Investigation of receptor association and tyrosine phosphorylation of ~~ribosomal protein~~ S6 kinases

by

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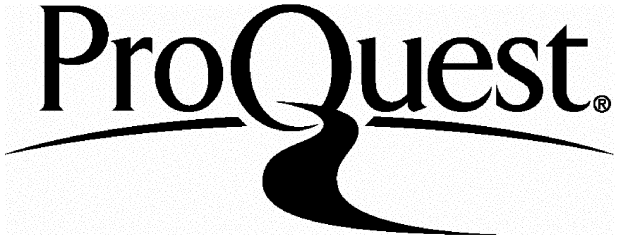

ABSTRACT

S6 kinase, a member of the AGC family of kinases, is activated by an array of mitogenic stimuli and is a key player in the regulation of cell growth and proliferation. The activation process involves a complex sequential series of ten or more serine and threonine phosphorylations.

In this thesis it was investigated if S6K activation would involve translocation to the plasma membrane and if tyrosine phosphorylation would occur as was previously shown for some other AGC kinases. It is demonstrated for the first time that S6 kinase is associated with receptor protein tyrosine kinases (RTKs) upon growth factor stimulation. The binding occurs via the kinase or kinase extension domain of S6K as shown by the use of truncation mutants in co-immunoprecipitation studies. Furthermore, both isoforms of S6 kinase, α and β, were shown to be phosphorylated on tyrosine in a RTK- and Src-dependent manner. Using mass spectrometry and truncation mutants, two phosphorylation sites were mapped: one is located in the activation loop and the second one in the N-terminus of the kinase. Inhibitor studies reveal that phosphorylation occurs independently of PI3 Kinase and mTor but is inhibited by Src family kinase inhibitors. It has already been established that expression of Src kinase leads to an activated S6K. However, as shown in this study, this effect is not mediated through tyrosine phosphorylation. Subcellular localisation and S6K stability are also not altered in a Src-dependent manner. Taken together, the presented work shows for the first time an additional mechanism of S6 kinase regulation via association with receptor tyrosine kinases and tyrosine phosphorylation. The phosphotyrosine site/s possibly create/s recognition sites for SH2 domain containing proteins and therefore may be important for recruiting S6K into a multienzyme complex with other signalling molecules at the plasma membrane.

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ABBREVIATIONS

AmBic	Ammonium bicarbonate
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
C18	Reverse phase packing material used in peptide separation
CAMP	Adenosine3`,5`-cyclic monophosphate
CBP80	80 kDa subunit of RNA cap-binding complex CBP80
CBC	Cap-binding complex
cDNA	Complementary DNA
cGMP	Guanosine3`,5`-cyclic monophosphate
CID	Collision-induced dissociation
CREM	cAMP-responsive activator modulator
Da	Dalton
DAG	sn-1,2-Diacylglycerol
DHB	Dihydrobenzoic acid
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
DTT	Dithiotreitol
4E-BP1	eukaryotic initiation factor-4E binding protein 1
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetra-acetic acid
eEF1A	Eukaryotic elongation factor 1A
eEF2k	Eukaryotic elongation factor 2 kinase
EGF	Epidermal growth factor
EGTA	Ethyleneglycol-bis(β-aminoethyl)-N,N,N`,N`-tetraacetic acid

ERK	Extracellular signal-regulated kinase
ESI	Electrospray ionization
EST	Expressed sequence tag
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FKBP12	FK506-binding protein 12
GAP	GTPase-activating protein
GEF	Guanosine nucleotide exchange factor
GSK	Glycogen synthase kinase
HPLC	High performance/pressure liquid chromatography
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor 1
IRS-1	Insulin receptor substrate 1
ISD	In-source decay
KHL	Keyhole limpet hemocyanin
LC/MS	Liquid chromatography- mass spectrometry
MALDI	Matrix-assisted laser desorption ionisation
MAPK	Mitogen-activated protein kinase
MEK	MAPK kinase
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger RNA
mRNP	Messenger ribonucleic acid protein
MS	Mass Spectrometry
mTOR/FRAP	Mammalian target of rapamycin/FK506-binding protein, rapamycin-associated protein
m/z	Mass-to-charge ratio
NEK	NIMA-related kinase
NIMA	Never-In Mitosis, gene A
Ni-NTA	Nickel-nitrilotriacetic acid
NLS	Nuclear localisation signal
NES	Nuclear export signal

OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PDK1	3`-Phosphoinositide-dependent kinase-1
PDZ	Postsynaptic density-95, discs large, zona occludens-1
PEG	Polyethylene glycol
PH	Pleckstrin homology
PIF	PDK1-interacting fragment
PI3K	Phosphatidylinositide-3`-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PLCγ	Phospholipase Cγ
PMA	Phorbol 12-myristate 13-acetate
PCR	Polymerase chain reaction
PIP_2	Phosphatidylinositol-3,4-bisphosphate
PIP_3	Phosphatidylinositol-3,4,5-trisphosphate
Pro	Proline
PP1	Protein phosphatase1
PP2A	Protein-serine/threonine phosphatase 2A
p90RSK	p90 Ribosomal S6 kinase
PSD	Post-source decay
PTEN	Phosphatase and tensin homologue, deleted on chromosome 10
PVDF	Polyvinyldene difluoride
RNA	Ribonucleic acid
rpS6	Ribosomal protein S6
rRNA	Ribosomal RNA
RTK	Receptor tyrosine kinase
S6K	Ribosomal protein S6 kinase

SDS	Sodium n-dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
Ser	Serine
SGK	Serum- and glucocorticoid-induced protein kinase
SH2	Src homology 2
SH3	Src homology 3
snRNA	small nuclear RNA
SRE	Serum response element
TEMED	N,N,N`N`-Tetramethylethylenediamine
Thr	Threonine
TOF	Time-of-flight
5`-TOP	5`-terminal oligopyrimidine tract
tRNA	Transfer RNA
TSC	Tuberous sclerosis complex
Tween 20	Polyoxyethylenesorbitan monolaurate
WW	Tryptophan-tryptophan

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CHAPTER 1: INTRODUCTION

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INTRODUCTION

1.1. INTRODUCTION

A cell must grow to a certain extent before it divides to ensure that its size and macromolecular composition remain constant at any given proliferation rate. In a complex multicellular organism, different tissues are composed of cells of characteristic size and shape, but within a tissue these parameters remain highly constant. To initiate symmetrical cell division, mitogens trigger a number of early signals that lead to the activation of cyclins which are required for G1-S transition and induce an increase in cell mass. This suggests that cell cycle progression is determined by cell size and indeed the two processes, growth and division, are strongly linked. Nevertheless, they are separable processes and regulated by different mechanisms. In early embryogenesis in organisms such as Drosophila and C. Elegans, for example, division occurs without growth, which dramatically increases cell number but strongly reduces the size of the cells. In contrast, during a process called endoreplication occurring, for example, in Arabidopsis or other plants, a cell doubles its DNA content without dividing, leading to cells that can contain over a thousand copies of their chromosomes and a corresponding enlargement of size (Saucedo and Edgar, 2002). Furthermore, nutrient deprivation was shown to prevent cellular growth and proliferation, but mutations that blocked cell cycle progression did not inhibit cellular growth (Johnston *et al.*, 1997).

Two major pathways have been identified as regulators of cell growth: the Ras/MAPK pathway and the PI3K/mTor pathway.

The Ras/MAPK pathway can regulate cell growth via the expression of transcription factors such as myc (Schulze *et al.*, 2001; Lavoie *et al.*, 1996) or by enhancing translation. C-myc is a transcription factor whose expression is triggered by a variety of pathways, such as downstream of receptor tyrosine kinases via Src and Erks (Davis,

1995; Courtneidge, 2002). C-Myc can also be activated through Erk-mediated phosphorylation (Davis, 1995). Once expressed or activated, it mediates the transcription of many growth-related genes such as cyclins D1, E and A, cdc2, eIF4e, eIF2a (Daksis *et al.*, 1994; Leone *et al.*, 1997; Galaktionov *et al.*, 1996; Rosenwald *et al.*, 1993). By enhancing transcription of cyclins and by leading to the degradation of the G1/CDK inhibitor $p27^{kip}$ or by inhibition of transcription of the CDK inhibitor $p15^{INK4b}$, myc helps to drive the cell cycle (O'Hagan *et al.*, 2000; Staller, 2001; Bouchard *et al.*, 2001). In Drosophila, partial loss of c-myc results in very small flies primarily due to cellular hypotrophy (Johnston *et al.*, 1999). Another report, however, based on a mouse model, attributes the action of myc rather to a reduction in cell number than cell size (Trumpp *et al.*, 2001). But Ras can also act independently of myc's transcriptional activity, because it stimulates the kinase Mnk1/2 via Erk1/2. Mnk1/2 activates the eukaryotic translation initiation factor 4E, which boosts cap-dependent translation and protein synthesis (Pyronnet, 2000; Scheper *et al.*, 2001).

The second major signalling pathway that controls cell growth is the PI3K/mTor pathway involving phosphoinositide 3-kinase (PI3K) and the mammalian target of rapamycin (mTor). While PI3K activation depends on signals from outside the cell such as mitogens, mTor can sense the nutrient status of a cell and is activated in a nutrient-rich environment independently of inputs via PI3K. As will be discussed in chapter 1.1.2., both enzymes feed into activation of ribosomal protein S6 Kinase, termed S6K. Another, differently regulated, ribosomal S6Kinase, p90RSK, will not be discussed in this report. Interestingly, in Drosophila, mutations of any of the members of this pathway downstream of the insulin receptor have a striking effect on cell size and number. Drosophila knockouts of the species' only isoform, dS6K, exhibit a decreased body size which is mainly due to reduced cell size rather than cell number (Montagne *et al.*, 1999). Knockout-studies in mice also provided the evidence that S6K is an important regulator of cell size and growth. S6Kα-deficient mice are viable, but strongly reduced in body size during embryogenesis. They also exhibit symptoms of diabetes mellitus type 2, which are mainly attributed to a reduction in size of the

pancreatic β cells and, consequently, lower insulin production (Pende *et al.*, 2000; Giordano *et al.*, 1993).

1.2. THE REGULATION AND FUNCTION OF RIBOSOMAL PROTEIN S6 KINASES

1.2.1. The S6K family

The p70 S6K is a serine/threonine kinase and was first cloned in 1990 by three different groups (Kozma *et al.*, 1990; Banerjee *et al.*, 1990; Grove *et al.*, 1991) from mitogen-treated Swiss3T3 cells and from rat liver. Previously, few groups had reported the purification of ribosomal protein S6 kinase activity from a variety of cells. It was found that EGF, Insulin, serum, phorbol ester and also v-Src were able to activate S6Kinase in fibroblasts (Swiss3T3, chicken embryo fibroblasts) and hence some knowledge of the regulation via receptor tyrosine kinases, PI3K, PKCs and v-Src had existed prior to the cloning (Stefanovic *et al.*, 1986; Blenis and Erikson, 1985; Blenis and Erikson, 1986). A second isoform, termed S6Kβ or S6K2 was found much later (Gout *et al.*, 1998; Shima *et al.*, 1998; Saitoh *et al.*, 1998). The two isoforms of S6 Kinase S6Kα and β, or S6K1 and 2, respectively, share a similar modular organisation as shown in Fig. 1.2.1: The kinase and kinase extension domains are flanked by N- and C-terminal regulatory regions. Both S6Kα and S6Kβ have two isoforms, which originate from different translational start sites and exhibit a different subcellular localisation. They will be referred to as p85 for the longer and p70 S6 kinases for the shorter isoforms of S6Kα but, for reasons of simplicity, also for S6Kβ isoforms even though these migrate at slightly lower positions than S6Ksα in SDS-PAGE gels. P70S6Kα is predominantly cytosolic, whereas p85S6Kα, like the p85 isoform of S6Kβ, possesses a nuclear localisation sequence (NLS) in the extended N-terminus making these isoforms constitutively located in the nucleoplasm (Coffer and Woodgett, 1994; Minami *et al.*, 2001). In contrast to p70S6Kα, p70 S6Kβ has an additional NLS in its C-terminus and

is also predominantly nuclear. Nucleocytoplasmic shuttling has been shown for both cytoplasmic forms of S6Ks. The amino acid sequences of the kinase domains of S6Kα and S6Kβ are over 80% identical. Similarly, the kinase extension or linker domains share strong homology of 76%. However, the regulatory N– and C-terminal regions have a relatively low homology of 38% and 12% respectively, and these sequences are mostly responsible for a differential regulation of the α and β isoforms (Valovka *et al.*, 2003; Martin *et al.*, 2001). For example, mitogen-induced PKC-dependent phosphorylation of a unique site in the C-terminus of S6Kβ masks its NLS and causes the retention of this isoform in the cytosol (Valovka *et al.*, 2003). In this study mainly the shorter p70 isoforms were investigated, thus, unless otherwise stated, they will simply be termed S6Kα and S6Kβ in this thesis.

Both kinases lack canonical protein-protein interaction domains such as SH2, PTB, SH3, WW domains and have no PH domain, which would enable lipid binding. However, in their C-terminal domains S6Kα and S6Kβ possess either a PDZ domain-binding motif or a proline-rich region, respectively, through which S6Ks could bind other signalling molecules. It has been reported that the PDZ domain-binding motif is essential for S6Kα binding to the PDZ domain of the F-actin binding protein, neurabin. Mutation of the five carboxy-terminal amino acids of S6K abrogates the interaction (Burnett *et al.*, 1998). Furthermore, a short proline rich motif PXXP has been identified in the C–terminal regulatory region of PKB/Akt (Jiang and Qiu, 2002). This sequence is also conserved among S6Kinases, suggesting that there may be an additional protein-protein interaction domain.

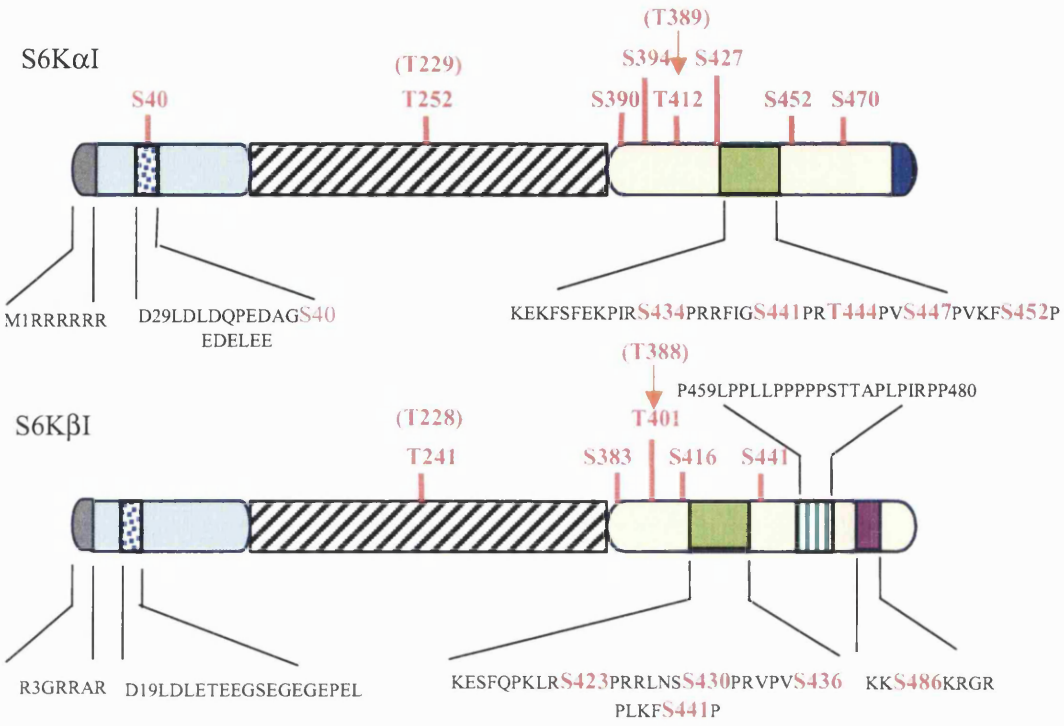


Fig. 1.2.1. Alignment of S6K domain structures.
Represented here are the long (p85) isoforms of S6Kα and S6Kβ. The centrally located kinase domain is shown as a hatched box, nuclear localisation sequences (NLS) are in dark grey and the acidic domains as dotted boxes. C-terminally adjacent to the kinase domain is the kinase extension domain which is followed by the autoinihibitory pseudosubstrate region (green boxes). The PDZ domain binding motif is unique for S6Kα and indicated in blue at the C-terminus, the proline-rich region which is solely found in the C-terminus of S6Kβ is marked in vertical stripes. The S6Kβ-specific NLS is in pink. Phosphorylation sites are marked in red. The numbering corresponds to the long isoforms. In this work the short p70 isoforms will be used, for which the numbering can be deduced from the p85 numbering by subtraction of 23 amino acids and 13 amino acids for p70S6Kα or β, respectively. The numbering of the major phosphorylation sites for the p70 isoforms is indicated in brackets. See text for details.

As early as 1990, it was proposed that in the inactive state of the kinase, the acidic N-terminus interacts with the basic residues of the pseudosubstrate region near the C-terminus, thereby preventing substrate binding (Banerjee *et al.*, 1990). Phosphorylation of sites in this pseudosubstrate region releases this inhibitory interaction and primes S6K for activation but not automatically activates it. Deletion of the C-terminal

regulatory domain consisting of the last 104 amino acids of S6Kα (p70S6KαΔC$_{104}$) including the pseudosubstrate autoinhibitory domain results in a kinase with low basal activity which can be stimulated by serum. Rapamycin sensitivity was slightly reduced in this mutant (Weng *et al.*, 1995b; Weng *et al.*, 1998). This finding was counterintuitive at the time, as the authors had anticipated that deletion of the autoinhibitory domain would produce an increase in basal activity and a consequent attenuation of the fold activation by serum. Similarly, a doubly truncated S6K mutant (ΔNΔC) has low basal activity and remains stimulatable. These findings lead to the conclusion that other major mitogen induced inputs must be directed to domains other than the autoinhibitory one. Interestingly, the doubly truncated mutant is rapamycin-insensitive as will be described in 1.2.3.4. (Weng 1995b).

The main physiological substrate for S6Ks is ribosomal protein S6. Besides p70/p85 S6Kinase, p90 ribosomal S6 Kinase (p90RSK) has been shown to phosphorylate S6 protein *in vitro*. P90RSK lies downstream of the p21 ras regulated MAPK pathway and is, therefore, also activated by mitogens. In addition, p90RSK mediated S6 phosphorylation appears to be important in Xenopus oocyte maturation, but is not essential in mitogen-stimulated S6 phosphorylation in somatic animal cells (Blenis *et al.*, 1991). It has recently been reported that in double knockout mice (S6Kα(-/-)S6Kβ(-/-) there is a residual S6 phosphorylation (on serines S235, S236) which is insensitive to rapamycin and sensitive to MAPK inhibitors. This finding indicates that under certain circumstances p90RSK may phosphorylate S6 protein also *in vivo* (Pende *et al.*, 2004)

1.2.2. Regulation of S6 Kinases

1.2.2.1. Canonical PI3Kinase pathway to S6K

S6 Kinases are activated through mitogen and nutrient-mediated pathways. Growth factors or mitogens activate receptor tyrosine kinases (RTKs) and lead to their dimerisation and autophosphorylation. A schematic representation can be found in Fig. 1.2.2.. Furthermore, the activated receptor molecules can phosphorylate other signalling molecules that have been recruited to the phosphotyrosine sites of the receptors via their SH2 domains. This mechanism will be discussed in more detail in chapter 1.3. In the case of insulin signalling, receptor-recruited insulin receptor substrate (IRS-1) becomes tyrosine phosphorylated and this generates a docking site for the p85 adaptor subunit δf class 1A PI3K at the membrane. Class 1A PI3 Kinases consist of the adaptor subunit and either isoform of the p110 catalytic subunits α, β and δ which all act downstream of RTKs, whereas the only member of class 1B family, PI3Kγ transmits signals downstream of G protein-coupled receptors (GPCRs). For mitogens other than insulin, such as PDGF, the p85 subunit can directly bind to the activated receptor. The PI3K catalytic subunit p110 is in complex with p85 and is activated upon docking to IRS1 or the RTK. It converts the lipid phosphatidyl-inositol 4,5 bisphosphate, PIP_2, into phosphatidyl-inositol 3,4,5 trisphosphate, PIP_3, by phosphorylation. This phospholipid is normally present at very low levels in unstimulated cells and its concentration rises quickly upon agonist induction (Hawkins, 1992). It acts as a second messenger and recruits several downstream signalling molecules such as protein kinase B (PKB/Akt) and 3'-Phosphoinositide-dependent kinase 1 (PDK1) via their PH domains to the plasma membrane (Blume-Jensen and Hunter, 2001; Hafizi Yacoub, 1997; Chung *et al.*, 1994). PDK1, which is constitutively active, phosphorylates a crucial residue in the activation loop of the kinase domain of PKB/Akt, S6K and other AGC kinases (Alessi *et al.*, 1997; Pullen *et al.*, 1998).

PKB/Akt is activated by PIP_3-mediated translocation to the membrane and PDK1 phosphorylation. To be fully active, PKB/Akt also needs to be phosphorylated on a

residue in the hydrophobic motif (S473), and, in turn, it leads to the inactivation of GSK3 but also to activation of the mammalian target of rapamycin, mTor (FRAP). It has been proposed that mTor is a direct target of PKB/Akt (Nave *et al.*, 1999). However, more recently a model has emerged which places the tumour suppressor complex Tsc1/2 in between. mTor or a mTor-dependent kinase then phosphorylates and thereby activates S6K on a crucial site in the hydrophobic domain. Another substrate of mTor is the translational repressor eIF4E binding protein, 4E-BP1. When phosphorylated by mTor on four proline-directed S/T sites, 4E-BP1 releases the translation initiation factor 4E and enables the formation of an active translation initiation complex (Raught *et al.*, 1999). As a part of a complex consisting of other initiation factors and an mRNA helicase, eIF4E specifically recognises and binds the mRNA cap structure (m^7GpppN with m=methyl and N=any nucleotide).

The role of PKB/Akt in the process of S6K activation is still controversial. Expression of a constitutively membrane-targeted and active gag-PKB (where the viral gag protein is fused to PKB/Akt similarly as in v-akt) activates S6K even in unstimulated cells (Burgering and Coffer, 1995). But PKB/Akt only activates S6K when it is tethered to the membrane while it still signals to 4E-BP when it is not membrane bound. This was assessed by the use of various PKB/Akt mutants. For example, a PKB/Akt mutant which is constitutively membrane-targeted due to an Lck myristylation and palmitylation signal at its N-terminus leads to S6K activation even in starved cells. In another membrane-targeted mutant the S473 hydrophobic motif site was mutated to alanine which yielded a kinase with low basal and strongly reduced insulin-mediated activity. In starved and stimulated cells this PKB/Akt mutant was able to activate S6K to a similar extent than wt PKB/Akt. In contrast, an activated PKB/Akt mutant (T308D/S473D) with no extra membrane-targeting domain only activates S6K after insulin stimulation, whereas 4E-BP is phosphorylated by expression of this mutant even in starved cells. In addition, a kinase dead (K179) PKB/Akt construct with a myristylation and palmitylation signal or a membrane targeting CaaX box from Ki-Ras is catalytically inactive as assessed by a reporter construct. Either of these two mutants of PKB/Akt block 4E-BP phosphorylation but not S6K activation (Dufner *et al.*, 1999). These findings demonstrate that 4E-BP phosphorylation is fully whereas S6K

activation is not necessarily dependent on PKB/Akt activity. However, the crucial factor for S6K activation seems to be membrane localisation of PKB/Akt. In studies using cells from PKBα (-/-)PKBβ(-/-) knock-out mice, the authors found phosphorylation of S6Kα is reduced however it is not impared after serum stimulation which could indicate that it may be phosphorylated via other mechanisms (Peng et al, 2003). Intriguingly, Drosophila mutants of PI3K and PKB/Akt do not have altered S6K activity (Radimerski *et al.*, 2002). This finding is in stark contrast to studies using cultured Drosophila cells where both PI3K and PKB/Akt activity are needed for insulin-induced S6K activation (Lizcano *et al.*, 2003). Pan and Gao argue in their review (Pan *et al.*, 2004) that this discrepancy is due to the different systems used. In cultured cells cross-talk between different pathways seems to be more extensive than in whole intact animals where hard-wired genetic circuits are analysed.

mTor can also be activated independently of the PI3K pathway because it senses the level of amino acids within a cell (Hara *et al.*, 1998). Thus, amino acid starvation results in rapid dephosphorylation of S6K and 4E-BP whereas re-addition of amino acids restores S6K and 4E-BP phosphorylation in an mTor–dependent manner. Overall, mTor can be considered a gatekeeper that couples nutrient availability with cell growth. The canonical pathway leading to S6K activation is depicted in Fig.1.2.2..

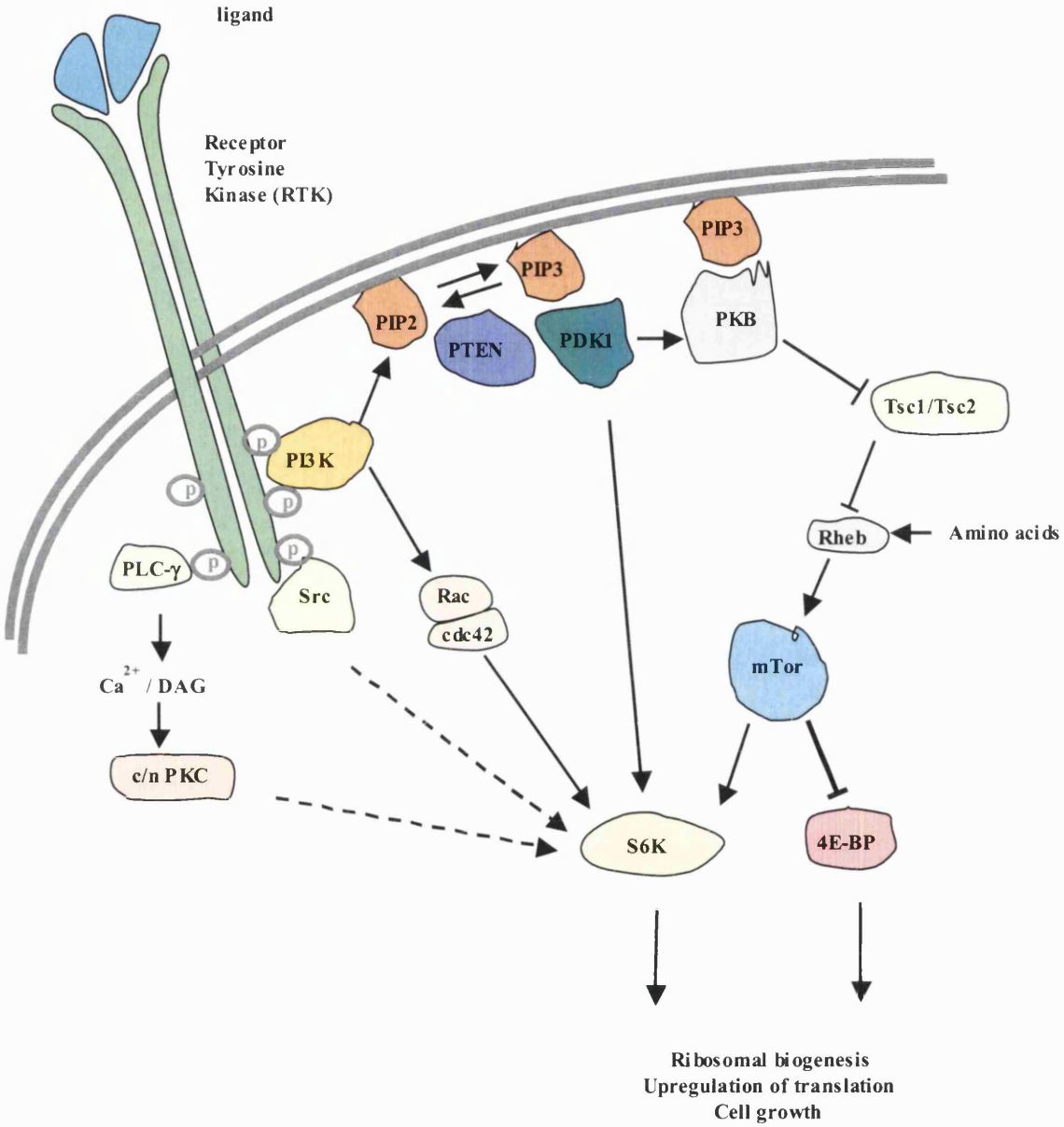


Fig. 1.2.2. RTK signalling pathways leading to S6K activation.
An arrowhead and a horizontal bar indicate activation and repression, respectively. A solid line represents an established signalling link, whereas a broken line marks an uncertain regulatory connection. n/c PKC: novel/classical PKCs. See text for details.

1.2.2.2. Other positive regulators of S6 kinases

Downstream of receptor tyrosine kinases but not part of the above described core pathway are a number of other signalling molecules which participate in S6 Kinase regulation.

In very early studies on S6Kinase it was observed that phorbol esters which mimic the effects of diacylglycerol (DAG) could stimulate S6K activity indicating that classical and novel protein kinases C (PKCs) are involved in the regulation of S6Ks. The PKC family is classified by the molecules which activate the various isoforms. Conventional PKCs are regulated by calcium, DAG and phosphatidylserine, novel PKCs do not require calcium but DAG and phosphatidylserine. Finally, atypical PKCs are regulated by phosphatidylserine but not calcium or DAG (Mellor and Parker, 1998). The phorbol ester PMA induces S6Kα activity which is reduced by rapamycin (Monfar *et al.*, 1995). The novel PKCδ was found in complex with mTor suggesting that it may act in concert with mTor to activate S6K. Furthermore, it was shown that PDK1 interacts with PKCs (Balendran *et al.*, 2000). Hence, PKC may not only bring mTor into close vicinity with S6K but also PDK1. The involvement of atypical PKCs which are not activated by phorbol esters, has recently been shown as they bind and activate S6Kα (Romanelli *et al.*, 1999), however it is unclear whether they directly phosphorylate S6K.

Furthermore, stimulation of cardiomyocytes with the adrenergic agonist phenylephrine (acting via G-protein coupled receptors) lead to S6Kα activation- an effect that could be blocked by MAPK inhibitors and enhanced by expression of a constitutively active MEK1 (Wang *et al.*, 2002). In another report, phenylephrine and insulin lead to S6Kβ activation in a MEK1-dependent manner. Dominant negative PKB/Akt could block the insulin-mediated but not the phenylephrine-mediated S6Kβ activation (Wang *et al.*, 2001a). Further, it was reported that S6Kβ but not S6Kα is situated within the MAPK pathway in FGF-2 stimulated small cell lung cancer cells (Pardo *et al.*, 2001). This indicates that at least one isoform of S6K, S6Kβ, may be under the control of MAPK after RTK stimulation and that both isoforms can be controlled by the MAPK pathway when cells are stimulated by GPCR agonists.

Even before S6K was cloned, studies performed with v-Src transformed cells indicated that Src could positively influence S6K activity (Blenis and Erikson 1986). More recently, the Src inhibitor PP1 was shown to interfere with S6K activity after insulin stimulation (Shah *et al.*, 2003). However, PP1 also inhibits PDGFR and Kit as well as Bcr-Abl and Abl, and therefore it might be causing inhibition of S6Ks indirectly. The Rho family of G proteins which consists of Rho, Rac and Cdc42 controls cytoskeletal organisation and cellular movement (Qiu *et al.*, 1995). Rac and Cdc42 have been shown to activate S6Kα. Overexpression of the activated alleles of Rac (V12) and Cdc42 (V12) but not Rho (V14) lead to activation of S6Kα under basal conditions (but could not be detected under conditions of serum stimulation). Furthermore, in lysates complemented with GTPγS, which should keep the small GTPases in an active state, a small amount S6Kα could be precipitated with Cdc42 and Rac but not Rho (Chou and Blenis, 1996).

1.2.2.3. Negative regulators of S6K signalling

Negative regulators upstream of S6K are the tumour suppressors PTEN and the Tsc1/2 complex, consisting of Hamartin and Tuberin respectively. Tsc2 encodes a GTPase activating protein (GAP) for the small GTPase Rheb (Tee *et al.*, 2003). Loss of function mutation of either Tsc1 or Tsc2 as found in tuberous sclerosis patients results in activation of Rheb which in turn activates mTor. Gigas or dTsc2 mutant cells in Drosophila are enlarged (Ito and Rubin, 1999) concomitant with an increased size of the nucleoli which is the locus for ribosome production. (Gao and Pan, 2001). Overexpression of either Tsc1 or Tsc2 does not lead to a decrease in cell size, however, co–overexpression of both in Drosophila lead to a 50% reduction of cell size (Potter et al, 2001). Epistasis studies in Drosophila have helped in establishing the genetic hierarchy in the insulin receptor pathway by gene suppression. It has been shown that dTsc1/2 acts epistatic to all the effectors of the insulin receptor/PI3K signalling pathway. Thus, disruption of any of the genes within this pathway generate a similar effect on the fly phenotype such as change in cell size. (Tapon *et al.*, 2001; Potter *et al.*, 2001).

Several recent studies point to Tsc1/2 as the integrator of insulin signalling and nutrient sensing upstream of mTor. PKB/Akt was shown to phosphorylate Tsc2 on two sites, S939 and T1462 (Inoki *et al.*, 2002; Potter *et al.*, 2002) and it was proposed that this phosphorylation drives the Tsc1/2 complex apart thereby inactivating it (Inoki *et al.*, 2002). It may not be a linear pathway linking PKB/Akt to mTor via Tsc1/2 as other studies suggest that knockout-mice lacking both PKB/Akt isoforms still exhibit phosphorylated Tsc but reduced 4E-BP phosphorylation (Peng *et al.*, 2003). Furthermore, genetic studies in Drosophila show that PTEN and Tsc1 double mutants show an additive increase in cell size which indicates that they must be situated in parallel pathways.

As previously mentioned, mTor can sense the nutrient or amino acid status of the cell and it was even proposed to be a sensor for the energy levels (Dennis *et al.*, 2001). ATP depletion decreases S6K and 4E-BP phosphorylation and inhibits translation. It has been questioned that mTor can directly sense the ATP level because of the abundancy of ATP in cells. The level of ATP is relatively high and therefore a drastic decrease in ATP would be required to affect the activity of mTor (Proud, 2002). AMP is a much more sensitive indicator for the cellular energy status. A relatively small decrease in ATP levels will result in a relatively large increase in the AMP level, which is sensed by the 5'AMP-activated protein kinase (Hardie *et al.*, 1998). A new model has recently emerged involving Tsc. Under starvation conditions and high AMP levels, the AMP-activated kinase (AMPK) phosphorylates S1345 of Tsc2 and enhances its activity, which would then negatively regulate mTor activity (Inoki *et al.*, 2003).

The tumour suppressor PTEN is a dual-specificity phosphatase, which is able to dephosphorylate lipid and protein substrates (similarly to PI3K being a lipid and a protein kinase). Its main role seems to be to dephosphorylate PI3K-generated PIP_3 and hence to downregulate the whole PI3K pathway (Myers *et al.*, 1997). It is mainly this lipid phosphatase function of PTEN which is critical for its tumour suppressor effect. PTEN is able to dephosphorylate RCML (carboxyamidomethylated and maleylated lysozyme) in an *in vitro* assay (Simpson and Parsons, 2001). However, the physiological relevance of its protein tyrosine phosphatase activity remains to be elucidated. PTEN is frequently mutated in cancers and mutations can be manifold

including deletions, missense or nonsense mutations leading to changes in expression and activity levels of the phosphatase.

1.2.3. Mechanism of activation and deactivation

1.2.3.1. Phosphorylation as a regulatory molecular switch

Protein phosphorylation is ubiquitous and is the most common post-translational modification mechanism of protein function regulation known to date. Protein kinases represent 2% of the proteins encoded by the eukaryotic genomes (Hunter and Plowman, 1997). They must be tightly regulated and be very specific in the selection of their substrates. S6 kinase belongs to the family of AGC kinases, which includes cAMP-activated protein kinase (PKA), PKB/Akt, cGMP-activated kinase (PKG), PKCs, serum-glucocorticoid induced kinase (SGK), p70S6K, p90S6K and PDK1. AGC kinases display a high degree of primary sequence conservation within their kinase domains, which can be subdivided into 12 subdomains according to Hanks and Hunter (Hanks and Hunter, 1995) and also in the C-terminal kinase extension domain. Yet, AGC kinases mediate a large variety of different functions. PKB/Akt and p70S6K even share the same substrate recognition motif (RXRXXS/T), but in cells they still act highly specific towards their unique substrates.

High specificity can be achieved by means of interaction with substrates via specific kinase-substrate recognition motifs, via a scaffolding protein, via subcellular co-localisation or via molecular docking interactions (Biondi and Nebreda, 2003). A basal but insufficient degree of specificity is generated by kinase-specific recognition motifs, which are short segments of the primary sequence located around the phosphorylation site (Kemp *et al.*, 1975). Not all kinases, especially tyrosine kinases, have a recognition motif. Furthermore, experiments with the peptides have shown that the kinetics *in vitro* do not properly reflect the *in vivo* kinetics (Kemp and Pearson, 1991). It has become more and more apparent that, in addition to the interaction via the substrate recognition

motif, secondary interaction sites between kinase and substrate may exist which are termed docking sites. Docking sites were first shown for transcription factor c-Jun binding to its kinase JNK2 (Adler *et al.*, 1992). With respect to S6K it has been shown that S6K is specifically phosphorylated by PDK1 and mTor (or and mTor-dependent kinase) and that two motifs on S6K are responsible for specific recruitments to these kinases: the PIF motif and the TOS signalling motif.

1.2.3.2. Activation mechanism shared among AGC kinases

All protein kinases catalyse the same reaction, the transfer of the gamma phosphate of ATP to the hydroxyl group of serine, threonine or tyrosine. In spite of the enormous diversity of their physiological function, protein kinases share a conserved catalytic domain that is characterised by highly conserved amino acids located in 12 major conserved subdomains (Hanks *et al.*, 1988). Thus, upon activation kinases all adopt catalytically active conformations and are structurally similar. The main conserved structural kinase module comprised of two subdomains, the N-lobe and the C-lobe, is shown in Fig. 1.2.3. (based on the PKA structure). The N-lobe is composed of 5 stranded β-sheets and one α-helix called αC-helix. The bigger C-lobe is predominantly helical. The P-loop is an important module as it binds and coordinates the phosphate of the ATP. The peptide substrate binds at one end of the nucleotide-binding pocket, close to the γ-phosphate of the ATP. The activation loop, typically 20-30 amino acids long, helps to recruit the peptide. Phosphorylation of a threonine (T229 for S6K, T308 for PKB/Akt) in the activation loop stabilises it in a conformation that is permissive for substrate binding. Positioned in the beginning of the activation loop is the crucial DFG motif. The aspartate binds a Mg2+ ion that is critical for binding the β and γ groups of the ATP (Adams, 1993) and is considered the key catalytic residue. The catalytic loop (RDLKPEN) in the large lobe is essential for peptide binding and catalysis (Knighton *et al.*, 1991) The basic residue R must be correctly positioned as it holds the phosphorylated threonine of the activation loop in place.

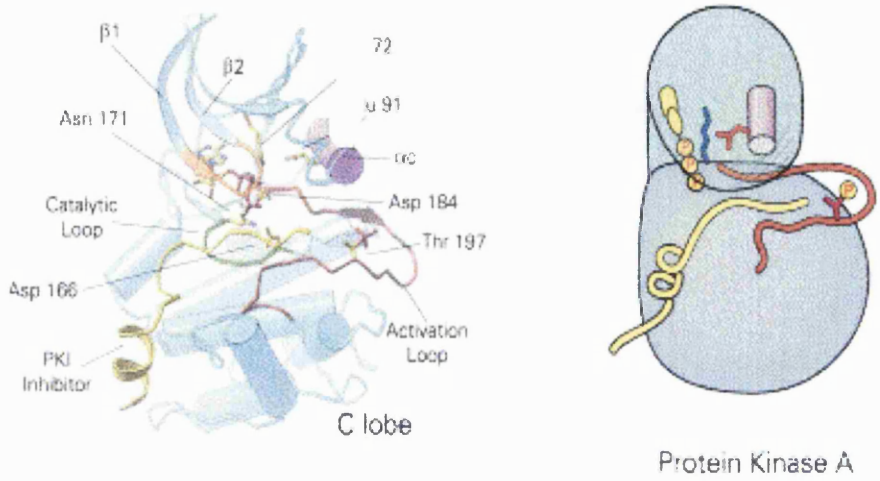


Fig. 1.2.3. The catalytically active conformation of PKA.
The left panel shows the crystal structure (as published by Zhen et al 1993) in a ribbon representation. Key structural elements are colored as follows: activation loop, red, αC-helix: purple, P-loop, orange and catalytic loop, green. In yellow the inhibitor peptide for PKA, called PKI is shown. The structure has been schematized on the right to clearly depict the major elements. Reproduced from Huse, M., and Kuriyan, J. (2002). Cell *109*, 275-282.

AGC kinases not only have strong sequence similarity in the amino acid sequence of their kinase domains, they also have a similar domain structure which consists of the kinase domains flanked by a hydrophobic motif (in the extension C-terminal to the kinase domain). Out of all AGC kinases, only PDK1 does not possess this motif. Within the hydrophobic motif lies a crucial phosphorylation site, which, when phosphorylated (i.e. by mTor) enables PDK1 to bind. Hence, PDK1 itself does not need such a site or indeed can afford to lack the whole hydophobic domain. Instead PDK1 has a PH domain C-terminally of the kinase domain, and all members of the PKB/Akt family possess this domain on the N-terminus. S6K is the sole AGC kinase with another domain C-terminally to the hydrophobic one, the autoinhibitory pseudosubstrate domain. All AGC kinases, with the exception of PDK1, have a similar mode of activation through phosphorylation of two key sites. Generally, but still controversially discussed, it is believed that first a threonine, or serine in the hydrophobic motif within the kinase extension domain becomes phosphorylated

followed by phosphorylation of a site located in the kinase activation loop. It is also known that phosphorylation of the site in the activation loop feeds back to the site in the hydrophobic motif and influences the efficieny of phosphorylation at this site.

Even though phosphorylation of the site in the hydrophobic motif is a common mechanism shared by AGC kinases, the kinase phosphorylating this site, has not been unambiguously identified for all AGC kinases. In the case of S6K, T389 is probably phosphorylated by mTor or a mTor-dependent kinase. Most members of the PKC family autophosphorylate the homologuous site (Cenni *et al.*, 2002), in novel PKCs this site seems to be under the control of mTor (Parekh *et al.*, 1999). Atypical PKCs have an acidic residue instead of a serine at the homologous site. In PKB/Akt this site may be subject to autophosphorylation and/or to action of other kinases. Very recently, DNA-PK, like mTor a member of Class 4 PI3K family, was purified from Hek293 cells as the activity towards S473 of PKB/Akt. The data could be strengthened by RNAi of DNA-PK, which inhibited S473 phosphorylation and DNA-PK deficient cells did not get phosphorylated at that site following insulin stimulation (Feng *et al.*, 2004). PKB/Akt is a family of kinases consisting of three isoforms, α, β and γ encoded by different genes. PKBα and β are ubiquitously expressed, and γ mainly found in the brain and testis. Interestingly, one splicing variant of the γ isoform does not possess the hydrophobic motif site indicating a different activation mechanism (Hanada *et al.*, 2004; Brodbeck *et al.*, 2001). The crystal structure of the kinase domain of active PKBβ/Akt (Yang *et al.*, 2002a) shows that phosphorylation of this hydrophobic motif site is required for an ordered conformation of the activation loop.

There is a plethora of data attributing a major role in growth regulation to PKB/Akt. Disruption of the most ubiquitously expressed isoform, PKBα, lead to smaller but viable mice. The cells have increased rates of apoptosis and die earlier when exposed to genotoxic stress (Chen *et al.*, 2001). However, in contrast to S6Kα knockout mice, they do not have a diabetic phenotype. Targeted gene disruption of PKBα lead to hypotrophy and structural abnormalities of the placenta (Yang *et al.*, 2003). PKB/Akt is involved in metabolism such as insulin signalling or glycolysis (Deprez, 1997), in the survival pathway for example by phosphorylation of Bad or of caspase 9 (Marte and

Downward, 1997; Cardone *et al.*, 1998), in enhancement of transcription via CREB (Du *et al.*, 1998) and in regulation of the cell cycle via phosphorylation of Mdm2 for example (Mayo and Donner, 2001).

PDK1 activates other AGC kinases such as RSK, PKA, S6K, SGK, PKB/Akt and PKC (Leslie *et al.*, 2001) by phosphorylation of the activation loop site. It therefore is a key enzyme in the insulin growth factor signalling pathway that lies downstream of PI3K. PDK1 was first found as the kinase responsible for T308 phosphorylation of PKB/Akt. PDK1 is constitutively active, but it is thought that docking interactions are important factors which determine when a substrate is phosphorylated. Interestingly, peptides derived from sequences around the activation loop site of various substrates are actually very poor *in vitro* substrates for PDK1, indicating that other structural determinants are required for full PDK1-mediated phosphorylation (Belham *et al.*, 1999). Within the hydrophobic motif of PDK1-substrates a PDK1 interacting fragment of approximately 76 amino acids was determined in PRK2 (protein kinase C-related kinase2) (Balendran *et al.*, 1999), termed the PDK1 interacting fragment, PIF. This conserved domain contains a stretch of amino acids, FXXFDY. In S6K the homologous sequence contains a threonine instead of the aspartic acid. This threonine represents the mTor phosphorylation site in the hydrophobic motif of the kinase extension domain. Peptide substrates, which encompass the hydrophobic motif sequence in addition to the PDK1 site, are much better substrates for PDK1 than short peptides only surrounding the phosphorylation site. Likewise, it was shown that prior phosphorylation of S6K at the hydrophobic motif site promotes its interaction with PDK1 and successively leads to enhanced activation loop phosphorylation (Biondi *et al.*, 2001).

The domain within the PDK1 kinase domain responsible for binding to PIF is termed PIF pocket. Mutation of leucine at position 155 to glutamic acid within the PIF pocket leads to a mutant that cannot phosphorylate S6K any more (Collins *et al.*, 2003).

However, the PIF pocket is not necessary for PKB/Akt activation. One explanation could be that PKB/Akt recruitment to PIP_3 via its PH domain leads to conformational change which is already sufficient for making it a suitable PDK1 substrate (Biondi *et al.*, 2001).

1.2.3.2. S6K activation mechanism

The activation of S6K consists of a complex series of phosphorylations. Altogether 10 or more sites are known to be phosphorylated by an array of independently regulated kinases (Weng *et al.*, 1998; Dennis *et al.*, 1998). In addition to the sites shared with other AGC kinases, it possesses many more sites, which makes the understanding of its regulation more complicitated. The autoinhibitory domain near the C-terminus for example adds four additional phosphorylation sites specific to S6Ks as indicated in Fig. 1.2.1.. The activation process can be divided into four main phases: Firstly, the presence of calcium primes S6K for activation (Hannan *et al.*, 2003). Calcium chelation reduces the overall levels of S6K phosphorylation. The fact that deletion of the last 104 amino acids renders the S6K mutant insensitive to calcium lead to the hypothesis that calcium may be necessary to release an inhibitory interaction between the N- and the C-terminus (Fig. 1.2.4). Secondly, a series of serines and threonines in the C-terminal autoinhibitory pseudosubstrate domain and carboxyterminal region juxtaposed to this sequence become phosphorylated (S411, S418, T421, S424 in S6Kα) by proline-directed kinases (Ferrari et al, 1992), which leads to a further change in S6K conformation. Numerous *in vitro* and *in vivo* studies indicate that this set of sites is phosphorylated by mitogen-activated kinases p42MAPK/p44MAPK, stress activated kinases and the cyclin-dependent kinase 2 (Papst *et al.*, 1998). Characteristic for these sites is the presence of a proline at +1 position and a hydrophobic residue at –2. Phosphorylation of these (Ser/Thr)-Pro sites helps in the activation process as shown by substitution with acidic residues. This enhances S6K activity modestly whereas mutation to alanines reduces it (Dennis *et al.*, 1998, Han *et al.*, 1995). S6Kα lacking the C-terminal 104 amino acids and, therefore, also the autoinhibitory domain, has low slightly lower basal activity, but normal activity when cells were serum-stimulated (Weng *et al.*, 1995b). This indicates that these phosphorylation events are not absolutely necessary for full S6K activity. In a third step, sites T389 and S404 within the hydrophobic linker domain become accessible for phosphorylation. These two sites are crucial for S6K activity (Alessi *et al.*, 1998; Pearson *et al.*, 1995). Finally, PDK1

phosphorylates T229 in the activation loop and hereby fully activates S6K (Pullen *et al.*, 1998; Biondi *et al.*, 2001; Balendran *et al.*, 1999).

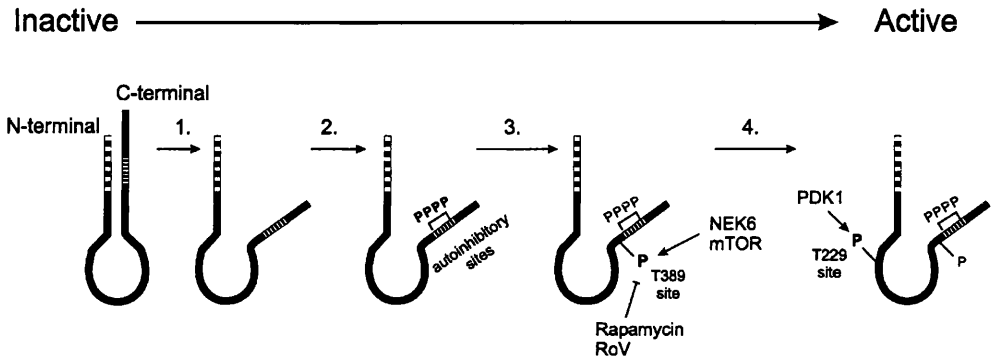


Fig. 1.2.4. Model of S6K activation via successive phosphorylation.
Four major steps are required to achieve maximal S6K activation: 1. Calcium priming; 2. phosphorylation of autoinhibitory pseudo-substrate region by proline-directed kinases; 3. phosphorylation of a threonine site in kinase extension domain by mTor or mTor-dependent kinase; 4. phosphorylation of a serine site in kinase domain by PDK1. See text for details. Reproduced from Hannan, K.M. *et al.* (2003). Biochem J *370*, 469-477.

Other sites in the kinase extension domain, T367, S371 and T447, which have a hydrophobic residue at -2 and a proline at +1 like the first set of sites, were also identified more recently. Out of the three, only one site, S371 was shown to be important for S6K activity (Moser *et al.*, 1997). *In vitro* studies and the fact that the T389 site is strongly rapamycin and less strongly wortmannin sensitive, suggest that mTor or an mTor-dependent kinase phosphorylate this site (Weng *et al.*, 1998; Isotani *et al.*, 1999). Mutation of this site to glutamic acid results in elevated T229 phosphorylation and, importantly, in a rapamycin-insensitive mutant (Dennis *et al.*, 1996; Pearson *et al.*, 1995; Pullen and Thomas, 1997). Besides, mTor was shown to phosphorylate T389 *in vitro* (Burnett *et al.*, 1998a; Isotani *et al.*, 1999). However, the identification of the T389 kinase still remains controversial. It has also been suggested that PDK1 may phosphorylate this site as well as the site in the activation loop *in vitro* and possibly also *in vivo* (Balendran *et al.*, 1999a). Other potential candidates for T389

kinases are NIMA-related kinases NEK6 and NEK7 which can activate S6K *in vitro* and *in vivo* (Belham *et al.*, 2001). Furthermore, it has been shown that T412 may also be a site of autophosphorylation (Romanelli *et al.*, 2002).

S6K contains a conserved five amino acid sequence in its very N-terminus (FDIDL), which is crucial for regulation by mTor, termed the TOS motif (Schalm and Blenis, 2002). When the whole motif was cut off or the phenylalanine mutated to alanine, the resulting S6K mutant lacked pT389 phosphorylation and was inactive, in a similar way as if the whole N-terminus had been truncated. This indicated that mTor must require this motif, which is also conserved in 4E-BP in the C-terminal region. For 4E-BP it was further shown that the TOS motif enables binding to raptor, a recently identified mTor binding protein (Schalm *et al.*, 2003; Hara *et al.*, 2002). It would be interesting to verify that S6K can recruit the Raptor/mTor complex in a similar way.

Much less controversy surrounds the identity of the kinase that phosphorylates the T229 site of S6Kα in the kinase domain, PDK1. Not only S6K but also other AGC kinases are phosphorylated by PDK1 at the homologous sites (Alessi *et al.*, 1998; Pullen *et al.*, 1998). Mutation of T389 to alanine strongly suppresses T229 phosphorylation. (Alessi *et al.*, 1998; Weng *et al.*, 1998). And substitution of T389 by an acidic residue leads to a higher basal T229 phosphorylation and enhanced activity (Dennis *et al.*, 1998). On the other hand, mutation of T229 to alanine also reduces T389 phosphorylation though less strongly, indicating a dependency in both directions. An S6K mutant where T389 and the four (S/T) P sites in the autoinhibitory pseudosubstrate domain have been changed to acidic residues is a very good PDK1 substrate. Taken together, these findings strengthen the hypothesis that T229 is the last phosphorylation to occur in the mitogen-induced S6K activation process.

Deletion mutants have been very useful in understanding the roles of specific domains in the activation process of S6 kinase. Truncation of the N-terminus (amino acids 2-46) leads to an inactive kinase, but this inhibition can be relieved by additional deletion of the C-terminus (the last 104 amino acids) and also mitogen-responsiveness can be restored (Weng *et al.*, 1995b). Deletion of the C-terminus alone yields an active kinase which is still wortmannin- and still, but to a lesser degree, rapamycin-sensitive (Weng *et al.*, 1995a; Weng *et al.*,1995b). These findings indicate that both domains are

important for activation and that the N-terminus may be needed to relieve the inhibitory effect exerted by the C-terminus.

1.2.3.4. Deactivation of S6K

While a lot of effort has been put into studying the activation process, considerably less is known about the deactivation of S6K. Two families of serine/threonine phosphatases, PP2A and PP1 have been found in complex with S6Ks (Bettoun *et al.*, 2002; Westphal *et al.*, 1999, Peterson *et al.*, 1999). PP2A has further been shown to be the major phosphatase responsible for the dephosphorylation and deactivation of S6K (Petritsch *et al.*, 2000). PP2A is a heterotrimeric protein complex consisting of a catalytic subunit (C), a scaffold protein (PR65 or A subunit) and one of many variable regulatory subunits (B). The B subunits are expressed differentially, control activity and specificity and target the holoenzyme to specific cellular compartments (Millward *et al.*,1999). Over the years, it has emerged that one of the major classes of PP2A substrates are protein kinases (Millward *et al.*, 1999). Interestingly, PP2A itself is inhibited by phosphorylation for example by Src upon stimulation with EGF, insulin or IGF1. PP2A was also shown to be under the negative control of mTor (Hartley and Cooper, 2002). The degradation of IRS1 correlates with its phosphorylation, which has been attributed to S6K activity (Harrington *et al.*, 2004) and is inhibited by rapamycin. Okadaic acid, which inhibits PP2A, also induces phosphorylation and degradation of IRS1. The fact that IRS1 degradation induced by okadaic acid could not be alleviated by rapamycin, suggests that the action of mTor in the degradation of IRS1 results from PP2A inhibition. Besides, mTOR can phosphorylate PP2A *in vitro* consistent with the model that this phosphorylation reduces PP2A activity and keeps S6K and 4E-BP in a phosphorylated state, whereas rapamycin or amino acid deprivation inhibit mTor's ability to restrain the phosphatase (Peterson *et al.*, 1999).

PP2A has been shown to associate with wild type S6K and the C-terminally truncated mutant but not with the rapamycin–insensitive N- and C-terminally deleted form of S6K. It was concluded that PP2A must bind via the first 46 amino acids (or 23 amino acids when taking the shorter isoform into consideration) of the N-terminus, because

when only the C-terminus is deleted the kinase is still rapamycin-sensitive and therefore the factor that binds to S6K in order to reduce its activity, presumably PP2A, must bind via the N-terminus of S6K (Peterson *et al.*, 1999).

1.2.4. S6 phosphorylation and function of S6 Kinases

1.2.4.1. S6 as major substrate and translational upregulation

The main known physiological substrate of S6 Kinases is the 40S ribosomal phosphoprotein S6. Phosphorylation occurs at sites S235, S236, S240, S244 and S247 located at the carboxy-terminus of the protein (Bandi *et al.*, 1993; Krieg *et al.*, 1988). S6 phosphorylation has been shown to correlate with translational upregulation of certain mRNAs. In cells where S6Kα and S6Kβ have been knocked out, two sites S235 and S236 which are the two sites to be phosphorylated first upon mitogenic stimulation, remain phosphorylated and insensitive to rapamycin indicating that another kinase, probably p90RSK may phosphorylate these two sites whereas S6K phosphorylates the following sites (S240, S244, S247) which are thought to be crucial for the regulation of translation initiation and also ribosomal biogenesis. The following findings support this hypothesis. In yeast (S.Cerevisae) the last three sites do not exist and there is no regulation of translation of ribosomal proteins. PKA has also been shown to phosphorylate the first two sites but fails, unlike S6K, to stimulate translation in a reconstituted system (Kozma *et al.*, 1989).

S6 protein is positioned at the interface of the 40S subunit and the large 60S subunit of the eukaryotic ribosome, in a region where mRNA and tRNA binding as well as association of many initiation and elongation factors occurs (Nygard and Nilsson, 1990). S6 protein, due to its position within the ribosome, may modulate the interaction of mRNAs with the ribosome and may be involved directly in the initation process of translation (Proud, 1992). S6 protein could furthermore be crosslinked to mRNA in

functional 80S ribosomes, indicating that there may be a direct interaction (Jefferies and Thomas, 1996).

It has been shown that S6K activity and phosphorylation of S6 correlate closely with the translational upregulation of a subset of mRNAs, termed 5'-TOP mRNAs due to an oligopyrimidine-rich tract at their 5' end (Jefferies *et al.*, 1997). The 5'-TOP sequence contains a cytosine followed by 6 pyrimidines at the 5'-terminus of mRNAs and is conserved in all mRNAs encoding ribosomal proteins and a number of elongation factors or proteins involved in the translational machinery. mTor inhibitor rapamycin abolishes S6K activity and reduces the translation of mRNAs encoding some ribosomal proteins S3, S6, S14 and S24, translation elongation factors (eEF1A and eEF2) and others, which all contain 5'-TOP elements. Rapamycin does not affect the mRNA translation of a variety of nonribosomal proteins, which were tested (Terada *et al.*, 1994, Jefferies *et al.*, 1994). It was further shown that a dominant negative allele of S6K was as efficient in blocking 5'-TOP mRNA translation as rapamycin (Jefferies *et al.*, 1997; Schwab *et al.*, 1999). And a rapamycin-insensitive S6K mutant was able to rescue the effect of rapamycin. S6K is activated by mTor, which also phosphorylates the binding partner of the eukaryotic translation initiation factor eIF4E, termed eIF-4E BP (Hara *et al.*, 1997). Upon phosphorylation by mTor the eIF4E-BP releases the translation factor, which leads to an increase in m^7G cap-dependent translation initiation. Therefore, activation of mTor by growth factors or nutrients increases a cell's capacity for protein biosynthesis via two complementary pathways, one leading to cap-dependent translation, the other to 5'-TOP–dependent translation.

How does S6 phosphorylation by S6K mediate 5'-TOP mRNA translation? So far, the mechanism has not been clearly elucidated. It has been found that ribosomes that contain phosphorylated S6 proteins are engaged in translation as polyribosomes to a greater extent than ribosomes in which S6 is not phosphorylated. Terada and co-workers reported that rapamycin inhibited the polysomal association of the affected mRNAs (Terada *et al.*, 1994). Very recently it was published that RNAi for S6Kα in a breast cancer cell line (MDA-MB-453) lead to a reduced translation of Cyclin D1 in response to FGFR and ErbB2 activation (Kozicak and Hynes, 2004). All these

observations hint that binding of mRNA to ribosomes may be enhanced when S6 protein is phosphorylated.

Lately, the necessity for S6K in 5'-TOP mRNA translation was questioned. In ES cells where S6 is constitutively unphosphorylated due to disruption of S6K, retinoic acid-induced translation of 5'-TOP mRNAs in non-dividing cells still occurs (Stolovich *et al.*, 2002; Tang *et al.*, 2001). In the S6Kα(-/-) S6Kβ(-/-) knock-out mice the translation of 5'-TOP mRNAs remains rapamycin-sensitive (Pende *et al.*, 2004). This further suggests that another rapamycin-sensitive enzyme/s might be involved in the translational upregulation of these mRNAs or that the regulation of their translation may be cell-type specific.

1.2.4.2. S6K and ribosomal biogenesis

The correlation between S6K activity and 5'-TOP mRNA translation not only means that more proteins are produced and hence a cell can accumulate mass, but also that a cell increases its overall translational capacity by the elevated production of mature ribosomes. Ribosomal biogenesis plays an important role in growth and proliferation and is tightly regulated at the transcriptional and translational level and at the stage of assembly of its many protein and rRNA counterparts. All ribosomal RNAs, with the exception of 5S, are transcribed as a polycistronic precursor rRNA (45S rRNA) by RNA Polymerase I in the nucleolus. The rRNA is then processed, most importantly by conversion of uridine to pseudouridine with the help of small nucleolar RNAs (snoRNAs). This modified transcript is subsequently cleaved to produce mature rRNAs, 18S, 5.8S and 28S (Larson *et al.*, 1991). More than 70 ribosomal proteins must assemble with the rRNA in the nucleolus to form the small and the large ribosomal subunits (40S and 60S). Ribosomal proteins are important in holding the structure together and are involved in co-ordinating the interaction between the ribosome, mRNA, the initiation and elongation factors. These particles are then exported via the nuclear pores into the cytoplasm where the mature ribosomes are formed.

Deletion of specific ribosomal proteins leads to a reduction in the number of polyribosomes and a defect in cellular growth. In Drosophila, mutations in one of the

ribosomal genes can generate the "minute" phenotype with strongly reduced body size and diminished fertility (Kongsuwan *et al.*, 1985). This is caused by a reduced number of ribosomes. In the same context it is important to note that conditional deletion of the ribosomal S6 protein in mice results in defective ribosomal biogenesis associated with reduced cell proliferation (Volarevic *et al.*, 2000).

S6 protein is one of the first proteins to be incorporated into the 45srRNA complex (Jefferies and Thomas, 1996) in the nucleoli. It has been demonstrated that, in addition to the cytoplasmic ribsosomal S6 protein, a nuclear and a nucleolar pool of S6 protein are phoshorylated upon hormone stimulation (Franco and Rosenfeld, 1990). Recently, it was shown that growth factors mainly stimulate S6 phosphorylation in the cytosol and nucleoli which matches the localisation of ribsomal subunits (Pende *et al.*, 2004). It is possible that the nuclear isoforms of S6K (both S6Kβs and the long isoform of S6Kα) are responsible for phosphorylation of the nucleolar pool of S6. However, to our knowlegde, the presence of S6Ks in nucleoli has not been explicitly described. Yet, it could be imagined that phosphorylation of S6 protein may not only stimulate binding of 5'TOP mRNAs and the initiation complex formation, but also may have a regulatory function for ribosomal biogenesis in the nucleoli.

Recently, it has been reported that S6K is involved in ribosomal biogenesis by enhancing 45S ribosomal gene transcription (Hannan *et al.*, 2003). rRNA synthesis is a major rate limiting step in ribosomal biogenesis and cellular growth and is tightly linked to cell cycle progression. Maximal rRNA synthesis occurs during S and G2 phases, almost none during M phase. The transcription factor UBF (upstream binding factor) is a key regulator for rRNA transcription (Cavanaugh *et al.*, 1995). It has been shown that phosphorylation of UBF upregulates rRNA synthesis and that a non-phosphorylatable UBF mutant leads to a decrease in rRNA synthesis and cell growth (Voit *et al.*, 1999). Phosphorylation at multiple sites enhances UBF's DNA binding capacity and its ability to participate in an active initiation complex (Kihm *et al.*, 1998, Tuan *et al.*, Voit and Grummt, 2001). A hypophosphorylated UBF for example is transcriptionally inactive (O'Mahony *et al.*, 1992; Voit *et al.*, 1992).

RNA Polymerase I, the polymerase that is responsible for rDNA transcription, binds to the same rDNA promoter as UBF and is regulated by UBF (Cavanaugh *et al.*, 1995). Casein kinase II or CDK4-cyclin D and CDK2-cyclin E were shown to phosphorylate UBF (Voit *et al.*, 1992). Furthermore, in response to EGF, ERK1/2 were shown to phosphorylate UBF. This finding places ribosomal transcription under the direct control of extracellular growth signals (Stefanovsky *et al.*, 2001). Recent evidence has suggested that S6K may participate in the regulation of UBF (Hannan *et al.*, 2003). Phosphorylation of UBF in the carboxy-terminal activation domain was rapamycin-sensitive and could be restored by the expression of a rapamycin-insensitive S6K. Taking into account that ribosomal biogenesis is a prerequisite for cellular growth, this finding attributes S6K a role in growth regulation independently of translation.

1.2.4.3. Cell cycle progression

In addition to the role of S6K in the regulation of translation and ribosomal biogenesis, it has been postulated that S6Kα is also involved in promoting cell cycle progression through G1. The first indication came from the inhibitory effect of rapamycin on the cell cycle. Chung and co-workers could show that in 3T3 fibroblasts a delay into S phase correlated with a block of phosphorylation and activation of S6Kα (Chung *et al.*, 1992). Next, it was shown that microinjection of neutralising antibodies against cytoplasmic p70S6Kα into the cytosol reduced S6K activity and lead to cell cycle arrest in G1 or a delay of entry into S phase depending on the cell type (Lane *et al.*, 1993). The same effect was found when antibodies against the nuclear p85 S6Kα isoform were microinjected into the nucleus but not into the cytoplasm (Reinhard *et al.*, 1994). Furthermore, it has been demonstrated that TGF-β, a physiological suppressor of cell cycle progression, induces G1 arrest through phosphatase PP2A-mediated inhibition of S6Kα (Petritsch *et al.*, 2000).

However, it is still unclear by what mechanism S6K influences cell cycle progression. As S6K upregulates ribosomal biogenesis by S6 phosphorylation (and 5'-TOP mRNA translation), the simplest explanation could be that a sufficient number of mature ribosomes is a requirement for cell cycle progression. The hypothesis that S6

phosphorylation is needed for cell cycle progression is consistent with studies reporting that conditional deletion of rS6 in the liver of adult mice results in abrogated 40S ribosome biogenesis and inhibited progression of the cell cycle at the G1/S transition (Volarevic *et al.*, 2000). The liver cells also failed to induce cyclin E expression after partial hepatectomy. Hence, rS6 protein or 40S ribosomal biogenesis may induce a checkpoint that is necessary for cell cycle progression.

S6 kinase may also promote cell cycle progression by controlling the transcriptional activity of E2F as shown in interleukin-2-stimulated T lymphocytes (Brennan *et al.*, 1999). E2F stimulates the transcription of many genes, which are involved in cell cycle regulation, such as cyclin E (Botz *et al.*, 1996). PI3 kinase activity is necessary for IL2-induced regulation of transcriptional E2F activity, which is inhibited by rapamycin. Expression of a rapamycin–resistant mutant of S6K in T-cells could restore rapamycin-suppressed E2F responses. Hence, S6K is able to regulate E2F's transcriptional activity and therefore controls the G1 checkpoint in T-cells.

The role of S6K in cell cycle regulation *in vivo* was again questioned by the double knockout S6Kα(-/-)S6Kβ(-/-). MEFs derived from these mice did not have altered doubling times (Pende *et al.*, 2004).

1.2.5. Other S6K substrates and potential functions

The recent report by Pende *et al.*(Pende *et al.*, 2004*)* suggested that S6 protein is not the major substrate for S6Kα. Knock-out studies have shown that in S6Kβ(-/-) cells S6 phosphorylation is strongly reduced whereas in S6Kα(-/-) cells there are no obvious changes. This finding indicates that S6 protein may not be the major substrate for S6Kα as it cannot compensate for the lacking S6Kβ activity in the S6Kβ(-/-) cells. It is possible to imagine that S6 kinase α exerts some effects via other, yet to be identified novel substrates. Since S6Ks have a similar substrate specificity motif as PKB/Akt, it is likely that novel S6K substrates may be found which are currently assigned to PKB/Akt. Almost all of the up to 50 substrates so far identified for PKB/Akt have a

minimal consensus of RXRXXS/T, which matches the sequence around the GSK3 phosphorylation site (Obata *et al.*, 2000).

The structure of active PKB/Akt kinase domain has been solved previously by X-ray crystallography (Yang *et al.*, 2002a; Yang *et al.*, 2002b). The authors used a constitutively active PKB/Akt mutant (S474D), a non-hydrolysable ATP analogue, AMP-PNP, and a substrate peptide derived from GSK3 (GRPRTTSFAE). PKB/Akt residues that form the GSK3 substrate binding sites are conserved in S6Ks (but also PKA, PKC and p90RSK) which provides a structural rationale for the potentially overlapping substrate specificities. Below, an alignment of the key residues in the activation segment that interact with the GSK3 peptide between PKB/Akt and S6K is given revealing the strong sequence conservation. The top line represents the peptide amino acids, which are complexed by the kinase residues.

Peptide	-5	-5	-5/-2	-5	-3	-3	+1	+1	+2
PKB	F_{238}	S_{242}	E_{279}	E_{342}	E_{236}	D_{440}	F_{310}	L_{317}	F_{359}
P70S6K	F_{170}	E_{174}	E_{211}	D_{274}	E_{168}	D_{372}	F_{242}	M_{249}	M_{291}

The numbering for PKB/Akt is based on PKBβ and the numbering for S6K is based on the nuclear long isoform of S6Kβ. Reproduced from Yang *et al.*, 2002a.

It may be that *in vivo* S6K has substrates, which are currently assigned to PKB/Akt. This possibility can be tested by studies using rapamycin as an inhibitor for mTOR/S6K but not for PKB/Akt, and LY or wortmannin acting on both, S6K and PKB/Akt. Furthermore, the fact that S6K is activated by amino acids (see chapter 1.2.4) whereas PBK/Akt needs mitogenic activation via PI3K might help to distinguish between the activities of the two kinases *in vivo.* Indeed, some such substrates have already been identified. Work in our laboratory shows that a serine site of MDM2, a protein involved in the cell cycle and p53 pathway, is an *in vivo* substrate for S6K (at least on one of the two sites) rather than for PKB/Akt (Wang, M.L., personal communication).

A published example is GSK3 (glycogen synthase 3), a well-described PKB/Akt susbstrate that is inactivated by phosphorylation. The branched amino acid leucine has been shown to activate S6K via both PI3K and mTor, however PKB/Akt and MAPK were not involved in the signal transduction. LY294002 and rapamycin both reduced

S6K activity upon stimulation with leucine. Concomittant with the S6K activation, GSK3 becomes inactivated (Peyrollier *et al.*, 2000).

One residue found to be a S6K *in vitro* and *in vivo* substrate is S136 of the pro-apoptotic Bad (Harada *et al.*, 2001). It is also a substrate for p90RSK but on another site (S112) (Bonni *et al.*, 1999) and for PKB/Akt on the same site (S136) (Datta *et al.*, 1997). Phosphorylation of this site inhibits Bad's pro-apoptotic activity by enhancing its affinity of Bad to 14-3-3, which sequesters it into the cytosol where it cannot induce apoptosis. By binding to 14-3-3, other members of the BCL-2 family with anti-apoptotic activity such as BCL-X_L or BCL-2 are freed and can exert their function.

It has been demonstrated that S6Kα can phosphorylate CREMτ, a transcriptional transactivator leading to the upregulation of genes controlled by CREs (cAMP responsive element) upon adenylyl cyclase activation (de Groot *et al.*, 1994). Phosphorylation of serine 117 is also mediated by PKA and correlates with an increase in CREMτ-mediated transactivation. Co-expression of S6Kα significantly stimulates transcriptional activity of a Gal-CREM fusion protein and this effect is rapamycin sensitive.

Another potential substrate of S6Kα is the 80kDa RNA cap-binding complex (CBP80) (Wilson *et al.*, 2000). This phosphorylation is growth factor dependent and rapamycin-sensitive. S6Kα was also shown to phosphorylate CBP80 *in vitro*. CBP80 is a subunit of the CBC complex (nuclear RNA cap binding complex) which plays a key role in an early step of RNA splicing, pre-mRNA splicing, snRNP production and polyadenylation by binding to the m^7G cap structure of RNAs. CBC is activated downstream of the small GTPase cdc42 in a rapamycin-dependent manner. It was therefore suggested that a cdc42-S6K pathway may be involved in the regulation of RNA splicing, mediated by an increase in capped RNA binding by the CBC. A connection between cdc42 and S6K had previously been made when it was reported that rac and cdc42 could activate S6K (Chou *et al.*, 1996)– an effect that could be abrogated by the expression of dominant negative alleles of cdc42 and rac. In addition, in this publication, a complex could be shown between S6K and GTP bound rac and cdc42.

SKAR, a nuclear protein which is potentially involved in pre-mRNA splicing and mRNA export is phosphorylated on two rapamycin-sensitive serine sites (S383 and S385) by S6Kα but not S6Kβ upon insulin-stimulation. RNAi-mediated reduction of SKAR leads to a reduction in cell size, however to a lower extent than RNAi of S6Kα (Richardson *et al.*, 2004).

One potential substrate which S6K shares with the p90 RSK is elongation factor 2 kinase (Wang *et al.*, 2001). eEF2K phosphorylates and inactivates eEF2. Elongation factor 2 mediates the translocation step of elongation (Redpath *et al.*, 1996). Both, S6K and p90RSK, in contrast to other tested AGC kinases, readily phosphorylate GST-eEF2K *in vitro*. Insulin induces inactivation of the eEF2K and thus dephosphorylation of eEF2, and these effects are blocked by rapamycin. As insulin-like growth factor does not activate Erk and hence does not activate p90RSK in Hek293 cells, it could be shown that S6K activity was needed for phosphorylation of a conserved serine (S366) residue of the eEF2K leading to inactivation of eEF2K. Alternatively, stimuli such as TPA, which activate the Erk pathway, were used to prove the involvement of p90RSK in the eEF2K phosphorylation. As TPA also induces S6K activation, rapamycin and MEK inhibitor PD184352 were employed. Rapamycin had almost no effect whereas the PD inhibitor reduced the phosphorylation of S366. This underscores the role of S6K in the regulation of translation but shows that the effect may be mediated by a different mechanism rather than the more intensely studied initiation of 5'-TOP mRNA translation.

S6K is also linked to translation initiation via another substrate, the eukaryotic translation initiation factor 4B (eIF4B). It was recently demonstrated that serine 422 is a serum-responsive, wortmannin and rapamycin-sensitive phosphorylation site necessary for the ability of the protein to stimulate a helicase that unwinds mRNA– a necessary step for translation initiation. A rapamycin resistant S6K mutant confers rapamycin resistance to this site (Raught *et al.*, 2004). Thus, more evidence is accumulating which places S6K in the regulation of translation independently of S6 protein phosphorylation.

Taken together, in addition to its role in translational initiation, ribosomal biogenesis and cell cycle progression, S6 kinase is potentially implicated in the regulation of

transcription, RNA processing and translational elongation. A schematic of the potential functions and mediators is depicted in Fig 1.2.5.

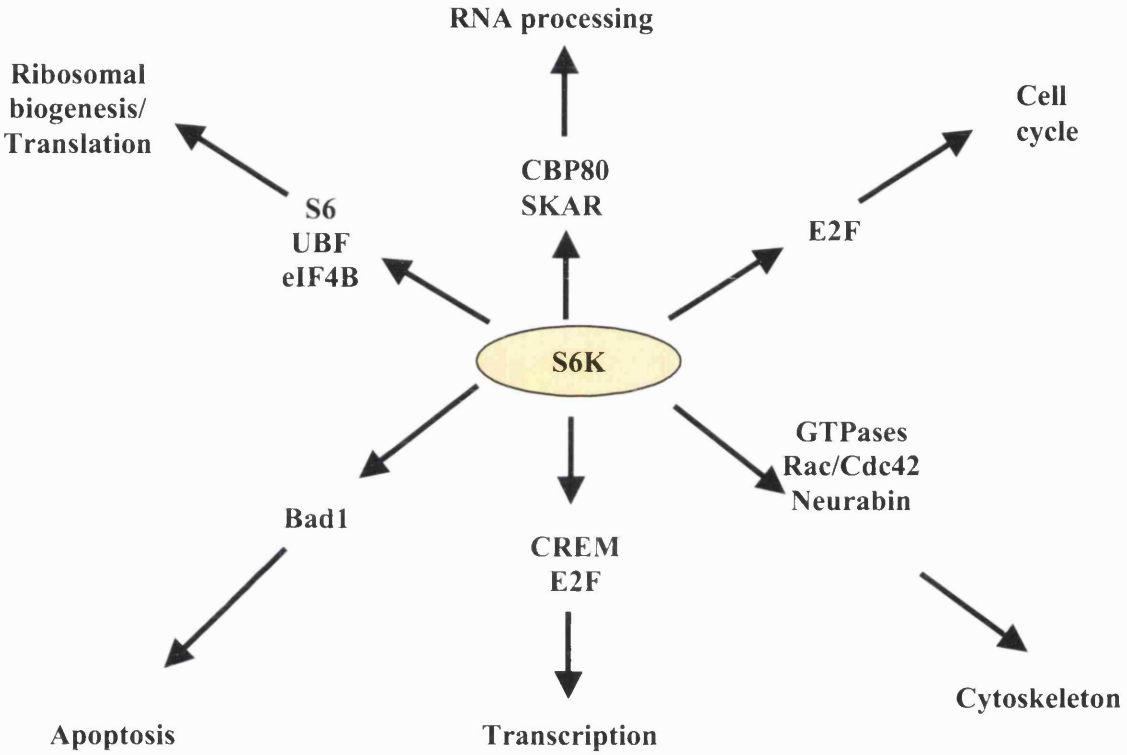


Fig. 1.2.5. Cellular substrates and functions of S6K.
Via its various substrates or binding partners S6K can influence different cellular processes. See text for details.

1.2.6. Deregulation of S6K signalling in animal models and disease

S6 becomes phosphorylated when quiescent cells are stimulated. The level of phosphorylated S6 increases in proportion to the level of protein synthesis. It has been shown that rapamycin strongly reduces phosphorylation of S6 protein and that this can be rescued by the overexpression of a rapamycin-insensitive S6K. To determine the

function in a physiological context the study of knockout animals is of great value. Furthermore deregulation of the S6K pathway occurring in certain pathological conditions reveals the function within a complex organ system.

1.2.6.1. Knockout models

Drosophila deficient in the species' only isoform, dS6K, exhibit an extreme delay in development and reduced body size. This effect was attributed to a smaller cell size rather than reduced cell number. The first S6K mouse knockout to be made was the S6Kα(-/-) mouse. The animals are viable but exhibit a strong reduction in body size (up to 20%) during embryogenesis (Fig. 1.2.6). This effect is largely overcome during adulthood (Shima *et al.*, 1998).

The S6Kβ knockout homozygous mice have been generated very recently (Pende *et al.*, 2004). They are viable and show no obvious phenotype. Contrarily to the smaller S6Kα(-/-) animals, they rather tend to be larger at later stages of development and during the postnatal period. In contrast, the double knockout S6Kα(-/-)/S6Kβ(-/-) mice (Fig. 1.2.6) were born at lower frequencies than expected. One third of the mice were born dead. Among the ones born alive the majority developed cyanosis and 50% died shortly after birth. The few surviving ones produced offspring with high perinatal lethality. In terms of growth rates, the double knockouts had similar growth rates as the S6Kα knockouts indicating that S6Kβ is not involved in the regulation of growth and proliferation. Surprisingly, in the S6Kα knockout MEFs, the level of phospho-S6 was not reduced in comparison to the wild type MEFs upon serum stimulation. S6Kβ expression was upregulated in these animals, suggesting a functional redundancy between the two isoforms. S6 protein was still phosphorylated in the S6Kβ knockouts, however to a markedly lesser extent than in S6Kα(-/-).

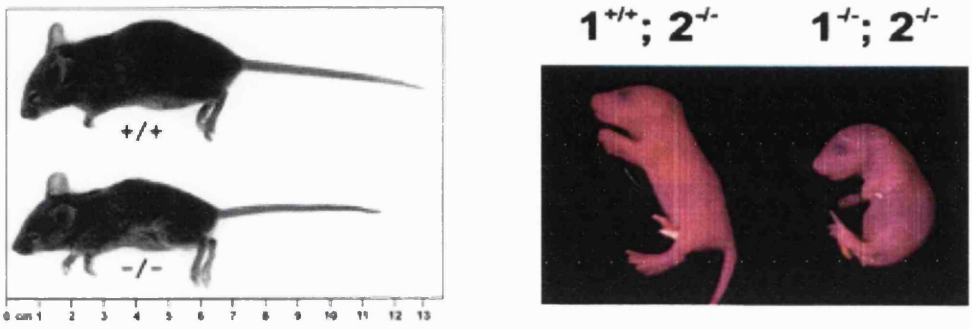


Fig. 1.2.6. S6K knockout mice.
The left panel shows the S6Kα or S6K1 knockout, the right panel the S6Kβ or S6K2 and the double knock-out. Left: Shima, H. *et al.* (1998). Embo J *17*, 6649-6659. Right: Pende, M. *et al.* (2004). Mol Cell Biol *24*, 3112-3124.

Rapamycin treatment lead to a reduction of phospho-S6 levels in both cell types, confirming that S6Kα, though much less efficiently can compensate for the lack of S6Kβ in cells. In the double knockout cells phosphorylated S6 (pS6) levels were strongly reduced and were at the same level as in rapamycin-treated cells. This indicated that S6Kα and β are the sole or major rapamycin-sensitive S6 kinases. The residual S6 phosphorylation after rapamycin treatment could be abolished by MAPK pathway inhibitor PD184352 which makes p90RSK a strong candidate kinase for the remaining S6 phosphorylation. 2D-PAGE analysis of the phosphoprotein S6 showed that S235 and S236 are still phosphorylated when both S6K isoforms are depleted, however phosphorylation of S240, S244, S247 disappears. One pool of S6 is localised in the nuleoli where ribosomal biosynthesis takes place and the other, bigger pool is in the cytosol as part of the 40S subunit. Therefore, a phospho-S6 blot from a whole cell lysate would mainly reflect the state of cytosolic S6 phosphorylation. In addition, three out of the four isoforms of S6K are predominantly nuclear. In order to investigate a potential difference in phosphorylation of specific S6 pools, fractionation of nuclear and cytosolic proteins was performed. However, a similar pattern of phospho-S6 phosphorylation for the different knockout hepatocytes was seen in both compartments.

One major question in need of clarification was the involvement of S6Ks in the regulation of 5'TOP mRNAs translation. The mRNA for EF1alpha was used as 5'TOP mRNA. Upon serum stimulation, the mRNA was recruited to a similar extent into polysomes in the wild type an in the double knockout MEFs. Rapamycin was able to abolish its translation in the double knockout cells. The same results were obtained for embryonic stem cells (ES) as these cells previously yielded contradictory results in terms of 5'TOP translation. Pende's findings suggest that another rapamycin-sensitive enzyme may be required for 5'TOP translation.

Cardiac hypertrophy is an important risk factor for cardiac morbidity and mortality (Levy *et al.*, 1990). A key feature of cardiac hypertrophy is increased protein synthesis and it has been suggested that S6 or phospho-S6 may play an important role in the stimulation of protein synthesis in the heart. In isolated cardiac myocyte models of hypertrophy, rapamycin inhibited protein synthesis (Sadoshima and Izuma, 1995). Rapamycin inhibits hypertrophy of cultured neonatal rat cardiac myocytes, but it also inhibited cardiac hypertrophy induced by pressure overload in intact rats (Boluyt *et al.*, 2004). Transgenic mice overexpressing either constitutively active PI3K or IGF1 receptor displayed cardiac hypertrophy and this was paralleled by enhanced S6K activity and S6 phosphorylation. In order to test if S6 kinases affect heart size, cardiac specific S6Kα and S6Kβ transgenic mice were generated (Mc Mullen *et al.*, 2004). It could be determined that overexpression of S6Kα and a rapamycin-resistent isoform of S6Kα induced a modest degree of cardiac hypertrophy (heart weight/body weight ratio was elevated by 10-15% compared to wild type mice at three months of age) whereas overexpression of S6Kβ did not yield an obvious cardiac phenotype. When the transgenics were treated with rapamycin the heart weight/body weight ratio of the S6Kα mice and S6K activity were reduced and similar to that of the wild type mice. When rapamycin was given to the rapamycin resistant transgenics the heart weight/body weight ratio was still slightly reduced. More interestingly, S6 phosphorylation was fully abolished. These results confirmed the findings of Pende *et al.*, that there may be and additional S6 kinase. Also, in the S6Kβ transgenics S6

phosphorylation is not elevated. This could mean that there is a maximal phosphorylation that is already achieved by endogenuous levels of S6Kβ.
In order to induce cardiac hypertrophy, mice can either be subjected to a pathological stimulus (pressure overload) or a physiological stimulus (chronic exercise such as swimming). Unexpectedly, deletion of either S6K isoform in knockout mice had no impact on the heart weight/body weight ratio when mice were subjected to hypertrophy-inducing stimuli. Overexpression of dominant negative PI3K in transgenics lead to reduced heart size. When these mice were crossed with S6Kα transgenics, S6K activity was rescued but not the heart size. Neither had crossbreeding of S6Kα knockout or S6Kα/β double knockouts with IGF1 receptor or PI3K transgenics an effect on the hypertrophy induced by IGF1 receptor and constitutively active PI3K (McMullen *et al.*, 2004).

Taken together, studies with knockout animals revealed that *in vivo* S6Kβ plays the major role in S6 phosphorylation and that a MAPK-dependent S6 kinase, probably p90RSK, is also involved in this process. In the S6Kα(-/-)/S6Kβ(-/-) knockout mice, the translation of 5'TOP mRNAs is not altered in comparison to the wild type mice and is still sensitive to rapamycin. S6K may therefore be not involved in 5'TOP mRNA translation or another kinase can compensate for the lack of both S6K isoforms. It had previously been shown that ectopic expression of the rapamycin-resistant form of S6K could rescue the rapamycin–induced reduction of 5'TOP mRNA translation (Jefferies *et al.*, 1997). To resolve this contradiction one could think of a scenario in which the ectopic but not endogenous S6K phosphorylates an enzyme involved in 5'TOP mRNA translation.

Finally, body size does not correlate with the extent of S6 phosphorylation. These data raise the interesting possibility that S6Ks also exert their specific functions via substrates other than S6 protein.

1.2.6.2. Diabetes Mellitus

The S6Kα(-/-) mice have a phenotype that resembles type 2 diabetes mellitus. This condition is generally characterised by imbalanced glucose homeostasis caused by either one of or a combination of the three following parameters: resistance to insulin action, impaired insulin action and deregulated insulin secretion (Kahn, 1998). S6Kα knockout mice in comparison to wild type mice exhibit hyperglycemia when injected with glucose and a sharply reduced glucose-induced insulin secretion. The isolated muscle cells from these animals, however, did not exhibit insulin resistence (Pende *et al.*, 2000). It was shown that the problem of glucose intolerance was caused by a 30% reduction of insulin content in the pancreas. The origin was that the size of the β cells which form the islets of Langerhans are reduced in mass and size, but not in number (Pende *et al.*, 2000). Such a reduction in cell size (up to 20%) was shown reduce insulin secretion by up to 40% (Giordano *et al.*, 1993). Very recently, it has been demonstrated that S6Kα(-/-) knock-out mice are protected from obesity when fed on a high-fat diet (Um *et al.*, 2004). After four months of high-fat nutrition the knockout mice heightened their body fat index by 20%, but the wild type by 100%. As previously mentioned, the S6Kα (-/-) knockouts do not become insulin resistant as shown by autophosphoylation of the insulin receptor and this is in contrast to wild type mice. S6K has been shown to negatively regulate insulin signalling by repression of IRS1 and via direct phosphorylation of IRS1 (Harrington *et al.*, 2004; Shah *et al.*, 2004). This finding was confirmed in the S6Kα(-/-) mice where phosphorylation of IRS1 (S636, S639, S307) was also reduced. Phosphoserine 473 of PKB/Akt was enhanced when compared to the wild type mice on a high fat diet, which is a good indicator that signalling downstream of PI3K was restored by loss of S6Kα. Similar results were obtained when S6Kα was downregulated by RNAi. Thus, nutritionally or genetically driven obesity leads to upregulation of S6Kα activity, which may in turn suppress PI3K signalling, contributing to insulin resistance and obesity.

1.2.6.3. Involvement of the S6K pathway in tumorigenesis

Tumour development proceeds via a process in which a succession of genetic changes, each conferring another type of growth advantage, leads to the progressive conversion of normal cells into cancer cells. Hanahan and Weinberg proposed that carcinogenesis involves DNA changes leading to alteration in six aspects of cell physiology that collectively dictate malignant growth (Hanahan and Weinberg, 2000).

1.Self-sufficiency in growth signals: Normal cells require growth signals to proliferate. Tumour cells, however, show a greatly reduced dependence on exogenous growth stimulation by expressing and responding to a panel of growth factors.

2. Insensitivity to growth-inhibitory signals: Within a normal tissue, multiple anti-proliferative signals operate to maintain cellular quiescence and halt the cell cycle. The Retinoblastoma (RB) protein plays a crucial role in cell cycle progression by sequestering E2F transcription factor, which controls the expression of S-phase genes (Weinberg, 1995). In many cancers, the RB pathway is not functional.

3. Evasion of apoptosis: The programme for cell death is intrinsic to all cells, but cancer cells have acquired a resistance to apoptosis, for example by the loss of the function of tumour suppressor protein p53 in over 50% of human cancers.

4. Limitless replicative potential: After a certain number of doublings, cultured cells enter the stage of senescence, which can be circumvented by cancer cells (Hayflick, 2003). Telomeres, the chromosome ends, are shortened with each replication due to the inability of DNA polymerase to replicate the 3' ends of the DNA. At a limiting point of telomere length, cells loose the ability to protect their DNA ends which then fuse, leading to an abnormal karyotype and cell death. Cancer cells however, have intact telomeres and elevated levels of telomerase, a DNA polymerase, which adds hexanucleotides to the ends of telomeric DNA (Shay, 1997). They can therefore proliferate endlessly.

5. Sustained angiogenesis: For survival of cells the oxygen and nutrient supply through a closeby blood vessel is crucial. The ability of tumour cells to induce angiogenesis may be achieved by their ability to change the balance between angiogenesis inducers

and inhibitors (Hanahan and Folkman, 1996), for example by enhanced expression of VEGF (vascular endothelial growth factor) or FGF (fibroblast growth factor).

6. Tissue invasion and metastasis: Metastasis underly 90% of human cancer deaths (Sporn 1996). Cell–cell adhesion molecules (CAMs) which mediate cell-to-cell interactions and integrins which mediate adhesion to the extracellular matrix have been shown to play a role in promoting metastatic growth. The function of E-cadherin, a member of the CAM family, is often lost in epithelial cancers (Christofori and Semb, 1999). Changes in expression of integrins and metalloproteases (which degrade the matrix) are also found in metastatic cells. This may facilitate detachment of cells and invasion into the nearby stroma (Coussens and Werb, 1996; Kleiner and Stetler-Stevenson, 1999).

One could imagine S6Kinases to be involved in the development of some of these hallmarks. Even if deregulation of S6K alone probably cannot override cell cycle check points and other growth regulatory control points, it may act synergistically with other molecules in influencing Weinberg's parameters 1 and 2: self–sufficiency in growth signals and insensitivity to growth-inhibiting signals. S6K also has been shown to phosphorylate and inactivate the pro-apoptotic protein Bad, hinting to a potential role in modulating the third hallmark, evasion of apoptosis. PKB/Akt is an important member of the survival pathway and it regulates S6K via Tsc and mTor.

As there is no published data on the oncogenic potential of S6K, a few strong indicators of the involvement of S6K in cancer will be brought together in the following chapter.

1.2.6.3.1. Ribosomal biogenesis and cancer

It has been known for almost 30 years that the rate of cell growth and proliferation is proportional to the rate of protein synthesis (Baxter and Stanners, 1978). In the last years it has become clear that deregulation of protein synthesis can affect tumorigenesis. Alterations in the expression of ribosomal proteins have been observed in cancer cells lines and primary tumours (Ferrari *et al.*, 1990; Loging and Reisman, 1999; Kondoh et al, 2001).

A number of proto-oncogenes and tumour suppressors have recently been shown to directly regulate ribosome production or protein translation (Ruggero and Pandolfi, 2003). For example, the tumour suppressor RB regulates the activity of RNA Polymerase Pol I (which synthesises rRNA in the nucleolus) and Pol III (which synthesises tRNAs and small stable RNA ribosome components). Furthermore, transcription factor c-Myc, which is frequently deregulated in tumours, acts as a regulator of ribosomal biogenesis and translation control.

Two syndromes shall be mentioned here which link ribosomal dysfunction to cancer. Dyskeratosis congenita is a condition in which modification of ribosomal RNA (the conversion of uridine into pseudo-uridine) is distorted (Ruggero *et al.*, 2003). This modification is thought to be important for mRNA and tRNA binding to the ribosome and therefore could imply impaired translation of specific mRNAs. Clinically, this results in a variety of symptoms such as premature aging, bone-marrow failure but also enhanced cancer susceptibility.

A second condition where ribosome function is compromised is called Diamond-Blackfan anaemia and is characterised by general growth retardation, anaemia and increased susceptibility to hematopoetic malignancies (da Costa *et al.*, 2001). This disease has been associated with a mutation in the ribosomal protein S19 (Draptchinskaia *et al.*, 1999). It is plausible to hypothesise that a mutation in the human S19 protein could lead to a downregulation of the translation of a specific set of mRNAs in a similar way as the S6 protein specifically upregulates the translation of 5'TOP mRNAs. One could imagine for example, that mRNAs encoding tumour suppressors may be translated to a lesser extent in this condition (Ruggero and Pandolfi, 2003). However, no research has been performed to tackle this question.

Similarly, by enhancing the translation of ribosomal proteins more ribosomes are produced and the cell is ready for growth and proliferation. Many ribosomal proteins are overexpressed in a variety of solid and hematopoetic tumours. It is imaginable that this may be due to enhanced S6K activity and enhanced S6 phosphorylation. Proteins with a 5'-TOP tract on their mRNA which are under the control of S6K are for example elongation factors 1A1 and 2 (or eEF1A1 and eEF2) and several other proteins which

are involved in either ribosomal biogenesis or translational control (Terada *et al.*, 1994; Jefferies *et al.*, 1997). The initiation factor eEF2 has been found amplified in ovarian tumours and acts as oncogene in fibroblast and xenograft tumour models (Anand *et al.*, 2002). Hence, S6K may be tumorigenic by enhancing ribosomal biogenesis and regulating translation of initiation or elongation factors.

S6K was further shown to have a positive effect in rDNA transcription by phosphorylating the rDNA transcription factor UBF (see chapter 1.2.4.2). In this context, it is worth noting that the RB tumour suppressor not only restricts the transcriptional activity of E2F (Classon and Harlow, 2002) but also of UBF (Hannan *et al.*, 2000). RB is frequently mutated in human tumours and this alleviates the tumour cells from transcriptional inhibition. Similarly, p53 was shown to inhibit Pol I activity and rRNA synthesis. Compared to wild type cells, p53 null cells exhibit increased Pol I activity. P53 acts by preventing complex formation on the rRNA promoter (Zhai and Comai, 2000). Both, RB and P53 also have an inhibitory effect on Pol III which is the polymerase responsible of rtRNA and small ribosomal rRNAs (Larminie *et al.*, 1997; Cairns and White, 1998).
Some indication of the effects of increased ribosome synthesis comes from work in Drosophila. Homozygous mutants for the Brat gene (brain tumour), which encodes a protein regulating rRNA synthesis, have malignant brain tumours (the brain is enlarged up to eight times), bigger cells and nucleoli. In C. elegans the homologue gene to brat was shown to negatively regulate Pol I and III and brat could rescue the mutant phenotype (Frank *et al.*, 2002). However, much more work needs to be done to clearly establish the link between ribosomal biogenesis and cancer.

1.2.6.3.2. S6K expression is upregulated in breast cancer

Gene amplification plays an important role in the progression and initiation of many solid tumours, including breast cancer and is often linked to poor prognosis. The long arm of chromosome 17 is most frequently affected and it contains two independently amplified regions, q21 and q22-24. The region q22-24 is amplified in about 20% of

breast cancer (Barlund *et al.*, 1997). It was found that this region, 17q23, comprises the gene for S6Kα (Couch *et al.*, 1999). Further, it was shown that S6K is amplified and highly overexpressed in MCF7 cells when compared to normal mammary epithelium, and that protein expression and activity are also increased (Barlund *et al.*, 2000). In 9% of analysed primary breast tumours, S6K was amplified. This and amplification of the 17q21 linked oncogene, HER-2/c-ErbB2 which is overexpressed at high levels in 20% of breast cancers, both imply poor survival (Gullick and Srinivasan, 1998).
Furthermore, S6K has been found to be overexpressed in papillary thyroid cancer (Miyakawa *et al.*, 2003), pancreatic, lung and ovarian cancer. However, it remains to be established, if S6K overexpression is a consequence of or a part of the many events causing transformation.

1.2.6.3.3. S6K is part of major cancer pathways

S6K is regulated by a variety of proto-oncogens and tumour suppressors, such as PI3K, Src, PTEN and Tsc1/2 and may therefore be a common downstream target which could lead to unregulated growth. The PTEN tumour suppressor gene is mutated in many malignancies. Germline mutations of PTEN cause a variety of syndromes (i.e.Cowden syndrome, Lhermitte-Duclose syndrome, Proteus syndrome) which all share the tendency to develop benign tumours. Cowden Syndrome in addition leads to the development of malignant tumours (Nelen *et al.*, 1996). Somatic mutations, particularly the loss of heterozygosity of the chromosome 10q where the PTEN gene is located, have been increasingly found in a variety of malignant tumours such as glioblastoma or endometrial carcinoma. PTEN is mutated in approx 50% of these tumours (Risinger *et al.*, 1997; Tashiro *et al.*, 1997). Furthermore melanoma, ovarian, cervical and prostate cancer had frequent mutations in PTEN underscoring the importance of this tumour suppressor. The tumour suppressor PTEN directly downregulates the PI3 Kinase pathway, and also S6K activity via mTor. In human tumour cell lines in which PTEN is lost, rapamycin leads to a reduction in cell size.
Furthermore, S6K activity is negatively regulated by the tumours suppressor termed tuberous sclerosis complex Tsc1/2. In tumours, which carry mutations in Tsc1 or 2,

S6K activity is strongly enhanced and correlates with hyperphosphorylation of S6 and increased cell proliferation (Goncharova *et al.*, JBC 2002). Tuberous sclerosis is an autosomal dominant disorder that affects 1 in 6000 individuals and is characterised by widespread development of benign tumours, termed hamartomas, of brain, eyes, skin, heart, lungs and kidneys which can lead to mental retardation, autism and an are accompanied by an increased risk of renal cell carcinoma.

The bacterial macrolide rapamycin is a specific inhibitor of mTor. Rapamycin is already successfully used in the clinic and helps to prevent the rejection of allografts due to its capacity to inhibit T-cell activation. Rapamycin binds to the immunophillin FK506 binding protein, FKBP12, which in turn binds mTor and inhibits its activity (Abraham, 1998). In low nanomolar doses, rapamycin is able to arrest cells in G1 phase of the cell cycle (Heitman *et al.*, 1991). In recent years rapamycin or its analogues (CCI-779, RAD001, AP23573) have come into focus as anti-cancer drugs and are currently tested for a variety of tumours. Transformation by PI3K or PKB/Akt correlates with mTor and S6K activation in primary cultures of chicken embryo fiboblasts. Rapamycin partially prevents the growth rates and colony formation in soft agar during v-Src transformation (Penuel and Martin, 1999). Rapamycin further reduces tumour proliferation and size in PTEN(-/+) mice and this effect was attributed to S6K downregulation, but it has no effect on tumours where PTEN activity is intact (Neshat *et al.*, 2001). As PTEN, PKB/Akt and TSC pathways all converge in mTor, downstream molecules S6K and eIF4E have emerged as interesting candidates for drug targeting.

Rapamycin analogues are currently in clinical trials for acute leukemia or non-Hodgkin's lymphoma, endometrial cancer, glioblastomas and possibly more (www.clinicaltrials.gov/). Particularly, renal cell carcinoma have become a target for rapamycin-based clinal trials. It has also been shown that PI3K, mTor and PKB/Akt are all activated downstream of VEGFR (Yu and Sato, 1999) and rapamycin might therefore be an anti-angiogenetic drug. Rapamycin was also tested in breast cancer cells lines. Although the effect of the drug could be reduced by RNAi of S6K, the level of S6K- and 4EBP-posphorylation is not a prognostic factor for rapamycin sensitivity.

This showed that rapamycin or mTor may have other yet to be identified targets in cells (Noh *et al.*, 2004).

1.3. PDGF RECEPTOR SIGNALLING

In multicellular organisms an elaborate intercellular network has developed that coordinates growth, differentiation and metabolism of a multitude of specialised cells. Communication can be exerted directly from one cell to the adjacent cell via specialised junctions in the plasma membrane or via specific receptors on the surfaces or in the cytoplasm of the receiving cell. Signalling can be endocrine and mediated by hormones, which reach their targets via the blood stream or paracrine where the signal is addressed to the adjacent cells. Autocrine signalling means that a cell responds to a signal that is released by itself. Hormones or other signalling factors can either diffuse across the plasma membrane, bind to receptors in the cytosol or the nucleus (which is the case for lipophilic hormones such as steroids) or they can bind to cell surface receptors which mediate the signal into the cell. There are four major classes of cell surface receptors:

1. Ion channel receptors: ligand binding changes the conformation of the receptor so that specific ions can flow through. 2. G-protein-coupled receptors recruit trimeric G-proteins. This either leads to the generation of second messengers and activation of signalling cascades or to the modulation of ion channels. 3. Tyrosine kinase linked receptors (i.e. for cytokines) lack an intrinsic kinase activity but form dimers upon ligand-stimulation and activate cytosolic tyrosine kinases. 4. Finally, receptors with intrinsic enzymatic activity, in most cases receptor tyrosine kinases (RTKs). When active, RTKs phosphorylate tyrosine residues of their own cytoplasmic domain or of other proteins (Lodish *et al.*, 1999).

1.3.1. Mechanism of receptor tyrosine kinase signalling

1.3.1.1. Receptor activation

Ligands for RTKs are usually soluble peptides or proteins including for example nerve growth factor (NGF), platelet-derived growth factor (PDGF), insulin, insulin-like growth factor-1 (IGF1), fibroblast growth factor (FGF) or epidermal growth factor (EGF). Upon ligand binding to the extracellular domains of RTKs, they dimerise, phosphorylate the adjacent receptor molecules, recruit and activate a variety of signalling molecules such as PI3K, Src, GAP and many others via their SH2 or PTB domains. Because these domains are highly specific in their recognition motifs, an activated RTK can trigger its responses via a well-defined network of cascades.

One layer of complexity is added by a mechanism called transactivation. Activated G-protein coupled receptors (GPCRs) can induce EGFR (epidermal growth factor receptor) activation for the propagation of mitogenic signals (Fischer *et al.*, 2003). Recent studies have also shown that not only EGFR but also PDGFR, neurotrophin and fibroblast growth factor receptors can be transactivated via GPCRs (Shah and Catt, 2004). But not only can GPCRs signal via RTKs, the reverse mechanism in which RTKs signal to GPCRs has also been observed (Mira *et al.*, 2001). In this report, IGF1 induces specific transactivation of the G_i-coupled chemokine receptor CCR5.

Two major deactivation pathways of an activated RTK exist. First, protein tyrosine phosphatases, PTPs, such as for example SHP2, dephosphorylate the receptor molecules. Secondly, receptor ubiquitination leads to downregulation, which involves ligand-induced endocytosis and subsequent degradation by the proteasome.

1.3.1.2. Phosphotyrosine recognition modules

SH2 domains are modules of approximately 100 amino acids that bind to specific phosphotyrosine containing peptide motifs (Pawson *et al.*, 2001). They have a conserved pocket that recognises phosphotyrosine and a more variable pocket that binds 3-6 residues C-terminal to the pY and confers specificity. However, some SH2

domains also recognise amino acids N-terminal of the pY site. Binding specificities were deduced from an oriented phosphopeptide library (Songyang *et al.*, 1993). The ideal peptide sequence for recognition by the Src SH2 domain could hereby be defined as pYEEI. Moreover, structural studies confirmed that this peptide fits well into an active Src structure (Waksman *et al.*, 1993).

Like SH2 domains, phosphotyrosine binding (PTB) domains also bind to phosphotyrosine containing sequences. They comprise 100-150 amino acids and commonly bind to NPXpY motifs. In contrast to SH2 domains, PTB domains have high specificity on the basis of sequences amino-terminal to the phosphotyrosine (Songyang and Cantley, 1995) Examples for PTB domain binding to a receptor tyrosine kinase are, for example, IRS1 binding to insulin receptor (motif LYASSNPEpY) and Shc docking protein binding to TrkA nerve growth factor receptor (IINPQpY).

The enzyme binding to an activated RTK very often is a kinase itself (such as Src, Yes, Fyn, PI3K) but it can also be an adaptor protein such as Grb2 which can recruit other molecules via its two SH3 domains or a scaffold/docking protein such as Shc which has one SH2 and one PTB domain and can be phosphorylated itself in order to bring for example Grb2 into a complex (Pawson and Scott, 1997). Furthermore, phosphatases such as SHP2 and transcription factors (such as STATs) can bind to the activated receptors and transmit the signal. Hence, one growth factor ligated to its receptor can trigger a variety of pathways leading to different cellular
responses (Pawson *et al.*, 2001).

1.3.2. Receptor tyrosine kinases involved in tumorigenesis

The erbB family is an important family of proto-oncogenes consisting of the epidermal growth factor receptor (EGFR or c-erbB1), c-erbB2 (or Her-2/neu), c-erbB3, c-erbB4 and their ligands EGF, heregulin, neu differentiation factor and gp30, to only mention a few. These RTKs and their ligands have been implicated in the development of breast cancer. Two of the most important intracellular pathways activated by ErbB receptors are those involving MAPK and PI3K (Yarden and Sliwkowksi, 2001). For example p85

of PI3K binds to a YXXM motif on ErbB3 receptor and activates the PI3K pathway. The adaptor shc binds to NPXY or Y1309 of ErbB3 to activate the MAPK pathway (Prigent and Gullick, 1994). EGFR itself does not contain the YXXM motif, yet can activate the PI3K pathway. To this end, it was found that upon ligand-induced EGFR stimulation, ErbB3 can recruit p85 subunit of PI3K in certain cells (such as A431) (Soltoff *et al.*, 1994) as PI3K was found to co-precipitate with ErbB3. Thus it was proposed that ErbB3 in relation to EGFR may act like IRS1 in relation to the insulin receptor.

ErbB receptors play a major role in the development of the mammary gland and therefore it comes as no surprise that overactive ErbB receptors are found in 25-30 % of human breast cancers and that this overexpression is linked to poor prognosis (Slamon *et al.*, 1987).

Many more receptor tyrosine kinase, when they are mutated, overexpressed or co-expressed with their ligands, have been linked to cancer. C-Met, the receptor for scatter factor of hepatocyte growth factor is overactive in renal papillary carcinomas (Danilkovitch-Miagkova and Zbar, 2002). In various types of cancer elevated levels of vascular endothelial growth factor (VEGF) have been detected which leads to elevated VEGF receptor activity and angiogenesis (Kleespies *et al.*, 2004). The role of PDGFR in cancer will be discussed in chapter 1.3.4..

1.3.3. PDGFR and downstream molecules

Platelet-derived growth factor (PDGF) was first identified in the search for serum factors that stimulate the proliferation of arterial smooth muscle cells (Ross *et al.*, 1974) and was found to be involved in the embryonic development, cell cycle regulation, angiogenesis, wound healing, fibroblast proliferation and migration (Heldin and Westermark, 1999).

There are two different platelet-derived growth factor receptors, α and β, which are, despite their high similarity in structure and function, encoded on different chromosomes. The receptors also function as homo- or heterodimers and have different

binding affinities to the ligands. There are four ligands, PDGFA to D (of a length of approx. 100-150 amino acids). All PDGFs function as secreted, disulfide-linked homodimers. PDGFA and PDGFB can also form functional heterodimers AB (Hoch and Soriano, 2003). PDGFA and B are both cleaved and activated by proteolytic processing before secretion, whereas PDGFC and D are first secreted and then processed by yet to be identified extracellular proteases (Heldin and Westermark, 1999). Knock-out mice have been generated for both receptors and for the two ligands PDGFA and PDGFB and lead to the conclusion that PDGFA and PDGFRα are more broadly required for embryogenesis, especially in the nervous system and organ development, whereas PDGFB and PDGFRβ are mainly important for the vasculature (Hoch and Soriano, 2003).

Each receptor molecule has five immunoglobulin repeats in the extracellular ligand-binding domain and the tyrosine kinase domain in the cytoplasmic region. Similarly to other receptor tyrosine kinases, PDGFRs dimerise upon ligand-binding. The juxtaposition of the kinase domains of two dimerised receptors allows phosphorylation in trans between the receptors on several tyrosine residues in the receptor cytoplasmic domain, also referred to as autophosphorylation. This leads to increased kinase activity and creates docking sites for downstream signal transduction molecules. One site in the kinase domain (i.e. Y857 for PDGFRβ) is essential for the kinase activity, mutation to phenylalanine strongly reduces it (Fantl *et al.*, 1989). The other autophosphorylation sites are spread over the cytoplasmic domain of the receptor. In total, 11 out of 15 tyrosine residues in the cytoplasmic domain of PDGFRβ become phosphorylated and at least 10 different molecules are recruited as shown in Fig. 1.3.1. and table 1.3.2. (in the case of PDGFRβ).

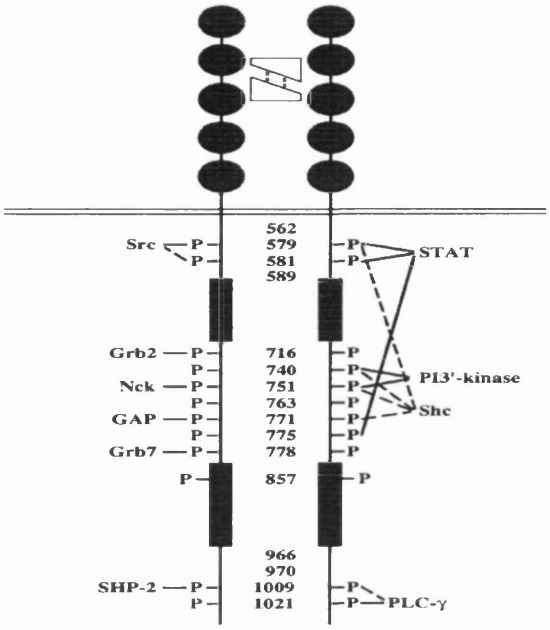


Fig. 1.3.1. Illustration of the interaction between PDGFβ R and downstream molecules.
Ig-like domains in the extracellular part are illustrated by filled circles. Tyrosine residues known to be auto-phoshorylated are indicated with P. The specificity of the interaction between SH2 domain molecules and the phosphotyrosines is indicated. Solid lines indicate high-affinity and broken lines interactions of lower affinity. Reproduced from Heldin, C.H. (1997). FEBS Lett *410*, 17-21.

Molecule	Class of molecule			Role in signalling
		PDGFα R	PDGFβ R	
Src, Yes, Fyn	Cytoplasmic tyrosine kinase	Tyr572, Tyr574	Tyr579, Tyr581	Mitogenicity
PI3-kinase	Lipid kinase	Tyr731, Tyr742	Tyr740, Tyr751	Mitogenicity, chemotaxis
RasGAP	GTPase-activating protein	No binding	Tyr771	Mitogenicity, chemotaxis
SHP-2	Tyrosine phosphatase	Tyr720, Tyr754	Tyr763, Tyr1009	Mitogenicity, chemotaxis
PLC-γ1	Lipase	Tyr988, Tyr1018	Tyr1009, Tyr1021	Mitogenicity, chemotaxis
Grb2	Adapter	No binding	Tyr716	Mitogenicity, chemotaxis
Nck	Adapter	No binding	Tyr751	?
Grb10	Adapter	?	Tyr771	Mitogenicity, chemotaxis?
Shc	Adapter	No binding	Tyr579, Tyr740, Tyr751	Mitogenicity, chemotaxis
Grb7	Adapter	No binding	Tyr775	?
Crk	Adapter	Tyr762	No binding	?
Stat5	Transcription factor	?	Tyr579, Tyr581, Tyr775	

Table 1.3.2. SH2 domain-containing signalling molecules binding to PDGF receptors
See text for details. Reproduced from Ronnstrand, L. and Heldin, C.H. (2001). Int J Cancer *91*, 757-762.

Both receptors can activate many of the same major signal transduction pathways such as Ras-MAPK, PI3K and PLCγ pathways. However, the array of interacting proteins differs slightly for PDGFRαα and ββ which leads to different *in vivo* responses (Rosenkranz and Kazlauskas, 1999; Klinghoffer *et al.*, 2001).

One class of molecules that bind to PDGFR are members of the Src family of tyrosine kinases, which bind to pY579 with high and pY581 with lower affinity (numbering for PDGFRβ) (Mori *et al.*, 1993). Src then becomes activated and phosphorylates its substrates, which also include the PDGFR itself (Heldin, 1997). As depicted in Fig. 1.3.1.and 1.3.2, the PI3K pathway is activated by binding of the p85 regulatory subunit of PI3K to the pY740/751 sites of the receptor (McGlade *et al.*, 1992). Phospholipase Cγ binds to pY1021 and pY1009 in the C-terminal tail and becomes activated (Roennstrand *et al.*, 1992). This leads to the cleavage of $PI(4,5)P_2$ into diacylglycerol (DAG) and IP_3, Ca^{2+} mobilisation from intracellular stores and activation of the classical and novel PKC families. Protein tyrosine phosphatase SHP2 binds to pY1009 and is activated by the binding (Valius *et al.*, 1993). This subsequently can lead to receptor dephosphorylation. The complex consisting of the adaptor protein Grb2 and the nucleotide exchange factor Sos1 can bind directly to pY716 of the PDGFRβ (Arvidsson *et al.*, 1994). As a consequence, Ras is recruited and converted from an inactive GDP-bound form to an active GTP-bound form. A GTP-bound Ras activates the MAPK pathway. Ras, in complex with Grb2/Sos, can furthermore be activated by binding to the adaptor molecule Shc which is also recruited to the activated receptor sites pY771, pY751 and pY740 (Salcini *et al.*, 1994). Alternatively, Grb2/Sos1 and ras can be recruited to the receptor by binding to SHP2 (Li *et al.*, 1994). An activated PDGF receptor can even bind and phosphorylate transcription factors of the STAT family which are mainly activated by cytokine receptors (Silvennoinen *et al.*, 1993). They interact with tyrosines pY579, pY581 and pY775, however the significance of this activation in cells remains to be determined. It is not known how many SH2 domain containing proteins can bind simultaneously to a dimerised receptor.

Crosstalk between the different pathways has been observed: some PI3K responses are mediated by Ras. Both molecules have been found to interact with each other

(Rodriguez-Viciana *et al.*, 1994; Hu *et al.*, 1995). Ras can activate Rho and Rac downstream of PI3K (Qiu *et al.*, 1995; Khosravi-Far *et al.*, 1995). Src can activate PKCs downstream of PLCγ. These are only a few examples to illustrate that a very complicated network of pathways exists.

To help to disentangle this network a set of PDGFR signalling mutants has proven helpful. These were point mutants in which specific tyrosine residues in the receptor cytoplasmic domains have been mutated to phenylalanines. The mutations disrupt the receptor's interaction with cytoplasmic signalling molecules and can abrogate one subset of receptor function (Heuchel *et al.*, 1999; Tallquist, 2000). Conversely, another approach has used complete mutation of all tyrosine sites in the cytoplasmic domain of PDGFR and "adding back" tyrosines one by one.

1.3.4. Deregulation of the PDGFR pathway

PDGFRs play a critical oncogenic role in a broad spectrum of hematological and solid tumours. In certain malignancies, characteristic genetic alterations have been identified which cause constitutive activation of PDGF receptors. The PDGFRα gene is amplified in some types of gliomas (Fleming *et al.*, 1992; Kumabe *et al.*, 1992). In certain cases of chronic myelomonocytic leukaemia the PDGFRβ gene is fused with other genes, which leads to its dimerisation and constitutive activation (Golub *et al.*, 1994). In addition to this selection of mutations, upregulation of PDGFR and its ligand are also well-documented (Ostman and Heldin, 2001). Dominant negative forms of receptors or ligands have shown to reduce the growth of glioma cells in culture.
A member of the same family of RTKs (class III) is Kit. Like PDGFR, it is involved in a multitude of cellular responses such as differentiation, proliferation, growth, survival, adhesion, chemotaxis and is highly critical in hematopoesis (Linnekin, 1999). A variety of Kit gain-of-function mutations were linked to cancer such as leukaemia or gastrointestinal tumours. One frequently occurring mutation is Kit D816V where an aspartate close to the kinase DFG motif is mutated to valine. This mutation renders Kit

constitutively active. In addition, this mutation alters the substrate specificity (Piao *et al.*, 1996). In many gastro-intestinal stromal tumours (GISTs) constitutive activation of Kit was found and this was often associated with gain-of-function mutations in Kit which are mainly localised in the juxtamembrane region responsible for dimerisation (Rubin *et al.*, 2000). Interestingly, in GISTs where Kit is not mutated, PDGFRα is mutated (Heinrich *et al.*, 2003). One mutation, D842V, is homologous to the well-described Kit D816V mutation (Hirota *et al.*, 2003). The overlap in the phenotypes of cells with PDGFRα and Kit mutations indicates the similar *in vivo* functions of the two different tyrosine kinases. Likewise, Kit and PDGFRβ have been observed to be expressed in a number of solid tumours including small cell lung cancer, prostate, ovarian, breast cancer and thyroid cancer.

Therefore, both tyrosine kinases are very promising candidates as drug targets. Indeed, the tyrosine kinase inhibitor Imatinib (STI571, Gleevec by Novartis) is being tested in a variety of tumours, which are linked to deregulated PDGFR activity. Kit and its ligand stem cell factor (SCF) are co-expressed in 40-70% of small cell lung cancer specimens and inhibition of Kit by Imatinib (STIO571, Gleevac) abrogates small cell lung cancer cell growth. Imatinib was first developed to inhibit Bcr-Abl and Abl by competitively binding to the ATP binding pocket with high affinity, preferentially when the kinase is in an inactive conformation. But during the development, the drug was also found to affect PDGFR and Kit (Krystal, 2004). It already proved successful in the treatment of chronic myelogenous leaukemia (CML), which are philadelphia chromosome positive (where the Bcr-Abl fusion generating translocation has occurred). There is also strong evidence for treatment of patients with GISTs, which are highly resistant to radiotherapy and conventional chemotherapy (Sawaki *et al.*, 2004). However, Kit mutations conferring drug resistance were already reported (Tamborini *et al.*, 2004), such as the D816V mutant, which does not bind Imatinib (Frost *et al.*, 2002).

1.4. SRC KINASE SIGNALLING

Src was the first oncogene to be discovered. In 1911, Peyton Rous reported that a microscopic particle, later shown to be a virus, could induce sarcoma (malignant tumours of connective tissues) in chicken (Rous, 1911). Later on, Src was found to be the transforming factor of Rous sarcoma virus. On a molecular level, this was confirmed by the identification of a temperature-sensitive mutant of Src. This mutant only failed to transform cells at the non-permissive temperature. When transformed cells infected with the temperature sensitive mutant of the virus were shifted to the non-permissive temperature they reverted to a normal phenotype. This indicated that Src was not only necessary for malignant transformation but also for the maintenance of this state (Martin, 1970).

1.4.1. Src family of tyrosine kinases: structure and activation

1.4.1.1. Src family of kinases and tissue distribution

Src is recruited via its SH2 domain to many receptor tyrosine kinases and plays a major role in mediating cell growth and division, motility and adhesion, angiogenesis and transformation (Frame, 2002). The Src family of tyrosine kinases consists of nine members with a similar primary sequence and structure. Src, Fyn and Yes are expressed in most tissues, while Blk, Yrk, Fgr, Hck, Lck, Lyn are mainly detected in hematopoetic cells (Courtneidge, 2002). Amongst them, Fgr is only expressed in monocytes, granulocyes and macrophages. However, Src has been the major focus of interest as it has been primarily implicated in the development of human cancer.

1.4.1.2. Structure and regulation of Src family kinases

All members of the Src kinase family share a similar modular structure as shown in Fig. 1.4.1. In addition to the kinase domain (also termed SH1 domain), they all have a SH2 domain, a SH3 domain, a N-terminal variable region where myristylation or palmitylation occurs and a flexible tyrosine containing tail (Abram and Courtneidge, 2000; Brown and Cooper, 1996). The SH2 domain binds phosphotyrosine containing peptide sequences, the SH3 domain to proline-rich regions, and the N-terminus due to its post-translational modifications is responsible for membrane recruitment. The activity of the kinase is regulated by protein-protein interactions (involving the above described domains) and by phosphorylation. The related tyrosine kinase Csk phosphorylates and inactivates Src at a site in the C-terminus. This phosphotyrosine acts as an intramolecular binding site to Src's own SH2 domain, hereby maintaining an inactive conformation of the kinase. A phenylalanine mutant to this site (Y527F) is constitutively active. Dephosphorylation of the Y527 site (numbering for the avian gene, 529 for the human gene) activates Src. It is unclear how the tyrosine kinase Csk is regulated. There is evidence that it may primarily be regulated on the level of expression: Csk levels are reduced in liver carcinoma when compared to normal liver tissue and this correlates with enhanced Src kinase activity (Masadi *et al.*, 1999). In addition, overexpression of Csk suppresses the metastasis of colon cancer as shown in mouse colon adenocarcinoma cells (Nakagawa *et al.*, 2000). However, the tumorgenicity of these cells is not affected.

Src can be kept in an active open conformation by binding of FAK and p130CAS via the SH2 and SH3 domains (Schaller *et al.*, 1994; Thomas *et al.*, 1998). In order to be fully active an autophosphorylation site, Y416 in the avian gene (and Y418 in human gene) must also be phosphorylated. Furthermore, the SH3 domain can interact with a linker sequence between the SH2 and the kinase domain, hereby also contributing to the negative regulation. Taken together, activation of Src can be achieved by dephosphorylation of the C-terminal site, by phosphorylation of the tyrosine site within the kinase domain and by displacement of internal interactions via higher affinity ligands.

The tyrosine phosphatase PTP-1A has been shown to dephosphorylate the Y527 site *in vitro* and *in vivo* (Zheng *et al.*, 1992; Su *et al.*, 1999), however other phosphatases, notably SHP1 have also been proposed to positively regulate src activation. A dominant-negative SHP1 mutant can inhibit Src dephosphorylation and kinase activity (Somani *et al.*, 1997). Furthermore, phosphatase PTP1B which is overexpressed in some breast cancer cells, was purified from breast cancer cells and capable of dephosphorylating a phosphotyrosine-527 containing peptide (Bjorge *et al.*, 2000).
One additional level of Src regulation is its stability: When Src is unphosphorylated at the Csk-dependent site it is more prone to ubiquitination and degradation by the proteasome (Hakak and Martin, 1999).

1.4.1.3. c-Src and v-Src

In 1976, it was shown that v-Src encodes an oncogene which was almost identical to a normal human gene (Stehelin *et al.*, 1976). v-Src was found to be a cytosolic tyrosine kinase with uninhibited, unregulated activity (Hunter and Sefton, 1980). The major difference between c-Src and v-Src is in the C-terminal region and a series of point mutations throughout the genes (Takeya *et al.*, 1982). c-Src possesses a negative regulatory phosphorylation site, Y527 or Y529 for the avian and mouse/human gene, which is lost in v-Src due to a C-terminal truncation (Fig. 1.4.1.). It has been shown that in late stages of colon cancer, a truncation of the c-Src genes occurs which activates the protein similarly to the truncation in v-Src (Irby *et al.*, 1999).

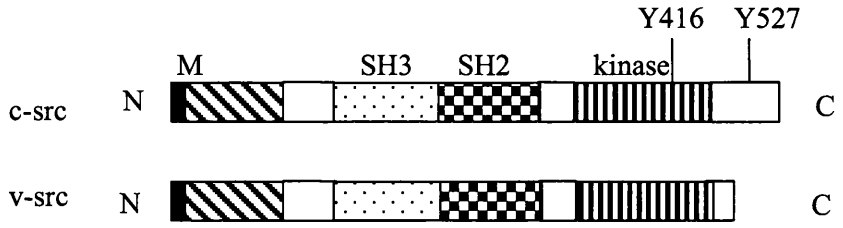


Fig. 1.4.1 Domain organisation and phosphorylation sites of c-Src and v-Src.
The Src molecule is composed of an amino-terminal myristylation sequence (M), followed by SH3 and SH2 interaction domains. The kinase domain (also termed SH1) contains the auto-phosphorylation site Y416. In the carboxy-terminus a negative-regulatory phosphorylation site Y527 is located. Csk (C-terminal Src kinase) is responsible for phosphorylation of the site which is missing in the truncated v-Src.

1.4.2. Function and substrates

1.4.2.1. Focal adhesions and adherens junctions

Focal adhesion kinase (FAK), an integrin binding tyrosine kinase, was one of the first identified Src substrates and is thought to regulate growth-factor and integrin-mediated cellular motility, adhesion and invasion. FAK has an autophosphorylation site (Y397) which constitutes a recognition site for Src's SH2 domain. Once recruited to FAK, Src is thought to mediate FAK phosphorylation on different tyrosine residues. When FAK is absent or when an inactive form of v-Src is expressed in cells, one can observe impaired migration and enlarged focal adhesions (Fincham and Frame, 1998). On the other hand, during transformation, v-Src leads to phosphorylation of FAK and disrupts the actin/focal adhesion network, a process which, though to a lesser extent, is also necessary for migration. Src activity is required to induce dissociation of the FAK-Src complex, degradation of FAK and for focal adhesion turnover (Fincham and Frame, 1998). Other Src substrates are p190RhoGAP, p120RasGAP and cortactin– all of which are known to affect cellular motility.

Focal adhesions are dynamic structures that assemble to allow cells to adhere to the extracellular matrix (ECM) and are composed of over 50 different proteins including

Src, FAK, Paxillin, CAS, α-actinin, talin, vinculin (Zamir and Geiger, 2001). Focal adhesions connect the ECM with the actin cytoskeleton and are disrupted due to Src activity when a cell is moving. Cells move directionally by forming protrusions with stable attachments at the leading edge. The release of adhesion and retraction at the rear end follows (Lauffenburger and Horwitz, 1996). Actin polymerisation then propels the cell forward. The release of focal adhesions is necessary for the forward movement. During transformation enhanced motility is also due to Src-mediated focal adhesion disruption via phosphorylation of RhoGAP, which inactivates the small GTPase RhoA. Activity of RhoA is absolutely required for formation of focal adhesions, thus RhoA inactivation disrupts these structures (Fujisawa *et al.*, 1998; Chang *et al.*, 1995).
Adherens junctions mediate cell-cell adhesion through homotypic binding between molecules of the transmembrane glycoprotein E-cadherin, located on adjacent cells. A cytoplasmic catenin complex links E-cadherin to the cytoskeleton. Src also associates with this complex and is able to promote the disruption of the adherens junction by supressing E-cadherin function. Likewise, Src-induced tyrosine phosphorylation of E-cadherin results in E-cadherin ubiquitination and endocytosis in transgenic mice (Fujita *et al.*, 2002). E-cadherin was shown to act as an invasion suppressor (Frixen *et al.*, 1991; Perl *et al.*, 1998). Besides, Src may affect the expression of metalloproteases (MMP2 and MMP9) via a FAK/c-Jun N-terminal kinase (JNK) pathway (Noritake *et al.*, 1999; Hsia *et al.*, 2003), thereby also affecting the invasive phenotype of cells. Taken together, Src promotes cell motility and invasion via the release of a cell from not only the matrix context but also from the cell–cell context.

1.4.2.2. AGC kinases

Among the numerous Src substrates are several AGC kinases. Phosphorylation of AGC kinases often results in enhanced activity of the substrate, but also has been found to influence stability and subcellular localisation.
For example, PDK1 is tyrosine phosphorylated upon pervanadate (a tyrosine phosphatase inhibitor) and insulin treatment in a Src-dependent manner (Grillo *et al.*, 2002). Three sites were identified, of which two, Y373/376, are located in the kinase

extension domain and are involved in the activation of the kinase (Park *et al.*, 2001). A member of the FAK family, Pyk 2, acts as a scaffold for Src to phosphorylate Y9, an event which permits the subsequent phosphorylation of the Y373/376 sites in response to angiotensin II in vascular smooth muscle cells. Mutation of Y9 abolishes focal adhesion formation (Taniyama *et al.*, 2003). This indicates that tyrosine phosphorylation of PDK1 may be important for maintaining the integrity of focal adhesions.

The fact that pervanadate is a very strong activator of PKB/Akt, suggested that tyrosine phosphorylation might be involved in the activation of PKB/Akt. Three phosphorylation sites have been identified which are either situated near the activation loop (Y315/326) or adjacent to the hydrophobic motif phosphorylation site (Y474) (Chen *et al.*, 2001; Conus *et al.*, 2002). These sites are necessary for PKB/Akt activity. In v-Src transformed cells PKB/Akt activity is enhanced (Datta *et al.*, 1996; Liu *et al.*, 1998). Syk and Btk-mediated tyrosine phosphorylation of PKB/Akt has been reported in B cells (Craxton *et al.*, 1999). The Y474 site is directly adjacent to the S473 site in the kinase extension domain which is crucial for PKB/Akt activation. It is therefore possible that tyrosine phosphorylation may affect S473 phosphorylation. It is argued that the Y315/326 sites may be too close to the catalytic and P-loop (which mediates ATP binding) and that, consequently, their involvement in the regulation of activity may have falsely been attributed simply because of misfolding.

Tyrosine phosphorylation was shown for all subtypes of PKCs. The novel PKCδ was the first PKC found to be functionally modulated by tyrosine phosphorylation. *In vitro*, Src is able to phosphorylate PKCδ (Gschwendt *et al.*, 1994) and PKCδ associates with v-Src in v-Src transformed cells and with c-Src in MCF7 cells (Denning *et al.*, 1996; Song *et al.*, 1998; Szallasi *et al.*, 1995). When tyrosine phosphorylated, PKCδ localises in the membrane fraction and has an increased activity (Li et al, 1994). PKCδ tyrosine phosphorylated sites are Y52, Y155, Y565 (Szallasi *et al.*, 1995), Y64, Y187 (Blass *et al.*; Li *et al.*, 1996), Y512 and Y523 (Konishi *et al.*, 1997), Y311 and Y332 (Konishi *et al.*, 2001). One site, Y311, was found to be necessary for full kinase activity. This site is located upstream of the kinase domain, in the hinge region between the regulatory and catalytic domain. However, other studies indicate that tyrosine

phosphorylation reduces PKCδ activity. These contradictory results can be re-conciliated by the hypothesis that tyrosine phosphorylation may alter substrate specificity (Haleem-Smith *et al.*, 1995). Additionally, the Y311 site was shown to be involved in downregulation of PKCδ. Src-mediated phosphorylation at this site lead to enhanced PKCδ degradation which could be attenuated by mutating the site to phenylalanine (Blake *et al.*, 1999). And last, Y64 and Y187 need to be phosphorylated so that PKCδ can mediate its apoptotic responses after etoposide treatment (Blass *et al.*, 2002).

Upon H_2O_2 treatment PKCα, β, γ (classical PKCs), ε (novel) and ζ (atypical) were also found to be activated and tyrosine phosphorylated (Konishi *et al.*, 1997).

Several Src phosphorylation sites in the atypical PKCι were identified, Y256, Y271, Y325 of which only the Y325F mutant exhibited significantly reduced activity (Wooten *et al.*, 2001). It was published that Y256 was important for mediating the nuclear import. Phosphorylation of this site enhanced binding to importin possibly via a conformational change that exposed the arginine-rich nuclear localisation sequence of PKC (White *et al.*, 2002).

PKD is a distant relative to PKCs (formerly termed PKCμ) and is regulated by DAG in the presence of phospholipids but does not bind calcium. In contrast to other PKCs, the N-terminal regulatory region possesses a PH domain (Iglesias and Rozengurt, 1998) and shows a unique substrate specificity within the PKC family (Nishikawa *et al.*, 1997). H_2O_2 activates PKD and leads to tyrosine phosphorylation, which can be inhibited to 45% by Src specific inhibitors PP1 and PP2 (Waldron and Rozengurt, 2000). Furthermore, tyrosine phosphorylation occurs at three sites located in the PH domain (Y432, Y463, Y502) within a Src-Abl pathway and phosphorylation of Y463 fully activates the kinase (Storz *et al.*, 2003).

To summarise, Src-mediated tyrosine phosphorylation of AGC kinases in response to a variety of growth factors and stimuli leads, in most cases, to enhanced activation. However, altered protein stability and subcellular localisation of some AGC kinases were also shown to be Src-dependent.

1.4.2.3. Transcription

c-Src can affect gene expression via a variety of transcription factors (such as STATs or c-Myc). Gene–expression profiling was performed when c-Src or v-Src were overexpressed. As a result, hypoxia-inducible factor (HIF) or cyclin D1 were found upregulated whereas other transcripts were downregulated. Moreover, transcription of a large number of genes regulating the cell cycle, the cytoskeleton, cellular transcription and lysosomal proteins are regulated by Src (Yeatman, 2004).

It has been shown that in v-Src transformed cells STAT3 activity and its DNA binding specificity are constitutively elevated (Yu *et al.*, 1995). STAT3, in turn, upregulates the expression of vascular endothelial growth factor (VEGF), which promotes angiogenesis (Niu *et al.*, 2002). When c-Src expression was reduced in colon cancer cells using antisense silencing, VEGF production was reduced (Ellis *et al.*, 1998).

1.4.2.4. Transformation

Transformation induced by v-Src is a visible process that can be observed under the microscope leading to morphological changes in transfected cells. They round up, disaggregate and begin to float in the culture medium as they loose the intercellular integrin-based cytoskeletal attachments that normally ensure that cells bind to the substratum in an ordered monolayer. Over the course of several weeks, v-Src transformation results in overgrown lumps of cells, known as foci that form when cells loose their density inhibition– a hallmark of a cancer cells. In addition, v-Src increases the proliferation rates. In vivo, transfected cells rapidly grow to form visible tumours within days of injection and metastasize.

Not only v-Src but also c-Src is involved in cancer. Overexpression of c-Src has been observed in numerous cancers (Yeatman, 2004). Like v-Src, activated mutants of c-Src can tranform cells in culture and induce tumours in chickens (Lin *et al.*, 1995; Levy *et al.*, 1986). In gastrointestinal tumours one can observe an increase of c-Src activity correlating with the progression of the disease (Talamonti *et al.*, 1993). However, it was shown that c-Src activity does not correlate with colon cancer cell proliferation *in vitro*

or tumour growth *in vivo*, but c-Src seems to act via an intregrin-mediated mechanism enhancing the ability of these cells to spread on substrate (Jones *et al.*, 2002).
c-Src is often activated in tumours where receptor tyrosine kinases are overexpressed. Similarly, the co-operation of c-Src and epidermal growth factor receptor (EGFR) regulates the invasiveness of colon cancer cells. When EGFR and c-Src are co-transfected into fibroblasts, their combined action results in increased proliferation, invasiveness and tumorigenesis (Tice *et al.*, 1999). One hypothesis is that in tumorigenesis Src promotes proliferation whereas in later stages it is responsible for invasiveness (Frame, 2002).

All the functions attributed to Src, disruption of adhesion, promotion of motility, invasion and angiogenesis clearly underscore that deregulated Src may play a major role in tumorigenesis and metastasis.

1.5. MASS SPECTROMETRICAL APPROACHES FOR PHOSPHOPEPTIDE IDENTIFICATION

Post-translational modifications modulate the activity, localisation and turnover of many eukaryotic enzymes. They are tightly regulated, selective and control many aspects of cell growth, metabolism, division, motility and differentiation (Hunter, 1995; Posada and Cooper, 1992). Phosphorylation occurs in as many as a third of proteins in mammalian cells (Hubbard and Cohen, 1993). Other important post-translational modifications are for example acetylation, methylation, acylation, glycosylation and ubiquitylation. Proteomics have proven successful in identifying proteins in complexes and organelles, usually by means of 2D-PAGE followed by excision and proteolytic digestion of the spots of interest and mass spectrometry (MS). Post-translational modification such as glycosylation, acetylation, ubiquitylation and especially

phosphorylation have been successfully determined by mass spectrometry. In the following chapter, mass spectrometrical approaches for phosphopeptide identification will be presented.

Mass spectrometers generally consist of three functional elements as can be seen in Fig.1.5.1: an ionisation source, a mass analyser and a detector. In the source, molecules are brought into the gaseous state and ionized, and in the mass analyser the actual separation according to the mass to charge ratio (m/z) takes place. Because the intensity of ions can be very low, in the detector the signal must be greatly amplified which generally is achieved by successive electron emissions caused by collision of the incoming ions with, for example, metallic dynodes.

1.5.1. Ionisation

In order for any molecule to be analysed, it must be ionised and brought into the gas phase (Gaskell, 1996). Two soft ionisation methods, developed in the 1980ies are now commonly in use: Electrospray ionisation (ESI) and matrix-assisted laser desorption ionisation (MALDI).

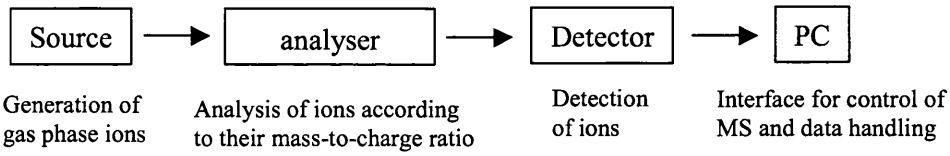


Fig. 1.5.1. Schematic diagram of the basic components of a mass spectrometer
All mass spectrometers consist of a source where ionisation occurs, a mass analyser where ions are separated according to their m/z ratio and a detector. The Mass Spec is operated by a PC and the generated data usually analysed using specific software and databases.

1.5.1.1. Electrospray Ionisaton (ESI)

In the 1980ies John Fenn demonstrated the use of ESI for the ionisation of high mass biological compounds and their subsequent analysis by mass spectrometry. In principle, the analyte in solution is introduced into a source e.g. via a pump or liquid chromatography. The analyte then passes through an electrospray needle that has a high potential difference in comparison to the inlet of the mass spectrometer. The electric field generates charged droplets. Through either the application of drying heat or gas the solvent evaporates, the droplet size decreases until desolvated ions are formed (Fenn *et al.*, 1989). Characteristically, highly charged ions form without fragmenting. This process has an enormous advantage as, due to the higher charge states, the m/z values decrease and the ions can be separated by different mass analysers. The m/z separation of the analyte's isotopes is indicative of the charge state (for example the isotopes of +2 ions are separated by an m/z value of 0.5) (Yates, 1998). Electrospray ionisation does not tolerate high levels of salt or detergents and therefore it is advisable that the analyte samples are previously cleaned up, either by HPLC or by using ZipTips (C18 resin fixed at the end of a 10µl pipette tip).

1.5.1.2. MALDI

This method of soft ionisation was introduced in 1988 by Karas and Hillenkamp (Karas and Hillenkamp, 1988). The sample is dissolved in a matrix (usually an aromatic acid), and irradiated with a pulsed nitrogen laser at 337nm. When the laser strikes, the matrix crystals heat up, sublimate and carry the analyte molecules into the gas phase as ions. This generates pulses of mainly singly charged ions, from each component of the sample. The ions may be formed through a gas-phase proton transfer, however the exact mechanism of ionisation in MALDI remains a matter of debate (Dreisewerd, 2003; Karas 2003). As predominantly singly charged ions are produced, a spectrum is generated that directly reflects the components in the analyte mixture, i.e. the molecular mass of the non-ionised molecule can be easily determined by substracting one mass unit from the usually positively charged ions. MALDI is more tolerant to the presence

of salts and buffers than ESI and this may be due to the process of crystallization within the matrix molecules when contaminants might be sequestered from the analyte (Yates, 1998).

1.5.2. Mass analysers

Within the mass analyser the ions generated in the ion source are separated according to their m/z ratio. Mainly three types of mass analysers are in widespread use: quadrupoles, ion trap and time-of-flight (TOF) mass analysers.

1.5.2.1. Quadrupole mass analysers

Mass separation in a quadrupole analyser is achieved by establishing an electric field in which ions of a certain m/z value have a stable trajectory through the field.
The analyser consists of four parallel rods. Opposite pairs of rods are connected electrically and a combination of DC voltage (U) and an oscillating r.f. voltage (a.c. voltage at r.f. radio frequencies) with opposite polarities are applied to each pair. This generates an electrical field in which ions move in complex trajectories down the axis of the array of rods. By stepwise increasing the voltages, stable trajectories for each m/z value across the scan range are created. Different ions, therefore, are sequentially allowed through the quadrupole array and exit at the detector. The quadrupoles can also simply act as mass filters allowing a range of m/z ions through the quadrupole. All ions above a set mass value are focused and transmitted through the quadrupole. This mass filter device is often used in combination with a gas phase collision cell between the first and a second scanning quadrupole in order to gain MS/MS data. Quadrupole mass analysers are usually limited to the separation of ions up to m/z of 4000. The quadrupole mass analyser generates spectra of a typically unit resolution. That means that the higher charged the ions are the closer the isotope points get to each other until cannot be distinguished any more. The speed at which the quadrupole scans over the set mass range determines the resolution. As the scan time increases, the resolution will increase, however, this will be at the expense of sensitivity. At a given point in time

only ions of a certain m/z value are stable and are detected, all the other ions are lost. For this reason a quadrupole is less sensitive than a non-scanning device such as a TOF.

1.5.2.2. Ion trap mass analysers

Ion trap mass analysers are scanning devices, which trap ions in a three-dimensional electrical field. Three hyperbolic electrodes consisting of a ring and two endcaps, form the core of this instrument. Injected ions are trapped in a radio frequency quadrupole field and then sequentially ejected from the ion trap into an electron multiplier detector. The scanning process is based on a gradual ramping of the radio frequencies which leads to destabilization of ions, one by one within an m/z range, their ejection from the trap and detection. Helium gas of one Torr in the trapping volume is used to improve the mass resolution. Collision with the helium molecules dampens the kinetic energy of the ions and contracts the stable trajectories towards the center of the trap.
The main advantage is that all ions of the m/z range of interest remain within the trap until they are ejected one by one, that means that none of the ions is lost and the signal to noise ratio is thereby increased. The biggest advantage of the Ion trap is the possibility to perform a multitude of tandem MS experiments and thereby gain structural information on large molecules. The resolution of the ion trap mass analyser is typically 0.3 m/z units at slower scan speeds.

1.5.2.3. Time-of-flight (TOF) mass analysers

The time-of-flight mass analyser is one of the simplest and most widely used mass analysing devices and is very often used in combination with MALDI (Mamyrin, 2001). Separation of ions is based on different flight time of ions, all of which have been accelerated up to the same kinetic energy before being pulsed out of the source into the flight tube. They travel the length of the flight tube at a velocity that can be calculated according to the classical equation for kinetic energy, E:

$E = (mv^2)/2$

$V = (2E/m)^{1/2}$

Thus, ions with greater mass will travel slower than the lighter ones and therefore reach the detector later. If they have the same charge, the flight times of two ions are directly proportional to the square root of their masses. The ions receive not a set value of kinetic energy, but rather a "spread" of initial kinetic energy which results in a "spread" of velocities for the same ions and, ultimately, in a broader peak for that ion.
In order to enhance resolution of the so-called linear mode, TOF instruments can also be run in the reflectron mode where ions are focused in an ion mirror or reflector (with a slightly higher voltage than the accelerating voltage) by slowing ions down until they stop and re-accelerating them into a second flight tube (Mamyrin, 1994). All ions deflected out from the mirror have exactly a full and exact kinetic energy of 25kV. Depending on the kinetic energies or velocities the ions posses, they will be penetrating the mirror more or less deeply and be turned around faster or slower. There is no theoretical limit to the maximum m/z value that can be analysed and resolution can be very high with mass accuracy better than 10ppm.

1.5.3. Tandem MS and hybrid instruments

It was already mentioned that some instruments such as the ion trap instrument are applicable for MS/MS experiments. Furthermore, the combination of different mass analysers such as quadrupole-TOF or triple-quadrupoles have also proven useful in obtaining more detailed spectra (Anderson and Mann, 2000). Discussed here will be the MS set-ups and hybrid instruments that were used for this study.

Ion trap tandem MS

Multiple stages of ion dissociation (MS^n) can be performed with the quadrupole ion trap instrument to obtain structural information of the analyte. Ions of specific m/z values can be isolated and the kinetic energy of these ions is increased which causes more energetic collisions with the helium gas until fragmentation, termed collision-induced dissociation (CID), occurs. Ions produced from this first MS/MS experiment can again be isolated and subjected to another collision-induced fragmentation, effectively performing (MS^3).

TOF-ISD and TOF-PSD

Fragmentation within a TOF instrument can occur in the field free region of the flight tube, hereby a metastable precursor ion, which is sufficiently stable to get out of the ion source but insufficiently stable to survive the flight to the detector, decomposes in the flight tube. This process of fragmentation is called post-source decay (PSD) (Kaufmann *et al.*, 1993). In order to maintain the conservation of energy, the fragment ions must have kinetic energies proportional to the ratio of their masses to the mass of the precursor ion, however, all the fragment ions will have the same velocity which means that they would all each the detector at the same time and therefore fragment ions would not be observed when the instrument is used in the linear mode. In the reflectron mode however, the energy will be focused by the reflectron to the original 25kV and after re-acceleration the fragment ions will reach the detector according to their m/z ratio (Annan and Carr, 1996).

The second and much less-well studied way of fragmentation in a TOF instrument is in-source–decay or ISD. The delayed extraction of ions before acceleration into the flight tube and the use of higher laser fluences can lead to fragmentation in the ion source itself. Because the fragmentation occurs before acceleration, the fragment ions will all have similar kinetic energy and therefore different velocities according to their mass, which makes it possible to record a reasonable spectrum in linear mode (Brown and Lennon, 1995). However, due to the energy distribution or "spread" as described previously, the data can be enhanced by using the machine in the reflectron mode.

Triple Quadrupole

In a series of three quadrupoles typically a gas collision cell is constructed from the middle quadrupole mass filter. Within this collision cell, filled with a noble gas, the pressure is raised which induces multiple low-energy collisions and will eventually lead to fragmentation of ions. In a triple quadrupole arrangement, the first quadrupole will be used as a mass filter which allows only one ion of the m/z of interest to pass into the collision cell, the third quadrupole then scans and performs the analysis of fragment ions (product ion scan). In another set-up, the first mass analyser scans over a range of m/z values, the second is a collision cell, while the third is set to transmit only one m/z value. This scan mode is called precursor ion MS/MS scan and can be use to identify

ions containing specific structural features as described by the chosen fragment ion (e.g. a phosphate).

Quadrupole-ToF

The quadrupole is operated as an ion guide in MS mode and as a mass selection device in MS/MS mode. A reflectron TOF analyser serves as mass resolving device. A collision cell is located between the quadrupole and the TOF analyser. The Q-TOF instrument has very high sensitivity, resolution and mass accuracy. It allows unambiguous charge state identification and isotope resolution. Peptides from a tryptic digest are particularly suitable for sequencing with the Q-TOF, mainly because of the basic residues (Lys or Arg) at their C-termini.

1.5.4. Phosphorylation studies using MS

The relative occurrences of the different phosphorylated residues show a striking dominance of phosphoserines which make up 90%, phosphothreonine 10% and phosphotyrosine only 0.05% (Hunter and Sefton, 1991). The high abundance of phosphoserine has partially to do with the fact that it is one of the four most abundant amino acids. Together with leucine, lysine and glutamic acid these four amino acids comprise 32 % of all the amino acid residues in a typical protein (Lodish *et al.*, 1999). Even though the number of ser/threonine kinases outnumbers the number of tyrosine kinases, of the 90 tyrosine kinases known in the human genome, 34 of them are involved in various types of cancer (Blume-Jensen and Hunter, 2001).

Conventionally, the identification of phosphorylated residues was carried out by radioactive labeling and Edman sequencing of the digested peptides carrying the radioactivity. Major technological advances in mass spectrometry and sample preparation such as 2D-PAGE or chemical derivatisation have lowered the detection limits considerably so that nowadays even phosphopeptides at low stoichiometry can be detected. In general, characterisation of phosphopeptides by MS requires two steps: first the selection of peptides from a complex mixture resulting from proteolysis, and second, the identification of the phosphorylated amino acid residue.

1.5.4.1. Methods of phosphopeptide enrichment

A crucial step determining the success of mass spectrometrical analysis is sample preparation. Particularly with respect to post-translational modifications, which often occur at low stoichiometries, it is important to maximally enhance the proportion of the modification. Obviously, this starts when the modification is generated, for example in an *in vivo* reaction that could mean that inhibitors for phosphatases or deacetylases would be added to cells, or, in an *in vitro* reaction, that the reaction time would be extended or the concentrations of certain adducts enhanced in order to shift the reaction towards the product side.

IMAC, or immobilized metal ion affinity chromatography exploits high electro-negativity of the phosphate groups on a phosphopeptide. This method is based on the affinity of lone electron pairs of proteins (from the phosphate groups) to metal ions (such as Ni^{2+} or Fe^{2+} or Fe^{3+}), which have been immobilised by chelation upon solid-phase beads to allow affinity capture of a subset of proteins. This is the same principle as that used for the purification of His-tagged proteins with Ni-NTA beads (Crowe *et al.*, 1994).

It has been described that a pan-posphotyrosine specific antibody was successfully used to immunoprecipitate phosphotyrosine containing proteins from a mixture and to identify them by mass spectrometry (Pandey *et al.*, 2002). Theoretically, this should mean that every affinity purified molecule carries the modification and therefore that a stiochiometry of 1:1 is achieved. The immunocomplexes can be speparated by SDS-PAGE and in-gel digested. Alternatively, one could elute the proteins from the antibody (for example by competitive elution using the antigenic peptide), which, however, is difficult to achieve to a satisfactory extent. One could also use the antibody-peptide complex for digestion although it is less desirable as it adds another set of peptides to the analyte mixture. In contrast to the pan-phosphotyrosine antibody, very few good antibodies raised against phosphoserine and phosphothreonine exist.

In a proteomic approach, i.e. where a whole cell lysate is analysed, it is advisable to fractionate the analyte mixture in order to increase sensitivity. Cellular fractionation is a means to split the many thousands of expressed proteins into subgroups, which then can be handled more efficiently. Typically, the protein content of a cell may be separated into a membrane, nuclear and cytosolic fractions.

In order to simplify any peptide mixture (even if it stems from a digest of one purified enzyme), gas chromatography (GC) or high-performance/pressure liquid chromatography (HPLC) are very often combined with mass spectrometry. The longer a gradient is run, the fewer peptides elute at one point in time. The eluate can either be injected directly into the instrument (online use) or fractions can be collected and analysed individually (offline).

1.5.4.2. Fragmentation of a phosphorylated residue

When phosphorylated peptides are fragmented both under PSD and CID conditions, loss of phosphate is frequently the primary mode of fragmentation (Mann *et al.*, 2002). This neutral loss of 80 (HPO_3) or 98 (H_3PO_4) is due to a cleavage at either side of the phospho-ester bond but in many cases additional sequence information is needed in order to clearly identify the phosphorylated residue particularly, for example, if there are multiple possible phosphoryl-accepting residues within the peptide. Phosphoserine and threonine readily loose phosphoric acid, H_3PO_4, generating dehydroalanine (69Da) and dehydroamino-2-butyric acid (83Da) respectively. Phosphotyrosine cannot loose phosphoric acid as that means disintegrating the aromatic ring, but it looses HPO_3 (Fig. 1.4.3.). Due to the aromatic ring and the energy distribution phosphotyrosines are more stable than phosphoserines and threonines. Therefore phosphotyrosine-peptide fragment ions are less abundant and more difficult to detect.

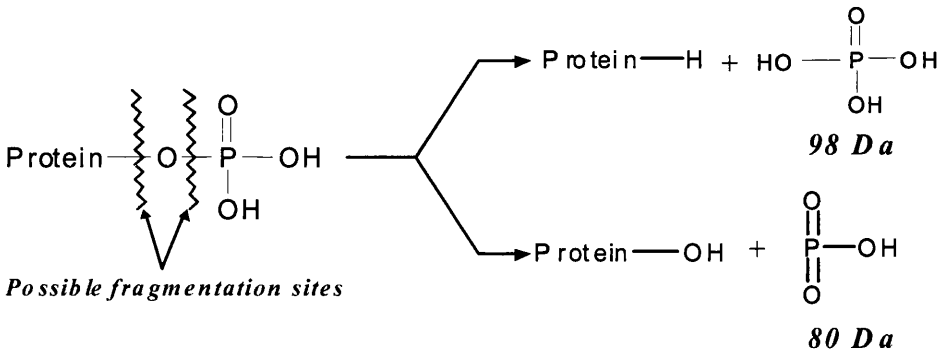


Fig. 1.5.3. Fragmentation of phosphopeptides.
In a fragmentation process either a phosphate or phosphoric acid are generated. In the case of phophoserine and phosphothreonine both fragments, 80 and 98, occur to a high extent generating dehydoalanine (mass of 69) and dehydroamin-2-butyric acid (mass of 83) at the place of serine and threonine, respectively. The resulting fragments in a positive ion scanning mode would be [MH-H3PO4]$^{+}$= MH-97 or [MH-HPO3]$^{+}$= MH-79 and a neutral H3PO4 (98kDa) or HPO3 (80kDa). A peptide containing a phosphotyrosine characteristically fragments into [MH-HPO3]$^{+}$ and HPO3 only.

In negative ion mode, phosphorylation-specific precursor ion scanning identifies parent ions generating a fragment ion of 79, which is characteristic for the loss of PO_3^-.

In positive ion mode, for higher charge states, the neutral loss would be 49 and 40 for the doubly, and 32.6 and 26.6 for the triply charged phosphopeptides (for losses of H_3PO_4 and HPO_3 respectively). To confirm the presence of a phosphopeptide, a subset of the sample can also be selectively dephosphorylated by the use of calf intestine phosphatase (CIP) (Liao *et al.*, 1994). The dephosphorylated sample should exhibit an ion peak 80 m/z lower when compared to the non-treated sample.

1.5.4.3. Digest and simulated digest

A protein must be digested by an endoprotease to yield fragments of a manageable size. The choice of protease can be critical for the identification process. Particularly, if one already has, due to biochemical analysis or sequence homology studies, a specific residue in mind it is important to chose the protease that generates a fragment suitable

for ionisation and detection in a mass spectrometer. Generally, fragments between 700 –3000m/z are suitable. Trypsin is the most efficient and commonly used protease, it specifically cuts C-terminally of arginine and lysine, but not when this residue is followed by a proline. A variety of other proteases are also commonly used such as Asp-N, Glu-C or Pro-C. Each protease will generate a specific set of peptide masses for a protein, termed a peptide mass fingerprint. This is exploited by a variety of algorithms which run a simulation of the digest, list the peptide masses and can be interrogated to match the peptides that were generated in the mass spectrometric experiments. These programmes can be linked to a database or the World Wide Web and pull out peptides that match the experimentally achieved peptide fingerprint for protein identification (MASCOT, Perkins *et al.*, 1999; ProFound, Zhang *et al.*, 2000; MS-Fit, Clauser *et al.*, 1999). In the case of protein phosphorylation the protein sequence is usually known and therefore the simulated digest of this individual sequence is performed taking into consideration chemical and post-translational modification such as methionine oxidation or phosphorylation (i.e. MS digest, Mass Lynx). This enables the researcher to quickly compare the peaks achieved for example in a MS experiment with the peptides from the simulated digest.

1.5.4.4. Precursor ion scanning

Triple quadrupole instrumentation is frequently used to perform precursor ion scanning for the detection of phosphopeptides. In the negative mode, as used for this study, fragmentation of the phosphorylated species provides a unique daughter ion of m/z 79 which is characteristic of a phosphate group stemming from either S,T or Y scanning (Annan *et al.*, 2001; Mann *et al.*, 2002). A precursor scan identifies the parent ion that yielded this 79 ion. Briefly, the first quadruple is scanned over the full mass range and CID is induced in the second quadruple filled with a noble gas. The third quadrupole is set to detect the fragment ions of 79. The spectrum shows the parent ions which are phosphopeptides that have lost a fragment of m/z=79. The m/z values are then compared with the simulated digest. A match is considered to be maximally 1 m/z apart from the value of the theoretical digest. Because of the relatively low resolution of a

triple quad instrument, the charge state of the precursor peptides cannot be assigned and therefore it is often difficult to match the peptide with the theoretical digest. The triple quadruple instrument is not ideal for sequencing due to its low resolution. Moreover, it is not possible to directly switch from a precursor scan to a sequencing mode because alkaline spray conditions (such as 5% NH3) are necessary for the precursor ion scan and this makes sequencing difficult. The fragmentation in negative ion mode does not occur in the same pattern along the peptide backbone than it does in a positive mode. Thus, additional (positive ion) experiments have to be performed for gaining sequence information.

However, this method is very useful for the selection of a phosphopeptide because of the sensitive MS detection of the PO_3^- anion. Synthetic peptides have been characterised at low concentration such as 10fmol/µl (Wilm *et al.*, 1996), and *in vivo* modifications have also been reported (Verma et al, 1997; Beuvink *et al.*, 2000).

More recently, a precursor ion scanning method in the positive mode specifically aimed at detecting phosphotyrosine containing peptides was developed by Mann and Steen (Steen *et al.*, 2001; Steen *et al.*, 2001), termed phosphotyrosine-specific immonium ion scanning (PSI). This is an important development because due to the relative low abundance of phosphotyrosine, signals from phosphoserine and phosphothreonine containing peptides often overshadow the detection of a phosphotyrosine containing peptide. In this method, the immonium ion of phosphotyrosine is detected at m/z 216.043. It is depicted in Fig.1.5.4. An immonium ion is an internal fragment with just a single side chain formed by cleavage leaving the amino acid without a carboxyl group. They have specific masses for every amino acid.

Due to the presence of numerous other fragment ions with the same nominal but slightly different exact masses, high resolution mass spectrometers such as Q-TOFs are required for this approach.

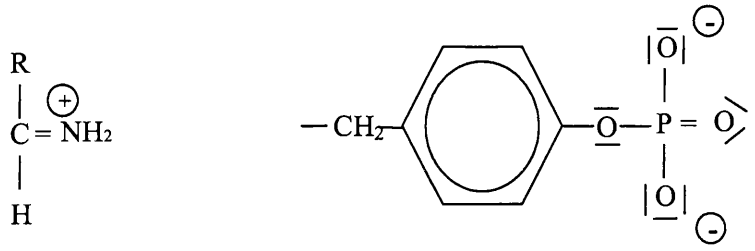


Fig. 1.5.4. Phosphotyrosine-specific immonium ion.
On the left the generic formula of an immonium ion is depicted. In the phosphotyrosine immonium ion the R is replaced by the side chain of phosphotyrosine, right panel.

1.5.4.5. Sequencing

Once a phosphopeptide is found by negative precursor ion scanning on the triple quadrupole instrument, additional sequence information needs to be acquired for the following reasons: First, the mass accuracy of the triple quad instrument is not sufficient for the unambiguous identification of a peptide, and secondly, a peptide often contains more than one theoretical phosphoacceptor residue. For this study sequencing was performed in positive mode using a Q-TOF instrument where the precursor ion of interest was singled out in the first quadruple, then subjected to collision-induced fragmentation and the fragment ions separated in the TOF tube. Fragmentation generally occurs by backbone cleavage as illustrated in Fig 1.5.5. In a product ion of a phosphopeptide one can usually detect a loss of 80m/z (or 40m/z for a doubly charged ion) in comparison to the precursor signal. Ladder sequencing of for example the Y-ions series is relatively simple and can be done manually, however software also exists which automatically assigns a sequence. The latter approach requires a very clear spectrum to prevent false sequence assignment. The difference between the major peaks represents the mass of one amino acid within one series. In our hands, during the fragmentation process the phosphoresidues remained partially intact which made the identification of the sites relatively easy: phosphotyrosine could be detected as a

difference of 163+80 m/z = 243m/z (=mass of tyrosine+phosphate) or phosphoserine as 87+80=167 m/z (=mass of serine+phosphate) within a series.

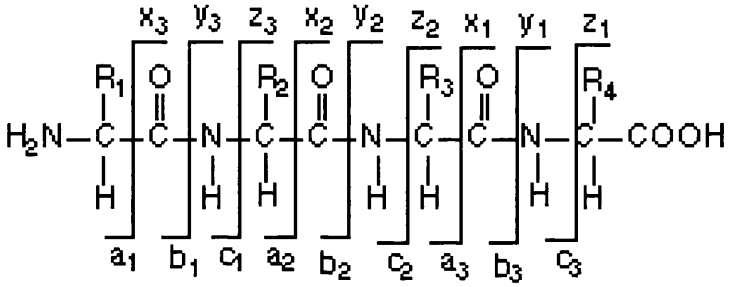


Fig. 1.5.5. Labeling of fragment ions.
Fragments formed through bond cleavages along the backbone of protonated linear peptides. The labeling of fragment ions in this study will be based upon the generally accepted nomenclature developed by Biemann. Biemann, K. (1990).Nomenclature for peptide fragment ions (positive ions). Mass Spectrometry, McCloskey, J.A., Ed., Methods in Enzymology 193, Academic Press, San Diego, 1990, Appendix 5, pp. 886-7.

1.5.4.6. Ion Trap mass spectrometry for phosphosite identification

The ion trap mass spectrometer has one major advantage for the identification of phosphorylation sites (Zhang *et al.*, 1998). Because ions can be trapped and excited in the spectrometer for much longer than in other mass analysers, fragmentation patterns that require longer timescales and less energy can also be observed. Generally, in an ion trap phosphoserines and threonines loose H_3PO_4 through β–elimination.
Phosphotyrosines can fragment into either 80 or a combination of 80 and 98, which is also accompanied by a high degree of backbone fragmentation. It has been shown that even though phosphotyrosine cannot undergo the same β-elimination reaction as phosphoserine and phosphothreonine, it can still loose a neutral fragment of 98 (Haller *et al.*, 1995). Another subset of phosphotyrosine containing peptides do not release a phosphate group at all and that seems to depend on the charge state: at a high charge state such as +4 no loss of phosphate is observed.

Ideally, the ion trap mass spectrometer is connected to an HPLC system. The incoming peptides with the highest intensity at a given retention time are subjected to tandem MS. Typically, in set-ups used for this study a gradient of 60-90 minutes was run. The QualBrowser software (ThermoFinnigan) detects all precursors that have lost either 98, 49 or 32.6 for the neutral loss of H_3PO_4 or 80, 40, 26.7 as the loss of HPO_3, from singly, doubly and tiply charged precursor ions respectively. MS/MS is performed on the three incoming peptides with highest intensity at a given point of elution.

1.5.4.7. MALDI-ISD

In-source-decay (ISD) is a relatively recent method for sequencing peptides and proteins at low picomol levels and was first described by Brown and Lennon (Brown and Lennon, 1995). One of the advantages of ISD is that the amino acid sequence of proteins can be determined without pre-digestion (Lennon and Walsh, 1997). Sequencing can be achieved either from the N- or the C-terminus. ISD is the most convenient method for the assignment of product ions because the nature of products is limited to c_n, b_n, a_n, y_n and z_n+2 series, with the c_n series being the most prominent. DHB was found to be the most suitable matrix for ISD (Takayama, 2001). ISD has not yet been described in the literature as a method for phosphopeptide identification. However, in this study it was successfully employed and an intact phosphotyrosine (243 m/z) was found within a c-ion series.

1.6. AIM OF THESIS

The regulation of S6K activity is very complex and involves multiple and successive serine and threonine phosphorylation events mediated by a variety of independently regulated kinases. More than 10 serine or threonine sites have been identified and almost all of them, with the exception of one, which mediates subcellular localisation in S6Kβ, are involved in the activation of the kinase or have a so far unknown function

The fact that other AGC kinases have been shown to be tyrosine phosphorylated prompted us to investigate if S6K could under certain circumstances become phosphorylated on tyrosines as well. The aim of this study was to establish the conditions under which this post-translational modification occurs and which kinase is responsible for phosphorylation. Furthermore, it was of interest to determine the site/s of phosphorylation by using mass spectrometry. The identified sites would then be useful in understanding the physiological relevance of the modification. First, phosphospecific antibodies could be generated and used in a variety of *in vivo* assays including confocal microspcopy. Secondly, site mutants could be overexpressed in cells and by comparison with the wild type S6K, one could address functional aspects such as activity, effects on cellular growth and the cell cycle.

As already discussed, Src-mediated tyrosine phosphorylation has mainly been shown to positively affect the activity of other AGC kinases. However, for atypical PKCs the subcellular localisation (White *et al.*, 2002) and for PKCδ the stability (Blake *et al.*, 1999) are altered by this modification. Thus, the presented study focussed on these functional readouts.

CHAPTER 2: EXPERIMENTAL PROCEDURES

CHAPTER 2

EXPERIMENTAL PROCEDURES

2.1. NUCLEIC ACID MANIPULATION

2.1.1. Expression vectors and viruses

2.1.1.1. Plasmids

For bacterial expression of the His-tagged N-terminal fragment, the pET 23d (+) vector (Novagen) was used. The mammalian expression vectors for a variety of cellular proteins were the following: for p70S6Kα and β wild type, point and truncation mutants: pcDNA3.1(+) (Invitrogen). The following expression constructs were given to our laboratory: PDGF receptor wild type and point mutants in pcDNA3 (Invitrogen), wild type Src in PSGT (from Lars Roennstrand, University of Malmo, Sweden), bovine wild type PKBα (from Len Stephens, Babraham Institute, Cambridge) and wild type PKCγ (present from Peter Parker, CRUK). Src529F in pUSEamp(-) and dominant negative Src: pUSEamp(+) were purchased from Upstate Biotechnology.

2.1.1.2. Baculoviruses for expression in SF9 cells

The viruses were previously constructed by Ivan Gout (LICR) previously using the Baculogold Expression Vector system (BD Biocsciences) (Savinska et a., 2004). Viruses containing the cDNA of p70S6Kα and p70S6Kβ, various receptor tyrosine kinases (HGFR, PDGFR, EGFR, CSFR, NeuR) and the cytosolic tyrosine kinase fyn were used in this study.

2.1.1.3. Oligonucleotide design

Oligonucleotide primers were designed using the known DNA sequence of the template and restriction enzyme sites created at the end of primers to facilitate subsequent subcloning of fragments. His-tag (6×His) was already present in the pET23(d) plasmid at the 3′ end in frame with the inserted sequence. Annealing temperatures (T_m) for each primer were calculated using the equation: $T_m(°C)=2(A+T)+4(G+C)$. The mutagenic oligonucleotide primers for use in site-directed mutagenesis were designed individually according to the desired mutation. The following formula was used for estimating the T_m of mutagenic primers: $T_m(°C)=81.5+0.41(\%GC)-675/N-\%mismatch$ (N is the primer length in bases, values for %GC and %mismatch are whole numbers).

2.1.1.4. DNA amplification by the polymerase chain reaction

The polymerase chain reaction (PCR) was used to amplify required regions of DNA from various templates. PCR was performed in a 50 µl volume containing 8 µl 1.25 mM dNTPs, 40 pmoles of each primer and 1U Vent polymerase (New England Biolabs) in 1X reaction buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl, pH 8.8, 0.1% Triton X-100, supplemented with 2 mM $MgSO_4$). The wild-type rat S6Kα cDNA clone and human S6Kβ cDNA clones were used as template DNA for the PCR reaction. This was already done previously by T. Valovka in the laboratory (Valovka *et al.*, 2003). For the construction of the bacterial expression vector containing the N-terminal fragment of S6Kα, the pcDNA3.1/ p70S6Kα was used as a template. PCR amplification was performed using a Thermal Cycler (PTC-200). Samples were denatured at 94°C for 30 sec, annealed at a temperature appropriate for the length and sequence of the oligonucleotide primers (56°C for the N-terminal fragment) for 30 sec, and extended at 72°C for 30 sec per 500 bp length. 25-30 cycles were used to amplify DNA fragments.

2.1.1.5. DNA digestion with restriction endonucleases

Restriction enzymes were obtained from standard commercial sources and digests were performed in the appropriate accompanying digestion buffer, as directed. 1 μg DNA was digested with 5 U of restriction enzyme in a volume of 20 μl. Reaction mixtures were incubated at 37°C for 1-2 h and DNA fragments were analysed as described in 2.1.1.6. If the restriction fragments were used for purification and ligation more than 3-5 μg were used.

2.1.1.6. Electrophoresis and purification of DNA fragments

The electrophoretic mobility of DNA molecules depends on their size and the concentration of agarose gel used. 1% (w/v) gels were generally used, although 1.5% (w/v) gels were employed for analysis of DNA fragments less then 500 bp in size and 0.8% (w/v) for preparative gels. The appropriate weight of agarose was added to TAE buffer (40 mM Tris-Acetate, 1 mM EDTA) and heated to allow the agarose to dissolve. The solution was cooled to approximately 60°C and ethidium bromide was added to a final concentration of 1 μg/ml. The melted agarose solution was then poured into a mold and allowed to harden at room temperature. DNA samples were mixed with 6X loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 30% (w/v) glycerol in water), loaded into the gel and fragments were separated by electrophoresis in TAE buffer at 90-100 V. Standard molecular weight markers (1 kb DNA ladder, Gibco) were electrophoresed alongside the samples. DNA was visualized and photographed under a long-wave UV light.

The DNA fragment of interest was excised from the gel with a scalpel and purified using the QIAEX DNA Gel Extraction kit (QIAGEN), according to the manufacturer's recommendations.

2.1.1.7. Ligation of DNA fragments

The LigaFast™ Rapid DNA Ligation System (Promega) was used to clone DNA fragments into plasmids. To perform ligation reactions and minimise self-ligation of plasmids a 1:3 molar ratio of vector:insert was used. Conversion of molar ratios to mass ratios was calculated using the following equation:

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{molar ratio of } \frac{\text{insert}}{\text{vector}} = \text{ng of insert}$$

The reaction was carried out at RT in buffer containing 30 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP and 10% polyethylene glycol–8000 (PEG) in the presence of 3 U of T4 DNA ligase. After incubation for 5 min, 10 µl of reaction solution was used to transform competent E.coli XL-1 Blue cells (section 2.1.3.4.).

2.1.2. Site-directed mutagenesis

Site-specific mutants were generated by using the QuickChange™ site-directed mutagenesis kit (Stratagene) as recommended by the manufacturer. The QuickChange site-directed mutagenesis method was performed using PfuTurbo DNA polymerase, which replicates both plasmid strands with high fidelity and without displacing the mutant oligonucleotide primers, and a thermal cycler. The basic procedure utilizes a supercoiled, double-stranded DNA (dsDNA) vector with an insert of interest and two synthetic oligonucleotide primers containing the desired mutation. The following considerations were made to design mutagenic oligonucleotide primers: mutagenic primers must contain the desired mutation and anneal to the same sequence on opposite strands of the plasmid. Primers should be between 25 and 45 bases in length, and the melting temperature (T_m) of the primers should be greater than or equal to 78°C (for estimating the T_m see section 2.1.1.3.). The desired mutation should be in the middle of the primer with 10-15 bases of correct sequence on both sides. The primers

optimally should have a minimum GC content of 40% and should terminate in one or more C or G bases. They must be purified either by high pressure liquid chromatography (HPLC) or by polyacrylamide gel electrophoresis (PAGE). The oligonucleotide primers, each complementary to opposite strands of the vector, were extended during PCR cycling by means of PfuTurbo DNA polymerase. Samples were denatured at 95°C for 30 sec, annealed at 55°C for 30 sec, and extended at 68°C for 30 sec per 500 bp of plasmid length. In the case of single amino acid changes 16 cycles were used to amplify DNA. On incorporation of the oligonucleotide primers, a mutated plasmid containing staggered nicks was generated. Following PCR cycling, the product was treated with 10U of DpnI endonuclease at 37°C for 1 hour to digest the parental non-mutated DNA template. The nicked vector DNA incorporating the desired mutation was then transformed into E.coli XL-1 Blue competent cells (section 2.1.3.2.), the presence of the desired mutation in selected clones was checked by DNA purification and sequencing (section 2.1.5.).

The primers used for site-directed mutagenesis in this work (described in chapter 6.2.) were the following:

1. S6Kα Y216F:

Forward primer CAAAAGGGATCATC**TTC**AGAGACCTGAAGCCGGAG

Reverse primer CTCCGGCTTCAGGTCTCT**GAA**GATGATCCCCTTTTG

2. S6α Y193D: the same primer sequences except the nucleotides in bold are GAC and GTC for forward and reverse primer, respectively
3. S6Kα Y193H: the same primer sequences except the nucleotides in bold are CAC and GTG for forward and reverse primer, respectively.
4. S6Kβ Y193F

Forward primer CCCAGGGCATCATC**TTC**CGGGACCTCAAGCCC

Reverse primer GGGCTTGAGGTCCGG**GAA**GATGATGCCCTGGG

5. S6Kβ Y192D: the same primer sequences except the nucleotides in bold are GAC and GTC for forward and reverse primer respectively.
6. S6Kβ Y192H: the same primer sequences except the nucleotides in bold are CAC and GTG for forward and reverse primer respectively.

7. PKBα Y192F

   Forward primer GGAAGTGGTG**TTC**AGGGACCTCAAGCTGGAG

   Reverse primer CTCCAGCTTGAGGTCCCT**GAA**CACCACTTCC

8. PKCγ Y478F

Forward primer CCAGGGCATTATC**TTT**CGGGACCTGAAACTGGAC

Reverse primer GTCCAGTTTCAGGTCCCG**AAA**GATAATGCCCTGG

9. S6Kα Y39F

Forward primer GGGGGAGTTGGACCA**TTT**GAACTTGGCATGG

Reverse primer CCATGCCAAGTTC**AAA**TGGTCCAACTCCCCC

10. S6Kα Y39D: the same primer sequences except the nucleotides in bold are GAT and ATC for forward and reverse primer respectively
11. S6Kβ Y45F: had already been made previously
12. PDGFRβ D850V

Forward primer GACTTTGGCCTGGCTCGA**GTC**ATCATGCGGGACTCG

Reverse primer CGAGTCCCGCATGAT**GAC**TCGAGCCAGGCCAAAGTC

2.1.3. Transformation of E.coli

2.1.3.1. Strains and media

Escherichia coli XL-1 Blue cells. Genotype: supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac-F[proAB$^+$lacI9 lacZΔM15 Tn10(tet^r)].

Escherichia coli BL21(DE3)pLysS cells. Genotype: F$^-$ ompT hsdS$_B$ (r_B^- m_B^-) gal dsm (DE3) pLysS (Cam^R). The DE3 designation means the strains contain the λ DE3 lysogen that carries the gene for T7 RNA polymerase under control of the *lac*UV5 promoter. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) is required to induce expression of the T7 RNA polymerase.

Luria Bertani (LB) medium contains 1% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl (pH 7.0 was adjusted with 5N NaOH). NZY$^+$ Broth (NZY) medium: 1% (w/v) NZ amine, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, 12.5 mM $MgCl_2$, 12.5

mM $MgSO_4$ and 20% (w/v) glucose. LB medium and LB agar were autoclaved at 121°C for 15 min. NZY medium was filter sterilised. Ampicillin and kanamycin were prepared as 100 mg/ml stock solutions in ddH_2O and stored at -20^0C. Ampicillin and kanamycin were used at a final concentration 50 µg/ml and 10 µg/ml respectively.

2.1.3.2. Transformation

Competent cells were thawed on ice and 150 µl cell suspension was mixed with 10 µl ligation mix or 50 ng plasmid DNA. After 10 min incubation on ice, the cells were induced to take up the DNA by heat-shock at 42°C for 2 min, cooled on ice for 2 min and allowed to recover in 1ml of LB medium for 45 min at 37°C in shaking incubator (225 rpm). The bacterial cells were then briefly centrifuged and pellet was resuspended in 100µl of LB medium. Cell suspension was spread onto pre-warmed LB agar plate containing the appropriate selective antibiotic (section 2.1.3.1.) and incubated overnight at 37°C.

2.1.4. Purification of plasmid DNA

Plasmid DNA was purified using QIAGEN Plasmid Purification kit (QIAGEN), according to the manufacturer's directions. The QIAGEN plasmid purification protocol is based on a modified alkaline lysis procedure, followed by binding of plasmid DNA to anion-exchange resin under appropriate low salt and pH conditions. The bacterial pellet from an overnight shaker-culture of XL-1 Blue *E. coli* was resuspended in resuspension buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 µg/ml RNase A) and an equal volume of Lysis buffer (200 mM NaOH, 1% Sodium n-dodecyl sulfate (SDS)) was added to the cell suspension. Following 5 min incubation at RT, the lysate was neutralized with 0.5 volume of chilled 3 M potassium acetate, pH 5.5 and incubated for 5 min. Cellular debris was removed by centrifugation at 13000 rpm for 10 min or filtering the lysate through the QIAfilter Cartridge. The supernatant was applied to a QIAGEN-tip containing anion-exchange resin, pre-equilibrated with QBT buffer

(50 mM MOPS, pH 7.0, 750 mM NaCl, 15% isopropanol, 0.15% Triton X-100), and allowed to move through by gravity flow. The resin was washed several times with Wash buffer (50 mM MOPS, pH 7.0, 1 M NaCl, 15% isopropanol) and DNA was eluted with an appropriate volume of Elution buffer (50 mM Tris-HCl, pH 8.5, 1.25 M NaCl, 15% isopropanol). The eluted plasmid DNA was desalted and concentrated by isopropanol precipitation. To precipitate the DNA, an equal volume of 100% isopropanol was added to the DNA solution and immediately centrifuged at 13000 rpm for 30 min. The DNA pellet was washed with 70% ethanol, air-dried for 5-10 min and redissolved in an appropriate volume of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Alternatively, the DNA was precipitated with a QIAGEN precipitator, and eluted with an appropriate volume of TE buffer. To determine the concentration of plasmid DNA, a 1µl aliquot was diluted in 100 µl of TE buffer and OD_{260} was measured. An OD_{260} value of 1 was taken as being equal to a double stranded DNA concentration of 50 µg/ml. Quantity and quality of DNA samples were also checked by gel electrophoresis (section 2.1.1.6.).

2.1.5. DNA sequencing

2.1.5.1. Principle

All generated mutations of constructs were checked by sequencing using Tag DyeDeoxy™ Terminator Cycle Sequencing kit for use with Automated Fluorescent Laser ABI Model 373A DNA sequencer™ (Applied Biosystems). This kit relies on four dye-labeled dideoxy nucleotides: G, A, T and C DyeDeoxy terminators. When these terminators replace standard dideoxy nucleotides in enzymatic sequencing, 3` end-labelled products are produced.

2.1.5.2. DNA sequencing reaction

For each template to be sequenced the following reaction mix was prepared: 8 µl sequencing reagent premix (contains DyeDeoxy™ dNTPs and AmpliTaq DNA polymerase), 0.2 pmole double-stranded template DNA and 5 pmole primer. The final reaction volume was made up to 20 µl with ddH_2O. The PCR cycle sequencing reaction was performed under the following conditions: denaturing at 95°C for 20 sec, annealing at 50°C for 15 sec and extension reaction at 60°C for 1 min. After 25 cycles the products of reaction were precipitated by adding 2 µl of 3 M sodium acetate, pH 4.6, 1 µl of 0.5 M EDTA and 80 µl of 95% ethanol. Mix was incubated on ice for 30 min and then centrifuged in a microcentrifuge at top speed for 15 min. The pellet was washed with 500 µl of 70% ethanol and vacuum-dried for 2-5 min.

2.1.5.3. Sample preparation and analysis

Sequencing gel loading buffer was made up using a 5:1 ratio of de-ionised formamide: 50 mM EDTA, pH 8.0. Blue dextran was added to give the buffer color and to make loading easier. 3 µl of gel loading buffer was added to each sample, vortexed and then heated at 90°C for 2 min and loaded onto the sequencing gel. The gel was run overnight in 1× TBE buffer (50 mM Tris-borate, pH 8.0, 2.5 mM EDTA) on Automated Fluorescent Laser DNA Sequencer™ according to the system's manual settings. Sequence data was analysed using AUTOASSEMBLER software (Applied Biosystems). This reaction was performed by Athena Nikitopoulou at LICR.

2.2. EXPRESSION OF RECOMBINANT PROTEINS IN E. COLI AND AFFINITY PURIFICATION

The Ni-NTA purification system (Qiagen) was used to purify recombinant proteins for *in vitro* studies. This system is based on the remarkable selectivity and affinity of nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatograghy matrices for

biomolecules which have been tagged with 6 consecutive histidine residues (6×His tag). The general strategy of protein expression consists of the construction expression clones, followed by the expression of His-tagged proteins in E.coli and purification on Ni-NTA matrices.

2.2.1. Culture growth for preparative purification

5 ml of LB medium containing appropriate selective antibiotic was inoculated with a single BL21(DE3) pLysS *E. coli* colony and grown overnight at 37°C with vigorous shaking (220-250 rpm). The overnight culture was then used to inoculate 100 ml of LB medium supplemented with selective antibiotic and incubation was continued until an OD_{600} of 0.6-0.8 was reached. To induce recombinant protein expression IPTG was added to a final concentration of 1 mM. After incubation for an additional 3-5 hours cells were harvested by centrifugation at 4000×g for 20 min and frozen in dry ice-ethanol bath and stored at -70°C.

2.2.2. Preparation of bacterial cell lysates under native conditions

The cell pellet was resuspended in 5 ml of lysis buffer (10 mM Tris, pH 8.0, 50 mM NaCl, 10 mM imidazole, 5 mM Benzamidine, 1 mM PMSF, 10 mM Leupeptin, 2 mg/ml Aprotonin, 1 mM Pepstatin). Lysate was incubated on ice for 30 min, sonicated (six 10 sec bursts at 200-300 W with 10 sec cooling period between each burst), and then centrifuged at 10000×g for 20-30 min at 4°C to pellet the cellular debris. Obtained supernatant was used to analyse expression and to purify recombinant protein.

2.2.3. Purification of His-tagged proteins on Ni-NTA agarose

To purify His-tagged recombinant protein, 125 µl of the 50% Ni-NTA slurry was added to 5ml of cleared lysate and mixed by rotation at 4°C for 60 min. After incubation the lysate-Ni-NTA mixture was centrifuged at low speed (2500rpm in table centrifuge).

The Ni-NTA pellet was twice washed with 4 ml of wash buffer (10 mM Tris, pH 8.0, 300 mM NaCl, 20 mM imidazole). The bound protein was eluted with 0.5ml of elution buffer containing 10 mM Tris, pH 8.0, 300 mM NaCl and 400 mM imidazole. The elution was repeated 3 times with 0.5 ml elution buffer as described above. The 4 eluates were then pooled. The eluate was dialysed in 1 l of 10 mM Tris, pH 7.5, 150 mM NaCl, 1 mM DTT for 6 hours at 4°C and then dialysed in 0.5 l of 10mM Tris, pH 7.5, 150 mM NaCl, 1 mM DTT and 50% glycerol. Protein concentration was measured as described in section 2.5.3. and by SDS-PAGE and samples were stored at -20°C.

2.3. PRODUCTION OF PHOSPHOSPECIFIC ANTI-S6K ANTIBODIES

2.3.1. Generation of rabbit antisera and affinity purification

Polyclonal phosphospecific antibodies to S6Kα pY193 and S6Kβ pY192 were raised by immunizing rabbits with one synthetic peptide (KGIIpYRDLKPENIC) coupled to keyhole limpet haemocyanin. One peptide could be chosen for both, S6Kα and S6Kβ, due to the very strong sequence homology which differs only by a Q instead of a K in S6Kβ for the whole peptide sequence. The synthetic peptide used for immunization was synthesised and coupled to activated keyhole limpet hemocyanin (KHL) by Eurogentech. The peptide coupled to KLH was used for the immunisation of two rabbits (performed by Eurogentec). Four bleeds were taken and the third was used for affinity purification of antibodies.

The antibodies generated were affinity-purified using antigenic peptides coupled to Actigel (Sterogene). 5 mg of the synthetic peptide were dissolved in 300 µl of coupling buffer (100 mM phosphate buffer, pH 7.8). Small portions of 5 M NaOH and/or 100% DMSO were added into the mixture to optimise peptide solubilisation. 0.5 ml of 50% Actigel beads, pre-washed several times with coupling buffer, were mixed with 250 µl of solubilised peptide. Coupling of the peptide to Actigel was initiated by adding 1 M

$NaCNBH_3$ (1/10 of final reaction volume), and carried out on a wheel for 4-6 hours at 4°C. After coupling, the beads were washed twice in buffer containing 100 mM Tris-HCl, pH 8.0 and 500 mM NaCl and once in 100 mM Tris-HCl, pH 8.0. To block uncoupled sites, the Actigel beads were finally incubated with 100 mM Tris-HCl, pH 8.0 for 2-4 hours and then stored at 4°C in the presence of 0.02% (w/v) sodium azide.
Polyclonal antiserum was centrifuged at 15000 rpm at 4°C for 10 min and loaded into a column prepared with peptide coupled-Actigel. The column was allowed to empty by gravity flow and then washed extensively with PBS. Bound antibody was eluted with 0.1 M glycine, pH 3.0 and collected as 1ml fractions into tubes containing 100 µl of 1M Tris-HCl, pH 8.0. Protein concentration was measured as described in section 2.5.3. Peak protein fractions were combined, dialysed twice against PBS and once against 50% glycerol/PBS and stored at -20°C.
Affinity purified antibodies were screened for antigen reactivity by immunoblot analysis.

2.4. CELL CULTURE METHODOLOGY

2.4.1. Tissue culture media and general cell culture technique

Human embryonic kidney HEK 293 cells, Cos7 cells and most other cell lines unless otherwise stated, were maintained at 37°C in humidified atmosphere containing 5% CO_2. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Inc.), 2 mM L-glutamine, 50 U/ml penicillin and 0.25 µg/ml streptomycin. NIH 3T3 cells were grown in DMEM medium supplemented with 10% donor calf serum (DCS; Life Technologies, Inc.), 2 mM L-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin. Monolayer cells were split (1/10) at 70-80% confluence. To dislodge cells medium was removed from tissue culture dish, cells were rinsed once with PBS and incubated in Trypsin-EDTA solution (Gibco BRL) for 2-4min at room temperature. Cells were then suspended in complete DMEM medium and split into 10cm dishes.

Wild type Swiss3T3 or F29 Swiss 3T3 stably expressing temperature-sensitive v-Src were cultured in the same media than Hek293 however they were grown at the permissive temperature of 35°C.
Subculturing procedures were carried out in a laminar flow hood in a sterile environment using media/reagents that were all pre-warmed to 37°C.

2.4.2. Transient transfection

Quality plasmid DNA constructs for transfection were prepared as described in section 2.1.4.. Cells were seeded at 1.2×10^6/60-mm dish for Hek293 and at 0.8×10^6/60-mm dish 12 hours prior to transfection. Transient transfection was performed with 2.5-10 μg of total DNA using LipofectAMINE reagent (Life Technologies, Inc.) or ExGen500 (Fermentas) according to the manufacturer's recommendations. For each transfection 2.5 μg plasmid DNA and 10 μl of LipofectAMINE reagent were separately diluted in 100 μl of Opti-MEM I Reduced Serum Medium (Gibco BRL). Mixtures were incubated for 10 min at room temperature (RT), combined and then left at RT for the next 30 min to allow the formation of DNA-liposome complexes. During this period of time cells were rinsed once with 4ml of serum-free DMEM medium and 2 ml of Opti-MEM I medium was added to the dish. DNA-liposome complex solution was overlaid onto the cells and dishes were incubated for 5 hours at 37°C in a CO_2 incubator. Following incubation the medium was replaced with complete DMEM medium and transfected cells were grown for the next 24-48 hours. Recombinant protein expression was analysed to optimize transfection conditions for individual plasmid DNA.
The ExGen transfection procedure is simpler than LipofectAMINE since the DNA/ExGen mixture can be delivered to cells with serum containing medium and therefore the medium does not need to be exchanged after 5 hours. Per 60mm dish, 3 μg of DNA were mixed by vortexing with 250 μl of sterile NaCl (150 mM) and 9 μl of Exgen reagent. After 10 min incubation at RT the mixture was distributed onto the cells which were covered by 2.5 ml complete media, then cells were treated as decribed above.

For transfection of NIH3T3 cells, Polyfect reagent (Qiagen) was used. For a 60 mm dishes 2.5 μg of DNA was incubated with 150 μl DMEM (unsupplemented) and 15 μl Polyfect reagent for 10 minutes at room temperature. To this mixture 1ml DMEM (supplemented with 2 mM L-glutamine, antibiotics and 10%DCS) was added and this mix then applied to cells covered by 3 ml full medium. After 5 hours the medium was changed and cells allowed to grow for 24-48 hours. In very few cases LipofectAMINE 2000 (Invitrogen) was also used for transient transfection of NIH3T3 cells. The procedure is similar to the standard LipofectAMINE protocol, including premixing of DNA and Opti-MEM I and combining this with a LipofectAMINE/Opti-MEM mixture, followed by incubation at room temperature. The DNA/LipofectAMINE complexes can then be added to serum-containing medium and no change of medium is necessary, although in some cases preferable.

2.4.3. Foci formation assay

One hallmark of transformation is the loss of contact inhibition which can be tested with the foci formation assay in a simple way. NIH3T3 cells were split into 60 mm dishes one day before transfection. 24 hours after transfection with S6K wild type or mutants and dominant negative or activated Src constructs, cells there were transferred into 100 mm dishes at a dilution of 1:4. When cells reached 80% confluency, they were subjected to G418 and 5% DCS containing selection media and grown for 2-3 weeks during which media was exchanged every third day. Cells were then rinsed once with PBS and stained with Crystal Violet to visualise formed foci.

2.5. ANALYSIS OF CELLULAR PROTEINS

2.5.1. Preparation of mammalian and insect cell lysates

Mammalian cells were washed twice with ice-cold PBS and extracted with lysis buffer containing 20 mMTris pH7.5, 150 mM NaCl, 1 % v/v Nonidet P-40 (NP-40), 5 mM EDTA, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM sodium

orthovanadate, 50 µg/ml leupeptin (Boehringer Mannheim), 0.5 % aprotinin (Sigma), 1 mM phenylmethylsulfonyl fluoride (Sigma), and 3 mM benzamidine (Sigma). Total cell extracts were incubated on ice for 30min and then centrifuged at 13000 rpm for 15 min at 4°C to pellet the insoluble cell debris. The supernatant fraction was then removed and used for cellular protein analysis.

Several buffers were tested to achieve co-immunoprecipitation of Src and S6K, and one buffer (10 mM K_3PO_4, 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 50 mM glycerosphosphate, 0.5% NP-40, 0.1% Brij 35, 0.1% Na-Deoxycholate, 1 mM Na-Orthovanadate, 50 µg/ml leupeptin , 0.5 % aprotinin, 1 mM phenylmethylsulfonyl fluoride and 3 mM benzamidine) was successfully used for the co-immunoprecipiation experiment of endogenous S6K and Src.

SF9 cells were lysed in the following buffer: 50 mM Tris pH 7.6, 150 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1% Triton X-100, 20 mM NaF, 1 mM Na-Orthovanadate, 50 µg/ml leupeptin, 0.5% aprotinin, 1 mM phenylmethylsulfonyl fluoride and 3 mM benzamidine.

Total protein concentrations of the lysates were determined by Bradford protein assay (section 2.5.3.).

2.5.2. Pervanadate treatment of cells

A 100 mM Na-Orthovanadate stock solution was prepared and adjusted to pH 10 by repeatedly heating and cooling until the solution was transparent and the pH could be stably adjusted with NaOH. When cells were treated with Na-Pervanadate (with a concentration of 1 mM final, unless otherwise stated), a 10X solution (10 mM Na-Pervanadate, 0.2% H_2O_2 in DMEM) was prepared and then distributed 1:10 into the medium-containing culture dishes for the indicated times. In a typical experiment, pervandate was added for 2-10 minutes after cells had been incubated already with either serum or PDGF for a certain amount of time.

2.5.3. Estimating of protein concentration by Bradford protein assay

To estimate protein concentration in cell lysates the colorimetric method with Coomassie Protein Reagent (Pierce) was used. The method is based on the absorbance shift from 465 to 595 nm which occurs when Coomassie brilliant blue G-250 binds to proteins in an acidic solution. 0.5 ml of Coomassie Protein Reagent was diluted in 0.5 ml of ddH_2O and 1 µl of cell lysate was added to the mixture. Upon up to 30 min incubation at RT the absorbance was measured at OD_{595} and compared with a blank. The protein concentration was then determined by comparison of absorbance values with a bovine serum albumin (BSA) standard curve.

2.5.4. SDS-PAGE

Cellular proteins were separated based on their molecular weight (MW) using the discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) system as described by Laemmli (Laemmli, 1970). In a discontinuous system, a non-restrictive large pore gel, called a stacking gel, is layered on top of a separating gel called a resolving gel. Each gel is made with a different buffer, and the tank buffers are different from the gel buffers. The proteins run through the stacking gel as tight bands and are only separated when they migrate through the resolving gel which is characterised by higher pH and acrylamide concentration. The percentage of acrylamide in the resolving gel may vary depending on the range of separation desired. 7.5%-12.5% acrylamide concentration was generally used. To prepare the resolving gel 30% acrylamide stock solution (acrylamide:N,N'-methylene bis-acrylamide 37.5:1) was diluted with appropriate volume of ddH_2O and 1.5 mM solution of Tris-HCl, pH8.8 and 10% SDS solution were added to the final concentration of 375 mM and 0.1% (w/v) respectively. Polymerization was initiated by the addition of ammonium persulfate (0.05% (w/v)) and TEMED (0.005% (v/v)). The gel mixture was then promptly poured into a glass plate assembly and overlaid with water-saturated butanol to ensure a flat surface and to exclude air. After polymerization butanol was removed and gel surface was washed

with water. The stacking gel mixture (4.5% (w/v) acrylamide) was prepared in the same way in 0.125 M Tris-HCl buffer, pH6.7, poured onto top of set resolving gel and left to polymerise with a spacer comb inserted into the mixture. Following polymerisation, the comb was removed and created wells were flushed and filled with SDS electrophoresis buffer (25 mM Tris-HCl, pH 8.3, 195 mM glycine, 0.1% (w/v) SDS). 1 part of cell lysates or protein solution was mixed with 1 part of 2× SDS-PAGE sample buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 0.2 M DTT, 0.1% Bromophenol Blue), heated for 5 min at 95°C and then samples were loaded under buffer into stacking gel wells. Electrophoresis was run in electrophoresis buffer at a fixed current of 20-30 mA per gel until the dye front reached the end of the gel. Visualisation and analysis of separated proteins were performed as described in sections 2.5.5. and 2.5.6.

2.5.5. Visualisation of proteins

2.5.5.1. Coomassie Blue staining

Following electrophoresis, some gels were stained for the presence of protein by soaking in Coomassie Blue stain (0.2% (w/v) Coomassie brilliant blue R-250, 45% (v/v) methanol and 10% (v/v) acetic acid) for 20 min, followed by destaining in 20% (v/v) methanol and 5% (v/v) acetic acid with agitation. The gel was then dried under vacuum at 80°C for 1 hour. Coomassie brilliant blue binds to proteins stoichiometrically, so this staining method is preferable when relative amounts of protein are to be determined by densitometry.

2.5.5.2. Silver staining

Silver staining is the most sensitive nonradioactive method of protein detection in gels which allows to analyse nanogram quantities of protein. In this study we have used method described by Shevchenko *et al.* (Shevchenko *et al.*, 1996) as it is compatible with mass spectrometry. After electrophoresis the polyacrylamide gel was fixed for 20

min in solution containing 50% methanol and 5% acetic acid, rinsed with 50% methanol for 10 min and ddH_2O for another 10 min. A sensitisation step was carried out for 1 min in 0.02% $Na_2S_2O_3$ solution, followed by two brief washes with ddH_2O. The gel was then stained in pre-chilled 0.1% $AgNO_3$ solution for 20 min at 4°C and developed in 2% Na_2CO_3/0.04% formalin solution to the desired degree. Development was stopped by incubation of gel in 5% acetic acid for 10 min. Gels were stored in 1% acetic acid at 4°C or vacuum dried as described above.

2.5.5.3. Ponceau staining

After tranferrral of separated proteins from an SDS-PAGE gel to a nitrocellulose membrane, the membrane was routinely stained with Ponceau Red (0.5 % (w/v) Ponceau Red Sigma, 1 %(v/v) acetic acid) and destained with ddH_20 in order to assess the amount of protein present on the membrane. This was particularly useful to see if immunoprecipitation of overexpressed proteins was successful.

2.5.5.4. Detection of radiolabeled proteins in gels

Samples, which consist of proteins that were radioactively labeled in an *in vitro* reaction (i.e. kinase assays) were separated by SDS-PAGE and analysed by autoradiography. For autoradiographic detection, the gel was simply dried and exposed to X-ray film (Fuji) or a storage phosphor screen (Bio-Rad).

2.5.6. Immunoblot analysis

2.5.6.1. Wet transfer of proteins

Following SDS-PAGE of protein samples the gel was equilibrated in transfer buffer (190 mM glycine, 25 mM Tris Base and 20% (v/v) methanol) for approximately 5 min. The gel was then placed on top of a nitrocellulose membrane (Schleicher and Schuell) or methanol-soaked polyvinyldene difluoride (PVDF) membrane (Millipore), both of

which were immersed in transfer buffer, and sandwiched in a compression cassette between several layers of pre-wetted Whatmann 3 MM. The transfer was performed in Trans-Blot^{TM} electrophoretic transfer cell, according to the manufacturer's instructions (Bio-Rad) at 60 V for a minimum of 90 min.

2.5.6.2. Enhanced Chemiluminescence (ECL) immunodetection

This method, developed by Amersham, was subsequently used for all antibody detection because of the speed of the reaction and the exclusion of radioactivity. ECL is a light emitting, nonradioactive method for the detection of immobilised specific antigens with antibodies conjugated to horseradish peroxidase. The system utilises a chemiluminescent reaction which takes place when the cyclic diacylhydrazide luminol is oxidised in the presence of the hydrogen peroxide (H_2O_2). Following oxidation, the luminol is in an excited state which decays to the ground state by emission of light.
After the wet transfer the membrane was incubated in blocking buffer (5% non-fat dry milk, 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.05% Tween 20) for 1 hour at RT to block non-specific binding sites. The membrane was then incubated with the primary antibodies in minimal volume of blocking buffer at the appropriate dilution (1/200-1/5000) for 1 hour at RT or overnight at 4°C. Excess antibodies were removed by washing in blocking buffer once and twice in in TBS-T buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.05% Tween 20) at RT. A species-specific horseradish peroxidase-conjugated second antibodies (Sigma) was then applied in a minimal volume of blocking buffer for 1 hour at RT at a dilution 1/4000. Following incubation with secondary antibodies the membrane was rinsed twice in blocking buffer and then washed three times in TBS-T buffer for 5 min at RT. Immunoreactive proteins were detected by enhanced chemiluminescence. Equal volumes of ECL detection solution 1 and 2 were mixed and added to the membrane. The reaction was allowed to proceed for 1 min at RT and excess of horseradish peroxidase substrate was removed. The membrane was wrapped in SaranWrap and exposed to X-ray film for various periods of time or scanned by fluoroimager (Bio-Rad). The signal was quantified using software provided by the manufacturer.

2.5.6.3. Stripping and reprobing

Immunoblot membranes may be stripped of bound antibodies and reprobed several times. Membranes should be stored wet wrapped in SaranWrap in a refrigerator (2-8°C) after each immunodetection. The membrane was submerged in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) and incubated at 50°C for 30 min under agitation. The membrane was then washed at least six times in TBS-T buffer for 5 min at RT, blocked for 1 hour at RT in blocking buffer, and immunoblotted as described above.

2.5.7. Immunoprecipitation and affinity purification of proteins

Lysates were prepared as described in section 2.5.1. and incubated with appropriate antibodies (1.5 μg of antibody per 1 mg of total protein) on a rotating wheel for 2 hours or overnight (in the case of detection by phosphotyrosine immunoblot) at 4°C. 20 μl of 50% protein A/G-Sepharose suspension was added to each sample to bind antibody-protein complexes and the incubation continued for a further 1 hour. Or, alternatively, antibody and protein A/G sepharose were added to lysates simultanously and incubated for 2 hours or overnight at 4°C. The immune complexes were then pelleted by low speed centrifugation (2500 rpm) and washed 3-4 times with ice-cold lysis buffer. The samples were boiled in 2× SDS-PAGE sample buffer for separation by gel electrophoresis or subjected to *in vitro* protein kinase assays.

2.5.8. Immune complex ribosomal protein S6 kinase assay

Recombinant or endogenous S6Ks were immunoprecipitated from cell lysates with appropriate antibodies immobilised on protein G-Sepharose beads. Immune complexes were washed two times with lysis buffer, once with lysis buffer containing 500mM NaCl, followed by a single wash with kinase assay wash buffer (20mM MOPS pH 7.2, 10mM β-glycerophosphate, 1mM PMSF). The kinase reaction was initiated by

resuspending the beads in 25µl of kinase assay buffer (50 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiotreitol (DTT), 10 mM β-glycerophosphate) supplemented with 1 µM protein kinase A inhibitor (PKI, Calbiochem), 100µM ATP, 5µCi of [γ-^{32}P]ATP (Amersham) and 20 µg of 40S ribosomes, isolated from rat liver (Thomas *et al.*, 1978). The reaction was carried out at 30°C for 10-30 min and terminated by addition of 5× SDS-PAGE sample buffer and boiling for 5 min. Samples were subjected to 10% SDS-polyacrylamide gel electrophoresis, and the amount of ^{32}P incorporated into S6 protein was assessed by autoradiography and quantified by phosphorimaging (Bio-Rad).

2.5.9. *In vitro* phosphorylation of S6K by tyrosine kinases

Recombinant EE-tagged S6Ks were immunoprecipitated from serum-starved HEK 293 cells with anti-EE monoclonal antibody immobilized on protein G-Sepharose. Immunocomplexes bound to beads were washed twice in lysis buffer followed by one wash in tyrosine kinase buffer (25 mM Tris HCL pH7.5, 30 mM $MgCl_2$, 0.5 mM EGTA, 10 mM $MnCl_2$, 0.12 M KCl, 0.05% TritonX100, 0.5 mM DTT). For assays using PDGFR as tyrosine kinase the following buffer was used as kinase buffer as recommended by the manufacturer (Upstate): 20 mM MOPS pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 0.5% Glycerol, 0.01% TritonX100, 30 mM $MnCl_2$. Per sample, 100 ng PDGFR or 7 picomol of purified tyrosine kinases were added to the kinase buffer including 0.5 mM Na-orthovanadate, 100 µM ATP plus 5µCi of [γ-^{32}P] ATP to give a final volume of 40 µl which were added to immune complexes. Incubation was 30 min (or when mass spectrometry samples were prepared 1hour) at 30°C. Reactions were stopped by one wash with cold 20 mM Tris HCl pH7.5/150 mM NaCl and the addition of SDS-PAGE sample buffer. Samples were subjected to 10% SDS-polyacrylamide gel electrophoresis, and the amount of ^{32}P incorporated into S6 protein was assessed by autoradiography and quantified by phosphorimaging (Bio-Rad).

For *in vitro* tyrosine phosphorylation of the His-tagged N-terminal fragment of S6Kα, purified tyrosine kinase and the S6Kα fragment were incubated in kinase buffer (25

mM Tris HCL pH7.5, 30 mM $MgCl_2$, 0.5 mM EGTA, 10 mM $MnCl_2$, 0.12 M KCl, 0.05% TritonX100, 0.5 mM DTT) including 0.5 mM Na-orthovanadate, 100 μM ATP plus 5 μCi of [γ-^{32}P] ATP in a volume of 25 μl for 1 hour at 30°C. Reaction was stopped by the addition of 2X Laemmli sample buffer and the proteins separated on a 4-12% Bis-Tris gradient gel (NuPage, Invitrogen) run with MES buffer and the dried gel analysed by autoradiography.

When samples were prepared for analysis by mass spectrometry, instead of [γ-^{32}P] ATP only cold ATP was used.

The cytosolic tyrosine kinases Src, Lyn, Btk and Syk were a present from Len Stephens. The recombinant cytoplasmic domain of PDGFRβ was purchased from Upstate.

2.5.10. PKC *in vitro* kinase assay

Myc-tagged PKC was overexpressed and immunoprecipitated from Hek293 cells. Beads were washed three times in lysis buffer and once in kinase buffer (20 mM Hepes pH 7.5, 10 mM $MgCl_2$. 100 μM $CaCl_2$). Immune complexes and 1 μg of histone H1 were incubated in 30 μl of kinase buffer containing additionally 100 μM ATP, 5 μCi of [γ-^{32}P]ATP, 0.03% Triton X100, 100 μg/ml phosphatidylserine and 20 μg/ml DAG. Reaction was terminated after 15 minutes at 30°C by the addition of Laemmli sample buffer and boiling the mixtures. The incorporation of ^{32}P into recombinant histone was determined by autoradiography after SDS-PAGE. Expression of Myc-PKC was assessed by immunoprecipitation and western blotting.

2.5.11. PKB/Akt *in vitro* kinase assay

HA-tagged PKB/Akt was overexpressed and immunoprecipitated from Hek293 cells. Lysates were split in two halves, one of which was used for immunoprecipitation and western blotting and the second half for immunoprecipitation and kinase assay. The latter immunoprecipitates were washed three times in lysis buffer and once in kinase

buffer (50 mM Hepes, 10 mM $MgCl_2$, pH 7.4). The reaction was carried out in in a final volume of 30 µl in 50 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 50 µM ATP, 2 µM cyclic AMP-dependent protein kinase inhibitor (PKI), and 5 µCi of [γ-^{32}P]ATP and 1 µg myelin basic protein, as a substrate at 30°C for 20 min. Reaction was stopped by the addition of 5X Laemmli sample buffer, samples were boiled and separated by SDS-PAGE. The gel was vacuum-dried and autoradiographed as previously described. The expression was assessed by western blotting with anti-HA antibody.

2.6. INVESTIGATION OF SUBCELLULAR LOCALISATION BY CONFOCAL MICROSCOPY

NIH3T3 or Swiss3T3 cells were plated onto poly-L-lysine coated coverslips in 24-well dishes at a density of 1.5 x 10^4 cells per well and cultured overnight. In some cases, cells were transfected then with 0.5 µg of expression vectors containing various S6K constructs. Twenty four hours post-transfection cells were starved in serum-free DMEM medium for 24 hours and then stimulated with 10ng/ml PDGF or serum for various times (5 minutes up to 1 hour). After a brief wash at room temperature with PBS, cells were fixed with 4% formaldehyde for 20 min and permeabilized with 0.2 % Triton-X100 in PBS for 5 min. Non-specific binding was blocked by incubation with 0.5 % bovine serum albumin in PBS for 30 min. The cells were then incubated with anti-EE (1:1500; mouse) for 2 hr at RT. After extensive washing with PBS, the samples were incubated for one hour with goat fluorescein isothiocyanate-conjugated (FITC) anti-mouse (1:200). After 50 min of incubation with the secondary antibody Rhodamin-Phalloidin was added at a final dilution of 1:2000 for 10 minutes. Finally, the coverslips were extensively rinsed with PBS, air-dried and mounted onto microscope slides using moviol mounting medium (Sigma). Immunofluorescent staining was analysed using Laser Scanning Microscope LSM510 (Zeiss, Germany), using 40×/1.30 oil Plan-Neofluar immersion objective (Zeiss, Germany). As a source of illumination for confocal microscopy Krypton/Argon laser, which emits at three wavelengths 488, 568 and 647 nm, was used.

For analysis of subcellular localisation of endogenuous S6Kα, NIH3T3, Swiss 3T3, Syf or SYF+Src fibroblasts were treated similarly with PDGF and stained with polyclonal anti-S6Kα antibody (1μg/ml) overnight. As secondary antibody anti-rabbit-FITC at a dilution of 1:200 was used.

2.7. MASS SPECTROMETRY

2.7.1. Sample preparation

2.7.1.1. *In vitro* kinase assay

SF9 cells were infected with viruses containing either EE-p70 S6Kα or S6Kβ, lysed and S6Ks immunoprecipitated using anti EE-antibody. The immunocomplexes or 1-2μg of recombinant protein were subjected to an *in vitro* kinase assay using tyrosine kinases as described (2.5.9.) but without [γ-^{32}P]ATP. The reaction products were separated by SDS-Page, Coomassie or silver-stained (Svechenko *et al.*, 1996) and digested using endoproteases trypsin or GluC.

In other experiments, S6K and receptor tyrosine kinases or the cytosolic tyrosine kinase fyn were co-expressed in SF9 cells. In this case, no *in vitro* assay was performed as it was assumed that overexpression of a tyrosine kinase would lead to its activation and subsequent tyrosine phosphorylation of *in vivo* substrates. S6K from these cells was immunoprecipitated, the immunocomplexes were separated on SDS-PAGE and the stained gel bands corresponding to S6K were used for the in-gel digest.

Alternatively, other *in vivo* derived samples were prepared. S6Kinases were overexpressed together with a wild type or activated mutant of Src or PDGFR in Hek293. Cells were starved and stimulated with either 20% FCS/pervanadate or PDGF/pervanadate for 15-30 minutes. Finally, S6Ks were immunoprecipitated using EE-antibody, SDS-PAGE purified and stained gel bands digested.

2.7.1.2. In-gel digest

Briefly, bands were excised from the gel with a clean scapel and cut into small $1mm^3$ cubes. In a wash step, 10 µl H2O was added and pieces left for 15 minutes. Then 10 µl acetonitrile were added for another 15 minutes to dehydrate the cubes. Liquid was removed and procedure repeated once. 20 µl of 50mM NH_4HCO_3 were added for 15 min, followed by 10 µl acetonitrile. After further incubation of 15 minutes gel pieces were shrunken in a vacuum centrifuge. When dry, trypsin (porcine sequencing grade, Promega) or Glu-C (Roche Biochemicals) was added in 50 mM NH_4HCO_3/5 mM $CaCl_2$ in an approximate ratio of (protein:endoprotease)=1:20 (w:w). The cubes were well covered in 25mMNH_4HCO_3 to avoid drying of the reaction mixture. Incubation took place overnight at 28°C. The supernatant contained the extracted S6K peptides and was directly used for mass spectrometry or desalted using C-18 resin loaded Zip-Tips (Millipore).

2.7.1.3. Desalting with Zip-Tip or Bruker C3 beads

After digestion with an endoprotease, the sample was usually desalted using ZipTips (Millipore). ZipTips are 10µl pipette tips, which have been loaded with a small volume of C18 resin at the end of the tip and, therefore, constitute small and fast-to-use reverse phase columns. Briefly, the tips were pre-wetted with 5 µl 50/50 CH_3CN/H_2O, then equilibrated with the same volume of 2/98 CH_3CN/H_2O. Typically, 2-5 µl of sample mixture was taken up and down the tip. The resin was washed once or twice with 10 µl of 2/98 CH_3CN/H_2O. Peptides were then eluted in 2-3 µl of elution solution. Depending on the scanning mode to be applied in the mass spectrometry experiment the elution solution was either 50/49/1 CH_3CN/H_2O/formic acid or 50/45/5 $CH_3CN/H_2O/NH_3$ for positive and negative ion scanning, respectively.

The 52 amino acid long N-terminal fragment of S6Kα was subjected to an *in vitro* kinase assay (2.5.9.) and was to be used directly for mass spectrometrical analysis, without proteolytic digestion. Consequently, all the buffer compenents, salts, inhibitors as well as Src kinase were still present in the mixture. In some cases, if low solubility

matrices are used, some impurities may be removed on target, but this was not satisfactory for analysis of the S6K fragment. Therefore, magnetic particles with a porous surface functionalised with hydrophobic coating (C3) (MB-HIC for Magnetic Beads based hydrophobic interaction chromatography, Bruker Daltonics) were used for purification of the sample. 10µl of binding solution, 2 µl sample and 5 µl beads were mixed. After one minute the beads (with bound peptides) were concentrated along the wall of the tube with a magnetic device, the remaining buffer taken off. Beads were then resuspended in 100 µl wash buffer which was again removed with the help of the magnetic device. This process was repeated 8 times. Finally the peptides were eluted in a small volume of 50% acetonitrile and used for MALDI analysis.

2.7.1.4. HPLC

High performance liquid chromatography (HPLC) was performed in combination with the ion trap instrument for neutral loss MS/MS analysis. A 75 µm x 5 cm picofrit C18 column (BioBasic) was used and peptides were separated using a linear gradient of a% to 65% solvent B over 60 or 90 minutes. Solvent A was 98/2/0.1 H_2O/CH_3CN/formic acid and solvent B was 95/5/0.1 CH_3CN/H_2O/formic acid. The flow rate was 200nl/min which was achieved by splitting the initial flow of 100µl/min. The eluting peptides were directly injected into the electrospray source.

2.7.2. Instrument settings

2.7.2.1 Triple Quad

The API3000 (SCIEX) instrument was used to perform precursor ion scans for the selection of ions m/z-79 which is characteristic for all phosphoamino acids. The operating parameters were optimised previously in the laboratory by Sarah Brooks (GSK) using 13pmol/µl of a commercial phosphopeptide (DpYVPML, Bachem) in 50/45/5 $CH_3CN/H_2O/NH_3$. The analyte mix was desalted with ZipTips and injected into

a nanospray needle (New Objective PicoTip; 2mm ID tip). First, a full scan was acquired in the negative mode then the instrument was switched into precursor ion mode, scanning over m/z 300-1500 in 5 seconds and potentiating the acquired spectra (MCA). The optimised instrument paramaters were the following: ion spray voltage – 300V; Nebuliser gas: 1 unit; curtain gas: 8 units; declustering potential –40 V; focusing potential –30 V; entrance potential –10 V. The analyte mix was desalted with ZipTips and injected into a nanospray needle (New Objective PicoTip; 4μm ID tip).

2.7.2.2. Ion Trap

The instrument, a LCQ Deca (ThermoElectron), was operated in a "double play" mode set to automatically acquire a full scan between m/z 350-1800 and a CID spectrum with a relative collision energy of 35% of the three most intense ions detected in the full scan. The source conditions were capillary temperature: 250 ^{o}C; sheath gas flow: 0 units; auxillary gas flow: 0 units; ESI voltage: 1.0 kV; capillary voltage: 28 V; tube lense offset: -60V. The mass isolation window was set to 4 m/z mass units. These parameters were set up previously in the laboratory by A. West (GSK).

2.7.2.3. Q-ToF

The instrument was a Q-Star Pulsar i (SCIEX) combined with an electrospray ion source (Protana) and it was used to sequence the peptides that had been detected as potential phosphopeptides by precursor ion scanning in the negative ion mode. The analyte mix was desalted with ZipTips and injected into a nanospray needle (New Objective PicoTip; 2μm ID tip). First, a full scan was acquired in the positive mode and by focusing into the region of interest, it was determined if the peptide was present in the spectrum and if the charge state could be confirmed. Then the instrument was switched to perform a product ion MS/MS scan (PIS). The Quadrupole was used to scan over a range of m/z 300-1500, CID was performed in the collision cell and the TOF tube was used to separate the fragment ions. The collision energy was manually ramped up (up to 45%) until suitable fragmentation was observed. The experiment was

performed using both the enhanced and MCA scan modes of the instrument. In some cases, however, the phosphopeptide could not be detected in a full scan, but still a product ion scan was performed.

The sequence was deduced manually. Alternatively, Mass Lynx software was used to simulate the fragmentation and to match the theoretical values with the peaks from the spectra. Sciex sequencing software was also used to pick the peaks from a spectrum which correspond to ions within a fragmentation series. The Q-Tof instrument was also used for manual protein identification by acquiring a peptide fingerprint in a Tof-MS scan and matching the peptides to a non-redundant protein in-house or NCBI database. The instrument settings were previously optimised by A.West and S.Brooks (GSK).

2.7.2.4. MALDI-TOF

MALDI samples were prepared using the „dried droplet" method, which involves mixing 0.5 µl of the analyte solution with 0.5-1µl of the matrix solution (mainly 2,5 Dihydobenzoic benzoic acid, DHB or 5-methoxysalicylic acid/2.5-dihydroxybenzoic acid 1/10, Super DHB) on the target and drying by means of a warm stream of air. Measurements were conducted on a Ultraflex TOF/TOF (Bruker Daltronics) instrument. The mass spectrometer was equipped with delayed extraction and a reflector analyser for improved mass resolution and accuracy. For the sequencing experiment the instrument was run in reflectron mode. The instrument was operated at 25kV source potential in positive ion mode with pulsed nitrogen laser at a wavelength of 337nm and a pulse width of 3 ns. The laser power was adjusted manually and the minimal laser fluency used to give a high signal to noise ratio whilst minimising metastable decay.

CHAPTER 3: ANALYSIS OF GENERAL S6K TYROSINE PHOSPHORYLATION AND RECEPTOR ASSOCIATION

CHAPTER 3

ANALYSIS OF GENERAL S6 KINASE TYROSINE PHOSPHORYLATION AND RECEPTOR ASSOCIATION

3.1. INTRODUCTION

As S6Ks become strongly phosphorylated upon mitogenic stimulation, and are also situated in a pathway, which involves tyrosine phosphorylation, we questioned if S6Ks themselves could become a substrate for tyrosine kinases. Numerous studies in various laboratories had only revealed that S6Ks were phosphorylated on serines and threonines. In a phosphoamino acid analysis of serum stimulated Swiss 3T3 cells no phosphotyrosine residues were found (Ballou *et al.*, 1988). However, recent publications showed tyrosine phosphorylation of other AGC kinases such as PKB/Akt, PDK1 or various isoforms of PKCs. S6Ks share important features with other AGC kinases, mainly their phosphorylation-mediated activation mechanism consisting of two crucial sites, one in the kinase extension and one in the kinase domain. Therefore, it was logical to investigate if tyrosine phosphorylation of S6Ks would also occur.

3.2. TYROSINE PHOSPHORYLATION OF S6KS

3.2.1. S6K tyrosine phosphorylation in Sf9 cells infected with tyrosine kinases

In a preliminary experiment we used a baculoviral expression system in Sf9 insect cells. We infected the cells with either cytoplasmic EE-tagged S6Kα or S6Kβ together with a receptor tyrosine kinase such as epidermal growth factor receptor (EGFR),

HER/neu receptor (NeuR), colony stimulating factor receptor (CSFR), hepatocyte growth factor receptor (HGFR), platelet-derived growth factor receptor (PDGFR) or the cytosolic tyrosine kinase Fyn.

In this study, mainly the cytoplasmic isoforms (p70) have been used in overexpression experiments. Therefore, in the course of this report, p70 S6Kα/β will simply be annotated as S6Kα or β.

We hypothesised that infection with viruses containing RTKs and strong overexpression of RTKs could lead to a constitutive activation of receptors and downstream pathways in Sf9 cells. The high concentration of the transmembrane monomer chains could facilitate dimerisation, trans-phosphorylation and finally activation of RTKs. Indeed, when we immunoprecipitated S6Ks with anti-EE-tag antibody and probed the membrane with phosphotyrosine antibody (4G10), we found that some tested kinases induced tyrosine phosphorylation of S6Kα and β (Fig. 3.2.1a.) in three individual experiments. Very strong phosphorylation of S6Kα and β was generated by expression of HGFR, PDGFR and CSFR. In the case of S6Kβ but not S6Kα, cytoplasmic tyrosine kinase fyn also induced tyrosine phosphorylation (as shown in Fig. 3.2.1a). Interestingly, the RTKs leading to tyrosine phosphorylation of S6K also co-immunoprecipitated specifically with S6Ks and not with protein G-sepharose beads alone (Fig. 3.2.1b). This pointed to an *in vivo* interaction between S6K and RTKs, which will be investigated in the next subchapter. In this preliminary experiment we did not test for the expression of NeuR and EGFR receptors that did not lead to tyrosine phosphorylation of S6K. However, the viruses were made and tested positively for expression previously. Furthermore, as this experiment was only thought to be a test of principle to find out if tyrosine phosphorylation of S6K could occur *in vivo* we did not intend to investigate the lack of S6K tyrosine phosphorylation in EGFR or NeuR expressing cells any further.

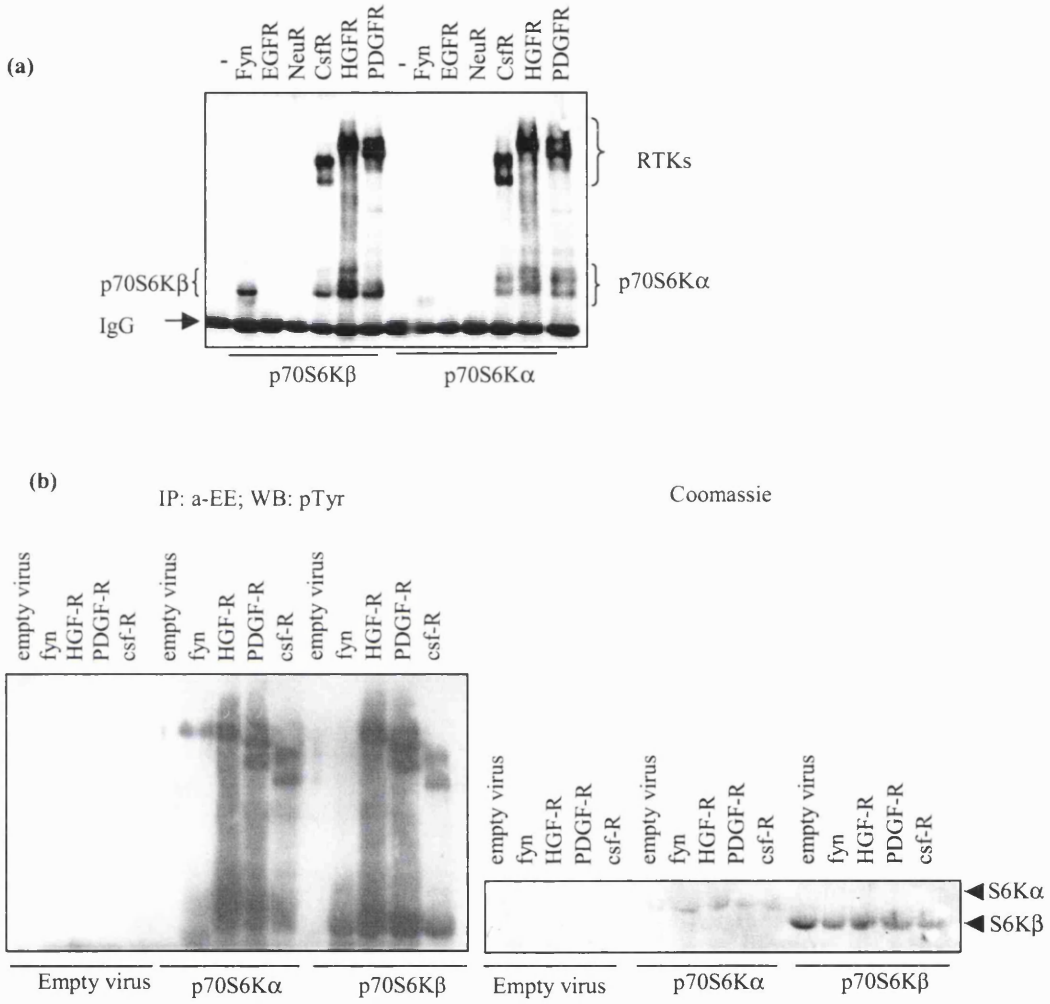


Fig. 3.2.1. Tyrosine phosphorylation and receptor association of S6Ks in Sf9 cells.
(a) Sf9 cells were infected with baculoviruses encoding either EE-tagged S6Kα or S6Kβ and RTKs (EGFR, NeuR, CSFR, HGFR, PDGFR) or Fyn. Cells were lysed two days post-infection and S6Ks were immunoprecipitated with anti-EE antibodies. Samples were resolved by SDS-PAGE, transferred onto membrane and analysed by immunoblotting with monoclonal antibodies against phosphotyrosine (4G10). **(b)** To ensure that tyrosine kinases co-immunoprecipitate specifically with S6Ks (and not with anti-EE antibody or beads alone), cells were infected with tyrosine kinases alone or with S6Ks and tyrosine kinases. All lysates were subjected to immunoprecipitation using anti-EE-antibody and the membrane was blotted with 4G10 antibody. The membrane was then Coomassie-stained.

3.2.2. Tyrosine phosphorylation of S6Ks *in vitro*

As PDGFR was among the RTKs which generated a strong phosphotyrosine signal on S6Ks and as stimulation of mammalian cells with PDGF leads to activation of S6Ks, we chose this receptor for the following experiments. First, we wanted to investigate if PDGFR could directly phosphorylate S6K in an *in vitro* kinase assay. We found that the recombinant cytosolic domain of PDGFRβ was able to phosphorylate S6Ks efficiently in three independent experiments (Fig. 3.2.2a.). We could also detect PDGFR autophosphorylation under conditions used but to a very low degree. This was due to the fact that the sepharose beads with immunopurified S6Ks were washed once after the kinase reaction with 20mM Tris/150mM NaCl and hence the majority of PDGFR molecules were washed away. We further tested a panel of non-receptor tyrosine kinases, such as Src and Fyn as members of the Src family tyrosine kinase, Bruton' s tyrosine kinase (Btk) and Syk in an *in vitro* kinase assay.

As shown in Fig. 3.2.2.b., all tested kinases could use, in four experiments, S6Ks as a substrate *in vitro*, but Src kinase had a higher phosphorylation efficiency. However, it is impossible to judge if this stronger phosphorylation by Src accurately reflects the processes in a cell, as *in vivo* phosphorylation may require co-factors and binding partners, which are absent in the *in vitro* assay.

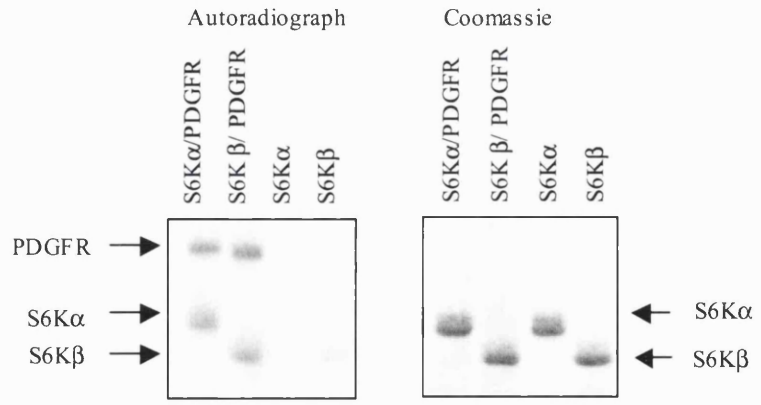


Fig. 3.2.2. Tyrosine phosphorylation of S6Ks *in vitro*

(a) The cytoplasmic domain of PDGFR phosphorylates S6Ks *in vitro*. S6Ks were immunoprecipitated from Sf9 cells with anti-EE antibody and subjected to a kinase assay with PDGFR as kinase for 1hour at 30°C. 100ng of PDGFRβ were used per sample. Autoradiograph and Coomassie–stained gel are shown.

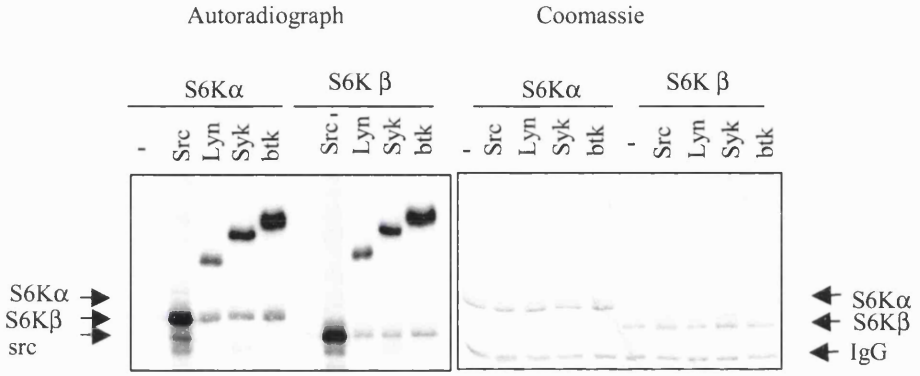


Fig. 3.2.2b. Cytosolic tyrosine kinases phosphorylate S6Ks *in vitro*.
S6Ks were immunoprecipitated from Sf9 cells and subjected to an *in vitro* tyrosine kinase assay for 30min at 30°C. 7 picomol of the different tyrosine kinases were used. Autoradiograph and the Coomassie–stained gel are shown.

Another explanation for differences in phosphorylation efficiency could simply be that the specific activity of Src was higher when compared to the activity of other kinases. As Src kinase with a molecular mass of 59 kDa migrates at the same spot in an SDS-PAGE gel as S6Kβ, the autoradiography signal from the samples S6Kβ+Src represents the combined signal from tyrosine-and autophosphorylated S6K and autophosphorylated Src. The intensity of autophosphorylated Src can be seen as the lower band in the autoradiography of the S6Kα plus Src sample and is very low. Therefore, the autoradiography signal in the Src plus β sample must mainly stem from S6Kβ itself.

3.2.3. Tyrosine phosphorylation of S6Ks in mammalian cells

3.2.3.1. Tyrosine phosphorylation of overexpressed S6Ks

To test whether tyrosine phosphorylation would not only occur in Sf9 cells or *in vitro*, but also in mammalian cells, we transiently transfected Cos7 or Hek293 cells with S6Ks and PDGFRβ. As hypothesised, we found PDGF-dependent tyrosine

phosphorylation of immunoprecipitated S6Kinases in at least four different experiments. Tyrosine phosphorylation reached its maximum at 30 minutes of stimulation and decreased after 1 hour (Fig. 3.2.3.). Similar results were also obtained for Hek293 or PAE cells (porcine aortic endothelial cells) but are not shown here.

An efficient way to enhance tyrosine phosphorylation is to treat cells with the tyrosine phosphatase inhibitor Na-Pervanadate. Vanadium was found to mimic the action of insulin in three ways, by direct insulin-mimetic action, enhanced insulin sensitivity and prolongation of the biological insulin response (Fantus and Tsiani, 1998). All three aspects were attributed to the inhibitory action of vanadate compounds towards phosphoprotein tyrosine phosphatases (PTPs).

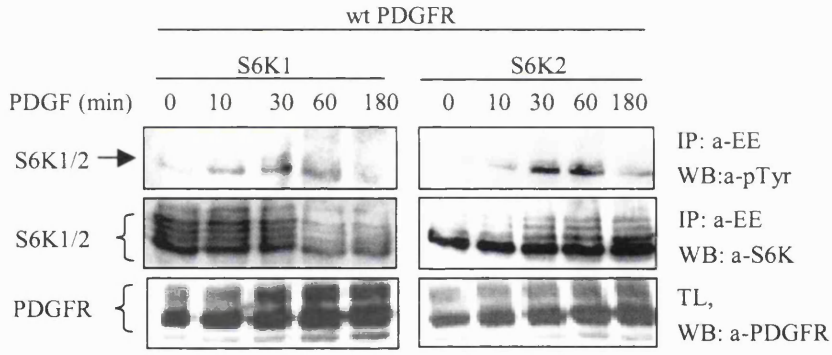


Fig. 3.2.3. S6Ks are tyrosine phosphorylated in transfected Cos7 cells.
PDGFR and S6Kα or S6Kβ were used for electroporation of Cos7 cells. 24 hours post transfection cells were starved for another 24 hours and then stimulated with 40ng/ml PDGF for the indicated times. S6Ks were immunoprecipitated from the lysates and analysed in a phosphotyrosine western blot, followed by stripping and reprobing the membrane with anti-S6K antibody. The soluble fraction of total lysate (TL) was also used for western blotting with anti-PDGFR antibody to assess PDGFR expression. In a SDS-PAGE gel PDGFR migrates at a position of ca. 135kDa of which is higher than expected (MW=123 kDa), this is probably due to post-translational modifications such as glycosylation

As shown in Fig. 3.2.1., in Sf9 cells the Src family tyrosine kinase Fyn induces tyrosine phosphorylation of S6Kβ in Sf9 cells. Furthermore, we found that both S6Kα and S6Kβ are very good substrates for Src kinase in an *in vitro* assay. Therefore, in the

experiment shown in Fig. 3.2.4., Src kinase was overexpressed together with S6K. Starved cells were stimulated with different doses of Na-pervanadate in combination with serum. In a dose-dependent manner, stimulation with pervanadate leads to an enhanced tyrosine phosphorylation of S6Ks. Three dose-course experiments were performed.

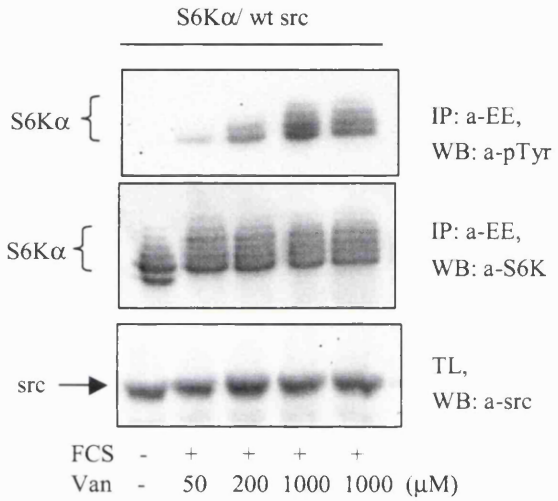


Fig. 3.2.4. Na-Pervanadate induces tyrosine phosphorylation of S6Kα.
Hek293 cells were transiently transfected with S6Kα and wt Src, starved for 24 hours before being stimulated with 10% FCS for 15 min and Na-pervanadate at the concentrations indicated for additional 10 min. S6K was immunoprecipitated with anti-EE antibody and analysed by western blotting with 4G10 antibody. Membrane was then stripped and reprobed with anti-S6K antibody. Total lysate (30μg) was used to test the expression of Src.

Our collaborators A. Romanelli and P. Blume-Jensen (Serono, USA) found that upon stimulation of Hek293 cells transiently transfected with constructs for S6Kα and Kit (stem cell factor receptor), a member of the PDGFR family, mainly involved in hematopoesis and differentiation, pervanadate can also induce tyrosine phosphorylation of S6K (Fig. 3.2.5.). This indicates that, in addition to PDGFR, other RTKs might also mediate tyrosine phosphorylation of S6Ks in mammalian cells. It was also shown that

addition of stem cell factor augmented the S6Kα-phosphotyrosine signal of pervanadate-treated cells (data not shown).

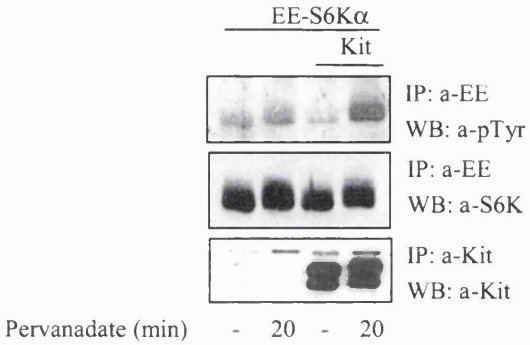


Fig. 3.2.5. Tyrosine phosphorylation of S6Kα when overexpressed with Kit.
Hek293 cellswere transiently transfected with S6Kα and Kit, starved for 24 hours before being stimulated with pervanadate for 20 min. S6K was immunoprecipitated with anti-EE antibody and analysed by western blotting using phosphotyrosine antibody (4G10). Membrane was then stripped and reprobed with anti-EE antibody. Additionally, Kit was immunoprecipitated from lysates and analysed by western blotting. This experiment was performed by A.Romanelli, Serono, USA.

3.2.3.2. Tyrosine phosphorylation of endogenous S6Kα

To strengthen our observation we wanted to examine whether endogenous S6K would also become tyrosine phosphorylated. Initially we tested tyrosine phosphorylation of endogenous S6Kα in MCF7, Hek293 and an ErbB2 overexpressing breast luminal epithelial cell line "5.2" (Timms *et al.*, 2002) but could not detect a specific signal. However, NIH3T3 cells which have relatively high levels of PDGFR and S6Kα (p70 and p85) gave a positive signal for S6K in the phosphotyrosine blot (Fig. 3.2.6.). Cells were starved and stimulated with serum for the indicated times. The result confirmed that endogenous S6Kα becomes tyrosine phosphorylated upon stimulation in a similar

time course as transiently expressed S6K in Cos7 cells. Interestingly, the phosphorylation of the nuclear isoform, p85 S6Kα is delayed by approx. 30 minutes compared to p70. In this experiment, the tyrosine phosphorylated bands of S6K corresponded to the activated, i.e. slower-migrating bands of S6Kinase. This indicates that S6K has to at least partially be activated and phosphorylated before tyrosine phosphorylation can occur. Three successful experiments were performed.

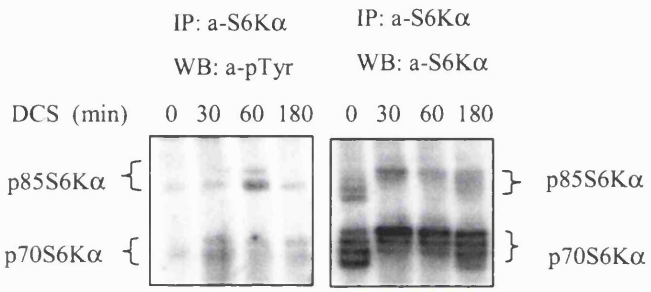


Fig. 3.2.6. Tyrosine phosphorylation of endogenous S6Kα.
NIH 3T3 cells were serum-starved and stimulated with 10% Donor calf serum (DCS) for the indicated times (30, 60, 180 min). Endogenous S6Kα was immunoprecipitated using antibody against its C-terminus. Denatured proteins were separated by SDS-PAGE, transferred onto nitrocellulose and blotted with phosphotyrosine antibody. The membrane was consequently reprobed with C-terminal S6K antibody. In this experiment we focused on S6Kα as NIH3T3 cells do not express a detectable amount of S6Kβ.

3.3. RECEPTOR ASSOCIATION OF S6KS

Having shown that S6Ks become phosphorylated on tyrosine upon mitogenic stimulation in mammalian cells, it remained to be established if S6Ks would also be in a complex with RTKs as the preliminary experiment in Sf9 cells had suggested.

3.3.1. Association of S6Ks with PDGFR in transiently transfected cells

The experiments were carried out in Cos7, Hek293 and NIH3T3 cells. We overexpressed PDGFR and S6Ks, starved and stimulated the cells with PDGF, immunoprecipitated S6K from the lysates and probed the membranes with antibodies against PDGFR.

PDGFR was found to co-immunoprecipitate with S6K in Cos7 cells overexpressing receptor and S6Ks in three independent experiments (Fig. 3.3.1). This association appears to be independent of PDGF, however, one has to take into account that the receptor is highly overexpressed, which leads to constitutive activation (as shown by the 4G10 blot) and may change its affinity to S6K. We had previously shown in Sf9 cells that PDGFR specifically binds to S6K and not to beads or antibody alone.

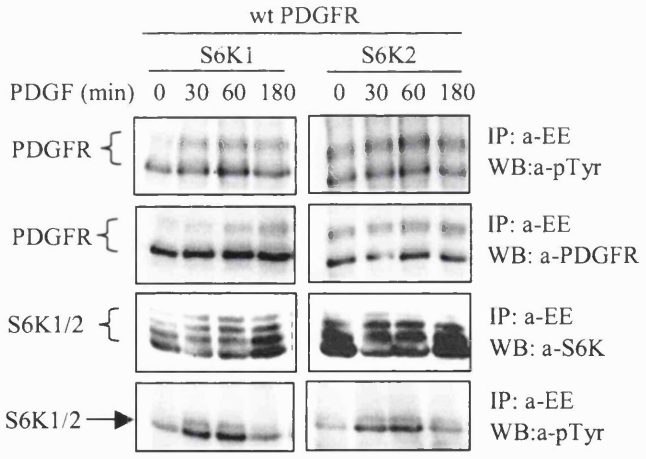


Fig. 3.3.1. PDGFR co-immunoprecipitates with S6Ks in Cos7 cells.
Cos7 cells were electroporated with PDGFRβ and wild type EE-tagged S6Ks, serum starved and stimulated with 40ng/ml PDGF for the indicated times. EE-tagged S6Ks were immuno-precipitated with the anti-EE antibody and immunoprecipitates analysed by western blot with 4G10, anti-PDGFR and anti-S6K antibodies.

3.3.2. Association of S6K with Kit in transiently transfected cells

In a similar set-up, our collaborators found that when Hek293 cells overexpressing S6Kα and Kit are stimulated with stem cell factor (ligand for Kit), the receptor can be-immunoprecipitated with specifically S6Kα (Fig.3.3.2.) in a stem-cell factor dependent manner.

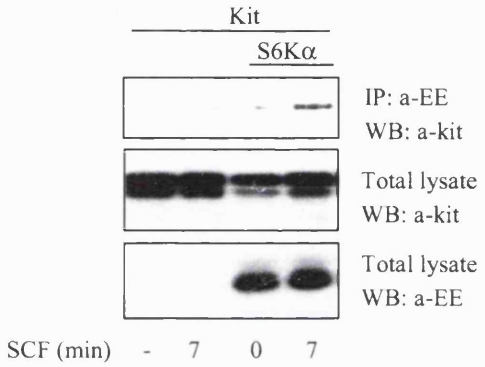


Fig. 3.3.2. Kit co-immunoprecipitates with S6K.
Hek293 cells, transfected with S6Kα and Kit as indicated, were starved for 24 hours and then stimulated with SCF for 7 min. Lysates were subjected to immunoprecipiation using either anti-EE antibody and analysed with anti-Kit antibodies. Total lysate was also tested for S6K and Kit expression. This experiment was performed by A.Romanelli, Serono, USA.

3.3.3. Association of endogenous S6K with insulin receptor

In order to test if S6Ks also associate with other RTKs such as the insulin receptor, CHO-IR cells were used. This chinese hamster ovary cell line stably overexpresses insulin receptor. Co-immunoprecipitation experiments were performed using antibodies against the C-terminus of endogenous S6K and anti-IR antibodies. In Fig. 3.3.3., the right panel indicates that S6K associates with insulin receptor in a stimulation–dependent manner whereas a small amount of insulin receptor seems to co-immunoprecipitate constitutively with S6K (left panel). The reason for this could for example be that insulin receptor is very abundant in these cells and sticks to protein A-

sepharose beads quite easily. However, this option was tested and no detectable amount of IR precipitates with beads alone. The small non-inducible amount of insulin receptor, which immunoprecipitates with S6K antibodies could represent non-specific binding to the antibody. We tested if the insulin receptor may be active even in starved cells due to overexpression by western blotting with pTyr antibody but found that it was inactive (data not shown). On the other hand, the lack of more specific inducible binding could be caused by the antibody blocking a domain on S6K, which is needed for the association with the insulin receptor. The presented data is only preliminary and was not confirmed, but we decided not to pursue this aspect but to focus on the PDGFR system.

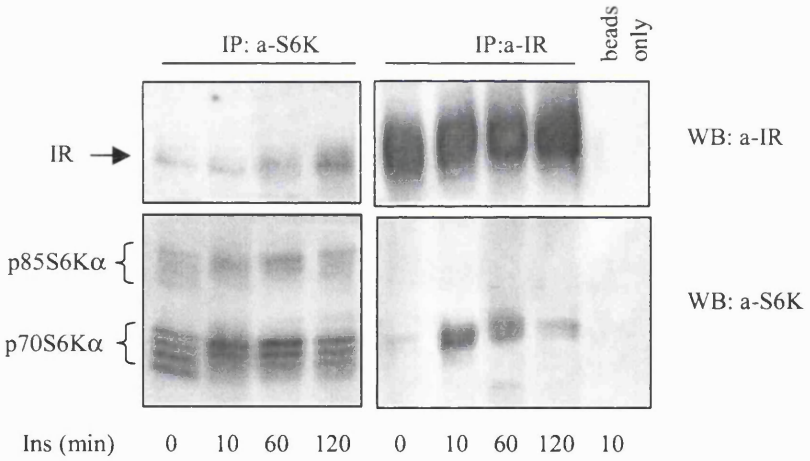


Fig. 3.3.3. Insulin receptor co-immunoprecipitates with S6K.
CHO-IR cells were starved for 20 hours and then stimulated with insulin (100nM) for the indicated times. Lysates were subjected to immunoprecipiation using either anti-S6K (C-terminal) antibody or anti-IR-antibody and analysed by immunoblotting with the indicated antibodies. As a negative control, lysates were incubated with ProteinA-sepharose beads only.

3.3.4. Association of endogenous S6K and PDGFR

Finally, we found that in serum-stimulated NIH3T3 cells, endogenous PDGFR is in complex with endogenous S6K (Fig. 3.3.4.). Here, the association is inducible. The association is strongest between 10 minutes and thirty minutes and already decreased after one hour of stimulation. This suggests that the constitutive complex seen in cells transfected with PDGFR was indeed an effect of the overexpression and that under physiological circumstances S6K would only be recruited to an activated RTK. Combining our findings, we found that overexpressed and endogenous S6Ks are in complex with receptor tyrosine kinases in an inducible manner and this association parallels the kinetics of tyrosine phosphorylation. This suggests that the physical association of receptor and S6Ks may lead to tyrosine phosphorylation of S6Ks.

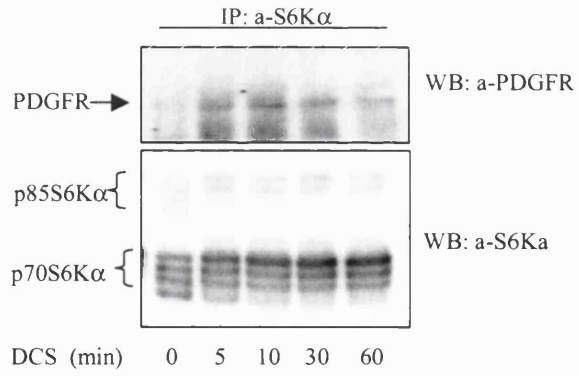


Fig. 3.3.4. Endogenous PDGF receptor co-immunoprecipitates with S6Kα.
NIH3T3 cells were starved with 0.3% DCS for 24 hours before stimulation with 10% DCS. Endogenous S6Kα was immunoprecipitated with antibody against its C-terminus and immunocomplexes analysed by immunoblotting using anti-S6K or anti-PDGFR antibodies.

3.3.5. Interaction with receptor is mediated via kinase/ kinase extension domain of S6Ks

To determine which domain of S6K interacts with the cytoplasmic domain of PDGFR we transfected Hek293 cells with PDGFR and either wild type or deletion mutants of S6K that lacked the regulatory N- or C-terminus or both. In the diagram (Fig. 3.3.5.) the different truncation mutants are shown, the C-terminally truncated mutants not only lack the far C-terminus but also the autoinhibitory pseudosubstrate motif. The ΔNΔC mutant is a serum-stimulatable, but rapamycin insensitive variant of S6K (Weng *et al.*, 1995b). Deletion of the N-terminus alone leads to an inactive kinase. Immunoprecipitating S6Ks with antibodies against their tags (EE and Flag) we could show that all mutants could associate with the receptor indicating that the interaction is exerted via the S6K kinase and/or the kinase extension domain (Fig. 3.3.6.). Repeatedly, in three independent experiments, the S6Kα ΔN and S6Kβ ΔC could immunoprecipitate more PDGFR, the reason for this is unclear as it does not correlate with the expression of the receptor or the expression of S6Ks.

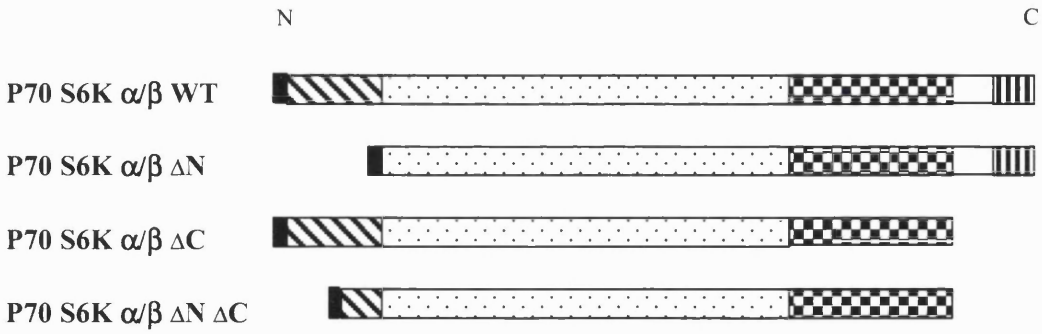


Fig. 3.3.5. Schematic representation of S6K truncation mutants.
The N-terminal tag (EE or Flag) is shown as a black box, the N-terminal regulatory domain as diagonally hatched and the kinase domain as dotted box. The kinase extension is represented in a chequed pattern, the autoinhibitory pseudosubstrate sequence in white and the C-terminal regulatory region as vertically striped. The truncation mutants were previously generated by T. Valovka and K. Yonezawa. In the EE-tagged p70S6Kα and S6Kβ ΔN 52 and 51 amino acids are missing from the N-terminus, while in the EE-tagged p70 S6Kα and S6Kβ ΔC 100 and 81 amino acids from the C-terminus are missing, respectively. 23 and 104 amino acids from N-and C-terminus in the doubly truncated flag-tagged p70 S6Kα ΔNΔC, whereas 23 and 85 amino acids in the flag-tagged p70 S6Kβ ΔNΔC are cut off.

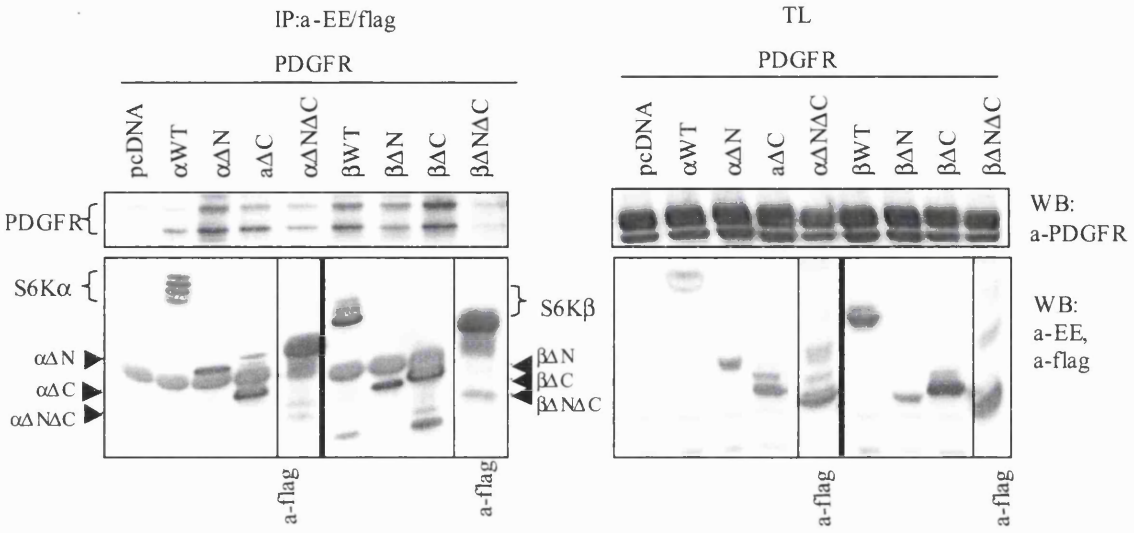
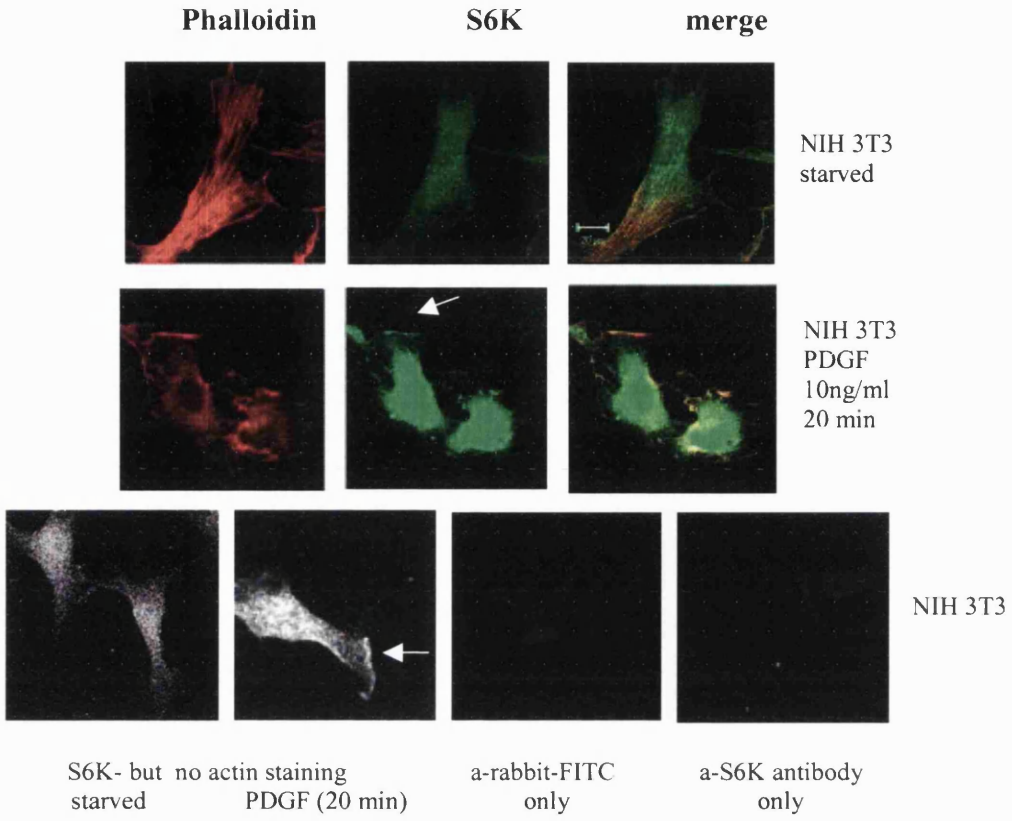


Fig. 3.3.6. Association with PDGFR is mediated by S6K kinase or kinase extension domain.
WT and truncated mutants of S6K (EE-S6Kα/β, EE-S6Kα/β ΔN, EE-S6Kα/β ΔC, Flag-S6Kα/β ΔNΔC) and PDGFR-WT were transiently expressed in Hek293 cells. S6Ks were immunoprecipitated using either anti-EE or anti-flag antibody and analysed by SDS-PAGE. Western blotting was performed with a-EE/anti-flag antibodies (lower panels) and anti-PDGFR antibody (upper panels). To unambigously identify the migratory position of S6Ks, the total lysates (30μg) were also run on the same gel. The positions of S6Ks are indicated with arrows.

3.4. ANALYSIS OF S6K LOCALISATION BY IMMUNOFLUORESCENCE MICROSCOPY

Not much is known about the detailed localisation of the different isoforms of S6Ks. In general, the long isoforms of S6Kα and S6Kβ are predominantly nuclear and this is due to the presence of an additional nuclear localisation sequence in the extended N-terminal stretch. The shorter p70 isoforms are found in the cytosol and in the nucleus (Coffer and Woodgett, 1994; Minami *et al.*, 2001), with S6Kα predominantly present in the cytosol and S6Kβ predominantly found in the nucleus (Koh *et al.*, 1999) due to an additional nuclear localisation sequence in the C-terminus. In our laboratory we have repeatedly observed a mitogen-mediated translocation of p70S6Kα into the nucleus.

Upon stimulation, p70S6Kβ can also be detected in the cytosol (Valovka *et al.*, 2003). This seems to be mediated by phosphorylation of a serine site (S473), which is located in the middle of a nuclear localisation sequence whose function is disrupted when S473 is phosphorylated. More recently, the F-actin binding protein Neurabin was found to bind to S6K (Burnett *et al.*, 1998b). Regulators of the actin cytoskeleton, cdc42 and rac (responsible for filopodia and ruffle formation respectively) were also shown to bind to and activate S6Kα in COS and NIH3T3 cells. (Chou *et al.*, 1996). Immunofluorescence microscopy revealed that S6Kα is localised along stress fibers which are induced by thrombin. Upon for example EGF or TPA stimulation S6Kα can be found in the actin arc which is a caveolin-enriched structure found solely in migrating cells. The pool of actin arc–localised S6Kα has a high activity whereas S6K along the stress fibers has low activity. Disruption of the cytoskeleton with Cytochalasin D resulted in S6K activation (Berven *et al.*, 2004; Crouch, 1999). Other AGC kinases (PDK1, PI3K and PKB/Akt) are well known to be localised at the membrane and are also found enriched in membrane ruffles or actin arc structures (Berven *et al.*, 2004). It was previously shown that, in fibroblasts, PKB/Akt is recruited to membrane ruffles upon mitogen treatment and this translocation was visualised by immunofluorescence microscopy (Watton and Downward, 1999). We therefore decided to investigate if membrane localisation of S6K could be detected in a similar way. NIH3T3 cells were stimulated with PDGF for 5, 10 or 20 minutes, fixed and stained with an antibody against the C-terminus of S6Kα. Using a FITC-labeled secondary anti-rabbit antibody and phalloidin actin staining in immunofluorescence microscopy, S6K was shown to be evenly distributed in the cytoplasm of starved cells, including the membrane region. We could also detect co-localisation with stress fibers (data not shown). Stimulation lead to a redistribution of the bulk of S6K towards the nucleus or the perinuclear region, however, a small fraction could be detected in membrane ruffles. In Fig. 3.4.1. (upper part) only one time point is shown (20 minutes), however, ruffling and the presence of S6K in ruffles could be detected earlier. Swiss cells transformed by v-Src exhibit very strong ruffles upon PDGF treatment. Therefore we assessed S6Kα localisation in these cells. As can be seen in Fig. 3.4.1 (lower part), S6Kα can be detected within the ruffles after 5 minutes of stimulation.



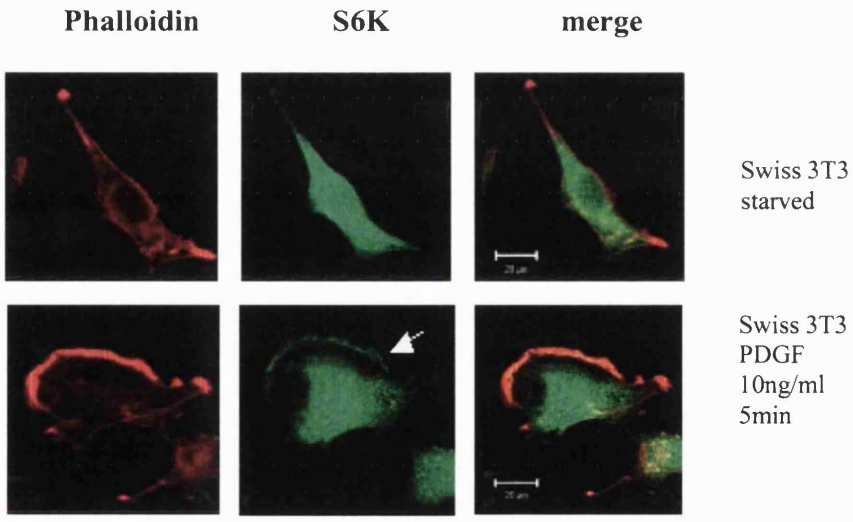


Fig. 3.4.1. S6Kα is localised in membrane ruffles upon PDGF stimulation in NIH3T3 and Swiss3T3 cells.
Cells were starved for 24 hours and stimulated with PDGF. Cells were fixed, permeabilised, blocked and probed with anti-C-terminal S6Kα- and secondary FITC-anti-rabbit antibody. Actin was visualised by phalloidin staining. White arrow indicates ruffles. Bar represents 20μm.

3.5. DISCUSSION

Membrane translocation in response to mitogenic stimulation has been shown for a variety of AGC kinases, including PKB/Akt, PDK1, PKD and various isozymes of the PKC family. This is mainly thought to occur via binding to second messengers, such as phospholipids or molecules downstream of the RTKs such as adaptors. For example, translocation can be mediated through PH domains (for PDK1 or PKB/Akt), which bind to phospholipids generated by PI3 Kinase. PKC translocation is mediated by a variety of isoform-specific RACKs (receptors for activated C-kinase) (Schechtman and Mochly-Rosen, 2001). Furthermore, many AGC kinases have been shown to be substrates for Src kinase, which can directly associate to an RTK such as PDGFR. We are not aware of any published co-immunoprecipitation studies of RTKs and AGC kinases.

So far, few potential mechanisms exist by which S6K could be integrated into multienzyme signalling complexes formed around activated RTKs. Firstly, the PIF pocket of PDK1 was shown to interact with the S6K motif surrounding phosphorylated T389, the PIF domain. Therefore, PDK1 could bring S6K to the plasma membrane. Secondly, small GTPases of the Rho family, Rac and Cdc42 were found to associate with and activate S6K (Chou *et al.*, 1996). Cdc42 and Rac are membrane-bound, thus they possibly could integrate S6K in signalling complexes on the cellular membrane (Del Pozo *et al.*, 2002). It has also been shown that the p85 adaptor subunit of PI3K is also in complex with S6K and that complex formation is necessary for mTor signalling (Gonzalez-Garcia *et al.*, 2002). We propose that S6K becomes recruited into complexes with receptor tyrosine kinases. *In vitro*, the recombinant cytosolic domain of PDGFR can phosphorylate S6Ks. This experiment, together with the co-immunoprecipitation studies could indicate a direct association between PDGFR and S6K. Using immunofluorescence microscopy in NIH3T3 cells we show that S6K is evenly distributed within the cytoplasm of starved cells and can also be detected along stress fibers. Upon stimulation the majority of S6K molecules translocates to the nucleus, whereas a small fraction can be found in membrane ruffles.

In addition to PDGFR, we performed co-immunoprecipitation experiments which indicated an inducible association of S6Kα with insulin receptor and Kit.

Using truncation mutants, we could show that the association with RTKs is mediated via the kinase or the kinase extension domain of S6K. Loss of the N- or C-terminus of S6K or both does not abolish the interaction of PDGFR with S6Ks.

Upon mitogenic stimulation, S6K becomes heavily phosphorylated on more than 10 residues. All of the phosphorylation sites are either serines or threonines and no tyrosine phosphorylation of S6K has been described so far. We could establish that both cytoplasmic isoforms of S6Kα and β and the p85 splicing variant of S6Kα (and possibly of S6Kβ) become tyrosine phosphorylated in stimulated cells. At least one splicing variant of each isoform, the p70 S6K, associates with a receptor tyrosine kinase when cells are stimulated. The phosphorylation occurs quickly, decreases after one hour and can be enhanced by Na-pervanadate. *In vitro*, in addition to PDGFR itself, a variety of cytosolic tyrosine kinases such as Src, Lyn, Syk and Btk were also shown to phosphorylate S6Ks.

It would be of interest to determine where tyrosine phosphorylated S6K accumulates. It is possible that tyrosine phosphorylated S6K may preferentially be found at the plasma membrane as membrane localised PDGFR and S6K (and possibly other tyrosine kinases as will be discussed in the following chapter) are in a complex. This would explain why tyrosine phosphorylation of S6K has not been reported before. It may only occur to the fraction of S6K that is targeted to the membrane and can be visualised in membrane ruffles. One way to test this would be to perform subcellular fractionation followed by western blotting with pTyr antibody.

CHAPTER 4: ANALYSIS OF SIGNALLING PATHWAYS LEADING TO S6K TYROSINE PHOSPHORYLATION

CHAPTER 4

ANALYSIS OF SIGNALLING PATHWAYS LEADING TO S6K TYROSINE PHOSPHORYLATION

4.1. INTRODUCTION

The activity of S6 Kinase is regulated by receptor tyrosine kinase signalling. PDGFR receptor is important for many cellular functions such as growth and motility, differentiation, tissue repair and inflammation (Ronnstrand and Heldin, 2001). S6K is involved in some of these functions as well. It has been established that signalling from an RTK to S6K is mediated via the PI3 kinase and mTor pathways but that there are also inputs from other signalling molecules including cdc42 and rac (Chou *et al.*, 1996) and Src kinase (Shah *et al.*, 2003) and more. S6Kβ but not S6Kα is also activated by the ras/MAPK pathway (Pardo *et al.*, 2001). Furthermore, upon G-protein coupled receptor (GPCR) activation (by hypertrophic agonists such as phenylephrine and endothelin-1) S6Kα activation via a MEK1 and mTor dependent mechanisms could be observed. Expression of a dominant negative isoform of ras (N17) inhibited regulation of S6Kα and protein synthesis stimulated by GPCR agonists in cardiomyocytes (Wang *et al.*, 2002).

Our findings presented in the previous chapter indicated a direct or indirect interaction between receptor tyrosine kinases such as PDGFR, insulin receptor, and Kit and S6Ks. The fact that PDGFR is capable of phosphorylating both S6K isoforms, strengthens the hypothesis that there may be a direct interaction between PDGFR and S6K at least for a short amount of time. However, many other molecules are complexed with an activated receptor, too, and they also could affect S6K tyrosine phosphorylation. We therefore wanted to establish which pathways downstream of PDGFR signalling would lead to tyrosine phosphorylation of S6K.

4.2. PDGFR MUTANTS REVEAL INVOLVEMENT OF SRC IN S6K TYROSINE PHOSPHORYLATION

PDGF receptor, when stimulated by its ligand PDGF, dimerises and cross-phosphorylates the adjacent receptor molecule within its cytoplasmic domain. The generated phosphotyrosine sites constitute recognition and binding sites for a variety of downstream proteins with SH2 domains.

A panel of PDGFR mutants has previously been generated by C.H. Heldin (LICR Uppsala). In these mutants the tyrosines which constitute recognition sites for SH2 domain containing proteins such as PLC, Ras-GAP, Grb2, Src, PI3K or SHP2 have been mutated to phenylalanines. In this study we used two PDGFR mutants, one deficient in Src-and one in SHP2 signalling. Src kinase binds to phosphotyrosines Y579/581 and SHP2 phosphatase to Y763/1009 on PDGFRβ. We transfected Cos7 cells with PDGFR Y579/581F and Y763/1009F mutants and S6Ks. The phosphotyrosine signal of immunoprecipitated S6Ks from cells expressing the Y763/1009F receptor was not altered. However, expression of the mutant Y579/581F receptor strongly reduced the phosphotyrosine signal of S6K to a basal level when compared to expression of the wild type receptor (Fig. 4.2.1.). In the control experiment with kinase dead receptor (PDGFRβY634F), no phosphotyrosine signal was detected. But, in all experiments performed, the expression of S6K when co-expressed with kinase dead PDGFR was rather low. Nevertheless, we can state confidently that the kinase dead PDGFR mutant prevented S6K, especially as can be seen for S6Kβ, from being tyrosine phosphorylated. In four independent experiments, using PDGFR point mutants we could establish that Src kinase downstream of PDGFR was involved in tyrosine phosphorylation of S6Ks.

A bigger panel of PDGFR mutants was originally tested including mutants which were deficient in signalling via PLCγ, PI3K, Grb10, Grb2, and Grb7 with mutations Y1021F, Y740/751F, Y771F, Y716F and Y775/778F, respectively. However, the expression of these mutants was so low that in a Western blot using 30μg of total lysate only a very weak signal appeared. In contrast to the four PDGFR constructs (WT, KD, Y579/581F

and Y763/1009F) which were cloned into pcDNA3.1, the other five constructs were pcDNA1/Neo or pBabe vectors. For this reason, their cDNAs were excised with the appropriate restriction enzymes and subcloned into pcDNA3.1 vector. However, the expression efficiency still remained very low. One would have to regenerate the mutants by site-directed mutagenesis using the well-expressing PDGFR wild type construct as template. However, because the PDGFR mutant that is deficient in Src signalling yielded an interesting result, we wanted to investigate this further.

(a)

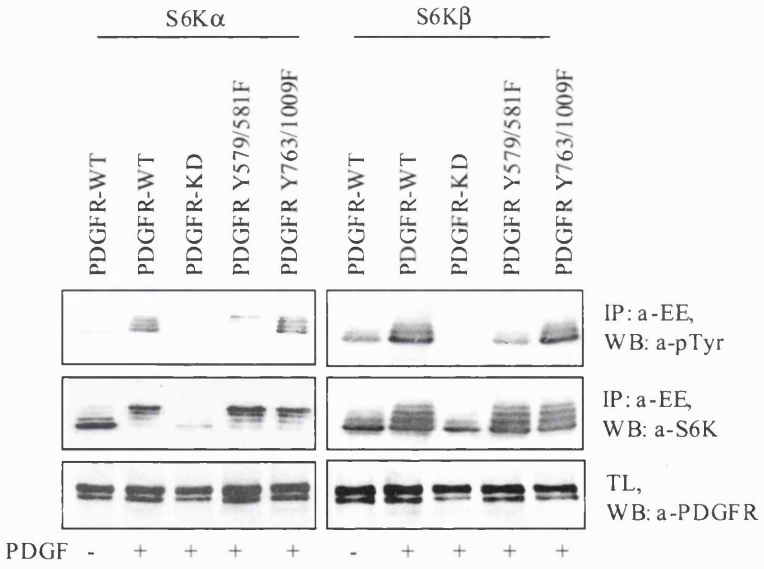


Fig. 4.2.1. Tyrosine phosphorylation of S6K is mediated via a PDGFR-Src pathway.
(a) Cos7 cells transfected with either wt or mutant forms of PDGFRβ (KD PDGFRβ K634A, PDGFRβ 579/581F, PDGFRβ Y763/1009F) and either EE-tagged S6Kα or β, were starved and stimulated with PDGF (40ng/ml) for 15 minutes. Lysates were incubated with anti-EE antibody bound to protein A-sepharose followed by western blot analysis using anti-phosphotyrosine antibody. The membrane was stripped and reprobed with anti–S6Kα/β antibodies. The total lysate (30μg) was also tested for PDGFR expression levels.

(b)

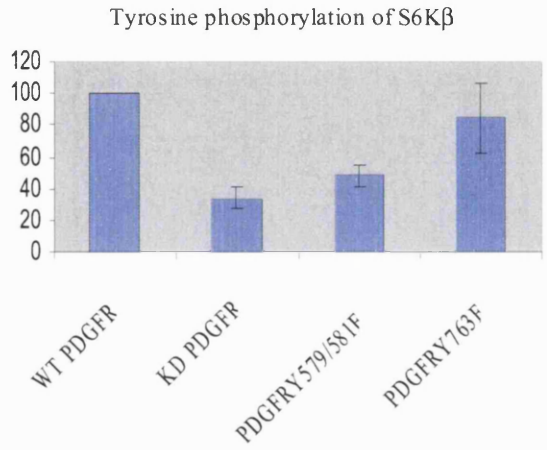


Fig. 4.2.1. Tyrosine phosphorylation of S6K is mediated via a PDGFR-Src pathway.
(b) Representation based on three individual experiments where tyr phosphorylation of S6Kβ co-expressed with wt PDGFR was set as 1. Phosphotyrosine levels were normalised with S6K expression. Error bars represent one standard deviation.

4.3. SRC OVEREXPRESSION LEADS TO TYROSINE PHOSPHORYLATION OF S6KS

To confirm the result obtained with the PDGFR mutants, we transiently expressed various mutants of Src together with S6K in Hek293 cells. Cells were starved for 24 hours, then serum-stimulated and exposed to a brief, 2 min treatment with pervanadate. Cells transfected with empty vector exhibited a very weak phosphotyrosine signal for S6K that could only be seen when it was enhanced with the fluoroimager software. A dominant-negative Src lead to a complete loss of the observed phosphotyrosine signal. This indicated that endogenous Src kinase or a Src-dependent kinase may have been responsible for the weak residual phosphorylation seen when empty vector was transfected. On the other hand, expression of wild type Src and a constitutively active Src (Y527F) enhanced tyrosine phosphorylation of S6K moderately and strongly, respectively (Fig. 4.3.1.). Similar results were obtained in two additional experiments.

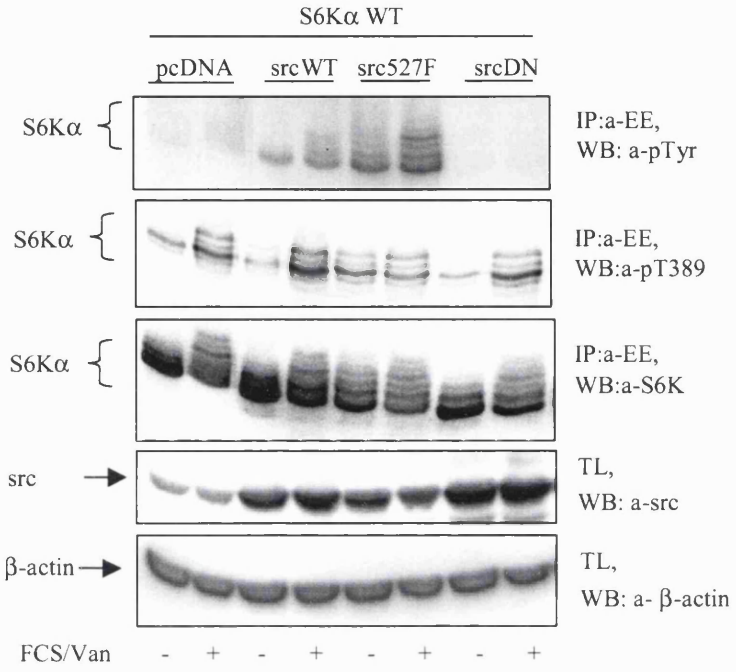


Fig. 4.3.1. Overexpression of Src induces tyrosine phosphorylation of S6K.
Hek293 cells were transfected with S6Kα and either pcDNA3.1 or wild type Src (wt), dominant negative Src (DN) or constitutively active Src (Y527F). Starved cells were stimulated with 10% FCS (15min) followed by a brief treatment with pervanadate (2 min). Phosphotyrosine levels of S6K were assessed by western blot using 4G10 monoclonal antibody. The membrane was then stripped and reprobed twice with antibody against pT389 and S6Kα. Total lysates (30μg) were probed with antibody against Src and β-actin.

When an activated form of S6K (T389D) was co-expressed with wild type Src the phosphotyrosine signal was elevated indicating that tyrosine phosphorylation might preferentially occur after serine/threonine phosphorylation in cells (Fig. 4.3.2.). This observation underscores the finding that, in NIH3T3 cells, the tyrosine phosphorylated fraction of endogenous S6K corresponds to the slower migrating, already partially S/T phosphorylated bands. However, the experiments performed so far are not sufficient to draw the conclusion that ser/thr phosphorylation occurs before tyrosine phosphorylation. To test this, a panel of S6K mutants where the major ser/thr sites are mutated to alanines could be made and analysed with 4G10 antibody. In a system overexpressing Src, even the lower S6K bands appear tyrosine phosphorylated. This could signify that we simply only detect a phosphotyrosine signal when a minimal

number of modified molecules is reached. Thus, when overexpressed, even the lower migrating forms of S6K are relatively abundant and the modification by the phosphorylation is detectable. In addition, we found that kinase activity of S6K was not necessary as a prerequisite for its tyrosine phosphorylation. A kinase–dead S6K still was phosphorylated to the same extent as wt S6K. This experiment was repeated once and yielded similar results.

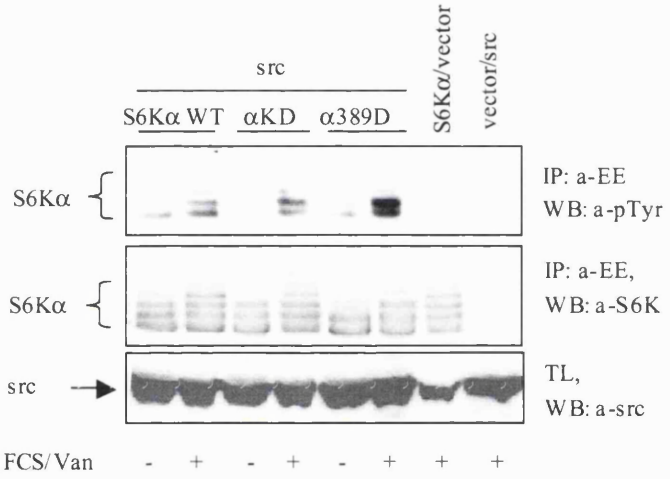


Fig. 4.3.2. Activated S6K is a better substrate for Src-induced tyrosine phosphorylation.
Hek293 cells were transfected with wt Src and either wt S6Kα or kinase dead (KD) S6Kα (K100R) or an activated S6Kα (T389D). Cells and lysates were then treated as described above. Total lysate (30µg) was probed for equal expression of Src.

V-src was the first oncogene to be discovered as the transforming protein of the Rous sarcoma virus (Rous, 1911; Martin, 2001). In comparison to its cellular homologue, v-Src it is constitutively active and this is mainly due to a C-terminal truncation of a regulatory region including a tyrosine site (Y527 or 529 for avian and human DNA respectively) which inactivates Src when phosphorylated. We obtained a temperature-sensitive v-Src transformed Swiss3T3 cell line. Src activity could be turned on by shifting the cells from the non-permissive temperature of 41°C to the permissive temperature of 35°C. In order to test the hypothesis that in these cells tyrosine phosphorylation would be elevated when compared to the parental cell line, we

immunoprecipitated S6Kα from the exponentially growing cells (at 35°C) and analysed S6K tyrosine phosphorylation by western blotting using the 4G10 monoclonal antibody. Interestingly, tyrosine phosphorylation occurred in v-Src transformed Swiss 3T3 but not in the parental cells (Fig. 4.3.3. left panel). A src–specific inhibitor SU6656 was added to these cells before lysis and lead to a significant reduction of tyrosine phosphorylation. We also tested the total lysate from these cells and could detect an enhancement of the phospho-T389 and phospho-S6 signal in the transformed cells (Fig. 4.3.3. right panel). This is in congruence with earlier findings by Blenis and Erikson who described that S6K activity could be stimulated by v-Src (Blenis and Erikson, 1985).

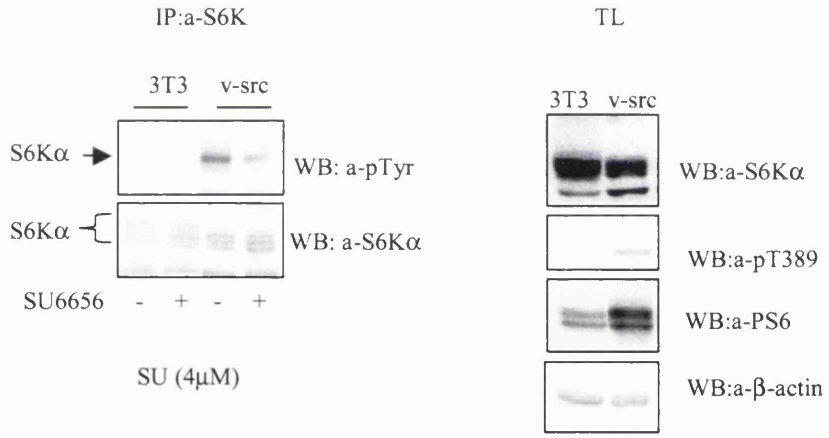


Fig. 4.3.3. S6K is tyrosine phosphorylated in v-Src transformed cells.
Exponentially growing v-Src transformed Swiss 3T3 and parental cells were treated with 4μM SU6656 for 16 hours and then lysed. S6Kα was immuno-precipitated and tested for phosphotyrosine in a western blot. The membrane was then stripped and reprobed with anti-S6Kα antibody. Total lysate was also analysed by western blotting using anti-phospho-S6, anti-pT389, anti-S6K and a-β-actin antibodies.

4.4. S6Kα ASSOCIATES WITH SRC KINASE *IN VIVO*

To strengthen the previous experiments which indicated that Src activity mediates S6K tyrosine phosphorylation, co-immunoprecipitation studies were performed. Kinase reactions can occur very rapidly and the on-off rate of substrate-kinase interaction is often very high. Possibly for this reason, in most previous papers which have described Src-mediated tyrosine phosphorylation of AGC kinases such as PKB/Akt and PDK1, no co-immunoprecipitations were presented. In this study, we observed only a very weak but reproducible association of endogenous Src with S6K. Lysates from exponentially growing Hek293 cells were used for immunoprecipitation with polyclonal antibody against S6K C-terminus. The separated and blotted immunocomplexes were tested for the presence of Src by western blotting with anti-Src antibody. In the negative control where beads only were used, no Src is visible in the blot, but a weak yet specific and reproducible band is present in the sample immunoprecipitated with anti-S6K antibody (Fig. 4.4.1a.). This co-immunoprecipitation was only achieved by using a buffer which is described in 2.5.1. in two individual experiments. This association buffer contained no NaCl and only low concentration of other salts (10mM K_3PO_4, 10mM $MgCl_2$).

Similar problems to show the association between S6Ks and Src kinase occurred in Hek293 cells transiently overexpressing S6Kα and Src. A significant enhancement of Src immunoprecipitated with EE-tagged S6K was visible only when cells were treated with Na-Pervanadate in addition to serum. This immunoprecipitation was performed in a standard lysis buffer. The S6K/Src association was pervanadate dose-dependent and was induced under the same conditions as S6K tyrosine phosphorylation (Fig. 4.4.1b.). Thus, tyrosine phosphorylation and successful co-immunoprecipitation correlate in this experiment. Similarly, it was recently published that PKD could associate with Src but this association was dependent on pervanadate treatment (Storz *et al.*, 2003).

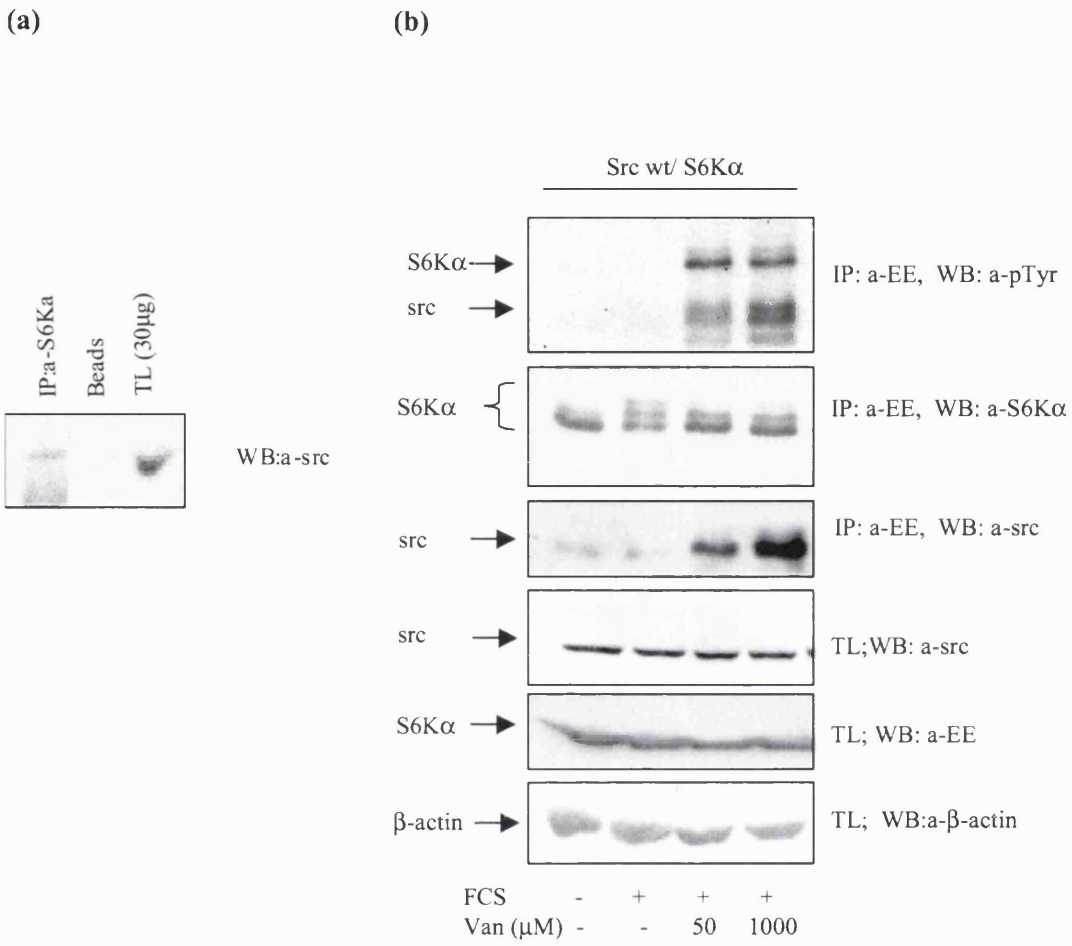


Fig. 4.4.1. Src co-immunoprecipitates with S6K in Hek293 cells.
(a) 1000µg of lysate from exponentially growing Hek293 cells was used for immunoprecipiation with anti-S6Kα antibody. The SDS-PAGE separated proteins were transferred to a nitrocellulose membrane and probed with anti-Src antibody. In the control experiment only protein A sepharose beads were used. Co-immunoprecipitation of native S6K and Src was achieved in two individual experiments.
(b) Hek293 cells were transiently transfected with wt Src and S6Kα, starved and stimulated with 10% serum followed by a 10minute pervanadate treatment. S6K was immunoprecipitated anti-EE antibody and immunocomplexes separated by SDS-PAGE. The membrane was probed with anti-pTyr antibody (4G10) and then reprobed for S6K and Src. The total lysate was also tested for expression of S6K and Src.

4.5. THE EFFECT OF SIGNALLING INHIBITORS ON S6K TYROSINE PHOSPHORYLATION

4.5.1. Tyrosine kinase inhibitors and S6K tyrosine phosphorylation

A panel of tyrosine kinase inhibitors was employed to verify the involvement of tyrosine kinases in the phosphorylation of S6Ks. The inhibitors were added to the starved cells 1 hour before stimulation with 40 ng/ml PDGF. Firstly genistein, a general inhibitor of tyrosine kinases was used at a concentration of 100 μM. This inhibitor is poorly dissolved in DMEM unless it is warmed up in a waterbath and vortexed several times. As depicted in Fig. 4.5.1a-c. it leads to a reduction of S6K tyrosine phosphorylation. PP1 inhibits Src but also PDGFR, Kit and c-abl (Waltenberger *et al.*, 1999). PP1 generated the strong reduction in tyrosine phosphorylation which can be explained by the fact that it exactly targets both the tyrosine kinases involved in the signal transduction leading to PDGF-induced S6K tyrosine phosphorylation (Fig. 4.5.1a-c). SU6656 is specific for Src or other members of the Src kinase family and was employed at a concentration of 4 μM. This inhibitor also reduced tyrosine phosphorylation of overexpressed S6K in Hek293 cells in three experiments (Fig. 4.5.1a and c). Furthermore, SU6656 was used in v-Src transformed Swiss 3T3 cells as shown in fig 4.3.3. and had a negative effect on the phosphotyrosine levels in these cells, too.

4.5.2. PI3K and mTor inhibitors and their effect on S6Ks

Next, it was of interest to test if the major activators of S6K, PI3K and mTor were also involved in mediating tyrosine phosphorylation to S6K. In three different experiments, treatment of cells with LY294002 as PI3K and rapamycin as mTor inhibitor, did not reduce the phosphotyrosine signal of S6Ks. Interestingly, as shown in Fig. 4.5.1b and 4.5.1c., in our case, the two inhibitors rather lead to an enhancement in the tyrosine

signal. This effect could be due to a compensatory mechanism but a proof for this cannot be given. It would be interesting to see if this finding can be validated by overexpression of a kinase-inactive form of mTor or a dominant negative isoform of PKB/Akt downstream of PI3K.

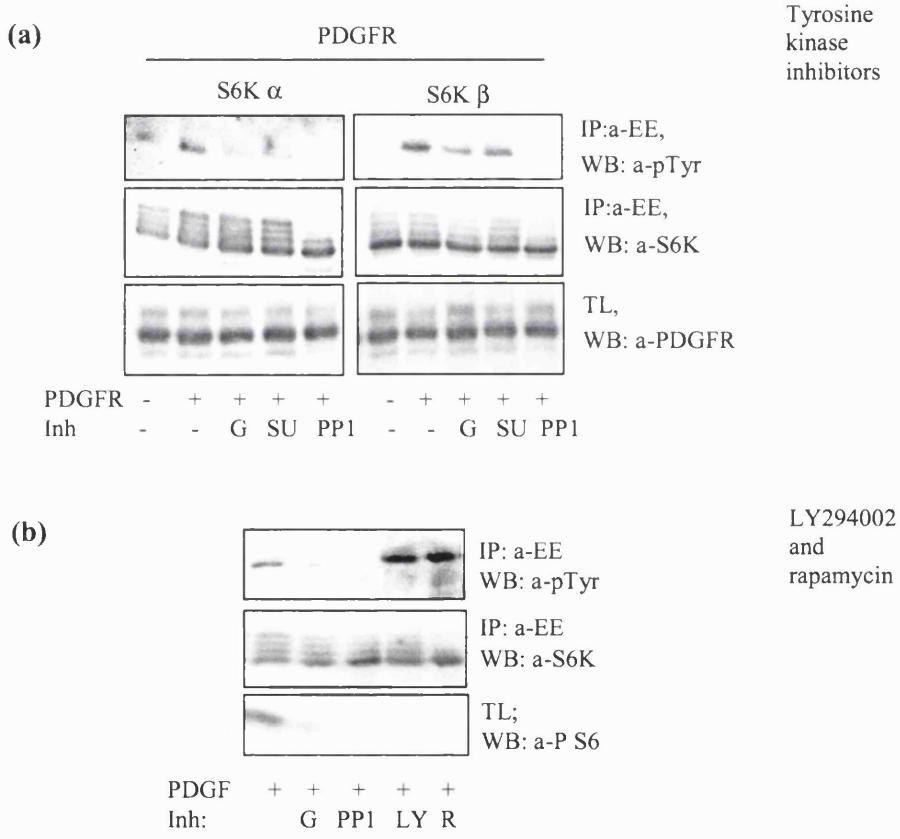


Fig. 4.5.1. Tyrosine kinase inhibitors reduce S6K tyrosine phosphorylation.
(a) Hek293 cells transiently expressing PDGFR and S6Ks were starved for 24 hours. 60 min. before stimulation with PDGF (40ng/ml) cells were incubated with inhibitors (Genistein 100uM, PP1 50μM and SU6656 4μM). S6Ks were immuno-precipitated, transferred to membrane and probed with 4G10 followed by anti–S6K antibody. Total lysate (30μg) was tested for equal expression of PDGFR.
(b) Hek293 were transfected with S6Kα and PDGFR. Cells were treated as described above, but LY 294002 (20μM) and rapamycin (30nM) were employed.

(c)

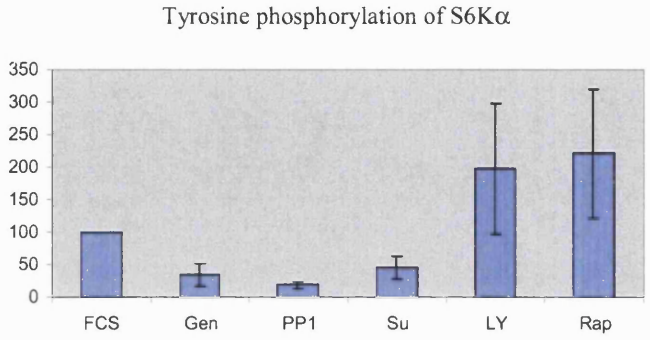


Fig. 4.5.1c. Representation of the above blots.
Tyrosine phosphorylation as derived from three individual experiments for S6Kα, error bars indicate one standard deviation. Tyrosine phosphorylation signal was normalised with S6K expression.

4.6. DISCUSSION

With the help of PDGFR mutants which are deficient in signalling to certain downstream molecules it was ascertained that both PDGFR and Src kinase activities are needed to achieve maximal tyrosine phosphorylation of S6K. This confirmed the preliminary experiments described in chapter 3 which indicated that Src kinase could tyrosine phosphorylate S6K *in vitro*. Overexpression of Src with varying degrees of activity (kinase dead, wt and activated forms) correlated with the amount of tyrosine phosphorylation of S6Ks. Other AGC kinases such as PKB/Akt, PDK1, PKCs and PKD were also found to be tyrosine phosphorylated in a Src-dependent manner. Unlike most other groups we were able to show a specific association between S6K and Src *in vivo*. However, to achieve strong association, serum stimulation was not sufficient but needed to be enhanced by the use of pervanadate.

In contrast to various tyrosine kinase and Src inhibitors which all reduce tyrosine phosphorylation of S6Ks, we have found that neither rapamycin nor LY294002 could reduce tyrosine phosphorylation indicating that mTor or PI3K are not involved in mediating the tyrosine phosphorylation. However, if one assumes that Src kinase directly phosphorylates S6K, then one might not expect to see an involvement of PI3K or mTor. Our finding that tyrosine phosphorylation of S6K is independent of PI3K is in congruence with the finding that PDK1 tyrosine phosphorylation is PI3K-independent (Park *et al.*, 2001). However, tyrosine phosphorylation of PKB/Akt was shown to be PI3K-dependent (Conus *et al.*, 2002). For PKB/Akt, it is plausible that PIP_3 must be generated by PI3K before PKB/Akt can translocate to the membrane and become activated. PDK1 however is constitutively active and might not be dependent on PIP_3 to a similar extent.

CHAPTER 5: PHOSPHOTYROSINE SITE IDENTIFICATION BY MASS SPECTROMETRY AND BY USING TRUNCATION MUTANTS

CHAPTER 5

PHOSPHOTYROSINE SITE IDENTIFICATION BY MASS SPECTROMETRY AND BY USING TRUNCATION MUTANTS

5.1. INTRODUCTION

In order to investigate the physiological relevance of the tyrosine phosphorylation and receptor association of S6K any further, it was necessary to determine the tyrosine phosphorylation sites. Once the sites are identified, point mutants that cannot be phosphorylated or mimic the phosphorylation, can be generated and expressed in cells in order to study the biological properties of the modified site. Furthermore, phosphospecific antibodies are a valuable tool to detect the presence of this post-translational modification under various physiological conditions and to study the subcellular localisation of the modified protein within a cell.

In general, characterisation of phosphopeptides by mass spectrometry requires two steps: firstly, the selection of phosphorylated peptides from a complex peptide mixture resulting from proteolysis of the protein, and secondly, the identification of the phosphorylated amino acid residue within the peptide which may contain more than one serine, threonine or tyrosine residue. For this study, the identification of a phosphopeptide was mainly carried out by precursor ion scanning on a triple quadrupole instrument, or, alternatively by LC/MS/MS and neutral loss experiments on an ion trap instrument. Both methods rely on the detection of phosphate-specific fragments (HPO_3/ PO_3^- and $H_3PO_4/H_2PO_4^-$) during collision-induced decomposition (CID). The identification of the modified amino acid residue was usually undertaken on a quadrupole-time of flight instrument (Q-TOF) by tandem MS. On one occasion, a MALDI-TOF instrument was used for the N-terminal sequencing of a large phosphopeptide.

Another means to identify a site of post-translational modification is to make deletion mutants of a protein, express these in cells and test, for example by western blotting, if the post-translation modification is lost. If one creates protein fragments which are successively shorter and the modification disappears between two mutants, it can be assumed that the lost fragment harbours the modification. However, this is a simplistic approach because it does not take into consideration protein folding and protein–protein interactions. For example, a domain may be needed for binding of the kinase, adaptor or scaffold, but the modification generated by the kinase may not be located within this interaction domain. In such a case, loss of the interaction leads to loss of the post-translational modification even though the actual site is still present in the truncation mutant. Therefore, this method can only be used to indicate the possibility of a post-translational modification and needs additional cell-based and biochemical experiments confirm the results.

5.2. DETECTION OF SERINE AND THREONINE PHOSPHORYLATION SITES IN S6K

The fragmentation pattern of phosphoserine, -threonine and –tyrosine residues is very similar. Whereas phosphoserine and -threonine easily loose HPO_3 and H_3PO_4 in a collision induced fragmentation, phosphotyrosine only readily looses HPO_3. The loss of H_3PO_4 would interfere with the aromatic ring and the stable electron system of tyrosine and therefore does not occur. When performing a neutral loss experiment, the loss of either HPO_3 or H_3PO_4 can be monitored within the same experiment. Performing a precursor ion scan, one has to decide which of the two fragment ions the instrument scans for. As we were mainly interested in phosphorylated tyrosine residues, the fragment of PO_3^- of an m/z value of –79 was chosen.

In the first mass spectrometry experiments in this study, S6K was immunoprecipitated from Sf9 cells that had been infected with viruses containing RTKs and S6Kα or S6Kβ. The immunocomplexes were separated by SDS-PAGE, the gel was stained with

Coomassie and the excised band subjected to an in-gel tryptic digest. The peptides were analysed by LC/MS/MS, a method where the mass spectrometer is online with the HPLC and set to generate MS/MS spectra of the three most prominent peptides at a given elution time over the full length of the gradient (usually 60 or 90 minutes). One peptide, RLNSSPRVPVVSPLK with a monoisotopic mass of 1549 units representing amino acids 426-439 of p70S6Kβ, was reproducibly found in S6Kβ samples derived from Sf9 cells overexpressing PDGFR. We found that the doubly charged ion corresponding to this peptide (of m/z 856.0) lost a neutral fragment of 49 twice during fragmentation, indicating its double phosphorylation (Fig. 5.2.1a). Furthermore, the same phosphopeptide was found in a precursor ion scan. In negative scanning mode it has a mass to charge ratio of 853.6 due to the loss of two protons (data not shown). Two of the three serine residues present in the peptide (indicated in bold in the above sequence) are well–known sites phosphorylated by proline-directed kinases. They are homologous to the sites in the autoinhibitory pseudosubstrate domain of S6Kα (Ferrari *et al.*, 1992; Mukhopadhyay *et al.*, 1992). The detection of known phosphorylated residues in S6K confirmed that the methods chosen by us delivered reliable data.

Another phosphopeptide dectected by LC/MS/MS was the S6Kβ peptide, AKIVRNAKDTAHTR (amino acids 103-117). This peptide was identified when S6Kβ was immunoprecipitated from Sf9 cells overexpressing HGF receptor. In the LC/MS/MS experiment this peptide was present as doubly and triply charged ions (m/z of 894.60 and 596.77) showing neutral losses of 49 and 32.7, respectively. The MS/MS spectrum of the triply charged ion is shown in Fig. 5.2.1b. Since trypsin does not usually cleave when the amino acid adjacent to the lysine or arginine is phosphorylated, we suggest T113 as the phosphorylated residue because T116 is followed by an arginine at which cleavage occurred. The peptide containing the homologous threonine residues in S6Kα was found in a precursor ion scan underscoring the probability that this phosphorylation is real and present in both isoforms. However, as we were mainly interested in identifying tyrosine phosphorylation sites we did not perform a MS/MS experiment with the Q-TOF instrument with this peptide to achieve detailed sequence information. The performed experiments showed that using a combination of precursor ion scanning and LC/MS/MS, we could identify known phosphopeptides as well as

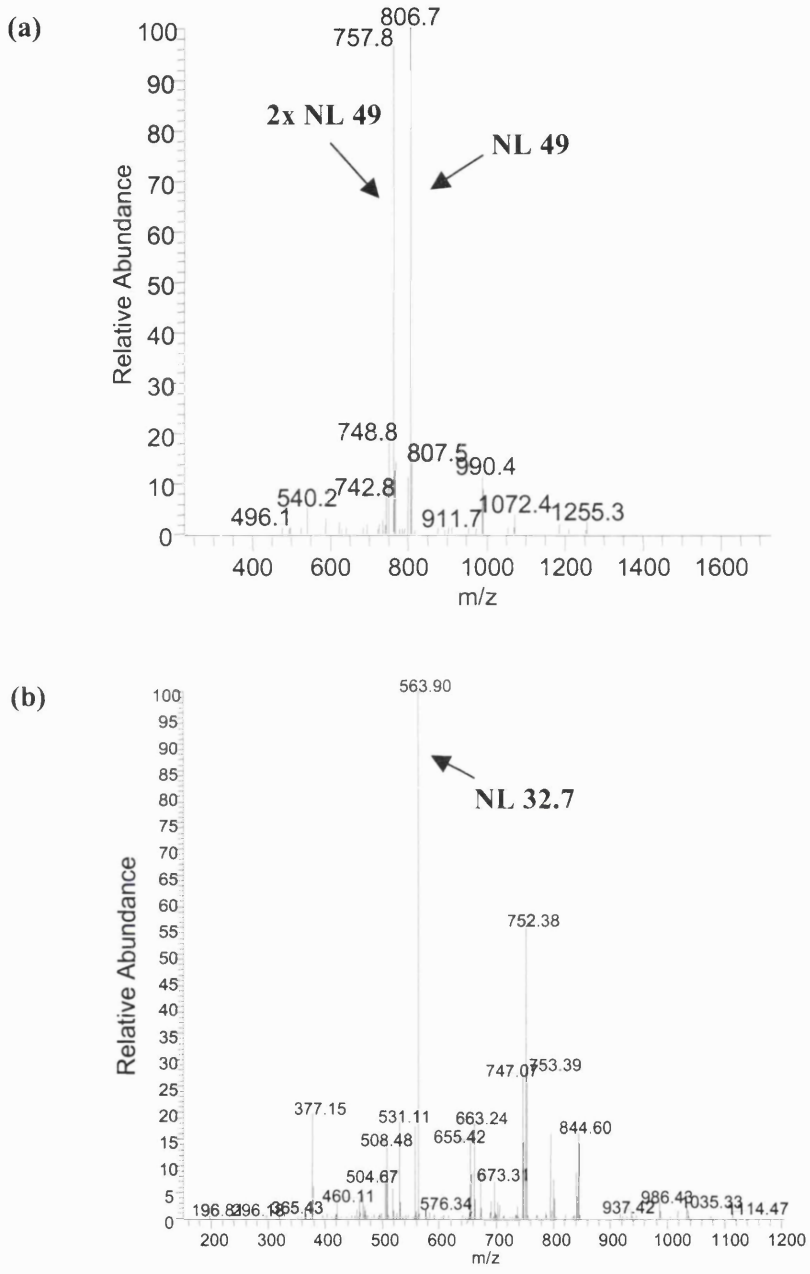


Fig. 5.2.1. MS/MS spectra from Ion trap instrument

(a) S6Kβ is phosphorylated on serines. MS/MS of m/z 856.0 $[M+2H]^{2+}$ phosphopeptide RLNSSPRVPVVSPLK. The ion is derived from S6Kβ overexpressed with PDGFR in Sf9 cells and is doubly charged. Therefore a loss of H_3P0_4 appears as a loss of 49. The monoisotopic mass of the peptide is 1548.9 Da (theoret.).

(b) S6Kβ is phosphorylated on threonine T113. MS/MS of m/z 596.8 $[M+3H]^{3+}$ phosphopeptide KAKIVRNAKDTAHTR of S6Kβ derived from Sf9 cells stimulated by co-expression of HGFR. The ion is triply charged, therefore a loss of H_3P0_4 appears as a loss of 32.7. The monoisotopic mass of mass the peptide is 1708.0 Da (theoret.).

novel ones. However, no tyrosine phosphorylated peptide was found. It is likely that under physiological conditions only a subset of S6K becomes tyrosine phosphorylated, otherwise phosphoamino acid analysis (Ballou *et al.*, 1988) would probably have detected the phosphotyrosine residue. Consequently, tyrosine phosphorylation of S6 kinase *in vivo* occurs at low stoichiometry and may be below the limit of detection of our approach. For this reason, all samples used in the following experiments were prepared by *in vitro* phosphorylation, mainly using Src as a tyrosine kinase but also btk, syk and the cytoplasmic domain of PDGFR.

5.3. IDENTIFICATION OF A PHOSPHOTYROSINE SITE WITHIN THE CATALYTIC LOOP OF S6Kα

In order to increase the stoichiometry of tyrosine phoshorylation of SF9-derived S6K, an *in vitro* kinase assay was optimised. The assay was performed as previously described (2.5.8.) without radioactivity and with the difference that the incubation time was increased to 1 hour and the ATP concentration elevated to 500μM. A panel of tyrosine kinases (Src, Syk, Btk or PDGFR) was used to phosphorylate S6K. The efficiency of phosphorylation was monitored by analysing S6K phosphorylation in a radioactive assay or by Western blot using the 4G10 anti-phospho-tyrosine antibody. Coomassie-stainable amounts of tyrosine kinase-treated S6K were used for in-gel digestion. On the Coomassie-stained gel (7.5%) one could detect an upwards gel shift in the Src-treated sample but not in the control and to a lesser extent in the samples treated with Syk and Btk.. The band corresponding to S6K was cut out of the gel, washed twice with a 50% acetonitrile solution and digested with trypsin (which cuts C-terminally of lysine and arginine), overnight.

5.3.1. Precursor Ion Scanning

Samples were desalted with ZipTips (Millipore) and inserted into a nanospray needle. First, a full scan in the negative mode was acquired of the samples digested with trypsin. The peptide mix was then analysed by static nanospray precursor ion scanning in negative ion mode. This scanning method was set to detect any peptide that produced an ion of -79 m/z (PO_3^-) after fragmentation in the collision cell. As shown in Fig. 5.3.1a, a major peak (m/z 699.4) could be seen in the sample of S6Kα treated with Src but not in the control (Fig.5.3.1b). An additional peak at m/z 349.5 represents the same peptide in the doubly charged state. It was putatively identified as phosphopeptide GIIpYR (amino acids 190-194) with a theoretical mass of 701 Da. The fact that the control spectrum displays a very low signal to noise ratio can be explained by the lack of phosphorylated peptides in this sample. We also tested S6Kα that had been *in vitro* phosphorylated with Btk and Syk kinases. The 699/349 ion pair was also detected in these samples suggesting that Y193 may be an *in vitro* phosphorylation site for various kinases (data not shown). We could not identify this site for S6Kβ using mass spectrometry. The amino acid sequence of S6Kβ does not contain arginines or lysines which are close to tyrosine Y192 on the N-terminal side, so the corresponding peptide (31 amino acids) was probably too large to be visible in the spectrum or it was poorly ionised. For this reason, we digested the S6Kβ sample with AspN (cuts N-terminal of aspartate) or GluC (cuts C-terminally of glutamate but also, with much lower specificity at aspartate) which should generate smaller peptides of a length of 25 and 21 amino acids, respectively. However, we were not able to detect the phosphopeptide with this approach either, possibly due to the lower proteolytic activity of these proteases. Besides, the peptides could still be poorly ionised during electrospray ionisation. The amino acid sequence closely around the tyrosine site in both isoforms only differs in a glutamine in place of a lysine at position -4. Thus, we concluded that S6Kβ might also be tyrosine phosphorylated at this site. Once we identified Y193 as an *in vitro* phosphorylation site, we aimed to determine by mass spectrometry if this site could be detected in an *in vivo* sample derived from Hek293 cells overexpressing S6Kα and constitutively active Src. Per sample, approx. $7x10^6$ serum-starved cells were

with serum and pervanadate and the immunoprecipitated S6Kα visualised after SDS-PAGE by Coomassie. The amount of S6K present was sufficient to stain 4 bands (according to a successively higher phosphorylation state) which were individually excised, digested in-gel (with Trypsin or GluC) and analysed by mass spectrometry. However, no tyrosine sites could be determined from such an *in vivo* sample.

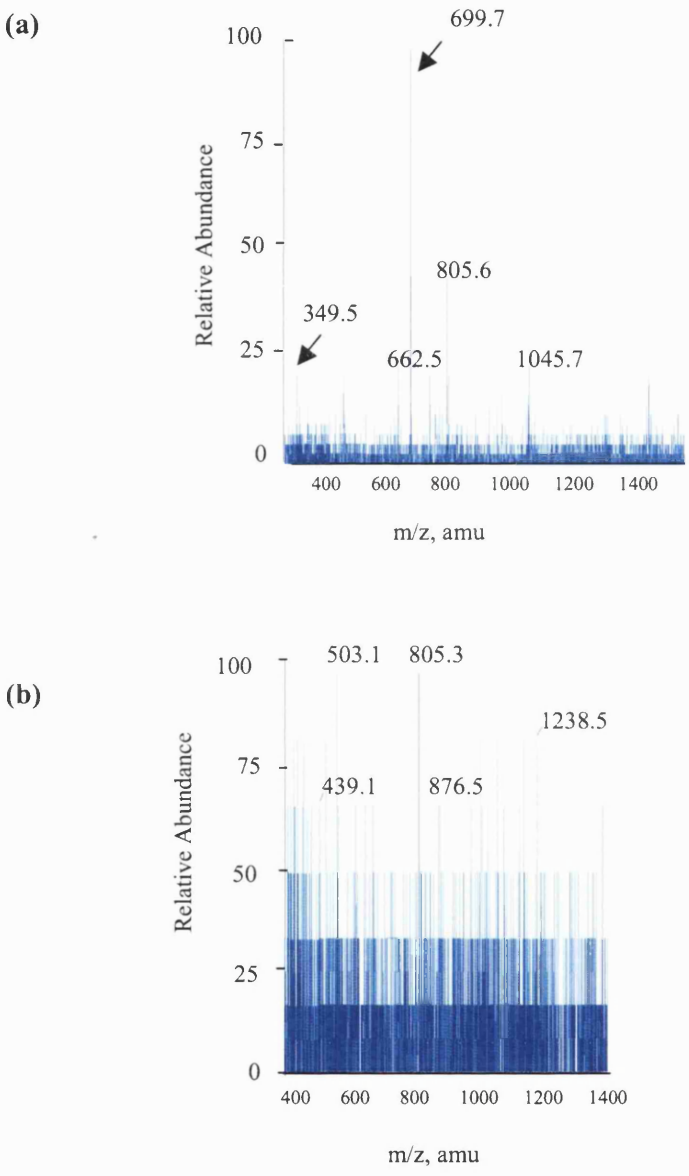


Fig. 5.3.1. SKα is phosphorylated on tyrosine Y193.
EE-tagged S6Kα was expressed in and immunopurified from Sf9 cell extracts, then subjected to *in vitro* phosphorylation by Src (as described above, but without [γ -32P] ATP). Coomassie-stained SDS-PAGE gel bands of S6Kα were digested with trypsin. Peptides were desalted on μC18 packed Zip Tips and eluted in 5% NH3/ 75%CH3CN/25%H2O. The peptide mix was analysed by electrospray ionisation mass spectrometry (ESI-MS) on a Sciex API3000 mass spectrometer with a NanoESI source. First, the mass spectrometer was operated in single quadrupole mode and a negative ion full scan was acquired, then a precursor ion scan was performed.
(a) Precursor Ion Scan of m/z-79 of S6Kα treated with Src kinase *in vitro*.
(b) Precursor Ion Scan of m/z-79 of S6Kα mock–treated sample.

5.3.2. LC/MS/MS on Ion trap instrument

To complement the identification of Y193 phosphotyrosine by another method, we used tandem mass spectrometry on an ion trap instrument connected to HPLC. Due to the HPLC separation prior to MS, this method can be more sensitive than a static nanospray experiment as it overcomes the relative suppression of weak peptides by those that are highly abundant and well ionised. The detection limit is less than 10 fmol for a test peptide, therefore, we hoped to be able to detect the GIIpYR phosphopeptide from an *in vitro* or even *in vivo* sample. HPLC was performed on a reverse phase C18 column over a 90 minute gradient using 0.1% formic acid in water or acetonitrile. Specific software ("Qualbrowser", ThermoFinnigan) was used to visualise the three sets of chromatograms shown in Fig. 5.3.2.. The neutral loss of 80 m/z chromatogram of the Src-treated sample clearly exhibits a strong peak at RT 30.91, which is not present (to the same extent) in the control sample. Similarly, there was a peak in the control sample at elution time 24.60, which was much stronger in the control sample. However, as this peptide was not treated with Src, the loss of 80 may not be due to a phosphate but to other fragmentation and was not inspected any further. The peptide, which was subjected to tandem MS at elution time 30.91, indeed was the GIIpYR peptide as is shown in the MS/MS spectrum (Fig. 5.3.3.). The fragmentation delivered a satisfactory amount of sequence information as the arrows and annotation within the spectrum confirm. Loss of H_2O (-18) and HPO_3 (-80) could readily be detected. Furthermore, the sequence ions z_2, z_3 and b_3 were present. Unfortunately, this peptide could not be detected in *in vivo* samples.

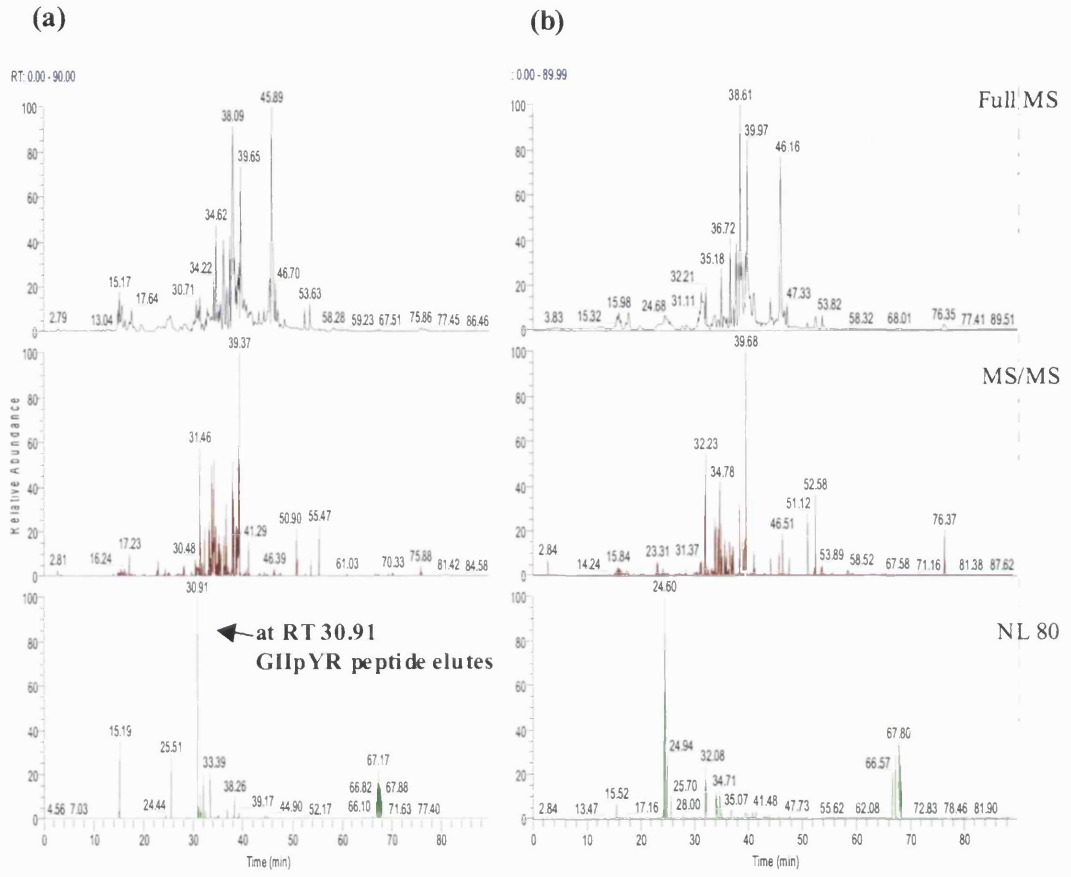


Fig. 5.3.2. A phosphopeptide is present in Src-treated sample.
Nanoflow LC/MS/MS on Electrospray Ion trap MS. Samples were prepared as described for Fig. 5.2.1. The tryptic peptide mix was injected onto a reverse phase C18-column (75µmx50mm). A 90 min gradient of 0.1% formic acid in either acetonitrile or water was run at a nanoflow rate of 200nl/min. Shown above are the chromatograms of the Src-treated sample on the left side (a) and the control sample (b). The top row chromatograms represent all the eluted peptides (Full MS), the middle two panels show the chromatograms of MS/MS. The lowest panel shows the chromatogram of peptides that lost 80m/z during fragmentation (NL 80). At retention time 30.91 a peptide elutes in the Src treated sample only. The MS/MS spectrum of this peptide is shown in Fig. 5.3.3. and 5.3.4..

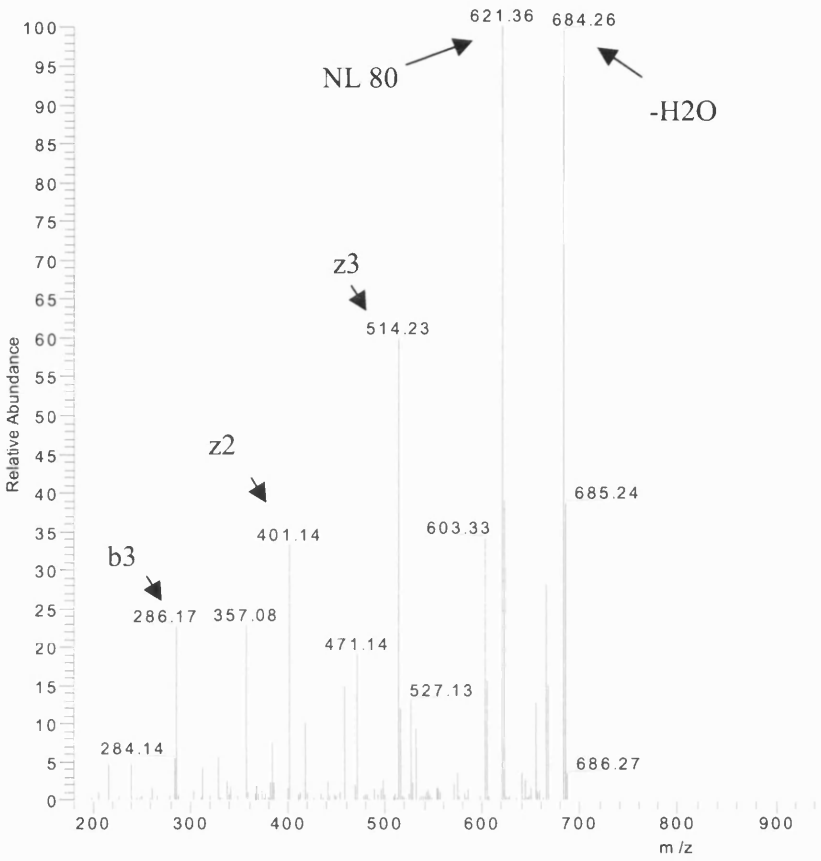


Fig. 5.3.3. TheS6Kα–derived phosphopeptide is GIIpYR.
LC/MS/MS of m/z 701.46. This MS/MS spectrum was acquired at retention time 30.91 on an LCQ Deca (ThermoElectron) ion trap mass spectrometer. The strongest peak (base peak) displays a loss of 80 m/z from the parent ion. Fragment ions are annotated. See text for details.

5.3.3. Sequencing by tandem MS on a Q-TOF instrument

Although we had already gained satisfactory data from the combination of precursor ion scanning and LC/MS/MS, we also wanted to investigate whether the peptide GIIpYR could be sequenced directly without separating the peptide mix through HPLC. Direct static nanospray sequencing (Tandem MS/MS) was performed using a Q-TOF instrument and confirmed the presence of the phosphorylated Y193 (Fig. 5.3.4.). The fragment peak of m/z 621.4 (-80) represents the loss of phosphate. The immonium ion

of m/z 216.04 derived from a phosphotyrosine was also present. Mainly z and y, but also b, series fragment ions could be found.

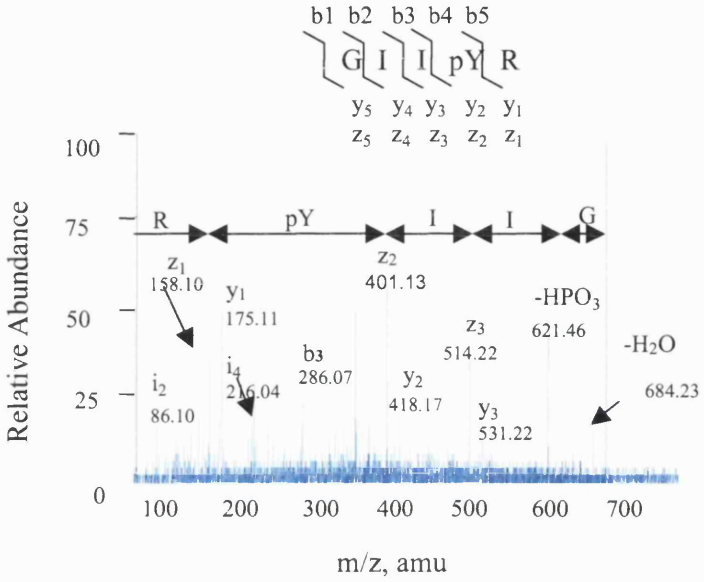


Fig. 5.3.4. MS/MS spectrum of m/z 701 GIIpYR on Q-TOF instrument.
For sequencing the phosphopeptide GIIpYR, tandem MS on a quadrulope time-of-flight mass spectrometer (Qstar, PE Sciex) equipped with a NanoESI source was performed. The tryptic digest was desalted on μC18 Zip Tips and eluted in 1% formic acid, 50% acetonitrile and 50% water. A few microlitres were loaded into gold-coated nanospray needles.

The identified Y193 is 19 amino acids upstream of the DFG motif and 35 amino acids upstream of the T229 site, which has been shown to be crucial for S6K activity. This tyrosine residue is conserved among all AGC kinases (with the exception of PDK1 and NDR) (Fig. 5.3.5.). It is located at the beginning of a highly conserved loop structure at the base of the active site, called the catalytic loop. The arginine, which is next to the phosphorylated tyrosine is believed to form an ion pair with a phosphate within the activation loop (usually the T309/308 for PKB/Akt or the T229 for S6K) (Russo *et al.*, 1996; Yamaguchi and Hendrickson, 1996). The ion pairing interaction is likely to be important for rotating the DFG motif into proper orientation for catalysis. The aspartate that follows the arginine interacts with the attacking hydroxyl side chain of the substrate (Huse and Kuriyan, 2002). The DFG motif itself, which is responsible for

binding the divalent cation (usually Mg2+), is involved in nucleotide recognition. These findings stress that the tyrosine in question is in an important position of the kinase and phosphorylation of it may have a strong impact on S6K kinase activity. This issue will be addressed in chapter 6.

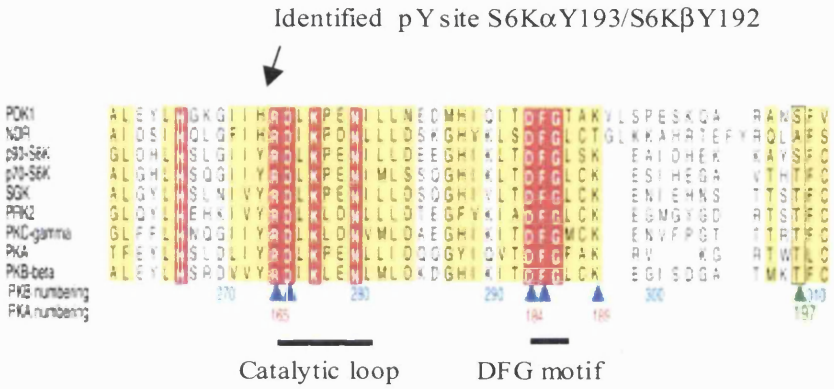


Fig. 5.3.5. Alignment of the sequence surrounding the catalytic loop in AGC kinases.
The identified site is marked with an arrow. The catalytic loop is underlined. Functionally important residues are indicated with an arrowhead. Invariant residues are coloured in red, conserved ones in yellow. Adapted from Yang *et al.* (2002). Mol Cell 9, 1227-1240.

5.4. IDENTIFICATION OF A TYROSINE PHOSPHORYLATION SITE IN THE N-TERMINUS OF S6KS BY THE USE OF TRUNCATION MUTANTS

5.4.1. Analysis of S6K truncation mutants

As will be described in further detail in chapter 6.3., it was not possible to rule out that the tyrosine phosphorylation site identified in the catalytic loop may be an *in vitro* rather than an *in vivo* site. It was further established that other phosphorylated tyrosine residues than pY193 have to exist *in vivo* since the Y193F mutant did not lead to a signal reduction when probed with 4G10 antibody. In order to determine the major

region of tyrosine phosphorylation, another approach based on the expression of deletion mutants of S6Kα and S6Kβ was used. For the schematic illustration of the truncations see Fig.3.3.5.. Analysing immunoprecipitates from Hek293 cells which transiently co-express an activated variant of Src (Y527F) and truncated S6Ks revealed that an S6KαΔN mutant was not whereas the S6KαΔC still was tyrosine phosphorylated (Fig. 5.4.1.). This indicated that the major phosphorylated tyrosine residue or the residue to be phosphorylated first must be located in the N-terminal domain of S6K. It could also be, however, that truncation of the N-terminus disrupts the structure of S6K or protein-protein interactions and therefore leads to the loss of the phosphotyrosine signal. Similarly, for S6KβΔN the phosphotyrosine signal is also abrogated. But this mutant is also very weakly expressed and this may cause the lack of signal. The experiment was repeated twice and similar results obtained.

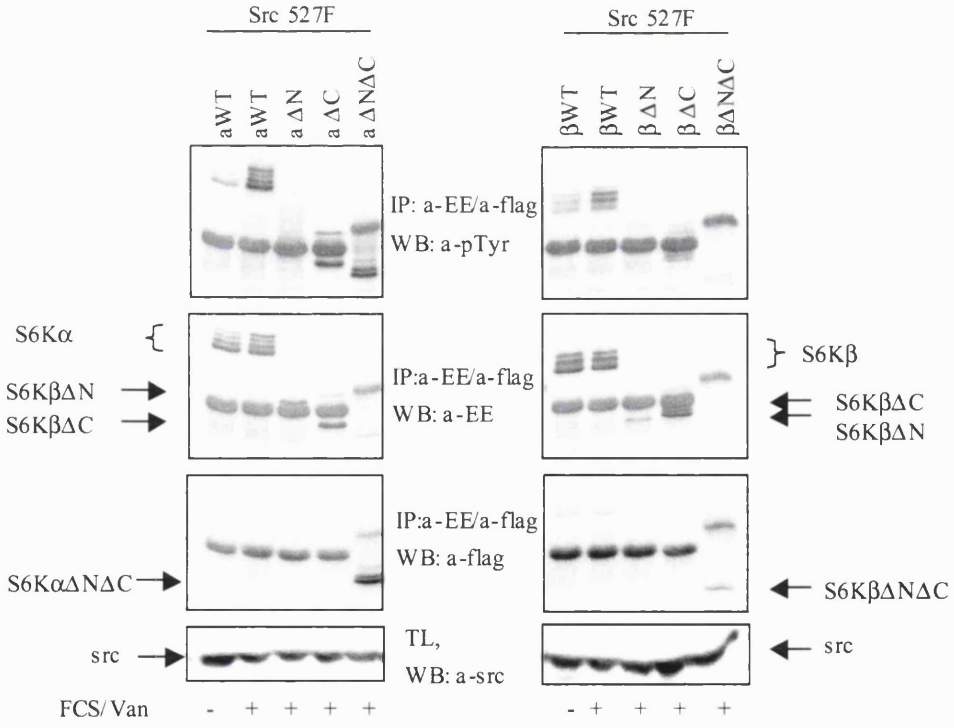


Fig. 5.4.1. Deletion of the N-terminus leads to a loss of phosphotyrosine in S6K.
Hek293 cells were transfected with wt and truncated mutants of S6K (EE-S6Kα/β WT, EE-S6Kα/β ΔN, EE-S6Kα/β ΔC, flag-S6Kα/β ΔNΔC) and Src 527F. Cells were starved for 24 hours and stimulated with 10% FCS for 15 min followed by a 2-min treatment with Pervanadate. S6Ks were immunoprecipitated with anti-EE or flag-antibodies. Immunocomplexes were separated via SDS-PAGE and blotted with 4G10 antibody. Membrane was reprobed with anti-EE or anti-flag antibody. Total lysate (30µg) was also analysed for Src expression. Arrows indicate the various truncated S6Ks.

The S6Kα/β ΔC mutants appeared to be tyrosine phosphorylated to the same degree as wt S6K indicating that the C-terminal region does not contain phosphorylated tyrosine residues. However, flag-tagged S6Kα ΔNΔC and β ΔNΔC mutants are still tyrosine phosphorylated. These conflicting results can easily be reconciliated because the doubly truncated mutant only lacks the first 23 amino acids whereas the S6KαΔN mutant lacks 52 and the S6KβΔN 51 amino acids (as depicted in Fig. 3.3.5.). Thus, the phosphorylated tyrosine residue must be located between amino acids 24 and 51/52.

5.4.2. Generation and purification of a bacterially expressed N-terminal S6Kα domain

Analysis of the S6Kα protein sequence revealed that there is only one potential tyrosine in the N-terminal domain, herein referred to as S6KαY39. To verify our hypothesis that the N-terminus harbours a major tyrosine phosphorylation site and to exclude that the lack of tyrosine phosphorylation in the S6KΔN mutants might be due to a conformational change that hinders the access of tyrosine kinases to their substrate residues, we next aimed to generate a His-tagged N-terminal domain.

To achieve this, we chose the bacterial expression vector pET42a (Novagen) because it hosts a His-tag that is placed C-terminally of the multiple cloning site (MCS). The vector is designed for high expression of sequences fused with a 220 amino acid GST-tag N-terminally of the MCS. For the purpose of this study, the N-terminal end of the domain was to be kept free like in the original kinase. This was possible by using a unique restriction site (NdeI) provided N-terminally of the GST-tag. We cut out the GST tag with NdeI and a restriction enzyme targeting a site in the multiple cloning site (Hind III). The C-terminal His-tag provided by the vector was already in frame with the S6K coding sequence. The S6K N-terminal fragment contained an internal NdeI site. This site was located in the vicinity of the C-terminal end of the domain. It was therefore possible to construct primers for PCR amplification with one nucleotide

change leading to a silent mutation at this restriction site and hereby preventing an internal restriction of the N-terminal S6Kα domain by NdeI.

The following primers were used:

Sense:

5'-GCAATAC**CATATG**GCAGGAGTGTTTGACATAGACCTGGA

The NdeI site is marked in bold, the start codon is underlined. The nucleotides 5' of the NdeI site have been added as spacer nucleotides according to the Novagen manual in order to allow for efficient digestion.

Antisense:

CCC**AAGCTT**TGAGATTTCAAATTTCTCACAATGTTCCATGCCAAGTTC*G*TAT GGTCCAACTCCC

The HindIII site is marked in bold, the underlined sequence was the former NdeI restriction site which was present in the S6Kα sequence, with the G mutated from the original A. The nucleotides 5' of the HindIII site have been added as spacer nucleotides according to the Novagen manual in order to allow for efficient digestion.

The N-terminal domain of S6Kα (1-52) was generated by PCR amplification using the pcDNA3.1 p70 S6Kα vector as template. It had previously been made by T.Valovka in our laboratory.

As shown in Fig. 5.4.2a., the PCR product of a calculated length of 178 base pairs was generated. It ran higher in an agarose gel than a mix of both primers but lower than the 250bp marker of the 1kb DNA gene ruler ladder. The product of this PCR amplification and the pET42a vectors were then digested with NdeI and HindIII. In a first round of restriction, ligation and transformation no clones with the right insert were produced. In a second round, the restriction was first performed with NdeI alone and the efficiency of restriction checked by analysing 1/10 of the digestion volume on an agarose gel. Once the full digestion was assured, the second restriction enzyme (HindIII) was added. The efficiency of digestion was examined by running the product on an agarose gel (Fig. 5.4.2.b.). The restricted PCR product (162bp), the cut vector (5050bp) and the insert (890bp) were present at their expected sizes. The digestion mixtures were run on a preparative 0.8% TAE agarose gel and PCR product and vector purified as

recommended by the manufacturer and described in 2.1.1.6. (QIAEX DNA Gel Extraction kit Qiagen). The T4 fast ligation kit (Promega) was used for ligation of 100 ng vector with 10 ng insert and, as a control, with vector only according to the manufacturer's instructions.

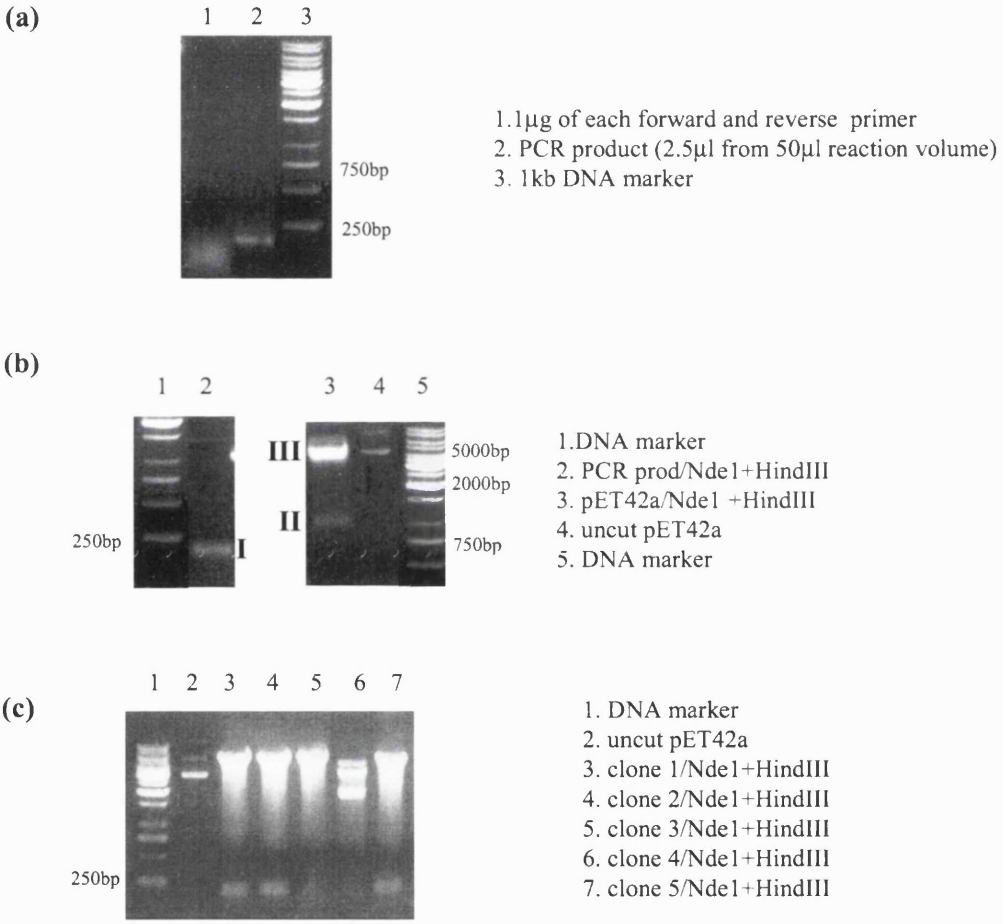


Fig. 5.4.2. Cloning of the N-terminal fragment of S6Kα.
The aim was to create a construct for bacterial expression of a 52 AA N-terminal domain of p70S6Kα with a C-terminal 6xHis tag. The His-tag followed by a stop codon was provided by vector pET42a (Novagen). **(a)** 1.5% TAE agarose gel electrophoresis of the PCR product of the first 156 nucleotides of S6Kα. A mixture of the original primers is in lane one, the 1 kb DNA marker in lane three. **(b)** Digestion of 5µg of PCR product and of pET42a with Nde1and HindIII endonucleases.Nde1/HindIII fragments used for ligation are shown by arrows. Lane one/arrow I: digested PCR fragment. Lane2 / III: digested empty vector. A long fragment of 890 bp (containing GST, a second His tag , and most of the multiple cloning site) of the vector had to be excised using HindIII and Nde1 as indicated by II. Bands I and III were cut out from the preparative 0.8%TAE agarose gel and purified.
(c) Restriction analysis of expression vector for recombinant N-terminal fragment of S6Kα.1.5% agarose gel. 5ml cultures of 5 kanamycin-resistant clones were grown and plasmids purified. 2µg of DNA were used for HindIII/Nde1 restriction and electrophoresed. Insert can be clearly seen for clones 1, 2 and 5.

XL1 Blue cells were transformed and selected with Kanamycin. The following day the control plate had 2 colonies, the sample plate had 6 colonies of which 5 colonies were picked in order to perform plasmid DNA purification, restriction analysis and PCR. Fig. 5.3.2c. shows the restriction analysis with NdeI and HindIII. Clones 1, 2 and 5 contained an insert of the right size.

Clone 1 and 2 were used for transformation of BL21 DE cells. A mini-batch purification of 5 ml showed the expression of a 61 aa peptide migrating at approx. 10 kDa in a gradient SDS-PAGE gel (Fig. 5.4.2d.).100 ml of these cells were used for purification of the His-tagged S6K with Ni-NTA agarose as described in 2.2..

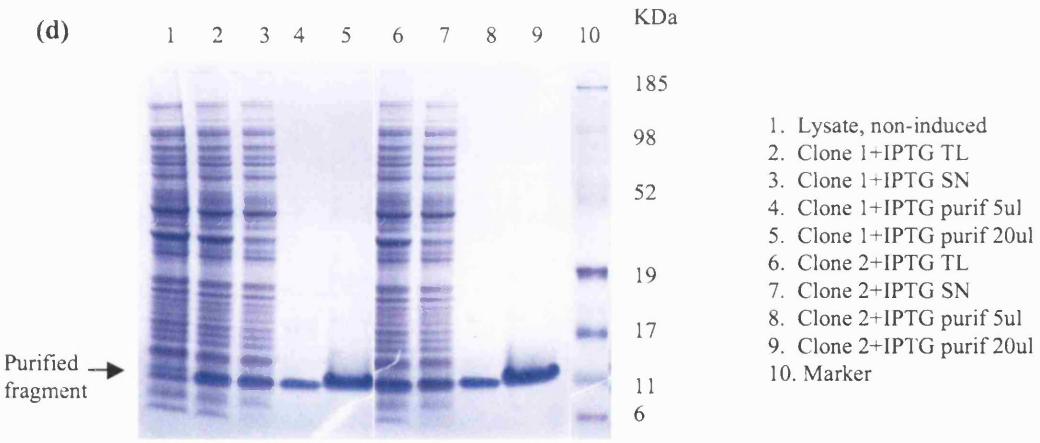


Fig. 5.4.2d. Purification of N-terminal fragment.
100ml of cells were grown from clone 1 and 2. At OD=0.8 expression was indcued with 0.4 mM IPTG for 3 hours at 370C. First a small batch was lysed and checked for expression, then the rest of the cells was lysed as described in chapter 2. Purified protein bound to NTA-agarose beads was eluted with 400mM imidazole in 2ml per 100 ml of cells and dialysed overnight. A 5-12% SDS-PAGE gel was run with samples as indicated above and Coomassie-stained. TL stands for the soluble fraction of the total lysate. Lanes 4, 5, 8, 9 show the purified fragment.

5.4.3. *In vitro* kinase assay with N-terminal domain as substrate

To verify that the tyrosine at position Y39 of this polypeptide is indeed a Src substrate, the purified N-terminal domain was subjected to an *in vitro* kinase assay with Src, Btk, Lyn or Syk as kinases as described in chapter 2.5.8.. The reaction mix was denatured by adding Laemmli sample buffer, boiled and separated on a 5-12% gradient Bis-Tris pre-cast gel using MES gel running buffer (Invitrogen NuPAGE). As a result, an almost complete mobility shift could be seen, presumably due to phosphorylation of the S6Kα N-terminal domain in the presence of Src (Fig. 5.4.3.). The other tyrosine kinases tested also phosphorylated this N-terminal S6K fragment, but with less efficiency. However, when the ratio between autophosphorylation and S6K phosphorylation is taken into account the other kinases may be almost as effective in phosphorylating the N-terminal domain.

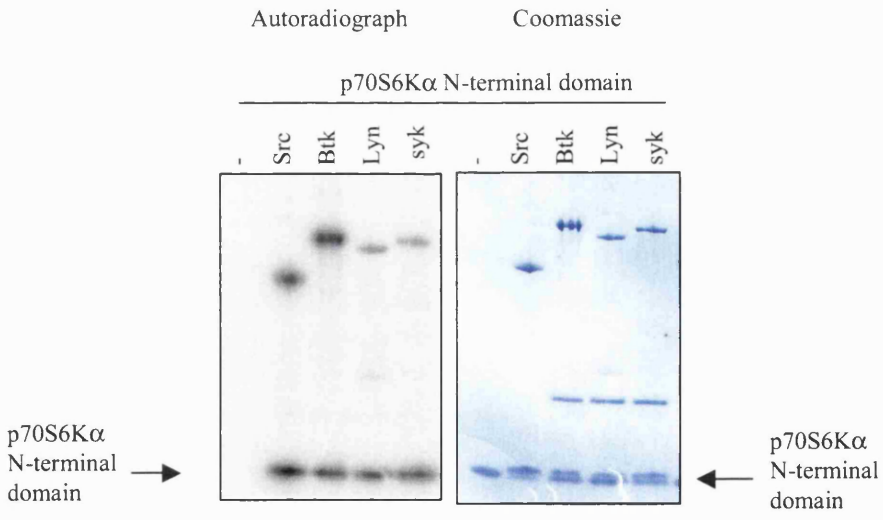


Fig. 5.4.3. The N-terminal domain of S6Kα is a substrate for tyrosine kinases.
2μg of the purified recombinant N-terminal fragment were used for an in-vitro kinase assay using cytosolic tyrosine kinases Src, Btk, Lyn and Syk. 200ng of tyrosine kinases were employed for 2hrs at 30°C, concentration of cold ATP was 0.5mM.

5.4.4. Mass spectrometrical analysis of the phosphorylated N-terminal domain

Furthermore, we performed mass spectrometry experiments using the *in vitro* phosphorylated N-terminal domain. For MALDI-ISD, no trypsin digestion of the peptide was performed but the sample mix derived from the *in vitro* kinase assay (using Src) was cleaned up on C3 magnetic beads (Bruker Daltonics) as described in chapter 2.7.1.3., and stabilised by the addition of DTT and EDTA. 0.5 µl of the sample solution was mixed with an equal amount of MALDI matrix (sDHB) and loaded onto a MALDI target. The MALDI instrument was used in reflectron mode. Sequencing from the N-terminus by MALDI-ISD lead to a clear peptide backbone fragmentation and c-ion series in which the phosphorylated tyrosine residue was still intact as a 243Da (mass of tyrosine plus phosphate) mass increment within the sequencing ladder (Fig. 5.4.4). These results confirmed that this N-terminal fragment is a very good Src substrate *in vitro*.

To complement the finding, we also analysed the phosphorylated peptide by precursor ion scanning mass spectrometry. The endoprotease of choice was GluC (cuts C-terminally of Glutamate, thereby generating a 14AA long peptide), since trypsin would not have generated a peptide of manageable size. Precursor ion scanning (m/z-79) resulted in a spectrum where the three major peaks all stem from one phosphopeptide with a theoretical monoisotopic mass of 1578.65 Da SMDHGGVPpYELGME and a measured mass of 1578.6 Da (as depicted in Fig. 5.4.5.). The peaks represent the peptide in a doubly, triply and quadrupoly charged states. The base peak in this figure is shifted approx. +8m/z when compared to the doubly charged phosphopeptide ion. Similarly as in the MALDI-ISD spectrum, this peak may stem from the same peptide with an oxidised methionine.

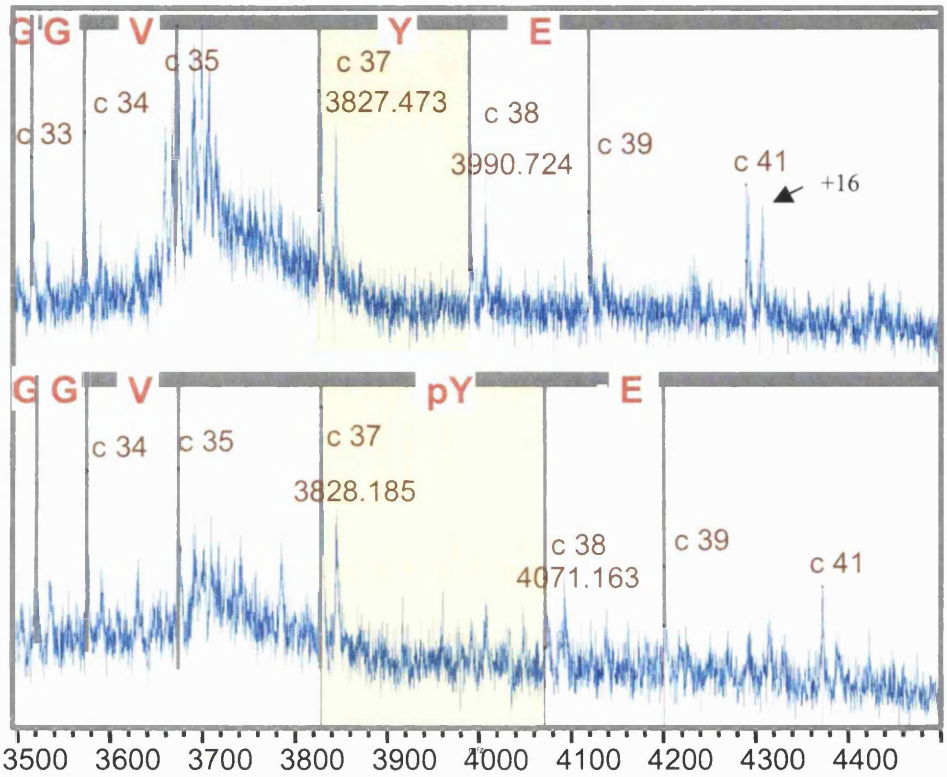


Fig. 5.4.4. Sections of an ISD-MALDI spectrum of N-terminal fragment of S6Kα.
Samples were prepared as described in Fig 5.3.3. DTT (final concentration 2mM) and EDTA (final concentration 5mM) were added to the *in vitro* kinase mixture. 1µl sample and 1µl superDHB matrix were mixed and loaded onto a MALDI target and anlysed by a Bruker Ultraflex Tof/Tof instrument in reflectron mode. The ISD spectrum of the control sample (mock-treated without Src kinase) is shown in the upper panel, the lower panel stems from the tyrosine phosphorylated S6K fragment. After phosphorylation all C-ions following C38 are shifted by +80. Phosphotyrosine remained stable and could be detected within the series as a 243 m/z fragment (163+80). Also, the doubling of signals is explained by partial methionine oxidation at M19. The arrow indicates the +16 shift caused by oxidised methionine.

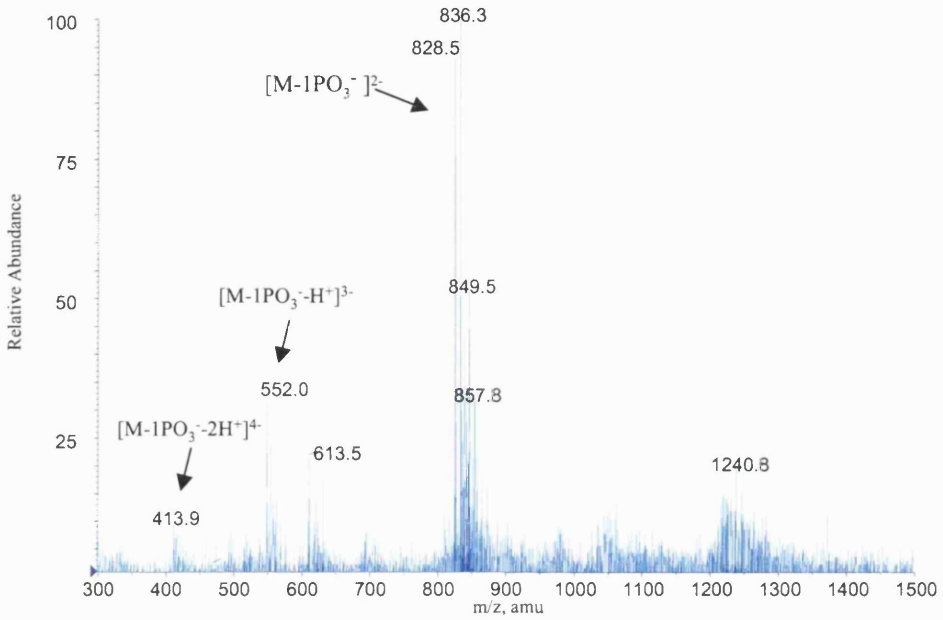


Fig. 5.4.5. Precursor Ion Scan of m/z79 of p70S6Kα N–terminal domain.
S6Kα N–terminal domain, *in vitro* tyrosine phosphorylated, was cut out from an SDS-PAGE gel and subjected to an in-gel digest with GluC endoprotease at 28°C overnight. The peptide mix was analysed by electrospray ionisation mass spectrometry (ESI-MS) on a Sciex API3000 mass spectrometer with a NanoESI source. Doubly, triply and quadrupoly charged phosphopeptides are indicated by arrows and correspond to a phosphopeptide SMDHGGVPpYELGME of a monoisotpic mass of 1579.7 Da (theoret.).

5.5. DISCUSSION

Using a combination of different mass spectrometrical methods we not only found phosphoserine sites on S6K which are already published but we also identified a novel phosphorylation site, T113 (p70S6Kβ numbering) in a S6Kβ sample derived from Sf9 cells overexpressing HGFR. This site is conserved in S6Kα and S6Kβ and also in p90RSK but not in any of the other AGC kinases. This could hint to a different regulatory mode for these S6Kinases in comparison to other AGC kinases. The

phosphothreonine site is positioned in the a-C helix of the N-lobe in the kinase domain. Two amino acids downstream of this site is the basic residue, histidine, which at least in the case of PKB/Akt (His 87) was shown to be important to hold the phosphorylated activation segment site T308 for PKB/Akt in position. If T113 really is phosphorylated *in vivo* than the introduction of the negative charge from the phosphate could possibly alter the structure of the activated kinase. But so far, no experiments with this site have been performed and the sequencing by mass spectrometry is also outstanding.

While mass spectrometry was successfully applied to detect phosphorylated serine and threonine sites, we initially were not able to detect any phosphotyrosine peptides from *in vivo* samples prepared by overexpression of receptor tyrosine kinases and S6Ks in Sf9 cells. We reasoned that the cause might be a very low level of tyrosine phosphorylation in our experimental set-up. However, we did not attempt a quantification of the stoichiometry achieved in Sf9 cells because it is a rather artificial system in comparison to a real *in vivo* setting with endogenous levels of protein. Yet, even though a strong signal was detected using the anti-phosphotyrosine 4G10 antibody in a western blot, in a precursor ion scan or in an LC/MS/MS experiment no phosphotyrosine containing peptide could be found. In order to raise the stoichiometry of tyrosine phosphorylation, we employed an *in vitro* kinase assay and enhanced the efficiency of phosphorylation by using high doses of ATP (250-500μM) and long incubation times (up to 1 hour). Samples prepared *in vitro* enabled us to identify phosphorylation of Y193 on S6Kα. This was found by precursor ion scanning as well as by LC/MS/MS. And finally, the peptide could be sequenced which resulted in the presence of characteristic fragment ions. As will be discussed in more detail in the next chapter this site is located at the beginning of the catalytic loop in the kinase domain and is highly conserved.

One way of achieving better sensitivity would have been the use [γ-^{32}P]ATP in the *in vitro* kinase assays, proteolytic digest and peptide separation by HPLC. The fractions containing the ^{32}P could then be analysed individually by precursor ion scanning. Similarly, isotopic labeling of cells with ^{32}P or ^{33}P could have been performed to detect *in vivo* phosphorylation sites. However, both these approaches were not pursued

because the no radioisotopes were to be used on the HPLC and mass spectrometry instruments in the laboratory.

A more biochemical approach yielded another phosphotyrosine site. S6K truncation mutants lacking the N-terminal regulatory domain failed to produce a signal in a phosphotyrosine western blot. This indicated that a major tyrosine site may be located in this domain. Indeed, a recombinant N-terminal fragment of S6Kα was a very good *in vitro* substrate for Src. Using MALDI in source decay (ISD) spectrometry, it was possible to sequence the S6Kα fragment from the N-terminus until reaching position Y39 where a phosphate group could be detected. In addition, the phosphopeptide containing phospho-Y39 could also be identified by a GluC digest followed by precursor ion scanning.

Taken together, through mass spectrometry and the use of truncation mutants it was possible to identify a novel threonine phosphorylation site, and two novel tyrosine phosphorylation sites. As will be discussed in chapter 6, one of the tyrosine sites (N-terminal site) is phosphorylated also *in vivo*, whereas the other site may only be an *in vitro* site.

CHAPTER 6: FUNCTIONAL ANALYSIS OF S6K TYROSINE PHOSPHORYLATION

CHAPTER 6

FUNCTIONAL ANALYSIS OF S6K TYROSINE PHOSPHORYLATION

1.1. INTRODUCTION

As described in chapter 5, two novel tyrosine residues on S6K, which are *in vitro* tyrosine phosphorylated by Src, have been identified. Site-directed mutagenesis of these residues could increase our understanding of the physiological importance of this modification. Src-induced tyrosine phosphorylation of AGC kinases was previously shown to affect three major functional aspects, including:

1. activity
2. subcellular localisation
3. stability

Tyrosine phosphorylation of several other AGC kinases was shown to lead to an increased kinase activity. In v-Src transformed cells PKB/Akt activity is enhanced, and this as attributed to elevated PIP_3 levels (Datta *et al.*, 1996; Liu *et al.*, 1998). Three PKB/Akt tyrosine phosphorylation sites have been determined which are either situated near the activation loop (Y315/Y326) or adjacent to the hydrophobic motif phosphorylation site (Y474) (Chen *et al.*, 2001; Conus *et al.*, 2002). These sites are necessary for PKB/Akt activity. In PDK1, three phosphorylation sites were also identified, of which two sites Y373/Y376 are located in the kinase extension domain and are involved in the activation of the kinase (Park *et al.*, 2001). Furthermore, in PKCδ, one out of three identified tyrosine phosphorylation sites (Tyr 311) was found to be necessary for full activity of the kinase. Y311 is upstream of the kinase domain, in the hinge region between the regulatory and catalytic domain. The two other phosphorylation sites Y332, Y512 have a not yet identified function (Konishi *et al.*,

2001). Furthermore, tyrosine phosphorylation in the PH domain of PKD leads to its activation (Storz *et al.*, 2003).

In the case of S6K, there is also evidence pointing towards Src-induced S6K activation: Firstly, phosphorylation of S6 protein in v-Src transformed cells is higher than in non-transformed cells and this finding was attributed to an elevated S6K activity (Blenis and Erikson, 1985). PI3K, which lies upstream of S6K, is involved in v-Src transformation and the level of PIP_3 is elevated in v-Src transformed cells (Penuel and Martin, 1999; Whitman *et al.*, 1986). These higher PIP_3 levels could also lead to stronger S6K activation in v-Src transformed cells. Furthermore, the Src inhibitor PP1 interferes with insulin, IGF1 and pervanadate-mediated S6K activation (Shah *et al.*, 2002).

Src-induced tyrosine phosphorylation of AGC kinases has also been shown to affect stability or subcellular localisation of the respective substrates. It has been shown for atypical PKCs that Src-induced tyrosine phosphorylation facilitates nuclear import, possibly by exposing the NLS through conformational changes (White *et al.*, 2002). Tyrosine phosphorylation can also affect the stability of a protein substrate. For example, Src-mediated tyrosine phosphorylation of PKCδ at Y311 by Src promotes its degradation (Blake et al, 1999). However, this finding has recently been questioned and it was suggested that phosphorylation of this site regulates the activity of PKCδ towards certain substrates, i.e. affects substrate specificity (Rybin *et al.*, 2004).

In this chapter the experiments are described which assess if tyrosine phosphorylation of S6K affects one of these different functional aspects.

6.2. SITE-DIRECTED MUTAGENESIS TO CREATE PHOSPHO-TYROSINE SITE MUTANTS

In order to create a residue that resembles tyrosine but cannot be phosphorylated it is generally agreed that phenylalanine is a good candidate as it is identical to tyrosine but lacking the hydroxyl-group at the 1' position of the aromatic ring. To mimic the phosphorylated tyrosine residue has proven more difficult as both naturally occuring

acidic residues, Glu and Asp, which can mimic a phosphoserine or phosphothreonine with their negative charge, are much smaller than tyrosine or a phosphotyrosine. This is probably the reason why in most reports only the phenylalanine mutant is used in functional assays.

A variety of different point mutants to both identified tyrosine sites were generated by site-directed mutagenesis as described in 2.1.2.. As the *in vitro* site in the catalytic domain was highly conserved among AGC kinases we also wanted to address the question if the same tyrosine site could play a role in the function of PKB/Akt and PKCs. Therefore we also generated phenylalanine mutants to both, PKBα and PKCγ. Mutation of such a crucial residue located at the mouth of the catalytic loop could change the conformation within the catalytic pocket so that a change in kinase activity in the Y to F mutant might be simply due to a disruption of the secondary and tertiary structure of the kinase. To address this, we also generated a Y-H S6K mutant. As shown in the alignment of AGC kinases (Fig. 5.3.5.), NDR and PDK1 have a histidine instead of a tyrosine at this position. Tyrosine kinases such as PDGFR also have a histidine. By mutating the S6K tyrosine residue to histidine we could be confident that the catalytically important loop structure remained intact but that no tyrosine phosphorylation could occur.

The templates used for PCR were vectors of wt S6Kα or S6Kβ, PKBα or PKCγ. Moreover, we generated a gain-of-function PDGFR mutant D850V. This mutant was able to further enhance tyrosine phosphorylation of S6K when expressed in cells. It was designed according to a natural gain-of-function mutant of Kit occurring in a variety of gastrointestinal stromal tumors. The rationale was that by strongly enhancing tyrosine phosphorylation we may be able to detect any functional relevance of the modification more easily.

The primers used for site-directed mutagenesis are listed in chapter 2.1.2.. All mutants were sequenced to ensure successful site-directed mutagenesis as shown for some but not all mutants in Fig. 6.2.1..

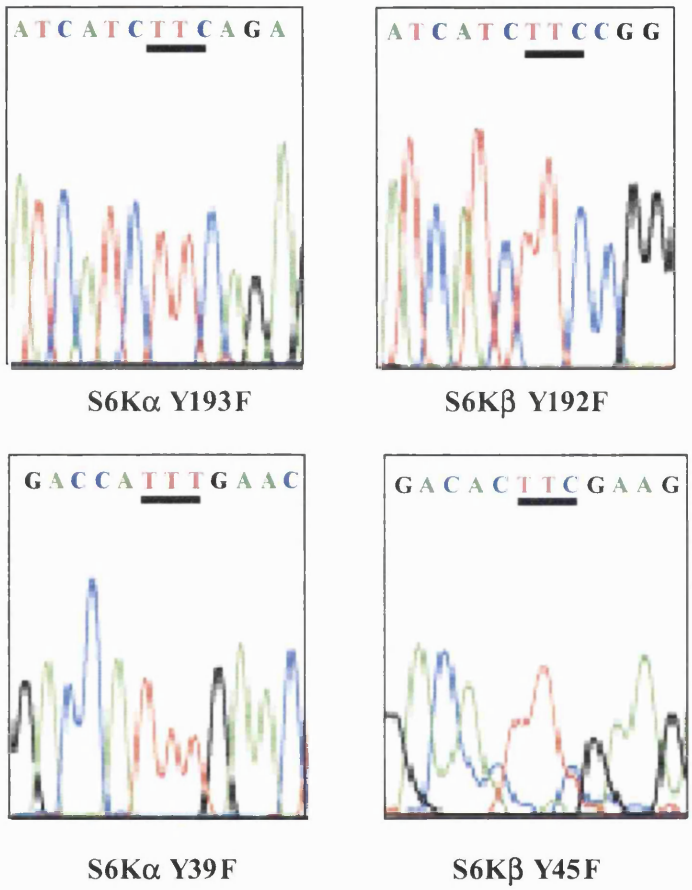


Fig. 6.2.1. DNA sequence analysis of S6K mutants.
A similar analysis was performed for all other plasmids which were subjected to site-directed mutagenesis but the graphs are not shown here.

6.3. GENERATION, PURIFICATION AND TESTING OF A PHOSPHO-SITE-SPECIFIC ANTIBODY

The S6KαY193 and S6KβY192 site were identified as phosphorylation sites for Src, Btk and Syk kinases *in vitro*. In order to obtain a powerful tool to use in *in vivo* studies we decided to generate a phosphosite-specific antibody. The antibodies were raised in rabbits and affinity purified as described in 2.3.. S6K was phosphorylated *in vitro* by various tyrosine kinases in order to assess if affinity-purified antibody could detect phospho–S6K at residues Y193 (for S6Kα) or Y192 (for S6Kβ). In the western blot,

the antibodies generated a stronger signal in the samples subjected to tyrosine phosphorylation than in the mock-treated samples (data not shown). To analyse if phosphorylation of tyrosine Y193/192 occurs *in vivo* after mitogenic stimulation we tested the purified antibody on S6K from stimulated MCF7 or NIH3T3 cells by western blotting. The antibody recognised endogenous S6Kα only from serum or pervanadate stimulated cells but not from starved cells. However, the Y193/192F mutants were still recognized to the same extent as the wild type (data not shown). That indicated that the affinity-purified antibody was a phosphotyrosine antibody that was not site-specific. These data are not shown because they did not clarify if the identified site is an *in vivo* site. Similarly, the 4G10 blot of the Y193/192F mutant does not exhibit a reduced phosphotyrosine signal. This indicates that the identified site may not a be a major tyrosine phosphorylation site *in vivo* even though *in vitro* it clearly was strongly present (as detected by mass spectrometry). It is possible, however, that the 4G10 antibody may not recognise this specific site and that we may not see a reduction in signal for this reason. This scenario is rather improbable as the antibody yielded a strong phosphotyrosine signal in a western blot of S6K that had been *in vitro* phosphorylated by Src. If one assumes that the Y193/Y192 site is a major *in vitro* phosphorylation site for Src then the 4G10 antibody seems to have recognised the phosphotyrosine. On the other hand, the fact that the peptide represented the major phosphopeptide peak found by mass spectrometry (in the precursor ion scan) does not give information about its relative occurence. This peptide could simply have a favourable charge distribution for ionisation in the mass spectrometer and may not be the major *in vitro* phosphorylation site for Src.

Another possibility to unify these findings is that the identified site is purely an *in vitro* site. *In vitro*, Src and other kinases might phosphorylate other sites than *in vivo* where co-factors and subcellular localisation also play a role.

Taken together, we are very confident that the Y193/192 site is an *in vitro* site but we currently do not have a proof that this site is phosphorylated *in vivo*.

6.4. ANALYSIS OF THE Y39/45F MUTANT

As we were not successful in determining whether the Y193/Y192 site is an *in vivo* site, it was necessary to establish whether the overall phosphotyrosine signal of S6K is diminished when the site in the N-terminus is mutated (S6KaY39F).

Even though N-terminal sequences of S6Kα and S6Kβ are only conserved to 38%, as can be seen in the alignment (Fig. 6.4.1.) a tyrosine residue is present in the S6Kβ (Y45) sequence very closely to the site identified for S6Kα (Y39), equally followed by a glutamate at +1. In order to determine if the S6Kα and S6Kβ sites are both major phosphorylation sites in full length S6K, EE-tagged phenylalanine mutants (S6KαY39F, S6KβY45F) were generated.

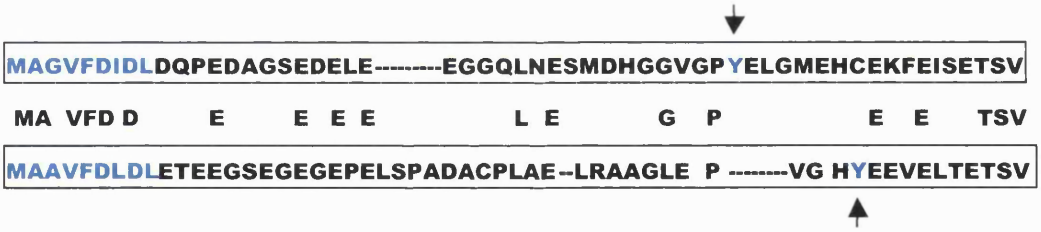


Fig. 6.4.1. Alignment of the N-terminal domains of S6Kα and S6Kβ.
The arrows indicate the potential and only tyrosine sites in the N-terminus. The amino acids in the middle row represents conserved residues. The amino acid homology between S6Kα and S6Kβ in this region of the kinase is 39%. The blue letters mark a putative nuclear export signal.

Mutants were expressed in Hek293 cells, immunoprecipitated and subjected to an *in vitro* kinase assay using Src where tyrosine phosphorylation was much reduced in the Y39/45F mutants when compared to wt S6Ks. Interestingly, *in vitro*, the cytoplasmic domain of PDGFR phosphorylated the mutant and wild type S6K to a similar extent which indicated that the identified site is a Src-specific but not a PDGFR- specific site (Fig. 6.4.2.). As Src and S6Kβ have the same molecular weight and cannot be separated by SDS-PAGE, Src alone also was tested to exclude that the signal for S6Kβ might be

overshadowed by Src autophosphorylation. But this was not shown to be the case. To exclude the possibility that the changes in S6K phosphorylation stem from a reduced autophosphorylation capacity of the Y-F mutant we tested this and found that autophosphorylation of WT and mutant are low and similar (lower panel, Fig. 6.4.2.).

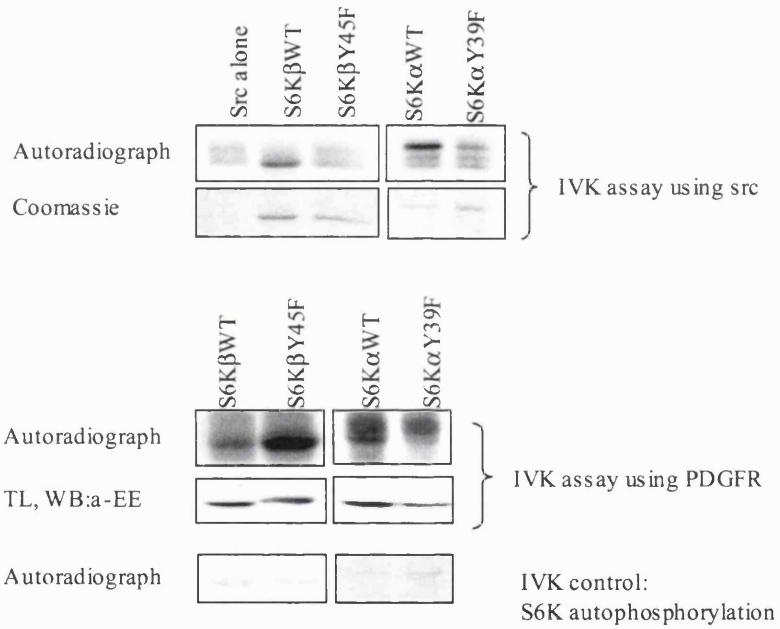


Fig. 6.4.2. S6Kα/β Y39/45 is a substrate for Src but not for PDGFR *in vitro*.
EE-S6K WT and mutants were immunopurified from Hek293 cells and subjected to an *in vitro* kinase assay using recombinant Src kinase or the cytosolic domain of PDGFR. Reaction products were analysed by autoradiography, Coomassie-staining and western blotting as indicated.

Fig. 6.4.3a. shows that *in vivo*, overexpression of either the S6KαY39F or S6KβY45F mutant together with activated Src in Hek293 cells similarly lead to a strongly reduced phosphotyrosine signal. Yet again, when PDGFR instead of Src was overexpressed and PDGF was used as mitogen, a reduction could not be observed. Work presented in chapter 3.3. established that S6K and PDGFR associated in a stimulation–dependent manner. Interestingly, PDGFR still co-immunoprecipitated with the Y-F mutant, but

possibly to a lesser extent (Fig. 6.4.3b.). One can conclude that S6K associates with PDGFR and is phosphorylated by it independently of Src-mediated phosphorylation. If Src-induced phosphorylation was needed for the recruitment to the receptor, then the Y39/45F mutant should not co-immunoprecipitate PDGFR. This is further confirmed by the finding that the PDGFR 579/581F-Src signalling deficient mutant is still complexed with S6K as it co-immunoprecipitates with S6K (data not shown). The residual tyrosine phosphorylation which is detected in S6K from cells overexpressing this receptor mutant therefore may be due to tyrosine phosphorylation mediated by the receptor itself (Fig. 4.2.1.).

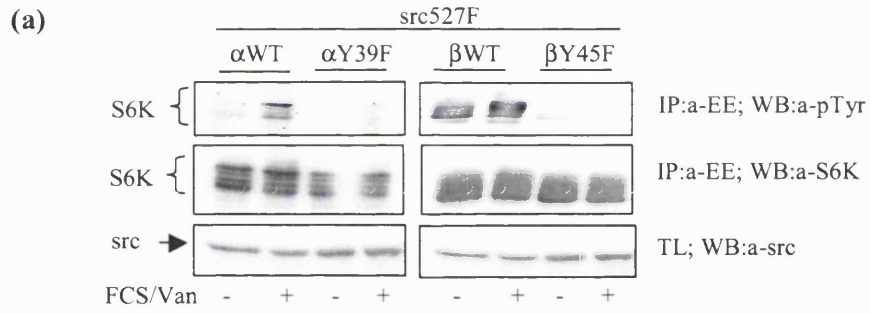


Fig. 6.4.3. S6Kα/β Y39/45 is a substrate for Src but not for PDGFR *in vivo*.
(a) S6K wt or mutants and Src kinase were overexpressed in Hek293 cells, which were starved and stimulated with 10% FCS for 15 min and for 5 min with pervanadate. Immunoprecipitated S6Ks were tested with anti-phospho-tyrosine antibody by western blotting and membrane was reprobed with S6K antibody. Total lysate (TL) was also analysed for Src expression.

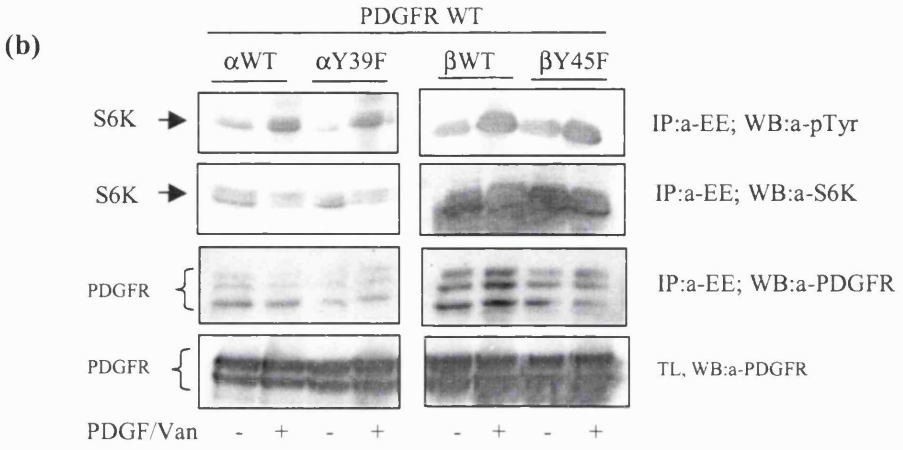


Fig. 6.4.3. S6Kα/β Y39/45 is a substrate for Src but not for PDGFR *in vivo*.
(b) Experiment was performed similarly to (a) but instead of Src, PDGFR was used.

6.5. INVESTIGATION OF THE INFLUENCE OF TYROSINE PHOSPHORYLATION ON S6K ACTIVITY

This chapter will address if tyrosine phosphorylation affects the activity of S6K. Various conditions which lead to changes in S6K tyrosine phosphorylation will be applied and it will be addressed if they alter S6K activity. The following conditions will be tested: overexpression of a signaling-defective PDGFR mutant (6.5.1.), use of inhibitors (6.5.2.), Src overexpression (6.5.3.) and finally overexpression of a gain-of-function PFGFR mutant (6.5.4.). Thereafter, the mutants to the tyrosine site in the kinase domain (S6KαY193, S6KβY192) will be tested for their activity (6.5.5. and 6.5.6.), followed by the same analysis for the mutants to the site in the N-terminal region (S6KαY39, S6KβY45) (6.5.7.).

6.5.1. Effect of PDGF receptor mutants on S6K activity

Src has been shown to positively regulate the activity of S6K. In v-Src transformed cells S6K activity is elevated and Src inhibitor PP1 reduces S6K activity (Blenis and Erikson, 1985; Shah *et al.*, 2002). However, it still is unclear if Src achieves this effect indirectly, by activating upstream signalling molecules such as PDK1, PI3K or PKB, or whether tyrosine phosphorylation of S6K might play a role.

First, we needed to assess if there was a correlation between tyrosine phosphorylation and S6K activity. To achieve this, the PDGFR point mutants were tested. As previously shown, tyrosine phosphorylation is strongly reduced when the src-signalling defective PDGFRY579/581F mutant is expressed (Fig. 4.2.1.). When the membrane is reprobed with a-S6K C-terminal antibody, the S6Kα signal still exhibits a mobility shift in the SDS-PAGE gel. This indicates that S/T phosphorylation and hence activity may be normal and not correlate with the phosphotyrosine signal. In the S6Kβ sample it is more difficult to assess the mobility shift which has occurred to a much lesser extent than in S6Kα (Fig. 4.2.1). To investigate this further, we assayed S6K in an *in vitro* kinase assay. When co-expressed with PDGFR Y579/581F, S6K remained fully active as shown in Fig. 6.5.1. Expression levels of S6Ks and PDGFR were also assessed by western blotting. Taken together, in this system, we were not able to correlate S6K tyrosine phosphorylation with its activity.

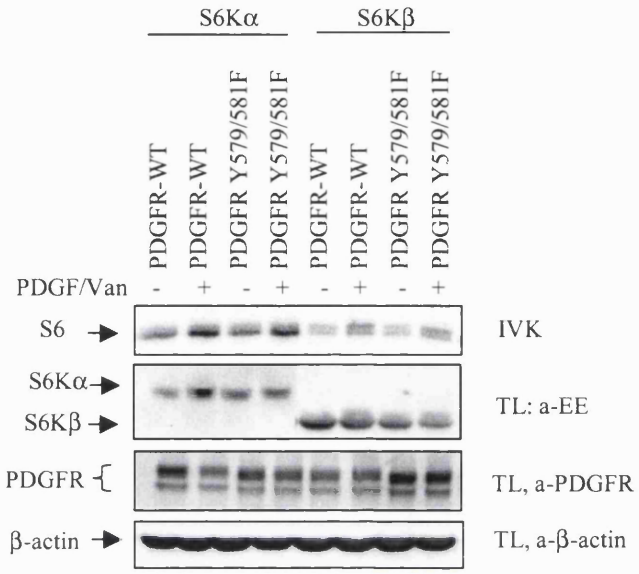


Fig. 6.5.1. S6K activity is not affected by PDGFR mutant deficient in Src signalling.
Cos7 cells were transfected with either WT or mutant PDGFRβY579/581F, and either isoform of S6Kα or β, starved and stimulated with PDGF (40ng/ml) for 15 min followed by pervanadate (5 min). S6Ks were immunoprecipitated with the EE-antibody, then used for an *in vitro* kinase assay (IVK) with ribosomal protein S6 as a substrate. The total lysate (TL, 30μg) was tested for S6K and PDGFR expression and β-actin.

6.5.2. Effect of inhibitors on S6K activity

Another way to test if S6K activity correlated with tyrosine phosphorylation was to employ the inhibitors which previously lead to a reduced phosphotyrosine signal in S6K. Hek293 cells were starved for 24 hours, the inhibitors were added to cells 60 minutes before serum stimulation with 10 % FCS. LY294002 and rapamycin, at concentrations of 20 μM and 10 nM respectively, were previously shown to inhibit S6K activity and, as can be seen in Fig. 6.5.2., this was indeed the case. Tyrosine kinase inhibitors PP1 which acts on Src and PDGFR, resulted in significantly reduced activity which is more prominent *in vivo* (assessed in the phospho-S6 blot) than *in vitro*. However, SU6656, a Src-specific inhibitor did not reduce S6K activity. From the

previous experiment (Fig. 4.5.1b.) we know that SU6656 can lead to a reduction in tyrosine phosphorylation of S6K. As a result, the use of tyrosine kinase inhibitors lead us to conclude that tyrosine phosphorylation does not correlate with S6K activity. Similar results were obtained when either serum or PDGF were used for stimulation.

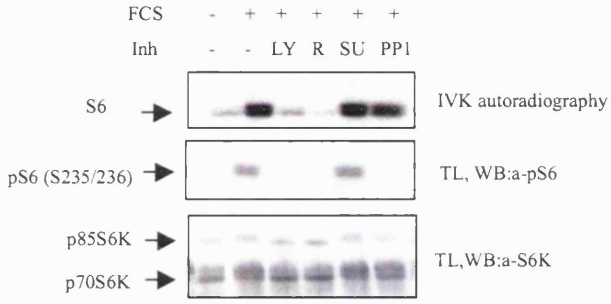


Fig. 6.5.2. S6K activity is not affected Src-specific inhibitor SU6656.
Inhibitors were added to starved Hek293 cells 60 minutes before serum-stimulation. Immunoprecipitated endogenous S6Kα was used in an *in vitro* kinase assay (IVK) with S6 as substrate. 30µg of total lysate were probed for S6Kα expression and levels of phospho-S6 (S235, S236). Concentrations used: LY20µM, Rapamycin10nM, SU6656 4µM, PP1 50µM.

Furthermore, in a few *in vitro* experiments, tyrosine phosphorylated S6K (derived from stimulated Cos7 cells) was dephosphorylated with recombinant tyrosine phosphatase PTPβ (Upstate) and the *in vitro* activity of S6K was subsequently assessed. But no difference in activity between de-phosphorylated and mock-treated samples could be observed. Western blotting with 4G10 antibody ensured that the PTPβ-mediated dephosphorylation was successful (data not shown).

6.5.3. Effect of Src overexpression on S6K activity

Because the findings with the Src-signalling-deficient PDGF receptor mutant (Y579/581F) and inhibitor SU6656 were different to our expectations or to findings in other AGC kinases, we also wanted to determine the influence of Src overexpression on S6K activity. In Fig. 4.3.1. it can be seen that there is no direct correlation between Src-induced tyrosine phosphorylation and S6K activity. For example, pT389 which is considered a hallmark of S6K activity, is not altered whether dominant negative Src or constitutively active Src or empty vector are expressed in stimulated cells. Interestingly, in starved cells, activation of S6K occurs when constitutively activated Src is overexpressed as indicated by the gel shift. From this experiment we can conclude that Src activates S6K (and this activation is mainly visible when other stimulatory inputs are shut down such as in starved cells), but that this activation is independent of tyrosine phosphorylation of S6K.

This experiment was performed with serum as stimulus, which activates many pathways including the PDGFR pathway. However, a specific PDGF-mediated pathway may be overshadowed by other pathways and hence it was important to assess the effect of Src on S6K activity with PDGF as stimulus. Therefore co-expression of PDGFR and Src with either S6Kα or S6Kβ in Hek293 cells was performed. It was tested if Src in comparison to empty vector expression would make S6K more active. As a result it can be stated that there was no difference in activity of S6K derived from the differently transfected cells, whether they expressed Src or not (Fig. 6.5.3.).

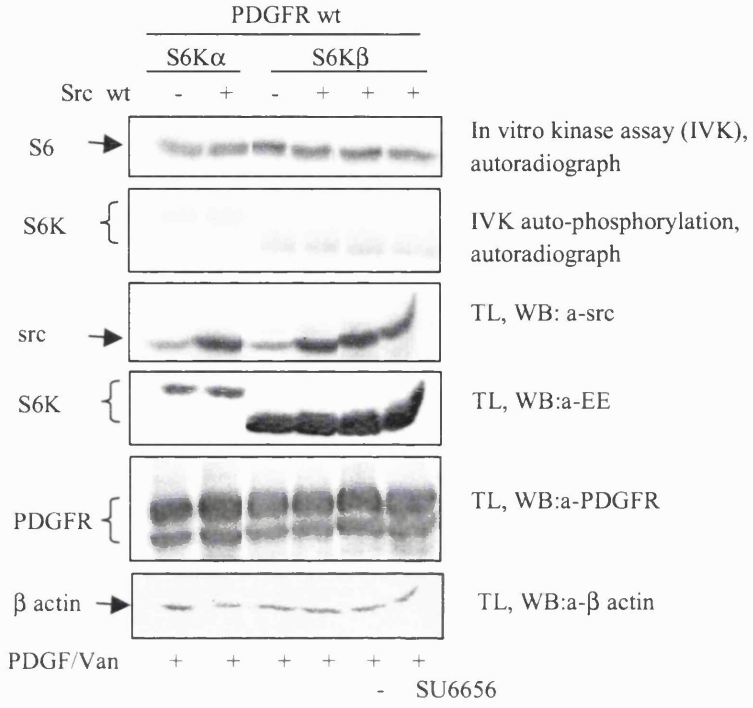


Fig. 6.5.3. Src overexpression does not alter S6K activity.
Hek293 cells were transfected with PDGFR, Src and either S6Kα or β, starved and stimulated with PDGF (40ng/ml) for 30 min/5 minutes pervanadate. S6Ks were immunoprecipitated with the EE-antibody, then used for an *in vitro* kinase assay (IVK) with S6 protein as a substrate. The total lysate (30μg) was tested for S6K, Src, PDGFR and β-actin expression.

However, the amount of endogenous Src may be sufficient to maximally activate S6K and thus a surplus of Src may not yield a detectable change in activity. In parallel, one set of samples was treated with SU6656 for 30 minutes before stimulation, but S6K activity was not reduced by the inhibitor. Taken together, in this experimental set-up, no obvious correlation between tyrosine phosphorylation and S6K activity could be detected.

6.5.4. Gain-of-function PDGFR mutant

In many gastrointestinal stromal cancers the kit gene was found to be mutated and the gene product Kit is strongly activated. One re-occuring mutation is the D816V mutation in the kinase domain. Kit is a member of the PDGFR family and the physiological functions of both receptors overlap. Hence, it is not surprising that mutation of the homologous site of the Kit D816V mutation is also found in a subset of gastrointestinal stromal tumours in PDGFRα, D842V (Hirota *et al.*, 2003). We reasoned that a mutation of the homologous site in PDGFRβ, which has been used as a system for all previous experiments, may be useful to test the effect of such an activated PDGFR mutant on S6K. We therefore generated the point mutant PDGFRβ D850V and co-expressed it together with S6Ks in cells. As expected, the level of tyrosine phosphorylation of S6K was much higher than when wt PDGFRβ was co-expressed with S6K (Fig. 6.5.4a.). This confirmed the hypothesis that this mutation strongly enhances PDGFRβ activity as seen for c-Kit and PDGFRα. The receptor mutant exhibits slightly faster migration in the polyacrylamide gel. The wt PDGFR migrates as two bands in the gel, the lower form represents an unmature, not fully glycosylated receptor, whereas the slower migrating one represents a mature and fully glycosylated form of the recptor (L. Ronnstrand, personal communication). Thus, the PDGFRβ D850V, which migrates even faster than the unmature wt receptor, may have a defect in glycosylation. It was not assessed if the naturally occurring mutant PDGFRα D842V does have a similar migration pattern in a SDS-PAGE gel as work with the PDGFRβ D850V mutant was not pursued. Surprisingly, the activity of S6K seems to be reduced when co-expressed with the D850V mutant (Fig. 6.5.4b.). This would indicate an inverse correlation between tyrosine phosphorylation and activity. It is known that this mutant has a defect in signalling to PDK1 (P. Blume-Jensen, unpublished data). Hence the reduced activity of S6K could be explained by a lack of PDK1 mediated S6K activation and therefore does not contradict our previous data.

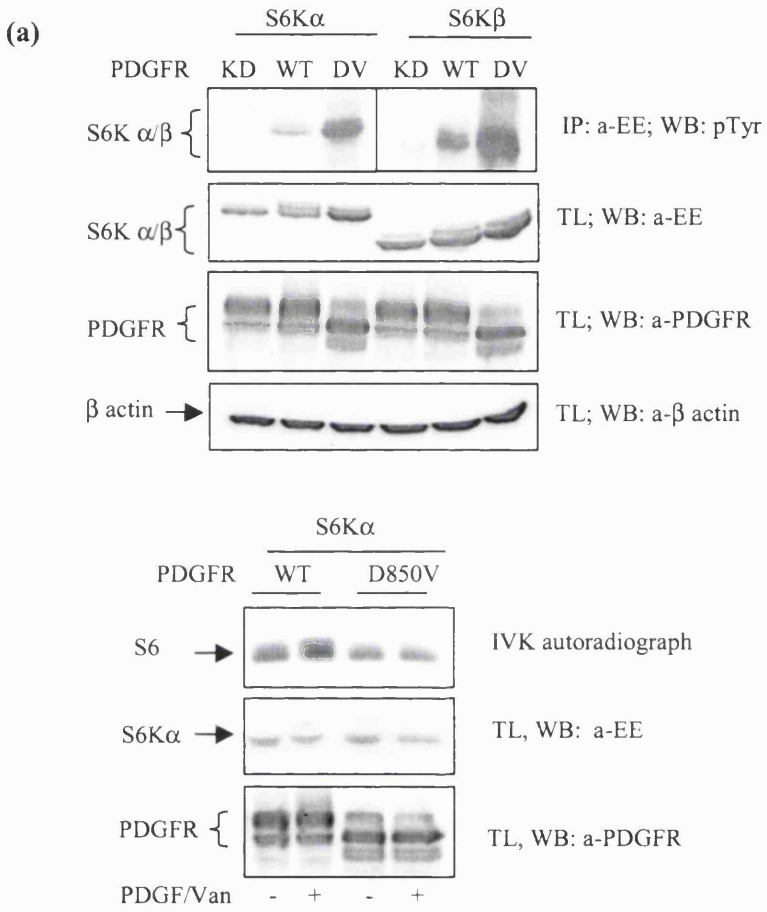


Fig. 6.5.4. Effect of PDGFR D850V on S6K tyrosine phosphorylation and activity.
Hek293 were transfected with PDGFR-WT or D850V and S6Kα or S6Kβ. **(a)** S6Ks were immunoprecipitated from exponentially growing cells and analysed by western blotting using the 4G10 antibody. 30μg of total lysate were tested by western blotting. **(b)** S6Ks were immunoprecipitated from starved and stimulated cells and and subjected to an *in vitro* kinase assay using S6 protein as substrate. Expression of S6K and PDGFR was assessed by probing 30μg of lysate.

6.5.5. Mutation of S6Kα/β Y193/192 affects activity

To further address the role of tyrosine phosphorylation in the regulation of S6K activity the Y193/192F mutants were tested. Wild type and mutant S6Ks were overexpressed in serum-stimulated Hek293 cells, then immunoprecipitated using anti-EE antibody followed by an *in vitro* kinase assay with S6 protein as substrate. Interestingly, the Y-F mutant exhibited a strongly reduced activity when compared to the wild type over long periods of activation. Four different time course experiments (up to 0.5, 2, 3 and 6 hours of serum simulation) all consistently showed reduced activity of the Y-F mutant. Fig. 6.5.5. shows one representative result as a blot/autoradiograph and one as a graph. Due to the different time points used in each experiment it is not possible to combine the results for the calculation of error bars, however due to the fact that the four experiments delivered similar results, we can conclude that the activity of the mutant really is reduced.

Tyrosine Y193/192 is in a crucial position within the catalytic loop (as shown in Fig. 5.3.5.). The crystal structure of activated PKA reveals that the arginine (and also the asparatate) following the tyrosine are involved in holding the phosphorylated threonine pT197 (which corresponds to T229 in S6Kα or T309 in PKB/Akt) in its position when the kinase is in an active conformation (Yang *et al.*, 2002b). Hence, we had to exclude that the loss in activity in the Y-F mutant was not caused simply by mutation of a crucial residue important for the protein folding. A variety of kinases (such as AGC kinases PDK1 and NDR but also PDGFR) have a histidine at the place of tyrosine. Hence, it was logical to generate S6Kα/βY193/192H mutants. One can assume that the conformation of the kinase domain in a Y-H mutated protein should not be distorted and that histidine can figure as a good replacement of a non-phosphorylatable tyrosine residue. When tested for its activity, the H mutant, similarly to the F mutant, exhibited reduced but not abolished S6K activity. The reduction was approx. 50% for S6KY-H mutants in comparison to wild type S6Ks after 10 minutes of serum-stimulation. Two individual experiments were performed. The result was taken as additional indication that this tyrosine site may be phosphorylated *in vivo* (Fig. 6.5.6.).

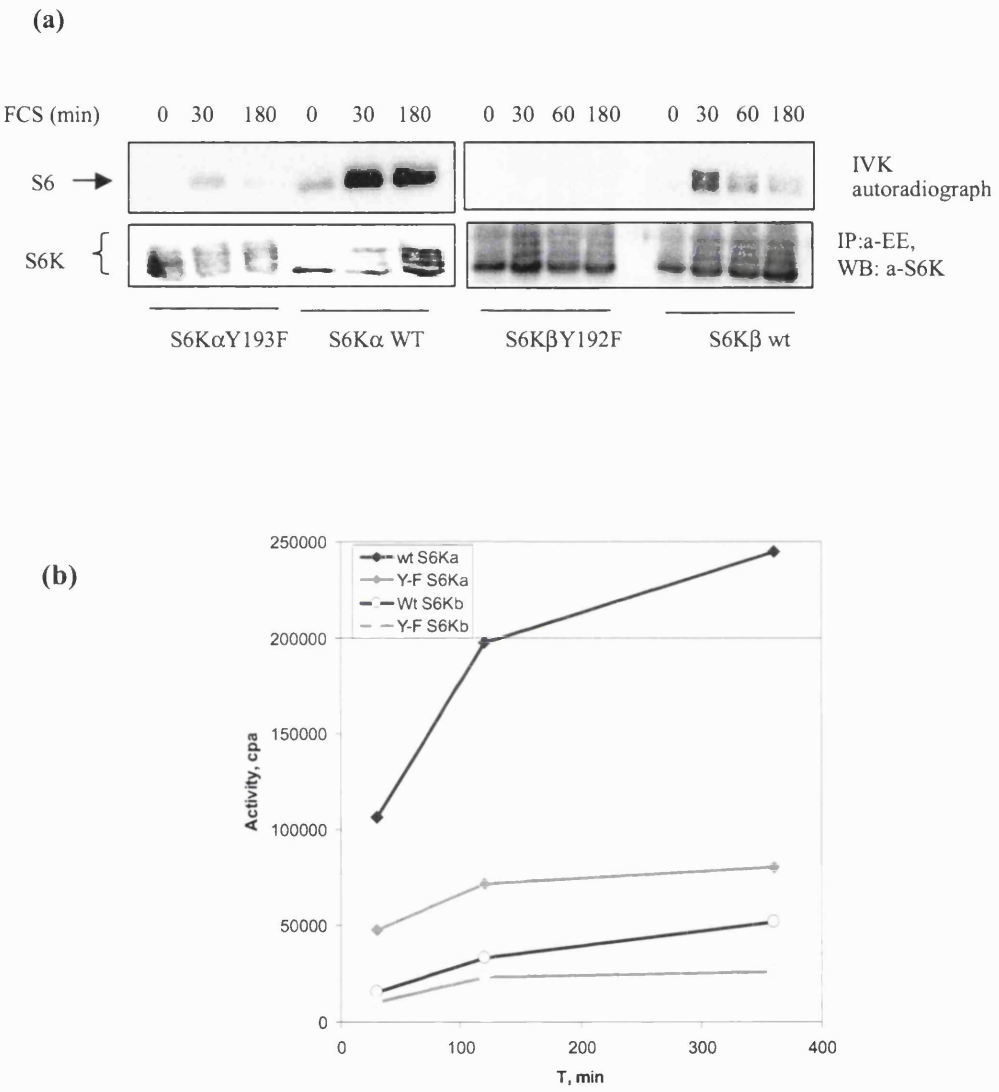


Fig. 6.5.5. Mutation of S6Kα/β Y19/192 reduces S6K activity.
S6K point mutants, expressed in and immunoprecipitated from starved and serum-stimulated Hek293 cells and used in an *in vitro* kinase assay with 40S ribosomal proteins as substrate. S6 phosphorylation and autophosphorylation activity were assessed and quantified with the BioRad phosphoimager software. **(a)** A three hour time course experiment is presented. Three different time course experiments were performed for S6Kα and β, however with different time points in each experiment. **(b)** A six hour time course experiment is represented.

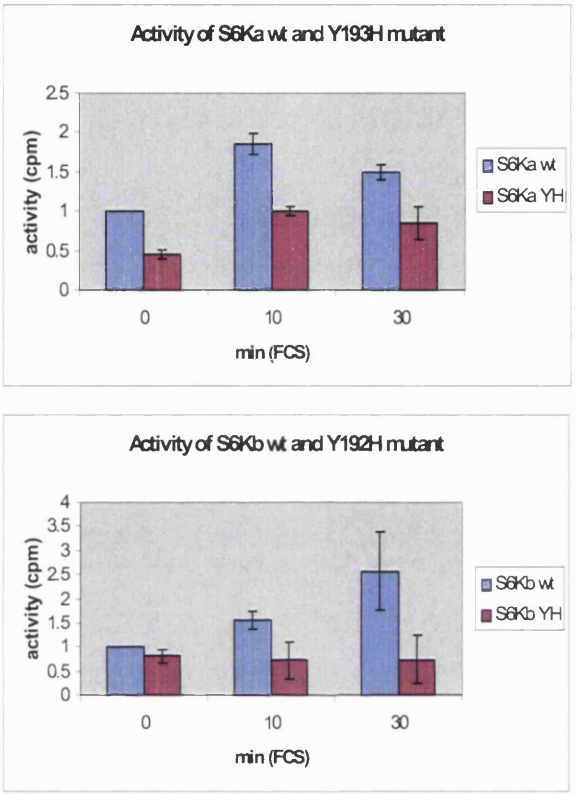


Fig. 6.5.6. S6K activity of wt S6K versus Y193/192H mutants.
Hek 293 cells were transfected with either wt or histidine mutants (Y193/192H) of S6Ks, starved and stimulated with 10% FCS for 10 oder 30 min. Immunoprecipitated S6Ks were subjected to an *in vitro* kinase assay. The top diagramm shows the activities of S6Kα wt and Y193H mutants, the lower diagram shows the activities of S6Kβ wt and Y192H mutants, based on two experiments. Activity was normalised with the amount of S6K expression and the basal activity of S6Kα/β wt was set as 1. The error bars represent one standard deviation

6.5.6. Activity of PKB/AKT and PKC point mutants

The tyrosine phosphorylation site (S6KαY193/S6KβY192) at the mouth of the catalytic loop is conserved among AGC kinases, with the exception of PDK1 and NDR. It was of interest to determine if tyrosine phosphorylation may also occur at homologous sites in other AGC kinases and if this also affected their activity. We chose PKBα and PKCγ and generated Y-F mutants which we expressed in Hek293. To test PKCγ activity, cells were starved and stimulated with TPA before lysis. Immunopurified myc-PKCγ was then subjected to a kinase assay using histone H1 as substrate (see 2.5.10.). The autoradiograph in Fig. 6.5.7a. clearly indicates that, similarly to S6K, the mutation of Y to F reduced the activity of PKCγ. However, when PKBα was assayed with myelin basic protein as substrate, the wt and mutant exhibited similar activities (Fig. 6.5.7b). PKB/Akt is membrane located and activated, whereas PKC and S6K are activated differently as they don' t bind to phosphatidylinositol second messengers. Thus, the activation process may be slightly different and not require tyrosine phosphorylation on this site. These arguments could explain why tyrosine phosphorylation at the corresponding PKB/Akt site is not part of the activation process. More experimental data is needed to confirm this hypothesis. In the two individual experiments that were performed for each kinase, no real stimulation could be achieved. This may be due to the quality of cells used, or possibly a longer starvation period should have been chosen. However, from these experiments, it is obvious that the activity of the PKC mutant is affected whereas the PKB/Akt mutant is not.

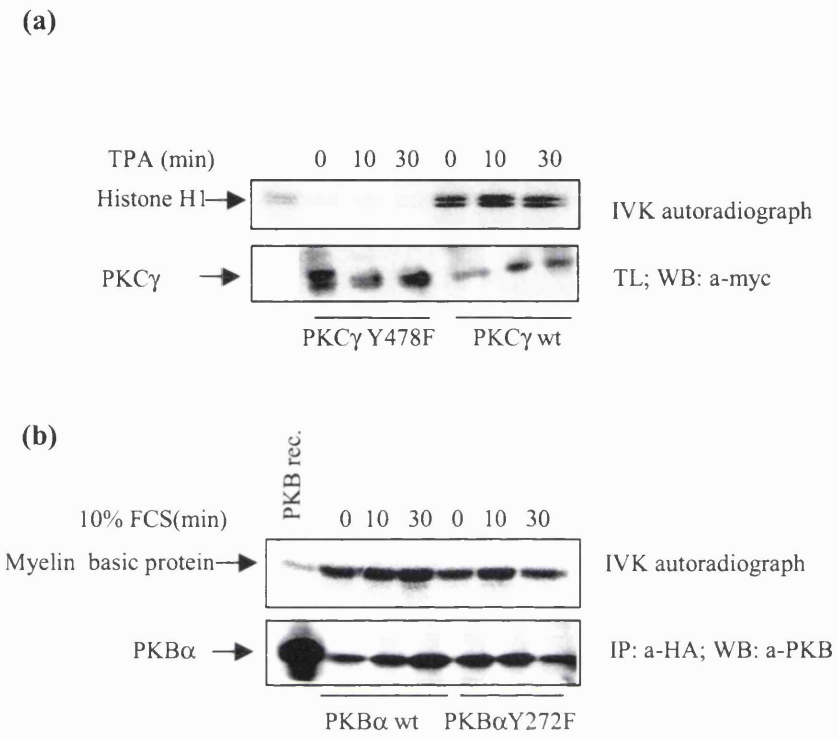


Fig. 6.5.7. PKCγ but not PKBα activity is reduced in the Y-F mutant.
(a) PKCγ wt and Y478F were transiently expressed in Hek293 cells. Starved cells were stimulated with TPA (1μM) for the times indicated, myc-tagged PKCγ was immunoprecipitated using flag-antibody and immunocomplexes were tested in an *in vitro* kinase assay with histone H1 as a substrate as described in chapter 2.5.10.
(b) Bovine PKBα wt and Y272F were transiently expressed in Hek293 cells. Starved cells were stimulated with serum for the times indicated, HA-tagged PKB was immunoprecipitated using HA-antibody and immunocomplexes were tested in an *in vitro* kinase assay with myelin basic substrate as described in chapter 2.5.11.

6.5.7. Activity of the S6Kα/β Y39/45F mutants

We have already established that the site Y39/45 is a major Src-dependent phosphorylation site in S6K. When the site is mutated to phenylalanine, tyrosine phosphorylation of S6Ks is abolished in the presence of transiently overexpressed Src kinase. Next, we wanted to see if this mutation would also affect the activity of the kinase. We expressed an activated Src and S6Kα wild type and Y39F or Y39D mutants

in Hek293 cells. As a result, in three experiments, no difference between wild type and mutant activities could be observed in stimulated or starved cells, *in vitro* or *in vivo* (phospho-S6), indicating that this site does not influence kinase activity (Fig. 6.5.8a.).

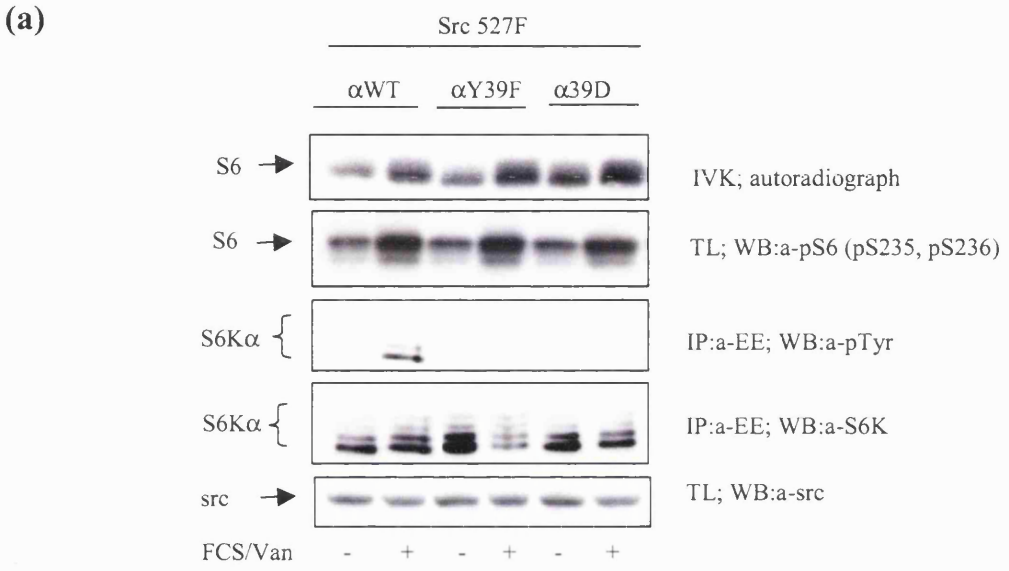


Fig. 6.5.8. The activity of S6Kα/β Y39F/Y45F or S6KαY39D mutants is not altered.
(a) Hek293 cells were transfected with S6K WT or mutants and activated Src (527F). Cells were starved and stimulated as indicated. S6K was immunoprecipitated from these cells, subjected to an *in vitro* kinase assay using S6 as a substrate or to phosphotyrosine western blotting. The membrane was also probed with anti-S6K antibody, total lysate was analysed using anti-Src antibody.

The N-terminus of S6K is a regulatory domain. It has been proposed that the serine/threonine phosphatase PP2A inactivates S6K through its interaction with the first 24 amino acids of the S6K N-terminus and dephosphorylation of crucial serine/threonine sites of S6K (Peterson *et al.*, 1999). Thus, it was plausible to imagine that phosphorylation on tyrosine Y39 or Y45 could alter the binding of PP2A to S6K, even though these two sites are not part of the direct PP2A binding domain but adjacent to it. Possibly, tyrosine phosphorylation could be linked to de-activation of S6K by affecting binding of PP2A. If the phosphorylation mediates PP2A binding, then a Y-F

mutant may be active for longer, as it cannot bind PP2A as efficiently. Fig. 6.5.8b. shows that there is no difference in the deactivation between wild type and mutant over a time course of 21 hours. Likewise, it was also tested if the mutant would be less sensitive to rapamycin. It is thought that rapamycin activates PP2A by inhibiting mTor-mediated phosphorylation and inactivation of PP2A (Peterson *et al.*, 1999). This was addressed by treating cells overexpressing S6Ks wt and Y39/45F mutants with 10 nM rapamycin and assessing the effect on phosphorylation of the rapamycin-sensitive site T389. As a result, no difference between S6K wild type and mutants after rapamycin treatment could be detected in a western blot using pT389 phosphospecific antibody, as both were similarly rapamycin-sensitive (data not shown). Besides, co-immunoprecipitation experiments were performed to address if PP2A would be in complex with wt S6Ks and to a different degree with the Y39/45F mutants. However, in the laboratory only a very old and non-specific antibody was present and due to time constraints this aspect was not pursued any further. With regards to unaltered deactivation kinetics and rapamycin sensitivity, we concluded that phosphorylation on the N-terminal site probably does not affect binding of the phosphatase PP2A.

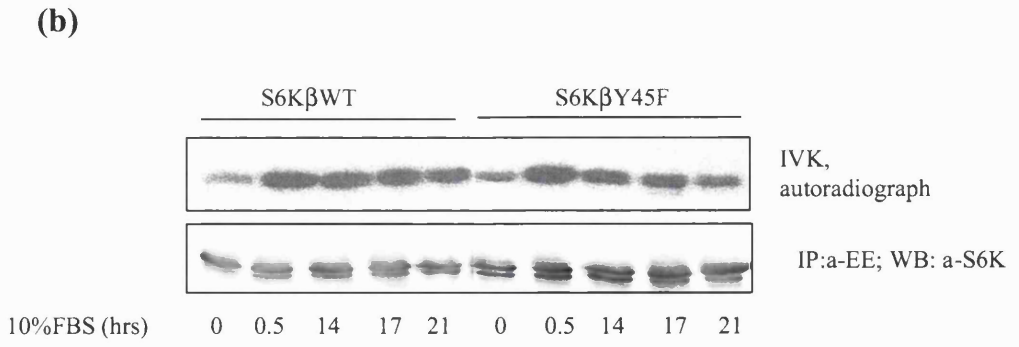


Fig. 6.5.8b. The activity of S6Kα/β Y39F/Y45F or S6KαY39D mutants is not altered.
Hek293 cells were transfected with S6KβWT or Y45F, starved and stimulated as indicated. S6Ks were immunoprecipitated from these cells and used in an *in vitro* kinase assay. S6K expression was also assessed by western blotting.

6.6. EFFECT OF SRC ON STABILITY OF S6K

Tyrosine phosphorylation was shown to have other effects on AGC kinases such as altering protein stability or subcellular localisation. We then wanted to see if S6K stability was affected by Src-induced tyrosine phosphorylation. PKCδ was shown to be degraded after Src-mediated phosphorylation. In NIH3T3 cells overexpressing activated Src there was significantly less PKCδ present. (Blake *et al.*, 1999). In the case of S6K we cannot see a reduction of S6K expression levels in v-Src transformed Swiss3T3 (Fig.4.3.3.) or wild type Src overexpressing Hek293 cells. Similarly, we could not detect any effects on S6K turnover in a cycloheximide assay when either dominant negative or activated Src (Y527F) mutants were overexpressed (Fig. 6.6.1.). Cycloheximide was shown to activate S6K (observations in our laboratory) which could also influence its stability. This could be confirmed by a mobility shift of S6K in an SDS-PAGE gel in the cycloheximide treated samples. Even though this experimental set-up was not ideal, and ^{35}S metabolic labelling would have given more accurate results, two individual experiments failed to reveal a difference in the protein turnover. Thus, this functional aspect was not pursued any further.

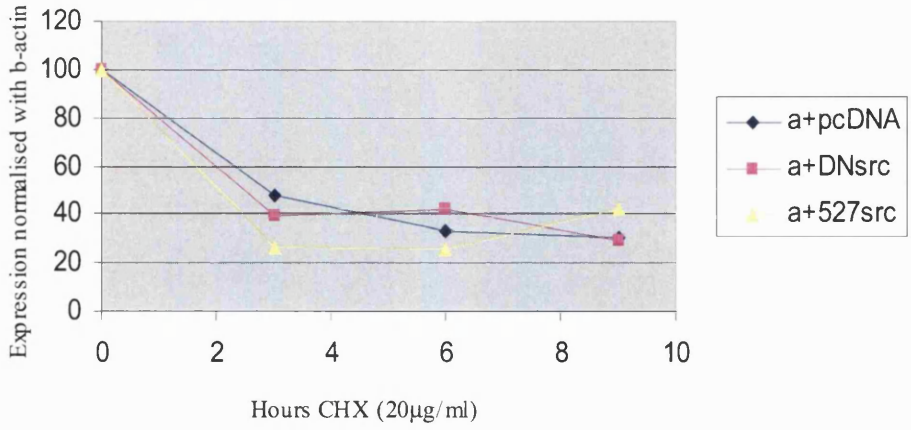


Fig. 6.6.1. Src expression does not alter S6K stability.
Hek293 cells were transfected with activated Src, dominant negative Src or empty vector and treated with 20µg/ml cycloheximide for the times indicated. Total lysates were then blotted with anti-EE antibody, the amounts of S6K quantified using the Bio-Rad Fluoroimager software. The experiment was performed twice and lead to the same result, but different time points were chosen.

6.7. EFFECT OF SRC ON LOCALISATION OF S6K

Src-induced tyrosine phosphorylation facilitated nuclear import of atypical PKC by exposing the NLS on the protein surface (White *et al.*, 2002). Hence, we were interested to find out if localisation of S6K could be altered in a Src-dependent manner. For this purpose, we used mouse embryonic fibroblasts which were deficient in all Src family kinases, Src, Yes, and Fyn, also termed "SYF" cells or a cell line which had Src put back, termed "SYF+Src" (Klinghoffer *et al.*, 1999). However, by immunofluorescence microscopy we could not detect any changes in subcellular localisation of S6K in the presence or absence of Src when stimulated with PDGF or serum (Fig. 6.7.1). The majority of S6K molecules translocated to the nucleus or perinuclear region after stimulation, whereas a small fraction could be detected in membrane ruffles.

Similarly, WT S6Kα/β versus point mutants (Y39/45F) did not localise in different subcellular compartments when NIH3T3 cells were treated with PDGF (data not shown).

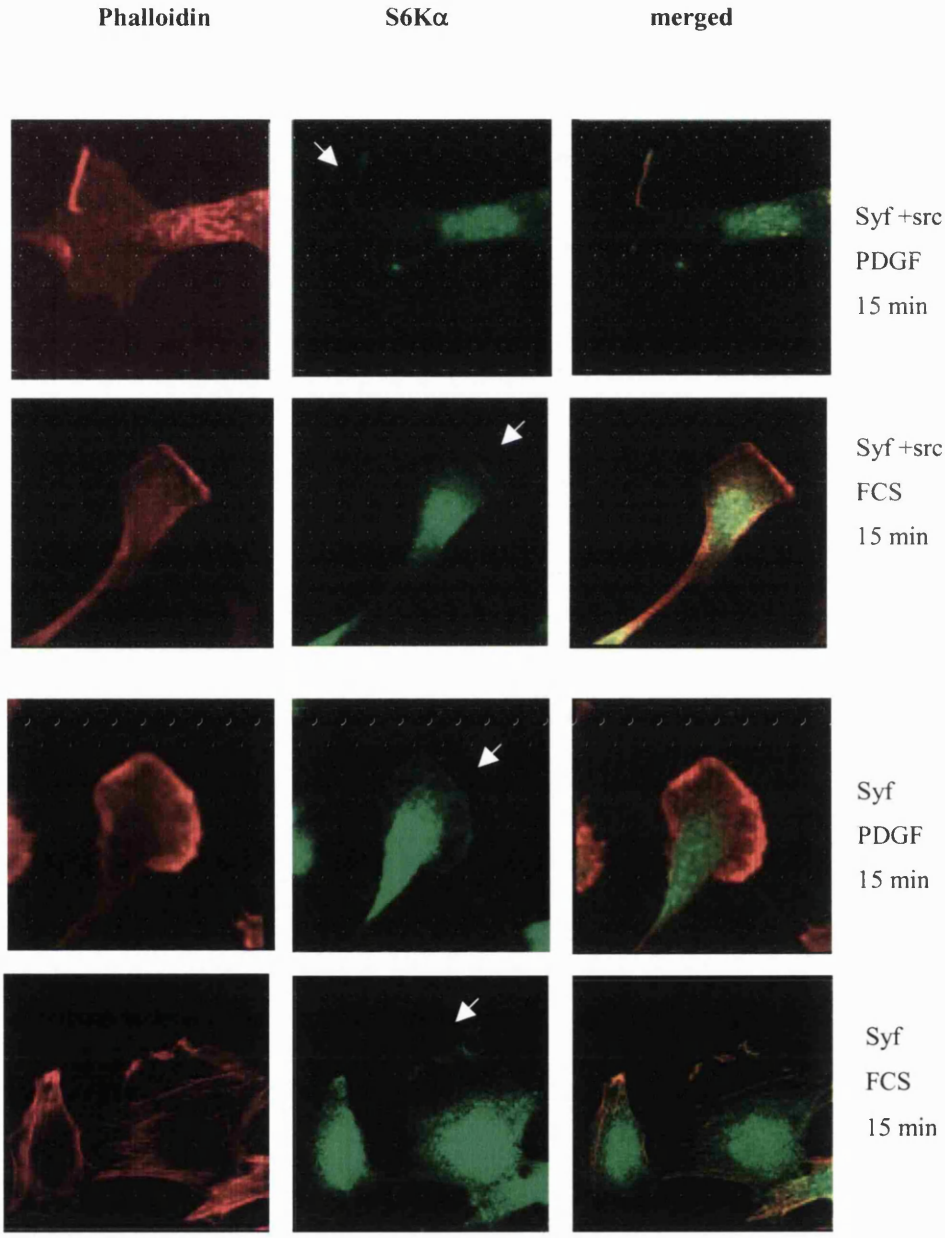


Fig. 6.7.1. S6K subcellular localisation is not Src-dependent.
Syf (depleted of Src, Yes and Fyn) or SYF+Src cells were starved and stimulated with 10% FCS or 30ng/ml PDGF for 15 minutes. After fixing, permeabilising and blocking, cells were stained with anti-S6Kα antibody and phalloidin. No difference in S6K localisation could be detected between the two cell lines, S6K was either situated in the nucleus or the perinuclear region or, to a minor extent, in membrane ruffles.

6.8. EFFECT OF S6K ON PDGFR

Receptor tyrosine kinase signalling can be controlled by serine/threonine phosphorylation. For example, it was shown that PKCs act in a negative feedback loop which controls Kit tyrosine kinase activity by directly phosphorylating two serine residues in the kinase insert of the receptor (Blume-Jensen *et al.*, 1993; Blume-Jensen *et al.*, 1995) in a stem cell factor-dependent manner. Furthermore, IRS1 is strongly Ser/Thr phosphorylated and, depending on the site, phosphorylation can have a positive or negative effect on insulin signalling: PKB/Akt induced phosphorylation acts positively on the signalling whereas mTor induced Ser307 phosphorylation acts negatively. Similarly, it was published that S6K activity is required within a negative feedback loop which downregulates insulin receptor signalling via phosphorylation of S302/307 of IRS1 (Harrington *et al.*, 2004; Radimerski *et al.*, 2002a; Shah *et al.*, 2004). In order to achieve this, S6K must be recruited to IRS1 and therefore be in membrane vicinity. We reasoned that S6K might have a similar function in PDGF signalling.
PDGFR does not have a consensus site for S6K, but it may still be possible that S6K is recruited to the receptor in order to negatively regulate the receptor's activity. *In vitro*, we were not able to detect any phosphorylation activity of S6K towards PDGFR in a preliminary experiment. Either activated (S6KαT389D, S6Kβ388D) or kinase dead S6Ks were immunoprecipitated from Hek293 and the cytoplasmic domain of PDGFR was added and an *in vitro* kinase assay performed (Fig. 6.8.1.). The reaction was analysed by autoradiography. If a significant phosphorylation of the receptor was mediated by S6K this should manifest itself in a stronger PDGFR radiography band in comparison to the samples treated with kinase-dead S6K or to the mock-treated PDGFR sample. The expression of S6Kα was low and may explain the lack of a difference. However, S6Kβ expression was sufficient to lead to a considerable S6Kβ autophosphorylation, but even here, the intensity of the signal derived from PDGFR was not affected. One would assume that *in vitro* these two isoforms α and β have very similar specificity and that if S6Kβ does not show activity towards PDGFR then S6Kα would possibly not phosphorylate the receptor either. However, more data is required to fully address this issue.

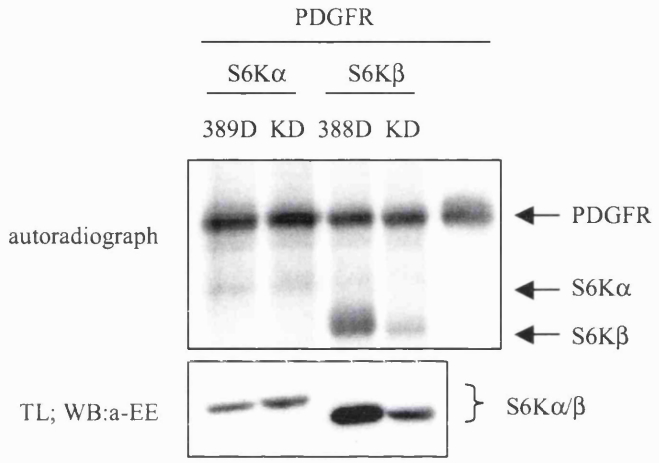


Fig. 6.8.1. S6K does not phosphorylate PDGFR.
S6Kα/β WT/KD were expressed in Hek cells. After 24 hours of expression cells were lysed and S6K was immunoprecipitated using a-EE antibody. Beads were washed, then S6K activity towards the cytoplasmic domain of PDGFR (0.8 µg PDGFR per sample) was assayed in the same buffer normally used for the S6K activity assay as described in chapter 2. Shown above are the autoradiograph of the kinase assay and the Western blot for determination of S6K expression levels.

To address the question if S6K can mediate the downregulation of PDGFR, experiments were performed to test if ubiquitination of PDGFR was altered in the presence of S6K. In our hands, receptor ubiquitination, induced by stimulation with PDGF, did not depend on S6K as shown in Fig. 6.8.2.. In a similar preliminary experiment, an activated form of S6Kβ (T388D) did not alter the ubiquintination of PDGFR. However, it is impossible to draw a definite conclusion as a time course experiment with longer stimulation times would be necessary. Likewise, to see an effect on ubiquitination it is often advisable to use a proteasome inhibitor such as MG132. This prevents the proteasome-mediated degradation and therefore helps to accumulate the ubiquitinated protein whereas it has no effects on proteins which are not primed for degradation.

It will be interesting to further elucidate the precise function of tyrosine phosphorylated S6K within these multiprotein complexes at the plasma membrane.

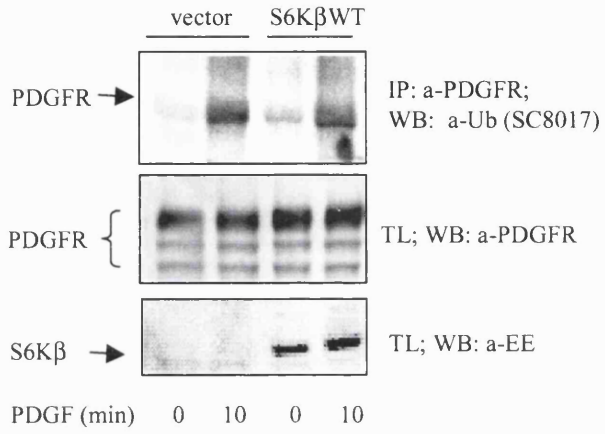


Fig. 6.8.2. S6K expression does not affect PDGFR ubiquitination.
NIH3T3 cells were transfected with empty vector, p70S6Kβ wt and HA-ubiquitin. Cells were starved for 24 hours before stimulation with PDGF (40ng/ml). PDGFR receptor was immunoprecipitated and analysed by western blotting with anti-ubiquitin antibody (Santa Cruz SC8017). Total lysates were used to assess expression of PDGFR and transfected S6Ks.

6.9. DISCUSSION

Already in the 1980ies it was published that S6K was activated in v-Src transformed chicken fibroblasts indicating for the first time that Src was involved in the regulation of S6 Kinase (Blenis and Erikson, 1985). It has been shown that v-Src leads to transformation via two major pathways, PI3K and MAPK pathways, which function in parallel and are both needed for full transformation. Rapamycin or wortmannin blocked some but not all parameters of transformation (such as morphological changes, increased hexose uptake, loss of contact inhibition, and anchorage-independent growth) (Penuel and Martin, 1999; Whitman *et al.*, 1986). With S6K being downstream of PI3K it does not come as a surprise that S6K is activated in v-Src transformed cells. Furthermore, Src inhibitor PP1 was shown to interfere with S6K activation after insulin, IGF1 and pervanadate treatment (Shah et al, 2003). We could confirm the

finding of elevated S6K activity in v-Src transformed Swiss 3T3 cells by detecting elevated levels of phosphorylated S6 protein in the transformed versus parental cells. Moreover, we generated additional data which supported the idea that Src activates S6K. When constitutively active Src (527F) is overexpressed in starved Hek293 cells, one can detect a mobility shift for S6K in the polyacrylamide gel accompanied by elevated levels of pT389 (which is considered an indicator for S6K activity).

Interestingly, we found that S6K from transformed cells is tyrosine phosphorylated whereas S6K from the parental cells is not and wanted to address the question whether Src-mediated tyrosine phosphorylation would enhance activity of S6K as has been shown for a number of other AGC kinases. Interestingly, S6K activity in v-Src transformed cells can be reduced to a level comparable to S6K derived from non-transformed cells by rapamycin (Tuhackova *et al.*, 1999). This indicated that the elevated activity of S6K in v-Src transformed cells must be mediated by mTor. We tested the effect of rapamycin and found that it did not reduce tyrosine phosphorylation. This finding argued that tyrosine phosphorylation was not mediated by mTor and, gave a first indication that the activation, which could be completely reduced with rapamycin, may not be linked to tyrosine phosphorylation.

Other findings substantiated the hypothesis that activity of S6K did not correlate with the state of tyrosine phosphorylation: a PDGFR mutant deficient in Src signalling lead to a reduced phosphotyrosine signal however did not affect the activity of S6Ks (or, to a very minor extent in the case of S6Kβ). Similarly, Src inhibitor SU6656 did not alter the activity of S6K after PDGF and serum stimulation under the conditions used. Likewise, in PDGF or serum-stimulated cells, S6K activity did not depend on Src expression as co-expression of S6K with a dominant negative, wild type Src or activated Src lead to similar pT389 levels and band shifts in SDS-PAGE gels (indicating various degrees of phosphorylation). In contrast to activity, tyrosine phosphorylation in stimulated cells varied significantly: WT Src lead to a moderate and activated Y527F Src to a strong tyrosine phosphorylation whereas DN Src completely abolished it. The effect described above where activated Src lead to S6K activation in starved cells, did not correlate with tyrosine phosphorylation because in starved cells none or almost no tyrosine phosphorylation was detectable.

So how, if not via tyrosine phosphorylation, can Src lead to S6K activation? Firstly, it has previously been shown that Src can exert many of its functions independently of its tyrosine kinase activity, for example via its SH2 or SH3 domains (Frame, 2002). Secondly, kinases upstream of S6K such as PI3K, PKB/Akt and PDK1 are activated by Src and could relay this to S6K. Thirdly, Src has been shown to phosphorylate and inactivate the serine/threonine phosphatases PP2A (at Y307 of its catalytic subunit) and PP1 upon mitogenic stimulation (Chen *et al.*, 1992). In this context, the activity of PP1 phosphatase derived from v-Src transformed cells was reduced 3-fold when compared to that from the parental cells (Belandia *et al.*,1994; Villa-Moruzzi *et al.*, 1996). This has an effect for example on the Jak pathway which is downregulated by PP2A. It was shown that wild type PP2A binds less well to Jak2 than a non-phosphorylatable mutant (PP2AY307F) indicating that Src-mediated phosphorylation abolishes binding of PP2A to Jak2 (Yokoyama *et al.*, 2003). In the context of S6K, the elevated phospho-S6 level in v-Src transformed cells could be due to the inhibited phosphatases which normally would bind to and dephosphorylate phospho-S6. It is important to note that the same phosphatases that dephosphorylate S6 protein are also responsible for S6K inactivation. It was published that PP2A and S6K can form a complex (Westphal *et al.*, 1999, Peterson *et al.*, 1999). So by inhibiting the phosphatase PP2A, Src would activate S6K and additionally slow down the dephosphorylation of S6 protein.

We have identified two tyrosine phosphorylation sites for Src kinase which are located in the N-terminal regulatory and in the catalytic region of S6Kα and S6Kβ. Whereas a phenylalanine mutant to the site in the catalytic loop (S6KαY193F, S6KβY192F) did not lead to a reduction of the overall tyrosine phosphorylation, the point mutant to the N-terminal domain site (S6KαY39F, S6KβY45F) clearly diminished the phosphotyrosine signal for both S6K isoforms in a western blot. The mutant to the site in the catalytic domain (S6KαY193F, S6KβY192F) exhibited a strongly reduced activity. In order to exclude the possibility that this reduction may be caused by structural changes due to mutation in the catalytic loop, we replaced the tyrosine by histidine (S6KαY193H, S6KβY192H). This was done because a histidine is found in

the homologous position in PDK1 or in some tyrosine kinases such as PDGFR. Mutation to histidine also reduced but did not abolish the activity. This validates our finding of tyrosine phosphorylation at this site being a requirement for full S6K activity. The homologous sites in PKBα and PKCγ were also mutated to phenylalanines and the activity of the mutants tested. Whereas the activity of the PKCγ mutant was, the activity of the PKBα mutant was not reduced. This finding not only hints that PKB/Akt and S6K may be regulated differently, it also argues that mutation to phenylalainine does not affect the kinase's activity by disturbing the protein structure. Nevertheless, we were not able to confirm that these phosphorylation sites (S6KαY193/S6KβY192) exist *in vivo*. In the light of previous experiments, where we looked for overall changes of S6K activity and could not detect a correlation between tyrosine phosphorylation and activity, it seems unexpected that mutation of the site in the kinase domain (S6KαY193/S6KβY192) would have an effect on activity. But the fact that this site may be phosphorylated only with low stiochiometry may reconciliate the seemingly contradictory results. Only a small fraction might become tyrosine phosphorylated on this site *in vivo* and, by immunoprecipitating all wt S6K molecules the effect of the tyrosine phosphorylated molecules, with possibly enhanced activity, may be overshadowed by the non-phosphorylated molecules. By immunoprecipitating the mutant via its EE-tag, it is possible to look at a homogenous S6K population, at least regarding this mutated residue. It is worth mentioning here that we have generated Y193/192D mutants which also have, unexpectedly, reduced activity. However, this result is not surprising, because, as previously mentioned, the aspartic acid cannot mimic a phosphotyrosine residue well.

Finally, the Y39/45F mutants of S6K are as active as wild type S6K, in starved and stimulated cells. We therefore conclude that tyrosine phosphorylation at this N-terminal site (S6KαY39, S6KβY45) does not play a role in the regulation of S6K activity.

Our data suggest that, under the conditions used, subcellular localisation is not changed as in Src-deficient cells, S6K is still recruited to membrane ruffles and, in its majority, translocates towards the nucleus. This conclusion is also supported by the analysis of the Y39/45F mutants which have a similar subcellular localisation than the wild types. Our experiments also pointed out that the stability of S6Ks is not affected by tyrosine

phosphorylation. The levels and turnover of S6Ks in a cycloheximide experiment were not altered in the presence or absence of Src.

The N-terminal domain of S6K has been reported to bind to PP2A. Deletion of the first 52 or 24 amino acids from N-terminus leads to the loss of activity (Weng *et al.*, 1995b). Deletion of the N-terminus and C-terminus restores activity, renders the kinase sensitive to stimulation and insensitive to rapamycin. However, the C-terminal deletion mutant retains its rapamycin sensitivity. Thus, the factor leading to sensitivity must bind to the to the first 24AA of the N-terminus. Peterson *et al.* proposed that the mode of rapamycin action on PP2A is via inhibition of direct phosphorylation of PP2A by mTor. When inhibited by the drug or by amino acid deprivation mTor cannot phosphorylate and hereby restrain the phosphatase any more (Peterson *et al.*, 1999). The identified site, Y39/45 does not lie in the binding domain of S6K to PP2A, but is 15 amino acids C-terminal to it. Nevertheless, we reasoned that PP2A binding could be modulated depending on the phosphorylation state of Y39/45 and that, if this was the case, one should see a difference in the deactivation kinetics between wild type and mutant. As PP2A does not have an SH2 domain itself, the binding could occur via an intermediate protein or other interactions than pY-SH2. In a time course experiment over 21 hours, no difference was visible in the *in vitro* kinase activity of S6K wild type and mutant. Besides, The Y39F mutant did not show an elevated resistance to rapamycin, which would also have indicated that phosphorylation of this site facilitates PP2A binding and ser/thr dephosphorylation.

Taken together, we were unable to determine the physiological role of the Y39/45 site, but found that the site in the catalytic loop is, if it were an *in vivo* site, involved in the activation of the kinase.

In the future, it will be very interesting to determine, if tyrosine phosphorylation in the N-terminus alters the protein-protein interactions of S6K. This could for example be tested with a synthetic phosphopeptide encompassing the respective phosphotyrosine versus non-phosphorylated peptide. The peptides could be cross-linked to a resin (such as Actigel) and pull–down experiments with total lysates could be performed. If proteins can be detected which specifically bind to the phosphopeptide, these could be identified using mass spectrometry. This method does not take into consideration the

structural context, but as the site (Y39/Y45) is located in the N-terminus which is not part of a complex structure such as the kinase lobes, 3-dimensional folding may not be so crucial. Furthermore, phosphotyrosines are recognised by either SH2 or PTB domains: SH2 domains gain their specificity by binding to residues which are 3-6 amino acids C-terminal of the phosphotyrosine site, and PTB domains recognise a sequence up to 5 amino acids N-terminal of the phosphotyrosine site. Therefore, only short sequences rather than 3-dimensional structures seem to be of major importance.
The identification of specific binding partners to the pTyr site at the N-terminus should allow a better understanding of the complex formation involving RTKs and S6K. Knowing the binding partners to the created pTyr site may yield information about potentially novel functions of S6K. The major known S6K substrate to date, S6 protein, has not been detected in ruffles or along the membrane but in the cytosol and the nuclei (or nucleoli). Thus, it is possible that S6K is recruited to the membrane to be close to novel substrates.

CHAPTER 7: GENERAL DISCUSSION

CHAPTER 7

GENERAL DISCUSSION

Upon mitogenic stimulation, S6K becomes heavily phosphorylated on more than 10 residues. In a complex and well-concerted manner, three different clusters of sites are phosphorylated, leading to a successive change in conformation: first, phosphorylation occurs in the autoinhibitory pseudosubstrate domain which releases an inhibitory interaction between N- and C-terminus. A set of phosphorylations in the kinase extension domain follows (T389, S371) which allows the site (T229) to be phosphorylated- an event which renders the kinase fully active. All of the phosphorylation sites are either serines or threonines and no tyrosine phosphorylation of S6K has been described so far. In this study we demonstrate for the first time that S6Ks become tyrosine phosphorylated upon mitogenic stimulation.

Membrane translocation in response to mitogenic stimuli has been shown for a variety of AGC kinases, including PKB/Akt, PDK1, PKD and various isozymes of the PKC family. This is mainly thought to occur via binding to second messengers such as phospholipids or molecules downstream of the RTKs such as adaptors. For example, translocation can be mediated through PH domains (for PDK1 or PKB/Akt) which bind to phospholipids such as PIP_3 (Lemmon and Ferguson, 2000). PKC translocation is mediated by a variety of isoform-specific RACKs (receptors for activated C-kinases) (Schechtmann and Mochly-Rosen, 2001). Binding to its respective RACK localises the PKC isozymes next to a subset of substrates and bestows functional specificity (Mochly-Rosen, 1995). PKA can be recruited to the membrane with the help of AKAP18 (A-kinase anchoring protein) (Fraser *et al.*, 1998). Furthermore, many AGC kinases are substrates for Src kinase which also associates to an activated RTK. To our knowledge, no co-immunoprecipitation studies of RTKs and AGC kinases have been published.

So far, few potential mechanisms exist by which S6K could be integrated into multienzyme signalling complexes formed around activated RTKs. Firstly, PDK1 directly phosphorylates S6K on the site in the activation loop. At least for a short

amount of time S6K must be complexed with PDK1 at the membrane. Interestingly, very recently the crystal structure of the PDK1 PH domain was solved and revealed that PDK1 may also be able to bind to cytoplasmic inositol phosphates (phosphorylated in D6 position) (Komander *et al.*, 2004). The authors found that only 10% of PDK1 in starved cells are membrane localised and they did not detect a marked translocation to the membrane upon IGF1 stimulation. Therefore, they propose that a pool of cytoplasmic PDK1 may be necessary to activate substrates which do not possess PH domain such as S6K or p90RSK. Using immunofluorescence microscopy in NIH3T3 cells, we show that in starved cells S6Kα is evenly distributed within the cytoplasm of the cells and could also be detected along stress fibers. Upon PDGF stimulation, the majority of S6Kα molecules translocate to the nucleus or the perinulear region, whereas a small fraction could be detected in membrane ruffles. Hence, in agreement with the structural data, the majority of S6K molecules may be activated via cytosolic PDK1, but the pool of S6K which is localised in ruffles maybe be phosphorylated by membrane bound PDK1. There are, however, additional reasons why S6K could translocate to the plasma membrane as other molecules which activate S6K are membrane localized. Rho family G proteins Rac and Cdc42, which control cytoskeletal organisation, were shown to associate with and activate S6K (Chou and Blenis, 1996). As these small GTPases are most active when they are membrane-bound, it would be logical for S6K to be co-localised with its upstream effectors (Del Pozo *et al.*, 2002). Besides, it was reported that S6K was in complex with the receptor-associated p85 subunit of PI3K and that this complex formation was needed for mTor and PI3K-mediated activation of S6K (Gonzalez- Garcia *et al.*, 2002). In this study we demonstrate that S6Kα and β, in addition to being tyrosine phosphorylated, are associated with RTKs in a ligand-induced manner. Both events, receptor association and tyrosine phosphorylation, occur simultanously and peak at around 30 minutes after stimulation. As model system for our experiments in this study, we have mainly used PDGFR, but also Kit (or stem cell factor receptor) which both associate with S6K in a stimulation-dependent manner.

Using truncation mutants, we could demonstrate that the association with RTKs is mediated via the kinase or the kinase extension domain of S6K. Loss of the N- or C-

terminus of S6K or both does not abolish the co-immunoprecipitation of PDGFR with S6Ks.

In vitro, the recombinant cytosolic domain of PDGFR can phosphorylate S6Ks. This experiment, together with the co-immunoprecipitation studies could point to a direct association between PDGFR and S6K. In addition, cytosolic tyrosine kinases such as Src are also capable of phosphorylating S6Ks on tyrosine. *In vivo*, by using PDGFR mutants which are deficient in signalling via Src kinase we found that both, PDGFR and Src kinase activity are needed to achieve maximal tyrosine phosphorylation of S6K. Inhibitor studies validated this finding. Genistein, a general tyrosine kinase inhibitor, PP1 an inhibitor of PDGFR and Src kinase and Src-specific inhibitor SU6656 all reduce tyrosine phosphorylation of S6K. PI3K and mTor do not influence tyrosine phosphorylation of S6K as the inhibitors LY294002 or rapamycin do not reduce the 4G10 signal in a western blot. This finding is in congruence with the finding that PDK1 tyrosine phosphorylation is independent of PI3K activity (Park *et al.*, 2001). However, phosphorylation of PKB/Akt was shown to be PI3K-dependent (Conus *et al.*, 2002). This difference may be explained by the slightly different PH domains of PDK1 and PKB/Akt. The PH domain of PDK1 has affinities to both phospholipids, $PI(3,4,5,)P_3$ and $PI(4,5,)P_2$ with a higher and a 15 times lower affinity, respectively (Komander *et al.*, 2004). The basal cellular levels of PIP_2 are 50-200 times higher than that of PIP_3 and this can explain why a part of PDK1 is already membrane localised in starved cells and it is still unclear how much more PDK1 can be recruited after stimulation. This is not the case for PKB/Akt which is markedly recruited to the membrane and to PIP_3 molecules in a stimulation-dependent manner (Komander *et al.*, 2004; Watton and Downward, 1999). Also, the PKBα PH domain shows a signifcianly lower affinity to inositol phosphates such as IP_6 than the PH domain of PDK1. But it is this binding capability that seems to retain PDK1 in the cytoplasm (Komander *et al.*, 2004). For PKB/Akt, it is plausible that PIP_3 must be generated by PI3K before PKB/Akt can translocate to the membrane and become activated.

For several other AGC kinases it was shown that tyrosine phosphorylation results in increased kinase activity. In v-Src transformed cells PKB/Akt activity is enhanced, due

to elevated PIP_3 levels (Datta *et al.*, 1996; Liu *et al.*, 1998). Three tyrosine phosphorylation sites in PKB/Akt were identified as regulators of activity (Chen *et al.*, 2001; Conus *et al.*, 2002). In PDK1, two phosphorylation sites in the kinase extension domain were found to be involved in the activation of the kinase (Park *et al.*, 2001). Furthermore, in PKCδ, one out of three identified tyrosine phosphorylation sites was required for full activity of the kinase (Konishi *et al.*, 2001). Furthermore, tyrosine phosphorylation of PKD mediates its activation (Storz *et al.*, 2003).

As described in the previous chapter, it would be logical that Src-induced tyrosine phosphorylation also leads to S6K activation: S6K activity was found to be higher in v-Src transformed cells than in non-transformed cells (Blenis and Erikson, 1984, 1985 and our data). Secondly, PIP_3 levels and therefore PI3K activity is elevated in v-Src transformed cells. (Penuel and Martin, 1999; Whitman *et al.*, 1986). We found that S6K from v-Src transformed cells is tyrosine phosphorylated but S6K from the parental cells is not. However, we have not found a correlation between S6K tyrosine phosphorylation and activity, by various different experimental approaches such as inhibitor or overexpression studies, phosphatase treatment and *in vitro* kinase assays.

Taking into account that the activities of PDK1, PKB/Akt, PI3K and PKC are activated by wt or oncogenic Src, S6K could be activated indirectly via the action of these upstream kinases. Furthermore, as described in the previous chapter, Src was found to phosphorylate and inhibit the phosphatases PP2A and PP1 (Chen *et al.*, 1992). Hence, the elevated phospho-S6 level in v-Src transformed cells could be due to the inhibited phosphatases which normally would rapidly dephosphorylate phospho-S6 and deactivate S6K.

We have identified the major tyrosine phosphorylation site for Src kinase which is located in the N-terminal regulatory region in S6Kα and S6Kβ. When Src is overexpressed together with the S6Kα/β Y39/45F mutant or wt, the mutant shows almost no tyrosine phosphorylation. Similarly, *in vitro*, Src phosphorylates the mutant to a lesser extent than the wild type. *In vitro* and *in vivo* PDGFR still phosphorylates the Y39/45F mutants and still co-immunoprecipitates with them. This indicates that S6K is recruited to RTKs independently of or before Src-mediated phosphorylation of the N-terminal site occurs. Finally, the Y39/45F mutants are as active as wild type S6K, in

starved and stimulated cells. Considering these and the previous results, we suggest that tyrosine phosphorylation should play another role than to enhance activity.

Src-induced tyrosine phosphorylation of AGC kinases has been shown to have other effects such as altered protein stability or subcellular localisation. Our data suggest that, under the conditions used, subcellular localisation of S6Kα is not changed as in Src-deficient cells no difference could be seen regarding translocation of S6K to the nucleus or recruitment to membrane ruffles upon PDGF stimulation. This conclusion is also supported by the analysis of the Y39/45F mutants which have a similar subcellular localisation as wt S6Kα and S6Kβ. Tyrosine phosphorylation can also affect the stability of a substrate. We could not detect that, under the conditions used, the stability of S6Ks is affected by increased tyrosine phosphorylation. In an experiment using cycloheximide (which inhibits the activity of the peptidyl transferase of the 60S ribosomal subunit during translation elongation), levels and degradation kinetics of S6Ks were not altered in the presence or absence of Src.

Furthermore, we tested if S6K could be involved in a negative feedback loop on PDGFR activity, in a similar way as S6K was shown to phosphorylate IRS1 and lead to donwregulation of the insulin pathway (Harrington *et al.*, 2004; Shah *et al.*, 2004). *In vitro*, we were not able to detect any phosphorylation activity of S6K towards PDGFR. Preliminary experiments were performed to test if ubiquitination and thus stability of PDGFR was altered in the presence of S6K. But receptor ubiquitination did not seem to depend on S6K.

Phosphotyrosine sites are important signalling modules that constitute recognition sites for proteins with SH2 or PTB domains (see also 1.3.1.2.). Changes in binding affinity due to Src-induced tyrosine phosphorylation were recently shown for cdc42 and PSD93 (Tu *et al.*, 2003; Nada *et al.*, 2003) but many more examples could be mentioned. For example, EGF-stimulated tyrosine phosphorylation of cdc42 by Src is accompanied by an enhancement in the interaction of cdc42 with the Rho-GDP dissociation inhibitor (RhoGDI). And the post-synaptic density protein PSD93 was found to bind to csk better after phosphorylation by fyn, a member of the Src tyrosine kinase family.

We have found that S6K and PDGFR are in a complex even if Src signalling is deficient or if the major Src phosphorylation site on S6K is mutated to phenylalanine. This finding indicates that S6K does not need to be phosphorylated by Src in order to be recruited to the membrane. This also suggests that activation of S6K might occur via the well-described pathways (via PI3K and mTor) before Src-induced tyrosine phosphorylation occurs. It was reported that S6K was in complex with the p85 subunit of PI3K and that this association was needed for mTor and PI3K-mediated activation of S6K (Gonzalez-Garcia *et al.*, 2002). This is in agreement with our findings. Recruitment to the receptor may occur and lead to S6K activation via the well-described pathways, and in addition, tyrosine phosphorylation on one or multiple sites by the receptor itself occurs before Src then phosphorylates the site in the N-terminal domain. This series of events is schematised in Fig. 7.1.1.

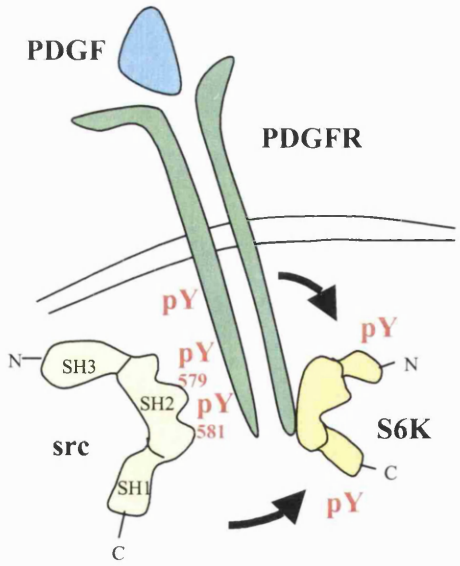


Fig. 7.1.1. Model of S6K and RTK association.
S6Kinase is in multienzyme complexes involving activated receptor tyrosine kinases. See text for details.

Our finding that an activated, and hence S/T phosphorylated, S6K (T389D) is a better Src substrate than WT S6K further supports this concept. Additionally, we have evidence that S6K becomes tyrosine phosphorylated by PDGFR on sites other than on the Src phosphorylation site in the N-terminus. When the Y39/45F mutants are transiently co-expressed with PDGFR, they are still tyrosine phosphorylated. This indicates that a sequence of events occurs which involves S6K recruitment to RTKs, phosphorylation on tyrosines, binding of Src followed by additional tyrosine phosphorylation and very possibly recruitment of a variety of binding partners via the acquired phosphotyrosine sites. The identified tyrosine phosphorylation of S6K may be required to create a nucleation site which assembles SH2 domain containing signalling molecules and potential novel substrates. It has been shown that some SH2 domain containing tyrosine kinases selectively phosphorylate sites recognized by their own or related SH2 domains (Songyang and Cantley, 1995). In the case of S6Kβ the sequence carboxy-terminal of the tyrosine residue (EEV) represents an optimal binding sequence for fgr, a member of the Src family of kinases (mainly expressed in hematopoetic cells) (Songyang and Cantley, 1995). Phosphorylation at this site could occur first and enhance binding of the Src kinase through its own SH2 domain, thus allowing multiple phosphorylation events to occur. A working model for the interaction between S6K and Src is depicted in Fig. 7.1.2.. It is possible that Src, after being recruited to the RTK via its SH2 domain, could bind via its SH3 domain to the PXXP motif in the kinase extension domain of S6Kα and β. This binding would enable Src to phosphorylate the site in the N-terminus of S6K, which, in turn, may constitute a binding site for Src's own SH2 domain and stablise the Src-S6K or the Src-S6K-PDGFR complexes. We are currently investigating whether the S6KαY39F mutant co-immunoprecipitates with Src less efficiently than wt S6Kα. It is possible that the phosphotyrosine site at the N-terminus leads to recruitment of other molecules than Src which stablise the PDGFR-S6K complex.

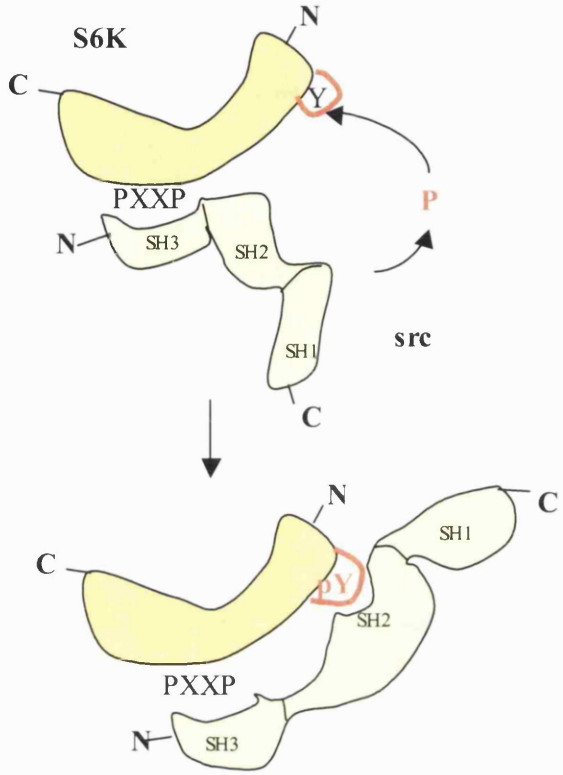


Fig. 7.1.2. Model of S6K binding to Src.
See text for details.

In a preliminary experiment, we have already detected that even though the S6KαY39F mutant is in complex with PDGFR after stimulation, it seems to co-immunoprecipitate less of the receptor than S6Kα wild type. However, this experiment needs to be repeated several times and subjected to quantification to enable us to draw a final conclusion.

In this study, we have characterised a novel post-translational modification of S6K, tyrosine phosphorylation. We have established that its' occurence after mitogenic stimulation co-incides with receptor association and is mediated by the action of a receptor tyrosine kinase (i.e. PDGFR) and Src. In addition, we have found that tyrosine

phosphorylation does affect neither the activity, the subcellular localisation nor the stability of the kinase. The phosphotyrosine site/s possibly create/s recognition sites for SH2 domain containing proteins and therefore is/are important for recruiting S6K into a multienzyme complex. The function of S6K in membrane ruffles has not been addressed so far. There are, however, several studies which have attributed S6K a role in the cytoskeletal organization and migration (Berven *et al.*, 2004; Qian *et al.*, 2004). The fact that Src, which is an important regulator of cell motilty, mediates tyrosine phosphorylation of S6K, argues for a possible role of S6K tyrosine phosphorylation in this context. With the recent findings that S6 protein may not be the major S6Kα substrate, an important task is to determine novel substrates (Pende *et al.*, 2004). One could imagine that S6K could be recruited to novel substrates which are membrane-localised. Based on the presented work, it will be interesting to further elucidate the precise function of tyrosine phosphorylated S6K within these multiprotein complexes at the plasma membrane.

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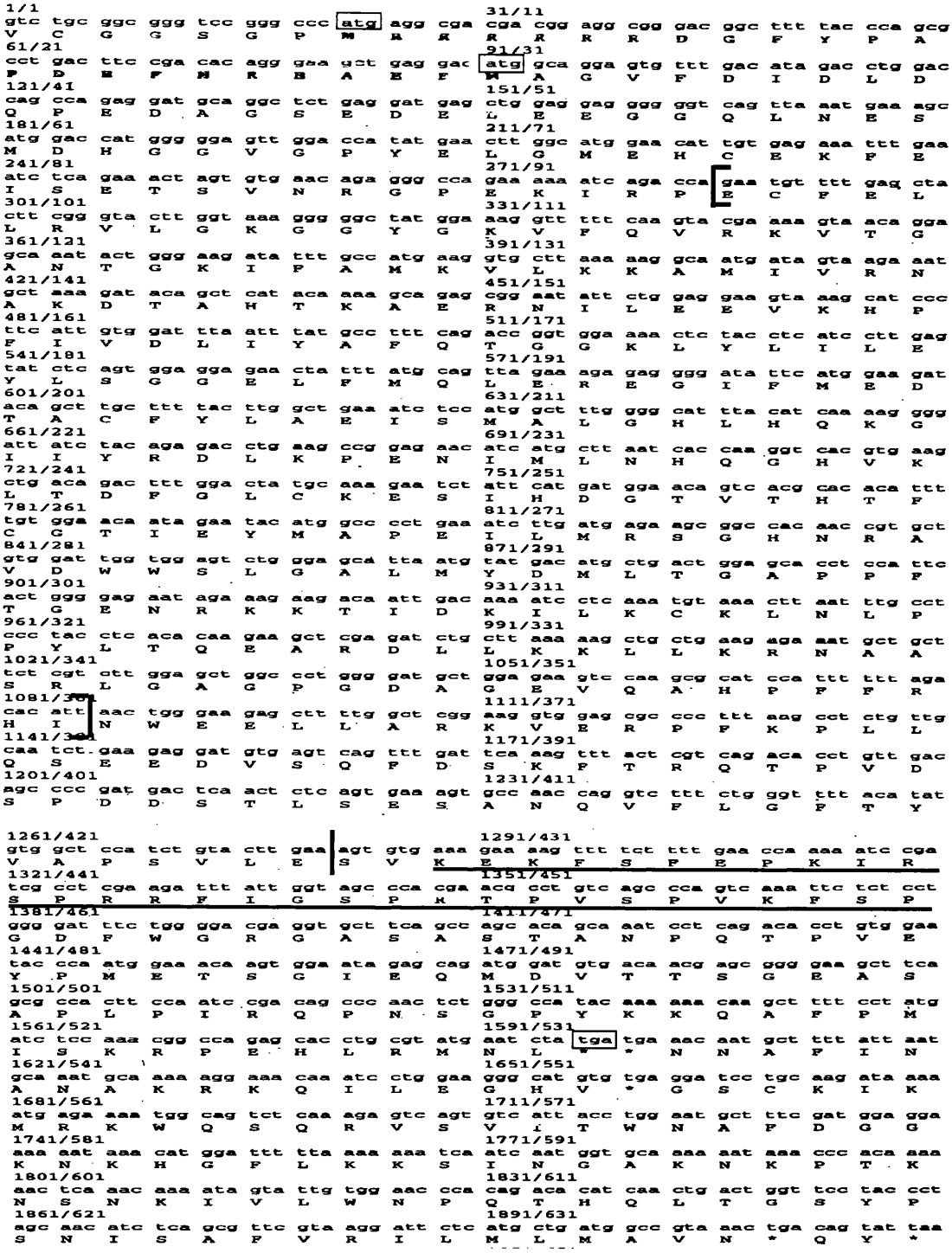
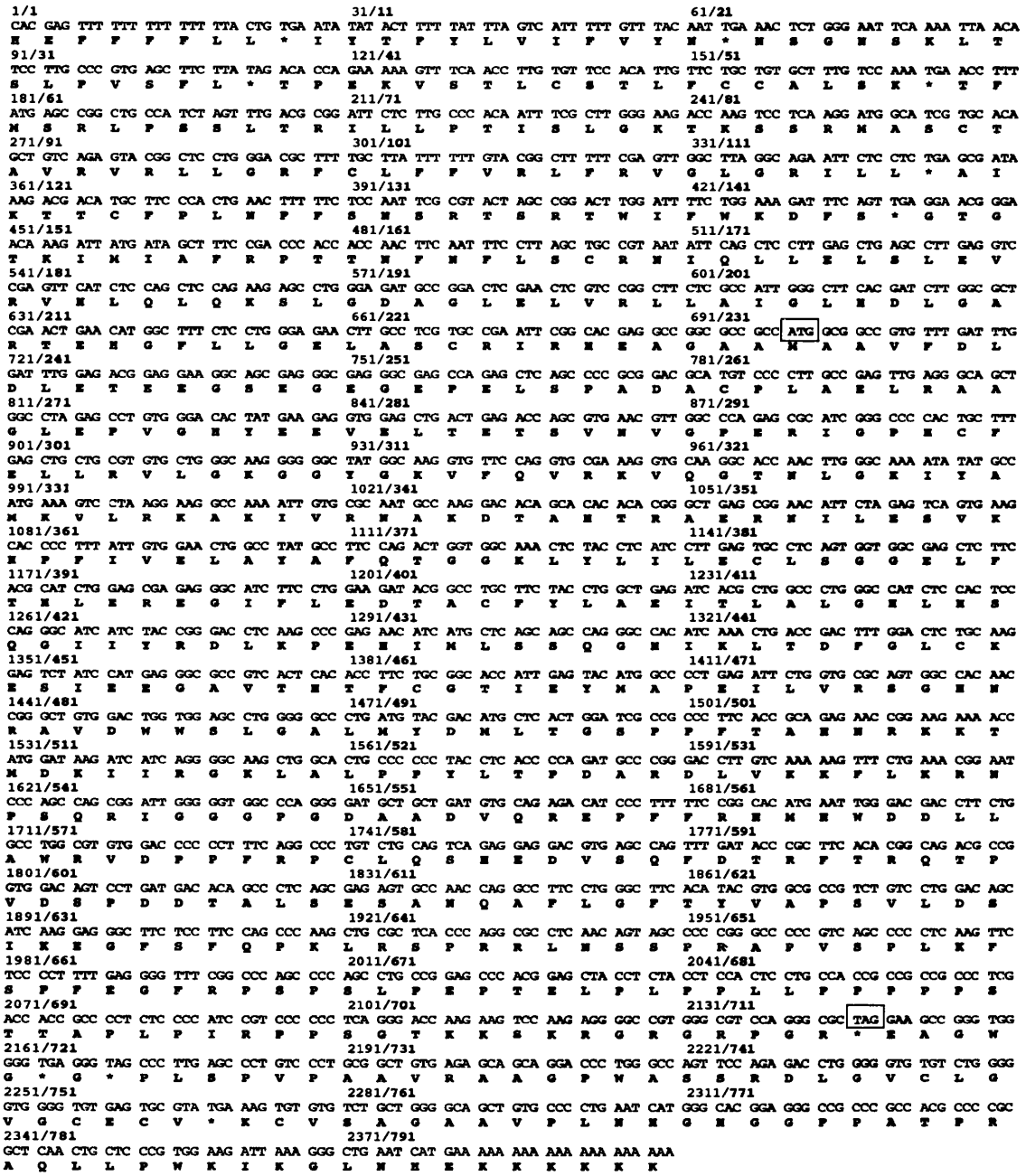


Appendix A. Nucleotide and predicted amino acid sequences of cDNA S6Kα.
The red and blue boxes outlined in the sequence refer to the translation initiation codon and stop codon, respectively. The sequence corresponding to the catalytic domain is enclosed in the brackets. Black bar indicates the end of linker domain

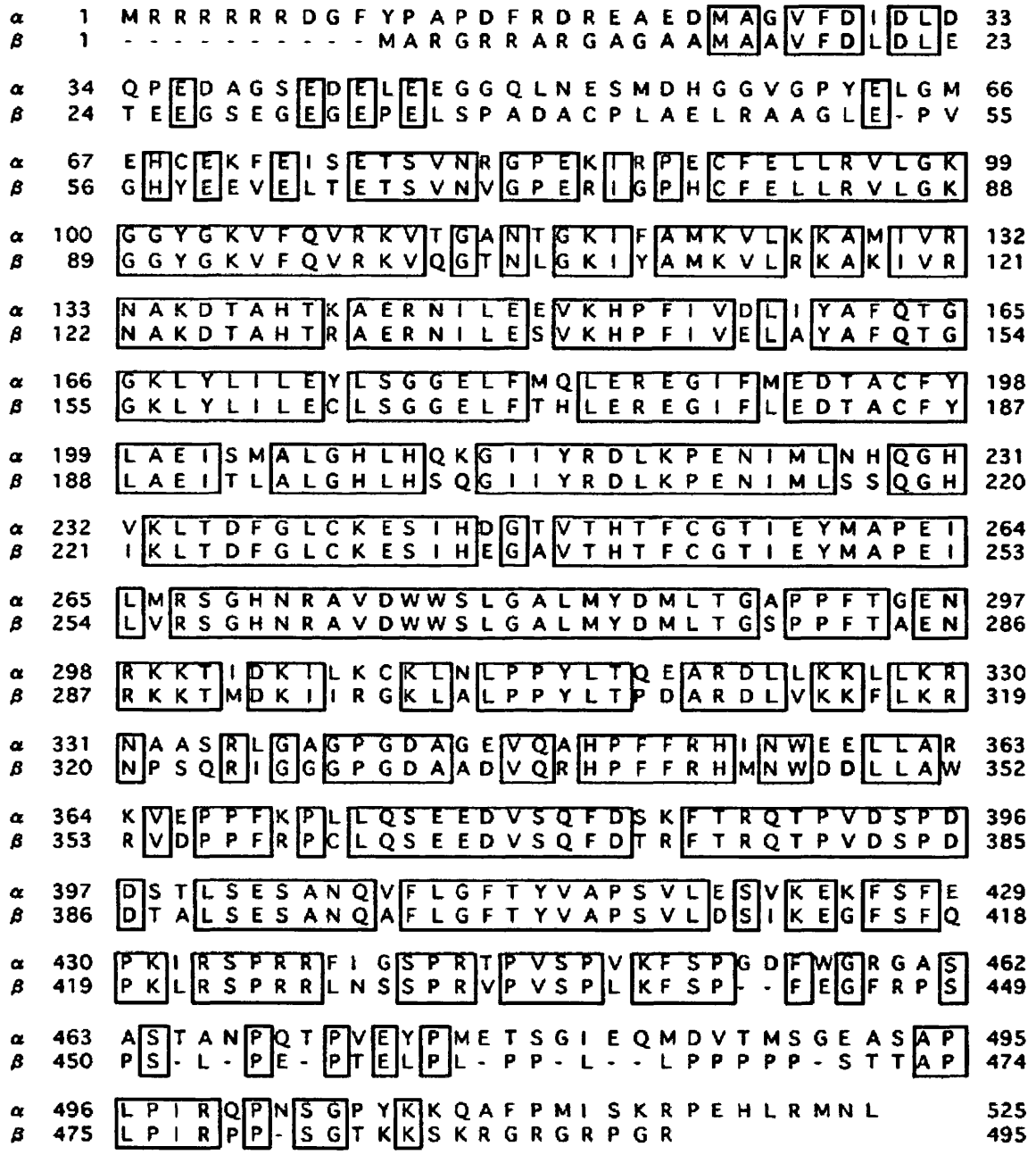
Appendix B

cDNA S6Kβ



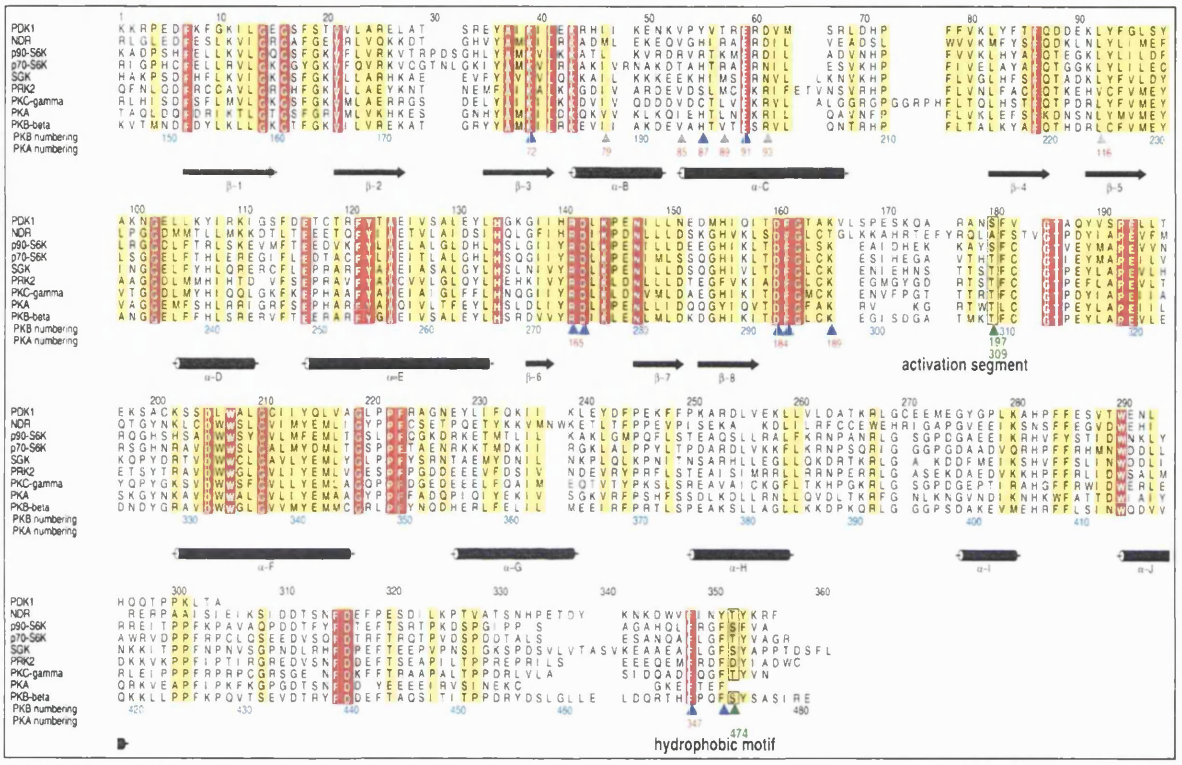
Appendix B. Nucleotide and predicted amino acid sequences of cDNA S6Kβ.
The red and blue boxes outlined in the sequence refer to the translation initiation codon and stop codon, respectively.

Appendix C



Appendix C. Comparison of amio aci sequences of S6Kα with S6Kβ.
Amino acid sequences of S6Kα and S6Kβ are aligned. Conserved residues are boxed.

Appendix D



Appendix D. Alignment of kinase and kinase extension domain of various AGC kinases
Reproduced from Yang *et al* .(2002). Mol Cell *9*, 1227-1240.

Appendix E

name	3-letter code	1-letter code	composition	Monoisotopic mass	Average Mass
Alanine	Ala	A	C_3H_5NO	71.03711	71.0788
Arginine	Arg	R	$C_6H_{12}N_4O$	156.10111	156.1876
Asparagine	Asn	N	$C_4H_6N_2O_2$	114.04293	114.1039
Aspartic acid	Asp	D	$C_4H_5NO_3$	115.02694	115.0886
Cysteine	Cys	C	C_3H_5NOS	103.00919	103.1448
Glutamine	Gln	Q	$C_5H_8N_2O_2$	128.05858	128.1308
Glutamic acid	Glu	E	$C_5H_7NO_3$	129.04259	129.1155
Glycine	Gly	G	C_2H_3NO	5702146	57.052
Histidine	His	H	$C_6H_7N_3O$	137.05891	137.1412
Ileucine	Ile	I	$C_6H_{11}NO$	113.08406	113.1595
Leucine	Leu	L	$C_6H_{11}NO$	113.08406	113.1595
Lysine	Lys	K	$C_6H_{12}N_2O$	128.09496	128.1742
Methionine	Met	M	C_5H_9NOS	131.04049	131.1986
Phenylalanine	Phe	F	C_9H_9NO	147.06841	147.1766
Proline	Pro	P	C_5H_7NO	97.05276	97.1167
Serine	Ser	S	$C_3H_5NO_2$	87.03203	87.0782
Threonine	Thr	T	$C_4H_7NO_2$	101.04768	101.1051
Tryptophan	Trp	W	$C_{11}H_{10}N_2O$	186.07931	186.2133
Tyrosine	Tyr	Y	$C_9H_9NO_2$	163.06333	163.176
Valine	Val	V	C_5H_9NO	99.06841	99.1326

Appendix E: The masses and compositions of the twenty commonly occurring amino acid residues