Elucidation of signaling pathways regulating ribosomal protein S6 kinases

by

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**ABSTRACT**

The ribosomal protein S6 kinase belongs to the AGC family of Ser/Thr kinases and is known to be involved in the regulation of translation and G1/S transition of the cell cycle. In addition to playing an essential role in regulating cell growth, S6K appears to be a multifunctional protein, involved in the control of other cellular processes such as transcription, RNA processing, and apoptosis.

Two human S6K forms have been identified, termed S6Kα and S6Kβ, which have cytoplasmic and nuclear splicing variants. Both kinases have very high level of homology in kinase domain, but differ significantly in the N- and C-terminal regulatory regions. These differences and some variations in the pattern of phosphorylation sites may predestine the involvement of both kinases in distinct signaling events and cellular responses.

This manuscript presents analysis of regulatory and functional properties of ribosomal S6Kα and its recently identified homologue S6Kβ. Recombinant full-length versions and a panel of deleted and point mutants of S6Kα and S6Kβ were created and used to study the mechanisms of mitogen-induced activation of these kinases. Furthermore, employment of specific inhibitors of mTOR/FRAP, PI-3'-K, PKC and MEK allowed us to study the contribution of these signaling molecules in the activation of S6Kα and S6Kβ by various mitogenic stimuli. Here we also present a detailed analysis of subcellular localisation of S6Kα and S6Kβ isoforms. Using confocal microscopy and a panel of mutants, we have described for the first time mitogen-regulated nucleocytoplasmic shuttling of S6KβII and addressed a critical role of PKC signaling in this process. A novel PKC phosphorylation site, specific for S6Kβ, was identified by mass spectrometry and found to be involved in the regulation of its subcellular distribution. Finally, we have developed tetracycline-inducible stable cell lines, overexpressing activated versions of four different isoforms of S6K. This model system allowed us to initiate studies on the role of individual S6K isoforms in the regulation of gene expression.

In conclusion, this study demonstrates the existence of distinct signaling mechanisms, involved in the regulation of S6Kα and S6Kβ.
With LOVE to MY WIFE,

ORYSYA
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ATM</td>
<td>Ataxia telangiectasia mutated</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Adenosine3',5'-cyclic monophosphate</td>
</tr>
<tr>
<td>CBP80</td>
<td>80 kDa subunit of RNA cap-binding complex</td>
</tr>
<tr>
<td>CBC</td>
<td>Cap-binding complex</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>cGMP</td>
<td>Guanosine3',5'-cyclic monophosphate</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CREM</td>
<td>cAMP-responsive activator modulator</td>
</tr>
<tr>
<td>DAG</td>
<td>sn-1,2-Diacylglycerol</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNA-PK</td>
<td>DNA-dependent protein kinase</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleoside triphosphate</td>
</tr>
<tr>
<td>2D-PAGE</td>
<td>Two-dimensional polyacrylamide gel electrophoresis</td>
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<tr>
<td>DTT</td>
<td>Dithiotreitol</td>
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<tr>
<td>4E-BP1</td>
<td>Eukaryotic initiation factor-4E binding protein 1</td>
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<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra-acetic acid</td>
</tr>
<tr>
<td>eEF1A</td>
<td>Eukaryotic elongation factor 1A</td>
</tr>
<tr>
<td>eEF2k</td>
<td>Eukaryotic elongation factor 2 kinase</td>
</tr>
<tr>
<td>E2F</td>
<td>Early promoter 2 factor</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<td>EGTA</td>
<td>Ethyleneglycol-bis(β-aminoethyl)-N,N,N',N'-tetraacetic acid</td>
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<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>EST</td>
<td>Expressed sequence tag</td>
</tr>
<tr>
<td>Fab</td>
<td>Antigen-binding fragment</td>
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<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>FKBP12</td>
<td>FK506-binding protein 12</td>
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<td>FTI</td>
<td>Farnesyltransferase inhibitor</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-activating protein</td>
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<td>GSK</td>
<td>Glycogen synthase kinase</td>
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<td>Immunoglobulin</td>
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<td>IPG</td>
<td>Immobilized PH gradient</td>
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<td>KHL</td>
<td>Keyhole limpet hemocyanin</td>
</tr>
<tr>
<td>LMB</td>
<td>Leptomycin B</td>
</tr>
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<td>MALDI TOF</td>
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</tr>
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<td>Messenger RNA</td>
</tr>
<tr>
<td>mRNP</td>
<td>Messenger ribonucleic acid protein</td>
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<td>Ni-NTA</td>
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<td>NLS</td>
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<td>PIF</td>
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<td>PKA</td>
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<td>PMA</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>Pro</td>
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<tr>
<td>S6K</td>
<td>Ribosomal protein S6 kinase</td>
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<td>SDS</td>
<td>Sodium n-dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>SGK</td>
<td>Serum- and glucocorticoid-induced protein kinase</td>
</tr>
<tr>
<td>SH3</td>
<td>Src homology 3</td>
</tr>
<tr>
<td>snRNA</td>
<td>small nuclear RNA</td>
</tr>
<tr>
<td>SRE</td>
<td>Serum response element</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N’N’'-Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TetO</td>
<td>Tetracycline operator</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>5'-TOP</td>
<td>5'-terminal oligopyrimidine tract</td>
</tr>
<tr>
<td>T-Rex</td>
<td>Tetracycline-regulated expression system</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer RNA</td>
</tr>
<tr>
<td>TSC</td>
<td>Tuberous sclerosis complex</td>
</tr>
<tr>
<td>Tween 20</td>
<td>Polyoxyethylenesorbitan monolaurate</td>
</tr>
<tr>
<td>Val</td>
<td>Valine</td>
</tr>
<tr>
<td>WW</td>
<td>Tryptophan-tryptophan</td>
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</tbody>
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CHAPTER 1

INTRODUCTION
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Unicellular organisms respond primarily to nutrient cues, many of which are able to cross the cell membrane. In multicellular organisms, complex intercellular signaling pathways have been developed to control and coordinate responses to altering physiological conditions. These pathways are regulated by a large number of polypeptide growth factors, cytokines and hormones which exert their biological effects by interaction with specific cell-surface receptors on responsive cells. Extracellular signals are then transmitted from the cell membrane to specific intracellular targets by signaling cascades that involve the formation of multienzyme complexes around the activated cell-surface receptors, generation of second messengers and protein phosphorylation events. These complex and often interacting signaling responses coordinate the multifunctional cellular programmes that culminate in cell growth, proliferation and/or differentiation (Egan and Weinberg, 1993).

All of these cellular processes require the expression of specific genes, precisely regulated at multiple levels: transcription, messenger RNA processing and stability, translation and post-translational modifications (Hershey et al., 1996). Regulation of gene expression at the level of protein synthesis allows cells to respond rapidly to extracellular stimuli, since activation or repression of mRNAs can occur essentially instantaneously, whereas regulation at the level of transcription entails a considerable time lag before a precursor RNA is processed and mRNA can accumulate or decay in the cytoplasm (Hershey et al., 1996; Morris, 1995). In recent years, considerable advances have been made in understanding the mechanisms of translational control. It has been estimated that translation proceeds through a set of steps involving a large number of translational components, including polypeptide initiation factors, tRNA$^{\text{Met}}$, mRNA and structural proteins of the 40S and 60S ribosomal subunits (Nygard and Nilsson, 1990; Preiss and Hentze, 1999). Furthermore, some of these components were shown to be directly regulated by distinct signaling pathways, suggesting their critical role in the control of translation. At the same time, signal transduction pathways, which transmit specific extracellular signals to the regulatory components of translational apparatus, are not well characterized. With regard to this, the study of signaling intermediates, which contribute to the protein synthesis regulation, presents a great scientific interest providing further insight.
into signaling mechanisms underlying the control of gene expression and cellular regulation.

1.1. Ribosomal protein S6 phosphorylation and mitogenic signal transduction

In the context of the mitogenic stimulation, the activation and maintenance of high rates of protein synthesis represents an obligatory anabolic response, which contributes to the ability of the cell to progress through the cell cycle, leading to cell growth, DNA synthesis, and cell division (Pardee, 1989). This response is associated with increased phosphorylation of serine/threonine residues in a number of specific translational components, including ribosomal protein S6 (Hershey, 1989; Kozma et al., 1989).

Ribosomal protein S6 (rpS6) is one of the 33 structural proteins which along with 18S rRNA comprise a mature 40S ribosomal subunit (Wool et al., 1996). Among these 40S ribosomal proteins, only S6 protein has been shown to undergo phosphorylation in vivo in response to growth factors and various mitogens (Jefferies and Thomas, 1996). A number of in vitro and in vivo studies have indicated that phosphorylation of five serine residues at the carboxyl terminus of S6 protein (see section 1.4.1.1.) is an important rate-limiting event in protein synthesis (Duncan and McConkey, 1982; Kreig et al., 1988; Païen and Traugh, 1987; Thomas et al., 1982). It has been also suggested that S6 phosphorylation may be involved in the regulation of selective translation of a specific mRNAs which encode for ribosomal proteins and other components of the translational apparatus (Jefferies and Thomas, 1996) (see section 1.4.1.1. and 1.4.1.2.). Therefore, S6 protein may represent an important signaling input for various signaling pathways involved in translational regulation.

The critical role of S6 protein in mitogen-stimulated proliferative responses has been shown by conditional deletion of the ribosomal protein S6 gene in the liver of adult mice (Volarevic et al., 2000). The lack of S6 protein expression in mouse hepatocytes abrogated ribosome biogenesis and as a result of that completely blocked induction of cyclin E expression and cell cycle progression after partial hepatectomy. It has also been shown that three independent P-element-induced mutations of the Drosophila S6 gene resulted in over-proliferation and abnormal development of the haematopoietic cells in the developing 3rd instar larvae as well as in mortality of the insect before the adult stage (Stewart and
Denell, 1993; Watson et al., 1993). Although these studies strongly implicate S6 protein in the regulation of cell proliferation, the actual mechanisms of this regulation remain to be elucidated.

Based on in vitro studies, several kinases were initially implicated in the phosphorylation of S6 protein, including cAMP and cGMP-dependent protein kinases, protein kinase C, Ca\(^{2+}\)/CaM-dependent protein kinase II, casein kinase I, p21-activated kinase II and p90 Ribosomal S6 kinase (Kozma et al., 1989). However, none of these kinases could fully phosphorylate S6 in vitro (see section 1.4.1.1. for the description of S6 phosphorylation sites) and for most, the kinetics of activation was not consistent with mitogen-induced phosphorylation of S6 protein in vivo. Later studies that use specific growth factors (Ballou et al., 1991) or inhibitors (Blenis et al., 1991, Chung et al., 1992; Kozma et al., 1990; Price et al., 1989) have identified p70/85S6K (also named S6KαI/II) as the physiological S6 kinase in mammalian cells.

The ribosomal protein S6 kinase α (S6Kα) is an important signaling component of mitogen-stimulated pathways involved in the regulation of protein synthesis and G1/S transition of the cell cycle (Proud, 1996; Berven and Crouch, 2000). This kinase integrates regulatory signals mediated by PI-3'-K-, mTOR/FRAP- and PKC-dependent signaling pathways and links them to the regulation of gene expression at the level of translation, presumably through the phosphorylation of ribosomal S6 protein (Peterson and Schreiber, 1998; Dufner and Thomas, 1999). The involvement of S6Kα in the regulation of transcription has also been proposed (de Groot et al, 1994; Brennan, et al., 1999) (see section 1.4.). The established role of S6K signaling in the control of such important cellular functions makes it an interesting target for further investigations.

1.2. Family of ribosomal protein S6 kinases

1.2.1. S6Kα and S6Kβ isoforms. Subcellular localisation and tissue distribution.

The ribosomal protein S6 kinase belongs to the AGC superfamily of structurally related Ser/Thr protein kinases which includes the PKA, PKB, PKCs, PDK1, SGKs and p90 RSK (Peterson and Schreiber, 1999). The family of S6Ks is currently represented by two homologous forms, S6Kα (initially termed as p70/85S6K) and the recently identified S6Kβ, which are the products of distinct genes (Gout et al., 1998; Lee-Fruman et al., 1999;
Saitoh, et al., 1998; Shima et al., 1998). Two isoforms of S6Kα, denoted S6KαI and S6KαII are known to be generated by the use of alternative translational start sites (Grove et al., 1991). The 525-amino acid S6KαI isoform differs from the 502-amino acid S6KαII isoform only at the amino terminus, where S6KαI contains a 23 amino acid extension that carries a polybasic nuclear localisation motif consisting of six consecutive arginine residues immediately after the initiator methionine residue (Coffer and Woodgett, 1994). Similarly to S6Kα, analysis of the isolated cDNA clones for S6Kβ revealed the presence of two potential alternative start codons suggesting existence of splice variants of the kinase (Gout et al., 1998). The S6KβI and S6KβII isoforms consist of 495 and 482 residues, respectively, and the sequence of S6KβII is identical to the 14-495 part of S6KβI. The expression of S6KβI and S6KβII isoforms as endogenous proteins has been recently confirmed by immunoblot analysis of various rat tissues using isoform specific anti-S6Kβ antibodies (Minami et al., 2001). The 13 amino acid extension of S6KβI contains a putative nuclear localisation sequence, RGRRAR, which is similar to that found within the 23 amino acid extension of S6KαI. Furthermore, the presence of a functional nuclear localisation signal at the C-terminus of S6Kβ, which is represented by a basic KKSKRGR stretch and found in both splice variants, has recently been reported (Koh et al., 1999). The functional importance of N- and C-terminal nuclear localisation sequences as well as the expedience of two nuclear signals in S6KβI remains to be investigated.

Immunofluorescence studies of the S6KαI isoform demonstrated its exclusive nuclear localisation in the cell. Furthermore, it has been shown that the nuclear targeting sequence of S6KαI, when fused to the bacterial protein CAT, was sufficient to constitutively target the chimeric protein to the nucleus (Reinhard et al., 1994). The S6KαII isoform is predominantly cytosolic, but can accumulate in the nucleus when cells are treated with leptomycin B, suggesting nucleocytoplasmic shuttling of this kinase (Coffer and Woodgett, 1994; Kim and Chen, 2000; Reinhard et al., 1994). In contrast, both S6KβI and S6KβII isoforms appear to be localised primarily to the nucleus because of the C-terminal nuclear localisation sequence (Koh et al., 1999; Lee-Fruman et al., 1999). The nuclear functions of S6Ks are not known so far. It has been proposed that in the nucleus, S6Ks may be responsible for the phosphorylation of the chromatin-bound form of S6 protein and the initiation of ribosome biogenesis (Franco and Rosenfeld, 1990; Jefferies and Thomas, 1996).

Both S6Kα isoforms and S6KβII were shown to be ubiquitously expressed having a similar pattern of expression in various cell lines and tissues (Gout et al., 1998; Saitoh, et
al., 1998). In contrast, S6KβI exhibits differential and limited expression and is not found in many tissues normally expressing other S6Ks (Minami et al., 2001). This may suggest that S6KβII shares some common functions with S6Kα isoforms, whereas S6KβI may play a distinct role in specific tissues. This is also supported by the fact that S6KβI resides in the particulate cellular fraction while S6KβII, S6KαII and S6KαI are found in the soluble fraction of the cells (Lee-Fruman et al., 1999).

1.2.2. Homology, structure and domain organization

Analysis of the amino acid sequence of S6Kα, along with functional studies, suggests modular organisation of this enzyme (Banerjee et al., 1990; Kozma et al., 1990; Cheatham et al., 1995; Weng et al., 1995a). Excluding the 23 amino acid extension containing a nuclear localisation sequence, both S6KαI and S6KαII can be dissected into four functional domains: the amino-terminal segment of 65 amino acid residues, the protein kinase catalytic domain, the kinase extension or linker domain and the carboxyl-terminal regulatory segment of 104 residues (Figure 1.1. and Appendix B).

The amino-terminal noncatalytic region is characterized by the presence of highly acidic sequence, wherein 10 out of 18 residues are aspartic acid or glutamic acid and basic residues are absent. This motif is thought to interact with basic carboxyl-terminal segment is the inactive enzyme (Banerjee et al., 1990). The catalytic domain contains all of 11 conserved subdomains found in various kinases and exhibits a consensus ATP-binding site (GKGGYG, in the single-letter amino acid code) followed by a lysine twenty residues thereafter. The characteristic motifs YRDLKPEN and G(S/T)XX(Y/F)XPÆE, corresponding to subdomains VI and VIII of S6Kα, are highly conserved between members of serine/threonine kinase family (Hanks et al., 1988). A unique feature of the carboxyl-terminal segment of S6Kα is the presence of a putative autoinhibitory pseudosubstrate region. This region constitutes a moderately basic serine/threonine rich sequence whose composition is reminiscent of the carboxyl-terminal region of S6 protein itself (Chan and Wool, 1988), that contains all the S6 kinase-directed phosphorylation sites (Krieg et al., 1988 and Figure 1.1. and Figure 1.2.). Support for the existence of an autoinhibitory region comes from the observation that synthetic peptides mimicking this region inhibit the activated S6Kα in the low micromolar range in vitro (Flotow and Thomas, 1992; Mukhopadhayay et al., 1992). Moreover, four of the major sites of serum-
Fig. 1.1. Domain organisation and phosphorylation sites of S6KαI and S6KβI isoforms. Structural features are indicated as follows: diagonally hatched box represents catalytic domain; striped box corresponds to kinase extension domain; grey box indicates autoinhibitory pseudosubstrate sequence; solid black boxes indicate nuclear localisation signals (amino acid sequences of NLS are shown above the diagrams); green boxes indicate acidic sequences and blue box means proline-rich sequence (amino acid sequences are shown in letters below each diagram); red box indicates PDZ domain binding motif; amino- and carboxyl-termini are left white and marked with NH₂ and COOH letters, respectively. Phosphorylation sites are shown with corresponding amino acid positions and surrounding sequences indicated.
Fig. 1.2. Comparison of S6 protein sequence to autoinhibitory pseudosubstrate sequences of S6Kα and S6Kβ. The carboxyl-terminal 41 amino acids residues of rat liver S6, which encompass all the reported (Krieg et al., 1998) sites of S6 phosphorylation (in bold text), are aligned with sequences from the carboxyl-terminal segments of rat S6Kα and human S6Kβ. Phosphorylation sites of S6Kα and S6Kβ are in bold and indicated by arrows. The leftmost bracket indicates the end of the kinase extension domain (see Appendix B and Appendix C). The S6Kα and S6Kβ sequences enclosed in brackets are the putative autoinhibitory pseudosubstrate regions. Identical amino acids are indicated by vertical lines, dots are employed to indicate amino acids of increasing similarity, scored by the mutational difference matrix of Schwartz and Dayhoff (Schwartz and Dayhoff, 1979). Identities score 1.5, one dot is >0.1, and two dots are >0.5.
induced phosphorylation associated with kinase activation are situated in this region (Ferrari et al., 1992). More recent studies with synthetic peptides demonstrate that substitution of these four residues with acidic amino acids relieves peptide inhibition (Jefferies and Thomas, 1996). The results are consistent with a model in which the putative autoinhibitory region directly interacts with the catalytic domain, blocking its ability to bind the substrate. Phosphorylation of the regulatory residues lying in the autoinhibitory domain would be hypothesised to participate in disruption of its interaction with the catalytic domain, allowing access to the substrate.

The overall sequence of S6Kβ is very close to that of S6Kα with 70% identity and 85% similarity at the protein level (Gout et al., 1998, Appendix C and Figure 1.1.). Moreover, S6Kα and S6Kβ share similar modular alignment of functional domains and conserved motifs described above. Despite the very high level of homology in the catalytic domain, kinase extension domain and autoinhibitory region (83%, 80% and 73%, respectively), the kinases exhibit major differences in their amino-terminal noncatalytic segment and in the carboxyl-terminal tail (share only 28% and 25% homology, respectively). In addition to the potential nuclear localization sequence at the carboxyl terminus, S6Kβ contains a specific proline-rich stretch of amino acids, extending from residues 459 to 480. This stretch exhibits similarity to docking sites for SH3 and WW domain-containing proteins and might be involved in mediating protein-protein interactions, which could attribute to S6Kβ functions distinct from those of S6Kα. The extreme carboxyl-terminal region of S6Kα forms a PDZ domain-binding motif which interacts with neurabin, described as a neural tissue-specific F-actin binding protein (Burnett et al., 1998a). S6Kβ does not contain a consensus sequence for PDZ domain recognition. Thus, the carboxyl termini of S6Kα and S6Kβ may target them to distinct signaling complexes and intracellular compartments. The regulatory and functional consequence of discrepancies in the primary sequences of S6Kα and S6Kβ awaits investigation.

1.2.3. Phosphorylation sites and molecular mechanism of activation

Mitogen-induced activation of S6Kα occurs through a complex series of serine/threonine phosphorylation events and requires precise interplay between specific domains and multiple phosphorylation sites (Dennis et al., 1998; Pullen and Thomas, 1997; Weng et al., 1998). To date, three sets of phosphorylation sites that undergo
mitogen-stimulated phosphorylation *in vivo*, have been identified in this kinase. Phosphorylation or mutation of amino acid residues that represent these phosphorylation sites, substantially affect S6 kinase activity suggesting their regulatory role in S6Kα activation. The first set of regulatory residues (Ser434, Ser441, Thr444, Ser447 and Ser452 in S6KαI) is situated within the autoinhibitory pseudosubstrate sequence and carboxyl-terminal region juxtaposed to this sequence (Ferrari et al., 1992; Price et al., 1991 and Figure 1.1.). These phosphorylation sites form a cluster of (Ser/Thr)-Pro motifs, which are characterized by a proline in the +1 position and a hydrophobic residue in the -2 position. Conversion of Ser434, Ser441, Thr444 and Ser447 to alanines significantly reduces S6Kα activation *in vivo*, whereas substitution with acidic residues, to mimic the phosphorylated state, produces a modest increase in basal S6 kinase activity (Dennis et al., 1998; Han et al., 1995). These (Ser/Thr)-Pro sites can be phosphorylated *in vitro* by an array of proline-directed kinases that are activated by diverse mitogenic stimuli (Mukhopadhayhay et al., 1992; Price et al., 1991); such kinases include mitogen-activated protein kinases (p42MAPK/p44MAPK or ERK1/2), stress-activated protein kinases and the cyclin-dependent kinase 2 (the latter acting particularly at S434 (Papst et al., 1998)). However, phosphorylation by these kinases fails to reactivate phosphatase 2A-treated S6Kα (Mukhopadhayhay et al., 1992), indicating that phosphorylation of the autoinhibitory pseudosubstrate region, although perhaps necessary, is not sufficient for S6Kα activation.

Consistently, it has been shown that S6KαΔC104 mutant, lacking the entire carboxyl-terminal segment, exhibited a low basal activity in quiescent cells and underwent phosphorylation-dependent activation in response to serum that was nearly identical to that of the full-length S6 kinase (Weng et al., 1995b). The activation of S6KαΔC104 was accompanied by a selective increase in phosphorylation of the second and third clusters of regulatory residues. These clusters are represented by Ser394 and Thr412 located in the kinase extension domain (Pearson et al., 1995) and Thr252 situated in the activation loop of the catalytic VIII subdomain (Weng et al., 1995b). More recent, studies have led to the identification of two additional mitogen-regulated phosphorylation sites, namely Thr390 and S427, which are located within the kinase extension domain (Moser et al., 1997). Based on mutational analysis and biochemical characterization, Thr252, in the activation loop, as well as Ser394 and Thr412, in the linker region, appear to make the most important contribution to S6 kinase activity (Pearson et al., 1995, Weng et al., 1995b). Furthermore, it has been argued that coordinated phosphorylation of both Thr252 and
Thr412 is absolutely critical for the full activation of S6Kα (Alessi et al., 1997a; Weng et al., 1998). At the same time, the role of Thr390 and S427 phosphorylation is not clear, since mutation of these sites had little or no effect on basal and serum-stimulated activities of the kinase (Moser et al., 1997; Pearson et al., 1995).

The activation loop phosphorylation site, Thr252, as well as Ser394 and Thr412, in the kinase extension domain of S6Kα, are common regulatory elements found in most members of the AGC family of serine/threonine kinases (Hanks and Hunter, 1995; Marshall, 1994). Furthermore, the motifs surrounding these sites appear to be conserved in all S6Kα homologs, including those from Drosophila melanogaster, Arabidopsis thaliana, and Saccharomyces cerevisiae, as well as in PKCα and PKCβII (Moser et al., 1997). Interestingly, this is not the case for Thr390, suggesting that it plays a role which is unique to the mammalian S6Kα. Similarly, the autoinhibitory pseudosubstrate sequence, along with the carboxyl-terminal segment of S6Kα, are conspicuously absent in other members of the AGC family of serine/threonine kinases (Hanks and Hunter, 1995).

Both critical phosphorylation sites, Thr252 and Thr412, are situated in relatively hydrophobic contexts and flanked by bulky aromatic amino acids (...VTHT252FCGT..., ...FLGFT412YVAP..., in the single-letter amino acid code). The kinase responsible for phosphorylation of Thr252 in vivo has been identified as PDK1 (Alessi et al., 1997a; Pullen et al., 1998; Williams et al., 2000), which is known to phosphorylate several other substrates, such as PKB, PKCs and PKA. It has also been demonstrated that PDK1 is capable of phosphorylating S6Kα at Thr412 in vitro and perhaps mediates phosphorylation of this residue in vivo (Balendran et al., 1999a). Nevertheless, it is not clear whether these two threonine residues are phosphorylated by a single, or by two different protein kinases. First, phosphorylation of Thr412 was shown to be completely abolished in the S6KαΔN54ΔC104 mutant by treatment of cells with wortmannin, a specific inhibitor of PI-3'-K, whereas phosphorylation of Thr252 remains unaffected under this conditions (Dennis et al., 1996). This correlates with the fact that PDK1 phosphorylates Thr252 in a PtdIns-3,4,5-P3-independent manner (Alessi et al., 1997a) but questions the role of this kinase in Thr412 phosphorylation. Second, although phosphorylation at both Thr252 and Thr412 residues is sensitive to rapamycin, a specific inhibitor of mTOR/FRAP, Thr412 appears to be the principal site of rapamycin-induced S6Kα inactivation (Weng et al., 1998). Mutation of Thr412 to glutamic acid results in an increase in basal S6 kinase activity and elevated Thr252 phosphorylation as well as conferring resistance of S6Kα to
inhibition by rapamycin (Dennis et al., 1996; Pearson et al., 1995; Pullen and Thomas, 1997). Moreover, it has been recently shown that Thr 412 in S6Kα can be directly phosphorylated by the mTOR/FRAP kinase in vitro and that this phosphorylation induces substantial increase in S6 kinase activity (Burnett et al., 1998b; Isotani et al., 1999). Subsequent phosphorylation of S6Kα by PDK1 gives a strong synergistic increase in S6 kinase activity (Burnett et al., 1998b). In addition, the NIMA-related kinases NEK6 and NEK7 have recently been identified and shown to phosphorylate Thr412, as well as some other sites, and activate S6Kα in vitro and in vivo, in a manner synergistic with PDK1 (Belham et al., 2001). However, confirmation of the identity of physiological kinase/s, which are responsible for phosphorylation of Thr412 in vivo, requires further study.

Based on several recent observations, it has been proposed that phosphorylation of S6Kα occurs in a hierarchical fashion, involving a potent cooperation and specific interactions between phosphorylation sites. Indeed, the ability of S6Kα to be phosphorylated at Thr252 by PDK1 and the relative and absolute extents of S6Kα activation caused by PDK1-catalysed phosphorylation are greatly altered by a variety of S6 kinase mutations (Alessi et al., 1997a). Thus the conversion of the five clustered (Ser/Thr)-Pro phosphorylation sites in the carboxyl-terminal segment to alanines strongly suppresses PDK1-catalysed Thr252 phosphorylation, whereas deletion of the entire carboxyl-terminal segment, to give the S6KαΔC_{104} mutant, increases both the rate and extent of Thr252 phosphorylation by PDK1 and also the degree of S6 kinase activation achieved at any level of Thr252 phosphorylation. Deletion of S6Kα amino-terminal noncatalytic residues 2-46, a modification previously shown to greatly inhibit S6 kinase activity (Weng et al., 1995a), abolishes the ability of the kinase to be phosphorylated by PDK1 in vitro, whereas the additional deletion of the carboxyl-terminal tail (to produce S6KαΔN_{2-46}ΔC_{104}), which restores S6 kinase activity and mitogen responsiveness in vivo (Dennis et al., 1996; Weng et al., 1995a), also restores the ability of PDK1 to catalyse Thr252 phosphorylation in vitro. Furthermore, the mutation of phosphorylation sites clustered in the autoinhibitory domain to acidic residues facilitates an in vivo phosphorylation of Thr412 (Moser et al., 1997). Together, these results indicate that the carboxyl-terminal segment exerts a strong influence on the phosphorylation of critical regulatory residues, Thr252 and Thr412, as well as the extent of S6Kα activation resulting from these phosphorylation events.
In addition to the ability of (Ser/Thr)-Pro sites to regulate Thr252 and Thr412 phosphorylation, it has been revealed that phosphorylation state of Thr412 itself strongly affects phosphorylation of Thr252 (Alessi et al., 1997a; Weng et al., 1998). Thus, conversion of Thr412 in S6KαΔC104 to glutamate facilitates PDK1-catalysed Thr252 phosphorylation in vitro. Consistently, S6KαT412A and S6KαT412AD3E mutants, the latter of which possesses substitution of the four sites in the pseudosubstrate region with acidic residues, fail to undergo any detectable Thr252 phosphorylation in vivo in response to serum stimulation (Dennis et al., 1998). Interestingly, mutation of Thr252 to alanine was also shown to partially suppress Thr412 phosphorylation in vivo, suggesting interdependent phosphorylation of these sites. Moreover, several studies have recently demonstrated that functional interaction of Thr252 and Thr412 is the major, perhaps predominant, factor in determining S6Kα activity (Alessi et al., 1997a; Dennis et al., 1998; Weng et al., 1998). It has been shown that selective phosphorylation of PP2A-treated S6KαΔC104 at Thr252 by PDK1 resulted in a 15 fold increase in S6 kinase activity to a level comparable to that exhibited by PP2A dephosphorylated S6KαT412EΔC104 mutant. This indicates that modification of either Thr252 or Thr412 is sufficient to increase the basal level of S6 kinase activity. This resulting activity, however, does not represent a maximal S6 kinase activity, since phosphorylation of PP2A-treated S6KαT412EΔC104 and S6KαT412ED3E mutants by PDK1 increases their activities by a further 20-30 fold, clearly demonstrating the strong positive cooperation between Thr252 and Thr412 in S6Kα activation.

Finally, the phosphorylation of Thr412 was found to be dependent upon Ser394, which is situated in close proximity to this site (Moser et al., 1997). Mutation of Ser394 to either an acidic or neutral residue potently suppresses phosphorylation of Thr412, suggesting structural requirement of serine in this position for normal Thr412 phosphorylation. However, the mechanism of this regulation is not clear. The site equivalent to Ser394 has been identified as a major autophosphorylation site in PKCβII (Keranen, et al 1995; Tsutakawa et al., 1995) and more recently in PKCα (Bornancin and Parker, 1996), Thr641 and Thr638, respectively. These sites were shown to be involved in the control of dephosphorylation and inactivation of these kinases (Bornancin and Parker, 1996; Dutil et al., 1994; Zhang et al., 1994). In contrast, Ser394 does not appear to be phosphorylated by an intra- or intermolecular autophosphorylation mechanism, since the kinase-dead S6KαK100Q mutant undergoes normal Ser394 phosphorylation in serum-stimulated cells.
Furthermore, the substitution of Thr412 or Thr252 to alanine or rapamycin treatment, which blocks their phosphorylation, has no effect on Ser394 phosphorylation suggesting the involvement of rapamycin-insensitive upstream kinase/s (Moser et al., 1997). In fact, Ser394, like phosphorylation sites in the autoinhibitory pseudosubstrate region, is followed directly by a proline residue and is likely to be phosphorylated by proline-directed kinase/s distinct from those acting on Thr252 and Thr412. The actual role of Ser394 phosphorylation in the control of S6Kα activation requires further study.

Based on these studies a model for S6Kα activation has been proposed which implies that the active conformation of the kinase is achieved by coordinated phosphorylations at regulatory residues clustered in the carboxyl-terminal autoinhibitory segment, the conserved kinase extension domain and the activation loop of the catalytic domain (Alessi et al., 1997a; Dennis et al., 1998; Pullen and Thomas, 1997). Regarding this model (Figure 1.3.), the process of mitogen-induced S6Kα activation can be divided into several steps, regulated by diverse a signaling inputs. The initial step involves the action of an array of proline-directed kinases which phosphorylate the S6Kα carboxyl-terminal segment (Ser434, Ser441, Thr444, Ser447 and Ser452), and perhaps Ser394 in the linker domain (Price et al., 1991). This disrupts the inhibitory interaction between the carboxyl and amino termini and subsequently releases the phosphorylated carboxyl-terminal autoinhibitory segment from the catalytic domain, providing access to Thr412 and Thr252. The existence of this step is inferred from the ability of the 2-46 amino terminal deletion to interrupt S6Kα activation at this point (Weng et al., 1995a). However, it is not known whether this step is regulated. The next step involves the phosphorylation of Thr412 by an unknown PtdIns-3,4,5-P3-dependent kinase. This alone produces only partial activation of S6 kinase, but facilitates access to Thr252 and sets up the conformation necessary for a concerted activation. Thus, Thr412 phosphorylation acts as rate-limiting intermediary step (Dennis et al., 1998; Weng et al., 1998) between autoinhibitory (Ser/Thr)-Pro sites and activation loop site phosphorylation the final step in mitogen-induced S6Kα activation (Figure 1.3.). This final step is mediated by PDK1 and results in full activation of the S6 kinase due to the strong positive cooperation between the phosphorylated Thr412 and Thr252 residues. Phosphorylation of critical sites, represented by Thr412 and Thr252, probably occurs in a concerted and interdependent fashion, because all of the components (Thr412 kinase, PDK1 and S6Kα) are likely assembled close together through the mediation of PtdIns-3,4,5-P3.

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Fig. 1.3. Model of mitogen-induced S6Kα activation. The schematic represents the proposed mechanism of mitogen-stimulated S6Kα activation, outlining the sequential steps in the process. See text for details.
Most of the phosphorylation sites identified in S6Kα are conserved in S6Kβ and include: a set of (Ser/Thr)-Pro motifs clustering in the autoinhibitory sequence and region located immediately carboxyl-terminal to this sequence (Ser423, Ser430, Ser436 and Ser441 in S6Kβ, which correspond to Ser434, Ser441, Ser447 and Ser452 in S6KαI, respectively); Thr379, Ser383, Thr401 and Ser416 located in the kinase extension domain, which correspond to Thr390, Ser394, Thr412 and S427 in S6KαI, respectively; and Thr241 located in the catalytic loop, which corresponds to Thr252 in S6KαI (Gout et al., 1998 and Figure 1.1.). However, one of the known S6Kα regulatory residues, represented by Thr444, is not conserved and substituted to valine in S6Kβ. Based on a comparison of the phosphorylation sites between these two kinases, it has been predicted that S6Kβ might be activated through mechanisms which are similar, but not identical, to those proposed for S6Kα (Gout et al., 1998; Shima et al., 1998).

1.3. Signal transduction and regulation of S6K activity

Identification of a number of regulatory phosphorylation sites involved in the activation of S6Kα, and most likely S6Kβ, indicates that this complex process requires multiple signaling inputs mediated by different Ser/Thr protein kinases. Recent studies have revealed some of the upstream regulators which indicate that at least two distinct signaling pathways influence S6Kα activation. One of these pathways is regulated by PI-3'-kinase and its downstream effectors, such as PDK1, PKB, atypical PKCs, Rac and Cdc42 (Toker, 2000). Consistent with the important role of this pathway in S6Kα regulation, growth factor-stimulated activation of S6 kinase is potently inhibited by pharmacological inhibitors of PI-3'-kinase, namely wortmannin and LY294002 (Cheatham et al., 1994; Chung et al., 1994).

A separate pathway contributing to S6Kα activation involves mTOR/FRAP kinase and can be selectively inhibited by rapamycin (Dufner and Thomas, 1999; Meyuhas, 2000; Shah et al., 2000a). The immunosuppressant rapamycin is the most potent indirect inhibitor of S6 kinase described, blocking its activation by all known stimuli (Pullen and Thomas, 1997). Thus, rapamycin was shown to strongly inhibit PMA-induced activation of S6Kα, mediated by conventional and novel PKCs, while wortmannin had little or no effect on PKC-dependent S6Kα activation in several cell lines (Han et al., 1995; Law et al., 2000;
Monfar, et al., 1995). The actual role of the c/nPKC-regulated pathway in the regulation of S6Ks and its dependence on mTOR/FRAP signaling remain unclear.

Although significant progress has been made in determining upstream modulators of S6Kα activity, the signaling mechanisms involved in this regulation are largely unknown. Regarding this point, the identification of immediate effectors of S6Ks is an important step in elucidating those events directly involved in the regulation of S6K signaling. Early in vitro studies implicated several proline-directed kinases, including mitogen-activated protein kinase (MAPK), in phosphorylation of the S6Kα carboxyl-terminal (Ser/Thr)-Pro sites, but their involvement in activation of S6Kα in vivo was not clearly demonstrated (Mukhopadhayhay et al., 1992; Price et al., 1991). To date, among PI-3′-K-regulated effector kinases, only PDK1 was found to phosphorylate and activate S6Kα directly in vitro and in vivo (Alessi et al., 1997a; Balendran et al., 1999a; Pullen et al., 1998; Williams et al., 2000). Furthermore, it has been shown that atypical PKC isoforms ζ and λ form a multimeric complex with both PDK1 and S6Kα, which enable PI-3′-K-dependent activation of S6 kinase in vivo (Akimoto et al., 1998; Romanelli et al., 1999). However, it is not yet known whether aPKCs directly phosphorylate S6Kα.

Two members of the Rho family of small G proteins, Rac1 and Cdc42, were also found in complex with catalytically inactive S6Kα, but contribution of these proteins to S6 kinase regulation is not clear (Chou and Blenis, 1996). In addition, the NIMA-related kinases NEK6 and NEK7 were recently shown to phosphorylate in vitro and in vivo Thr412 and other sites in S6Kα, producing a significant increase in S6 kinase activity (Belham et al., 2001). However, their location within specific pathways leading to activation of S6Kα is yet to be elucidated.

Therefore, the activation of S6Kα is determined by multiple signaling mediators which are integral parts of PI-3′-K and mTOR/FRAP-regulated signal transduction pathways (Figure 1.4.). Mechanisms of coordination of multiple S6Kα activating inputs as well as the relevance of these signaling pathways in the regulation of the homologous S6Kβ are currently of great scientific interest.
Fig. 1.4. Schematic representation of S6K signaling pathway. A horizontal bar and an arrowhead indicate activation and repression, respectively. A solid line indicates established signaling link, whereas a broken line represents putative/uncertain regulatory connection. Question marks indicate unknown effectors. See text for details.
1.3.1. PI-3'-K and its downstream effectors

Phosphatidylinositide-3'-kinase (PI-3'-K) comprises a family of lipid kinases that phosphorylate phosphatidylinositol (PtdIns) and some specific phosphoinositides at the 3'-OH position of the inositol ring, generating PtdIns(3)P, PtdIns(3,4)P2, PtdIns(3,5)P2 and PtdIns(3,4,5)P3 products (Shepherd et al., 1998; Toker et al. 2000; Vanhaesebroeck and Waterfield, 1999). In mammalian cells, three distinct classes of PI-3'-Ks (class I (A and B), II and III) have been characterized and shown to differ in their subcellular and tissue distribution, substrate specificity and mechanisms of activation by extracellular agonists (Domin and Waterfield, 1997). It is commonly accepted that class III PI-3'-K is responsible for the in vivo synthesis of PtdIns(3)P, which is constitutively present at a relatively high level in eukaryotic cell membranes (Rameh and Cantley, 1999). This lipid product specifically interacts with FYVE finger domain-containing proteins many of which are involved in different membrane trafficking processes (Mills et al., 1998; Simonsen et al., 1998; Wiedemann and Cockcroft, 1998). Conversely, PtdIns(3,4)P2 and PtdIns(3,4,5)P3, generated by class I PI-3'-K, inositol polyphosphate-5'-phosphatase, and probably class II PI-3'-K, are present at a low level (less than 0.25 % of the total inositol-containing lipids), which rapidly rises upon agonist stimulation (Hawkins et al., 1992; Stephens et al., 1991). These two phospholipids were suggested to function as second messenger molecules which initiate class I PI-3'-K-dependent signaling cascades via interaction with certain PH domain-containing proteins (e.g., protein kinases, phospholipases, adaptor proteins and G-proteins) (Leevens et al., 1999; Rameh and Cantley, 1999).

Class I PI-3'-K is involved in receptor-induced growth factor and hormonal responses and the mechanism of activation of this enzyme differs depending on the particular extracellular stimuli. For tyrosine kinase-coupled receptor systems, p110 catalytic subunits α, β and δ (belonging to class Ia) are stimulated through interaction with the p85 adaptor subunit, which binds selectively through its SH2 domain to phosphorylated tyrosines on the activated receptor (Fruman et al., 1998). For G-protein-coupled receptor systems, the signaling for PI-3'-kinase activation is less clear and several mechanisms have been suggested. A number of reports have identified a p110γ catalytic subunit (belonging to class Ib), which is activated through binding either directly to the βγ subunits of activated trimeric G-proteins (Leopoldt et al., 1998) or through interaction with a p101 adaptor
protein (Krugmann et al., 1999; Stephens et al., 1997). Other studies have suggested alternative mechanisms involving G-protein-mediated activation of receptor tyrosine kinase activity (Daub et al., 1997) or through the p110β isoform, which can also be activated directly by G-protein βγ subunits (Maier et al., 1999).

For growth factor- and hormone-induced responses in vivo, PtdIns(4,5)P₂ is the primary substrate for class I PI-3'-K, leading to the production of PtdIns(3,4,5)P₃. PtdIns(3,4,5)P₃ is also rapidly dephosphorylated by 5'-phosphatase, giving rise to the production of PtdIns(3,4)P₂ (Stephens et al., 1991; Jefferson et al., 1997). The lipid products of class I PI-3'-K serve as both membrane anchors and allosteric regulators for many PH domain-containing signaling proteins, including PDK1 and PKB, and were shown to be indirectly involved in the regulation of some other AGC family-related Ser/Thr-kinases (Vanhaesebroeck and Alessi, 2000). Among them, S6Kα was the first AGC kinase identified as a physiologically relevant downstream target of PI-3'-K IA. Thus, F740/F751 and kinase-inactive R635 PDGF-R mutants, unable to bind and activate PI-3'-K IA, were found to abrogate the activation of S6Kα stimulated by PDGF (Chung et al., 1994). Consistently, the activation of S6Kα in response to various mitogenic stimuli is potently inhibited by two unrelated PI-3'-K inhibitors, wortmannin and LY294002 (Cheatham et al., 1994; Monfar et al., 1995; Myers et al., 1994; Weng et al., 1995b), and by overexpression of dominant negative forms of the PI-3'-K adapter/regulatory subunit (Burgering and Coffer, 1995). Additionally, the activity of S6Kα is significantly increased by overexpression of constitutively active class IA p110 catalytic subunits (Weng et al., 1998). Several in vivo studies have identified Thr252, Thr412 and Ser427 as wortmannin sensitive sites in wild-type S6Kα (Han et al., 1995; Weng et al., 1998), suggesting a critical role for PI-3'-K signaling in the activation of S6 kinase. Furthermore, the ability of neutralising antisera against S6Kα to inhibit the mitogenic activity of class I PI-3'-Ks provided further evidence supporting the physiological role of S6Kα as a downstream effector of PI-3'-K-regulated pathways (Mcllroy et al., 1997).

Ribosomal protein S6 kinase does not contain a PH domain, suggesting indirect regulation of the kinase by PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃. Generation of these phospholipids and subsequent activation of PDK1/PKB and/or PDK1/aPKC systems were proposed to provide an important signaling link between class I PI-3'-Ks and S6K (Figure 1.4.).
1.3.1.1. PDK1 and its role in PI-3'-K-dependent and independent regulation of S6K

Considering the importance and diversity of cellular processes regulated by 3'-phosphoinositide-dependent kinase-1 (PDK1) substrates, many of which are PI-3-K-dependent, it is clear that this enzyme is a critical element of PI-3'-K signaling. However, the role of PtdIns(3,4)P2 and PtdIns(3,4,5)P3 in the regulation of PDK1 activity and its cellular location is not entirely understood. Initial studies indicated that none of the PI-3'-K lipid products could directly activate purified PDK1 in vitro (Alessi et al., 1997b), despite the fact that the PDK1 PH domain has a remarkably high affinity for PtdIns(3,4,5)P3 (K_D = 1.6 nM) (Stephens et al., 1998). More recent studies have in fact shown that partially purified PDK1 is activated 3 fold by PtdIns(3,4,5)P3 (Stephens et al., 1998), and also that binding of PIF fragment of PRK2 to PDK1 converts it into a PtdIns(3,4,5)P3-activated enzyme (Balendran et al., 1999b). Similarly, the PtdIns(3,4,5)P3 requirement for PDK1 to phosphorylate PKCζ in vitro is lost when the PDK1 PH domain is deleted (Le Good et al., 1998). These studies suggest that PtdIns(3,4,5)P3 is required for PDK1 specific activity either by directly increasing its intrinsic kinase activity, or by promoting access to the substrate. At the same time, PDK1 appears to possess a constitutively active conformation in unstimulated cells, probably due to the autophosphorylation of its activation loop in a PI-3'-K-independent manner (Casamayor et al., 1999), and does not require PtdIns(3,4)P2 and PtdIns(3,4,5)P3 to phosphorylate some of its substrates. The regulatory factor of PDK1-mediated phosphorylation of these specific substrates in vivo is a limited access to the phosphorylation motif in targeted proteins rather than dependence of PDK1 activity on agonist stimulation. The access to substrates can be controlled by the specific subcellular localisation of proteins and/or conformational restrictions in the structure of these proteins. Activation of PKB is a classical example of such regulation where ability of PDK1 to phosphorylate this kinase is regulated by PtdIns(3,4)P2- and PtdIns(3,4,5)P3-mediated membrane localisation of both proteins and by conformational changes in PKB (Vanhaesebroeck and Alessi 2000). In contrast, S6Kα associates with PDK1 in a phosphoinositide-independent manner, but requires agonist-stimulated conformational changes to expose PDK1 phosphorylation site/s (Alessi et al., 1997a; Romanelli et al., 1999; Weng et al., 1998).

There is much evidence indicating that PDK1 is directly responsible for the in vitro and in vivo phosphorylation of Thr252 in the activation loop of S6 kinase (Alessi et al., 1997a;
Pullen et al., 1998). Cotransfection of S6Kα with PDK1 results in S6 kinase activation, concomitant with increased phosphorylation at Thr252. Reciprocally, kinase-inactive ATP site mutants of PDK1 blocks serum-stimulated phosphorylation of S6Kα at this regulatory residue. Consistent with these data, embryonic stem cells lacking PDK1 show no IGF-1 stimulated phosphorylation of Thr252 in S6Kα (Williams et al., 2000). In addition, phosphorylation of Thr412 in the hydrophobic motif of S6Kα was similarly abolished in these cells. Recently, it has been shown that PDK1 can phosphorylate S6Kα at Thr412 in vitro (although at a rate less than 5% of that for Thr252) and mediate the phosphorylation of Thr412 in vivo (Balendran et al., 1999a). However, it is questionable whether PDK1 participates directly as a Thr412 kinase in vivo or instead, if it is necessary for the activation of other Thr412 kinases (see section 1.2.3.).

In agreement with the constitutively active state of PDK1 Thr252 phosphorylation was found to be insensitive to wortmannin in the S6KαΔN54ΔC104 mutant, in which conformational restrictions towards Thr252 site are eliminated by deletion of N- and C-terminal regulatory segments (Dennis et al., 1996). However, phosphorylation of this site in wild type S6Kα exhibits dependence on class I PI-3'-K phospholipid products and can be blocked by wortmannin treatment (Han et al., 1995; Weng et al., 1998). As described in section 1.2.3., the phosphorylation state of Thr252 in S6Kα is largely dependent on the phosphorylation of other regulatory sites, including Thr412 in the hydrophobic motif of the linker domain. Phosphorylation of Thr412 is absolutely dependent on PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ production (Dennis et al., 1996) and is likely regulated by wortmannin-sensitive protein kinase/s distinct from that for Thr252. Therefore, phosphorylation of the wortmannin-sensitive Thr412 site, which governs accessibility of Thr252, determines the PI-3'-K-dependent nature of Thr252 phosphorylation.

Therefore, PDK1 may be involved in PI3K-dependent and independent regulation of S6Kα activation. The PDK1 mediated control of Thr412 phosphorylation is PI-3'-K-dependent (Williams et al., 2000), whereas constitutive activity of PDK1 towards Thr252 may represent a PI-3'-K-independent mechanism of S6Kα activation. Complexity of S6Kα regulation by PDK1 is also determined by involvement of this kinase in the regulation of PKB and aPKCs (Vanhaesebroeck and Alessi 2000), which are known to participate in PI-3'-K-dependent activation of S6Kα (Akimoto et al., 1998; Burgener and Coffer, 1995; Dufner et al., 1999; Romanelli et al., 1999).
1.3.1.2. Possible role of PKB in S6K regulation

Protein kinase B, the cellular homologue of the retroviral oncoprotein v-Akt, is a PH domain-containing kinase which belongs to the AGC family of serine/threonine kinases and is regulated by a range of growth factors (Burgering and Coffer, 1995; Coffer et al., 1998). The agonist-induced activation of PKB is mediated by PI-3′-K produced phospholipids and subsequent phosphorylation of Thr308 in the activation loop by PDK1 and Ser473 in the C-terminal hydrophobic motif possibly by a PDK1/PRK2 complex (Alessi et al., 1997b; Balendran et al., 1999b; Stokoe et al., 1997). Constitutively active PDK1 can only phosphorylate PKB in the presence of PtdIns(3,4,5)P3, and thus a model has been proposed in which binding of PtdIns(3,4,5)P3 to the PH domain of PKB causes a conformational change that allows PDK1 access to Thr308 and Ser473. The other major function of PtdIns(3,4)P2 and PtdIns(3,4,5)P3 binding in this system is likely to be in promoting co-localisation of PKB, PDK1 and PRK2 following growth factor stimulation and thereby facilitating efficient phosphorylation of PKB at the regulatory sites. This model is in agreement with data indicating that activation of class I PI-3′-K is both necessary and sufficient for the full activation of PKB in response to growth factor stimulation (Anjelkovic et al., 1997; Burgering and Coffer, 1995; Franke et al., 1995).

Several studies support the idea that PKB may play a role in the activation of S6Kα in response to phospholipid generating agonists. Although PKB has not been shown to directly phosphorylate S6Kα, expression of constitutively active gag-PKB, which is targeted to the membrane, activated S6Kα in unstimulated cells (Burgering and Coffer, 1995; Kohn et al., 1998). These observations have led to the hypothesis that PKB is a mediator conveying signals from PI-3′-K to S6Kα. However, the requirement of wild-type PKB for S6Kα activation is controversial and not well understood. Thus, depletion of intracellular calcium store by EGTA pretreatment has no effect on growth factor-induced PKB activation but completely abolishes S6Kα stimulation (Conus et al., 1998). Conversely, ionomycin-induced increase in intracellular calcium results in wortmannin-sensitive full activation of S6Kα, whereas little or no activation of PKB is observed. These findings led to the conclusion that activation of signaling to PKB is insufficient for the activation of S6Kα, which can be achieved independently of PKB. Consistent with this co-expression studies demonstrated that a constitutively active wortmannin-resistant form of PKB, harboring T308D and S473D substitutions (Alessi et al., 1996), is sufficient to
induce PKB-mediated responses, such as GSK-3 inactivation and 4E-BP1 phosphorylation, but not S6Kα activation (Dufner et al., 1999). In parallel, a membrane-targeted dominant interfering PKB variant did not prevent insulin-induced S6Kα activation, whereas it blocked GSK-3 and 4E-BP1 phosphorylation. This implies that activation of S6Kα in the system described is strictly dependent on the membrane localisation of PKB rather than its specific activity. Regarding this point, it is important to notice that constitutive membrane targeting of PKB promotes system A amino acid transport (Hajduch et al., 1998), which could provide a mechanism by which the membrane-targeted mutant of PKB induces S6Kα activation. Indeed, amino acids were shown to stimulate S6Kα activity by means of mTOR/FRAP signaling (see section 1.3.2.). Furthermore, disruption of the Drosophila genes encoding dS6K or PKB (dAkt1) led to a similar phenotype, suggesting that S6K and PKB may share a pathway that controls cell size (Montagne et al., 1999; Verdu et al., 1999). Given the role of mTOR/FRAP in the control of cell growth (Thomas and Hall, 1997), it is attractive to speculate that PKB may regulate S6Kα activity via the mTOR/FRAP pathway. This hypothesis is partially supported by studies which indicate that PKB regulates 4E-BP1 phosphorylation in rapamycin-sensitive manner and lies upstream of mTOR/FRAP kinase (Gingras et al., 1998). Recent studies have also revealed that PKB phosphorylates in vitro and in vivo tumor suppressor TSC2 (see section 1.3.4.) and releases its inhibitory function on S6Kα signaling, probably by derepression of TSC1-TSC2-mediated inhibition of mTOR/FRAP kinase (Inoki et al., 2002; Manning et al., 2002). These studies suggest strong requirement for PKB specific activity in the regulation of S6Kα. However, this contradicts earlier observations indicating that membrane localisation, but not kinase activity of PKB, is a predominant requirement for S6Kα activation (Dufner et al., 1999). Thus, further studies will be necessary to elucidate an actual role of PKB in the regulation of S6K activity.

1.3.1.3. Atypical PKCζ and PKCλ

Atypical isoforms of protein kinase C, PKCζ and PKCλ, appear to be an important link between PI-3-K and S6Kα. Unlike other PKC isotypes (see section 1.3.3.), atypical PKCs are not regulated by calcium or diacylglycerol and are insensitive to activation by phorbol esters both in vitro and in vivo (Mellor and Parker, 1998; Nishizuka, 1992). Several studies have shown that PKCζ is activated in vitro by the class I PI-3′-K lipid product.
PtdIns(3,4,5)P₃ (Nakanishi et al., 1993), and that PKCζ, as well as PKCζ, is regulated in vivo through a pathway involving PI-3'-K (Akimoto et al., 1996; Standaert et al., 1997) and PDK1 (Chou et al., 1998; LeGood et al., 1998). Because a PtdIns(3,4,5)P₃-binding site of PKCζ and PKCλ has not been described, the PtdIns(3,4,5)P₃ requirement for their activation remains elusive.

The involvement of aPKCs in S6Kα regulation has recently been suggested based on two independent studies, which demonstrate the ability of PKCζ or PKCλ to bind S6Kα contributing to its activation in vivo (Akimoto et al., 1998; Romanelli et al., 1999). Thus, coexpression of a dominant interfering allele of PKCζ antagonized S6Kα activation by EGF, PDK1, and activated Cdc42 or p110 catalytic subunit of PI-3'-K. A myristoylated constitutively active PKCζ induced a modest activation of S6Kα and showed functional cooperation with PDK1-mediated activation of S6Kα. Furthermore, all three components, PDK1, PKCζ and S6Kα, were coimmunoprecipitated in a growth factor-independent manner suggesting the existence of preassembled complexes of PI-3-K-regulated intermediates. Consistent with these findings, it has been demonstrated that the amino-terminal region of S6Kα, including the kinase domain, interacts with the kinase domain of PKCζ, whereas the carboxyl-terminal region of S6Kα is required for interaction with the regulatory domain of PKCζ (Akimoto et al., 1998). The authors also present evidence that overexpression of the kinase domain without kinase activity or regulatory domain of PKCζ significantly suppresses the serum-induced activation of S6Kα. However, the constitutively active form of PKCζ had no obvious effect on basal or stimulated S6Kα activity. These latter findings suggest that activity of PKCζ, and likely PKCζ, is indispensable but not sufficient for the activation of S6Kα.

The mechanism of S6Kα regulation by aPKCs is not presently clear. Although atypical PKCs have been found in complexes with S6Kα, it is not yet known whether they can phosphorylate S6 kinase and directly modulate its activity. Curiously, the activation of PKCζ, by PKCζ, is mediated through rapamycin-sensitive phosphorylation of the hydrophobic site S662 in PKCζ, which is analogous to Thr412 of S6Kα (Parekh et al., 1999; Ziegler et al., 1999). This led to the hypothesis that aPKCs may be directly involved in the phosphorylation of T412 in the hydrophobic motif of S6Kα as such, cooperate with PDK1 to promote Thr252 phosphorylation and S6K activation (Dufner and Thomas, 1999). Nevertheless, several other possible roles for aPKCs in S6Kα activation are
foreseen. Thus, PKCζ and PKCλ may be activators of S6Kα upstream kinases, e.g. the Thr412 kinase. Alternatively, but not exclusively, aPKCs, through their ability to stably associate with S6Kα, may serve as a shuttle to convey S6 kinase to sites of PtdIns(3,4,5)P3 synthesis, where S6 kinase is phosphorylated by the Thr412 kinase and PDK1 (Akimoto et al., 1998). Similar membrane targeting roles have also been proposed for Rac and Cdc42 known to form GTP-dependent complexes with catalytically inactive S6Kα (Chou and Blenis, 1996).

1.3.1.4. Rho family of small GTPases: Cdc42 and Rac

The Rho family of G proteins, including Rho, Rac, and Cdc42, controls various aspects of cytoskeletal organisation (Kozma et al., 1995; Nobes and Hall, 1995; Ridley et al. 1992) as well as serum response element (SRE)-regulated gene expression (Hill et al., 1995). Recently, they have also been shown to function in the regulation of signaling via JNK, p38 mitogen-activated protein kinase and p21-activated kinases (PAKs) (Bagrodia et al., 1995; Coso et al., 1995). Now it is evident that Rho family G proteins and PI-3'-K reside on a common signaling pathway (Reif et al., 1996), and most studies indicate that PI-3'-K functions upstream of these GTPases. For example, expression of activated p110 catalytic subunit of PI-3'-K increases the level of GTP-bound, active form of Rac, which in turn induces the changes in the actin cytoskeleton responsible for membrane ruffling (Hawkins et al., 1995). The precise mechanism through which PI-3'-Ks activate G-proteins has not been elucidated, but some data suggest that GTP/GDP-exchange factors for these GTPases can be directly stimulated by PtdIns(3,4)P3 and PtdIns(3,4,5)P3 (Han et al., 1998).

Recent studies have demonstrated that activated alleles of Cdc42 and Rac1 (Cdc42V12 and Rac1V12), but not RhoA, stimulate S6Kα activity under the basal conditions, whereas dominant negative mutants of these GTPases (Cdc42N17 and Rac1N17) abrogate growth factor-induced activation of S6Kα in various cell types (Chou and Blenis, 1996). In addition, cotransfection of S6Kα with Dbl, an exchange factor for Cdc42, activates S6 kinase to levels comparable to those induced by EGF. Another important observation of these studies is that Rac1 and Cdc42 form a GTP-dependent complex with the hypophosphorylated S6Kα both in vitro and in vivo. This interaction alone is not sufficient to activate S6Kα and was proposed to facilitate S6Kα recognition by upstream activating kinase/s, which are also regulated by PI-3'-K (Chou and Blenis, 1996). Interestingly,
Cdc42 and PKCζ or PKCλ have recently been shown to associate (Coghlan et al., 2000), suggesting a possible recruitment of this GTPase to PI-3-K-regulated complexes described for PDK1, PKCζ and S6Kα (Romanelli et al., 1999). Furthermore, the effect of Rac1/Cdc42 overexpression on S6Kα activity strongly requires isoprenylation of these G proteins, indicating that membrane targeting is important in this system.

1.3.2. TOR/FRAP kinase and nutrient input

As mentioned above the immunosuppressant rapamycin potently blocks S6Kα activation by all known stimuli without obvious effect on PI-3-K or PDK1 activities (Pullen and Thomas, 1997). This is achieved indirectly by the ability of rapamycin to form a gain-of-function complex with the peptidyl-prolyl-cis-trans isomerase FKBP12, which selectively binds to the mammalian target of rapamycin mTOR/FRAP (homologous to yeast proteins Tor1p and Tor2p) and blocks its specific functions (Brown et al., 1994; Sabatini et al., 1994). mTOR/FRAP is a high molecular weight protein, which contains a C-terminal region with strong homology to the catalytic domain of phosphatidylinositol-3'-kinase and phosphatidylinositol 4'-kinase (Keith and Schreiber, 1995; Kunz et al., 1993). Because of this homology, mTOR/FRAP has been classified as a member of the family of PIK-related kinases. Among other members of this family several crucial cell cycle regulators were characterized in yeast and mammals, including MEC1, TEL1, ATM, and DNA-PK. Despite the presence of C-terminal lipid kinase domains, none of the PIK-related kinases has been demonstrated to have lipid kinase activity (Schmelzle and Hall, 2000). Moreover, both yeast Tor1,2p and mammalian mTOR/FRAP are functional serine/threonine kinases.

In mammalian cells mTOR/FRAP controls the rapamycin-sensitive phosphorylation of at least two translational regulators: S6Kα and 4E-BP1, an inhibitor of the cap binding protein eIF-4E (Chung et al., 1992; Brown et al., 1995; Hara et al., 1998). mTOR/FRAP mutants unable to bind the rapamycin-FKBP12 complex can protect S6Kα and 4E-BP1 from rapamycin-induced dephosphorylation but only if the mTOR/FRAP kinase domain is intact (Hara et al., 1998). There is evidence suggesting that mTOR/FRAP-dependent control of S6Kα phosphorylation and activity can be mediated by two independent, but nonexclusive mechanisms. First, mTOR/FRAP has been reported to directly phosphorylate bacterially expressed recombinant fragments of S6Kα in vitro at sites important to
activation, including Thr412 (Burnett et al., 1998b). Later studies have demonstrated the ability of mTOR/FRAP kinase to phosphorylate mammalian expressed full-length S6Kα in vitro predominantly at Thr412 and activate its kinase activity towards S6 protein (Isotani et al., 1999). However, a number of observations are not consistent with the idea that mTOR/FRAP phosphorylates this site in vivo. For instance, a rapamycin-resistant S6KαΔN2,46ΔC104 mutant exhibits unimpaired insulin-stimulated phosphorylation at the Thr412 site in the presence of rapamycin at concentrations sufficient to fully inhibit the activity of the endogenous mTOR/FRAP kinase (Hara et al., 1998). Furthermore, the kinase activity of mTOR/FRAP is not appreciably stimulated under growth-promoting conditions, e.g. after insulin treatment, and physiological mitogen-responsive regulators of the mTOR/FRAP kinase are not known (Brunn et al., 1997; Hara et al., 1997; Peterson et al., 2000). A second possibility is that mTOR/FRAP may regulate S6Kα activation indirectly by restraining a specific phosphatase (Figure 1.4.). Several studies have demonstrated that protein phosphatase 2A (PP2A) directly associates with S6Kα and selectively dephosphorylates this kinase (Ballou et al., 1988; Peterson et al., 1999; Westphal et al., 1999). It has also been reported that lysate from rapamycin-treated cells contains an elevated activity of PP2A and that rapamycin-induced inactivation of S6Kα is partially relieved by calyculin A, a phosphatase inhibitor. Furthermore, PP2A fails to associate with S6KαΔN2,46ΔC104, suggesting that the rapamycin insensitivity of this mutant may reflect its inability to interact with PP2A (Peterson et al., 1999). The same authors have also reported that mTOR/FRAP kinase has a potential to directly phosphorylate PP2A phosphatase in vitro. However, the actual mechanism of PP2A inactivation by mTOR/FRAP in mammalian cells is not entirely clear. In Saccharomyces cerevisiae, Tor1p and Tor2p control protein synthesis through inhibition of Pph21/22 and Sit4, the yeast equivalents of the catalytic subunit of PP2A and PP6, respectively. The ability of Tor1,2ps to regulate the activities of Pph21/22 and Sit4 depends on the rapamycin-sensitive phosphorylation of an intermediary protein, Tap42 (Jiang and Broach, 1999). Phosphorylated Tap42 competitively excludes Pph21/22 from its regulatory subunits Tpd3 and Cdc55 (homologs of mammalian A and B subunits, respectively), thereby reducing protein phosphatase activity of Pph21/22 (di Como and Arndt, 1996; Jiang and Broach, 1999). A similar mechanism of phosphatase 2A regulation in mammalian cells may be operational. In fact, α4, the mammalian homolog of Tap42 (Onda et al., 1997), associates with the catalytic subunits of PP2A, PP4, and PP6 in a
rapamycin-sensitive manner (Chen et al., 1998; Murata et al., 1997; Nanahoshi et al., 1998). Furthermore, the formation of PP2A-α4 complex leads to the inhibition of PP2A phosphatase activity towards 4E-BP1 and myelin basic protein (Murata et al., 1997; Nanahoshi et al., 1998). To date, however, a demonstration of α4 phosphorylation by mTOR/FRAP is lacking. Therefore, the mechanism of mTOR/FRAP-mediated regulation of PP2A phosphatase remains to be elucidated.

Several studies indicate that the metabolic state of the cell and the availability of nutrients, independent of insulin or growth factors, regulate the activity of S6Kα through the mTOR/FRAP signaling pathway (Blommaart et al., 1995; Denis et al., 2001; Hara et al., 1998; Wang et al., 1998). It has been shown that amino acid deprivation blocked S6Kα activity in various cell lines, whereas reintroduction of amino acids restored this activity to levels induced by mitogens. Moreover, withdrawal of amino acids from cells renders S6Kα refractory to stimulation by insulin (Campbell et al., 1999), epidermal growth factor and nerve growth factor (Kleijn and Proud, 2000). The involvement of mTOR/FRAP in the regulation of S6 kinase by amino acid availability has been demonstrated by studies using rapamycin, which blocks the activation of S6Kα by amino acid re-addition (Hara et al., 1998; Wang et al., 1998). Expression of a rapamycin-resistant mutant of mTOR/FRAP was sufficient to prevent rapamycin-induced inhibition of S6Kα activity in this system (liboshi et al., 1999). Furthermore, the rapamycin-insensitive S6KαΔN246ΔC104 mutant exhibits resistance to amino acid deprivation, indicating that both amino acids and mTOR/FRAP transmit signals to S6Kα through common signal effectors (Hara et al., 1998). Similar to amino acids, decreasing the concentration of intracellular ATP negatively influences mTOR/FRAP signaling and phosphorylation of both S6Kα and 4E-BP1 (Denis et al., 2001). Moreover, considerable evidence supports the role of mTOR/FRAP as a cellular sensor of ATP, establishing a signaling link between energy-producing processes and protein synthesis regulation.

Taken together, these data support a critical role for mTOR/FRAP kinase in the regulation of S6Kα, but the mechanisms by which mTOR/FRAP contributes to S6K activation remain elusive.

1.3.3. Classical and novel PKCs
The protein kinase C (PKC) family of serine/threonine kinases has been implicated in cellular responses to various agonists including hormones, neurotransmitters, and some growth factors (Nishizuka, 1992). There are multiple PKC isoforms (Mellor and Parker, 1998; Newton, 1995) which can be classified into several distinct subgroups on the basis of structural and regulatory differences: the conventional PKCs (α, βI, βII, and γ), novel PKCs (δ, ε, η, and θ), atypical PKCs (ζ and ι/λ), and the more distantly related PKCμ/PKD and PKCν. The conventional PKC enzymes have four conserved (C1 to C4) and five variable (V1 to V5) regions and are regulated by calcium, diacylglycerol (DAG) and phosphatidylserine. The activation of cPKC by these allosteric regulators can be further enhanced by cis unsaturated fatty acids and lysophosphatidylcholine. The novel PKC isoforms, which lack the C2 region, do not require calcium and are regulated by DAG and phosphatidylserine. On the other hand, the atypical PKCs, which have only one cysteine-reach motif in the C1 region, are dependent on phosphatidylserine but not affected by DAG or calcium. The DAG-regulated PKC isoforms all bind phorbol esters and are the major cellular targets for this allosteric regulator, which mimics DAG-mediated effects. In addition to allosteric regulators, PKCs are regulated by phosphorylation at their activation loop and hydrophobic motif, which are highly conserved between PKC isoforms and other AGC kinases (Parekh et al., 2000). All PKCs share a highly conserved catalytic domain, although each isoform has a different optimal substrate specificity (Nishikawa et al., 1997), supporting the idea that each isoform has specific functions in vivo. The recently described PKC-related kinase, PKCμ/PKD, also contains a cysteine-rich C1 domain that binds DAG and phorbol esters but lack the C2 calcium binding domain seen in the cPKCs (Johannes et al., 1994; Valverde et al., 1994). In contrast to other PKCs, the N-terminal regulatory region of PKCμ/PKD contains a PH domain that regulates its enzyme activity (Iglesias and Rozengurt, 1998). Furthermore, the catalytic domain of PKCμ/PKD shows optimal specificity for an unique peptide substrate unrelated to those identified for other PKC isoforms (Nishikawa et al., 1997).

Several studies have demonstrated the role of PLCγ/DAG/1,4,5,-inositol triphosphate/PKC pathway in the mitogen-induced activation of S6Kα (Chung et al., 1994; Kahan et al., 1992; Susa et al., 1989). PKC-dependent and wortmannin-insensitive activation of S6Kα was also shown in calcium pyrophosphate dihydrate crystal-stimulated neutrophils (Tudan et al., 1998). Consistent with these data, S6Kα activity is stimulated
when cells are treated with PMA, an activator of classical and novel PKCs (Susa et al., 1989). Furthermore, after long-term treatment with PMA to down-regulate c/n PKCs, the S6 kinase is no longer activated by the drug and its response to mitogens is partially or completely abrogated (Blenis and Erikson, 1986; Pelech and Krebs, 1987; Susa et al., 1989). These studies indicate the involvement of DAG-dependent PKCs in the control of S6Kα activity. However, the links between c/n PKCs and S6 kinase regulation remain to be established.

Recent evidence has demonstrated a functional interaction between mTOR/FRAP and novel PKCδ in the regulation of 4E-BP1 phosphorylation (Kumar et al., 2000). Although regulatory signal from mTOR/FRAP to S6Kα and 4E-BP1 bifurcates upstream of S6 kinase (Sonenberg and Gingras, 1998), there is a possibility that PKCδ may similarly contribute to mTOR/FRAP-dependent activation of S6K. This possibility was raised by the fact that PMA-induced activation of S6Kα is potently inhibited by rapamycin (Chung et al., 1994; Law et al., 2000; Monfar, et al., 1995).

Finally, the role of PKC in S6Kα regulation may be indirect, since none of the PKC isoforms have been reported to directly phosphorylate S6Kα.

1.3.4. Negative control of S6K pathway

The evolution of mitogenic pathways has led to the parallel requirement for negative control mechanisms, which prevent aberrant growth and development of cancer. One such regulatory mechanism is represented by dephosphorylation/inactivation of signaling kinases and is mediated by various protein phosphatases (de Paoli-Roach et al., 1994).

In regard to S6Kα regulation, the protein phosphatase PP2A has been identified as a major phosphatase involved in the inactivation of this kinase (Ballou et al., 1988; Janssens and Goris, 2001; Millward et al., 1999). PP2A is a multimeric serine/threonine phosphatase, comprising an of evolutionarily conserved catalytic subunit (PP2Ac) and a regulatory subunit, termed PR65 or the A subunit. These two associated proteins form the core dimer which is able to further interact with variable B subunits in order to form trimeric holoenzymes attributed with distinct substrate specificity and targeted to different subcellular compartments. Moreover, the catalytic subunit is subjected to two types of post-translational modifications, phosphorylation and methylation, which are also thought to be important regulatory devices. Studies on PP2A implicate this phosphatase in the
regulation of metabolism, transcription, RNA splicing, translation, cell cycle progression, oncogenic transformation and signal transduction (Janssens and Goris, 2001; Wera and Hemmings, 1995). The ability of PP2A to participate in different signaling complexes may explain in part such pleiotropic effects of this phosphatase. However, the mechanisms by which PP2A affects diverse cellular functions are not well understood.

The involvement of PP2A in the negative control of S6Kα has been demonstrated in various cells treated with growth inhibitory agents, such as glucocorticoids and TGFβ (Monfar and Blenis, 1996; Petritsch et al., 2000; Shah et al., 2000b). It has been shown that TGFβ-induced repression of S6Kα coincides with its dephosphorylation and association with three subunits of PP2A: PP2AαC and the two regulatory subunits PP2A-αβ and PP2A-βα. PP2A-βα interacts with TβRI and thereby physically links TGFβ to the control of PP2A and S6Kα. On receptor activation, PP2A-βα specifically binds the activated TβRI and then recruits the PP2AαC/PP2A-αβ core dimer to form a trimeric holoenzyme. The activated holoenzyme is then released from the receptor to specifically bind and dephosphorylate S6Kα. Consistent with these findings, PP2A was found in complexes with S6Kα and p21-activated kinases PAK1 and PAK3 (Westphal et al., 1999). The mechanism of glucocorticoid-induced suppression of S6Kα activity is less clear than that described for TGFβ. Quiescent L6 skeletal myoblasts pre-treated with the synthetic glucocorticoid dexamethasone exhibit a marked attenuation of basal and IGF-1-induced S6Kα activity, although PI-3'kinase signaling remains unchanged (Shah et al., 2000b). This deactivation of S6Kα correlates with dephosphorylation of the kinase at Thr412 and Thr444/Ser447, whereas phosphorylation of Ser434 was unaffected. These data indicate that glucocorticoids deactivate S6Kα through site-selective dephosphorylation. Okadaic acid and calyculin A correct the dexamethasone-induced dephosphorylation of the kinase, implicating PP2A- and/or PP1-like protein phosphatase/s in this process (Ballou et al., 1988; Shah et al., 2000c). Moreover, the mechanism of dexamethasone action is reminiscent of classical transcriptional regulation by steroid hormone receptors in that these effects were preceded by a temporal lag and were sensitive to inhibitors of glucocorticoid receptor function as well as transcriptional and translational inhibition (Shah et al., 2000a; Shah et al., 2000c).

In contrast to steroids and TGFβ, growth-promoting stimuli, such as growth factors and nutrients, stimulate S6K phosphorylation by activation of specific upstream kinases and simultaneous inhibition of PP2A activity (Chen et al., 1994; Dufner and Thomas, 1999;
Guy et al., 1995; Srinivasan and Begum, 1994). Although the mechanisms by which growth factors and nutrients control PP2A specific activity toward S6K are currently unknown, considerable evidence supports a role for mTOR/FRAP in this regulation (see section 1.3.2. and Figure 1.4.). A calyculin A-sensitive phosphatase is required for the rapamycin- or amino acid dephosphorylation-mediated dephosphorylation of S6Kα, and treatment of Jurkat I cells with rapamycin increases the activity of PP2A toward 4E-BP1, a downstream target of mTOR/FRAP kinase (Peterson et al., 1999). Furthermore, PP2A was shown to associate with wild-type S6Kα but not with a mutated kinase that is resistant to rapamycin- and amino acid dephosphorylation-mediated dephosphorylation. mTOR/FRAP directly phosphorylates PP2A in vitro, consistent with a model in which phosphorylation of PP2A by mTOR/FRAP prevents the dephosphorylation of S6Kα and 4E-BP1, whereas amino acid deprivation or rapamycin treatment inhibits the ability of mTOR/FRAP to restrain the phosphatase. Further studies are needed to clarify molecular mechanisms of mTOR/FRAP-dependent PP2A regulation.

Recent research in both mammalian cells and Drosophila melanogaster have identified tuberous sclerosis complex tumour suppressor, TSC1-TSC2, as a negative effector of S6Kα (Marygold and Leevers, 2002). This complex comprises of TSC1 and TSC2 gene products, namely hamartin and tuberin, respectively (van Slegtenhorst, et al., 1998). Individually, hamartin (TSC1) contains a carboxyl-terminal ezrin-radixin-moesin-binding domain and has been implicated in signaling to the actin cytoskeleton, whereas tuberin (TSC2) has an amino-terminal leucine zipper necessary for hamartin binding and a carboxyl-terminal domain similar to that found in Rap GTPase-activating protein (GAP) (Jaeschke et al., 2002). Although TSC2 exhibits weak GAP activity towards Rap and Rab5, its significance in TSC1-TSC2 complex function remains unclear (Wienecke et al., 1995; Xiao et al., 1997). Since TSC1 and TSC2 exist as a TSC1-TSC2 protein complex in vivo, mutations in one gene result in phenotypes that are identical to those caused by mutations in the other (Gao et al., 2002).

Studies of Drosophila dTsc1 and dTsc2 identified a specific function for TSC1-TSC2 in the control of cell growth, with loss of TSC1-TSC2 resulting in an increase in cell size. Epistasis analysis in Drosophila melanogaster also demonstrated that TSC1-TSC2 functions in the insulin/PI-3'K/PKB-regulated pathway, which controls cell size upstream of Drosophila S6K (Potter et al., 2001). These data were confirmed by using an array of biochemical and molecular assays. For example, decreasing TSC2 expression by RNA
interference increases S6K phosphorylation on the critical Thr412 residue, whereas overproduction of TSC1-TSC2 is sufficient to inhibit S6K phosphorylation and activation (Gao et al., 2002; Inoki et al., 2002). Moreover, mammalian cells harbouring tuberous sclerosis complex disease-associated mutations in either TSC1 or TSC2 have constitutively high phosphorylation of both S6Kα and its substrate S6 (Goncharova et al., 2002; Inoki et al., 2002).

As mentioned above the TSC1-TSC2 complex resides downstream of PI-3'-K/PKB signaling. In addition to genetic analyses in Drosophola melanogaster (Gao and Pan, 2001; Potter et al., 2001), this was demonstrated by the findings that PKB phosphorylates TSC2 in a PI-3'-K-dependent manner and that this phosphorylation inhibits TSC1-TSC2 function (Inoki et al., 2002; Manning et al., 2002). Phosphorylation of TSC2 by PKB affects its function through several mechanisms, including PKB-dependent interaction of TSC2 with members of the 14-3-3 protein family, inhibition of TSC2 activity and dissociation of TSC1-TSC2 complex followed by ubiquitination of the free TSC2 (Marygold and Leevers, 2002; Inoki et al., 2002). At the same time, it is not clear how TSC1-TSC2 functions to inhibit S6Kα activity. Several observations suggest that TSC1-TSC2 may act upstream of TOR to block both its kinase activity and its ability to promote growth in vivo (Marygold and Leevers, 2002). Thus, TSC1-TSC2 was shown to physically associate with dTOR, but not S6K, and overexpression of recombinant TSC1 and TSC2 attenuates phosphorylation of mTOR/FRAP in a dose-dependent manner (Gao et al., 2002; Inoki et al., 2002). These effects strongly correlated with decrease in S6K phosphorylation at Thr412 and its specific activity, suggesting an intermediary role for TOR kinase in the TSC1-TSC2-mediated regulation of S6K. Consistent with this idea, rapamycin-resistant S6KαΔN2,46ΔC104 mutant also exhibits resistance to inhibition by TSC1-TSC2 (Inoki et al., 2002). Furthermore, the rapid and sustained decrease in S6Kα phosphorylation caused by amino acid deprivation is overcome by reducing TSC1-TSC2 levels (Gao et al., 2002). However, Jaeschke et al. (Jaeshke et al., 2002) provide evidence that TSC1-TSC2 does not inhibit S6 phosphorylation in vivo via mTOR/FRAP pathway, suggesting existence of alternative mechanisms of TSC1-TSC2-mediated S6K regulation.

Since the inactivation of S6Kα is thought to be regulated by complex formation with PP2A phosphatase and subsequent dephosphorylation of active kinase at regulatory sites, it is important to investigate the involvement of PP2A or other serine/threonine phosphatases in TSC1-TSC2-mediated S6Kα inhibition. Conceivably, S6Kα phosphorylation/activation...
could be inhibited through other mechanisms, including changes in subcellular localisation of S6Kα and protein-protein interactions that physically or otherwise impede its activation.

1.4. **Cellular function of ribosomal S6 kinases**

1.4.1. **Protein synthesis**

The most studied function of S6K is the multiple phosphorylation of 40S ribosomal protein S6 in response to a variety of growth-promoting stimuli. The ability of S6K to phosphorylate both cytoplasmic and nuclear pools of a S6 protein implicates this kinase in the regulation of protein synthesis at the level of translation initiation and suggests its involvement in the control of ribosome biogenesis (Jefferies and Thomas, 1996) (Figure 1.5.).

1.4.1.1. **S6 phosphorylation and initiation of translation**

S6 represents one of the major ribosomal phosphoproteins which along with 18S rRNA and other structural proteins comprises the 40S ribosomal subunit. Cross-linking and chemical protection studies have localized S6 protein to the small head region of the mature 40S subunit. Moreover, S6 resides at the interface of the 40S subunit juxtaposed to the large 60S subunit, where it apparently makes direct contact with 28S rRNA of 60S ribosomal particle. (Nygard and Nilsson, 1990). In this region many processes central to translation occur, such as mRNA and tRNA binding and the transient association of many initiation and elongation factors. Importantly, S6 protein has been directly cross-linked to mRNA in functional 80S ribosomes, suggesting direct interaction between these translational components. These findings raise the possibility that S6 protein may influence the binding of mRNA to the 40S subunit. Indeed, Fab fragments derived from antibodies generated against a carboxyl-terminal peptide of S6 potently block mRNA binding to 40S subunits (Jefferies and Thomas, 1996).

In intact cells, phosphorylation of S6 is one of the earliest events detected after mitogenic stimulation of quiescent cells to reenter the cell cycle and is closely associated
Fig. 1.5. The role of S6K in the regulation of protein synthesis. A solid arrow indicates established positive effect, whereas a dashed arrow represents putative/uncertain regulatory connection. A bar shows negative effect. See text for details.
with increased rates of protein synthesis (Stewart and Thomas, 1994). Phosphorylation sites of S6 are represented by serine residues (Ser235, Ser236, Ser240, Ser244 and Ser247) which are located at the carboxyl-terminus of the protein (Bandi et al., 1993; Krieg et al., 1988). Phosphate release studies have shown that phosphorylation of S6 proceeds in the order, Ser236>Ser235>Ser240>Ser244>Ser247, which might be determined by the specific conformation of this region (Jefferies and Thomas, 1996). Several lines of evidence allow speculation that the latter sites of phosphorylation contribute most to the regulation of protein synthesis. First, phosphorylation of S6 protein by two different protein kinases, the cAMP-dependent protein kinase and mitogen-stimulated S6 kinase, has different effects on translation of globin mRNA in a reconstituted reticulocyte system (Palen and Traugh, 1987). The cAMP-dependent protein kinase efficiently phosphorylates Ser235 and Ser236 in S6, but not other sites (Kozma, et al., 1989), and fails to stimulate protein synthesis in the reconstituted system. In contrast, phosphorylation of S6 by a mitogen-stimulated S6 kinase purified from rabbit liver produces four-fold stimulation of translation in this system. Second, the Saccharomyces cerevisiae homolog of S6 protein, termed S10, lacks the last three sites of phosphorylation corresponding to Ser240, Ser244 and Ser247 in the mammalian S6 proteins. This parallels the fact that unlike higher eukaryotes (see below), yeast does not regulate synthesis of ribosomal proteins at the translational level. Furthermore, protein synthesis and growth rate appear to be normal in a yeast strain expressing an S10 mutant in which two carboxyl-terminal serines equivalent to Ser235 and Ser236 in mammalian S6 are replaced with alanines (Johnson and Warner 1987).

Upregulation of protein synthesis by mitogens is primarily controlled at the level of initiation and can be observed as the recruitment of inactive 80S ribosomes into actively translating polysomes. It has been shown that polysomes have higher percentage of phosphorylated S6 than 80S ribosomes or free 40S subunits following mitogenic stimulation in HeLa cells. However, the level of S6 phosphorylation declined to basal levels 6 hours after addition of serum, when protein synthesis reached maximum rates (Stewart and Thomas, 1994). Based on these data and earlier data indicating that S6 directly interacts with mRNA on 40S subunit, it may be postulated that S6 phosphorylation increases the affinity of 40S subunits for stored messenger ribonucleic acid protein (mRNP) particles in a primary initiation event.

Several conjectural mechanisms were proposed to explain how S6 phosphorylation regulates translational activity of ribosomes. Ribosomes are highly flexible structures that
can fluctuate between pre- and post-translational conformations. This flexibility requires the presence of both 60S and 40S subunits, suggesting that altered contact between the two subunits may be involved (Nygard and Nilsson, 1990). Phosphorylation of S6 located at the interface of 40S subunits may affect conformation of ribosomes in the region of contact between 60S and 40S subunits. Additionally, translation is a dynamic process in which mRNAs, tRNAs and non-ribosomal proteins (e.g. initiation factors) are transiently associating with the ribosome (Nygard and Nilsson, 1990). Thus, phosphorylation of S6 may serve to induce or stabilize functional conformational changes in the ribosome which could allow differential association of the ribosome with proteins or mRNAs. Alternatively, due to its location, differentially phosphorylated forms of S6 could interact directly with mRNAs or with their associated proteins and lead to altered affinity of the translational machinery for specific messages. Consistent with the latter hypothesis selective translational upregulation of mRNA encoded for elongation factor 1α (eEF1α) has been shown to correlate with S6 phosphorylation in mitogen-stimulated Swiss 3T3 cells (Jefferies and Thomas, 1994a). Notably, the transcripts for eEF1A contains a 5'-terminal oligopyrimidine tract (5'-TOP) at its transcriptional start site, an element known to act as a translational regulator (Hammond et al., 1991; Levy et al., 1991). All mammalian ribosomal protein mRNAs in which the 5' transcriptional start site has been mapped, as well as elongation factor 1B and 2 (eEF1B and eEF2) mRNAs, contain this motif and are apparently upregulated in a manner similar to that of eEF1α (Meyuhas, 2000). These studies indicate that production of components of the translational apparatus is regulated at the level of translation. However, the involvement of S6 phosphorylation in the regulation of 5'-TOP mRNA has to be carefully interpreted due to the lack of direct evidence (see section 1.4.1.2.). Elucidation of the mechanisms underlying the translational control of 5'-TOP mRNAs will provide better understanding of the regulation of ribosome biogenesis and its critical role in mitogenic responses.

1.4.1.2. Ribosomal biogenesis

The biogenesis of ribosomes plays an essential role in the growth and proliferation of cells in response to a mitogenic signal (Nasmyth, 1996). This process is regulated at many levels including transcription, translation and formation of pre-ribosomal particles both in the nucleus and cytoplasm. The involvement of S6Ks and rpS6 phosphorylation in the regulation of ribosome biogenesis has recently been suggested based on several
observations. First, inhibition of S6Ks, and consequently of S6 phosphorylation by rapamycin led to a selective suppression of the mitogenic activation of 5'-'TOP mRNA translation in a selected set of cell lines (Hornstein et al., 2001). Indeed, rapamycin treatment of serum-stimulated fibroblasts caused an approximately 10-15% inhibition of global protein synthesis, whereas recruitment of 5'-TOP mRNAs into polysomes was severely repressed (~50% inhibition) in the presence of the macrolide (Jefferies et al., 1994b; Jefferies et al., 1997). Observed dissociation of 5'-TOP mRNAs from polysomes and their redistribution to monosomes/disomes and mRNP coincided with dephosphorylation of rpS6. However, no obvious effect was observed on the distribution of transcripts that lacked the 5'-TOP such as β-actin, eIF4A, and β-tubulin (Jefferies et al., 1994b). Second, overexpression of a dominant-interfering mutant of S6Kα (S6Kα T252A) has been shown to exert an inhibitory effect (~35% of inhibition) on the translational activation of 5'-TOP mRNAs following mitogenic stimulation. Moreover, 5'-TOP mRNA translation is not suppressed by rapamycin in cells that express a rapamycin-resistant form of S6Kα (S6Kα T412E) (Jefferies et al., 1997). Taken together, these studies suggest the importance of S6Kα activity and rpS6 phosphorylation in the translational efficiency of 5'-TOP mRNAs. However, it is necessary to note that the selective translational upregulation of these mRNAs is not completely abolished by rapamycin or S6Kα T252A overexpression, arguing for an additional pathway which is rapamycin and S6Kα independent. Consistent with this notion, mitogen-induced translational activation of 5'-TOP mRNAs was shown to be fully reliant on the PI-3'-K-mediated pathway but independent on S6Kα activity and rpS6 phosphorylation in PC12 and lymphoblastoid cell lines (Stolovich et al., 2002).

Franco and Rosenfeld (Franco and Rosenfeld, 1990) have demonstrated the existence of a nuclear pool of rpS6 that is phosphorylated in response to hormone stimulation. The nuclear function of S6 protein is unknown, although it was speculated that it may play a role in the regulation of ribosome biogenesis. Initial steps of ribosome biogenesis take place in the nucleolus (Jefferies and Thomas, 1996). A nascent 80S pre-ribosomal particle, containing a single strand of 45S rRNA, is processed to 40S and 60S pre-ribosomal particles that consist of either a single strand of 18S rRNA or a strand of both 28S rRNA and 5.8S rRNA, respectively (Larson et al., 1991). The pre-ribosomal particles are then exported to the cytoplasm, where the final stages of the assembly process are completed and mature ribosomal subunits are formed. Importantly, S6 was shown to be one of the
first proteins to be assembled into the 45S rRNA precursor (Jefferies and Thomas, 1996). The nuclear pool of phosphorylated S6 can be separated into nucleoplasmic and nucleolar fractions. Immunofluorescence studies show that both S6KαI and S6KαII are apparently excluded from the nucleolus (Coffer and Woodgett, 1994; Kim and Chen, 2000; Reinhard et al., 1994), suggesting that S6 is phosphorylated as a free nucleoplasmic protein before it is assembled into 80S pre-ribosomal particles or that S6 may have a separate function in the nucleus. Both possibilities could be envisaged in a model in which nuclear S6 phosphorylation is involved in regulating one or more specific steps of ribosome biogenesis. Based on this model, it has been proposed that in the nucleus, S6Ks may be responsible for the phosphorylation of the nucleoplasmic form of S6 protein and the initiation of ribosome biogenesis.

Upregulation of ribosomal biogenesis is an obligatory mitogenic-induced event which provides an increase in general protein synthesis needed for normal cell growth and proliferation.

1.4.2. Control of cell cycle progression, cell growth and proliferation

The importance of S6Kα in cell cycle control has been inferred from either the use of rapamycin (Chou and Blenis, 1995) or through microinjection of neutralizing antibodies into cells (Lane et al., 1993; Reinhard et al., 1994), both of which selectively suppress mitogen-induced S6K activation and lead to cell cycle arrest in G1 or a delay of entry into S phase depending on the cell type (Chung et al., 1992, Price et al., 1992). Furthermore, it has been demonstrated that TGF-β, a physiological suppressor of cell cycle progression, induces G1 arrest through PP2A-mediated inhibition of S6Kα in EpH4 cells (Petritsch, et al., 2000) (see section 1.3.4.). However, the mechanism through which S6Kα regulates cell cycle progression is not entirely clear. Given the obligatory role of translation upregulation for cell growth and proliferation (Meyuhas, 2000), it has been proposed that S6Kα may regulate cell cycle progression via phosphorylation of rpS6 and initiation of ribosome biogenesis. This hypothesis is consistent with studies demonstrating that conditional deletion of rpS6 in the liver of adult mice results in abrogated 40S ribosome biogenesis and complete inhibition of G1/S progression (Volarevic et al., 2000). Although rpS6-deficient hepatocytes were stimulated to proliferate and progress to late G1 phase after partial hepatectomy, progression beyond this point and entry into S phase were
blocked due to the abolished expression of cyclin E. In addition to its role in the initiation of translation and ribosome biogenesis, S6Kα has been implicated in the regulation of E2F transcriptional activity in interleukin-2-stimulated T lymphocyte proliferation (Brennan et al. 1999). Thus, ectopic expression of wild-type S6Kα, but not a kinase dead mutant, induced substantial increase of both basal and IL-2-stimulated E2F activity in Kit225 cells. Moreover, expression of a rapamycin-resistant mutant of S6Kα rescued the rapamycin-mediated inhibition of E2F transcriptional activity in these cells. The activation of transcription factor E2F is a critical cell cycle checkpoint (Helin, 1998) and, therefore, may link S6Kα to the regulation of G1/S progression.

As was shown in yeast and Drosophila models, a minimum cell size must be achieved before cells initiate S phase and divide, whereas mutations that block cell cycle progression do not inhibit cell growth (Conlon and Raff, 1999; Su and O’Farrell, 1998). These findings clearly demonstrate that cell growth is an important determinant of the cell cycle progression. Knock-out of the S6Kα gene in mice and dS6K gene in Drosophila indicates that the kinase is a key player in the regulation of cell growth. Mice deficient for the S6Kα gene exhibit a significant reduction in body size during embryonic development (Shima, et al., 1998). This effect is largely corrected by adulthood possibly via compensatory function of S6Kβ, whose expression is elevated in mice deficient for S6Kα. Furthermore, S6Kα−/− murine embryonic stem cells were shown to proliferate at a slower rate than parental cells due to the inhibition of G1/S progression of the cell cycle (Kawasome et al., 1998). Deletion of dS6K in Drosophila, which possess only one gene for the kinase, leads to a high incidence of embryonic lethality, an extreme delay in development and a severe reduction in body and cell size (Montagne, 1999). Interestingly, the loss of dS6K function leads to cell proliferation at a smaller size and at a reduced rate, without affecting any specific stage of the cell cycle.

Therefore, S6Ks appear to be an important signaling effectors involved in the control of cell size, growth, and proliferation, all of which must be regulated in coordinated fashion to ensure the normal development of the organism.

1.4.3. Novel substrates and multifunctional potential of S6K

In addition to the role of S6Kα in translation initiation, ribosome biogenesis and cell cycle progression, this kinase is potentially implicated in the regulation of transcription,
RNA processing and apoptosis. This became evident from the recent identification of several novel substrates for S6Kα (Figure 1.4.). It has been demonstrated that S6Kα phosphorylates the cAMP-responsive activator CREMα at Ser17 in vitro (de Groot et al., 1994). Phosphorylation of this residue is also mediated by PKA and correlates with increase in CREMα transactivation. Coexpression of recombinant S6Kα significantly stimulates transcriptional activity of GAL4-CREM fusion and this effect is sensitive to rapamycin. The role of S6Kα in transcriptional control is also supported by studies demonstrating the involvement of S6Kα in the regulation of E2F in T lymphocytes (see section 1.4.2.) and in the autocrine signaling of insulin gene transcription in pancreatic β cells (Leibiger et al., 1998).

Another potential substrate of S6Kα is the 80 kDa subunit of RNA cap-binding complex (CBP80) (Wilson et al., 2000). Nuclear cap-binding complex (CBC) binds to RNAs containing a m^7G cap structure and is involved in a number of fundamental aspects of RNA processing including pre-mRNA splicing, U snRNA export, and polyadenylation (Wilson et al., 2000). S6Kα was shown to phosphorylate CBP80 in vitro at a site that undergoes a rapamycin-sensitive, growth factor-dependent phosphorylation in vivo. Furthermore, S6Kα was demonstrated to cooperate with Cdc42 and PI-3'-K to activate RNA binding activity of CBC in coexpression experiments.

The critical role of S6K signaling in translational control is further supported by the findings that S6Ks phosphorylate elongation factor 2 kinase (eEF2k) and modulate its activity toward eEF2 (Wang et al., 2001). These data implicate S6Ks in the regulation of elongation phase of translation.

Finally, an anti-apoptotic function of S6Kα has been suggested based on its ability to phosphorylate and inactivate pro-apoptotic protein BAD (Harada et al., 2001). This protein forms a heterodimer with Bcl-2 and Bcl-XL at the mitochondrial membrane to promote intrinsic apoptosis which is mediated by mitochondria through release of apoptosis-potentiating factors. (Green et al., 1998; Zha et al., 1996). When phosphorylated on Ser112 or Ser136, BAD no longer interacts with Bcl-2 and Bcl-XL, allowing them to inhibit cell death. Mitochondria-associated fraction of S6Kα was purified and shown to specifically phosphorylate BAD at Ser136. Rapamycin, which blocked membrane-associated S6Kα activity, potently inhibited IGF-1-induced BAD Ser136 phosphorylation and cell survival. Moreover, IGF-1-induced phosphorylation of BAD was abolished in S6Kα-deficient ES cells.
Taken together, these observations suggest a role for S6Ks in the regulation of gene expression at the level of transcription, messenger RNA processing and stability, translation and post-translational modifications. Coordination of these processes is required for the proper control of cell survival, growth and proliferation.

1.5. Deregulation of S6K pathway: cancer and diabetes

Among other criteria, autonomously deregulated cell growth and a capacity of cells to metastasize are the main defining features of malignant transformation (Blume-Jansen and Hunter, 2001). The transformation of cells arises as a result of perturbed signal transduction, which leads to an unbalance between the rate of cell cycle progression (cell division) and cell growth (cell mass) on one hand, and differentiation (cell specialisation) or programmed cell death (apoptosis) on the other. Indeed, many of the known proto-oncogenes encode proteins involved in transduction of growth, differentiation and developmental signals and are often mutated in cancers (Hanahan and Weinberg, 2000). They belong to different classes of signaling proteins and include receptor tyrosine kinases (e.g. EGFR/ErbB2, Kit/SCFR), non-receptor tyrosine kinases (Src, Fgr, Abl), small GTPases (Ras), serine/threonine kinases (PKB), phosphatases (PTEN) and transcription factors (Myc, Jun).

The increasing evidence implicating S6K in the control of cell survival, growth and proliferation (see section 1.4.), along with its participation in signaling pathways frequently deregulated in transformed cells, suggests a possible role for S6K in cancer development. Although an oncogenic version of S6K has yet to be found, many of the proteins directly linked to S6K regulation, such as Ras, PI-3'-K and PKB have been identified in oncogenic versions or demonstrated to have transforming potential (Blume-Jansen and Hunter, 2001; Kenerson et al., 2002). Furthermore, elevated activity of S6Kα (Grewe et al., 1999; Kwon et al., 2002), as well as S6Kβ (I. Gout, unpublished data), has been found in many cancer cells. For example, S6Kα is constitutively phosphorylated and activated to various degrees in serum-deprived MiaPaCa-2 and Panc-1 human pancreatic cancer cell lines, whereas treatment with rapamycin abrogates the transformed phenotype in these cell lines (Grewe et al., 1999). Similar effect of rapamycin has been reported for lung cancer cell lines, which exhibit anchorage-independent proliferation mediated by constitutively activated phosphoinositide-3'-kinase in a PKB and S6Kα-dependent manner.
In addition, the action of a peptidomimetic farnesyltransferase inhibitor (FTI or L-744,832), a compound that induces rapid growth arrest in many cancer cell lines, is associated with dephosphorylation and inactivation of S6Kα in v-K-Ras transformed mouse keratinocytes (Law et al., 2000).

Identification of tuberous sclerosis complex tumour suppressor (TSC1-TSC2) as a negative regulator of S6Kα provides further evidence implicating S6 kinase in oncogenesis (see section 1.3.4.). Both tumour suppressors, hamartin (TSC1) and tuberin (TSC2), are demonstrated to function downstream of the insulin/IGF-1 receptor/PI-3'-K/PKB pathway and upstream of S6Kα in the control of cell growth (Marygold and Leevers, 2002). It is well documented that mutations in either TCS1 or TSC2 are associated with widespread medically distinct tumours of the brain, eyes, skin, heart, lung and kidneys (Young and Povey, 1998). Moreover, disease-associated mutations of TSC1 or TSC2 have been shown to correlate with constitutively high phosphorylation of both S6Kα and its substrate S6 protein (Goncharova et al., 2002; Inoki et al., 2002).

Significantly, the analysis of genetic abnormalities in breast carcinoma has identified S6Kα gene amplification in both breast tumour tissues and cell lines (Couch et al., 1999). The S6Kα gene was localised to the chromosome region 17q23, which is a frequent site of gene amplification in this type of cancer (Barlund, et al., 1997; Courjal and Theillet, 1997). Furthermore, Northern and immunoblot analyses of MCF-7, BT-474 and MDA-361 breast cell lines revealed that amplification of S6Kα gene was accompanied by corresponding increases in mRNA and protein expression. In addition, tissue microarray analysis showed overexpression of S6Kα in primary breast tumours and its correlation with lower survival rates of patients compared to those without S6K amplification (Barlund et al., 2000). These studies provide the first evidence that S6Kα itself can exhibit oncogenic activity rather than merely being hyper-activated as a consequence of enhanced upstream regulators.

Finally, several studies have proposed a role for S6Kα in cytoskeleton reorganisation and, potentially, cell motility (Berven and Crouch, 2000). Cell migration is a crucial process in cancer metastasis (Hanahan and Weinberg, 2000) and S6Kα was shown to associate with and be activated by the small GTPases Rac1 and Cdc42 (see section 1.3.1.4.), which are known to participate in membrane ruffling, migration and actin polymerisation. Furthermore, in migrating Swiss 3T3 cells, S6Kα is co-localised with...
actin-based structures of the leading edge, formation of which is required for the cell motility (Berven and Crouch, 2000).

There is also evidence suggesting an important role for S6Kα signaling in the control of glucose homeostasis (Kozma and Thomas, 2002). In addition to growth defects, particularly during embryogenesis, S6Kα deficient mice display a phenotype that closely parallels that of preclinical type 2 diabetes mellitus (Pende et al., 2000). Detailed analysis of glucose homeostasis in S6Kα⁻/⁻ mice revealed a selective decrease in pancreatic β-cell size, which was accompanied by hypoinsulinaemia and glucose intolerance. The pathophysiology of S6Kα deficient mice clearly demonstrates the importance of cell growth control in disease development and suggests critical role for S6Kα in these processes.
1.6. Aim of thesis

Accumulating evidence suggests that ribosomal protein S6 kinase regulates a wide range of cellular processes which together represent an essential biochemical basis of cell survival, growth and proliferation. The contribution of S6K to such fundamental aspects of cell functioning makes it an attractive target for investigation and development of novel therapeutic agents. The elucidation of signaling mechanisms involved in the regulation of S6K represents an important subject of research carried out in this field. Despite a significant progress made in determining upstream effectors and signaling pathways, which control S6Kα activity, many details of this regulation have yet to be explored. Moreover, recent identification of homologous S6Kβ, which is unable to entirely compensate physiological functions of S6Kα, adds another layer of complexity to the regulation and functioning of S6K signaling pathway.

The aim of the thesis therefore, was to study signaling pathways and mechanisms that regulate individual S6Ks and their specific physiological responses to diverse mitogenic stimuli.

Special objectives were:

I. To create a strong base for the study of S6Ks by generating:
   a) bacterial, mammalian and baculoviral expression systems for all isoforms of S6K;
   b) a panel of S6Kα and S6Kβ mutants possessing amino acid substitutions at regulatory residues;
   c) specific antibodies directed towards S6Kα or S6Kβ isoforms.

II. To perform functional analysis of recombinant wild-type and mutated versions of S6Kα and S6Kβ transiently expressed in HEK293 cells.

III. To study signaling pathways involved in the regulation of S6Kα and S6Kβ:
   a) to compare the kinetics of S6Kα and S6Kβ activation in HEK293 cells stimulated by different mitogenic stimuli;
   b) to investigate differences in regulation of S6Kα and S6Kβ activities by employing specific signal transduction inhibitors;

IV. To characterize subcellular localization of S6Kα and S6Kβ isoforms and explore the specific mechanisms involved in the regulation of their subcellular distribution.
V. To initiate studies on the physiological function of S6Ks by generating and characterizing of tetracycline-inducible stable cell lines, which overexpress activated mutants of cytoplasmic and nuclear S6Kα and S6Kβ isoforms.
CHAPTER 2

EXPERIMENTAL PROCEDURES

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CHAPTER 2

EXPERIMENTAL PROCEDURES

2.1. Nucleic acid manipulation

2.1.1. Construction of expression vectors

2.1.1.1. Plasmids

The following plasmids were used to generate recombinant constructs:

- bacterial expression system – pET 23d(+) (Novagen)
- mammalian expression system – pcDNA3.1(+), pcDNA4/TO (Invitrogen)

2.1.1.2. 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) or EE-tag epitope (MEFMPME) sequences were introduced into the sense oligonucleotide primers in-frame with the protein coding sequence. Annealing temperatures (Tm) for each primer were calculated using the equation: \( Tm^{(\circ C)} = 2(A+T)+4(G+C) \). The mutagenic oligonucleotide primers for use in site-directed mutagenesis were designed individually according to the desired mutation (section 2.1.2.). The following formula was used for estimating the Tm of mutagenic primers: \( Tm^{(\circ C)} = 81.5+0.41(\%GC)-675/N-%mismatch \) (N is the primer length in bases, values for \%GC and \%mismatch are whole numbers).

2.1.1.3. 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 (NH₄)₂SO₄, 20 mM Tris-HCl, pH 8.8, 0.1% Triton X-100, supplemented with 2 mM MgSO₄). The wild-type rat S6Kα cDNA clone and human S6Kβ cDNA clones 53 or 23 were used in PCR as template DNA. 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 (52-64°C) for 30 sec, and extended at 72°C for 30 sec per 500 bp length. 25-30 cycles were normally used to amplify DNA fragments.

2.1.1.4. 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. 1μg 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 2.1.1.5.

2.1.1.5. 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) gels were used for DNA fragments larger than 4 kb. 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 RT. DNA samples were mixed with 6 X 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.
2.1.1.6. 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 next equation:

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

The reaction was carried out at RT in buffer containing 30 mM Tris-HCl (pH 7.8), 10 mM MgCl₂, 10mM 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 2.1.1.2.)
- 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
- primers 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 nonmutated DNA template. The nicked vector DNA incorporating the desired mutations was then transformed into E.coli XL-1 Blue competent cells (section 2.1.3.4.), the presence of the desired mutation in selected clones was checked by DNA sequencing (section 2.1.5.).

2.1.3. Transformation of E.coli

2.1.3.1. Bacterial strains

*Escherichia coli* XL-1 Blue cells. Genotype: supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac-F[proAB lacI9 lacZAM15 Tn10(ter)].

*Escherichia coli* BL21(DE3)pLysS cells. Genotype: F' ompT hsdS' B (rB' mB') gal dsm (DE3) pLysS (Cam'). The DE3 designation means the strains contain the λ DE3 lysogen that carries the gene for T7 RNA polymerase under control of the *lacUV5* promoter. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) is required to induce expression of the T7 RNA polymerase.

2.1.3.2. Growth media and antibiotics

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 MgCl2, 12.5 mM MgSO4 and 20% (w/v) glucose. TYM medium: 2% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 0.1 M NaCl and 10 mM MgSO4. To prepare LB agar bacto-agar (1.5% (w/v)) was
added to LB medium. LB medium, TYM medium and LB agar were autoclaved at 121°C for 15 min. NZY medium was filter sterilised.

Ampicillin, kanamycin and chloramphenicol were prepared as 100 mg/ml stock solutions in ddH2O and stored at -20°C. Ampicillin, kanamycin and chloramphenicol were used at a final concentration 50 μg/ml, 10 μg/ml and 34 μg/ml, respectively.

2.1.3.3. Preparation of competent E. coli

Highly competent cells were prepared by the following procedure: 20 ml of TYM medium was inoculated with a single colony of bacterial strain and growth shaking at 37°C in a 250 ml flask, until the OD_{600} was between 0.2 and 0.8. The culture was transferred to a 2 liter flask containing 100 ml TYM and agitated at 37°C, until OD_{600} reached 0.5-0.9. A further 500 ml TYM was added and the incubation continued until the OD_{600} reached 0.6. The culture was cooled rapidly by swirling the flask in an ice bath and the bacteria were then pelleted (5000 rpm, 15 min) and resuspended in 100 ml ice-cold TfibI (30 mM potassium acetate, 50 mM MnCl₂, 100 mM KCl, 10 mM CaCl₂, 15% (v/v) glycerol) by gentle shaking/pipetting. The bacteria were repelleted (5000xg, 8 min) and resuspended in 20 ml TfibII (10 mM NaMOPS, pH 7.0, 75 mM CaCl₂, 10 mM KCl 15% (v/v) glycerol). Finally, 0.6 ml aliquots were “snap-frozen” in liquid nitrogen in pre-chilled screw-cap microcentrifuge tubes and stored at -70°C.

2.1.3.4. Transformation

Competent cells were thawed on ice and 150 μl cell suspension was mixed with 15 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 10 min and allowed to recover in 1 ml 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 100 μl of LB medium. Cell suspension was spread onto pre-warm LB agar plate containing the appropriate selective antibiotic (section 2.1.3.2.) 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. Bacterial pallet from 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 on ice for 5 min. Cellular debris was removed by centrifugation at 13000 rpm for 10 min. 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). To determine the concentration of plasmid DNA, a 1μl aliquot was diluted in 1ml 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 2.1.1.5.).

2.1.5. DNA sequencing

2.1.5.1. Principle

All generated 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 ddH₂O. The PCR cycle sequencing reaction was performed using a Peltier Thermal Cycler (PTC-200) 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 3M sodium acetate, pH 4.6, 1 μl of 0.5M 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).

2.2. Expression of recombinant His-tagged proteins in E.coli and affinity purification

The QIAexpress® system was used to express and purify recombinant proteins for in vitro studies. The QIAexpress system is based on the remarkable selectivity and affinity of
QIAGEN’s nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography matrices for biomolecules which have been tagged with 6 consecutive histidine residues (6xHis tag). The general strategy of protein expression with the QIAexpress system begins with constructing expression clones, followed by the expression of His-tagged proteins and purification on Ni-NTA matrices.

2.2.1. Culture growth for preparative purification

20 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 1 liter of LB medium supplemented with selective antibiotic and incubation was continued until an OD_{600} of 0.6 was reached. To induce recombinant protein expression IPTG was added to a final concentration of 1 mM. After incubation for an additional 4-5 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 (50 mM NaH_{2}PO_{4}, pH 8.0, 300 mM NaCl, 10 mM imidazole, 1% glycerol) and lysozyme was added to 1 mg/ml. 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 the Ni-NTA Agarose

To purify His-tagged recombinant protein, 1 ml of the 50% Ni-NTA slurry was added to 5 ml of cleared lysate and mixed by rotation at 4°C for 60 min. After incubation the lysate-Ni-NTA mixture was loaded into a column and the lysate was filtrated by gravity flow. The Ni-NTA pellet was twice washed with 4 ml of Wash buffer (50 mM NaH_{2}PO_{4}, pH 8.0, 300 mM NaCl, 20 mM imidazole). The bound protein was eluted with 2 ml of
Elution buffer containing 50 mM NaH₂PO₄, pH 8.0, 300 mM NaCl and 250 mM imidazole. The second round of recombinant protein purification was performed by incubation of the eluate with 50% Ni-NTA slurry in the presence of 0.1% Triton- X100, 0.5 M NaCl, 1% Glycerol. The bound protein was then eluted as described above. The eluate was dialysed in 1 liter of 50 mM NaH₂PO₄, pH 7.5, 150 NaCl, 1 mM DTT for 6 hours at 4°C and then dialysed in 0.5 liter of 50 mM NaH₂PO₄, pH 7.5, 150 NaCl, 1 mM DTT and 50% glycerol. Protein concentration was measured as described in section 2.5.2.1. and samples were stored at -20°C.

2.3. Production of anti-S6K antibodies

2.3.1. Generation of rabbit antisera

Polyclonal antibodies to S6Kα and S6Kβ were raised by immunizing rabbits with synthetic peptides (S6Kα - CKQAFPMISKRPEHLRMNL and S6Kβ - CRPPSGTKKKRGKRGPGR) coupled to keyhole limpet haemocyanin. Polyclonal antiserum that recognizes a specific phosphorylation site at the C-terminus of S6Kβ was raised against a phosphopeptide SGTKKpS⁴⁸⁶KRGRG.

2.3.1.1. Coupling of peptides to keyhole limpet haemocyanin

Synthetic peptides used for immunization were synthesised by Alta Bioscience and coupled to activated keyhole limpet hemocyanin (KHL) using the method described by Sambrook et al., 1989 with some modifications. 20 mg of KHL (Calbiochem) were resuspended in 1 ml of phosphate-buffered saline (PBS) adjusted to pH 6.0 with concentrated HCl, dialysed against 1 liter PBS, pH 6.0 for 20 hours at 4°C, transferred to a flat-bottomed 5 ml polystyrene tube (Bibby Sterilin Ltd.) and the volume of the mixture was adjusted to 2 ml with PBS, pH 6.0. KHL mixture was stirred vigorously on a magnetic stirrer and 3 mg of m-maleimidobenzoic acid N-hydroxysuccinimide ester (MBS, Sigma) in 100 μl of dimethyl sulfoxide (DMSO) was added. The mixture was stirred at RT for the next 30 min and became blue/grey as coupling occurred. Then the KLH-MBS was separated from unconjugated MBS and DMSO using a Sephadex G-25 (Sigma) column. 5 mg of synthetic peptide were dissolved in 400 μl of PBS, pH 7.4, 1 ml of KLH-MBS was
added to peptide solution, then the mixture was vortexed vigorously and stirred for 1.5 hour. The volume of peptide-KLH solution was adjusted to 3 ml with PBS, pH 7.4 and stored at -20°C in 1 ml aliquots. Each aliquot contained approximately 3.3 mg of KLH coupled to 1.7 mg of peptide and was used for single immunization of two rabbits.

2.2.1.2. Immunization schedule

Peptides coupled to KLH were used for the immunisation of two rabbits (performed by Eurogentec). The animals were boosted every month and the serum tested by immunoblot analysis (section 2.5.6.). Four bleeds were taken and the third was used for affinity purification of antibodies.

2.3.2. Affinity purification

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 5M 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 NaCNBH₃ (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 1 ml fractions into tubes containing 100 µl of 1 M Tris-HCl, pH 8.0. Protein concentration was measured as described in section 2.5.2.1. 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 (section 2.5.6.).
2.4. Cell culture methodology

2.4.1. Tissue culture media and general cell culture technique

Human embryonic kidney HEK 293 cells and human breast cancer MCF-7 cells were maintained at 37°C in humidified atmosphere containing 5% CO₂. 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 2-4 min at RT. Cells were then suspended in complete DMEM medium and split into 10-cm dishes. Adult rat ventricular cardiomyocytes (ARVC) were isolated from hearts of adult rats as described previously (Wang et al., 2000). After isolation, cells were washed three times with DMEM. Cells were then resuspended in medium 199 containing 1 g/l glucose, 0.68 mmol/l glutamine, 5 mmol/l creatine, 2 mmol/l carnitine, and 5 mmol/l taurine and were washed three times in this medium. Cells were cultured on laminin-coated 60- or 100-mm diameter dishes seeded at a density of 1.4 × 10⁴ cells/cm² for 1-2 h in medium 199 at 37°C with the above additions. Plates were rinsed once with the same modified medium 199, and nonadherent cells were discarded. Attached cells were cultured overnight in modified medium 199 without serum but with penicillin (50 U/ml) and streptomycin (50 μg/ml) before further treatment.

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. and DNA was re-precipitated under sterile conditions. Cells were seeded at 1.2×10⁶/60-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.)
according to the manufacturer's recommendations. For each transfection 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 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 2 ml 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 3-5 hours at 37°C in a CO2 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.

2.4.3. Generation of stable cell lines

To generate stable cell lines a Tetracycline-Regulated Expression System for mammalian cells (T-Rex System, Invitrogen) was used. This system includes the T-Rex HEK293 cell line stably expressing the tetracycline (Tet) repressor and pcDNA4/TO inducible expression plasmid. T-Rex HEK293 cells were cultured as described in 2.4.1., except of the presence of selective antibiotic blasticidin at the final concentration of 5 µg/ml. Genes of interest were cloned into pcDNA4/TO vector as described in section 2.1.1. and resulting plasmid DNAs were used for transfection.

The T-REX HEK293 cells were split (1/5) at 70-80% confluence and transfected the next day with pcDNA4/TO vectors containing coding sequences for recombinant proteins of interest as described previously (see 2.4.2.). 48 hours after transfection, cells were split 1/20 into complete medium containing 5 µg/ml of blasticidin and 100µg/ml of zeocine. Selective medium was changed every 3-4 days and cell survival was monitored over the period of two weeks to identify colonies that had integrated a gene construct. Discrete colonies were selected and isolated in individual plastic chambers to allow them to be trypsinised and removed. For each gene 20 clones were selected and screened for tetracycline-regulated protein expression. To induce protein expression tetracycline was added into each dish to a final concentration of 1µg/ml and cells were incubated for 24 hours at 37°C. Those cell clones that tested positive for expression were frozen in DMEM medium containing 50% FBS and 10% dimethyl sulfoxide and stored in liquid nitrogen.
2.4.4. Metabolic labeling with $[^{35}\text{S}]$Methionine/Cysteine or $[^{33}\text{P}]$Orthophosphate

T-Rex HEK293 cells were grown in complete DMEM medium containing 5 $\mu$g/ml of blasticidin and 100$\mu$g/ml of zeocin to approximately 60-70% confluency. Recombinant protein expression was induced with 1 $\mu$g/ml tetracycline 12 hours prior to labeling. Cells were washed twice with methionine/cysteine or sodium phosphate free DMEM medium and incubated in this medium for 1 hour at 37°C. After incubation the medium was removed and fresh methionine/cysteine or sodium phosphate free DMEM medium, supplemented with 10% dialysed FBS and radioactive Pro-mix-$[^{35}\text{S}]$ (100-200 $\mu$Ci/ml, Amersham Pharmacia Biotech) or $[^{33}\text{P}]$Orthophosphate (50 $\mu$Ci/ml, Amersham Pharmacia Biotech), was added to the cells. Cells were then incubated for 6 hours (short-term labeling) at 37°C. After labeling, cells were washed twice with ice-cold PBS and lysed on ice in appropriate buffer for further studies.

2.5. Analysis of cellular proteins

2.5.1. Preparation of mammalian cell lysates

HEK 293 and MCF7 cells were washed twice with ice-cold PBS and extracted with lysis buffer containing 50 mM Hepes, pH 7.5, 150 mM NaCl, 1% v/v Nonidet P-40 (NP-40), 2 mM EDTA, 50 mM sodium fluoride, 10mM sodium pyrophosphate, 1mM sodium orthovanadate, 50 $\mu$g/ml leupeptin (Boehringer Mannheim), 0.5% aprotinin (Sigma), 1mM phenylmethylsulfonyl fluoride (Sigma), and 3 mM benzamidine (Sigma). Total cell extracts were incubated on ice for 30 min and then centrifuged at 13,000 rpm for 15 min at 4°C to pellet the NP-40 insoluble cell debris. The supernatant fraction was then removed and used for cellular protein analysis.

ARVC cells were washed two times with ice-cold PBS and homogenized in ice-cold extraction buffer containing 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM Na$_2$VO$_3$, 50 mM NaF, 5 mM sodium pyrophosphate, 270 mM sucrose, and 1 mM DTT and also 1% (v/v) Triton X-100, 1 $\mu$mol/l microcystin-LR, 5 $\mu$g/ml leupeptin, 5 $\mu$g/ml pepstatin, 5 $\mu$g/ml antipain, and 200 $\mu$mol/l phenylmethylsulfonyl fluoride (added immediately before use). Postmitochondrial/nuclear supernatants were prepared by centrifugation of the total cell extracts at 13,000 rpm for 10 min at 4°C.
Total protein concentrations of the lysates were determined by Bradford protein assay (section 2.5.2.1.).

2.5.2. Estimating of protein concentration

2.5.2.1. Bradford protein assay

To estimate protein concentration in cell lysate the colorimetric method, involving 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 ddHiO and 1 µl of cell lysate was added to the mixture. Upon 30 min incubation at RT the absorbance was measured at OD595 and compared with a blank. The protein concentration was then determined by comparison of absorbance values with a bovine serum albumin (BSA) standard curve.

2.5.2.2. BCA protein assay

To estimate protein concentration in solution, containing high concentration of SDS and NaOH, BCA Protein Assay Reagent Kit (Pierce) was used. The method combines the reduction of Cu\(^{2+}\) to Cu\(^{1+}\) by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu\(^{1+}\)) using a unique reagent containing bicinchoninic acid. The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562 nm that is nearly linear with increasing protein concentrations over a broad working range (20–2,000 µg/ml). To prepare Working Reagent 1 part of Reagent B (containing 4% cupric sulfate) was mixed with 50 parts of Reagent A (containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide). 50 µl of protein solution were added to 1 ml of Working Reagent, thoroughly mixed, and incubated at 37°C for 30 min. After incubation all samples were cooled down to RT and absorbance was measured at OD562 versus blank sample. Finally, protein concentration was then determined based on the absorbance value as described in 2.5.2.1.
2.5.3. SDS-PAGE protein electrophoresis

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 characterized by higher pH and acrylamide concentration. The percentage of acrylamide in 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 ddH₂O 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 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 polymerize with spacer comb inserted into the mixture. Following polymerization, 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.

2.5.4. Two-dimensional protein electrophoresis using immobilized pH gradient

Two-dimensional electrophoresis (2D-electrophoresis) is a widely used method for the analysis of complex protein mixtures extracted from cells, tissues, or other biological
samples. This technique sorts proteins according to two independent properties in two discrete steps: the first-dimension step, isoelectric focusing (IEF), separates proteins according to their isoelectric points (pI); the second-dimension step, SDS-polyacrylamide gel electrophoresis, separates proteins according to their molecular weights.

2.5.4.1. Sample preparation

For standard analytical loading of the first-dimensional gel a volume of lysate, corresponding to 120 µg of total protein (generally 50-100µl), was made up to 350 µI with solubilizing solution (8 M urea, 4% CHAPS, 65 mM DTT, 0.8% ampholine pH 3.5-10 (Amersham Pharmacia Biotech)).

2.5.4.2. IPG strip rehydration

Solubilised samples were then applied to the ready-made Immobiline DryStrip gels with linear pH gradient 3-10 (IPG strips, Amersham Pharmacia Biotech). Samples were pipetted into separate reservoir slots of the reswelling tray and IPG strips were placed gel-side down onto the solution. To minimize evaporation and urea crystallization each IPG strip was overlaid with 2-3 ml of mineral oil and allowed to rehydrate overnight at RT.

2.5.4.3. First-dimensional isoelectric focusing

Before removing the IPG strips from the reswelling tray, the Multiphor II cooling system (MultiTemp III Thermostatic Circulator) was set to 20°C. DryStrip aligner was placed groove-side up into the DryStrip tray of Multiphor II on top of the mineral oil. DryStrip tray is located on the cooling plate of Multiphor II and therefore has determined temperature. The IPG strips were lift out of the reswelling tray, rinsed briefly in a stream of ddH₂O and transferred to adjacent grooves of the DryStrip aligner. 110 mm electrode strips, pre-wetted with ddH₂O, were placed across the cathodic and anodic ends of the aligned IPG strips and fixed on the top of gel-side by electrodes. Finally IPG strips were completely covered with mineral oil. IEF was performed according to the following profile: 300V for 2 hours, ramping to 3500V over 3 hours; hold at 3500V for a further 19 hours. Wattage and current limit were set to 10 W and 5 mA respectively.
2.5.4.4. IPG strip equilibration

The equilibration step that saturates IPG strip with the SDS buffer system is required for the second-dimension separation. The IPG strips were prepared for SDS-PAGE by immersion in the equilibration solution containing 50 mM Tris-HCl, pH 6.8, 6 M urea, 2% SDS, 1% (w/v) DTT, 30% (v/v) glycerol. The strips were placed in the equilibration tray and secured at either end in order to avoid the strips moving during equilibration. 100 ml of the equilibration solution was added to the tray, and the IPG strips were incubated for 20 min at RT.

2.5.4.5 Second-dimension SDS-PAGE

The Tris-Glycine system described by Laemmli (Laemmli, 1970) was used for second-dimension SDS-PAGE. Vertical gradient second-dimension gels were usually casted several at a time, in a multiple gel caster. 5% and 16% acrylamide gel mixtures, containing 375 mM Tris-HCl, pH 8.8 and 0.05% (v/v) TEMED, were made up in two separate chambers of a vacuum flask. Ammonium persulphate was added into each chamber to the final concentration of 0.035% and the gel solutions were then mixed in the front chamber and poured into glass plate assemblies and overlaid with water-saturated butanol. After polymerization, butanol was removed and gel surface was washed with buffer containing 0.375M Tris-HCl, pH 8.8 and 0.1% SDS. Equilibrated IPG strips were washed down with SDS electrophoresis buffer, positioned between the plates on the surface of the second-dimantion gels, and sealed with pre-heated to 50°C 0.5% agarose solution in electrophoresis buffer containing trace of bromphenol blue. Gels were run at about 30-40 mA per gel for 1 hour (until the dye-front has passed the IPG strip) and then run overnight at 5-10 mA per gel at 10°C. Visualization and analysis of separated proteins were performed as described in sections 2.5.5. and 2.5.6.

2.5.5. Visualization 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 distaining 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 gel which allows to analyse nanogram quantities of protein. In this study we have used method described by Shevchenko et al. (Shevchenko et al., 1996). 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 ddH2O for another 10 min. Sensitization step was carried out for 1 min in 0.02% Na2S2O3 solution, followed by two brief washing with ddH2O. The gel was then stained in pre-chilled 0.1% AgNO3 solution for 20 min at 4°C and developed in 2% Na2CO3/0.04% formalin solution to the desired degree. Development was stop by incubation of gel in 5% acetic acid for 10 min. Gels were stored in 1% acetic acid at 4°C or dried as above.

2.5.5.3. Detection of radiolabeled proteins in gels

Samples, which consist of radiolabelled in vivo proteins (section 2.4.4.), 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. In the case of [35S]Methionine labeling, the signal was enhanced by immersing of gel in fluorographic scintillant (2,4-diphenyloxazole, Amersham) prior to drying.

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 or methanol-soaked
polyvinylidene 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™ electrophoretic transfer cell, according to the manufacturer’s instructions (Bio-Rad) at 60 V for 90min.

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 (H₂O₂). Following oxidation, the luminol is in an excited state which decays to the ground state via a light emitting pathway.

After wet transfer procedure, 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 (1/200-1/2000) for 1 hour at RT or overnight at 4°C. Excess antibodies were removed by washing in blocking buffer. At this step membrane was rinsed once in blocking buffer and then washed once for 15 min and twice for 5 min with fresh changes of the buffer at RT. A species-specific horseradish peroxidase-conjugated second antibodies (Sigma) were then applied in a minimal volume of blocking buffer for 1 hour at RT at a dilution 1/2000. Following incubation with second antibodies the membrane was rinsed twice in blocking buffer and then washed three times in TBS-T buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.05% Tween 20) for 5 min at RT. Immunoreactive proteins were detected by enhanced chemiluminescence. Equal volumes of ECL detection solution 1 and 2 (Amersham) 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 period of time or scanned by fluoroimager (Bio-Rad). The signal was quantified using manufacturer provided software.
2.5.6.3. Stripping and reprobing

Immunoblot membrane 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 with occasional agitation. The membrane was then washed twice in TBS-T buffer for 10 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 at 4°C. 20 μl of 50% protein G-Sepharose suspension was added to each sample to bind antibody-protein complexes and the incubation continued for a further 1 hour. The immune complexes were then pelleted by low speed centrifugation (2500 rpm) and washed 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 immobilized on protein G-Sepharose beads. Immune complexes were washed three times with lysis buffer, followed by a single wash with kinase assay buffer (50 mM Hepes, pH 7.5, 10 mM MgCl₂, 1 mM dithiotreitol (DTT), 10 mM β-glycerophosphate). The kinase reaction was initiated by resuspending the beads in 25 μl of kinase assay buffer supplemented with 1 μM protein kinase A inhibitor (PKI, Calbiochem), 50 μM ATP, 5μCi of [γ-32P]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 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 PKC

Recombinant EE-tagged S6Ks were immunoprecipitated from serum-starved HEK 293 cells with anti-EE monoclonal antibody immobilized on protein G-Sepharose. Beads were washed twice with lysis buffer containing 0.5 M NaCl and three times in PKC kinase buffer (20 mM Hepes, pH 7.5, 10 mM MgCl$_2$, 100 μM CaCl$_2$). Immune complexes, 1 μg of recombinant His-S6KαC, His-S6KβC, histone H1 or ε-peptide were incubated with 0.5 units/ml of different PKC isoforms (Calbiochem) at 30°C in buffer containing 100 μM ATP, 5μCi of [$γ$-$^{32}$P]ATP, 0.03% Triton X-100, 100 μg/ml phosphatidylycerine and 20 μg/ml diacylglycerol. After incubation for 10 min, reactions were terminated by the addition of 5× SDS-PAGE sample buffer and boiling for 5 min. Incorporation of $^{32}$P into recombinant EE-S6KαII, EE-S6KβII, His-S6KαC, His-S6KβC, histone H1 and ε-peptide was determined by phosphorimager analysis following 5-17.5% gradient SDS-polyacrylamide gel electrophoresis.

2.5.10. Mass spectrometry

Recombinant EE-S6KβII or His-S6KβC were phosphorylated with PKCs as described above, but without [$γ$-$^{32}$P]ATP. The products of the reaction were either directly analysed by IR and UV MALDI MS or first digested using modified trypsin (Promega) or endoproteinase Lys-C (Roche, Lewes, East Sussex, U.K.) in 25mM ammonium bicarbonate buffer (pH 8) at 37°C and then analysed. Prior to proteolysis some samples were separated on 1D SDS-PAGE and an in-gel digest (Rosenfeld et al., 1992) was performed on excised bands of interest.

MALDI samples were prepared using the "dried droplet" method, which involves mixing 0.5 μl of the analysed solution with 0.5-1 μl of the matrix solution on the target and drying by means of a warm stream of air. For all measurements external calibration was performed using the calibration mixture 2 of the Sequazyme™ peptide mass standards kit (5 peptides/proteins in the 1-6 kDa mass range) from Applied Biosystems, Warrington, Cheshire, U.K.
All measurements were conducted on a Voyager Elite XL (Applied Biosystems, Framingham, MA, USA) MALDI time-of-flight mass spectrometer equipped with delayed extraction and a reflector analyser for improved mass resolution and accuracy. The instrument has been modified to enable IR MALDI measurements at \(2.94 \, \mu \text{m}\) with a Q-switched Speser 15Q (Spektrum GmbH, Berlin, Germany) Er:YAG laser as well as UV MALDI measurements at 337 nm utilizing a VSL-337ND nitrogen laser (Laser Science, Inc., Franklin, MA, USA) as supplied by the manufacturer of the mass spectrometer. The technical details regarding the experimental set-up have been reported elsewhere (Cramer et al., 1998).

### 2.6. Investigation of subcellular localization

#### 2.6.1. Subcellular fractionation

HEK293 cells were washed twice with ice-cold PBS, scraped away from their culture dishes, and transferred to microcentrifuge tubes. The cells were then centrifuged at 1000 rpm for 5 min at \(4^\circ\text{C}\). After centrifugation, the supernatant was discarded, and the pellet was resuspended in 400 \(\mu\text{l}\) of cold hypotonic buffer containing 10 mM Hepes, pH 7.9, 10 mM KCl, 1 mM dithiothreitol (DTT), 10 mM sodium pyrophosphate, 1x protease inhibitor cocktail (Boehringer Mannheim Corp.). After incubation on ice for 15 min, 12.5 \(\mu\text{l}\) of 10% Nonidet P-40 was added, and the mixture was vortexed briefly. The cytoplasmic fraction was obtained as supernatant after centrifugation at 4000 rpm for 4 min at \(4^\circ\text{C}\). The pellet was washed in hypotonic buffer and resuspended in 50 \(\mu\text{l}\) of ice-cold hypertonic buffer (20 mM Hepes, pH 7.9, 0.4 M NaCl, 1 mM dithiothreitol (DTT), 10 mM sodium pyrophosphate, 1x protease inhibitor cocktail) followed by incubation on ice for 30 min. The mixture was then centrifuged at 13000 rpm for 5 min at \(4^\circ\text{C}\), and the supernatant was collected as a nuclear extract.

#### 2.6.2. Immunofluorescent staining and confocal microscopy

HEK 293 cells were plated onto poly-L-lysine coated coverslips in 24-well dishes at a density of \(2.5 \times 10^4\) cells per well and cultured overnight. The cells were transfected then with 0.5 \(\mu\text{g}\) of expression vectors (section 2.4.2.) containing various S6K constructs. Twenty-four hours post-transfection cells were starved in serum-free DMEM medium for
24 hours and then stimulated with 1 μM PMA for 30 min. Leptomycin B-treated cells were cultured in the presence of 10 ng/ml LMB for 16 h before stimulation. 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) or rabbit anti-pS486 (1:800) antibodies for 2 hr at RT. After extensive washing with PBS, the samples were incubated for 45 min with goat fluorescein isothiocyanate-conjugated (FITC) anti-mouse or anti-rabbit antibodies (1:200), respectively. Finally, the coverslips were extensively rinsed with PBS, air-dried and mounted onto microscope slides using mowiol mounting medium. 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.
CHAPTER 3

EXPRESSION AND FUNCTIONAL CHARACTERIZATION OF RECOMBINANT S6Kα AND S6Kβ ISOFORMS
CHAPTER 3

EXPRESSION AND FUNCTIONAL CHARACTERIZATION OF RECOMBINANT S6Kα AND S6Kβ ISOFORMS

3.1. Introduction

The ribosomal protein S6 kinase (p70/85S6K, also named S6Kα) is a serine/threonine kinase, which phosphorylates 40S ribosomal protein S6 and is involved in the initiation of protein synthesis induced by mitogenic stimuli. Two isoforms of the enzyme, denoted S6KαI and S6KαII (or p85S6 kinase and p70S6 kinase, respectively), are known to be generated from a single gene by the use of alternative translational start sites (Grove et al., 1991). The 525-amino acid S6KαI isoform differs from the 502-amino acid S6KαII isoform only at the amino terminus and contains 23 amino acid extension. Recent analysis of EST database (performed by Dr. I.Gout, LICR; Gout et al., 1998) revealed several human EST cDNA clones that were highly homologous to the S6Kα sequence. The full-length cDNA clones corresponding to the identified EST clones were isolated from HEK293 cells cDNA library using the insert from the EST clone (DBEST accession number AA410355) as a probe. Among isolated overlapping clones, clone 53 (cl.#53) was found to contain an open reading frame of 482 amino acids. The protein encoded by this clone has high level of amino acid sequence homology but is not identical to S6KαII and therefore was named as S6KβII. Another clone (cl.#23) was found to contain coding sequence of 495 amino acids and differed from clone 53 by the presence of additional 13 amino acids at the N-terminus. This clone encodes a long isoform of S6Kβ, named S6KβI. However cl.#23 has an internal G/T point mutation which introduces a stop codon at 1093 bp from the start codon (Gout at al., 1998). Other groups have also reported the isolation of S6KβII clones from cDNA libraries of different cell lines (Lee-Fruman et al., 1999; Saitoh, et al., 1998; Shima et al., 1998). cDNA clones described above were used in this study to construct and subclone into expression vectors recombinant S6KβI and S6KβII isoforms.

The predicted amino acid sequence encoded by the S6Kβ clones is very close to that of S6Kα with 70% identity and 85% similarity at the protein level (Appendix C). In addition, structural features common to both S6Kα and S6Kβ such as a conserved catalytic domain,
a kinase extension domain and an autoinhibitory pseudosubstrate region have been identified based on their homology. Moreover, most known regulatory residues of S6Kα are conserved in S6Kβ and include: a set of (Ser/Thr)-Pro motifs clustering in the carboxyl-terminal autoinhibitory segment (Ser423, Ser430, Ser436 and Ser441 in S6KβI, which correspond to Ser434, Ser441, Ser447 and Ser452 in S6KαI); Ser383 and Thr401 located in the kinase extension domain, which correspond to Ser394 and Thr412 in S6KαI; and Thr241 located in the catalytic loop, which corresponds to Thr252 in S6KαI (Gout et al., 1998). These suggest that S6Kβ may be regulated through a multisite phosphorylation mechanism, similar to that proposed for S6Kα (Dennis et al., 1998; Pullen and Thomas, 1997; Weng et al., 1998). However, both kinases differ significantly in their N- and C-terminal regulatory regions, sharing only 28% and 25% homology, respectively. In addition, one of the phosphorylation sites, which corresponds to Thr444 in S6KαI, is not conserved and substituted to Val in S6Kβ. Taken together, these findings indicate that there might be similarities and differences in the regulation of both forms of S6K.

To study and compare the functional and regulatory properties of S6 kinases, recombinant full-length and deleted mammalian expression constructs of S6Kα and S6Kβ isoforms were created. Recombinant kinases were transiently expressed in HEK293 cells and their activity towards S6 protein was characterized. Mutational analysis of regulatory residues was applied in this study to elucidate the molecular mechanisms of S6Kα and S6Kβ activation. This chapter also describes construction of bacterial expression vectors for recombinant S6Kα and S6Kβ C-terminal domains. Recombinant His-tag fusion proteins were expressed in E.coli, affinity purified using Ni-NTA-sepharose, and applied for in vitro phosphorylation studies.

3.2. Results

3.2.1. Construction of mammalian expression vectors containing full-length and deleted constructs of S6Kα and S6Kβ isoforms

To create mammalian expression constructs for recombinant S6K isoforms pcDNA3.1(+) and pcDNA4/TO mammalian plasmids were used. Both plasmids are
designed to provide high-level expression of recombinant proteins in a wide range of mammalian cells. In pcDNA3.1(+) vector the inserted cDNA sequence is under the control of human cytomegalovirus (CMV) immediate-early promoter/enhancer which permits high-level non-induced expression in mammalian hosts (see Invitrogen guide). The presence of bovine growth hormone (BGH) polyadenylation signal, which is located immediately after multiple cloning sites, allows efficient transcription termination and polyadenylation of mRNA. pcDNA4/TO expression vector is a part of the T-REX expression system and additionally to the CMV promoter utilises regulatory elements from the E.coli Tn10-encoded tetracycline resistance operon (see T-REX Invitrogen guide). This regulatory element enables tetracycline-inducible expression of recombinant proteins.

The full-length coding sequences corresponding to S6KαII and S6KβII isoforms were amplified by polymerase chain reaction (PCR) using rat S6Kα and human S6Kβ cl.#53 cDNAs (Appendixes B,C) as templates, respectively (Figure 3.1.B, lines 1 and 2). The following primers were used:

Sense :  
S6KαII – 5’-GAATTCGATCCGCCACCAGTGGATTCATGCAGTGGAGAGGCCAGGAGTGTTT GACATAGAC-3’; S6KβII – 5’-GAATTCGATCCGCCACCAGTGGATTCATGCAGTGGAGGCCC GCCGTGTGGATTTGAGAT-3’ (BamHI site is underlined, the translation initiation codon is underlined and written in bold text, EE-tag sequence is in bold text).

Antisense:  
S6KαII – 5’-CGGGAATTCTCATAGATGGGAGCAGGTTG-3’; S6KβII – 5’-CGGGAATTCTCAGCCACGCCCACG-3’ (EcoRI site is underlined, the translation termination codon is in bold text).

Amino- and carboxyl-terminal truncation mutants S6KαΔN75, S6KαΔC100, S6KβΔN64 and S6KβΔC81 were generated by PCR modification of full-length coding sequences of S6KαII and S6KβII, respectively (Figure 3.1.B, lines 3, 4, 5 and 6). The oligonucleotide primers complementary to the sequences, which are located immediately after truncation point, were used:

Sense :  
S6KαΔN75 – 5’-TTCGGATCCGCCACCAGTGGAGTTCATGCAGTGGAGGAAACTAGTGAACA GAGGGCCA-3’; S6KβΔN64 – 5’-TTCGGATCCGCCACCAGTGGAGTTCATGCAGTGGAGGAGAC CAGCGTGAACGTTG-3’ (BamHI site is underlined, the translation initiation codon is underlined and written in bold text, EE-tag sequence is in bold text).
Fig. 3.1. Generation of recombinant full-length and deletion constructs of S6KαII and S6KβII. (A) Schematic representation of recombinant S6KαII and S6KβII constructs and their deletion mutants, lacking amino- and carboxyl-terminal sequences. Structural features are indicated as follows: grey box indicates autoinhibitory domain and diagonally striped box corresponds to catalytic domain. All recombinant constructs carry an N-terminal EE-tag sequence shown as dotted box. (B) 1% TAE agarose gel electrophoresis of PCR products generated from rat S6Kα and human S6Kβ c.l.#53 cDNAs. Lanes 2 and 3 correspond to amplified DNA encoding recombinant S6KαII and S6KβII, respectively. Lanes 4, 5, 6 and 7 represent amplified DNA fragments of S6KαΔN64, S6KαΔC81, S6KβΔN64 and S6KβΔC81, respectively. 1 kb DNA ladder was run in parallel (lane 1). (C) Restriction analysis of created expression vectors for S6K recombinant constructs. Expression vectors for recombinant S6KαII and S6KβII constructs and their deletion mutants were digested using BamHI and EcoRI endonucleases. BamHI/EcoRI digests were separated by 1% TAE agarose gel electrophoresis. Lanes 2, 3, 4, 8, 9 and 10 correspond to untreated expression vectors for S6KαII, S6KαΔN75, S6KαΔC100, S6KβII, S6KβΔN64 and S6KβΔC81 constructs, respectively. Lanes 5, 6, 7, 11, 12, 13 represent digested expression vectors for the constructs described above (the same order). 1 kb DNA markers are shown in lane 1.
Antisense:

\[ S6K_{\alpha}^{\Delta C_{100}} \rightarrow 5' - CGGGAATTCCTACTTTTCTTTCACACTTTCAAGTACAGATG - 3' \; S6K_{\beta}^{\Delta C_{81}} \rightarrow 5' - CGGGAATTCCTAGCCCTCCTTGATGCTGTCCAG - 3' \] (EcoRI site is underlined, the translation termination codon is written in bold text).

All described sense primers contain a BamHI restriction site and a coding sequence for a seven amino acid epitope (MEFMPME, named as EE-tag epitope), which is in frame with the S6K protein coding sequence. The presence of the EE-tag epitope enables immunodetection of expressed recombinant protein using a specific monoclonal anti-EE-tag antibody. The antisense primers contain an EcoRI restriction site placed after the stop codon. The recombinant constructs created are schematically represented in Figure 3.1.A.

As shown in Figure 3.1.B the size of PCR products generated corresponded to calculated values 1557 bp, 1401 bp, 1326 bp for S6K_{\alpha}^{II}, S6K_{\alpha}^{\Delta N_{75}}, S6K_{\alpha}^{\Delta C_{100}} and 1497 bp, 1344 bp, 1293 bp for S6K_{\beta}^{II}, S6K_{\beta}^{\Delta N_{64}} and S6K_{\beta}^{\Delta C_{81}} recombinant constructs, respectively. The products of PCR amplification were then digested with BamHI and EcoRI restriction endonucleases and subcloned into pcDNA3.1(+) digested with the same enzymes. Figure 3.1.C represents restriction analysis of created expression vectors for S6K recombinant constructs using BamHI and EcoRI endonucleases. The results of restriction analysis clearly indicate the presence of the BamHI/EcoRI inserts of correct sizes for S6K constructs in pcDNA3.1(+) expression vectors.

The full-length coding sequence of a long S6K_{\alpha}^{I} isoform was simply amplified by PCR (Figure 3.2.A, line 3) using rat S6K_{\alpha} cDNA as a template and forward primer complementary to the 5'-terminal sequence which encode the 23 amino acid extension (Appendix A, underlined). Both forward and reverse primers used in this reaction were designed as described above and contain appropriate endonuclease restriction sites to enable subcloning into the plasmid.

Sense:

\[ S6K_{\alpha} \rightarrow 5' - GAAATTCCGGATCCGCCACCATTGCCATGCGATGAGAGGCGACGAGCGGGCG-3' \] (BamHI site is underlined, the translation initiation codon is underlined and written in bold text, EE-tag sequence is in bold text).

Antisense:

\[ S6K_{\alpha} \rightarrow 5' - CGGGAATTCCTACATAGCTAGAGCGAGGTG-3' \] (EcoRI site is underlined, the translation termination codon is in bold text).
Fig. 3.2. Generation of recombinant full-length constructs of nuclear S6KαI and S6KβI isoforms. (A) 1% TAE agarose gel electrophoresis of PCR products generated. Lane 1 correspond to amplified DNA fragment of nucleotides 4-978 of cl. #23 cDNA. Amplified DNA encoding recombinant S6KαI is shown in lane 2. Lane 3 represent 1 kb DNA markers. (B) Digestion of PCR product encoding 4-978 nucleotides of S6KβI (lane 1) and pcDNA3.1(+)/S6KβII expression vector (lane 2) with BamHI and EcoNI endonucleases. BamHI/EcoNI DNA fragments, used to create expression vector for S6KβI construct, are shown by arrows (I - 1-361 bp BamHI/EcoNI fragment; II - pcDNA3.1(+)/S6Kβ (362-1506 bp) fragment). 1 kb DNA ladder was run in lane 3.
Subcloning of the S6KαI insert into pcDNA3.1(+) plasmid was performed as described for S6KαII and resulting expression vector was analysed by restriction and DNA sequencing (Figure 3.4.A, line 1 and BII).

Since the isolated cl.#23, encoding a long S6KβI isoform, contained an internal point mutation, the construction of a pcDNA3.1(+)S6KβI expression vector necessitated the use of an alternative subcloning strategy (Figure 3.3.). The first step of the subcloning procedure involved amplification of nucleotides 4-978 of cl.#23 cDNA by PCR (Figure 3.2.A, line1 and 2) using the following primers:

Sense:
**S6KβI** — 5’-GAATTCGATCGTGCGATGAGTTCATGCCGATGGAGGCACGAGGCCGACGGGCCCGC-3’ (BamHI site is underlined, the translation initiation codon is underlined and written in bold text, EE-tag sequence is in bold text).

Antisense:
**S6KβI** — 5’- CCCAATCCGCTGGCTGGGATTCC-3’

In this reaction the first three ATG nucleotides of cl.#23 cDNA were eliminated by replacement with an EE-tag coding sequence which contained an alternative translation initiation ATG codon in frame with nucleotides 4-978 of the S6KβI cDNA sequence. The resulting PCR product was digested with BamHI and EcoNI endonucleases to obtain 4-361 bp fragment (Figure 3.2.B, line1), which coded for the N-terminal 13 amino acid extension of S6KβI. Digestion of pcDNA3.1(+)S6KβII expression vector with the same enzymes generated pcDNA3.1(+)S6KβII (362-1506 bp) construct (Figure 3.2.B, line2). Finally, the pcDNA3.1(+)S6KβI (1-1506 bp) expression vector, which contained full-length non-mutated S6KβI coding sequence, was created by ligation of the 1-361 bp fragment into the pcDNA3.1(+)S6Kβ (362-1506 bp) construct. The presence of the S6KβI insert was confirmed by restriction enzyme digestion and DNA sequence analysis (Figure 3.4.A, line2,3 and B1).
Fig. 3.3. Subcloning strategy used to generate recombinant full-length construct of nuclear S6KβI isoform. See text for details.
Fig. 3.4. Restriction and DNA sequence analysis of expression vectors for recombinant S6KαI and S6KβI isoforms. (A) Restriction analysis of expression vectors for S6KαI and S6KβI recombinant constructs. Lane 1 represents DNA fragments obtained after digestion of expression vector for S6KαI with BamHI and EcoRI endonucleases. Lane 2 and 3 correspond to BamHI/EcoRI and BamHI/EcoRI fragments of pcDNA3.1(+)/S6KβI vector respectively. 1 kb DNA markers are shown in lane 4. (B) DNA sequence of recombinant S6KαI (I) and S6KβI (II) N-terminal regions. N-terminal coding sequences for S6KαI (I) and S6KβI (II) are underlined.
3.2.2. Generation of S6Kα and S6Kβ mutants with amino acid substitutions at regulatory residues

It is well established that S6Kα is activated by multiple Ser/Thr phosphorylation at specific regulatory residues. The simultaneous phosphorylation of two of them, Thr 252 and Thr 412, was shown to be a critical event directly linked to the full activation of S6Kα (Alessi et al., 1997a). To study the relative importance of homologous Thr241 and Thr401 residues in the regulation of S6Kβ activity, mutational analysis of these sites has been employed in this work.

Substitution of phosphorylated sites for acidic amino acids mimics, in many cases, the phosphorylation of that site in the protein of interest and therefore provides an excellent model for functional studies. Taking this into account, we have generated S6KαI/II(T252D), S6KαI/II(T412D), S6KβI/II(T241D) and S6KβI/II(T401D) mutants by substituting Thr252 or Thr412 in S6Kα and Thr241 or Thr401 in S6Kβ for aspartic acid (Figure 3.5.A). The double-site mutants, holding aspartic acid substitution at both Thr252/Thr412 in S6Kα and Thr241 or Thr401 in S6Kβ, have been also created. In order to generate kinase inactive S6Kα and S6Kβ mutants, Lys123 and Lys112 residues, which are located in the ATP-binding pocket of both enzymes, were mutated to arginines, respectively. To achieve that, the expression vectors containing S6KαI, S6KαII, S6KβI, and S6KβII full-length inserts were modified by PCR using mutated oligonucleotide primers. All primers used for site-directed mutagenesis were designed as described in chapter 2, section 2.1.2. and contained appropriate nucleotide changes in the position of mutation.

Inspection of amino acid sequence of S6Kβ revealed a potential PKC phosphorylation site, Ser486, which is located within the C-terminal regulatory region of the kinase. In contrast, S6Kα, which exhibits low homology to S6Kβ in this region, does not contain consensus sequence for phosphorylation by PKC. To study this potential phosphorylation site a panel of S6KβII mutants, with substitution of Ser486 either to alanine or glutamic acid, were created. We also made several double mutants of S6KβII, bearing T401D and S486A or S486E substitutions. Later on, these mutants proved to be valuable tools for studying the mechanism of PKC regulated nucleocytoplasmic shuttling of S6KβII (chapter 5, section 5.2.7.).
Fig. 3.5. **Site-specific mutants of S6Kα and S6Kβ.** (A) Summary diagram of generated mutants of S6Kα and S6Kβ. Major domain boundaries are indicated as described in the legend for Fig.3.1. Amino acid substitutions and their positions are shown. (B) DNA sequence analysis of generated mutants.
The S6KαI/II(T412D) and S6KβI/II(T401D) mutants were simply subcloned from pcDNA3.1(+) to pcDNA4/T0 vector using BamHI and EcoRI cloning sites. These expression constructs were used to generate tetracycline-inducible stable cell lines as described in chapter 6.

Finally, the presence of mutations in S6K constructs generated was verified by DNA sequence analysis (Figure 3.5.B).

3.2.3. Functional analysis of recombinant S6Kα and S6Kβ constructs transiently expressed in HEK293 cells

To verify expression and to facilitate subsequent characterization of recombinant S6Ks in vivo, pcDNA3.1(+)/S6K constructs were transiently transfected into HEK293 cells using the LipofectAMINE reagent. Initially, optimization of transfection conditions, essential for the highest efficiency transfections and the lowest toxicity, was performed. The parameters that should be optimized include lipid concentration, cell number and time exposure to DNA-liposome complexes and depend on the cell type. In this work we generally used transfection parameters provided by LipofectAMINE manufacturer (Life Technologies, Inc.), which were specially designed for HEK293 cells. Another crucial factor which may influence the transfection efficiency include the quality of DNA and the size of the construct. To optimise the amounts of plasmid DNA, 80%-confluent HEK293 cells were transfected with various concentrations of pcDNA3.1(+)/S6KβII as indicated (Figure 3.6.A). pcDNA3.1. (+) vector without an insert was used as a negative control. After 24 hours incubation, the expression of recombinant protein was analysed in total cell lysates by immunoblotting with anti-EE-tag antibody. As shown in Figure 3.6.A there was no expression of recombinant protein when HEK293 cells were transfected with empty vector. At the same time, cells transfected with pcDNA3.1(+)/S6KβII construct exhibited different level of recombinant protein expression depending on the amount of plasmid DNA used for transfection. The most efficient expression of EE-S6KβII was observed when cells were transfected with 6 µg of expression vector, whereas lower or higher amounts of plasmid DNA significantly reduced recombinant protein expression. The optimal transfection conditions determined were used for all further experiments.

Next, we analysed basal and serum-stimulated activities of recombinant S6Ks towards S6 protein. The full-length recombinant EE-S6KαII and EE-S6KβII were transiently
Fig. 3.6. Expression and functional characterization of recombinant S6KαII or S6KβII. (A) HEK293 cells were transiently transfected with either empty pcDNA3.1 vector (lane 1) or various concentrations of pcDNA3.1/S6KβII construct (lanes 2, 3, 4 and 5 correspond to 2.5, 4, 6 and 10 µg of DNA, respectively). After 24 hours, the level of recombinant protein expression was analysed in total cell lysates by immunoblotting with anti-EE-tag antibody. (B) HEK 293 cells were transfected with EE-S6KαII or EE-S6KβII, serum-starved, and stimulated with 10% FCS for 15 min. Recombinant S6Ks were immunoprecipitated from lysates with anti-EE-tag antibody and their activities were analysed in immune complex S6 kinase assay as described in Experimental procedures. 32P incorporated into S6 protein was assessed by autoradiography. (C) The same lysates, used for the kinase assay, were subjected to SDS-PAGE and analysed by immunoblotting with anti-EE-tag
transfected into HEK293 cells. Twenty four hours post-transfection the cells were starved in serum-free DMEM medium for 24 hours and then stimulated with 10% FBS for 15 min. Recombinant S6Ks were immunoprecipitated from cell lysates with anti-EE-tag antibody and immune complex S6 kinase assay was performed. As shown in Figure 3.6.B both kinases efficiently phosphorylated S6 protein in vitro and their activities were increased by 3-4.5 fold after serum stimulation. Activation of both S6KαII and S6KβII coincides with the decrease in electrophoretic mobility of recombinant proteins as determined by immunoblot analyses (Figure 3.6.C.). However, the pattern of mobility shift of S6KβII was found to be different from that of S6KαII. The presence of multiple immunoreactive bands is a well-documented characteristic of activated S6Kα and reflects phosphorylation status of this enzyme (Ballou et al., 1988; Chung et al., 1992; Han et al., 1995). Thus, the results obtained suggest that S6KβII similarly to S6KαII undergoes an agonist-induced phosphorylation in vivo.

As mentioned above, phosphorylation of Thr252 and Thr412 is a critical regulatory event directly linked to the activation of S6Kα. This fact and the results presented above prompted us to investigate whether phosphorylation status of Thr241 and Thr401, which correspond to the regulatory Thr252 and Thr412 in S6Kα, had a similar impact on S6Kβ activity. To answer this question, wild-type and mutated forms of S6KαII and S6KβII were transiently expressed in HEK293 cells and their activities were assessed in an in vitro S6 kinase assay (Figure 3.7). The results indicated that the conversion of Thr412 and Thr401 to aspartic acid in S6Kα and S6Kβ, respectively, potentates activities of both kinases. We observed approximately 2.5 for S6Kα and 3.0 for S6Kβ fold increase of activity when the mutated forms were compared with the wild-type kinases. These results are in agreement with previously reported data for T412E mutant of S6Kα analysed in several cell lines (Denis et al., 1998). In contrast, substitution of aspartic acid for Thr252 and Thr241, which are located in the activation loop of S6Kα and S6Kβ, respectively, completely abolished kinase activities of these mutants. Moreover, simultaneous mutations of Thr252 and Thr412 in S6Kα or Thr241 and Thr401 in S6Kβ to aspartic acid had similar inhibitory effect on the activities of both S6 kinases. These effects were mostly expected, as it had previously been shown an absolute requirement for Thr or Ser at position 252 for S6Kα activity (Pearson et al., 1995; Weng et al., 1995). These results suggest a critical role for both Thr252 and Thr241, but then prohibit assessment of the importance of phosphorylation at these residues by mutational analysis. We observed complete inhibition
Fig. 3.7. Effects of various site-specific mutations on S6KαII and S6KβII activities. HEK293 cells were transfected with recombinant wild-type and mutated constructs of S6KαII and S6KβII as indicated. After 24 hours incubation in DMEM medium containing 10% FCS, cells were extracted with lysis buffer as described in Experimental procedures. Lysates were normalised for total protein content following Bradford Assay, and equal EE-S6K expression levels were confirmed by immunoblotting. Equal amounts of protein were subjected to immune complex S6 kinase assay as described for Fig.3.6. Activities of mutants are expressed relative to those of corresponding wild-type kinases (normalised to 100%).
of S6 kinase activity when the ATP binding site of S6Ka represented by Lys123 was mutated to arginine. Residue Lys112 in the structure of S6Kβ is an analog of Lys123 in S6Kα and therefore was predicted to serve as an ATP binding site. Indeed, we found that the mutation of Lys112 to arginine abolished S6Kβ activity.

Taken together, these results demonstrate that similarly to Thr252 and Thr412 in S6Kα, homologous Thr241 and Thr401 are principle regulatory sites of S6Kβ activity. The importance of Lys112 as the putative ATP binding site in S6Kβ was also established since mutation of this residue to arginine inactivated completely its activity.

3.2.4. Construction of bacterial expression vectors containing S6Kα and S6Kβ C-terminal domains. Expression in E.coli and affinity purification of the recombinant His-tag fusion proteins.

The C-terminal regions of S6Kα (453-525) and S6Kβ (442-495) were PCR amplified using rat S6Kα and human S6Kβ cl.#53 cDNAs as templates, respectively (data not shown). The following primers were used:

Sense:
S6KαC - 5'-ATTCCATGAGCACCACCACCACCACGCGGTTTCTGGGGAAGAGGTG-3';
S6KβC - 5'-ATTCCATGAGCACCACCACCACCACCATTTGAGGGGTTTCGGCCCAGC-3' (NcoI site is underlined, the translation initiation codon is written in bold text).

Antisense:
S6KαC - 5'-CGGGAAATTCTCATAGATCAGCAAGGGTT-3';
S6KβC - 5'-CGGGAAATTCTCAGCGCCCTGGACGCCCACG-3' (EcoRI site is underlined, the translation termination codon is in bold text).

The products of PCR amplification were cloned into pET23d(+) vector in frame with 6xHis sequences using NcoI/EcoRI restriction sites.

Recombinant His-tagged C-terminal domains of S6Kα and S6Kβ (His-S6KαC and His-S6KβC) were expressed in BLR21 DE3 cells and affinity purified on Ni-NTA matrices using the two step purification protocol described in chapter 2, section 2.2.3. As shown in Figure 3.8., the recombinant His-S6KαC and His-S6KβC peptides were purified to almost 95-97% of homogeneity. These preparations of recombinant proteins were used for in vitro phosphorylation studies (section 5.2.2.).
Fig. 3.8. Affinity purification of recombinant His-S6KαC and His-S6KβC domains expressed in E.coli. Recombinant His-tagged C-terminal domains of S6Kα and S6Kβ were expressed in BLR21 DE3 cells and affinity purified on Talon beads as described in Experimental procedures. Recombinant proteins after first (1) and second (2) stage of purification as well as unbound material (3) were analysed by gradient SDS-PAGE and Coomassie Blue staining.
3.3. Discussion

Initial studies aimed at identifying possible S6Kα isoforms led to the isolation of cDNA clones encoding a novel homologous kinase, termed S6Kβ. The cDNAs containing coding sequences for full length S6KβI and S6KβII were used in this study to produce recombinant expression constructs of S6Kβ. These constructs allowed us to express and subsequently characterize S6Kβ in vivo and in vitro.

The results presented here demonstrate the ability of S6KβII isoform to efficiently phosphorylate ribosomal S6 protein in vitro. Hence, it is possible that S6Kβ can phosphorylate S6 protein in vivo. This hypothesis is strongly supported by the fact that S6 phosphorylation was found to be normal in fibroblasts derived from mice lacking S6Kα gene (Shima et al., 1998). We have also shown that similarly to S6KαII, the catalytic activity of S6KβII towards S6 protein is rapidly activated by serum in transiently transfected HEK293 cells. The extent of S6KβII activation following serum stimulation was commensurate to that observed for S6KαII. However, we found that, unlike S6Kα, S6Kβ did not undergo a significant mitogen induced mobility shift, lacking at least two of the most slowly migrating electrophoretic forms. This could be due to a different pattern of phosphorylation as a result of distinct regulatory mechanisms and/or structural particularities of S6Kβ.

Studies from several laboratories demonstrated that activation of S6Kα requires multiple phosphorylations at specific serine and threonine residues (Pullen and Thomas, 1997). The phosphorylation of two of these regulatory residues, namely Thr252 and Thr412, which correspond to Thr241 and Thr401 in S6Kβ, appear to make the most important contribution to the activation of S6Kα (Alessi et al., 1997a). In addition, it has been shown that phosphorylation of Thr252 and Thr412 is largely responsible for the mobility shift following mitogenic stimulation, whereas phosphorylation at other sites had little effect on the mobility of S6Kα (Weng et al., 1998). At the same time, very little is known about the molecular mechanisms of S6Kβ activation. Based on the homology of its potential phosphorylation sites, it was proposed that activity of S6Kβ could be regulated similarly to S6Kα. However this hypothesis required experimental confirmation. Furthermore, as discussed above the pattern of S6Kβ mobility shift was found to be different from that for S6Kα. To explore these questions, the effect of point mutations of
Thr241 and/or Thr401 on S6Kβ activity was analysed and compared to that of equivalent mutations in S6Kα. We observed that the mutation of Thr401 to aspartic acid, which mimics the presence of a phosphate group, increased the kinase activity compared to wild-type S6Kβ. This effect was found to be equal to that observed for a T412D mutant of S6Kα. At the same time, equivalent mutation of Thr241 severely decreased the kinase activity to a level which was similar to that obtained by the ATP binding site defective K112R mutant. This phenomenon was previously described for S6Kα in which mutation of homologous Thr252 to either glutamic acid or alanine, but not Ser, completely abolished kinase activity (Weng, et al., 1995). It is believed that threonine or serine residue at this position is structurally required for intact kinase activity, but only threonine can serve as a phosphorylation site in situ.

Taken together, our results support the hypothesis that S6Kα and S6Kβ utilize similar molecular mechanisms of activation based on phosphorylation of regulatory residues conserved in these kinases. However, the data presented here do not exclude the possibility of differential regulation of S6Kα and S6Kβ through alternative signaling mechanisms. This issue was extensively investigated and discussed in the next chapter.
CHAPTER 4

INVESTIGATION OF SIGNALING PATHWAYS INVOLVED IN THE REGULATION OF S6Kα AND S6Kβ
CHAPTER 4

INVESTIGATION OF SIGNALING PATHWAYS INVOLVED IN THE REGULATION OF S6Kα AND S6Kβ

4.1. Introduction

The activity of S6Kα is regulated by phosphorylation/dephosphorylation events in cellular responses to various extracellular stimuli. Treatment of cells with growth factors, cytokines and hormones leads to a rapid activation of S6Kα (Chou and Blenis, 1995), while growth inhibitory agents, such as steroids and TGFβ suppress kinase activity (Petritsch et al., 2000; Shah et al., 2000). The mechanism of activation of S6Kα has been the subject of detailed investigations in various laboratories and was shown to be a multi-step phosphorylation process involving several Ser/Thr kinases (Dennis et al., 1998; Weng et al., 1998). The use of specific inhibitors such as wortmannin, LY294002 and rapamycin, demonstrated that signals from both PI-3'-K and mTOR/FRAP pathways are crucial for full activation of S6Kα. Several downstream effectors of PI-3'-K, including PDK1, PKB, aPKC, Rac and CDC42, were shown to be important for S6Kα activation (Meyuhas et al., 2000). However, only PDK1 was found to phosphorylate S6Kα at Thr252 and perhaps Thr412 residues, which are situated in the catalytic and kinase extension domain, respectively, and activate the kinase in vitro and in vivo (Alessi et al., 1997a; Balendran et al., 1999a; Pullen et al., 1998; Williams et al., 2000). Other phosphorylation sites involved in the activation of S6Kα lie in its C-terminal domain and are followed by Pro residues, suggesting that they may be targets for proline-directed kinase(s). Although S6Kα can be phosphorylated in vitro by MAPK (also known as ERK1/2), early studies indicated that S6Kα resides on a signaling pathway distinct from the Ras/MAPK pathway (Ballou et al., 1991; Mukhopadhyay et al., 1992). This view has been recently challenged by studies which show that S6Kα can be modestly inhibited by pharmacological inhibition of MEK activity in several cell types (Lehman and Gomez-Cambronero, 2002; Scott and Lawrence, 1997). It have been also shown that S6Kα is activated when cells are treated with PMA, an activator of classical and novel PKCs (Susa et al., 1989). Furthermore, prolonged treatment of cells with PMA, which leads to down-regulation of DAG-activated PKCs, partially or completely abrogated mitogenic activation of S6Kα in several cell types (Kanda et al., 1997; Susa et al., 1992). These data indicate that PKCs transduce signal(s) in
activated cells to ribosomal S6 kinase, but the regulatory mechanisms and functional importance remain unclear.

Despite significant progress made in determining upstream effectors and signaling pathways which control S6Kα activation, many questions still need to be explored. These include the identity of the direct S6K activating kinases, mechanisms by which mTOR/FRAP, PKB, PKC, Rac and Cdc42 contribute to S6K activation, coordination of multiple S6K activating inputs, the role of various domains of S6K in modulating its activity, and identification of novel regulatory elements in the structure of this enzyme.

Identification of S6Kβ, a mitogen-responsive S6Kα homologue, adds another layer of complexity to the regulation and functioning of S6K signaling pathway. S6Kα and S6Kβ are highly homologous, but differ substantially in their N- and C-terminal domains, two regions which are involved in the modulation of S6Kα activity. Moreover, unlike S6Kα, which has cytosolic and nuclear (S6KαII and S6KαI, respectively) isoforms, both S6KβI and S6KβII are primarily nuclear, because of the presence of a putative C-terminal nuclear localisation signal sequence (Koh et al., 1999). These differences and some variations in the pattern of phosphorylation sites may predestine the involvement of both kinases in distinct signaling events and cellular responses. Given the differences in subcellular localisation, as well as regions of divergence in primary sequence, it is important to understand the upstream signaling pathways and intermediates that regulate each S6 kinase to coordinate their specific physiological responses.

In this chapter we have compared the kinetics of mitogen-induced activation of S6KαII and S6KβII and analysed signaling pathways involved in the regulation of both kinases in HEK293 cells.

4.2. Results

4.2.1. Kinetic analysis of mitogen-induced activation of S6K isoforms in HEK293 cells.

Previous studies have shown that S6Kα activity is increased when cells are stimulated by various growth factors such as epidermal growth factor (EGF), platelet derived growth factor (PDGF), insulin, and serum. The activation of S6KαII stimulated by these agonists
was found to be biphasic and characterized by the presence of an early transient peak of kinase activity, followed by a sustained late phase of activation (Kahan et al., 1992; Oliver et al., 1988; Susa et al., 1992). The kinetics of S6Kα activation depends on the cell type and extracellular stimuli so that the appearance of an early peak of S6 kinase activity may vary from 5 up to 30 min in different cell lines. For example, the early peak of S6KαII activation in HepG2 cells (Chung et al., 1994) was observed 30 min following PDGF stimulation while a rapid activation of this kinase reached a maximum at about 10-15 min in PDGF-stimulated Swiss 3T3 cells (Susa et al., 1992). The ability of α6β1 and α6β1 integrins to activate S6Kα in response of P388D1 macrophages and REF52 fibroblasts to laminin or fibronectin adhesion has recently been reported (Malik and Parsons, 1996; Wei et al., 1998). However, the kinetics of integrin-dependent activation of S6Kα have not been reported to date. Integrin-dependent signaling is characterized by a weak mitogenic response and may represent a signaling system distinct from those utilized by serum and the growth factors mentioned above.

In the present study, we have analysed and compared the kinetics of S6KαII and S6KβII activation in HEK293 cells stimulated by serum or fibronectin. The full-length recombinant EE-S6KαII and EE-S6KβII were transiently transfected into HEK293 cells. After 24 hours starvation in serum-free DMEM medium cells were either stimulated with 10% FBS or removed from tissue culture dishes by trypsinization, incubated in suspension for 60 min, and then replated onto fibronectin coated dishes for the indicated period of time. Recombinant S6Ks were immunoprecipitated from cell lysates with anti-EE-tag antibody and S6 kinase activities were assessed in immune complexes. As shown in Figure 4.1., stimulation of quiescent HEK293 cells with 10% FBS resulted in a time-dependent biphasic activation of both S6KαII and S6KβII isoforms. Time course of activation of exogenously expressed S6KαII in response of HEK293 cells to serum correlated closely with that previously described for endogenous S6Kα (Ballou et al., 1991). An early phase of S6KαII activation reached a maximum at 20 to 30 min and then S6 kinase activity significantly declined by 60 min. After this stage a sustained phase of late activation was observed over the next several hours. In contrast to S6KαII, the activation of S6KβII was more rapid reaching a maximum at about 10 min. The late phase of S6KβII activation occurred between 30 and 90 min and was a significantly shorter than relevant phase of S6KαII activation. Therefore, the curves which describe S6KαII and S6KβII activation are
Fig. 4.1. Kinetics of S6KαII and S6KβII activation in serum-stimulated HEK 293 cells. HEK 293 cells were transiently transfected with EE-S6KαII or EE-S6KβII, serum-starved, and stimulated with 10% FCS for the times indicated. Recombinant S6Ks were immunoprecipitated with anti-EE-tag antibody and their activities were analysed in immune complex S6 kinase assay as described in Experimental procedures. $^{32}$P incorporated into S6 protein was assessed by autoradiography (B) and quantified by phosphorimaging (A). Typical results from three independent experiments are shown. Expression levels of transiently expressed S6Ks were analysed in cell lysates using anti-EE-tag antibody (C).
shifted in time so the peaks of S6KαII activity coincide with inactivation stages of S6KβII suggesting time-dependent differential regulation of these kinases upon serum stimulation.

In case of fibronectin-induced stimulation, the activity of S6KαII increased slowly by reaching a maximum at 90 min (Figure 4.2.). In contrast, the kinetics of S6KβII activation was characterized by the presence of two peaks of specific activity: an early phase, reaching a maximum at about 40 min and a late phase appearing 90 min after adhesion to fibronectin. We have also found that integrin-mediated activation of S6KβII during the late phase was substantially higher when compared to that of early response. At the same time serum-stimulated activation of S6KβII was always higher at the early stage of activation. Moreover, integrin-dependent S6KαII activation possessed only one peak which corresponded to the late phase of S6KβII activity. These findings may indicate that serum and fibronectin have different potential to induce early stages of S6KαII and S6KβII activation.

In conclusion, the results of kinetic analysis demonstrate that agonist-induced activation of S6KαII and S6KβII differs in time. S6KβII is activated earlier in response to serum and fibronectin stimulation and may be involved in the regulation of early cellular responses to these agonists.

4.2.2. The effect of specific mTOR/FRAP and PI-3'-kinase inhibitors on mitogen-induced activation of S6Kα and S6Kβ

4.2.2.1. Dose-dependent inhibition of S6Kα and S6Kβ by rapamycin and wortmannin

Specific inhibitors of mTOR/FRAP and PI-3'-K pathways, namely rapamycin and wortmannin respectively, have been shown to inhibit agonist-induced activation of S6Kα. To investigate the sensitivity of S6Kβ to these drugs, HEK293 cells were transiently transfected with either EE-S6KαII or EE-S6KβII, serum-starved for 24 hours and then stimulated with 10% FBS for 15 min. Various concentrations of rapamycin and wortmannin were added 20 min prior to the stimulation. The recombinant kinases were then immunoprecipitated with anti-EE-tag antibodies and their specific activities were assessed in an in vitro S6 kinase assay. As shown in Figure 4.3, serum-induced activation
**Fig. 4.2.** Kinetics of integrin-dependent S6KαII and S6KβII activation in HEK 293 cells. HEK 293 cells were transiently transfected with EE-tagged S6KαII or S6KβII. After 24 hours starvation in serum-free medium cells were removed from tissue culture dishes by trypsinization, incubated in suspension for 60 min, and then replated onto fibronectin coated dishes for indicated periods of time. Anti-EE immunoprecipitates were analysed for S6 kinase activity (A, B) as described in the legend for Fig. 4.1. Typical results from three independent experiments are shown. Protein levels of S6Ks in immune complexes were assessed in parallel by Coomassie staining (C).
of S6KαII and S6KβII was inhibited by rapamycin and wortmannin in a dose-dependent manner. The results indicated that pretreatment of HEK293 cells with wortmannin prior to serum stimulation had a similar inhibitory effect on activation of both kinases (Figure 4.3.A.): 65-70% inhibition by 100 nM wortmannin and 92-97% inhibition by 1000 nM wortmannin. These responses are similar to those reported previously for endogenous S6Kα in several cell lines as well as recombinant S6KαII expressed in NIH 3T3 and HepG2 cells (Chung et al., 1994; Weng et al., 1995). However, we found that serum-stimulated S6KβII activity was less sensitive to high doses of rapamycin than S6KαII. The extent of inhibition of S6KαII compared with that of S6KβII was about 83% versus 60% by 20 nM rapamycin and 98% versus 67% by 200 nM rapamycin (Figure 4.3.B.).

Taken together, these results suggest that S6Kβ is regulated by upstream signals dependent on mTOR/FRAP and PI-3'-kinase similarly to S6Kα. However, the potency of inhibition by rapamycin for S6KβII was significantly lower than that for S6KαII. This suggests that mTOR/FRAP-dependent inputs may regulate S6KαII and S6KβII activation via alternative mechanisms.

4.2.2.2. The effect of N- and C-terminal deletions on the sensitivity of S6Kα and S6Kβ to inhibition by rapamycin and wortmannin

Carboxyl-terminal deletion of S6KαI and S6KαII was shown to reduce the extent of maximal inhibition produced by rapamycin from about 95% in the full-length S6Kα to 60-80% in the C-terminal deletion mutant, and additional truncation of the N-terminus conferred total rapamycin resistance of doubly truncated mutant (Cheatham et al., 1995; Weng et al., 1995). However, these mutants of S6Kα were shown to have an intact sensitivity to inhibition by wortmannin. These data allowed us to hypothesize that the differences in sensitivity to rapamycin between S6KαII and S6KβII could be assigned to their amino- and carboxyl-terminal regulatory regions, which share low level of homology.

To verify this hypothesis, the effects of N- and C-terminal truncations on the activity of S6KαII and S6KβII were investigated. HEK293 cells transiently expressing the full-length recombinant S6KαII and S6KβII polypeptides and each of the deletion variants (S6KαΔN75, S6KαΔC100, S6KβΔN64 or S6KβΔC81) were treated with 200 nM rapamycin or 1000 nM wortmannin for 20 min prior to serum stimulation. After 15 min of stimulation, cells were lysed and recombinant kinases immunoprecipitated and analyzed
Fig. 4.3. Dose-dependent inhibition of S6KαII and S6KβII by rapamycin and wortmannin. Serum-starved HEK 293 cells expressing EE-S6KαII and EE-S6KβII were treated with 0.01% Me₂SO vehicle or indicated concentrations of wortmannin (A) and rapamycin (B) for 20 min prior to stimulation with 10% FCS. Cells lysates were normalised for total protein content following Bradford Assay, and equal EE-S6K expression levels were confirmed by immunoblotting. Equal amounts of protein were subjected to immune complex S6 kinase assay. The results are presented as the percentages of serum-stimulated activity in the absence of inhibitor (normalised to 100%).
for their specific activities towards ribosomal S6 protein. We have found that deletion of 75 or 64 amino acids from the amino-terminus of S6KαI and S6KβI respectively had a strong inhibitory effect on S6 kinase activity of deletion mutants generated. The basal and serum-stimulated activities of both S6KαΔN75 and S6KβΔN64 were reduced over 90% when compared to that of full-length recombinant S6KαII and S6KβII (data not shown). It has previously been described that the deletion of 46 and 54 amino acids from the N-terminus of S6Kα has strong inhibitory effect on its kinase activity (Cheatham et al., 1995; Weng et al., 1995). These findings suggest that the N-terminal fragment of S6Kβ as well as S6Kα is essential for full activation of these kinases. Thus, the sensitivity of S6KαΔN75 and S6KβΔN64 to wortmannin and rapamycin could not be correctly tested and interpreted since their activities are already suppressed by N-terminal deletion. By contrast, serum-induced activation of S6KαΔC100 and S6KβΔC81 deletion mutants is similar to that of wild-type kinases. We observed that their activities are stimulated by 4.7 and 6.8 folds, respectively, upon serum induction (Figure 4.4.). Consistent with previous reports, the S6KαΔC100 mutant possessed less sensitivity to rapamycin when compared to the full-length kinase. The serum-induced activities of S6KαII and S6KαΔC100 were inhibited by 97% and 62%, respectively, following treatment with 200 nM rapamycin. Interestingly, the removal of the S6Kβ C-terminal region did not significantly alter the sensitivity of the kinase to rapamycin. Thus, extent of inhibition of S6KβΔC81 by 200 nM rapamycin was about 60% that is comparable to those observed for S6KβII and S6KαΔC100.

The impact of carboxyl-terminal deletion on the ability of wortmannin to inhibit S6KαΔC100 and S6KβΔC81 activation was also examined. As shown in Figure 4.4., both S6KαΔC100 and S6KβΔC81 mutants exhibited over 90% of inhibition by 1000 nM wortmannin. The extent of inhibition of deletion mutants was comparable to that observed for the full-length S6KαII and S6KβII. These results clearly indicate that the C-terminal domains of both kinases do not mediate their sensitivities to inhibition by wortmannin.

In conclusion, we present the evidence that C-terminal truncation of S6KαII, but not S6KβII, significantly reduces its sensitivity to rapamycin. Since the activity of S6KαΔC100, S6KβII and S6KβΔC81 were inhibited to the same extent, we concluded that loss of the C-terminus of S6KαII is sufficient to eliminate differences in sensitivity to rapamycin between S6KαII and S6KβII isoforms.
Fig. 4.4. Sensitivity of S6Kα and S6Kβ C-terminal truncation mutants to rapamycin and wortmannin. HEK 293 cells were transiently transfected with EE-tagged S6KαII, S6KαΔC100, S6KβII or S6KβΔC81. Cells were serum-starved and then pre-treated with 0.01% Me2SO, 200 nM rapamycin or 1μM wortmannin for 20 min prior to stimulation with 10% FCS. S6 kinase activity of each recombinant construct was assayed after immunoprecipitation as described in Experimental procedures and in the legend for Fig 4.3. Results are expressed as described for Fig 4.3., except that each value is adjusted to that for corresponding mutant in the presence of serum alone.
4.2.3. The role of conventional and novel PKCs in the regulation of S6K signaling pathway

4.2.3.1. Analysis of phorbol ester induced activation of S6K isoforms

In many cell types, phorbol esters were demonstrated to induce PKC-mediated activation of S6Kα (Chung et al., 1994; Herbert et al., 2000; Law et al., 2000; Monfar et al., 1995). A detailed analysis of time-course stimulation revealed that PMA induces a delayed response when compared with serum, EGF or PDGF induction, suggesting that PKC may exert its effect on the late phase of S6Kα activation (Susa et al., 1989; Susa et al., 1992).

To examine the effect of phorbol esters on S6Kβ activity, recombinant EE-S6KαII and EE-S6KβII were transiently expressed in HEK293 cells. The transfected cells were serum-starved and then treated with 1 μM PMA for up to 150 min. The kinase activities of recombinant S6KαII and S6KβII were monitored over this time period. As shown in Figure 4.5., the kinetics of PMA-induced activation of S6KαII and S6KβII was similar and characterized by slow increase of kinase activity with the peak of activation at 60 min. Deactivation of both kinases was also slow by getting to the basal level within 90 min. Thus, the kinetics of PMA-stimulated activation of S6KβII is similar to that for S6KαII and characterized by the single peak of activity.

4.2.3.2. The effect of rapamycin, wortmannin and GF109206X on phorbol ester-induced activation of S6Ks

Next, we investigated signaling pathways by which phorbol esters stimulated S6KαII and S6KβII in HEK293 cells. To explore this question the cells transiently expressing EE-S6KαII and EE-S6KβII were treated with 1 μM GF109206X, 50 nM rapamycin or 100 nM wortmannin for 15 min prior to PMA stimulation. As shown, pretreatment of cells with a PKC inhibitor GF109203X completely blocked PMA-induced activation of S6KαII and S6KβII (Figure 4.6). This is consistent with the involvement of PKC in PMA-induced activation of both isoforms of S6 kinase.

In agreement with previous studies (Herbert et al., 2000; Law et al., 2000; Monfar, et al., 1995), rapamycin showed strong inhibitory effect on PMA-induced activation of
Fig. 4.5. Kinetics of phorbol easter induced activation of S6KαII and S6KβII in HEK 293 cells. HEK 293 cells were transiently transfected with EE-S6KαII or EE-S6KβII, serum-starved for 24 hours, and stimulated with 1 μM PMA for the indicated times. Cell lysates were analysed for S6 kinase activity (A, B) as described in the legend for Fig.4.1. Typical results from three independent experiments are shown. Protein levels of S6Ks in immune complexes were assessed in parallel by Coomassie staining (C).
Fig. 4.6. Effects of rapamycin, wortmannin and GF109203X on PMA-stimulated activities of S6KαII and S6KβII. HEK 293 cells were transiently transfected with EE-S6KαII or EE-S6KβII, serum-starved and stimulated with 1μM PMA for 60 min. 1μM GF109203X, 50 nM rapamycin or 100 nM wortmannin were added for 15 min prior to PMA stimulation. Lysates were normalised as described for Fig. 4.3. and used for immune complex S6 kinase assay. The kinase activities are expressed relative to that of PMA-stimulated control (100%).
S6KαII (Figure 4.6). In addition, we found that rapamycin also potently inhibited PMA-stimulated activity of S6KβII. The extent of inhibition of S6KαII and S6KβII were about 92% and 65%, respectively. These results are consistent with the lower sensitivity of S6KβII to the inhibition by rapamycin observed in serum-stimulated HEK293 cells (section 4.2.2.1., Figure 4.3.). Inhibitory effect of rapamycin on PMA-induced activation of S6KαII and S6KβII strongly suggests that mTOR/FRAP activity is essential for PKC-dependent activation of both S6 kinases.

Phorbol esters were shown to activate PI-3-kinase in a number of cell types, including Swiss 3T3 (Petritsch et al., 1995), 3T3-L1 adipocytes (Lin and Lawrence, 1996; Nave et al., 1996), and JB6 cells (Huang et al., 1998). However, the activation of S6Kα in response to PMA stimulation seems to be independent of PI-3-kinase. It has previously been reported that wortmannin at concentrations 50-200 nM had no obvious effect on PMA-induced activation of S6Kα in several cell types (Monfar et al., 1995; Han et al. 1995). At the same time, 100 nM wortmannin was shown to partially inhibit phorbol ester-mediated activation of S6Kα in HepG2 cells (Chung et al., 1994) and Balb/c-3T3 fibroblasts (Conus et al., 1998), suggesting possible involvement of PI-3'-K in this process. Therefore, the actual role of PI-3'-K in the PMA-induced activation of S6Ks remains to be elucidated.

To address this issue, we have investigated the effect of wortmannin on PMA-mediated activation of S6KαII and S6KβII in HEK293 cells. HEK293 cells represent an interesting cellular model for this study since phorbol esters do not stimulate PI-3-K Iα activity in these cells (Herbert et al., 2000). The ability of PMA to activate S6KαII and S6KβII in these cells indicates that cPKC and nPKC might activate S6Ks by a mechanism independent on PI-3'-K Iα activation. Paradoxically, we found that pretreatment of the cells with 100 nM wortmannin had partial inhibitory effect on the activation of both S6KαII and S6KβII isoforms in response to PMA stimulation (Figure 4.6.). Moreover, although potential of 100nM wortmannin to inhibit activation of both S6Ks by PMA was 25-30 % less when compared to that observed in serum-stimulated cells, 1000 nM wortmannin inhibited their activation by over 90% (data not shown and Figure 5.7.). The inhibitory effect of wortmannin on S6K activation in response to PMA most likely reflects the inhibition of basal PI-3'-K Iα activity which may contribute to the total S6K activity in these cells. However, it does not exclude a possibility that a wortmannin-sensitive PI-3-K
isoform distinct from the class Ia might be implicated in S6KαII and S6KβII activation by PMA in these cells.

Taken together, the results demonstrate that activation of S6KαII and S6KβII by PMA is mediated by PKCs and requires signaling input from mTOR/FRAP. Our results also suggest that PI-3-K signaling might be involved in c/n PKC-dependent activation of these kinases in HEK 293 cells.

4.2.3.3. PKC-mediated activation of S6Kβ is dependent on MEK/ERK signaling.

Evidence for the differential regulation of S6Kα and S6Kβ

Because phorbol esters are known to activate ERK1/2 in HEK293 cells (Herbert et al., 2000), we decided to examine whether the MEK/ERK pathway contributes to the c/nPKC-mediated activation of S6Ks. To determine this, we studied the effect of two structurally unrelated inhibitors of MEK, PD098059 and U0126, on PMA-induced activation of recombinant S6KαII and S6KβII in HEK293 cells. Both inhibitors are highly selective for MEK and block effectively signal transduction via the MEK/ERK pathway. As shown in Figure 4.7., pre-treatment of the cells with neither 25 μM PD098059 nor 5 μM U0126 had any obvious effect on S6KαII activation by PMA. In contrast to that, 25 μM PD098059 and 5 μM U0126 suppress PMA-induced activation of S6KβII by 54% and 68%, respectively. These results indicate that MEK/ERK signaling makes an important contribution to S6KβII, but not S6KαII, activation in response to PMA and therefore provide evidence of differential regulation of these S6K isoforms in HEK293 cells.

4.3. Discussion

To better understand the regulation of S6 kinase signaling we have studied the dynamics of S6KαII and S6KβII activation in response to diverse mitogenic stimuli, such as serum, fibronectin and PMA. All these stimuli potently activated both S6KαII and S6KβII, suggesting that these S6K isoforms can be regulated via common signaling pathways. However, kinetics of their activation in response to serum and fibronectin was found to be distinct. In agreement with other reports (Kahan et al., 1992; Oliver et al., 1988; Susa et al., 1992), we have shown serum-stimulated biphasic activation of S6KαII, characterized by the presence of early and late peaks of S6K activity. Both phases of S6K
Fig. 4.7. Effects of PD098059 and U0126 on PMA-stimulated activities of S6KαII and S6KβII. HEK 293 cells were transfected and stimulated with PMA as described for Fig. 4.7. 25 μM PD098059 or 5 μM U0126 were added for 30 min prior to stimulation. Cell lysates were prepared as described in the legend for Fig. 4.3. Recombinant S6Ks were immunoprecipitated with anti-EE-tag antibody and used for in vitro S6 kinase assay. The results are expressed as described for Fig 4.6.
activation were shown to be important for mitogenesis, however the late kinase activity was found to have the major impact on mitogenic response to growth factors (Susa et al., 1989; Simm et al., 1998). The activation profile of S6KβII was similar to that of S6KαII but the relative peaks of their activities were shifted in time. Activation of S6KβII occurred earlier and was more transient than activation of S6KαII suggesting time-dependent differential regulation of these kinases upon serum stimulation. Differences between profiles of S6KαII and S6KβII activation were also observed in the early period of fibronectin stimulation. Integrin-dependent activation of S6KβII was characterized by the presence of two peaks of specific activity whereas S6KαII activation possessed only one peak of activity which corresponded to the late phase of S6KβII activation. Therefore, we found that S6KβII was activated earlier in response to serum and fibronectin stimulation and might be involved in the regulation of early cellular responses to these agonists. A possible explanation of these results is that an additional mitogen-stimulated signaling pathway(s), which is not specific for S6KαII, contributes to the activation of S6KβII in response to these stimuli. The MEK/ERK pathway could be a good candidate for such regulation. Indeed, all three stimuli tested in this study were reported to efficiently activate ERK1/2 in several cell lines, even though their upstream signaling events are distinct (Ballou et al., 1991; Herbert et al., 2000; Wei et al., 1998; Zhu and Assoian, 1995). It has previously been shown that activation of ERK1/2 reaches a peak at about 5-10 min in serum-stimulated HEK293 cells (Burnett et al., 1998b) and at 40 min in P388D1 macrophages stimulated by adhesion to laminin (Wei et al., 1998). These responses correlate very well at early phase with serum- and fibronectin-induced activation of S6KβII, whereas no obvious correlation is found regarding S6KαII activation (Figure 4.1., Figure 4.1. and Burnett et al., 1998b; Ballou et al., 1991). Another important point which support this hypothesis is that activation of S6Kβ was found to be dependent on MEK/ERK signaling. We have demonstrated that PMA-induced activation of recombinant S6KβII, but not S6KαII, was abrogated by MEK inhibitors (PD098059 and U0126) in HEK293 cells. We also observed a similar inhibitory effect of PD098059 on FGF-2-induced activation of endogenous S6Kβ in H-510 SCLC cells (collaborative work, Pardo et al., 2001). Consistent with our data, the involvement of the MEK/ERK pathway in the regulation of S6Kβ activation by EGF, phenylephrine and endothelin-1 was recently described by other groups (Martin et al., 2001b; Wang et al., 2001). Controversially, although PMA was shown to activate ERK1/2, we do not observe significant differences in
kinetics of PMA-induced activation of S6KαII and S6KβII in HEK293 cells. Furthermore, neither S6KαII nor S6KβII exhibited early phase of activation in response to PMA. This is probably due to the late activation of ERK1/2 induced by PMA which coincides with the late PMA activation of both S6Ks in these cells (Figure 4.5. and Herbet et al., 2000). Taken together, these results suggest that MEK/ERK pathway modulates activation of S6KβII and probably cooperates with other signaling pathways (e.g. PI-3K, mTOR and PKC) involved in this process (see further discussion and Wang et al., 2001).

The activity of S6Ks is most likely determined by a balance between activation/inactivation events. Therefore, the kinetics of S6KαII and S6KβII activation may also depends on sensitivity to and/or specificity of phosphatases which dephosphorylate and inactivate these kinases. Recent reports provided direct evidence implicating protein phosphatase 2A (PP2A) and protein phosphatase 1 (PP1) in the regulation of S6Kα activity (Peterson et al., 1999; Bettoun et al., 2002). However, it remains to be investigated whether these phosphatases are involved in the regulation of S6Kβ activity and how they are regulated by mitogenic stimuli.

To get further insight into the regulation of S6KαII and S6KβII we have studied in details signaling pathways involved in the activation of these kinases. To this end, we have analysed and compared the effects of specific mTOR/FRAP and PI-3'-'-kinase inhibitors, namely rapamycin and wortmannin respectively, on the serum-stimulated activation of S6KαII and S6KβII. Both kinases were similarly inhibited by wortmannin suggesting a resemblance of their regulation by PI-3'-K and its downstream signaling effectors. In agreement with these data, it has been demonstrated that S6Kα and S6Kβ receive an input signals from common effectors of the PI-3'-'-K pathway, including PDK1, PKCζ, Rac and Cdc42 (Martin et al., 2001a). On the other hand, Minami at al. reported that S6KβI was significantly less sensitive to wortmannin than S6KαI (Minami et al., 2001). These discrepancies could be explained by different approaches used by these authors. In their experiments, HEK293 cells transiently expressing S6KαI and S6KβI were maintained all the time in the medium completed with 10% FCS. Then cells were treated with various concentrations of wortmannin in the presence of serum, so S6Ks were already active prior to the treatment with this inhibitor. Therefore, their results may reflect efficiency of dephosphorylation and subsequent inactivation rather than direct effect of PI-3'-'-K inhibition on the activation of S6KαI and S6KβI. However, the activation of phosphatases by wortmannin has not been demonstrated to date. By contrast, we treated serum-starved
cells with wortmannin prior to the stimulation. This approach allowed us to ascertain the actual effect of PI-3'-K inhibition on serum-induced activation of both S6 kinases.

We also demonstrated that rapamycin potently blocked activation of both S6KαII and S6KβII suggesting mTOR/FRAP-dependent regulation of these kinases. However, the ability of rapamycin to suppress activation of S6KαII and S6KβII was different. We found that serum-stimulated activity of recombinant S6KβII was less sensitive to high concentrations of rapamycin when compared to that of recombinant S6KαII. In addition to that, a higher resistance of endogenous S6Kβ to rapamycin was shown in FGF-2-stimulated H-510 SCLC cells (collaborative work, Pardo et al., 2001). Similarly to our findings, S6KβI overexpressed in HEK293 cells was also reported to be less sensitive to rapamycin (Minami et al., 2001). Moreover, the authors demonstrated that phosphorylation at Thr401 in S6KβI, which is closely related to its catalytic activity, was less sensitive to inhibition by rapamycin than phosphorylation at the relative Thr412 in S6KαI. The same group has also shown that activity of S6KβI was inhibited by amino acid withdrawal to a lesser extent than S6KαI activity (Minami et al., 2001). Since amino acid sufficiency regulates S6K activity via mTOR/FRAP pathway (Hara et al., 1998), these findings strongly support the results which indicate the differential rapamycin sensitivity of S6Kα and S6Kβ. However, the resistance of S6Kβ to rapamycin is still controversial as some other reports described strong inhibition of this kinase upon rapamycin treatment (Lee-Frumen et al., 1999; Shima et al., 1998). The failure of these groups to detect higher rapamycin resistance of S6Kβ may be due to the differences in experimental conditions, e.g. duration of rapamycin treatment (time-dependent effect of rapamycin was described by Pearson et al., 1995), time of cell stimulation and/or nature of stimulus, along with a complexity of rapamycin effect. It is well established that rapamycin affects S6Kα activity by inhibition of mTOR/FRAP signaling (Pullen and Thomas, 1997). At the same time it is not clear whether mTOR/FRAP can directly phosphorylate S6Kα at Thr412 in vivo or whether it operates through inhibition of PP2A, a major phosphatase for S6Kα (section 1.3.2.). It is possible that rapamycin may affect both of these mechanisms of mTOR/FRAP-mediated S6Kα activation. However, further analysis is required to elucidate whether these mTOR/FRAP-dependent signaling mechanisms can be applied regarding S6Kα and S6Kβ activation.
Based on the differences in rapamycin sensitivity of S6KαII and S6KβII, we propose that mTOR/FRAP-dependent inputs may differentially regulate activation of these kinases. The inhibitory effect of rapamycin requires intact amino- and carboxyl-terminal domains of S6Kα suggesting their role in mTOR/FRAP-dependent regulation of this kinase (Cheatham et al., 1995; Weng et al., 1995). This fact prompted us to investigate whether N- and C-terminal regions of S6KβII, which differ substantially from those of S6KαII, may contribute to its lower rapamycin sensitivity. For this propose we generated and characterized N- and C-terminal truncation mutants of S6KαII and S6KβII. In agreement with other studies (Cheatham et al., 1995; Weng et al., 1995), we found it difficult to assess rapamycin inhibition of S6KαΔN75, and S6KβΔN64, since their basal and serum-stimulated activities were strongly suppressed by the amino-terminal truncation. This implies importance of the N-terminal domain for the function of S6Kα and S6Kβ. In contrast to the N-terminal truncation mutants, both S6KαΔC100, and S6KβΔC81 efficiently phosphorylated S6 protein and responded to mitogenic stimuli. However, the ability of rapamycin to suppress S6KαΔC100 activity was significantly reduced in comparison with full-length enzyme. This effect was previously described for S6KαI and S6KαII isoforms by other groups (Cheatham et al., 1995; Weng et al., 1995). At the same time, similar deletion of the C-terminal region of S6KβII did not affect the susceptibility of the kinase to inhibition by rapamycin. Thus, we found that serum-induced activity of S6KαΔC100 and S6KβΔC81 were inhibited similarly by rapamycin. These data allowed us to conclude that truncation of the C-terminus of S6KαII is sufficient to eliminate differences in sensitivity to rapamycin between the S6KαII and S6KβII isoforms. One potential explanation of these findings could be the existence of an additional regulatory element in the S6Kα C-terminus that is controlled by the rapamycin sensitive upstream effector and is not present in S6Kβ. The possible candidate for such element is Thr444 in the autoinhibitory segment of S6Kα, which is not conserved and substituted to Val in S6Kβ. It was shown that phosphorylation of Thr444 correlates with S6Kα activation and is sensitive to rapamycin treatment (Weng et al., 1998). However, it remains to be determined which regulatory element in the C-terminus of S6Kα contributes to the differences in rapamycin sensitivity between S6Kα and S6Kβ.

We have also shown that unlike rapamycin, deletion of the carboxyl-terminal region in both S6KαII and S6KβII isoforms did not alter the sensitivity of these kinases to
wortmannin. These results clearly indicate that the PI-3-K-regulated input modulates activation of both S6KαII and S6KβII in similar mode and does not operate through their C-terminal regulatory domains.

Previous studies on signaling by the PDGF receptor demonstrated PI-3-K-independent activation of S6Kα via the PLCγ/DAG/1,4,5-inositol triphosphate/PKC pathway (Chou and Blenis, 1995; Chung et al., 1994). Mitogen stimulation of PLCγ leads to a rapid and transient increase of intracellular calcium through the second messenger inositol triphosphate (Berridge, 1993), as well as the generation of diacylglycerol (DAG) with subsequent activation of PKC (Nishizuka, 1992). However, signaling mechanisms of PKC-dependent regulation of S6Ks remain unclear. In the present study we have investigated signaling pathways by which phorbol ester activate S6KαII and S6KβII in HEK293 cells. PMA offers a paradigm for stimuli acting via PLCγ and is believed to mediate its effect through the activation of the DAG-dependent conventional and novel PKCs. In agreement with this assertion we demonstrated that the PKC inhibitor GF109203X blocked completely PMA-induced activation of S6KαII and S6KβII expressed in HEK293 cells. A similar inhibitory effect on PMA-induced activation of S6KαII and S6KβII was also observed when cells were treated with rapamycin. These results indicate that mTOR/FRAP activity is essential for PKC-dependent activation of these kinases.

Because PI-3'-K plays a crucial role in the activation of S6Ks in response to growth factors, we examined its contribution to PKC-dependent S6K regulation. Concentrations of PMA, sufficient to fully activate S6KαII and S6KβII, do not stimulate PI-3'-K IA in HEK293 cells as was previously shown in anti-phosphotyrosine immunoprecipitate assay (Herbert et al., 2000). This suggests that c/n PKC-mediated activation of S6Ks in HEK293 cells occurs through a mechanism which is independent of PI-3-K IA activation. However, we found that pretreatment of cells with wortmannin or structurally unrelated inhibitor LY294002 (data not shown), resulted in the inhibition of PMA-stimulated activity of S6KαII and S6KβII, despite the lack of PMA-induced activation of PI-3-K IA in these cells. This might be due to the inhibition of basal PI-3'-K IA activity and therefore elimination of its contribution to the total S6K activity. The sufficiency of basal PI-3'-K activity to promote wortmannin-sensitive activation of S6Kα by activated GTPase-deficient alleles of RacV12/Cdc42V12 has been demonstrated in NIH 3T3 cells (Chou and Blenis, 1996). Similarly to these findings, basal activity of MEK has been recently
suggested to be important for insulin-stimulated 4E-BP1 phosphorylation, eIF4F assembly and protein synthesis in HEK293 cells (Herbert et al., 2000). However, it is also possible that wortmannin-sensitive PI-3′-K isoform distinct from the class IA might be implicated in S6KαII and S6KβII activation by PMA in these cells. Wortmannin inhibition of MAPK activation by platelet-activating factor (PAF) through a mechanism independent of the conventional p85/p110 heterodimeric PI-3′-K has already been shown in a microphage cell line (Ferby et al., 1996). Taken together, these data indicate that PI-3-K signaling is essential for c/n PKC-dependent activation of ribosomal S6 kinases in HEK 293 cells.

Another important finding of this study is that PKC-mediated activation of S6KβII, but not S6KαII, depends on MEK/ERK signaling. As mentioned above, PMA-induced activation of S6KβII was blocked by treatment with specific MEK inhibitors, PD098059 and U0126, whereas PMA-stimulated activity of S6KαII was not sensitive to this treatment. These results uncover a novel regulatory connection between MEK/ERK and S6Kβ signaling pathways providing further evidence of the differential regulation of S6Kα and S6Kβ.

In summary, we have demonstrated that S6KαII and S6KβII are regulated by common signaling intermediates, such as PI-3′-K, mTOR/FRAP and c/n PKCs. At the same time, the differential regulation of these S6 kinase isoforms by distinct signaling mechanisms was shown in this work. Further studies will be required to ascertain whether selective regulation of S6KαII and S6KβII determine their specific physiological functions.
CHAPTER 5

SUBCELLULAR LOCALISATION OF S6Ks. REGULATION OF S6K NUCLEOCYTOPLASMIC SHUTTLING BY PKC
CHAPTER 5

SUBCELLULAR LOCALISATION OF S6Ks. REGULATION OF S6K NUCLEOCYTOPLASMIC SHUTTLING BY PKC

5.1. Introduction

As mentioned above, S6Kα and S6Kβ are represented by two splice variants with distinct subcellular distribution. The 23- and 13-amino acid extensions at the N-termini of S6KαI and S6KβI contain nuclear localisation signals that target these isoforms constitutively to the nucleus (Coffer and Woodgett, 1994; Minami et al., 2001; Reinhard et al., 1994). The cytoplasmic isoform of S6Kα is predominantly cytosolic, but can also accumulate in the nucleus when cells are treated with Leptomycin B, suggesting nucleocytoplasmic shuttling of this kinase (Kim and Chen, 2000). In contrast to S6KαII, the S6KβII isoform is localised mostly in the nucleus. Furthermore, the presence of a functional nuclear localisation signal at the C-terminus of S6Kβ, which is found in both splice variants, has recently been reported (Koh et al., 1999). Because Cdc42, Rac, and PDK1, thought to be cytosolic and associated with membranes, are potent activators of S6KβII, it was hypothesized that S6KβII may shuttle between the nucleus and the cytosol during the course of its activation (Martin et al., 2001). However, this assumption required experimental confirmation. Furthermore, characterization of the subcellular distribution of individual S6K isoforms would provide more information on their regulation and physiological functions.

Here we present detailed analysis of subcellular localisation of S6Kα and S6Kβ isoforms. Molecular mechanisms regulating nucleocytoplasmic redistribution of S6KβII in response to mitogenic stimuli were studied using confocal microscopy and site-directed mutagenesis.
5.2. Results

5.2.1. The effect of PMA and leptomycin B on subcellular localisation of S6Ks.

Initially, we examined subcellular localisation of EE-S6Ks in transiently transfected HEK293 cells non-stimulated or stimulated with PMA. As shown in Figure 5.1, S6KαII was mainly localised in the cytoplasm of serum-starved cells and PMA stimulation did not change its pattern of distribution. However, pretreatment of cells with leptomycin B (LMB), an inhibitor of Crm1-dependent nuclear export, leads to accumulation of S6KαII in the nucleus, suggesting dynamic nucleocytoplasmic shuttling of this isoform. These data are in agreement with studies carried out by other groups (Kim and Chen, 2000; Koh et al., 1999).

By contrast, S6KβII was found predominantly in the nucleus of serum-starved cells and shifted to the cytoplasm after PMA treatment (Figure 5.1.). Moreover, leptomycin B prevents PMA-stimulated accumulation of S6KβII in the cytoplasm as seen by the retention of the kinase in the nucleus. This finding indicates that S6KβII may shuttle between the nucleus and the cytosol during the course of its activation. The effect of PMA on subcellular localisation of S6KβII strongly suggests the involvement of PKCs in the regulation of its nucleocytoplasmic shuttling. In contrast, the nuclear localisation of S6KβI, which contains two nuclear localisation signal (NLS) sequences, one at the N- and another at the C-terminus, is not affected by treatment with PMA or Leptomycin B.

Taken together, these results indicate the existence of LMB-sensitive nucleocytoplasmic shuttling for S6KβII which can be specifically regulated by mitogenic stimulus, such as PMA. We also found that the presence of the additional N-terminal NLS in S6KβI is sufficient to prevent cytoplasmic redistribution of this S6Kβ isoform in response to PMA stimulation. In contrast to S6KβII, nucleocytoplasmic shuttling of S6KαII was shown to be not affected by PMA treatment suggesting differential regulation of their subcellular localisation.

Given the effect of PMA on redistribution of S6KβII between cytosol and nucleus, we aimed to examine a possible role of PKCs in the control of subcellular localization of this isoform of S6 kinase.
Fig. 5.1. Analysis of subcellular localisation of S6Kα and S6Kβ by confocal microscopy. HEK 293 cells were transiently transfected with wild-type EE-S6KαII, EE-S6KβI or EE-S6KβII, serum-starved for 24 hours and stimulated with 1 μM PMA for 30 min or vehicle alone. Treatment of cells with leptomycin B (10 ng/ml) was carried out for 16 hours before the stimulation with PMA. Cells were fixed, probed with anti-EE antibody and FITC-labelled anti-mouse IgG and analysed by confocal microscopy.
5.2.2. S6Kβ but not S6Kα is phosphorylated in vitro at the C-terminus by different isoforms of PKC

Inspection of the amino acid sequence of S6Kβ revealed a potential PKC phosphorylation site located within the C-terminal nuclear localisation signal (Figure 5.2.A). S6Kα displays a low level of identity with S6Kβ at the C-terminus and does not contain consensus sequences for phosphorylation by PKC. To test whether PKC phosphorylates S6Kβ, we initially employed an in vitro kinase assay. The C-terminal regions of S6Kα and S6Kβ (His-S6KαC and His-S6KβC), expressed in bacteria as His-tag fusion proteins (chapter 3, section 3.2.4.), were used as substrates in a PKC phosphorylation assay. As shown in Figure 5.2.B, all PKC isoforms tested efficiently phosphorylated His-S6KβC, whereas no significant phosphorylation of His-S6KαC was observed under similar conditions. The activities of the PKC isoforms were analysed using histone H1 or ε-peptide as substrates (Figure 5.3.). It should be noted that the efficiency of His-S6KβC phosphorylation by PKCs correlated with their specific activities.

Next, we investigated whether full-length S6KαII and S6KβII could serve as substrates for PKCs in an in vitro kinase assay. In this experiment, transiently expressed EE-tagged forms of S6KαII and S6KβII were immunoprecipitated from serum-starved HEK293 cells and subjected to in vitro phosphorylation by different isoforms of PKC. The results demonstrated that all isoforms of PKC readily phosphorylated full length S6KβII, but failed to use S6KαII as a substrate (Figure 5.2.C). We have also observed higher efficiency of S6KβII phosphorylation by PKCβI, PKCβII and PKCδ (2, 1.5 and 4 fold increase, respectively), when compared with other isoforms.

To confirm that the PKC phosphorylation site is located within the C-terminus of S6Kβ, we created N- and C-terminal deleted mutants and tested whether they were phosphorylated by PKCs under the conditions described for the full-length kinases. As shown in Figure 5.2.C, deletion of the N-terminal region of S6Kβ did not affect the efficiency and the pattern of phosphorylation by PKC isoforms. However, the removal of the C-terminus completely abolished PKC-mediated phosphorylation of S6KβII. The data presented above clearly indicate that S6Kβ can be phosphorylated by PKC in vitro and that the site/s of phosphorylation is/are located at the C-terminus.
Fig. 5.2. **S6KβII, but not S6KαII, is phosphorylated at the C-terminus by different PKC isoforms in vitro.** (A) Schematic representation of S6KβI and S6KβII and their deletion mutants, lacking amino- and carboxyl-terminal sequences. Major domain boundaries are indicated. Structural features are indicated as follows: diagonally hatched box represents catalytic domain; grey box indicates unique proline-rich sequence of S6Kβ; solid black boxes indicate nuclear localisation signals (NLS1 and NLS2); striped box corresponds to potential nuclear export signal (NES). The N- and C-terminal amino acid sequences, containing NES and NLS, are shown above the diagrams. All recombinant constructs carry an N-terminal EE-tag sequence and deleted amino acids are indicated. (B) **In vitro** phosphorylation of bacterially expressed His-S6KαC and His-S6KβC by various PKCs. Affinity purified His-tagged S6Kα and S6Kβ C-terminal peptides were incubated in the presence of different recombinant PKC isoforms and [γ-32P]ATP. The reaction mixtures were separated by SDS–PAGE and stained with Coomassie. The dried gel was analysed by autoradiography. (C) **In vitro** phosphorylation of recombinant full-length S6KαII,S6KβII and deleted S6KβII mutants by PKCs. HEK 293 cells transiently transfected with wild-type EE-S6KαII, EE-S6KβII, EE-S6KβIIΔN or EE-S6KβIIΔC, were serum-starved for 24 hours and recombinant proteins immunoprecipitated with anti-EE-tag antibody. The immunoprecipitates were incubated with [γ-32P]ATP in the absence or presence of different recombinant PKC isoforms. The reaction mixtures were analysed as described above.
Fig. 5.3. Analysis of enzymatic activities of recombinant PKC isoforms. *In vitro* kinase assays were performed as described in “Material and Methods”. Reaction products were analysed by SDS-PAGE followed by autoradiography and Coomassie staining.
5.2.3. Identification of PKC phosphorylation sites in S6Kβ by mass spectrometry. Generation, affinity purification, and characterization of phospho-specific S6Kβ antibody

The precise identification of PKC phosphorylation site/s in S6KβII was carried out by mass spectrometry. Affinity purified His-S6KβC was used as a substrate for PKCβII in the presence of cold ATP. The products of the reaction were digested by trypsin or endoproteinase Lys-C and resulting peptides were analysed by mass spectroscopy. Initial MALDI MS analysis of the intact or trypsin in-gel digested His-S6KβC was inconclusive with regard to PKC phosphorylation. However, proteolysis with the endoproteinase Lys-C produced phosphorylation indicative peptides (Figure 5.4.A). The recorded peptide ions from the MALDI MS analysis show that the main phosphorylation is located in the KS486K sequence stretch suggesting serine as the phosphorylation site. The stoichiometry of S6KβII phosphorylation by PKCβII was found to be approximately 1 mole of phosphate per mole of S6KβII.

Phosphospecific antibodies are a powerful tool in investigating the physiological importance of protein phosphorylations. We therefore generated an antibody that specifically recognizes S6Kβ phosphorylated at Ser486. The antibodies were raised in rabbits and affinity purified on Actigel beads coupled with antigenic peptide as described in chapter 2, section 2.3. The quality of antibody purification was tested in each elution fraction as shown in Appendix D, Supplementary Figure 4. Affinity purified antibodies from fraction 2 and 3 were combined, screened for antigen reactivity by immunoblot analysis, and used in this study. To test the specificity of the antibody generated we used recombinant His-S6KβC pre-phosphorylated with PKCβII. As shown in Figure 5.4.B and C, affinity purified anti-pS486 antibody specifically recognized His-S6KβC only when it was pre-phosphorylated by PKCβII. Furthermore, the recognition of phosphorylated His-S6KβC by anti-pS486 antibody was abolished by pre-incubation with the phosphorylated, but not with the non-phosphorylated form of the antigenic peptide (data not shown).
Fig. 5.4. Identification of PKC phosphorylation site and characterisation of phospho-specific S6Kβ antibody. (A) Mass-spectroscopy analysis of PKC phosphorylation site in S6KβII. Amino acid sequence of His-S6KβC is shown on top. (B)/(C) Analysis of specificity of anti-pS486 antibody. Bacterially expressed His-S6KβC was incubated with [γ-32P]ATP in the presence or absence of recombinant PKCβII. Samples were resolved by SDS–PAGE, transferred onto nitrocellulose membrane, and analysed by autoradiography (B) or by immunoblotting with anti-pS486 antibody (C).
5.2.4. Analysis of S6KβII phosphorylation at Ser486 in cellular responses to mitogenic stimuli

The availability of a phosphospecific antibody has allowed us to study the phosphorylation status of S6KβII at Ser486 in response to various extracellular stimuli. We found that treatment of HEK293 cells transiently overexpressing EE-S6KβII with PMA induced a significant (up to 15 fold) increase in Ser486 phosphorylation (Figure 5.5.A). A time-course stimulation of cells with PMA demonstrates that phosphorylation of Ser486 is very rapid and reaches a peak at 30 minutes, but is still detectable even 24 hours after induction (Figure 5.5.B). Noticeably, phosphorylation at Ser486 parallels the activation profile of S6KβII, as seen from the mobility shift of activated forms of the kinase (Figure 5.5.B).

We consistently observed an increase (1.5-3 fold) in Ser486 phosphorylation when starved HEK293 cells were treated with FCS, insulin or PDGF (Figure 5.5.A). In the case of FCS stimulation, the changes in Ser486 phosphorylation followed a time-course similar to that seen for PMA (Appendix E, Supplementary Figure 5.). When we compared the increase of S6 kinase activity of exogenously expressed S6KβII with the extent of Ser486 phosphorylation in response to PMA, FCS, insulin and PDGF, no obvious correlation was observed (Figure 5.5.A and C). These results suggested that phosphorylation of S6Kβ at Ser486 might not affect its kinase activity or its activation by other kinases.

It was recently demonstrated that S6KβII is expressed at high level in cardiomyocytes (ARVC) and is activated by treatment with insulin or phenylephrine (Wang, et al., 2001a). In contrast to S6Kα, which is known to be activated in cardiomyocytes via the PI3-K and mTOR signaling pathways, the activity of S6Kβ can also be regulated in a MEK-dependent manner. Moreover, studies from other laboratories show that treatment of cardiomyocytes with insulin and phenylephrine induces rapid activation of PKC (Pellieux et al., 2000; Puceat et al., 1994). Therefore, this cellular model was used to investigate whether endogenous S6Kβ is phosphorylated at Ser486 in response to insulin and phenylephrine. We treated ARVC with 20 nM insulin or 10 μM phenylephrine for 30 min and the endogenous S6Kβ was immunoprecipitated with C-terminal polyclonal antibodies. Western blot analysis of immune complexes, resolved by SDS-PAGE, with anti-pS486 antibodies indicated that S6Kβ is specifically phosphorylated at Ser486 in cardiomyocytes treated with insulin and phenylephrine (Figure 5.5.D). Thus endogenous S6Kβ in primary
Fig. 5.5. S6KβII is phosphorylated at Ser486 in response to different mitogenic stimuli. HEK 293 cells were transiently transfected with wild-type EE-S6KβII, serum-starved and stimulated with 10% FCS, 1μM PMA, 100nM insulin, 50ng/ml PDGF or vehicle alone. Recombinant S6KβII was immunoprecipitated with anti-EE antibody and used for in vitro S6 kinase assay (B) or analysed by Western blotting with anti-pS486 antibody (A). (C) Time-course phosphorylation of S6KβII at Ser486 in PMA-treated HEK293 cells. HEK 293 cells were transiently transfected with wild-type EE-S6KβII, serum-starved for 24 hours, and stimulated with 1 μM PMA for the indicated period of time. Cell lysates were analysed by Western blotting with anti-pS486 or anti-EE antibodies. (D) Phosphorylation of endogenous S6Kβ at Ser486 in PE-stimulated cardiomyocytes. Isolated cardiomyocytes were treated with 10 μM phenylephrine, 10 nM insulin or vehicle alone for 30 min. Native S6Kβ was immunoprecipitated from lysed cells with anti-C-terminal antibodies. Immune complexes were separated by SDS-PAGE and immunoblotted with anti-pS486 antibody. The results presented have been reproduced in three independent experiments.
cells undergoes phosphorylation at Ser486 in response to a physiological agonist that activates PKC.

5.2.5. PKC mediates phosphorylation of S6KβII at Ser486 and rpS6 in vivo

The in vitro phosphorylation studies and the ability of PMA to induce S6KβII phosphorylation at Ser486 strongly suggested the involvement of PKC. In order to examine whether PKC could mediate phosphorylation of S6KβII at Ser486 in vivo, the EE-S6KβII was transiently co-expressed with various myc-tagged PKCs in HEK293 cells. Two days after transfection, S6KβII was immunoprecipitated with anti-EE antibodies, resolved by SDS-PAGE and immunoblotted with anti-pS486 antibodies. The results indicated that co-expression of any PKC isoform with S6KβII induces strong phosphorylation of Ser486 (Figure 5.6.A). Coomassie staining of the PVDF membrane showed that an equal amount of EE-S6KβII was immunoprecipitated from all transfected cells. Western blotting of total cell lysates with anti-myc antibodies confirmed that all PKC isoforms were expressed at approximately equal levels (Figure 5.6.A, bottom section).

To further establish that S6KβII is a target for PKC mediated phosphorylation in vivo, we tested the effect of a PKC inhibitor, GF109203X on Ser486 phosphorylation in response to PMA. As shown in Figure 5.6.B, treatment of HEK293 cells expressing EE-S6KβII with 1μM concentration of GF109203X completely abrogated PMA-induced phosphorylation at Ser486. Collectively, the results presented above strongly suggest that PKCs mediate in vivo phosphorylation of S6KβII at Ser486.

Ribosomal protein S6 is thought to be a physiological substrate for both S6Kα and S6Kβ (Shima et. al., 1998 and section 3.2.3.). Phosphorylation of rpS6 is one of the earliest events detected following mitogenic stimulation and correlates with polysome formation and initiation of protein synthesis (Stewart and Thomas, 1994). Multiple studies have shown that different mitogenic stimuli employ distinct signaling pathways to mediate rpS6 phosphorylation and the initiation of protein synthesis. Taking this into account, it was interesting to examine the contribution of PKC signaling to in vivo phosphorylation of rpS6. MCF7 cells were chosen for this study since they express large quantities of both S6KαII and S6KβII, as determined by immunoblot and Northern blot analysis (data not shown). Treatment of serum-starved MCF7 cells with PMA induces 5 fold increase in the
Fig. 5.6. *In vivo* phosphorylation of S6KβII at Ser486 and ribosomal protein S6 phosphorylation are mediated by PKC. (A) Co-expression of various PKCs with S6KβII induces phosphorylation at Ser486 in HEK 293 cells. HEK 293 cells were co-transfected with EE-S6KβII and various Myc-PKCs. Recombinant S6Kβ was immunoprecipitated with anti-EE-tag antibody and analysed by Western blotting with anti-pS486 antibody. Expression levels of transiently expressed PKCs were analysed in whole-cell extracts with anti-Myc antibody. (B) Effect of PKC inhibitor GF109203X on Ser486 phosphorylation. HEK 293 cells were transiently transfected with wild-type EE-S6KβII, serum-starved and stimulated with 1μM PMA. 1μM GF109203X was added for 30 min prior to stimulation. (C) Effect of GF109203X on PMA-stimulated phosphorylation of rpS6. MCF7 cells were serum-starved for 24 h and then treated with 1 μM PMA or vehicle alone for 30 min. 1 μM GF109203X was added for 30 min prior to stimulation. Phosphorylation of S6 protein was analysed in whole-cell extracts with anti-phospho rpS6 (Ser235) antibody.
level of rpS6 phosphorylation at S235 (Figure 5.6.C). This increase was completely inhibited by 1µM GF109203X, strongly indicating that signaling via PKC is important for rpS6 phosphorylation in response to PMA. Further studies are required to ascertain whether PKC-mediated phosphorylation of S6KβII at Ser486 might be important for rpS6 phosphorylation stimulated by PMA.

5.2.6. PKC-mediated phosphorylation of S6KβII at Ser486 does not affect S6 kinase activity

Since S6K is activated by multiple Ser/Thr phosphorylations, it was important to investigate the effect of Ser486 phosphorylation on S6Kβ activity. In order to answer this question and to study the upstream regulation of Ser486 phosphorylation we used two indirect inhibitors of S6K, rapamycin (mTOR pathway) and wortmannin (PI3-K pathways).

Treatment of serum-starved HEK293 cells with PMA induced a 4 fold increase in the activity of recombinant S6KβII towards ribosomal S6 protein (Figure 5.7). As expected, pre-treatment of cells with rapamycin or wortmannin blocked PMA-induced activation of S6KβII. Noticeably, rapamycin did not exert any obvious effect on PMA induced phosphorylation of Ser486, while wortmannin showed slight inhibition at very high concentrations (Figure 5.7).

These results have also been confirmed by in vitro studies. In these experiments, EE-S6KβII was immunoprecipitated from serum-starved HEK293 cells and phosphorylated with different PKC isoforms in the presence of cold ATP. After washing, S6 kinase activity towards ribosomal S6 protein was measured. This experiment revealed that pre-phosphorylation of S6KβII by PKCs does not affect its S6 kinase activity (Appendix F, Supplementary Figure 6).

To gain further insight into the importance of PKC-mediated phosphorylation of S6KβII, we mutated serine 486 to alanine. It is important to note that anti-pS486 antibodies did not recognize the mutated form of S6KβII overexpressed in HEK293 cells, confirming their specificity. Moreover, the activity of the S486A mutant was found to be similar to that of the wild type kinase in HEK293 cells treated or non-treated with PMA (Figure 5.7).
Fig. 5.7. PKC-mediated phosphorylation of S6KβII at Ser486 is insensitive to specific TOR/FRAP and PI-3' kinase inhibitors and does not effect S6 kinase activity. HEK 293 cells were transiently transfected with wild-type EE-S6KβII or EE-S6KβII S486A and incubated in the presence or absence of 1µM PMA for 30 min after 24 hours starvation. Rapamycin or wortmannin were added for 30 min before cell stimulation. Recombinant S6KβII was immunoprecipitated with anti-EE-tag antibody and used for in vitro S6 kinase assay or analysed by immunoblotting with anti-pS486 antibody.
Taken together, the results demonstrate that PKC-mediated phosphorylation of S6KβII at Ser486 does not effect the activity of the kinase in response to mitogenic stimuli.

5.2.7. Phosphorylation at Ser486 abrogates the function of the nuclear localisation signal in S6KβII.

Since Ser486 is located within the C-terminal nuclear localisation sequence, it was reasoned that PKC-mediated phosphorylation of this site might modulate the subcellular localisation of S6KβII. To test this possibility, we examined the subcellular distribution of pS486-S6KβII in PMA-stimulated cells using the phosphospecific antibody. Confocal immunofluorescence microscopy clearly indicated that pS486-S6KβII is localised exclusively in the cytoplasm of PMA-treated cells (Figure 5.8.A). No signal was detected in serum-starved cells, confirming once again the specificity of the phosphospecific antibodies. It was interesting to study whether blocking of nuclear export with leptomycin B affected the subcellular localisation of pS486-S6KβII. As shown in Figure 5.8.A, the pattern of pS486-S6KβII distribution did not change when cells were treated with both PMA and leptomycin B. These results have also been confirmed by cell fractionation experiments (Appendix G, Supplementary Figure 7.). These results strongly suggest that PMA-induced phosphorylation of S6KβII at Ser486 takes place in the cytoplasm and prevents translocation of the kinase to the nucleus. Moreover, we have analysed subcellular localisation of pS486-S6KβII in NIH 3T3 cells stimulated with other mitogenic stimuli. Figure 5.8.B shows that pS486-S6KβII is localised in cytoplasm of NIH 3T3 cells stimulated with EGF, IGF-1, insulin or PDGF. However, the signal is significantly weaker when compared with PMA stimulation. These data are in agreement with anti-pS486-S6Kβ immunoblot analysis presented in Figure 5.5.A.

Substitution of phosphorylated site for acidic amino acids mimics, in many cases, the phosphorylation of that site in the protein of interest and therefore provides an excellent model for functional studies. To this end we have generated a Ser486 to E mutant of S6KβII and analysed its subcellular localisation with the expectation that it would be present only in the cytoplasm of transfected cells. Unexpectedly, the S486E mutant behaved similarly to the wild type protein in serum-starved and PMA-stimulated cells (Figure 5.9.). However, we found that the S486A mutant is predominantly localised in the nucleus of serum-starved cells and does not accumulate in the cytoplasm in response to
Fig. 5.8. Subcellular localisation of pSer486-S6KβII in HEK 293 and NIH 3T3 cells. (A) HEK 293 cells were transfected with EE-S6KβII and treated in the same way as described for Fig. 5.1. After fixation and probing with anti-pS486 antibody, confocal microscopy analysis was carried out. (B) Subcellular localisation of pSer486-S6KβII in NIH 3T3 cells treated with PMA, EGF, IGF-1, insulin or PDGF. Transient transfection and stimulation of NIH 3T3 cells were performed as described for Fig. 5.5. Immunofluorescent analysis was carried out as described above.
Fig. 5.9. Subcellular localisation of S6KβII mutants in HEK 293 cells. Plasmids, carrying EE-S6KβII, EE-S6KβII S486E, EE-S6KβII S486A, EE-S6KβII T401D/S486E or EE-S6KβII T401D/S486A were transfected into HEK 293 cells. After 24 h cells were serum-starved and stimulated with 1 µM PMA for 30 min or vehicle alone. Fixed cells were incubated with anti-EE antibody and analysed by immunofluorescence.
PMA. A possible explanation of observed differences in subcellular localisation of S486E and S486A mutants could be that regulation of nucleocytoplasmic shuttling by phosphorylation of S6KβII at this site requires the kinase to be in an activated state. To test this hypothesis, we created a double mutant of S6KβII, bearing T401D and S486E substitutions. We have previously demonstrated that a T401D (equivalent to Thr412 in S6Kα) mutant is constitutively active and possesses 3-4 times higher S6 kinase activity than the wild type S6KβII (chapter 3, section 3.2.3.).

The results of immunofluorescence analysis unambiguously demonstrate that the T401D/S486E mutant is retained in the cytoplasm of serum-starved and PMA-treated cells (Figure 5.9.). The importance of Ser486 phosphorylation in controlling nuclear shuttling of the activated form of S6KβII was further confirmed with the use of a T401D/S486A mutant. This mutant was found to be localised predominantly in the nucleus of serum-starved cells and did not accumulate in the cytoplasm in response to PMA.

Taken together, the results of immunofluorescence microscopy clearly demonstrate that PMA-mediated phosphorylation of S6KβII at Ser486 regulates nucleocytoplasmic shuttling of an activated form of the kinase. Since Ser486 is located in the middle of the C-terminal nuclear localisation signal, we propose that phosphorylation of this residue abrogates its function.

5.3. Discussion

In this study we have addressed the role of PKC signaling in the regulation of nucleocytoplasmic shuttling of S6KβII. We found that S6Kβ, but not S6Kα, is phosphorylated by PKCs in vitro and in vivo. The site of phosphorylation was identified by mass spectrometry as Ser486, which is located in the C-terminal regulatory domain. Furthermore, the use of phosphospecific antibodies indicated that Ser486 phosphorylation is induced by various mitogenic stimuli, including PMA, FCS, EGF, IGF-1, insulin and PDGF.

Studies from different laboratories demonstrated that activation of S6Kα is a multi-step phosphorylation process, involving at least 9 sites and various S/T kinases (Dennis et al., 1998; Weng et al., 1998). Most of these sites are conserved in S6Kβ, with the exception of one (equivalent to T444 in S6Kα), indicating a very similar mode of activation. In
contrast, this study clearly demonstrates that PKC-mediated phosphorylation of S6KβII at Ser486 is not involved in the regulation of its kinase activity.

What is the importance of Ser486 phosphorylation for the cellular functions of S6KβII? In agreement with previous reports, we detected S6KαII mainly in the cytoplasm, while S6KβII was predominately nuclear (Kim and Chen, 2000; Koh et al., 1999). The presence of a functional nuclear localisation signal at the C-terminus of S6KβII has been recently reported by Koh et al. (Koh et al., 1999). The authors also found that substitution of Lys487 to Met in the KKS487RGR sequence of S6KβII relocates the kinase from the nucleus to the cytoplasm. Since Ser486 is located in the middle of the C-terminal NLS, we focused our efforts on elucidating the effect of PKC-mediated phosphorylation of this site on subcellular localisation of S6KβII. Following this assumption, we found that treatment of cells with PMA induced rapid translocation of S6KβII from the nucleus to the cytoplasm, while no changes in the subcellular localisation of S6KαII were observed. Furthermore, this translocation was blocked completely by leptomycin B, a specific inhibitor of CRM1-mediated nuclear export, indicating the existence of nucleocytoplasmic shuttling for S6KβII. Interestingly, neither PMA nor leptomycin B affect subcellular localisation of S6KβI, whose exclusive nuclear distribution is determined by the presence of two NLSs.

A continuous shuttling of S6KβII between the nucleus and the cytoplasm may require the presence of both NLS and NES sequences in S6KβII. During the last few years, a short leucine-rich consensus has been identified in a variety of signaling molecules and shown to possess nuclear export properties (Gorlich and Mattaj, 1996; Nigg, 1997). Inspection of amino acid sequence allowed us to identify a potential nuclear export signal (NES) located at the N-terminus of S6KβII (Figure 5.2.A). This sequence resembles the Crm1 consensus, which is known to be leptomycin B-sensitive. Additional studies employing mutational analysis and confocal microscopy will be required to investigate whether the N-terminal region of S6KβII possesses a functional NES which is leptomycin B-sensitive. The nuclear export receptor for S6KβII also remains to be identified.

Many proteins are transported constitutively into and out of the nucleus by members of the β-importin family of nuclear transport receptors (Gorlich and Kutay, 1999). In contrast to constitutive transport, regulated transport occurs only in response to specific cellular signals and involves a specific NLS receptor, usually α-importin (Kaffman and O'Shea,
The docking of proteins that contain classical or bipartite types of NLS to the cytoplasmic side of the nuclear pore is mediated by an importin-α/importin-β heterodimer and Ran GTPase. The formation of this multiprotein complex can be influenced directly by post-translational modifications, such as phosphorylation, acetylation and methylation (Jans and Hubner, 1996). Is nuclear transport of S6KβII driven by this mode of regulation?

Mutational analysis of the Ser486 site allowed us to gain insight into the regulation of S6KβII nucleocytoplasmic shuttling by PKCs. We observed that a S486A mutant of S6KβII does not accumulate in the cytoplasm in response to PMA, indicating that phosphorylation of Ser486 might be necessary for this event to occur. However, when we tested the subcellular localisation of the S486E mutant, we found unexpectedly that it behaves similarly to the wild type S6KβII. Therefore, phosphorylation of S6KβII at Ser486 is not sufficient on its own to confer cytoplasmic localisation to the kinase. Further mutational studies of Ser486 and T401 (equivalent to T412 in S6KaII) uncovered the dependence of nucleocytoplasmic shuttling of S6KβII on the activated state of the kinase.

A possible explanation of these findings is that S6KβII has to be in an activated state, in which the structure unfolds making both NES and NLS operational. The structure of S6K has not been solved and in the absence of crystallographic data, the primary structure of S6K has been functionally dissected into four domains. Based on these studies a model for S6K activation has been proposed which implies that active conformation of the kinase is achieved by coordinated phosphorylations at three regions: the C-terminal autoinhibitory domain, by Ser-Pro directed kinases; the activation loop in the kinase domain by PDK1; and the conserved hydrophobic site in the kinase-extension domain (Pullen and Thomas, 1997). It is believed that in unstimulated cells, the interaction between the N- and C-terminal regulatory domains keeps the kinase domain in a locked conformation. Following mitogen stimulation, multiple phosphorylations open the structure by unlocking initially the N-terminal domain and subsequently releasing the C-terminal autoinhibitory domain. In agreement with this model, PMA-induced activation of S6KβII may release the N-terminal domain, making the nuclear export signal operational. This may shift the steady-state constants for nuclear export and import, establishing an equilibrium in nucleocytoplasmic shuttling of S6KβII.

Using pS486 phosphospecific antibodies, we discovered that pS486-S6KβII is exclusively localised in the cytoplasm of PMA-treated cells and that leptomycin B does not alter its localisation. These data strongly suggest that phosphorylation of S6KβII at
Ser486 occurs in the cytoplasm of PMA-stimulated cells. Moreover, phosphorylation of S6KβII at Ser486 coincides with the depletion of the kinase from the nucleus and subsequent accumulation in the cytoplasm. We propose that phosphorylation of S6KβII at Ser486 abrogates the function of its sole NLS and, as a result, the kinase is confined to the cytoplasm. This mode of regulation ("NLS-masking") is common among signaling molecules and has been reported for diacylglycerol kinase ζ, Ca2+/Calmodulin-dependent protein kinase II and the forkhead transcription factor AFX (Brownawell et al., 2001; Heist et al., 1998; Topham et al., 1998).

What is physiological relevance of S6KβII translocation from the nucleus to the cytoplasm in response to mitogenic stimuli? One possible explanation is that it brings the kinase in close vicinity to its substrate/s, such as ribosomal protein S6. Knock-out of the S6Kα gene in mice showed that S6 protein is a physiological substrate for S6Kβ (Shima et al., 1998). Mitogen-induced phosphorylation of ribosomal S6 protein is thought to be associated with the initiation of protein synthesis of a specific pool of mRNA, whose gene products are involved in ribosomal biogenesis (Jefferies et al., 1997).

Based on the data presented here and current knowledge on signaling via S6Ks, we propose a model to explain nucleocytoplasmic shuttling of S6KβII in response to mitogenic stimuli, such as PMA (Figure 5.10.). In unstimulated cells, S6KβII adopts an inactive conformation and is mainly localised in the nucleus. In this state, S6KβII import may be faster than export or the kinase may be in complex with an anchoring protein in the nucleus. Treatment of cells with PMA triggers the activation of classical and novel PKCs and downstream signaling molecules, including S6KβII. The fact that exclusively nuclear forms of S6K, S6KαI and S6KβI, are activated in response to mitogenic stimuli suggests that all components required for multi-step phosphorylation/activation of S6KβII are present in the nucleus (Minami, et al., 2001; Reinhard et al., 1994). Activation of S6KβII may unfold the kinase, releasing the N-terminal NES from its intramolecular interactions. In this state, the kinase may be transported to the cytoplasm by Crm1-facilitated nuclear export. Phosphorylation of S6KβII by activated forms of PKC may be essential for inactivating the function of its C-terminal NLS. The addition of negative charges within the NLS or flanking regions may abrogate the interaction with the NLS receptor. Given that negatively charged sequences of the NLS-R are thought to bind to the positively charged NLS of nuclear-targeted proteins for nuclear import to occur (Silver, et al., 1991), it is not surprising that the presence of a negative charge within the NLS may
Fig. 5.10. Subcellular localisation of S6KβII is regulated by PKC. See text for details.
inhibit this interaction. Retention of the activated form of S6KβII in the cytoplasm could be required for phosphorylation of ribosomal S6 protein and initiation of protein synthesis. It is well documented that PMA-activated protein synthesis is a key event for the induction of cell growth and proliferation (Brostrom et al., 1987; Morley and Traugh, 1990; Nishizuka, 1984; Nishizuka, 1992). Dephosphorylation of Ser486 in response to environmental changes can unmask the C-terminal NLS, making it available for importin-independent nuclear import.

In conclusion, we have described for the first time mitogen-regulated nucleocytoplasmic shuttling of S6KβII and deciphered a critical role of PKC signaling in this process.
CHAPTER 6

INTRODUCTION OF THE CELLULAR MODEL DESTINED FOR THE STUDY OF PHYSIOLOGICAL FUNCTIONS OF INDIVIDUAL S6K ISOFORMS
CHAPTER 6

INTRODUCTION OF THE CELLULAR MODEL DESTINED FOR THE STUDY OF PHYSIOLOGICAL FUNCTIONS OF INDIVIDUAL S6K ISOFORMS

6.1. Introduction

Ribosomal protein S6 kinase signaling plays a crucial role in the initiation of protein synthesis induced by growth factors, hormones and oncogenes. Recent studies have also provided strong evidence for the role of S6Ks in ribosome biogenesis, cell growth, and proliferation (Dufner and Thomas, 1999; Jefferies et al., 1997; Thomas and Hall, 1997).

There are two forms of S6K, S6Kα and S6Kβ, which have cytoplasmic (S6KαII and S6KβII) and nuclear (S6KαI and S6KβI) variants derived from alternative splicing at the N-terminus. Since S6Kα was identified more than a decade ago, and S6Kβ only recently, most functional studies have involved alpha isoforms of the kinase. The importance of S6Kα in proliferation of cells has been inferred from either the use of the immunosuppressant rapamycin or through microinjection of neutralizing antibodies into cells, both of which suppress mitogen-induced S6Kα activation and lead to the cell cycle arrest (Chung et al., 1992; Lane et al., 1993; Price et al., 1992, Reinhard et al., 1994). Consistent with these findings, knock-out studies showed that deletion of S6Kα gene in mice and dS6K in Drosophila had a significant effect on cell growth (Montagne et al., 1999; Shima et al., 1998).

The ribosomal protein S6 is the most widely studied physiological substrate of S6Kα. A direct link between the phosphorylation of S6 protein and the induction of translation in cells stimulated with various extracellular stimuli has been demonstrated. Further studies have also shown that phosphorylation of S6 correlates with an increased selectivity of 40S ribosomal subunit to interact with specific mRNAs which contain an oligopyrimidine tract at their 5′ untranslated region (5′-TOP mRNAs) (Jefferies and Thomas, 1996). The family of 5′-TOP mRNAs constitutes as few as 100 to 200 transcripts, which encode essential components of the translational apparatus, including ribosomal proteins and some of the elongation factors, and make up to 20-30% of the total cellular mRNA (Meyuhas et al., 1996) (see section 1.4.1.1.). In this way, activation of the S6K signaling pathway could conceivably augment ribosome biogenesis, induce gene expression at the level of
translation and thereby lead to the stimulation of cell growth. However, the exact
correction of each S6K isoform to the regulation of these processes is not clear.
Furthermore, the importance of S6K signaling in the control of 5'-TOP mRNA translation
has recently been challenged by studies which show S6Kα-independent translation of
these messages in several cell lines (Stolovich et al., 2002).

Within the few past years several approaches have been developed to study the
physiological importance of individual gene products. These include targeted disruption of
the gene of interest and subsequent generation of gene deficient organisms, down-
regulation of gene expression with small interfering RNA, generation of transgenic
organisms, and stable expression of dominant negative or constitutively activated mutants
in mammalian cells. To date, there are no reports in the literature on the physiological role
of S6Kβ isoforms and none of these methodologies have yet been applied to explore this
issue. By contrast, deletion of the S6Kα gene in mice has been recently described by G.
Thomas' group (Shima et al., 1998). Although analysis of S6Kα/− mice revealed some of
the physiological assignments of this kinase, isoform specific functions of S6KαI and
S6KαII were not addressed in this study. Furthermore, the effect of S6Kα deficiency on
translation initiation in mouse embryo fibroblasts was difficult to assess, since S6Kβ
seems to partially compensate for the lack of S6Kα. It was shown that S6 phosphorylation
and 5'-TOP mRNA translation were normal in fibroblasts derived from S6Kα/− mice,
strongly suggesting the existence of a compensatory mechanism for these S6Kα functions.
Moreover, generation of S6Kα/−/ S6Kβ/− mice, which lack both S6 kinases, could be
hampered due to the possible negative effect on their viability. Indeed, deletion of S6K in
Drosophila, which possess only one gene for the kinase, leads to a high incidence of
embryonic lethality (Montagne et al., 1999). Thus, other approaches have to be undertaken
to investigate physiological functions of individual S6K isoforms. To facilitate these
studies we have generated and characterized tetracycline-inducible stable cell lines,
overexpressing activated mutants of cytoplasmic and nuclear S6Kα and S6Kβ isoforms.

This chapter describes generation of stable cell lines expressing constitutively activated
versions of all four isoforms of S6K under the control of a tetracycline-inducible promoter.
The profiles of newly translated and in vivo phosphorylated proteins of metabolically
labeled cells ([35S]Methionine/Cysteine or [33P]Orthophosphate, respectively) were
analysed by two-dimensional electrophoresis.
6.1.1. Tetracycline-inducible expression system for mammalian cells

The tetracycline-regulated expression system (T-Rex System) in mammalian cells uses regulatory elements from the *E.coli* Tn10-encoded tetracycline (Tet) resistance operon (Hillen and Berens, 1994). Tetracycline regulation in the T-Rex System is based on the binding of tetracycline to the tetracycline repressor (Tet repressor) and derepression of the promoter controlling expression of the gene of interest (Yao et al., 1998).

The major component of the T-Rex System is the pcDNA4/TO inducible expression plasmid. In pcDNA4/TO, expression of the gene of interest is controlled by the strong human CMV promoter (Andersson et al., 1989) into which two copies of the tetracycline operator (TetO2) sequence have been inserted in tandem. The TetO2 sequences in the pcDNA4/TO promoter consist of two copies of the 19 nucleotide sequence, 5'-TCCCTATCAGTGATAGAGA-3' separated by a 2 base pair spacer. Each 19 nucleotide TetO2 sequence serves as the binding site for two molecules of the Tet repressor. The second major component of the T-Rex System is the pcDNA6/TR regulatory vector which constitutively expresses high levels of the Tet repressor under the control of the human CMV promoter.

Figure 6.1. schematically represents the regulatory mechanism which is involved in the control of gene expression in the T-Rex System. In the absence of tetracycline, the Tet repressor forms a homodimer that binds with extremely high affinity to each TetO2 sequence in the pcDNA4/TO promoter. Binding of the Tet repressor homodimers to the TetO2 sequences represses transcription of the recombinant gene. Upon addition, tetracycline binds with high affinity to each Tet repressor homodimer in a 1:1 stoichiometry and causes a conformational change in the repressor that renders it unable to bind to the Tet operator. The Tet repressor/tetracycline complex then dissociates from the Tet operator allowing RNA polymerase II-directed transcription from the gene of interest.

To create stable cell lines the gene of interest had to be cloned into the multiple cloning site of the inducible expression vector, pcDNA4/TO, and the resulting construct cotransfected with the regulatory plasmid pcDNA6/TR into mammalian cells. The production of stable cell lines depends on a random homologous recombination event to integrate the plasmid into the host genome, so that it replicates in tandem with the chromosomal DNA. The pcDNA4/TO plasmid contains a zeocin resistance gene that can be used to select for clones that contain the recombinant construct.
Fig. 6.1. Schematic representation of a tetracycline-regulated expression system for mammalian cells. See text for details.
6.2. Results

6.2.1. Generation and characterization of tetracycline-inducible HEK293 stable cell lines, overexpressing constitutively activated mutants of S6Kα and S6Kβ

To produce stable cell lines pcDNA4/TO plasmids, encoding EE-tagged S6KαI(T412D), S6KαII(T412D), S6KβI(T401D), and S6KβII(T401D) mutants, were independently transfected into T-Rex HEK293 cells\(^1\) as described in chapter 2, section 2.4.3. Initially, the sensitivity of the T-Rex HEK 293 cells to a selective antibiotic zeocin was determined by the treatment of non-transfected cells with different concentrations of the antibiotic. The 100 µg/ml concentration of zeocin was determined as that which caused over 90% inhibition of the cell growth within 10 days after addition of the antibiotic (data not shown). This concentration was used to select stably transfected cell clones. The selection and maintenance of transfected cells were carried out according to the protocol described in chapter 2, section 2.4.3.

For each gene twenty zeocin resistant clones were separated and screened for tetracycline-regulated protein expression by immunoblot analysis (data not shown). Based on this analysis, we selected four cell lines (αI(6), αII(1), βI(2), βII(20)), which express almost equal levels of corresponding recombinant S6Ks when stimulated with tetracycline under similar growing conditions. As shown in Figure 6.2.A, immunoblot analysis with anti-EE-tag antibody revealed no immunoreactive signal in parental T-Rex cells treated or non-treated with tetracycline. By contrast, strong induction of recombinant protein expression was observed in αII(1), βI(2) and βII(20) cell lines after tetracycline treatment (Figure 6.2.A). It is important to note that despite the presence of EE-tag epitope at the N-terminus of S6KαI, the anti-EE-tag antibody could not detect this isoform in the corresponding cell line lysate. However, further analysis, employing anti-S6Kα polyclonal antibodies raised against the C-terminal sequence of S6Kα (for generation and characterization of the antibodies see chapter 2, section 2.3.1. and Appendix H, Supplementary Figure 8), confirmed tetracycline-induced overexpression of recombinant S6KαI isoform in αI(6) cell line (Figure 6.2.B). It is most likely that EE-tagged S6KαI adopts a conformation, due to the presence of the 23 amino acid extension at the N-terminus of the kinase that precludes the binding of the anti-EE-tag antibody to the epitope. We have also detected a low level of non-induced expression of recombinant

\(^1\)T-Rex HEK293 cell line stably expresses the tetracycline repressor
Fig. 6.2. Analysis of tetracycline-inducible expression of S6K isoforms in stable cell lines. Parental T-Rex cells and αI(6), αII(1), βI(2) and βII(20) stable cell lines were incubated in the presence or absence of 1 μg/ml tetracycline for 24 hours. Cell lysates were analysed by immunoblotting with anti-EE-tag (A) or anti-S6Kα and anti-S6Kβ polyclonal antibodies (B).
proteins in these cell lines (Figure 6.2.A and B). This finding makes it impossible to use non-induced \( \alpha I(6) \), \( \alpha I(1) \), \( \beta I(2) \) and \( \beta I(20) \) cell lines as negative controls. Therefore, the parental T-Rex cells were considered as an appropriate control for all further studies.

Next, we compared the expression level of recombinant S6 kinase isoforms versus native kinases by immunoblot analysis using anti-S6K\( \alpha \) and anti-S6K\( \beta \) polyclonal antibodies (Figure 6.2.A and B, expression levels were quantified as described in chapter 2, section 2.5.6.2.). The results indicate that these cell lines express approximately 4.5-6.5 times higher levels of recombinant kinases after induction with tetracycline, when compared to the level of native forms. These findings were also confirmed at the level of S6K mRNA transcripts by microarray hybridisation studies (I.Gout, unpublished data).

Another important aspect for subsequent experiments includes the quantification of S6 kinase activity of the recombinant and the cognate endogenous kinases as well as elimination of the kinase activity originating from the native forms in these cell lines. To test for S6 kinase activity endogenous and recombinant kinases were immunoprecipitated with anti-S6K\( \alpha \) or anti-S6K\( \beta \) polyclonal antibodies from tetracycline-treated parental T-Rex cells and the stable cell lines. Immune complex S6 kinase assay was performed as described in chapter 2, section 2.5.8. As shown in Figure 6.3., the kinase activities precipitated from \( \alpha I(1) \), \( \alpha I(6) \), \( \beta I(20) \) and \( \beta I(2) \) cell lines were respectively 5.4, 4.6, 4.8 and 4.0 times higher, when compared to those in parental T-Rex HEK293 cells. Nevertheless, the activity of endogenous kinases represents 20-25% of total S6 kinase activity precipitated from these cells. Such high endogenous activity may mask the effect of overexpressed recombinant isoforms of S6K on cellular functions, including translation and transcription.

Since T412D and T401D substitutions in S6K\( \alpha \) and S6K\( \beta \), respectively, confer partial rapamycin resistance of these kinases (Pearson et al., 1995 and data not shown), low concentration of the inhibitor was used to selectively inhibit endogenous S6 kinase activity in stable cell lines. As shown in Figure 6.3., 5 nM rapamycin suppressed significantly the activity of endogenous S6K\( \alpha \) and S6K\( \beta \), whereas recombinant kinases were still highly active in stable cell lines. We found that the activity of recombinant kinases in stable cell lines treated with rapamycin still remained 2.5-3.5 times higher than corresponding activity of native forms in non-treated parental cells. Since rapamycin was shown to inhibit 4E-BP1 phosphorylation and protein synthesis (Herbert et al., 2000), the effect of this inhibitor on expression of recombinant kinases was also tested. We observed only modest
Fig. 6.3. The level of recombinant and endogenous S6 kinase activities in parental and stable cell lines treated or non-treated with rapamycin. Parental T-Rex cells and αI(6), αII(1), βI(2) and βII(20) stable cell lines were induced with 1 μg/ml tetracycline for 24 hours in the presence or absence of 5 nM rapamycin. Recombinant and endogenous S6Ks were immunoprecipitated with anti-S6Kα and anti-S6Kβ polyclonal antibodies and their specific activities were assessed in an in vitro S6 kinase assay.
decrease of the expression level of recombinant kinases in cells treated with low concentration of rapamycin (Appendix I, Supplementary Figure 9). Therefore, 5 nM rapamycin was used in all further experiments to inhibit endogenous S6 kinase activity in these cell lines.

In conclusion, we have successfully generated tetracycline-inducible stable cell lines expressing activated mutants of cytoplasmic and nuclear S6Kα and S6Kβ isoforms. The level of expression of recombinant constructs and their S6 kinase activities were examined in generated cell lines. Furthermore, in order to eliminate the effects originating from the activity of native kinases, the concentration of rapamycin sufficient to inhibit significantly endogenous S6 kinase activity, but not recombinant kinases, was determined for these cell lines.

6.2.2. The role of S6K isoforms in the regulation of protein expression

The establishment of stable cell lines has allowed us to study the effect of individual S6K isoforms on protein expression in HEK293 cells. For this propose we have analysed and compared the pattern of newly translated proteins between parental T-REx HEK293 cells and established cell lines upon induction with tetracycline. Total protein extracts from the cell lines, metabolically labelled with $[^{35}S]$Methionine/Cysteine, were analysed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) followed by autoradiography and silver staining of the separated proteins (Figure 6.4. and data not shown, respectively). As shown in Figure 6.4., the general profile of $[^{35}S]$Methionine/Cysteine labelled proteins in the T-Rex cells and the cell lines was similar and position of individual proteins was well reproducible on the gels. However, visual analysis of radioactive and stained images revealed a number of spots which were differentially expressed in T-Rex cells and stable cell lines. For the further analysis we selected only six differentially expressed spots, because intensity of their radioactive signal correlated well with amount of protein quantified by silver staining (Figure 6.5. and 6.6.). Of the six selected spots, two were present in T-Rex cells (N3 and N6), but not in the cell lines expressing activated S6K isoforms. In contrast, spots N2 and N4 were present in protein extracts obtained from tetracycline-induced stable cell lines, but not in parental cells. This finding suggests that S6K down-regulated the expression of proteins corresponding to spots N3 and N6 and up-regulated the expression of proteins represented by spots N2 and N4. We also found that expression of two proteins, corresponding to spots
Fig. 6.4. Analysis of translational profiles of $^{35}$S-labeled stable cell lines (Part I). Parental T-Rex cells and $\alpha$(I), $\alpha$(II), $\beta$(I), $\beta$(II) stable cell lines were induced with 1 $\mu$g/ml tetracycline and labelled with 200 $\mu$Ci/ml Pro-mix-$^{35}$S as described in Experimental procedures. Total cell lysates containing 120 $\mu$g of protein were separated by 2D PAGE and analysed by autoradiography. The boxes outlined in the autoradiograms refer to regions of altered patterns of newly translated proteins in the cell lines analysed (see Fig. 6.5. and Fig. 6.6.).
Fig. 6.5. Analysis of translational profiles of $^{35}$S-labeled stable cell lines (Part II). The details of cell treatment and analysis are described in Fig. 6.4. Differentially expressed proteins are shown by arrows and numbers. Red and blue arrows indicate increase or decrease in radioactive signal, respectively.
Fig. 6.6. Analysis of translational profiles of $^{35}$S-labeled stable sell lines (Part III). The fragments of silver-stained gells correspond to regions of divergence outlined in the autoradiograms (see Fig. 6.4. and Fig. 6.5.). Positions of differentially expressed proteins, identified in corresponding autoradiograms, are designated by arrows and the same numbers on the stained gells.
Table 6.1.

Predicted molecular weights of differentially expressed proteins

<table>
<thead>
<tr>
<th>Spot number</th>
<th>Predicted Mr</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>43000</td>
<td>αI(6), βI(2), βII(20)</td>
</tr>
<tr>
<td>2</td>
<td>24000</td>
<td>αI(6), αII(1), βI(2), βII(20)</td>
</tr>
<tr>
<td>3</td>
<td>23500</td>
<td>T-Rex</td>
</tr>
<tr>
<td>4</td>
<td>42000</td>
<td>αI(6), αII(1), βI(2), βII(20)</td>
</tr>
<tr>
<td>5</td>
<td>34000</td>
<td>αII(1)</td>
</tr>
<tr>
<td>6</td>
<td>18000</td>
<td>T-Rex</td>
</tr>
</tbody>
</table>
N1 and N5, was regulated in an S6K isoform specific manner. Protein N1 was highly expressed in αI(6), βI(2) and βII(20) cells and not found in T-Rex and αII(1) cells, whereas protein N5 was selectively translated only in αII(1) cell line. The results are summarized in Table 6.1. in which molecular size of differentially expressed proteins, predicted from the location of the spots on silver stained gels, are also presented.

Taken together, these results demonstrate the involvement of S6K in the regulation of protein expression in HEK293 cells. Furthermore, the profile of differentially expressed proteins, observed in stable cell lines versus T-Rex cells, was found to be different between αII(1) and the rest of the stable cell lines, suggesting S6K isoform specific regulation of protein synthesis. Studies, involving quantitative computational analysis of images, creation of two-dimensional representative protein maps and protein microsequencing by mass spectrometry are currently in progress.

6.2.3. Comparative analysis of in vivo phosphorylated proteins derived from the stable cell lines

Generated stable cell lines also present an interesting model system for the search of novel S6Ks substrates, identification of which may provide a link to the physiological functions of these kinases. In attempt to explore this issue, we have examined the pattern of in vivo phosphorylated proteins derived from the parental T-Rex cells and stable cell lines labelled with [33P]Orthophosphoric acid upon induction with tetracycline. The [33P]-labelled proteins were separated by 2D PAGE, transferred to PVDF membrane, and analysed by autoradiography and Coomassie Blue staining (Figure 6.7. and data not shown, respectively). Two zones (A and B), with different patterns of [33P] incorporation between the investigated cell lines, were found by visual analysis of autoradiograms (Figure 6.7.). The first zone A was a series of basic spots (~pI 10) at approximately 25-30 kDa (N1, N2 and N3) which were relatively heavy labelled in αI(6), αII(1) and βII(20) stable cell lines and only faintly labelled in parental T-Rex cells. In the case of βI(2) cells only spot N2 had a high intensity of [33P] incorporation whereas spots N1 and N3 were labelled to the same extend as the relative spots in control cells. These spots likely represent phosphorylated isoforms of ribosomal S6 protein, because their predicted pI and molecular weights correspond to those identified for S6 protein (pI 11.32 and Mr 28-33 kDa (Patel et al., 1996; Wool et al., 1996; Zhang et al., 2000)). Further studies, involving
Fig. 6.7. Comparative analysis of phosphorylated proteins from $^{33}$P-labeled stable cell lines. Parental T-Rex cells and $\alpha$(I), $\alpha$(II), $\beta$(II) and $\gamma$(II) stable cell lines were induced with 1µg/ml tetracycline and labelled with 50 µCi/ml [$^{33}$P]Orthophosphate as described in Experimental procedures. Total cell lysates containing 120 µg of protein were separated by 2D PAGE, transferred to PVDF membrane, and analysed by autoradiography. Differentially labeled protein spots are designated by arrows and numbers. Red arrows indicate increase in $^{33}$P-incorporation whereas blue arrows show decrease in radioactive signal.
two-dimensional electrophoresis adapted for resolution of basic ribosomal proteins and immunoblot analysis, will be required to confirm this assumption. The second zone B, in the acidic portion of the gel (~ pI 3-5), contained three labelled spots N4 (~45kDa), N5 (~35kDa) and N6 (~27kDa) specific for βI(2) cells, but not observed in parental and other cell lines. Despite relatively strong radioactive signal obtained from these spots, none of them were detected by Coomassie Blue staining (data not shown), indicating the presence of a low amount of corresponding proteins. Differential phosphorylation of these proteins suggests that they may represent unique S6Kβ1 substrates. However, it remains to be elucidated whether their phosphorylation is rapamycin sensitive and can be regulated by mitogenic stimuli.

We have attempted to identify the sequence of the differentially labelled proteins found in zone B. The areas of PVDF membrane, which overlap with radioactive spots of interest, were cut out and analysed by Edman degradation. No detectable amount of material was recovered from the spots N4 and N6 making it impossible to identify the corresponding proteins. The 21 amino acids sequence DXLARTPADAFYQSQAIASLS (X represents unidentified residue and small size letters were used to match very weak inconclusive sequence) was obtained for the spot N5 and subsequently used for the search of homology in the SWISS-PROT databank. However, we were not able to find any significant homology to this sequence. Similar problems were described by many other groups (Arthur et al., 2002; Cremona et al., 1995; Li et al., 2002) and explained by insufficient sensitivity of the techniques applied. Thus, other approaches have to be used to obtain sufficient amount of the protein for mass spectrometry analysis. For example, cell fractionation followed by preparative 2D PAGE can be applied to enrich analysed material with the protein of interest. Since, differentially phosphorylated proteins were observed in βI(2) cell line, overexpressing nuclear isoform of S6Kβ, it is reasonable to expect that these proteins may represent a nuclear pool of cellular proteins.

In conclusion, we have found that overexpression of activated S6K isoforms affected the pattern of protein phosphorylation in HEK293 cells. Moreover, the profile of 33P labelled proteins appeared to be different between βI(2) and other cell lines, suggesting the existence of potential substrates which are specific for S6Kβ1 isoform.
6.3. Discussion

Here we describe for the first time generation of stable cell lines, expressing in an inducible manner constitutively activated mutants of S6Kα and S6Kβ isoforms. These cells represent a novel model system well designed for the study of the role of individual isoforms of S6K in cellular functions. In contrast to a transient transfection approach, this system allows us to achieve a desire level of recombinant S6Ks expression simply by induction of the cells with tetracycline for various periods of time (Figure 6.2. and data not shown). Furthermore, it circumvents some other problems of transient expression, especially heterogeneity of a cell population, high cytotoxicity and low reproducibility of experimental conditions.

We have created a panel of the stable cell lines for each isoform of S6K and screened them for the expression of recombinant proteins. Four cell lines, which express the similar level of recombinant S6 kinases under the same inducible conditions, were selected for further studies. The S6 kinase activity in the tetracycline-induced cell lines was about 4-5.5 times higher than relative activity originated from the native kinases in parental T-Rex cells. Moreover, treatment of these cells with 5 nM rapamycin allowed us to eliminate activity of endogenous S6Ks, whereas activity of recombinant kinases was still very high due to the presence of T412/401D mutations in the recombinant constructs (Pearson et al., 1995 and Figure 6.3.). Therefore, the established cell lines provide an excellent experimental system for the study of physiological significance of individual S6K isoforms.

The cell lines generated are used in several studies (Figure 6.8.), including elucidation of the role of various S6Ks in the regulation of gene expression at the level of translation and transcription, identification of novel S6K substrates and investigation of oncogenic potential of activated forms of S6K. These studies employ proteome and DNA microarray analysis of the stable cell lines as well as several classical methodologies developed for investigation of cell transformation. As mentioned above, stable cell lines possess several practical advantages that are very important for methodological approaches applied in these studies. Moreover, their validity as an experimental system for such studies is also supported by the fact that similar systems were used to explore the effect of other genes involved in signal transduction and oncogenesis (Harkin et al., 1999; Mayeur and Hershey, 2002; Medina et al., 2002; Wu et al., 2002).
Fig. 6.8. Stable cell lines as a cellular model for the physiological studies of S6Ks.
Having established and characterized this cellular model we have initiated studies on the regulation of gene expression by ribosomal S6 kinases. The profile of newly translated proteins in the parental T-Rex cells and the stable cell lines, metabolically labelled with $^{35}$S Methionine/Cysteine, was analysed by high-resolution 2D-PAGE. Preliminary analysis of radioactive and silver stained two-dimensional images has shown a number of spots differentially expressed in these cells. Since their predicted molecular weights and pI were significantly different, we have referred to them as individual proteins rather than posttranslational modifications. However, this issue has to be properly addressed by mass spectrometry analysis and subsequent identification of these proteins. We revealed that some of these spots were up-regulated upon induction of expression of the recombinant S6Ks whereas several others were considerably down-regulated in these cell lines. Moreover, the profile of differently translated proteins was found to be different between αII(1) and the rest of the stable cell lines. This may suggest S6K isoform specific regulation of expression of individual proteins. Consistent with these data, deferentially regulated genes were recently identified in these cell lines by DNA microarray analysis. The results of microarray analysis clearly indicate that S6KαI, S6KβI and S6KβII isoforms upregulate a similar pattern of genes, while the transcriptional profile for the S6KαII is distinct (I.Gout, unpublished data). Regarding this point, it is important to note that S6KαII is mainly cytosolic, whereas S6KαI, S6KβI and S6KβII are localised predominantly in the nucleus of HEK293 cells (see chapter 5, section 5.2.1. and Coffer and Woodgett, 1994; Reinhard et al., 1994; Koh et al., 1999). Altogether, these findings support the hypothesis that the physiological functions of S6Kα and S6Kβ do not overlap entirely. This hypothesis was first raised in knock-out studies demonstrating that small size phenotype and hypoinsulinaemia persist in S6Kα−/− mice despite the presence of S6Kβ, normal protein S6 phosphorylation, and 5′-TOP mRNA translation (Shima et al., 1998).

Although the proteome analysis of the stable cell lines demonstrated that S6Ks are involved in the regulation of selective protein expression, it is not clear whether this regulation occurs at the level of translation or transcription. Several recent studies have implicated S6Kα in the regulation of CREMτ- and E2F-dependent transcription (de Groot et al, 1994; Brennan, et al., 1999). This issue can be addressed either by using of actinomycin D, a potent transcription inhibitor, or by gene profiling analysis of generated cell lines using DNA microarray, which would allow comparison of changes in the transcription of genes with changes in the abundance of the corresponding proteins.
Alternatively, given the technical limitation of proteomic studies, analysis of the actively translated mRNAs can be achieved by isolation of polysomes (Pradet-Balade et al., 2001) and comparison of the polysome-associated mRNAs in the cells expressing recombinant kinases with the polysomal fraction of the parental T-Rex cells. These approaches would allow the assessment of the relative contribution of S6K isoforms into transcriptional and translational control of protein expression.

The stable cell lines generated were also used in this study as a model system for the search of novel physiological substrates for S6Ks. The identification of these substrates might provide important information about cellular functions of S6 kinases. This issue presents a great interest, since S6Kα appears to be a multifunctional kinase that in addition to S6 protein has a potential to phosphorylate some other regulatory proteins, namely transcriptional activator CREMt, elongation factor 2 kinase, ribosomal protein S17, CBP80 subunit of RNA cap-binding complex, and the regulator of apoptosis Bad 1 (de Groot et al, 1994; Harada et al., 2001; Patel et al., 1996; Wang et al., 2001b; Wilson et al., 2000). However, the physiological relevance of these phosphorylations requires further investigation, since other protein kinases can phosphorylate these molecules at identical sites. Furthermore, it is not clear whether S6KαI and S6KαII phosphorylate the same substrates in vivo. In addition to that, the identity of physiological S6KβI and S6KβII substrates remains to be elucidated. To explore these issues, we have investigated the pattern of in vivo phosphorylated proteins derived from parental T-Rex cells and stable cell lines labelled with $[^{32}P]$Orthophosphate upon induction with tetracycline. Induction of recombinant S6K expression in αI(6), αII(1) and βII(20) cell lines correlated with a massive increase in phosphorylation of several closely located spots. The predicted pI and molecular weights of these spots correlated to those identified for phosphorylated forms of S6 protein. Furthermore, three additional $^{33}P$-labelled proteins were found in total lysates derived from the tetracycline-treated βI(2) cells, but not in the other cell lines. Predicted molecular weights of these proteins do not match the molecular weight of any known S6Kα substrate, suggesting that they may represent unique protein targets for S6KβI or downstream kinase/s activated by S6KβI. Further studies will be required to identify differentially phosphorylated proteins as well as the importance of their phosphorylation in the context of cell regulation.
Finally, the establishment of inducible stable cell lines for various S6Ks will facilitate studies on the physiological role of individual S6K isoforms.
GENERAL DISCUSSION
GENERAL DISCUSSION

Stimulation of cell growth and proliferation is initiated at the cell surface by growth factor-mediated activation of receptor-coupled G proteins or receptor-tyrosine kinases (Bourne et al., 1990, Cantley et al., 1991). This extracellular mitogenic signal is then converted and transmitted through tightly controlled protein phosphorylation cascades to specific intracellular targets which are involved in the regulation of a number of metabolic events. Phosphorylation of ribosomal protein S6, which is an integral part of the translational machinery, is one of the earliest events detected following mitogenic stimulation (Stewart and Thomas, 1994). This phosphorylation correlates with initiation of protein synthesis and is important for the biogenesis of translational components (Jefferies and Thomas, 1996). This critical translational response is thought to be mediated by two closely related kinases, ribosomal protein S6 kinase α (S6Kα) (Jefferies et al., 1997) and its recently identified homologue S6Kβ (Gout et al., 1998; Shima et al., 1998).

This manuscript presents comparative analysis of regulatory and functional properties of S6Kα and S6Kβ and investigation of signaling mechanisms involved in the control of their specific responses to diverse mitogenic stimuli.

Two human cDNA clones encoding S6KβI and S6KβII were isolated and used in this work to generate expression constructs for bacterial and mammalian expression studies (section 3.2.1.). Both S6Kβ isoforms were transiently expressed in HEK293 cells and shown to efficiently phosphorylate ribosomal S6 protein in vitro (section 3.2.3. and 6.2.1.), suggesting their potential to use rpS6 as a substrate in vivo. This hypothesis is supported by knock-out studies which demonstrated that S6 phosphorylation and 5'-TOP mRNA translation were normal in fibroblasts derived from mice lacking S6Kα gene. The presence of S6Kβ was suggested to be sufficient to compensate for these S6Kα functions in S6Kα−/− MEFs (Shima et al., 1998). However, to directly address this issue generation of S6Kα−/− /S6Kβ−/− mice will be required. Alternatively, the stable cell lines expressing activated mutants of S6K isoforms (described in chapter 6) could be used to answer this question.

The results presented in this thesis also demonstrate that both S6KαII and S6KβII have similar mode of activation in response of HEK293 cells to various mitogenic stimuli, including serum, insulin, PDGF and PMA (section 3.2.3., 4.2.1., and 5.2.4.). Furthermore, both S6KαII and S6KβII were potently activated by integrin-mediated adhesion of HEK293 cells to fibronectin (section 4.2.1.). The activation of S6Kα is a complex event
requiring a precise interplay between specific domains and multiple phosphorylation sites. It has been proposed that hyperphosphorylation of sites residing in the autoinhibitory region of S6Kα, combined with phosphorylation of Thr412, in the kinase extension domain, induces conformational changes which disrupt an inhibitory interaction between the carboxyl and amino termini. These conformational changes expose the activation loop allowing subsequent phosphorylation at Thr252 and full activation of the kinase (section 1.2.3.). However, the structure of S6Kα has not been solved so far and the proposed model of S6Kα activation is mainly based on functional studies employing mutated forms of this kinase (Pullen and Thomas, 1997). Similar approaches were used in this study to ascertain the molecular mechanisms of S6Kβ activation. Inspection of the amino acid sequence of S6Kβ revealed domain organisation and pattern of phosphorylation sites similar to S6Kα (Gout et al., 1998). Functional resemblance of S6Kβ domains was confirmed here by construction and characterization of deletion mutants of S6Kα and S6Kβ isoforms (section 3.2.1. and 4.2.2.2.). As in the case of S6KαΔN75 mutant, both basal and serum-stimulated activities of S6Kβ were strongly suppressed by the amino-terminal truncation of 64 amino acid residues (section 4.2.2.2.). The similar inhibitory effect of 46 and 54 amino acid deletions from the N-terminus was previously described for S6Kα by other groups (Cheatham et al., 1995; Weng et al., 1995a). These findings suggest that the N-terminal region of S6Kβ as well as S6Kα is essential for the function of these kinases, albeit the means through which these regions contribute to the kinase activity are not clear. It is proposed that the N-terminal region may interact with an upstream activator of S6K which modulates the inhibitory effect of the C-terminal autoinhibitory domain (Cheatham et al., 1995). However, additional studies are required to prove this assumption. It has also been shown that deletion of the C-terminus did not produce a constitutively active form of S6Ks (section 4.2.2.2., Weng et al., 1995a and Minami et al., 2001). Both mutants, S6KαΔC100 and S6KβΔC81, exhibited dependence on mitogenic stimulation, suggesting an inhibitory effect of the N-terminal domain. Thus, the analysis of S6Kα and S6Kβ deletion mutants demonstrated functional similarities of regulatory domains in these kinases. Furthermore, it has been shown that S6KβII similarly to S6KαII undergoes an agonist-induced phosphorylation in vivo (section 3.2.3. and Pardo et al., 2001). Mutational analysis of potential regulatory residues in S6Kβ presents evidence that Thr241 and Thr401, which are homologous to Thr252 and Thr412 in S6Kα, represent principle regulatory sites of this kinase. Taken together, these results support the idea that S6Kα and S6Kβ utilize similar
molecular mechanisms of activation and allow to apply the hypothetical model of S6Kα activation to S6Kβ (see section 1.2.3. and Figure 1.3.).

At the same time, the studies of signaling pathways have revealed some differences in the regulation of S6KαII and S6KβII isoforms (chapter 4). The kinetics of their activation in response to serum and fibronectin were found to vary in time, indicating that S6KβII was activated more rapidly and might be involved in the control of early cellular responses to these agonists. To explain these results we propose that an additional mitogen-stimulated signaling pathway, which is not specific for S6KαII, contributes to the activation of S6KβII in response to these stimuli. Regarding this issue it has been disclosed that serum- and fibronectin-induced early activation of S6KβII, but not S6KαII, correlated closely with changes in ERK1/2 activity stimulated by these agonists in several cell lines (section 4.2.1. and Ballou et al., 1991; Burnett et al., 1998; Wei et al., 1998). Similar correlation was previously reported for p90RSK, which is in agreement with an upstream regulatory role of ERK with respect to this kinase (Murphy et al., 2002). In support of this hypothesis others and we showed that S6KβII, but not S6KαII, is activated by MEK/ERK pathway. Therefore, S6KβII and S6KαII share common inputs from PI-3'-K, mTOR/FRAP and PKC-dependent pathways, but MEK signaling is specific only for S6Kβ (section 4.2.2.1., 4.2.3.2., 4.2.3.3. and Pardo et al., 2001).

The PI-3'-K pathway is critical to activation of S6 kinases, because wortmannin, a specific inhibitor of PI-3'-K, potently inhibits growth factor-stimulated activation of both S6KαII and S6KβII (section 4.2.2.1.). The role of PI-3'-K activation in the regulation of S6Kα was previously demonstrated by many other groups (section 1.4.1.). Moreover, our results indicate the involvement of PI-3'-K in the c/n PKC-mediated activation of both S6Ks, despite the lack of PMA-induced activation of PI-3-K Ia in the cell line investigated (section 4.2.3.2.). This may reflect a requirement for basal PI-3'-K Ia activity or PI-3'-K signaling complexes formation for the PMA-induced activation of S6KαII and S6KβII. Similar situation was earlier described for ionomycin-induced activation of S6Kα in Balb/c-3T3 fibroblasts (Conus et al., 1998). However, it is also possible that a wortmannin-sensitive PI-3'-K isoform distinct from the type Ia isoforms might be implicated in S6KαII and S6KβII activation by PMA in these cells. In agreement with data presented here, it was recently demonstrated that S6Kβ is activated similarly to S6Kα by PI-3'-K signaling intermediates, such as PDK1, PKCζ, Rac and Cdc42 (Akimoto et al.,
This study also demonstrates that rapamycin potently blocks mitogen-stimulated activation of both S6KαII and S6KβII suggesting mTOR/FRAP-dependent regulation of these kinases (section 4.2.2.1.). However, the exact mechanism of mTOR/FRAP-mediated regulation of S6Ks is not clear. Several studies have implicated regulatory residue Thr412 as a principal target of rapamycin-induced S6Kα inhibition (Dennis et al., 1996; Pearson et al., 1995). We have shown here that mutation of Thr412 and Thr401 to aspartic acid in S6Kα and S6Kβ respectively, confer a partial resistance of both kinases to rapamycin treatment (section 6.2.1.). These results are in agreement with previously reported data for the T412E mutant of S6Kα analysed in several cell lines (Pearson et al., 1995). Therefore, we provide evidence that Thr401, similarly to Thr412, is a principle rapamycin-sensitive site in S6Kβ. It has recently been shown that S6Kα can be directly phosphorylated at Thr412 by the mTOR/FRAP kinase in vitro, and that this phosphorylation induces substantial increase in S6 kinase activity (Burnett et al., 1998b; Isotani et al., 1999). However, it remains to be investigated whether mTOR/FRAP is an in vivo Thr412/401 kinase. Alternatively, mTOR/FRAP may regulate S6Kα and S6Kβ activation by inhibiting their specific phosphatases, e.g. PP2A and PPl (section 1.4.2. and 1.4.4.). Earlier studies demonstrated that S6Kα associates directly with PP2A and is selectively dephosphorylated by this protein phosphatase (Ballou et al., 1988; Peterson et al., 1999). It has also been reported that insulin-stimulation leads to a general inhibition of PP2A activity, which can be blocked by pre-treatment with rapamycin (Begum and Ragolia, 1996).

Although activation of both S6 kinases requires regulatory inputs from mTOR/FRAP, S6KβII is found to be less sensitive to rapamycin, when compared to S6KαII (section 4.2.2.1. and 4.2.3.2.). Based on this finding we suggest that mTOR/FRAP may differentially regulate activation of S6KαII and S6KβII in vivo. However, it is not clear whether this reflects the ability of mTOR/FRAP to differentially regulate phosphorylation of Thr412 and Thr401 in S6KαII and S6KβII, respectively, or different sensitivity of these S6 kinases to phosphatases activated by rapamycin.

The inhibitory effect of rapamycin requires intact amino- and carboxyl-terminal domains of S6Kα suggesting their role in mTOR/FRAP-dependent regulation of this kinase (Dennis et al., 1996; Weng et al., 1995a). Consistent with these findings, we have demonstrated that susceptibility of the S6KαΔC100 mutant to inhibition by rapamycin was
significantly reduced in comparison with the full-length enzyme (section 4.2.2.2.). However, similar deletion of the C-terminal region of S6KβII did not affect the sensitivity of the kinase to inhibition by rapamycin. Moreover, it was shown that activities of S6KαΔC100, S6KβΔC81 and S6KβII were inhibited by rapamycin to the same extent. (section 4.2.2.2.). These observations allow us to conclude that truncation of the C-terminus of S6KαII is sufficient to eliminate differences in sensitivity to rapamycin between S6KαII and S6KβII isoforms. Further studies are required to explain the functional role of the C-terminal domain of S6Kα and S6Kβ in mTOR/FRAP-dependent regulation of these kinases.

The subcellular localisation of components that compose signal transduction pathways has become an important focus in elucidating the mechanisms by which this pathways are regulated. Specific targeting of signaling protein kinases to subcellular compartments offers an additional level of regulation in which the accessibility of their specific activators and substrates can be spatio-temporally limited. Here we present a detailed analysis of subcellular localisation of S6Kα and S6Kβ isoforms and investigation of specific mechanisms involved in the regulation of their subcellular distribution. Dynamic nucleocytoplasmic shuttling has been demonstrated for S6KαII and S6KβII, whereas S6KβI isoform was shown to be constitutively localised in the nucleus (section 5.2.1. and Kim and Chen, 2000). Nevertheless, S6KαII and S6KβII were characterized by distinct pattern of their subcellular localisation. S6KαII was found to be predominantly cytosolic independently of mitogenic stimuli, while S6KβII resided in the nucleus of serum-starved cells, but shifted to the cytoplasm after PMA stimulation. The presence of a functional nuclear localisation signal (NLS) at the C-terminus of S6KβII, which is not found in S6KαII, is thought to determine its predominantly nuclear localization (Koh et al., 1999). In contrast to S6KβII, nuclear localisation of S6KβI, which contains two NLS sequences, one at the N- and another at the C-terminus, is not affected by PMA stimulation. Therefore, we present clear evidence that subcellular localisation of S6KαII, S6KβII and S6KβI isoforms is differentially regulated.

The ability of PMA to induce cytosolic accumulation of S6KβII suggests a possible role of PKCs in the control of subcellular localisation of this S6 kinase. We report here that S6KβII, but not S6KαII, is specifically phosphorylated by PKCs at Ser486 which is located in the middle of its C-terminal nuclear localisation signal (section 5.2.2. and
5.2.3.). Using phosphospecific antibodies, we found that phosphorylation of S6KβII at this site is strongly induced by PMA and to a lesser extent by insulin, PDGF, EGF and FCS (section 5.2.4. and 5.2.7.). Furthermore, we found that Ser486 phosphorylation does not effect S6 kinase activity, but abrogates the function of the C-terminal nuclear localisation signal (section 5.2.6. and 5.2.7.). Mutational analysis of S6KβII provided evidence that Ser486 phosphorylation results in retention of an activated form of the kinase in the cytoplasm, possibly via blocking its nuclear import. Further studies are required to ascertain whether PKC-mediated phosphorylation of S6KβII at Ser486 is important for ribosomal protein S6 phosphorylation and initiation of protein synthesis in response to mitogenic stimuli.

Thus, we have described for the first time mitogen-regulated nucleocytoplasmic shuttling of S6KβII and deciphered a critical role of PKC signaling in this process. Based on these data and current knowledge on signaling via S6Ks, a conjectural model of nucleocytoplasmic shuttling of S6KβII in response to mitogenic stimuli has been proposed (section 5.3.).

Differential regulation of S6Kα and S6Kβ isoforms, along with discrete subcellular localisation, may confer specific cellular functions to these S6 kinases. This idea is strongly supported by knock-out studies demonstrating that small size phenotype and hypoinsulinaemia persist in S6Kα−/− mice despite an elevated expression of S6Kβ isoforms (Shima et al., 1998). To elucidate the contribution of individual S6Ks in the regulation of cellular functions, we have generated and characterized tetracycline-inducible stable cell lines, overexpressing activated mutants of cytoplasmic and nuclear S6Kα and S6Kβ isoforms (section 6.2.1). The involvement of all four S6Ks in the regulation of protein expression has been demonstrated in generated cell lines (section 6.2.2.). Furthermore, the profile of differentially expressed proteins, observed in the stable cell lines versus T-Rex cells, do not overlap entirely in the cell lines, expressing different S6K isoforms. Therefore, it is possible that expression of individual proteins might be specifically regulated by different S6Ks. This hypothesis is supported by results of DNA microarray studies performed on these cell lines (I. Gout, unpublished data).

Another important aspect of these studies is a search for novel S6Ks substrates, identification of which may provide a link to the involvement of S6Ks in distinct cellular processes. We have initiated these studies by investigating the profile of 33P labelled proteins in generated stable cell lines. The profile of 32P labelled proteins appeared to be
different between the investigated cell lines, suggesting the existence of potential substrates, which are specific for individual S6K isoforms. Further studies will be required to identify differentially expressed and phosphorylated proteins as well as an involvement of these proteins in cell regulation.

The ribosomal protein S6 kinase appears to be a multifunctional regulator involved in the control of many cellular processes, including protein synthesis, cell cycle progression and apoptosis. Deregulation of these processes may contribute to a variety of diseases and therefore presents a great interest for investigators. The importance of deregulated S6K signaling in cancer, diabetes and developmental abnormalities is increasingly apparent (see section 1.5.). This makes it important to better understand complex mechanisms of S6K regulation and its cellular functions. Such studies may provide a basis for development of novel therapeutic agents to correct mentioned disorders.

In this study we have explored several important aspects of regulation and functioning of individual members of S6K family and developed a novel model system for the investigation of the role of S6K isoforms in cell regulation.
REFERENCES


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gamma sensitivity of a PI3K is dependent upon a tightly associated adaptor, p101. Cell, 89, 105-114.


van Slegtenhorst, M., Nellist, M., Nagelkerken, B., Cheadle, J., Snell, R., van de
between hamartin and tuberin, the TSC1 and TSC2 gene products. Hum. Mol. Genet., 7,
1053-1057.


Volarevic, S., Stewart, M.J., Ledermann B., Zilberman F., Terracciano, L., Montini E.,


myocytes by insulin involves multiple rapamycin-sensitive steps. Am. J. Physiol. Heart.

Wang, L., Gout, I., Proud, C.G. (2001a). Cross-talk between the ERK and p70 S6 kinase
(S6K) signaling pathways. MEK-dependent activation of S6K2 in cardiomyocytes. J Biol
Chem., 276, 32670-32677.

of elongation factor 2 kinase by p90(RSK1) and p70 S6 kinase. EMBO J., 20, 4370-4379.

the human S6 ribosomal protein is required for tumor supression in the hematopoietic

kinase activation by the cytoplasmic domain of the α6 integrin subunit. J. Biol. Chem.,
273, 5903-5907.

independent inputs are required for activation of the p70 S6 kinase. Mol. Cell. Biol., 15,
2333-2340.


Appendix A

cDNA S6Kβ Cl.#53

Supplementary Fig. 1. Nucleotide and predicted amino acid sequences of cDNA S6Kβ cl.#53 and cl.#23. The red and blue boxes outlined in the sequence refer to the translation initiation codon and stop codon, respectively.

cDNA S6Kβ Cl.#23

Supplementary Table 1. Nucleotide and predicted amino acid sequences of cDNA S6Kβ cl.#53 and cl.#23. The red and blue boxes outlined in the sequence refer to the translation initiation codon and stop codon, respectively.
Appendix B

cDNA S6Kα

Supplementary Fig. 2. Nucleotide and predicted amino acid sequences of cDNA S6Kβ cl.#53 and cl.#23. The red and blue boxes outlined in the sequence refer to the translation initiation codon and stop codon, respectively. The sequence corresponding to catalytic domain enclosed in the brackets. Black bar indicates the end of linker domain. Putative autoinhibitory pseudosubstrate region is underlined.

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### Appendix C

#### Supplementary Fig. 3. Comparison of S6Kα with S6Kβ.

Amino acid sequences of S6Kα and S6Kβ are aligned. Conserved residues are boxed.
Supplementary Fig. 4. Affinity purification of anti-pS486 antibody. Anti-pS486 antibody were purified on Actigel beads coupled with antigenic peptide and eluted as described in Experimental procedures. Aliquote from each elution fraction was analysed by SDS-PAGE and Coomassie Blue staining.
Appendix E

Supplementary Fig. 5. Time-course phosphorylation of S6KβII at Ser486 in serum-stimulated HEK293 cells. HEK 293 cells were transiently transfected with wild-type EE-S6KβII, starved for 24 h, and stimulated with 10 % FCS for the indicated period of time. Cell lysates were analysed by Western blotting with anti-pS486 or anti-EE antibodies.
Supplementary Fig. 6. The activity of recombinant S6KβII towards ribosomal protein S6 is not affected by pre-phosphorylation with different PKCs. HEK 293 cells were transiently transfected with wild-type EE-S6KβII and maintained in the medium supplemented with 10% FBS for 24 h. Following 24 h starvation recombinant S6KβII was immunoprecipitated with anti-EE-tag antibody and resulting immunoprecipitates were incubated with non-radioactive ATP in the absence or presence of different recombinant PKC isoforms. After 30 min of incubation, immunoprecipitates were washed with kinase buffer and used in S6 kinase assays.
Supplementary Fig. 7. Subcellular localisation of pSer486-S6KβII in HEK 293 cells treated with PMA and leptomycin B. HEK 293 cells were transfected with EE-S6KβII and treated in the same way as described for Fig.5.8. Cells were fractionated as described in Experimental procedures and cytoplasmic (C) and nuclear (N) fractions were analysed by immunoblotting with anti-pS486 or anti-β actin antibodies.
Supplementary Fig. 8. Characterization of affinity purified anti-S6Kα and anti-S6Kβ polyclonal antibodies. (A) Specificity of the antibodies generated was tested by immunoblotting of cell lysates obtained from HEK 293 cells transiently expressing recombinant S6KαII (1) and S6KβII (2) proteins. (B) Immunoprecipitation of recombinant kinase with anti-S6Kα and anti-S6Kβ polyclonal antibodies. Recombinant S6KαII (1) and S6KβII (2) were immunoprecipitated from cell lysates mentioned above using anti-S6Kα and anti-S6Kβ polyclonal antibodies, respectively. Immune complexes were resolved by SDS-PAGE and analysed by Coomassie Blue staining.
Supplementary Fig. 9. The effect of rapamycin on tetracycline-induced expression of recombinant S6Ks in stable cell lines. Parental T-Rex cells and stable cell lines were induced with 1 μg/ml tetracycline for 24 hours in the presence or absence of rapamycin. Cell lysates were analysed by immunoblotting with anti-S6Kα and anti-S6Kβ polyclonal antibodies.