acts at the level of Raf-1 we assayed the effect of PP5 on serum-induced activation of Raf-1. We coexpressed wild-type Raf-1 with wild-type or phosphatase-dead PP5, then determined Raf-1 kinase activity using a two-step assay. Expression of wild-

type PP5 reduced Raf-1 kinase activity, whereas the vector and phosphatase-dead PP5 had no effect of Raf-1 activity (Figure 5.4A). To rule out the possibility that PP5 might be acting on a protein required for the activation of Raf-1 by serum, we performed the same analysis in serum-starved cells on a mutationally activated mutant of Raf-1, Raf-$1^{YY340/341DD}$. Once again, wild-type PP5 inhibited the activity of this Raf-1 protein in comparison to vector-transfected cells. Interestingly, phosphatase-dead PP5 was able to further enhance the basal activity Raf-$1^{YY340/341DD}$ mutant (Fig. 5.4B). These results demonstrate that PP5 exerts a direct inhibitory effect on Raf-1, possibly by dephosphorylating an activating site.

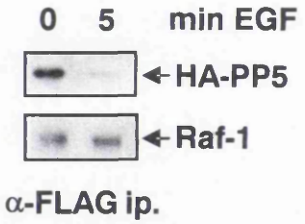

Figure 5.2: PP5 dissociates from Raf-1 upon stimulation with mitogens. FLAG-Raf-1 was co-expressed with HA-PP5 in Cos-1 cells. Cells were starved overnight and stimulated for 5 min with EGF. Raf-1 was immunoprecipitated and the levels of co-precipitating HA-PP5 were determined by immunoblotting.

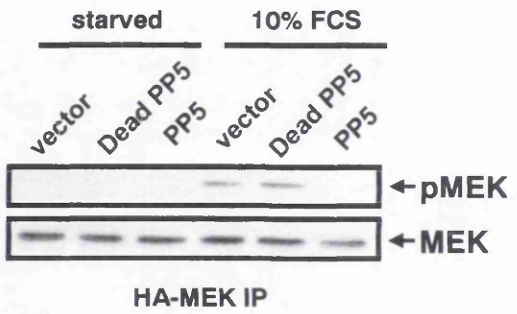

Figure 5.3: PP5 reduces serum-dependent activation of MEK. Wild-type PP5 or phosphatase-dead PP5 was coexpressed with Raf-1 and HA-MEK in Cos-1 cells. 24h post-transfection the cells were serum-starved overnight and stimulated with 10% FCS for 5min. HA-MEK was immunoprecipitated and the phosphorylation status of MEK was revealed by immunoblotting with activation specific antibodies.

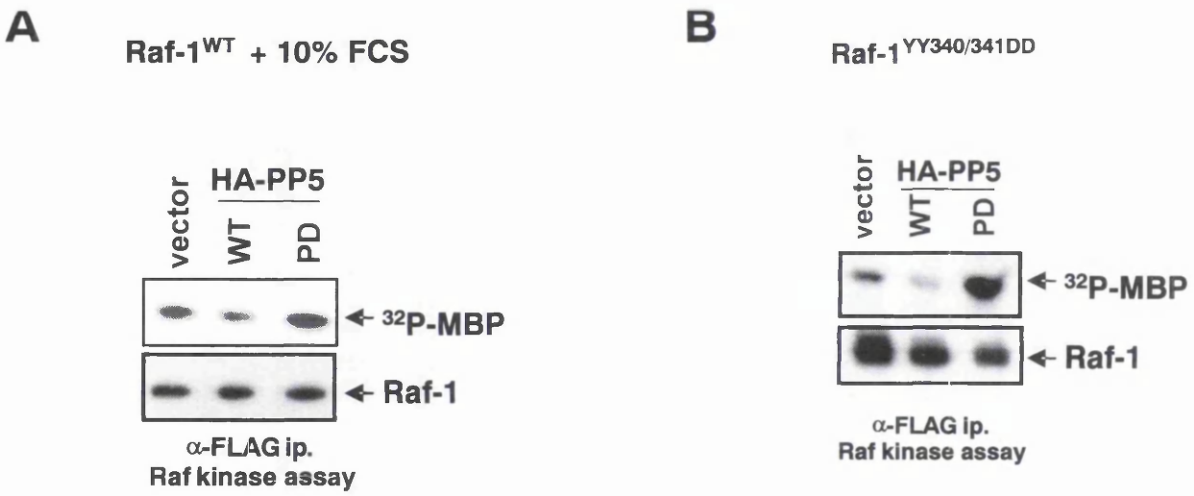

Figure 5.4. PP5 reduces Raf-1 kinase activity: A) Wild-type PP5 or phosphatase-dead PP5 and Raf-1 were coexpressed in Cos-1 cells with FLAG-Raf-1. 24h post-transfection, the cells were serum-starved overnight and stimulated with 10% FCS for 5min. Raf-1 kinase activity was assayed as ^{32}P incorporation in MBP using a 2-step kinase assay. B) Wild-type PP5 or phosphatase-dead PP5 were coexpressed in COS-1 with Raf-1$^{YY340/341DD}$. 24h post-transfection the cells were serum starved overnight. Raf-1 kinase activity was assayed as ^{32}P incorporation in MBP using a 2-step kinase assay.

5.3 Identification of the PP5-regulated site(s) on Raf-1

5.3.1 PP5 dephosphorylates pS338 but not pS621 or pS259 in-vitro

As PP5 appeared to directly reduce Raf-1 activity in cells we next tested if Raf-1 was a substrate for PP5 by assaying its ability to dephosphorylate several major Raf-1 phosphorylation sites in vitro. In order to generate Raf-1 that was highly phosphorylated on activating sites we coexpressed FLAG-Raf-1 with Ras^{G12V} , a GTPase inactive, therefore constitutively active Ras mutant, and immunoprecipitated Raf-1 using an anti-FLAG antibody coupled to agarose beads. The Raf-1 bound to the beads was subsequently incubated with purified recombinant wild-type GST-PP5 or a mutant lacking the phosphatase domain (GST-ΔPP5). In order to increase the phosphatase activity of PP5 100μM of arachidonic acid, a potent PP5 activator (Chen and Cohen, 1997), was added in the buffer. After 30 minutes the reaction was stopped and the Raf-1 immunoprecipitates were immunoblotted and probed with antibodies against three phosphorylation sites. Two of them, pS338 and pS621, are located in the kinase domain of Raf-1 while the other, pS259, is in the regulatory domain. Wild-type PP5 had no effect of the level of phosphorylation either S259 or S621 but dramatically reduced the level of S338 phosphorylation. By contrast, the phosphatase-inactive PP5 protein did not affect the phosphorylation levels of any of the sites. These results show that PP5 has a remarkable specificity in-vitro for pS338 of Raf-1 (Figure 5.5 A).

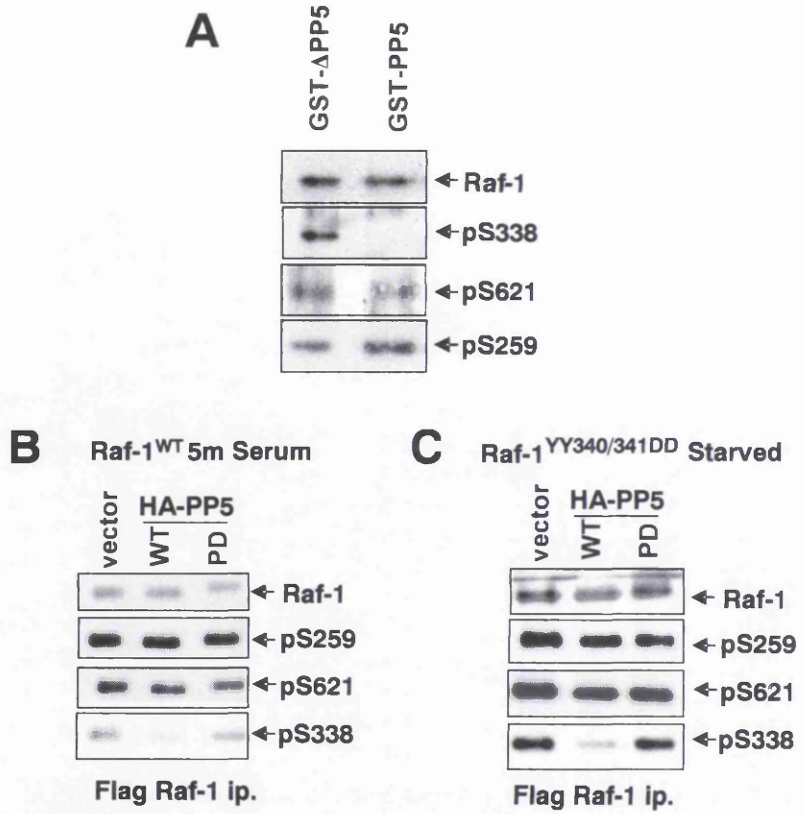

Figure 5.5: PP5 dephosphorylates pS338 on Raf-1. A) Raf-1 was co-expressed with Ras^{G12V} in Cos-1 cells and immunoprecipitated. Immunoprecipitated Raf-1 was incubated for 30min with bacterial expressed GST-PP5 or phosphatase-dead GST-ΔPP5 in presence of arachidonic acid. Levels of Raf-1 phosphorylation were determined by immunoblotting. B) PP5 specifically reduces S338 phosphorylation of serum stimulated Raf-1 without affecting pS621 or pS259. Wild-type PP5, phosphatase-dead PP5 and Raf-1 were coexpressed in Cos-1 cells. 24h post-transfection the cells were serum-starved overnight and stimulated with 10% FCS for 5min. Raf-1 was immunoprecipitated and its phosphorylation status was revealed by immunoblotting. C) PP5 specifically reduces S338 phosphorylation of the $Raf\text{-}1^{YY340/341DD}$ mutant without affecting pS621 or pS259. Wild-type PP5, phosphatase-dead PP5 and $Raf\text{-}1^{YY340/341DD}$ were coexpressed in Cos-1 cells. 24h post-transfection the cells were serum-starved overnight. Raf-1 was immunoprecipitated and its phosphorylation status was revealed by immunoblotting.

5.3.2 Expression of PP5 reduces pS338 but not pS621 or pS259 levels in cells

To determine if PP5 could also dephosphorylate pS338 in-vivo FLAG-tagged Raf-1^{WT} and Raf-1$^{YY340/341DD}$ were coexpressed with the empty vector, wild-type PP5 or phosphatase-dead PP5 in COS-1 cells. The FLAG-tagged immunoprecipitates were immunoblotted and probed with the Raf-1 phospho-specific antibodies described above. The result of this experiment was fully consistent with that obtained in the in-vitro experiments (Figure 5.5B & C). Both the pS259 and pS621 levels were not affected by coexpression of wild-type PP5 whereas pS338 levels were reduced in both Raf-1 proteins. The vector and phosphatase-dead PP5 mutant had no effect on the levels of phosphorylation on any of the sites. These results provide strong evidence that pS338 is a genuine in-vivo target of PP5.

5.3.3 The kinase activity of the Raf-1$^{SS338/339DE}$ mutant is not affected by PP5

To further test if other sites on Raf-1 other than S338 were regulated by PP5 we mutated both serines 338 and 339 to either alanines to prevent phosphorylation or to phosphomimetic glutamic and aspartic acid and then assessed the effect of PP5 on Raf-1 kinase activity. We used a one-step kinase assay to test the ability of Raf-1 to phosphorylate MEK. COS-1 cells coexpressing the Raf-1 mutants and PP5 were stimulated for 5 minutes with 10% FCS before Raf-1 was immunoprecipitated to determine its kinase activity (Figure 5.6). The Flag-Raf-1$^{S338A/S339A}$ mutant was devoid of kinase activity, confirming that the 338 site is essential for Raf-1 activity whereas the Flag-Raf-1$^{S338D/S338E}$ mutant had a slightly higher activity than wild-type Raf-1. As shown previously in Figure 6.4A, PP5 reduced the activity of FLAG-Raf-1^{WT} whereas the phosphatase-dead PP5 mutant had no effect (Fig. 5.6). By contrast, the activity of the Flag-Raf-1$^{SS338/339DE}$ mutant was not affected by either wild-type or phosphatase-dead PP5. These results show that PP5 inhibits Raf-1 activity by specifically dephosphorylating S338.

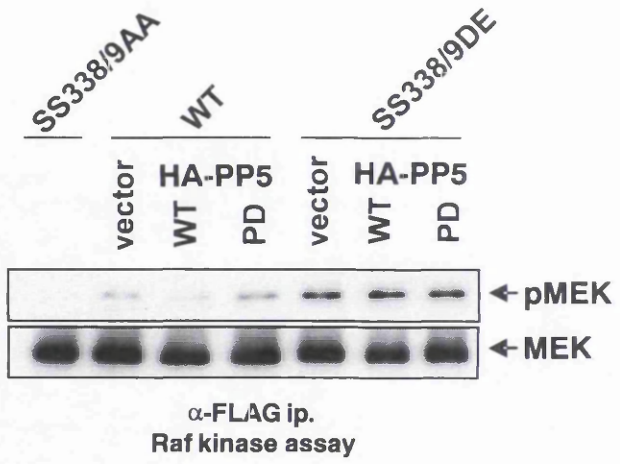

Figure 5.6. PP5-mediated inhibition of Raf-1 is prevented by phosphomimetic mutation of S338. Wild-type PP5, phosphatase-dead PP5 and one of the three Raf-1 mutants were transfected in Cos-1 cells. 24h post-transfection the cells were serum-starved overnight and stimulated with 10% FCS for 5min. Raf-1 was immunoprecipitated and its kinase activity determined by using Sf9 expressed GST-MEK as a substrate and determining pMEK levels by immunoblotting

5.4 Gα12-dependent regulation of Raf-1 S338 phosphorylation by PP5

5.4.1 Active Gα12 binds PP5 in COS-1 cells

Recent reports have indicated that active Gα12 increases binds to PP5 and recruits it to the cell membrane (Yamaguchi et al., 2002). To verify this report we coexpressed HA-tagged PP5 and with wild-type Gα12 and or the activated mutant $G\alpha12^{QL}$ in COS-1 cells. This mutant is defective in its GTPase activity and is so retained in an active conformation. We serum-starved the cells overnight and immunoprecipitated Gα12 with a Gα12 specific antibody. We then blotted for co-precipitating PP5 and found that active Gα12 bound considerably stronger to PP5 confirming the previously published results (Figure 5.7A).

5.4.2 Active $G\alpha12^{QL}$ impairs PP5 dependent pS338 dephosphorylation

Having established that PP5 is binds active Gα12 we wanted to investigate the effect of this interaction on the ability of PP5 to dephosphorylate pS338. We therefore coexpressed FLAG-Raf-$1^{YY340/341DD}$ and $G\alpha12^{QL}$ in COS-1 cells with either wild-type PP5 or the phosphatase-dead mutant. We used the FLAG-Raf-$1^{YY340/341DD}$ mutant for this experiment because it has a high basal level of S388 phosphorylation in starved cells and therefore no induction by growth factors was needed. Using growth factors to induce S338 phosphorylation could also activate Gα12 signalling, therefore making the interpretation of the results less definitive. We immunoblotted the Raf-1 immunoprecipitates with antibodies against two phosphorylation sites, pS338 and pS621 (Figure 6.7 B). pS621 was not affected by either PP5 or $G\alpha12^{QL}$. On the other hand the PP5-mediated reduction in S338 phosphorylation was prevented by coexpressing $G\alpha12^{QL}$. Phosphatase-dead PP5 on its own, or when coexpressed with $G\alpha12^{QL}$, slightly increased pS338 phosphorylation. These results suggest that Gα12 signalling can positively regulate Raf-1 activity by

antagonising PP5-mediated dephosphorylation of pS338. This effect of active Gα12 may in turn be mediated by its ability to sequester PP5 from Raf-1.

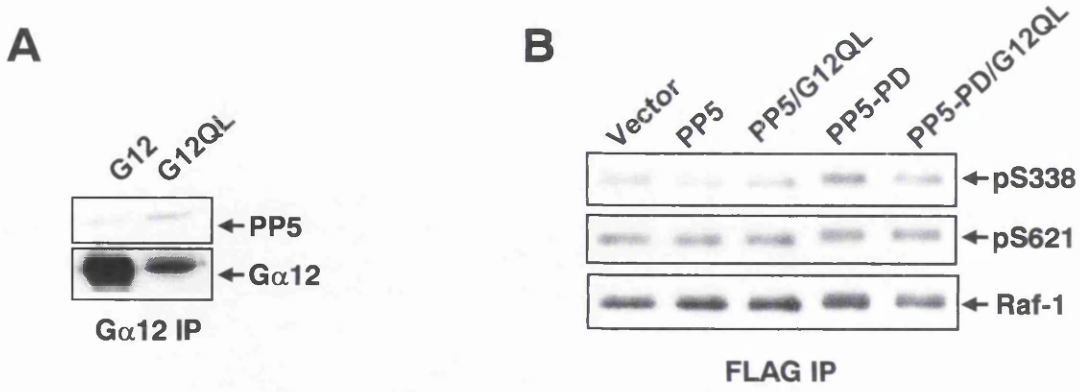

Figure 5.7. Activated Gα12 QL prevents PP5 from de-phosphorylating pS338. A) Mutationally activated Gα12 binds stronger to PP5 in co-immunoprecipitations. PP5, Gα12 and mutationally active $G\alpha12^{QL}$ were co-transfected into Cos-1 cells. 48h post transfection the cells were lysed and Gα12 was immunoprecipitated. Levels of co-precipitating PP5 were determined by immunoblotting; B) PP5 dephosphorylates pS338 of $Raf\text{-}1^{YY340/341}$ in starved cells but cotransfection of $G\alpha12^{QL}$ antagonises the PP5 induced dephosphorylation of pS338. Wild-type PP5, Raf-1 and mutationally active $G\alpha12^{QL}$ were co-transfected into Cos-1 cells. 48h post transfection the cells were lysed and Raf-1 was immunoprecipitated. Phosphorylation levels of Raf-1 were assayed by immunoblotting.

Chapter 5: Discussion

The experiments described in this chapter were aimed at characterising the interaction between PP5 and Raf-1 and to decipher its functional consequences. We found that the interaction is regulated by growth factor signalling and have identified pS338 on Raf-1 as a genuine in-vitro and in-vivo substrate of PP5. S338 is one of the sites whose phosphorylation is induced by Ras and mitogens and is required for Raf-1 activation by these signals (Diaz et al., 1997). The identity of the kinase which phosphorylates S338 has been controversial, but several studies have suggested that this kinase is PAK1 or PAK3 (Chaudhary et al., 2000; King et al., 1998; Sun et al., 2000). The results presented here provide strong evidence that PP5 is the S338 phosphatase. We found that PP5 showed a remarkable specificity for dephosphorylating pS338 both in vitro and in vivo. Most importantly, phosphomimetic substitutions of S338 rendered Raf-1 no longer susceptible to inhibition by PP5. We could also show that Raf-1 dependent activation of MEK in cells was reduced through PP5-dependent inhibition of Raf-1 kinase activity.

While we have shown that PP5 associates with the catalytic domain of Raf-1, we did not identify the region of PP5 to which Raf-1 binds. Thus far, most proteins that interact with PP5 do so via its TPR domain (Chen et al., 1996; Galigniana et al., 2002; Kono et al., 2002; Lubert et al., 2001; Ollendorff and Donoghue, 1997; Russell et al., 1999; Shao et al., 2002; Silverstein et al., 1997; Yamaguchi et al., 2002; Zhao and Sancar, 1997). Further studies using a TPR domain truncated PP5 mutant are required to determine if Raf-1 also binds to this domain. We also did not determine if PP5 bound directly to Raf-1 or if it did so via an adaptor. It is possible that proteins that bind to both PP5 and to Raf-1 could function as linkers. These include the regulatory subunits of PP2A (Lubert et al., 2001), HSP90 (Chen et al., 1996), and possibly 14-3-3. However our finding that the Raf-1^{S259A}, which is deficient in its 14-3-3 binding capacity (Rommel et al., 1996), interacts equally well with PP5 as wild-type Raf-1 argues against 14-3-3 as being a potential adaptor.

Another question to be resolved is the mechanism by which PP5 dissociates and re-associates to Raf-1. One elegant way this could occur would be if the catalytic subunit of PP2A would replace PP5 from the two regulatory subunits of PP2A that are constitutively bound to Raf-1. It has been shown that PP5 can bind to these subunits (Lubert et al., 2001) and, though it could be coincident, PP5 dissociates from Raf-1 when the catalytic subunit of PP2A is reported to associate.

We identified Gα12 signalling as one pathway that could regulate PP5-dependent pS338 dephosphorylation. This may provide a link between two distinct GTPase pathways (Ras and Gα12). Active Gα12 is an oncogene and strong inducer of cell transformation (Chan et al., 1993; Xu et al., 1993). It is thought that it main role is to activate the JNK pathway through a Rho dependent mechanism. Gα12 and Gα13 have been both found to interact and activate p115RhoGEF (Hart et al., 1998), thus increasing Rho-GTP levels and inducing Rho-dependent signalling pathways. Gα12 signalling has also been shown to increase Ras activity by binding to rasGAP (Jiang et al., 1998) and by activating PLCε(Lopez et al., 2001). Increasing both Ras and Rho activity in $G\alpha12^{QL}$-transfected HEK293 cells resulted in phosphorylation of JNK but not ERK (Collins et al., 1996). It was shown in the same cells that activation of JNK could be blocked by transfecting dominant negative Ras, demonstrating that both Ras and Rho are necessary for JNK activation. The lack of ERK phosphorylation is quite puzzling in respect to our results as we have found that active Gα12 inhibits PP5 dependent dephosphorylation of pS338 of Raf-1. This in combination with active Ras should result in increased signalling through the ERK pathway. This discrepancy can be partially explained by the finding that active Gα12 inhibits the Ras/Raf/MEK/ERK pathway at the level of MEK in COS-7 cells (Voyno-Yasenetskaya et al., 1996). On the other hand studies assaying the transformation potential of Gα12 and known proto-oncogenes in rodent fibroblasts have shown that there is a striking cooperative effect on focus formation when Gα12 and Raf-1 are co-transfected into NIH3T3 cells (Zhang et al., 1996). Co-expression of these two proteins also results in

the constitutive activation of ERK. These discrepancies in the outcome could be cell type dependent. It is possible that in a certain cell types MEK inhibition overrides the increase in Ras and Raf-1 activity and thereby prevents an increase of ERK phosphorylation. Clearly these discrepancies should be resolved by examining the effects of active Gα12 on Raf-1 activity and ERK phosphorylation in several different cell lines.

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