Critical Reviews in Biochemistry and Molecular Biology, 29(6):385–413 (1994)

S6 Phosphorylation and the $p70^{s6k}/p85^{s6k}$

Stefano Ferrari[1] *and George Thomas*[2]

[1]Tumor Biology Center, PO Box 1120, 79011 Freiburg, Germany; [2]Friedrich Miescher Institute, PO Box 2543, 4002 Basel, Switzerland

Referee: Dr. J. Schlessinger, Dept. of Pharmacology, New York University Medical Center, 550 First Ave., New York, NY 10016

ABSTRACT: Activation of cell growth leads to the multiple phosphorylation of 40S ribosomal protein S6. The kinase responsible for controling this event is termed $p70^{s6k}/p85^{s6k}$. Both isoforms of the kinase are derived from a common gene activated by a complex set of phosphorylation events; each resides in a unique cellular compartment: the $p70^{s6k}$ in the cytoplasm and the $p85^{s6k}$ in the nucleus. Although $p70^{s6k}/p85^{s6k}$ represent the first mitogen-activated serine/threonine kinase described, the signaling pathway leading to activation of both isoforms remains obscure. Recent studies have shown that this pathway is distinct from that of $p21^{ras}$ and the $p42^{mapk}/p44^{mapk}$, and that bifurcation of these pathways takes place at the level of the receptor. Experiments with point mutants of the PDGF receptor and inhibitors of phosphatidyl-inositol-3-OH kinase have implicated the latter molecule in this signaling event, but more recent findings suggest an alternative route may be employed. The $p70^{s6k}$ signaling pathway can also be ablated by the immunosuppressant rapamycin, which blocks $p70^{s6k}$ activation and S6 phosphorylation without affecting the other kinases whose activation is triggered by mitogen treatment. In parallel, rapamycin suppresses the translation of a family of mRNAs that contain a polypyrimidine tract at their 5′ transcriptional start site. The implication is that this event is mediated by the phosphorylated form of S6 that may either (1) directly interact with the polypyrimidine tract or (2) alter the affinity of the 40S ribosome mRNA binding site for polypyrimidine tract mRNAs, or (3) recognize proteins that directly bind to the polypyrimidine tract.

KEY WORDS: signal transduction, translation, cell growth.

I. INTRODUCTION

Activation of protein synthesis plays a key role in the control of cell growth and proliferation. Increased rates of translation are associated with meiotic maturation,[1] exit from mitosis,[2] wound healing,[3] and the activation of cell growth by mitogens and oncogenes.[4–7] The importance of this event in the control of cell growth has been made strikingly evident by a number of recent observations. These include the finding that overexpression of specific translational

1040-9238/94/$.50

components or obstruction of key gene products that regulate their function can render cells more transformed,[8,9] increase their susceptibility to transformation[10] or block their ability to proliferate.[11] In most systems characterized to date, control is exerted at the level of initiation of protein synthesis.[12] This process proceeds through a complex set of steps involving a large number of translational components, including initiation factors, $tRNA^{met}$ mRNA and the 40S and 60S ribosomal subunits. This review will focus on one such component in the mitogenic response, 40S ribosomal protein S6, the kinases that control its level of phosphorylation and the intracellular signaling pathway that couples kinase activation with growth factor-activated tyrosine kinase- and G protein-coupled receptors (see Figure 1).

II. LOCATION OF S6 IN THE 40S SUBUNIT

The 40S ribosomal subunit is composed of a single molecule of 18S rRNA and 30 different proteins, one of which is S6.[13] The 40S subunit can be roughly divided into two domains termed the head and body.[13] The head also contains a protrusion commonly referred to as the beak or bill. Immunoelectron microscopy studies had initially mapped S6 to two locations, on the beak and backside of the body.[14] However, these earlier studies were invalidated when the use of the same antibodies, following affinity purification, failed to react with intact 40S ribosomal subunits.[15] In the absence of specific antibodies, researchers have relied on chemical cross-linking between proximal proteins or between proteins and RNA as well as protection studies to, roughly map S6 to the small head region of the 40S ribosomal subunit on the inner side of the beak, in a position juxtaposed to the larger 60S subunit.[16,17] This area of the 40S ribosomal subunit is implicated in mRNA binding and is thought to reside near or at the tRNA acceptor site.

III. S6 PHOSPHORYLATION AND THE INITIATION OF PROTEIN SYNTHESIS

The only protein in the 40S ribosomal subunit which has been reported to undergo phosphorylation *in vivo* in response to a number of mitogens is S6. Increased S6 phosphorylation in relation to the activation of protein synthesis and increased cell growth were initially observed in regenerating rat liver after partial hepatectomy.[18] These studies suggested that S6 phosphorylation might be a ubiquitous response when cells are induced to transit the G_o/G_1 boundary and re-enter the cell cycle by different mitogens. This hypothesis was supported by studies in tissue culture model systems which demonstrated increased S6 phosphorylation in chicken embryo fibroblasts,[4] Swiss mouse 3T3 fibroblasts[19] and human HeLa cells[20] following stimulation to proliferate by a number of mitogens. Multiple phosphorylation of S6 was subsequently found to be associated as well with the activation of protein synthesis in a number of developmental systems.[21,22]

The question of causality was initially addressed *in vivo* by exploiting the properties of cycloheximide, a potent inhibitor of

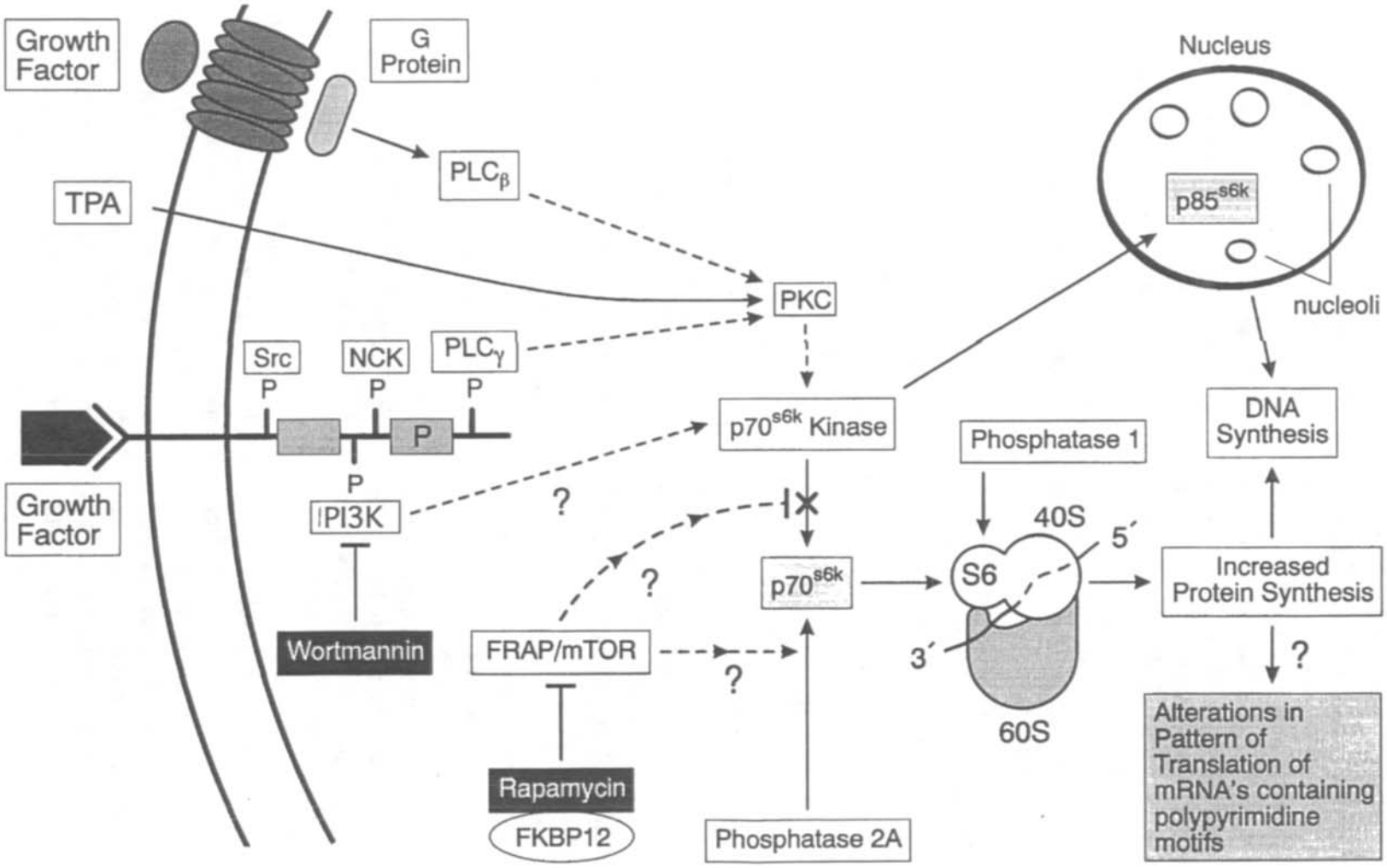

FIGURE 1. $p70^{s6k}/p85^{s6k}$ signalling pathway.

protein synthesis,[23] and theophylline, an amino-purine known to inhibit S6 phosphorylation.[5] Preincubation of cells with cycloheximide had no significant effect on serum-induced S6 phosphorylation at concentrations that blocked the shift of 80S monosomes into polysomes.[24] In contrast, theophylline inhibited both S6 phosphorylation and the activation of protein synthesis in a concentration-dependent manner.[24] These results demonstrated that increased S6 phosphorylation did not require protein synthesis, whereas the theophylline data suggested that S6 phosphorylation may be a prerequisite for the activation of protein synthesis.

The increase in protein synthesis during the G_o/G_1 transition is controlled at the level of initiation, indicating that S6 phosphorylation may exert its effect on translation at one of the steps involved in this process. Support for this hypothesis was provided by results from Duncan and McConkey[25] and Thomas and collaborators,[26] demonstrating that during the mitogen-induced shift of inactive 80S ribosomes into polysomes, ribosomes in polysomes have a higher percentage of phosphorylated S6 than either 80S ribosomes or 40S ribosomal subunits, at early times following mitogenic stimulation. At the time this difference was argued not to be due to preferential phosphorylation of polysomal ribosomes, as kinase activity was distributed equally between polysomes, 80S ribosomes, and 40S ribosomal subunits. Indeed, further studies in this laboratory suggested that S6 does not serve as a substrate for kinases or phosphatases when present in an 80S complex,[27] consistent with its location at the interface of the 40S subunit. This finding would support the interpretation that 40S subunits containing highly phosphorylated S6 have a selective advantage in entering polysomes over nonphosphorylated 40S ribosomal subunits.[25,26]

Though these observations indicated a role for S6 phosphorylation in the initiation of translation, the question arose as to its involvement in sustaining high rates of protein synthesis throughout G_1 prior to entry into S phase. In some systems, phosphorylation of S6 occurs rapidly in response to serum stimulation but declines to basal levels within 6 to 8 h, at a time when protein synthesis is still maximum.[20] However, in other systems it remains high throughout entry into S phase (G. Thomas, unpublished observations). These differences may be due to the cell type and the extent to which cells have quiesced. In the first case it was envisioned that S6 phosphorylation would exert an effect on a primary event in the initiation process but have little effect on the maintenance of the activated state.[25] Recent results[11] would instead suggest that S6 phosphorylation is important throughout G_1 for entry into S phase (see below).

IV. *IN VITRO* PROTEIN SYNTHESIS

Direct evidence for a link between S6 phosphorylation and the initiation of translation was provided by *in vitro* studies carried out by Burkhard and Traugh.[28] Initially, the effect of S6 phosphorylation on binding of poly(A,U,G) to 40S ribosomal subunits and on its translation were examined in an *in vitro* reconstituted protein-synthesizing system. Binding and transla-

tion of synthetic mRNA was enhanced by addition of 40S ribosomal subunits phosphorylated by a partially purified kinase referred to as protease-activated kinase 2 (PAK2). The stoichiometry of phosphorylation was up to 4 mol of phosphate/mol of 40S subunit. Conversely, phosphorylation by the cAMP-dependent protein kinase, with the incorporation of 2 mol of phosphate/mol of 40S subunits, had a small inhibitory effect in comparison to nonphosphorylated ribosomes.[28] A later investigation on the binding and translation of native messages in a similarly reconstituted reticulocyte protein-synthesizing system confirmed these initial observations.[29] In this set of studies phosphorylation of S6 with the mitogen-stimulated PAK2, in contrast to the cAMP-dependent kinase, stimulated globin synthesis by up to fourfold. Surprisingly, the effect was more pronounced for β- than for α-globin, suggesting that S6 phosphorylation also leads to a selective alteration of the ability of the 40S ribosomal subunit to recognize specific mRNAs. One possible criticism of this study is that the reconstituted system employed was low in total activity as compared to the unfractionated reticulocyte lysate. It will be of interest to determine whether similar results can be obtained employing a recently described reconstituted protein synthesis system that is up to 70% as efficient as the intact reticulocyte lysate.[30] It should also be noted that more recent studies indicate S6 is involved in the selective translation of a family of mRNA transcripts, characterized by a polypyrimidine tract at their 5′ transcriptional start site (see below, Rapamycin).

An alternative approach to establishing a role for S6 phosphorylation took advantage of homologous recombination in yeast.[31] *Saccharomyces cerevisiae* contains two gene copies for many of its ribosomal proteins, including S10,[32] which is the yeast homolog of mammalian S6. Although the overall amino acid identity is 60%, S10 lacks the last ten carboxyl terminal residues, including the last three phosphorylation sites that occur in mammalian S6. One gene for S10 was isolated by a random screening of recombinant λ phage clones containing *S. cerevisiae* sequences complementary to moderately abundant mRNA.[32] The second copy was isolated from an *S. cerevisiae* genomic DNA bank.[31] Employing gene disruption and site-directed mutagenesis, Kruse and co-workers[31] developed a strain of *S. cerevisiae* in which the only gene for S10 codes for a protein with alanines in place of the two serines suspected to be sites of phosphorylation. Cells of this strain can grow, albeit somewhat more slowly, even though they are unable to phosphorylate S10. From these results and a follow-up study, the authors concluded that S10 phosphorylation is not essential for normal growth.[33] However, the same conclusion may not be applicable to higher eukaryotes.[11,34] Indeed, the same two sites are phosphorylated in mammalian S6 by the cyclic AMP-dependent protein kinase and have little effect on the *in vitro* reconstituted protein synthesis system described above as compared to nonphosphorylated S6.[29] Further, the apparent ability of 40S ribosomal subunits to enter polysomes is unaffected by phosphorylation of these two sites.[26] Thus it may be that only the late sites of S6 phosphorylation are required for increased translation in higher eukaryotes, sites that are absent from *S. cerevisiae*.

Paradoxically, S6 appears to play the role of a tumor suppressor gene in *Droso-*

phila, at least in some tissues. In two recent publications[35,36] it has been shown that P element insertions in the promoter regions of *Drosophila* S6 lower the expression of the transcript, delaying larval development and leading to lethality by the third larval instar. This is not a surprising finding, as such a depletion of S6 transcripts would probably have direct impact on ribosome biogenesis. However, these mutations cause overgrowth of hematopoietic organs, abnormal blood cell differentiation, and melanotic tumors, indicating that S6 may play a role as a tumor suppressor gene. It is difficult to envision a scenario to explain this result. It has been shown that S6 is both in the nucleus, in association with chromatin, and in nucleoli.[37] Possibly the tumor suppressor-like activity is exerted at this level. It should also be noted that Stewart and Denell[36] reported the existence of two additional third exons in the S6 gene that if expressed would lead to alternative S6 phosphorylation sites. Further studies should resolve their functional importance as potential developmental switches.

V. IDENTIFICATION OF S6 PHOSPHORYLATION SITES

The amino acid sequence of 40S ribosomal protein S6 was first deduced from the nucleotide sequence of a mouse recombinant cDNA[38] and subsequently from a rat liver[39] and human[40] cDNA. All three cDNAs encoded an identical protein, which was also equivalent to the amino acid sequence of rat S6 as determined by direct protein sequencing.[41] The yeast homolog of S6, S10, displays 60% identity with mouse S6,[38] whereas the *Drosophila* counterpart is 75% identical.[36]

Studies carried out by Wettenhall and colleagues[41,42] first pointed to the existence of eight potential phosphorylatable residues in a 17-amino acid tryptic peptide of S6 thought to be at or near the carboxyl terminus of the protein. As an initial approach to identify the sites affected, Krieg et al.[43] isolated 40S ribosomal subunits from the livers of rats injected with cycloheximide and ^{32}Pi to obtain sufficient amounts of highly phosphorylated S6, which was then cleaved with cyanogen bromide. The cyanogen bromide fragment accounting for all of the radioactivity incorporated into S6 was purified and shown to contain only phosphoserine. The phosphorylated residues were converted to the stable analog S-ethyl cysteine,[44] and the five sites of phosphorylation were shown to be S_{235}, S_{236}, S_{240}, S_{244}, and S_{247} by direct Edman degradation. However, cycloheximide did not induce full S6 phosphorylation in rat liver[45] and apparently only activated one of the S6 kinases that had been implicated in S6 phosphorylation,[46] (see below, Rapamycin). In addition, *in vitro* studies[47] argued for other potential sites of S6 phosphorylation that are distinct from those identified following cycloheximide induction. Together, these findings placed in question the identity of the phosphorylation sites induced by mitogens. However, Bandi et al.[48] have recently demonstrated that S6 obtained from ribosomes of 3T3 cells stimulated with 10% fetal calf serum is phosphorylated at the same sites as those induced by cycloheximide. This suggested that phosphorylation of the five residues at the carboxyl terminus of S6 is a mechanism common to all growth stimuli. Furthermore, it had been shown earlier by tryptic phosphopeptide

analysis of the increasingly phosphorylated derivatives of S6 that the reaction proceeds in an ordered fashion.[49] These results were supported by the studies of Krieg et al.[43] which in addition indicated that the order of phosphorylation is $S_{236} \rightarrow S_{235} \rightarrow S_{240} \rightarrow S_{244} \rightarrow S_{247}$. However, more recent studies demonstrate that the order *in vivo* is $S_{236} \rightarrow S_{235}$ or $S_{240} \rightarrow S_{244} \rightarrow S_{247}$ (H. R. Bandi, S., Ferrari, R. E. Wettenhall, and G. Thomas, unpublished data). Based on the studies described above,[26,29] it would appear that the late sites of phosphorylation, i.e. S_{244} and S_{247}, would contribute the most to the stimulation of protein synthesis.

VI. MITOGEN-INDUCED S6 KINASE ACTIVITY

The search for an S6 kinase began ten years ago. Preliminary studies showed that S6 could serve as a substrate *in vitro* for the cAMP- and cGMP-dependent protein kinases,[50] protein kinase C,[51,52] calmodulin-dependent protein kinase II,[53] casein kinase I,[54] and the protease-activated kinases PAK II[55] and histone 4-protein kinase (H4PK).[56] However, none of the above kinases was able to fully phosphorylate S6 to the extent observed *in vivo*, nor in most cases were their mechanisms of activation consistent with a signaling pathway modulated by mitogens. The first report of a mitogen-activated serine/threonine S6 kinase, came from studies by Novak-Hofer and Thomas[57] in Swiss 3T3 cells. They used extraction conditions previously employed to isolate maturation promoting factor (MPF) from unfertilized *Xenopus* eggs,[58] as preparations of MPF were known to induce S6 phosphorylation when injected into immature oocytes prior to meiotic maturation or when incubated with 40S ribosomal subunits *in vitro*.[22] The key components of the extraction buffer appeared to be β-glycerol phosphate and EGTA, both being required for maintainance of full S6 kinase activity in extracts of serum-stimulated cells, with only the EGTA effect being reversible. The authors speculated that a phosphorylation-dephosphorylation mechanism was involved in the regulation of an S6 kinase activity, and that β-glycerol phosphate was acting as a competitive phosphatase inhibitor preventing dephosphorylation and inactivation of this activity. Based on this hypothesis, several potential phosphatase inhibitors were tested for their ability to maintain S6 kinase in its active form.[57] All protected S6 kinase activity to a large extent, with the exception of NaF, which inhibited the activity of the kinase *in vitro*. These studies indicated that either the S6 kinase itself or a regulatory component was under the control of phosphorylation and provided the first evidence for the existence of kinase cascades in mitogen-induced intracellular signaling.

At the same time, growing interest in the phosphorylation of S6 as a point of convergence of many mitogen-induced signaling pathways led to the identification of a number of agents able to trigger this response. Decker[7] observed that S6 was phosphorylated in a serum independent manner in cells transformed with a temperature-sensitive mutant of avian sarcoma virus (ASV) when grown at permissive temperature. Smith and collaborators[6] reported that physiological concentrations of insulin enhanced the incorporation of ^{32}Pi into ribosomal protein S6 in 3T3-L1 adipocytes. Thomas et al.[26] found that the ability of serum, epidermal growth factor (EGF),

prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and insulin in eliciting S6 phosphorylation paralleled their ability to activate protein and DNA synthesis. Blenis et al.[59] reported that S6 appeared to be highly phosphorylated in cell cultures transformed with RNA or DNA tumor viruses in the absence of the mitogen, whereas the parental cell lines required the presence of serum. Moreover, the tumor promoter 12-O-tetradecanoyl-phorbol-13-acetate, or PMA, when added to serum-starved chicken embryo fibroblasts resulted in increased phosphorylation of S6.[60] Employing extraction conditions similar to those described by Novak-Hofer and Thomas[57] a number of these workers subsequently demonstrated the existence of an apparently equivalent S6 kinase activity in other systems.[61–66]

VII. IDENTIFICATION OF THE p70^{s6k}

Purification of the mitogen-activated S6 kinase to homogeneity was achieved by Jenö et al.[67,68] following treatment of Swiss 3T3 cells with vanadate, a protein tyrosine phosphatase inhibitor. Activation by vanadate was chosen because it appeared to be as efficient as the most potent mitogens tested, including serum and platelet-derived growth factor (PDGF), and activation was persistent rather than transient. The protocol for purification of the mitogen-activated S6 kinase was based on the finding by Ballou et al.[69] that the active form of the enzyme could be resolved from putative inactivating phosphatases on cation exchange columns. That inactivation was due to phosphatases was suggested by a number of observations: (1) the kinase was stable in preparations lacking phosphatase activity; (2) the inactivating agent co-eluted with phosphorylase A phosphatase activity during anion exchange- and gel filtration-chromatography; (3) phosphatase inhibitors protected the kinase from inactivation; and (4) the pure catalytic subunits of phosphatase 2A and 1 catalyzed the inactivation of the kinase. Therefore, the first of the eventual five steps employed in the purification was cation-exchange chromatography to remove phosphatases. The purified molecule appeared to be a single polypeptide chain with an apparent M_r of 70,000 as judged by SDS polyacrylamide gel electrophoresis,[68] and was subsequently termed p70^{s6k}. When corrected for losses at each step of the purification, the amount of protein present revealed that p70^{s6k} is a rare molecule.[68] The kinase was clearly distinct from the cAMP-dependent protein kinase,[50] protein kinase C[51,52] and the protease-activated kinases PAK II[55] and H4PK.[56] The pure enzyme had a final specific activity toward S6 of ≈1.0 μmol per min per mg of protein, underwent autophosphorylation, was inactivated by phosphatase 2A, and incorporated 4 to 5 mol of phosphate into S6 *in vitro* in the same tryptic phosphopeptides as observed *in vivo*.

VIII. ACTIVATION OF p70^{s6k} IS ASSOCIATED WITH PHOSPHORYLATION

The results above demonstrated that phosphorylation was required for maintenance of the activated state, but did not demonstrate that p70^{s6k} activation by mitogens was associated with phosphorylation. To test this possibility, Ballou et al.[70] puri-

fied the enzyme from quiescent or 60-min serum-stimulated Swiss 3T3 cells labeled with $^{32}P_i$. A single radioactively labeled protein of M_r 70,000 that displayed kinase activity toward S6 and autophosphorylated, was obtained from serum-stimulated cells but not from quiescent cells. These results suggested that $p70^{s6k}$ from quiescent cells had a low basal activity which was greatly enhanced following mitogenic stimulation and that this increased activity was associated with increased phosphorylation of the kinase. Phosphoamino acid analysis revealed that the enzyme contained predominantly phosphoserine, to a lesser extent phosphothreonine, and no phosphotyrosine. Moreover, treatment of the *in vivo* labeled material with the free catalytic subunit of phosphatase 2A led to a loss of kinase activity concomitant with the release of $^{32}P_i$ from the kinase and a stepwise increase in its electrophoretic mobility on SDS-PAGE. These results clearly indicated that phosphorylation at multiple sites *in vivo* is associated with $p70^{s6k}$ activation. They also indicated that if the $p70^{s6k}$ lies on a signaling pathway initiated by activation of the tyrosine kinase receptor there has to be at least one additional kinase in the cascade that is activated by tyrosine phosphorylation but which phosphorylates serine/threonine residues. However, it has not yet been demonstrated that phosphorylation induces kinase activation (see below).

IX. OTHER MITOGEN-ACTIVATED S6 KINASES

As previously mentioned, numerous systems have been reported to contain kinases that phosphorylate S6. However, based on their modes of activation and their ability to generate S6 phosphopeptide maps equivalent to those observed *in vivo*, only two other kinases appear to be comparable to the $p70^{s6k}$ enzyme: a M_r 90,000 kinase (referred to as S6 kinase II or $p90^{rsk}$, rsk = ribosomal S6 kinase) initially isolated from unfertilized *Xenopus* eggs[62,71] and a M_r 67,000 kinase from bovine liver.[72] Both enzymes have been purified to apparent homogeneity but display a lower specific activity toward S6 in 40S ribosomal subunits than $p70^{s6k}$. The $p90^{rsk}$ is a member of the rsk family of kinases and was first designated S6K II since it elutes as the second peak of S6 kinase activity from the initial column used in its purification.[62,71] The first peak, S6K I, is related to S6K II and also has a M_r 90,000.[73] Based on partial amino acid sequences of tryptic peptides from $p90^{rsk}$, oligonucleotide probes were designed in order to clone the gene from a *Xenopus* cDNA library. The sequences of the cDNAs obtained predicted a M_r 83,000 molecule, though only four of the eight tryptic peptides sequenced were found in this clone. The existence of the four additional peptides may be due to the fact that the $p90^{rsk}$ appears to be one member of a large gene family.[74] The $p90^{rsk}$ displays the novel feature of having two apparent catalytic domains: the amino-terminal half of the molecule is related to the catalytic subunit of the cAMP-dependent protein kinase, whereas the carboxyl-terminal half is related to the catalytic subunit of phosphorylase kinase.[75] Despite the homology in the carboxyl-terminal domain with phosphorylase kinase, $p90^{rsk}$ is not able to phosphorylate phosphorylase b. Likewise, phosphorylase b kinase is unable to phosphorylate S6.[76] This finding led to the suggestion that it may only be the amino-ter-

minal domain that is catalytically active. Complementary cDNA clones predicted similar gene families in chicken, mouse, and humans, suggesting that the rsk family of kinases is highly conserved.[77] Antiserum to recombinant *Xenopus laevis* $p90^{rsk}$ protein was employed to identify $p90^{rsk}$ in chicken embryo fibroblasts, Swiss mouse 3T3 fibroblasts, and human HeLa cells.[78] The M_r 67,000 S6 kinase has been purified from bovine liver.[72] The purification protocol yielded an amount of kinase comparable to that obtained for the $p70^{s6k}$ from liver of cycloheximide-injected rats (see below) but displayed a low specific activity toward S6. It seems likely that this enzyme was equivalent to $p70^{s6k}$ and that its low activity was due to the fact that animals were not pretreated with any agents that would have induced activation of the kinase *in vivo* prior to purification. The bovine enzyme was partially characterized with regard to its substrate specificity and ion requirements,[72] but no further studies have been reported.

X. $p70^{s6k}$ FROM RAT LIVER

The $p70^{s6k}$ appeared to be much less abundant than the $p90^{rsk}$, leading to the speculation[77] that it may have been a degradation product of the $p90^{rsk}$. To resolve this question, a more abundant source of the enzyme was required to generate protein sequence data. In preliminary experiments it was shown that liver extracts from partially hepatectomized rats contained two- to eightfold more S6 kinase activity than extracts from sham-operated animals.[79,80] This treatment was not suitable for purification purposes because of the inherent loss of tissue. To circumvent this problem, the potency to stimulate S6 kinase activity in rat liver was tested by refeeding starved animals or injecting them with either cycloheximide, insulin, or vanadate.[81] The most potent method for eliciting $p70^{s6k}$ activation appeared to be cycloheximide injection of rats. This finding allowed purification of $p70^{s6k}$ to homogeneity.[81–83] The strategy employed by Kozma and collaborators was largely based on the protocol established for purification of the 3T3 cell enzyme. However, the initial protocol for purification[81] was subsequently facilitated by the addition of two affinity steps.[83] The advantage of the modified protocol described by Lane and Thomas[83] is that the purification can be carried out in 2 to 3 d with a final recovery of 4%. The specific activity of the final preparation is ≈1 μmol/min/mg protein. The large-scale purification was also facilitated by the use of preparative columns that could be used at high flow rates.[84] In combination the two affinity steps of threonine-Sepharose and a 32 amino acid peptide, based on the S6 substrate sites of phosphorylation, coupled to Sepharose, gave a 1000-fold purification.[83] The purified kinase preparation yielded a single band migrating at M_r 70,000. In contrast, Price et al.[82] detected in addition to the M_r 70,000 protein a second band of M_r 93,000/95,000. This higher molecular weight band did not react with azido-ATP, contrary to the M_r 70,000 protein. Therefore, the authors concluded that the M_r 93,000/95,000 kDa polypeptide was a contaminating protein that is structurally and functionally unrelated to the $p70^{s6k}$ (see below). The M_r 70,000 enzyme purified from the liver of rats injected with cycloheximide had the identical M_r as the

Swiss 3T3 cell kinase and could undergo autophosphorylation on serine/threonine residues. The two enzymes were judged to be the same based on their equivalent sensitivity to phosphatase type 2A and their phosphopeptide maps.[81]

XI. CHARACTERIZATION OF p70^{s6k}

The homogeneously purified rat liver p70^{s6k} was further characterized in terms of substrate specificity, following the development of optimal conditions for S6 phosphorylation.[85] These studies took into account the effects of different cations as well as the effect of autophosphorylation on kinase activity. The p70^{s6k} is dependent on Mg^{2+} for activity and no other cation can be substituted for Mg^{2+}. Indeed, all other divalent cations tested were potent inhibitors of p70^{s6k} activity in the nanomolar to micromolar range. Autophosphorylation was found to be intramolecular and led to a modest 25% reduction in kinase activity toward S6. Employing optimal conditions for S6 phosphorylation *in vitro*, four sites of phosphorylation were identified, S_{235}, S_{236}, S_{240}, and S_{244}. These are equivalent to four of the five sites observed *in vivo*,[43,48] with no phosphate detectable in S_{247}. However, analysis by isoelectric focusing of the endoproteinase Lys-C phosphopeptide, which contained all of the radioactivity incorporated into S6, revealed the presence of a minor isoform of the peptide that contained 5 mol of phosphate. This result supported the existence of a fifth site, possibly S_{247}, which was phosphorylated at low stoichiometry, but whose location could not be unambiguously assigned due to the presence of two additional serines in the endoproteinase Lys-C peptide.[85] Interestingly, in agreement with the above data, Wettenhall et al.[86] recently reported that the four sites phosphorylated *in vitro* by p90^{rsk} in S6 are equivalent to those described for the p70^{s6k} and that no phosphate was detectable in S_{247}.

In an attempt to understand the phosphorylation reaction mechanism and why S_{247} is such a poor substrate *in vitro*, the primary structure surrounding the sites of phosphorylation was examined.[85] No consensus motifs for phosphorylation could be detected at this level. Therefore, a number of secondary structural models of S6 were constructed beginning with K_{230} and extending through K_{249}. The structure that best fit the order of phosphorylation was an α-helix refined by energy minimization. Support for this model came from examination of the X-ray crystal structures of known proteins such as subtilisin.[87] Subtilisin contains a stretch of sequence that is almost identical to a large portion of the S6 peptide. Employing energy minimization, the α-helix appeared to be stable from K_{230} through E_{245}, with no possibility of assigning a definitive structure to the last four amino acids that included S_{247}. However, in the 40S subunit it may be that this last stretch of amino acids is held in the helix by interaction with neighboring proteins or with 18S rRNA. Examination of the model shows that S_{236}, S_{240}, S_{244}, and S_{247} would all lie on the same side of the helix. Although the aliphatic backbone of S_{235} is not on the this side of the helix, its hydroxyl group does face to this side in the same direction as the others. In contrast, S_{242} and S_{246} lie on the opposite side of the helix. Based on this structure, a progressive model

of phosphorylation was postulated in which the enzyme first catalyzes the phosphorylation of S_{236} and S_{235} and then moves along the helix phosphorylating S_{240}, S_{244}, and S_{247}. The order of phosphorylation is also consistent with the presence of three arginines at the amino terminus of the peptide, which create a positively charged environment around the first two serines. The electropositive amino terminus of the peptide and the permanent dipole generated by the helix introduce strong coulombic interactions that render the first sites of phosphorylation very favorable; in contrast, S_{247} becomes less accessible. The model is also consistent with recent studies aimed at defining the $p70^{s6k}$ recognition determinants.[88] The $p70^{s6k}$ has an apparent K_m for 40S ribosomal subunits of 0.25 μM. Moreover, employing a synthetic peptide derived from the carboxyl terminus of S6, it was possible to narrow down the substrate recognition determinants for the preferred site of phosphorylation, S_{236}, to a block of basic residues lying just upstream of the S6 phosphorylation sites. Interestingly, the K_m for this peptide was almost as good as it was for S6 in intact ribosomes, arguing that all the recognition determinants for $p70^{s6k}$ resided in this sequence. Based on this study, the $p70^{s6k}$ consensus recognition sequence was determined to be RXRXXSX. All the other kinases tested that are known to partially phosphorylate S6 displayed considerably higher K_m values toward this peptide, except for $p90^{rsk}$ whose affinity for this peptide was surprisingly much higher than for 40S ribosomal subunits. Although this model is consistent with *in vivo* and *in vitro* data, its validity must be tested by means of physical chemical techniques.[85]

XII. CLONING OF $p70^{s6k}$

To obtain protein sequence data from the $p70^{s6k}$, it was first treated with cyanogen bromide, as the amino terminus was apparently blocked. The cyanogen bromide cleavage products were resolved on a polyacrylamide gradient gel and electroblotted onto PVDF. Two of the resulting peptides were subjected to microsequencing using a sequencer equipped with a miniaturized reaction cartridge, employing rapid cycle chemistry and on-line analysis programs.[89,90] A second approach took advantage of trypsin digestion and a totally inert HPLC system fitted with a glass-lined C_{18} reverse phase column.[90] More than 50 tryptic peptides were resolved with 18 of them undergoing microsequencing, either directly or following further purification. Three of the initial peptides sequenced had evident homology to subdomains VI, IX, and XI of the conserved catalytic domain of the Ser/Thr kinases.[91] Degenerate oligonucleotides based on the peptide sequence in subdomains VI and XI were synthesized and a 0.4-kb DNA fragment was generated by PCR using rat embryo cDNA as template. This DNA fragment was then labeled to high specific activity in a PCR reaction and used as a probe to screen a rat liver poly(A)$^+$ mRNA Northern blot, which revealed the presence of four transcripts of 2.5, 3.2, 4.0, and 6.0 kb. Based on the complexity of this blot, it was initially predicted that $p70^{s6k}$ might represent a multigene family.[89]

To isolate cDNA clones encoding the S6 kinase, a rat liver cDNA library was screened with the same labeled cDNA fragment as described above. Five positive clones were obtained with two being full-length and identical in size, probably re-

flecting the fact that the library had been amplified once. The full-length clones were 2.8 kb in size. This cDNA is referred to as Clone 1,[89] or 70αII.[92] The two first ATGs encountered at the 5′ end of Clone 1 were followed by stop codons. The third ATG from the 5′ end of Clone 1 was assumed to be the translation start site since it had a G in positions -3 and +4, indicative of a strong Kozak translation start sequence.[93] The 5′ untranslated region was 133 nucleotides long, which was also consistent with the average size found in mRNA sequenced to date.[93] The open reading frame was 1506 nucleotides long, coding for a protein of 502 amino acids, and was terminated by two consecutive translation stop codons. This clone would encode a protein of 56,160 Da, which is substantially smaller in size than the M_r 70,000 obtained by SDS-PAGE for the $p70^{s6k}$. However, this anomaly could be explained in part by the gel system used for separating the $p70^{s6k}$ and by phosphorylation, which retarded the migration of the protein on SDS-PAGE.[89] The cDNA clone appeared to have been initiated by random priming since sequencing from the 3′ end of the gene did not reveal a poly(A)$^+$ tail. After aligning the 17 internal peptides sequenced, all but one were found in the cDNA sequence.[89]

In parallel studies, an almost identical cDNA was cloned in two independent laboratories.[92,94] One clone, isolated from an H4 hepatoma cDNA library, was 2.3 kb in size. The first ATG is at position 22 and it is followed by an open reading frame encoding a protein of 525 amino acids, terminating with a tandem pair of TGA stop codons. In contrast to the clone isolated from the rat liver library, this clone contained a polyadenylation signal at nucleotide 2259 followed by a poly(A)$^+$ tail starting 27 base pairs downstream. This same clone was found by screening a rabbit cDNA library at low stringency with a probe based on the sequence of phosphorylase kinase.[94] These two cDNAs are referred to as Clone 2[95] or 70αI.[96] Comparison of Clone 1 and Clone 2 revealed that they are identical except that (1) Clone 2 from rat H4 hepatoma cells contains one amino acid difference in the common coding region as compared to Clone 1 from rat liver or Clone 2 from rabbit liver, (2) Clone 2 contains a 23 amino acid extension at its amino terminus, and (3) both Clones 1 and 2 from rat contain distinct 5′ untranslated sequences. By using specific probes to the 5′ untranslated region it was shown that both Clones 1 and 2 are present in the 6.0 kb transcript, only Clone 1 is present in the 4.0 and 3.2 kb transcripts, and only Clone 2 is present in the 2.5 kb transcript.[95] Furthermore, Southern blots combined with a genomic PCR analysis argued that (1) both clones are derived from a common gene, (2) the untranslated region of Clone 2 is contiguous with the common coding region, and (3) transcripts for Clone 1 must be generated by differential splicing.[95] These indications have recently been confirmed in mouse by carrying out a genomic Southern blot analysis employing as a probe an exon spanning part of the 5′ untranslated region of Clone 2 and the beginning of the common coding region (Y. Chen, G. Thomas, and S. C. Kozma, unpublished data). Consistent with the data mentioned above, these two cDNA clones have also been isolated from a human cDNA library.[96] Clone 1 and Clone 2 products from humans differ from the rat proteins only by two amino acids, one in the amino-terminal extension and one in the common coding region of the protein.[96] More recently,

a homologous sequence was shown by polymerase chain reaction to be present as a maternal transcript in stage VI *Xenopus* oocytes.[97] The sequence appears to be 97% identical at the amino acid level. The authors suggested that considering the presence of activated $p70^{s6k}$ in *Xenopus* oocytes following progesterone induction, the lower affinity of $p90^{rsk}$ for S6 compared to $p70^{s6k}$ [88,98] and the finding that full S6 phosphorylation can be obtained by $p70^{s6k}$ activation without $p90^{rsk}$ activation,[46] $p70^{s6k}$ may be the sole enzyme responsible for S6 phosphorylation during meiotic maturation (see below, Rapamycin).

XIII. A NOVEL ISOFORM OF THE $p70^{s6k}$, THE $p85^{s6k}$

To determine which of the two clones was responsible for encoding the $p70^{s6k}$, both clones were translated *in vitro* and the products immunoprecipitated with specific antibodies.[95] The results demonstrated that Clone 1 yielded a protein identical in size to the purified-dephosphorylated $p70^{s6k}$, whereas Clone 2, with a larger open reading frame, gave rise to a product of M_r 85,000 in addition to the $p70^{s6k}$.[95,96] A monospecific polyclonal antibody generated against the 23 amino acid extension of Clone 2 specifically immunoprecipitated the M_r 85,000 product, leading to the conclusion that the M_r 85,000 protein is generated from the first translational start site of Clone 2 and the M_r 70,000 S6 kinase is derived from the second translational start site. To determine whether the M_r 85,000 exists *in vivo*, this same monospecific polyclonal antibody was used to probe Western blots of highly purified $p70^{s6k}$. The results showed that the M_r 85,000 was present, albeit in very low amounts. Moreover, in an immunocomplex assay the M_r 85,000 displayed activity toward S6.[95] More strikingly, it was also activated following mitogenic stimulation of quiescent cells in culture.[95] Therefore, there appear to be two isoforms of the enzyme, the $p70^{s6k}$ and a second form, now termed the $p85^{s6k}$. Closer inspection of this 23 amino acid extension reveals that it contains all the hallmarks of a nuclear targeting sequence, consistent with the fact that S6 becomes phosphorylated in the nucleus following stimulation of pituitary cells with phorbol esters or EGF.[37] Immunofluoresence studies employing a specific antibody to the 23-amino-acid extension of $p85^{s6k}$ showed exclusive localization in the nucleus.[120] This finding was supported by three independent lines of evidence. First, microinjection into cells of a mammalian expression vector encoding only the $p85^{s6k}$ isoform led to its exclusive accumulation in the nucleus. Second, microinjection of an expression vector containing the 23-amino-acid extension of $p85^{s6k}$ fused to the bacterial protein CAT also led to exclusive accumulation of the chimeric protein in the nucleus. Finally, microinjection of $p85^{s6k}$ affinity-purified antibodies into the nucleus, but not the cytoplasm, blocked G_1 progression. This last effect could be rescued by co-injection of the $p70^{s6k}$ isoform.[120] Indeed, in contrast to recent suggestions,[121] this last result, combined with quantitative confocal microscopy,[120] strongly supports the argument that $p70^{s6k}$ is a resident cytoplasmic protein.

XIV. TOPOGRAPHY OF $p70^{s6k}$/ $p85^{s6k}$

Excluding the 23 amino acid extension at the amino terminus of the $p85^{s6k}$, the

$p70^{s6k}/p85^{s6k}$ can be roughly divided into three domains: (1) An amino-terminal stretch of 65 amino acids containing only a single basic residue and a high content of acidic residues, ≈40%. Given that S6 is a highly basic protein, this domain may be involved in substrate recognition[88,92]; (2) A catalytic domain spanning the region from E_{66} to I_{332}, which displays all the highly conserved motifs typical of a number of the second messenger family of Ser/Thr protein kinases.[91] Among the five major subfamilies defined by such an amino acid sequence comparison, $p70^{s6k}$ would appear to belong to the protein kinase C subfamily. An obvious exception to the general trend of this subfamily is the presence of a tyrosine at position 79 instead of phenylalanine. A similar tyrosine in this position has only been found in the $p34^{cdc2}$ subfamily, where phosphorylation of this site keeps the protein in an inactive state. Within the second messenger subfamily, $p70^{s6k}$ appears to be closely related to the first catalytic domain of the α-form of $p90^{rsk}$ from *Xenopus* (56%),[75] yeast protein kinase 1 from *Saccharomyces cerevisiae* (49%),[99] and protein kinase C_ε (44%)[100]; (3) A carboxy-terminal domain from N_{333} to L_{502} that, due to the three-fold higher density of phosphorylatable residues and the presence of a putative autoinhibitory sequence,[92] was initially presumed to be the regulatory domain. This hypothesis was strengthened by the finding that all the phosphorylation sites associated with mitogen-activation of the kinase apparently reside in this domain.[90] The strategy employed to identify these sites was based on the observation of Roach[101] that enzymes whose activity is under the control of multiple phosphorylation tend to have their sites of phosphorylation closely clustered near one another. After activating $p70^{s6k}$ with serum in the presence of $^{32}P_i$, the immunoprecipitated ^{32}P-labeled $p70^{s6k}$ was selectively cleaved with cyanogen bromide followed by endoproteinase Lys-C. This protocol localized all the phosphorylation sites within a small endoproteinase Lys-C peptide of M_r 2,400. Subsequent digestion of the endoproteinase Lys-C peptide by trypsin was followed by the separation of the tryptic peptides by two-dimensional thin-layer electrophoresis/chromatography. Analysis of their migration patterns, employing a computer program developed by Hunter and colleagues,[102] revealed a single candidate for the endoproteinase Lys-C peptide fragment within the $p70^{s6k}$ protein sequence. Synthetic peptides based on the sequence of three tryptic peptides derived from the M_r 2,400 fragment were phosphorylated either chemically or enzymatically and found to comigrate in two-dimensional thin-layer electrophoresis/chromatography with the four major *in vivo*-labeled tryptic phosphopeptides, with one peptide either singly or doubly phosphorylated. Three of the phosphorylation sites were equivalent to those identified by direct sequencing[89] of internal tryptic peptides of rat liver $p70^{s6k}$. All four sites displayed the motif Ser/Thr-Pro typical of cell cycle-regulated sites. Moreover, this same endoproteinase Lys-C peptide contained part of the putative pseudosubstrate sequence for the $p70^{s6k}/p85^{s6k}$. This hypothesis was based on ≈30% homology of this sequence to the C-terminus of S6.[92] Banerjee and colleagues[92] initially envisioned that this sequence binds to the negatively charged amino terminus of the $p70^{s6k}/p85^{s6k}$, occluding the substrate-binding site and maintaining the kinase in its basal inactive state. Following phosphorylation of sites surrounding the

pseudosubstrate regulatory domain, the negative constraint is released, allowing the catalytic site to interact with the substrate. Subsequently it was found that a peptide spanning residues 400–432 of the $p70^{s6k}$ is not phosphorylated by the kinase,[88,92] but inhibits the phosphorylation of S6, displaying a $K_i \simeq 30\ \mu M$. By aligning peptide 400–432 with S6, Flotow and Thomas[88] showed that it lacks an arginine in position 233, one of the essential recognition determinants for phosphorylation of S6. They suggested that this could explain the 60-fold difference in inhibition observed with this peptide as compared to the S6 peptide containing R_{233} and A_{235}. The results support the model of regulation of $p70^{s6k}$ by an autoinhibitory domain, which itself is regulated by phosphorylation. However, it seems more likely that in the inactive state this domain does not interact with the amino terminus of $p70^{s6k}$ as initially predicted,[92] but with the catalytic site.

XV. BIPHASIC ACTIVATION OF $p70^{s6k}$

Early data had suggested that activation of $p70^{s6k}$ and S6 phosphorylation following addition of EGF were rapid and transient events.[103] We found that S6 phosphorylation increased more slowly than $p70^{s6k}$ activity and remained at its maximal level for up to 4 h post-induction when the kinase activity had already decayed to basal levels.[103] In these studies, $p70^{s6k}$ activity reached a maximum between 5 and 15 min before slowly returning to basal levels by 120 min. However, more detailed kinetic studies indicated that the time course of activation was more complicated, revealing two phases of activation. An early peak at 10 to 15 min was followed by a longer, late phase reaching a maximum between 30 and 45 min before returning to basal levels by 120 min.[104] This same behavior was observed following stimulation of quiescent cells to proliferate with other mitogens such as PDGF or insulin.[105] The possibility that two different enzymes could be activated sequentially or that a single kinase could be activated by tyrosine phosphorylation in the first phase and by serine/threonine phosphorylation in the second was investigated by purifying and characterizing the kinase activity from the two peaks following treatment with EGF.[104] Irrespective of the activation period, both activities displayed the same behavior on anion-, cation-, or hydrophobic-exchange interaction chromatography columns and both forms were equally sensitive to treatment with phosphatase 2A. These results strongly suggested that the same S6 kinase was responsible for both phases of activation, and indicated that a single mitogen acting through its corresponding receptor activated two signaling pathways that converge on $p70^{s6k}$ at different times.[106]

Previous studies carried out by Blenis and colleagues[60,61] had shown that long-term pretreatment of cells with the phorbol ester PMA leads to down-regulation of protein kinase C and a partial attenuation of the serum-induced S6 kinase response. The results obtained were consistent with part of the $p70^{s6k}$ response being regulated by a protein kinase C-dependent signaling pathway. To test this possibility, cells were pretreated for 24 h with PMA and rechallenged with EGF. Under these conditions, the second phase of S6 kinase response was almost totally abolished.[104] This finding was also consistent with the obser-

vation that stimulation of quiescent cells by PMA only results in a late-phase $p70^{s6k}$ response. Thus, the second phase of $p70^{s6k}$ activation induced by EGF appeared to be largely under the control of protein kinase C. Down-regulation of this second phase also led to a significant reduction in the ability of EGF to induce S6 phosphorylation, increase protein synthesis, (as measured by percentage of inactive 80S ribosomes shifting into polysomes), and stimulate cell growth. The authors showed that the effects observed were not due to loss of high-affinity EGF receptor binding sites through phosphorylation of the receptor by protein kinase C, as these sites returned within 2 h post-stimulation.

To ensure that the effects of PMA on $p70^{s6k}$ activation were through protein kinase C[105], a specific inhibitor of protein kinase C modeled on the structure of staurosporin was employed, as well as the growth factors PDGF, which is known to be strongly coupled to the protein kinase C signaling pathway, and insulin, which is not. It was found that the abolition of the second phase of EGF-induced S6 kinase activation was mimicked by pretreatment of cells with the active analog of staurosporin, termed CGP 41 251, supporting the hypothesis that the second phase is under the control of protein kinase C. In the case of PDGF and insulin, no inhibitory effect of CGP 41 251 was elicited on either phase of $p70^{s6k}$ activation. In the case of PDGF, this was a surprising finding, as this growth factor is known to be linked to the protein kinase C signaling pathway. To establish the validity of this result, cells were pretreated with PMA to down-regulate protein kinase C and then rechallenged with PDGF. Under these conditions, no inhibitory effect was seen on either phase of $p70^{s6k}$ activation, supporting the studies with CGP 41 251. Thus, unlike EGF, PDGF does not appear to activate $p70^{s6k}$ through a protein kinase C-dependent pathway. In fact, this result is consistent with the finding that down-regulation of protein kinase C inhibits EGF[104] but not PDGF-induced cell growth,[105] suggesting that PDGF induces mitogenesis via pathways alternative to protein kinase C activation or that PDGF activates a different protein kinase C isoform that is not down-regulated by PMA and is insensitive to the inhibitor CGP 41 251. Although the mechanisms controlling the two phases of $p70^{s6k}$ activation have not been clearly identified, the observation that all mitogens to date induce a biphasic response implies a basic biological principle in the activation of cell growth whose importance must still be evaluated.

XVI. IDENTICAL $p70^{s6k}$ IN BOTH PHASES OF THE BIPHASIC RESPONSE

The finding that $p90^{rsk}$, originally described in extracts of *Xenopus* eggs by Erikson and Maller,[62,71] was also activated within minutes of EGF stimulation in A431 cells[107] raised again the question of the identity of the S6 kinase activity in both phases and the point at which the early and late signaling pathways converge to induce S6 kinase activation. To address this question, Šuša and Thomas[106] purified the enzymes present in the two phases of the S6 kinase response induced by EGF. In both cases, a single ^{32}P-labeled protein was observed at M_r 70,000. Moreover, the authors showed by autophosphorylation of their

peak fractions that the only kinase present was $p70^{s6k}$ and that the autophosphorylated M_r 70,000 band purified from both phases gave rise to the same cyanogen bromide cleavage products. They also analyzed the phosphoamino acid content of the *in vivo*-labeled M_r 70,000 protein and showed that in both cases phosphoserine and to a much lesser extent phosphothreonine were present, but no phosphotyrosine. Finally, they showed that the same tryptic phosphopeptide map was generated from the two preparations of *in vivo* ^{32}P-labeled M_r 70,000 protein, with the exception of one phosphopeptide that was present only in the M_r 70,000 protein isolated from the late phase. From these data, the authors concluded that both the early and late signaling pathways converge on the same kinase, though the site of activation may in part be distinct. These results appear to rule out the phosphorylation and direct activation of the $p70^{s6k}$ by the EGF receptor tyrosine kinase in the first phase and again support a model where at least one kinase couples the EGF receptor with $p70^{s6k}$ during the early phase of activation.

Although in the studies mentioned above no $p90^{rsk}$ enzyme was detected under the conditions described, others have clearly shown that this protein is also rapidly activated following mitogenesis. However, from the studies of Chen and Blenis[78] as well as those of Ahn and co-workers,[108] it is clear that it is difficult to detect $p90^{rsk}$ activity in fibroblasts when employing 40S ribosomal subunits as substrate. Recently, two lines of evidence suggest that the $p90^{rsk}$ is not involved in regulating S6 phosphorylation; (1) Cycloheximide treatment of fibroblasts leads to activation of the $p70^{s6k}$ with no apparent effect on $p90^{rsk}$ or $p42^{mapk}$/$p44^{mapk}$, the enzymes involved in activating $p90^{rsk}$. Under these conditions cycloheximide induces complete S6 phosphorylation[46]; and (2) The immunosuppressant, rapamycin, has been shown to selectively inactivate $p70^{s6k}$/$p85^{s6k}$ with no effect on $p74^{raf}$, $p42^{mapk}$/$p44^{mapk}$, or $p90^{rsk}$. Under the last conditions S6 phosphorylation is totally abolished (see below, Rapamycin). It will be of interest to determine the effect of rapamycin on S6 phosphorylation and meiotic maturation in progesterone-treated *Xenopus* oocytes where the time course of $p90^{rsk}$ activation more closely fits the kinetics of S6 phosphorylation than does the time course of $p70^{s6k}$ activation.

XVII. S6 KINASE KINASE

Metabolic labeling of mitogen-stimulated cells yields $p70^{s6k}$ phosphorylated only at serine and threonine residues, suggesting that an intervening kinase transduces the signal from receptor-associated tyrosine kinases. Cytosolic serine/threonine protein kinases activated at an early stage by phosphorylation on tyrosine, such as raf-1 and $p42^{mapk}$ were at first considered to be strong candidates for $p70^{s6k}$ kinase. Sturgill et al.[109] reported that $p42^{mapk}$ could partially reactivate phosphatase-treated $p90^{rsk}$ from *Xenopus* unfertilized eggs. In quiescent Swiss 3T3 cells, serum or EGF cause a rapid and transient activation of $p42^{mapk}$ activity, with activation of $p70^{s6k}$ following more slowly. These kinetics of activation are consistent with a role for the $p42^{mapk}$ in the activation of $p70^{s6k}$. To test this possibility, $p42^{mapk}$ was purified from Swiss 3T3 cells[110] and appeared to be identical to the enzyme first described in insulin-treated

adipocytes by Ray and Sturgill[111] based on antibody cross-reaction studies.[110] The $p42^{mapk}$ isolated from Swiss 3T3 cells was unable to reactivate or phosphorylate $p70^{s6k}$ treated with phosphatase 2A, suggesting that $p42^{mapk}$ was not involved in regulating $p70^{s6k}$ activation.[110] The failure of $p42^{mapk}$ to reactivate the $p70^{s6k}$ *in vitro* led Ballou and colleagues[110] to search for corroborating *in vivo* evidence. Based on the assumption that $p42^{mapk}$ lies directly upstream of $p70^{s6k}$, it was reasoned that the ability of different mitogenic agents to activate both kinases should be equivalent. However, this was not the case. A number of agents were tested, among which PDGF, bombesin, EGF, PMA, and serum appeared to be the most potent with respect to $p42^{mapk}$ activation. However, the order of effectiveness was distinct for $p70^{s6k}$ activation. More surprisingly, insulin had no effect on $p42^{mapk}$ in 3T3 cells, although it led to a potent activation of the $p70^{s6k}$. Together these results supported the argument that $p42^{mapk}$ did not appear to be the $p70^{s6k}$ kinase, and further indicated that activation of the two enzymes is accomplished through distinct signaling pathways. Recently Mukhopadhayay et al.[112] identified four peaks of kinase activity toward a $p70^{s6k}$ peptide (K_{400}–D_{432}) after fractionation of cell extracts from insulin-treated hepatoma cells on a Mono Q column. By a number of criteria, they argued that three of the peaks contained $p42^{mapk}/p44^{mapk}$ and the fourth contained an activated cdc2-like kinase. Based on this data, they tested the ability of purified $p42^{mapk}/p44^{mapk}$ and $p34^{cdc2}$ to phosphorylate recombinant $p70^{s6k}$ from COS cells and from bacteria. In addition, they compared the two-dimensional tryptic phosphopeptide maps generated *in vitro* with the *in vivo* maps of $p70^{s6k}$. In brief, the authors showed that: (1) $p42^{mapk}/p44^{mapk}$ does not phosphorylate the rat liver $p70^{s6k}$ *in vitro* regardless of whether or not it is first treated with phosphatase 2A; (2) $p42^{mapk}/p44^{mapk}$ phosphorylates recombinant $p70^{s6k}$ *in vitro*, but does not activate it; (3) $p34^{cdc2}$ kinase phosphorylates rat liver $p70^{s6k}$ *in vitro* but does not activate it; (4) both $p42^{mapk}/p44^{mapk}$ and $p34^{cdc2}$ generate a pattern of $p70^{s6k}$ tryptic phosphopeptides *in vitro* that could partially account for the *in vivo* pattern. The authors argued from these studies that $p42^{mapk}$ is involved in the regulation of the $p70^{s6k}$.

In contrast to the conclusions of Mukhopadhayay,[112] the results with cycloheximide[46] along with those of Ballou et al.,[110] argued against $p42^{mapk}/p44^{mapk}$ playing a role in $p70^{s6k}$ activation. A principal pathway leading to $p42^{mapk}/p44^{mapk}$ has been established in the last year that involves the seqential activation of GDP-GTP exchange factor Sos, the GTP binding protein $p21^{ras}$ and protein kinases $p74^{raf}$ and $p47^{mek}$.[122-126] Since H-ras transformed cells contain increased levels of S6 phosphorylation,[127] this finding suggested that the $p42^{mapk}/p44^{mapk}$ pathway bifurcates from that of the $p70^{s6k}$ at some point between $p21^{ras}$ and $p42^{mapk}/p44^{mapk}$. To test this possibility Ming et al.[128] employed interfering mutants of $p74^{raf}$ and $p21^{ras}$ co-expressed with epitope-tagged $p44^{mapk}$ and $p70^{s6k}$ as well as specific mutants of the platelet derived growth factor (PDGF) receptor. In brief, they found that (1) interfering mutants of $p74^{raf}$ and $p21^{ras}$ blocked mitogen activation of $p44^{mapk}$ but had no effect on $p70^{s6k}$, (2) that bifurcation of these pathways was at the level of the receptor, and (3) the critical docking site in the PDGF receptor for $p70^{s6k}$ activation was tyrosine(Y) 751.[128] In parallel studies, Chung

et al.[129] demonstrated that the double point mutant of the PDGF receptor, Y740 and Y751, failed to bind phosphatidyl-inositol-3-OH kinase (PI3K) and in parallel failed to activate $p70^{s6k}$ in response to PDGF. They next demonstrated that the antibiotic wortmannin, which ablates PI3K activity, also blocked $p70^{s6k}$ activation. The conclusion from these studies was that activated receptor recruits PI3K to the membrane where it is activated and then signals to $p70^{s6k}$. However, in analyzing the individual point mutants Y740 and Y751, Ming et al.[128] found that, although both were severely depressed in PI3K binding, only the Y751 mutant blocked $p70^{s6k}$ activation. Thus, they concluded receptor associated PI3K was not critical for $p70^{s6k}$ activation They also concluded that PI3K activity downstream of $p21^{ras}$, was not implicated in $p70^{s6k}$ activation based on the use of interfering mutants of $p21^{ras}$.[130] The obvious challenge at this juncture is to establish the identity of the molecule that binds at tyrosine 751 leading to $p70^{s6k}$ activation.

XVIII. RAPAMYCIN

In lymphocytes, progression through the G_0 and G_1 phases of the cell cycle can be blocked by specific complexes between three distinct microbial products interacting with the endogenous immunophilins, cyclophilin, or FK506-binding protein(s) (FKBP). The natural products, termed immunosuppressants, are cyclosporin A, FK506, and rapamycin.[113] Cyclosporin A is used after transplantation surgery as an immunosuppressant to inhibit graft rejection. Cyclosporin A activity is thought to block the ability of T cells to exit G_0 and enter G_1, or the activated state, which is required to initiate the immune response. This effect is elicited through the inhibition of T cell receptor-activated early gene products.[114] The cytosolic target of the cyclosporin A-cyclophilin and FK506-FKBP complexes appears to be the Ca^{2+}/Calmodulin-dependent protein phosphatase calcineurin, termed protein phosphatase 2B, or PP2B. However, this is not the case for the rapamycin-FKBP complex. In contrast to cyclosporin and FK506, this complex exhibits its inhibitory effects during G_1 or at the time of T cell clonal expansion, after the genes encoding IL-2 and IL-2 receptor have been expressed.[113] Recently it was shown that rapamycin, but not FK506, inhibits the IL-2-stimulated phosphorylation and activation of $p70^{s6k}$.[115,116]. Moreover, Price et al.[117] and Chung et al.[118] provided evidence for inhibition of insulin-stimulated $p70^{s6k}$ in a hepatoma cell line and serum-stimulated $p70^{s6k}$ in fibroblasts following rapamycin treatment, without blocking the activation of $p42^{mapk}$/$p44^{mapk}$, $p74^{raf}$, or $p90^{rsk}$. The latter group also showed that rapamycin can delay the onset of DNA synthesis and severely inhibit the rate of cell growth in Swiss 3T3 cells. From *in vitro* studies, $p70^{s6k}$ does not appear to be a direct target of rapamycin. Instead, it would appear that the target is a M_r 220,000 protein, termed FRAP or mTOR which has structural homology to PI3K.[134] Recent results from this laboratory show that rapamycin treatment leads to a selective dephosphorylation of a novel set of phosphorylation sites[119] in the $p70^{s6k}$ that were not detected in the initial analysis.[90] The results would suggest that rapamycin elicits its inhibitory effects through the activation of a phosphatase or inhibition of a kinase that is not only specific for the $p70^{s6k}$

but for a subset of sites in the enzyme.[119] Recent studies also suggest that the rapamycin sensitive sites as well as the initially identified mitogen activated sites represent two independent pathways required for $p70^{s6k}$ activation (R. B. Pearson, J-W. Han, P. Dennis and G. Thomas, unpublished data).

In parallel studies it was recently shown that rapamycin also inhibits the upregulation of a family of mRNAs which are characterized by a polypyrimidine tract at their 5′ transcriptional start site.[131] Earlier studies had shown that these mRNAs are under translational control[132,133] and that mitogenic stimulation leads to their selective translational upregulation[133] in parallel with increased $p70^{s6k}$ activation[131] and S6 phosphorylation. Preliminary data employing mutants of the PDGF receptor, as well as chimeric mRNAs in which the polypyrimidine tract has been altered, support the hypothesis that rapamycin exerts its inhibitory affects on cell growth by sequentially blocking $p70^{s6k}$ activation, S6 phosphorylation and the translational upregulation of the polypyrimidine tract mRNAs (C. Reinhard, H. B. J. Jefferies, C. B. A. Berry, S. Shama, O. Meyuhas and G. Thomas, unpublished data).

XIX. INHIBITORY ANTIBODIES TO $p70^{s6k}$ BLOCK G_1 PROGRESSION

The availability of inhibitory monospecific polyclonal antibodies raised against mammalian $p70^{s6k}$ in conjunction with microinjection techniques has recently led to functional studies on the role of $p70^{s6k}$ during cell cycle progression. Lane et al.[11] found that serum-induced entry into S phase was severely depressed by microinjection of any one of three distinct, affinity purified, monospecific polyclonal antibodies to the $p70^{s6k}$, into quiescent rat embryo fibroblasts.[52] Consistent with the finding that $p70^{s6k}$ activity remains high throughout G_1, the inhibitory effect could be elicited at any time before entry into S phase. These antibodies also depressed the activation of protein synthesis, whereas no effect was observed after microinjection of the corresponding negative IgG, which had passed through the peptide affinity column. These data are consistent with the effect of rapamycin on cell growth, suggesting that $p70^{s6k}$ plays an important role in mitogenesis both at the early G_0/G_1 transition and throughout G_1.

XX. FUTURE PERSPECTIVE

Studies to date on S6 phosphorylation and the $p70^{s6k}$ have revealed a complex network of phosphorylation events which are strongly implicated in mitogenesis through the activation of protein synthesis. However, many questions remain unresolved concerning, (1) the signaling pathway leading to $p70^{s6k}/p85^{s6k}$ activation, (2) the role of the $p85^{s6k}$ in the nucleus, (3) the mechanism by which multiple phosphorylation leads to kinase activation, and (4) the role of S6 phosphorylation in exerting the mitogenic effects of the kinase through the translational upregulation of polypyrimidine tract mRNAs. Obviously this will be a fruitful area for young researchers in the coming years.

ACKNOWLEDGEMENTS

We would like to thank Drs. S. C. Kozma, R. B. Pearson, M. J. Stewart, and

P. King for their critical reading of the manuscript. In addition, we are indebted to C. Wiedmer and D. Schofield for their typing and editing expertise.

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