

## The RSK factors of activating the Ras/MAPK signaling cascade

Audrey Carriere<sup>1</sup>, Hind Ray<sup>1</sup>, John Blenis<sup>2</sup>, Philippe P. Roux<sup>1</sup>

<sup>1</sup>Institute for Research in Immunology and Cancer (IRIC), and Department of Pathology and Cell Biology, Faculty of Medicine, Université de Montréal, Montreal, Quebec H3C 3J7, Canada, <sup>2</sup>Harvard Medical School, Department of Cell Biology, Boston, Massachusetts, 02115, USA

### TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Structure and activation mechanisms
4. Functional interaction between ERK and RSK
5. Expression and subcellular localization
6. Genetic analysis of RSK function
7. RSK function and substrate phosphorylation
  - 7.1. Nuclear signaling
  - 7.2. Cell cycle progression and cell proliferation
  - 7.3. Cell growth and protein synthesis
  - 7.4. Cell survival
8. Perspectives
9. Acknowledgments
10. References

### 1. ABSTRACT

The p90 ribosomal S6 kinase (RSK) constitute a family of serine/threonine kinases activated downstream of the Ras/mitogen-activated protein kinase (MAPK) pathway. In mammals, four *RSK* genes have been identified (*RSK1*, *RSK2*, *RSK3* and *RSK4*), and RSK orthologues have also been described in *D. melanogaster* and *C. elegans*, but not in yeast or plants. The RSK isoforms are composed of two distinct and functional kinase domains that are activated in a sequential manner by a series of phosphorylation events. These enzymes were among the first substrates of extracellular signal-regulated kinase (ERK) to be discovered and have proven to be ubiquitous and multifunctional mediators of ERK signal transduction. While the RSK isoforms promote cell survival through the inactivation of several apoptotic effectors, they also appear to mediate cell growth and proliferation by simultaneously regulating substrates involved in gene transcription and mRNA translation. RSK1-4 are ubiquitously expressed in cell lines and tissues, and at present, little is known about specific and overlapping functions of individual RSK isoforms. The upregulation of RSK1 and RSK2 expression in different types of cancer suggest that they may be involved in oncogenesis and could potentially be targeted in anti-cancer therapies. The recent identification of specific RSK inhibitors will likely help addressing the biological functions of the RSK isoforms and their contributions in pathological conditions.

### 2. INTRODUCTION

The Ras/mitogen-activated protein kinase (MAPK) pathway regulates a variety of cellular processes that include cell proliferation, survival, growth and motility (1). The wide range of MAPK functions are mediated through the phosphorylation of several substrates, including members of the MAPK-activated protein kinase (MK) family (reviewed in 2), such as the p90 ribosomal S6 kinase (RSK) family of serine/threonine protein kinases (Figure 1A). The RSK family contains four human isoforms, RSK1 (3), RSK2, RSK3 (4) and RSK4 (5), which share 65-73% amino acid (aa) identity (Figure 1B). The RSK isoforms are directly activated by extracellular signal-regulated kinases 1 and 2 (ERK1/2) in response to growth factors, many polypeptide hormones, neurotransmitters, chemokines and other stimuli. RSK1 was the first identified member of this family and was purified from *Xenopus laevis* extracts as a kinase that phosphorylated the 40S ribosomal subunit protein S6 (6). RSK-related molecules have also been identified in *Caenorhabditis elegans* (T01H8.1) and *Drosophila melanogaster* (RPS6-protein kinase-II), which share around 50% aa identity to human RSK1 (7) and also contain the two kinase domains typical to RSK family members (Figure 1). No RSK homologues have been found in yeast or plant.

### 3. STRUCTURE AND ACTIVATION MECHANISMS

A unique feature of the RSK family is that, during evolution, the genes for two distinct protein kinases have fused, generating a single kinase capable of receiving an upstream activating signal from ERK to the RSK carboxyl-terminal kinase domain (CTKD) and transmitting, with high efficiency and fidelity, an activating input to the RSK amino-terminal kinase domain (NTKD). Both kinase domains are distinct and functional (8, 9) and are connected by a regulatory linker region of about 100 aa (Figure 1). The NTKD belongs to the AGC kinase family which also includes protein kinase C (PKC), Akt, and the p70 ribosomal S6 kinases 1 and 2 (S6K1 and S6K2) (Figure 2). The NTKD is responsible for phosphorylation of exogenous substrates and is also involved in an autophosphorylation event that regulates ERK-RSK interaction (Figure 3). The CTKD belongs to the  $\text{Ca}^{++}$ /calmodulin-dependent kinase (CaMK) family, which also includes all MK family members, such as the MAPK-interacting kinases 1 and 2 (MNK1 and MNK2), the MAPK-activated protein kinases 2 and 3 (MK2 and MK3), and MK5 (also known as PRAK) (Figure 2). The mitogen- and stress-activated kinases 1 and 2 (MSK1 and MSK2) are close relatives to the RSKs as they also contain two functional kinase domains, but these proteins have been shown to be responsive to both mitogenic and stress stimuli via their interaction with ERK1/2 and the p38 MAPKs. Although some groups have reported that RSK can be activated by stress stimuli such as UV light (10, 11), the RSK isoforms do not interact with p38 MAPK in cells.

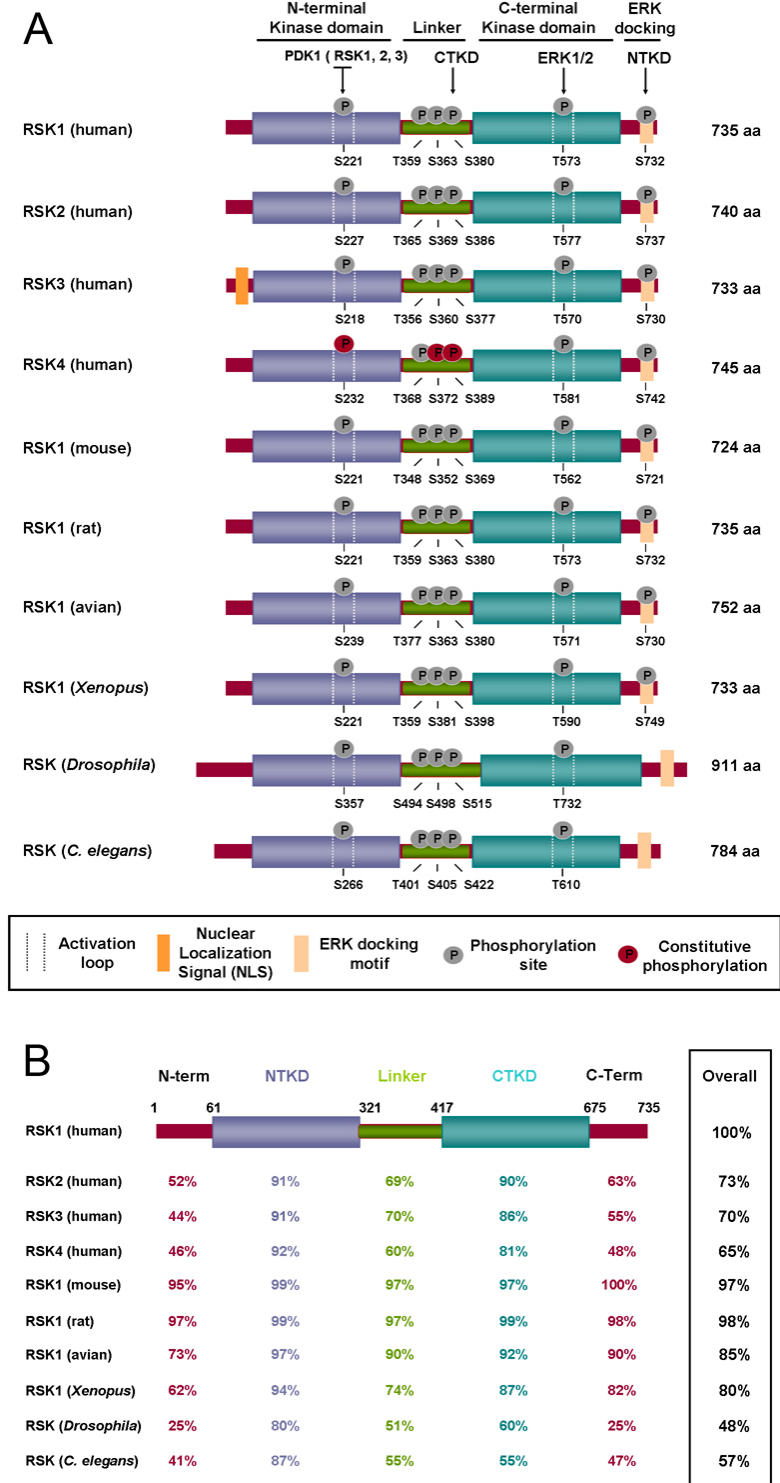
The only known purpose of the CTKD of RSK is to activate the NTKD via autophosphorylation (9, 12, 13) (Figure 3). All RSK isoforms contain a C-terminally located ERK docking site, also known as D domain that is required for docking and activation by ERK1/2 (14, 15). The D domain in RSK1-4 consists of Leu-Ala-Gln-Arg-Arg, where only the leucine and arginine residues were found to be essential for ERK1/2 docking (16). Two basic residues located C-terminal to the D domain also contribute to ERK1/2 binding, but their presence is not essential for RSK1 activation (16). The RSK isoforms and ERK1/2 have previously been shown to interact in cells and to dissociate upon activation (17-19). RSK1 was found to dissociate from ERK1/2 in response to the autophosphorylation of a serine residue located near its D domain (16), providing a mechanism for the regulated release of ERK1/2 (Figure 3). While RSK1 and RSK2 were shown to rapidly dissociate from ERK1/2 following stimulation with growth factors, RSK3 was found to remain associated longer to ERK1/2 which also correlated with sustained activation of this RSK isoform. RSK4 was shown to have high basal activity but the regulation of ERK1/2 docking to RSK4 remains unknown. These findings suggest that the regulated release of ERK1/2 through the phosphorylation of the D domain controls the duration of RSK activation (16).

Activation of RSK N-terminal phosphotransferase activity requires the successive interplay between both phosphorylation and autophosphorylation events (Figure 3). All RSK isoforms,

including the *C. elegans* and *D. melanogaster* orthologues of RSK (with the exception of S732), contain six phosphorylation sites that have been shown in RSK1 and RSK2 to be responsive to mitogenic stimulation (20). Mutational analysis revealed that four of these sites (S221, S363, S380, and T573 in human RSK1) are essential for RSK1 activation (20). Of these, S221 (activation loop of the NTKD), S363 (turn motif), and S380 (hydrophobic motif), are located within sequences highly conserved in other AGC kinases (21) (Figure 2). The process of RSK activation is closely linked to ERK1/2 activity, explaining why the MEK inhibitors U0126 and PD98059 have been widely used to inhibit RSK activation. As ERK1/2 activate several other protein kinases, the use of inhibitors of RSK phosphotransferase activity will likely be useful in the attribution of specific biological functions to the RSK isoforms. Three different classes of RSK inhibitors targeting the NTKD (22, 23) or the CTKD (24) have been identified in the last few years and tested for their specificity against a panel of protein kinases (25). While BI-D1870, SL0101 and FMK were found to be relatively specific against the RSK isoforms, all three compounds also inhibit additional protein kinases albeit with lower potencies. Since FMK inhibits the CTKD of RSK1 and RSK2, this compound may not be useful under circumstances where the NTKD of RSK is activated by a mechanism that is independent of the CTKD, as has recently been observed (26).

The current model of RSK activation suggests that ERK1/2 and RSK1/2 form an inactive complex in quiescent cells (18, 19). After mitogenic stimulation, ERK1/2 phosphorylate T573 located in the activation loop of the CTKD (14, 27) and possibly T359 and S363 in the linker region between the two kinase domains (20). Recent evidence indicates that ERK5 may also contribute to RSK1 activation by phosphorylating T573, but the specific function of ERK5-mediated RSK activation remains unknown (28). Activation of the CTKD by ERK1/2 leads to autophosphorylation at S380 (13), which is located in the linker region within a hydrophobic motif of aromatic residues. Mutational inactivation of the CTKD was shown to only partially inhibit activation of the NTKD of RSK1 (16, 29), suggesting that hydrophobic motif phosphorylation may also occur in a CTKD-independent manner (30, 31). Interestingly, the related MK2 and MK3 enzymes were found in dendritic cells to bypass the need for ERK1/2-mediated activation of the CTKD and to phosphorylate the hydrophobic motif of RSK2, which may explain how various stresses that stimulate p38 MAPK lead in some cases to RSK activation (26, 32). Aside from being involved in RSK phosphorylation, ERK1/2 may also promote RSK1 activation by facilitating its recruitment to the plasma membrane. Indeed, addition of a myristoylation sequence at the N-terminus of RSK1 was shown to constitutively activate RSK and promote phosphorylation at its hydrophobic motif. These findings suggest that a constitutively-activated membrane-associated kinase may be involved in RSK1 activation, but its identification has remained elusive (31).

## The RSK factors of activating the Ras/MAPK signaling cascade



**Figure 1.** Schematic representation of RSK orthologues with their functional domains and phosphorylation sites. A) Four functional domains are conserved in the human RSK isoforms and in other species: the N-terminal kinase domain (NTKD), the linker domain, the C-terminal kinase domain (CTKD) and the ERK docking site (D domain). All vertebrate RSK isoforms contain six phosphorylation sites that are regulated by mitogenic stimulation. Both the *Drosophila* and *C. elegans* RSK orthologues lack the C-terminal phosphorylation site located near the D domain. B) Percentage aa identities between human RSK1 and other RSK orthologues.

## The RSK factors of activating the Ras/MAPK signaling cascade

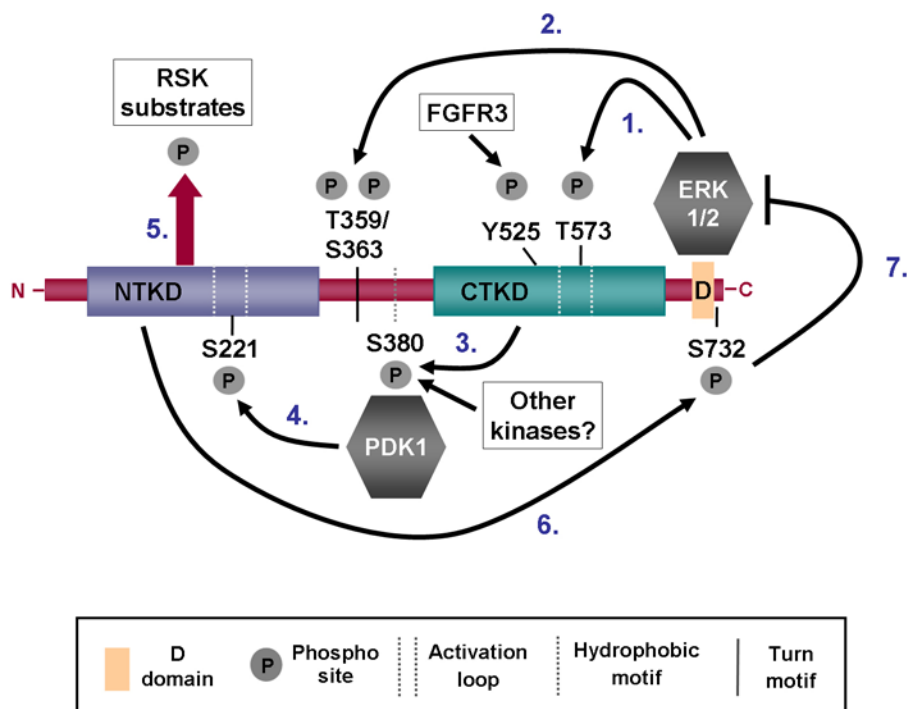


**Figure 2.** Schematic representation of RSK1 compared to related AGC and CaMK family of protein kinases. RSK1 and MSK1 are composed of two non-identical kinase domains, whereas MNK1, MK2, -3, -5 contain a single kinase domain that shares homology to the CTKD of RSK1 and MSK1. The structural alignment of RSK1 with major AGC family kinases shows that the NTKD of RSK1 and MSK1 is homologous to Akt, PKC and S6K1. These proteins also share two regulatory features: a phosphorylation site in the activated loop segment and two phosphorylation sites in the linker region. The MK2B and MK5B splice variants were used in this diagram to demonstrate domain homology to the RSK1 and MSK1 kinases.

Phosphorylation of the hydrophobic motif (S380) creates a docking site that recruits phosphoinositide-dependent protein kinase 1 (PDK1) (33). PDK1 is required for the activation of many AGC kinases as demonstrated using PDK1-null embryonic stem (ES) cells. PDK1 was found to be required for mitogenic stimulation of RSK1, Akt and S6K1 (34), but surprisingly, RSK4 does not appear to abide to this rule as its catalytic activity was found to be normal in PDK1-null ES cells (35). These findings suggest that another kinase or RSK4 itself may substitute for PDK1 in the phosphorylation and subsequent activation of the NTKD of RSK4. Unlike other RSK isoforms, RSK4 activity is constitutively high and inhibition of the MAPK cascade using the U0126 inhibitor does not fully suppress its activity (35). Nevertheless, PDK1 association to the phosphorylated hydrophobic motif in RSK1, RSK2 and RSK3 leads to the phosphorylation of S221 in the activation loop of the NTKD (36, 37). After dissociation of

PDK1, phosphorylated S380 binds to a phosphate-binding site in the NTKD, which enables the aromatic residues of the hydrophobic motif to interact and stabilize a nearby hydrophobic pocket (33). Phosphorylated S363 was found to interact with a distinct phosphate binding site in the NTKD, thereby promoting zipper-like binding of the linker region to the NTKD. Association of the phosphorylated linker region to the NTKD was found to stimulate kinase activity and suggested to protect RSK from dephosphorylation (38). PP2C $\delta$  was found to associate with RSK1-4, suggesting that this phosphatase regulates RSK dephosphorylation (39). Inactivation of RSK1 involves the NTKD-catalyzed autophosphorylation at S732 in the C-terminal tail of RSK1 (16), which was found to promote the release of ERK1/2 from RSK1 and RSK2, and to correlate with a reduction of kinase activity. Recent evidence indicates that RSK2 is also phosphorylated on a tyrosine residue in response to FGF receptor activation. This

## The RSK factors of activating the Ras/MAPK signaling cascade



**Figure 3.** Ordered phosphorylation events regulate RSK activation. Activation of RSK is achieved by successive protein-protein interactions and phosphorylation/autophosphorylation events. The binding of ERK1/2 to the D domain of RSK1 enables these upstream activating kinases to phosphorylate T573 (1) located in the activation loop of the CTKD, as well as two other residues located in the linker region (2). This event induces CTKD-mediated phosphorylation of S380 (3), which was also shown to be regulated by CTKD-independent mechanisms. This site is located within the hydrophobic motif, which leads to the recruitment of PDK1. PDK1 then phosphorylates S221 located in the activation loop of the NTKD (4). This event activates the NTKD which then phosphorylates exogenous substrates (5). The NTKD also phosphorylates S732 in the D-domain (6), resulting in ERK1/2 dissociation and RSK inactivation (7). It has been recently shown that Y529 in mouse RSK2 (homologous to Y525 in human RSK1) is phosphorylated by FGFR3, this event facilitating ERK1/2 binding to the D domain and subsequent RSK2 activation.

phosphorylation event was found to stabilize ERK1/2 binding to RSK2 and to promote its subsequent activation, suggesting an alternative mechanism for RSK activation in human tumors with activated FGFR3 signaling (40).

#### 4. FUNCTIONAL INTERACTION BETWEEN ERK AND RSK

While ERK is required for the phosphorylation and subsequent activation of RSK, the latter also plays some role in the regulation of ERK activity and function. When inactive, RSK1-3 have been shown to interact with ERK1/2 (16, 18, 19). This interaction allows ERK1/2 to phosphorylate the CTKD of RSK upon stimulation, the first step in the RSK activation process. One of the first evidence implicating RSK in the regulation of the Ras/MAPK pathway came from the analysis of *rsk2<sup>-/-</sup>* mice, which were found to display higher and more sustained phosphorylation of ERK1/2 in response to both exercise and insulin (41). This finding strongly suggested that RSK2 is part of a negative feedback loop that regulates Ras/MAPK pathway activation, and a possible mechanism may involve phosphorylation of SOS (son of sevenless), a guanine nucleotide exchange factor for Ras. RSK2 was found to phosphorylate SOS within a region that normally

interacts with the adaptor protein Grb2 (42), which is responsible for connecting the Ras/MAPK cascade to activated receptor tyrosine kinases. It is currently unknown whether all RSK isoforms negatively regulate ERK activation, but overexpression of RSK1-4 in HEK293 cells was found to antagonize growth factor-mediated ERK phosphorylation (P.P. Roux, unpublished observation). *Drosophila* RSK was also shown to inhibit the Ras/MAPK pathway using a second mechanism. While deletion of RSK does not appear to affect ERK activity in *Drosophila*, increased nuclear accumulation of ERK was found in RSK null flies (43). This effect was rescued by either wt or a kinase deficient form of RSK, suggesting that at least in *Drosophila*, RSK inhibits ERK function by acting as a cytoplasmic anchor for ERK (43). It is currently unknown whether the mammalian RSK isoforms play a similar function, but the presence of four RSK isoforms is likely to present a challenge in trying to determine this.

#### 5. EXPRESSION AND SUBCELLULAR LOCALIZATION

The expression profile of genes during development can give useful and interesting information regarding their potential roles and functions. Although little

## The RSK factors of activating the Ras/MAPK signaling cascade

is known about the specific and potential overlapping functions of the individual RSK isoforms, their differential expression profiles support the idea that they serve specific functions at a given time and a given environment. While both *rsk1* and *rsk3* genes give rise to only one transcript, northern analysis of *rsk2* expression revealed the alternative use of two different polyadenylation sites giving rise to two transcripts of 3.5 and 8.5 kb (44). Similarly, two secondary *rsk4* transcripts (5 and 9 kb) also exist, but whether these transcripts result from alternative splicing or alternative polyadenylation remains unknown (45).

During mouse development, *in situ* hybridization revealed that the *rsk2* gene is expressed at very low levels in contrast to *rsk4* for example, whose mRNA is very abundant in fetal tissues (45-47). However, at late embryonic stages, *rsk2* expression is weakly enhanced in some tissues including sensory ganglia, skeletal muscle and some peripheral organs, but not in the central nervous system. In contrast to *rsk2*, the *rsk3* gene shows high levels of expression during development with prominent expression from E11.5 and onwards, almost exclusively restricted to the developing central and peripheral nervous system (46). *Rsk3* expression is, in particular, detected in the ventricular zone bordering the lateral ventricle, a site of high proliferative activity. Conversely, the *rsk1* mRNA is strongly expressed in the neuroepithelium of the neural tube very early and until late midgestation, whereas at later stages it decreases dramatically and becomes undetectable in the nervous system. These results are consistent with a temporal regulation of the *rsk1* and *rsk3* genes and support the requirement of RSK1 in early and RSK3 in later development of the nervous system. At late stages of development, *rsk1* is highly expressed in regions harboring highly proliferating cells. These include liver, lung, thymus, olfactory and gut epithelia. From an expression pattern point of view, RSK1 seems to be more strictly linked to cell proliferation and RSK3 to cellular differentiation, particularly in the nervous system.

In adult tissues, northern analyses and RNase protection assays revealed that the *rsk1* mRNA is expressed in many tissues, with higher levels in kidney, lung and pancreas (44, 48, 49). Interestingly, expression of both *rsk2* and *rsk3* is most abundant in the heart, skeletal muscles and the pancreas, suggesting that they may have overlapping functions in these organs. In adult mouse brain, *rsk1* expression was clearly detected only in the granular cell layer of the cerebellum (44, 50). In contrast, high levels of *rsk3* expression were observed in various regions of the adult mouse brain, suggesting a specific function for RSK3 in nervous system maintenance and/or in neural signal transmission. In particular, the strong expression observed in structures that can be related to cognitive function, such as the cerebral cortex, the dentate gyrus and the amygdala suggest that RSK3 is a good candidate for disorders displaying involvement of the central nervous system, including mental retardation. In adult mouse brain, the highest levels of *rsk2* expression were observed in regions with high synaptic activity, including the neocortex, the hippocampus and Purkinje cells. These structures are essential components in cognitive function and learning,

which is consistent with the association between the loss of RSK2 and the mental retardation syndrome called Coffin-Lowry. *Rsk4* mRNA is present in many tissues, with higher levels in adult brain and kidney (45-47). Detection of RSK4 protein levels corroborated these findings, by showing that RSK4 is highly expressed in the adult mouse brain, heart, kidney and skeletal muscles (35).

At the subcellular level, RSK1, RSK2, RSK3 proteins are usually present in the cytoplasm of quiescent cells, but upon stimulation, a significant proportion of these proteins was reported to translocate to the nucleus of activated HeLa, COS-7 and HEK293 cells (31, 51-54). RSK4 also appears to be predominantly cytoplasmic (35) but contrary to the other RSK isoforms, no translocation into the nucleus following mitogenic stimulation has been reported for this isoform. Within minutes of stimulation, RSK1 was shown to accumulate transiently at the plasma membrane, where it presumably receives additional inputs necessary for activation before nuclear translocation (31). The small death effector domain protein PEA-15 has been shown to inhibit RSK2 nuclear translocation, but the biological function of this interaction remains unknown (54). Activated RSK2 is also present in the cytoplasm of stimulated cells, suggesting that RSK2 substrates exist in both nuclear and cytoplasmic compartments (51). While the mechanisms responsible for the nuclear translocation of the RSK isoforms remain unknown, RSK3 is the only human isoform to possess a nuclear localization signal (NLS), consisting of Lys-Lys-Xaa(10)-Leu-Arg-Arg-Lys-Ser-Arg, but the functionality of this domain has never been tested.

## 6. GENETIC ANALYSIS OF RSK FUNCTION

Phenotypic analyses of knockout mice for each RSK isoform will be informative and extremely helpful to understand the isoform-specific roles of RSK *in vivo*. The *rsk1*<sup>-/-</sup> and *rsk3*<sup>-/-</sup> knockout mice as well as *rsk1*<sup>-/-</sup> *rsk2*<sup>-/-</sup> *rsk3*<sup>-/-</sup> triple knockout mice appear to be viable, but no phenotype information has yet been reported for these animals (55). Contrary to the other RSK isoforms, *rsk2*<sup>-/-</sup> knockout mice have been extensively studied. It was reported that *rsk2*<sup>-/-</sup> knockout mice have impaired learning and cognitive functions, as well as poor coordination compared to wt littermates (41, 56). Interestingly, mutations in the human *rsk2* gene resulting in reduced kinase activity have been shown to cause Coffin-Lowry syndrome (CLS)(57-60). This X-linked mental retardation syndrome is often accompanied by facial and digital dimorphisms, as well as progressive skeletal deformations (61). In addition to their impaired learning abilities, *rsk2*<sup>-/-</sup> knockout animals develop a progressive skeletal disease, osteopenia, due to cell-autonomous defects in osteoblast activity (62). Both c-Fos and ATF4 transcription factors have been proposed to be critical targets of RSK2, mediating its effects in osteoblasts functionality (62, 63). The *rsk2* and *rsk4* genes are located on chromosome X and it was suggested that *rsk4* may be involved in non-specific X-linked mental retardation, but definitive evidence remains to be provided (45). *Rsk2*<sup>-/-</sup> knockout mice display other phenotypes. They are approximately 10 to 15% smaller than their wt littermates, with a specific loss of

## The RSK factors of activating the Ras/MAPK signaling cascade

white adipose tissue that is accompanied by reduced serum levels of the adipocyte-derived peptide, leptin (64). Interestingly, deletion of *Drosophila* RSK was found to result in defects in learning and conditioning (65), but whether these deficits result from the loss of RSK activity or deregulated ERK activation or function will require further investigation.

### 7. RSK FUNCTION AND SUBSTRATE PHOSPHORYLATION

Little is known about the specific and overlapping functions of the RSK isoforms, but the recent identification of specific RSK inhibitors (22-24) and the use of RNA interference should help shed some light on the functional contribution of each RSK isoform. RSK1 specificity for target phosphorylation has been determined using synthetic peptide libraries and was found to require the minimum sequences Arg/Lys-Xaa-Arg-Xaa-Xaa-pSer/Thr or Arg-Arg-Xaa-pSer/Thr (66). These analyses also revealed that RSK1 prefers to phosphorylate serine rather than threonine residues by a factor of about five fold, and consistent with this, the large majority of RSK substrates found to date are phosphorylated on Ser residues (Table 1). A number of RSK functions can be deduced from the nature of their substrates and data from many groups point towards roles for the RSK isoforms in transcriptional and translational regulation, cell growth, motility, proliferation and survival (Figure 4 and 5). While a majority of substrates have been identified for either RSK1 or RSK2, most studies have not determined if there was functional overlap between the RSK isoforms, indicating that many currently known substrates of RSK1 or RSK2 may be phosphorylated by more than one RSK family member.

#### 7.1. Nuclear signaling

Activated RSK phosphorylates a growing list of nuclear substrates including several transcription factors also regulated by ERK1/2, such as c-Fos, ER $\alpha$ , and ER81 (67-69). This dual regulation is thought to serve as a mechanism to decipher between activation of the Ras/MAPK pathway and activation of a kinase that may share substrate specificity with either RSK or ERK. RSK regulates the immediate-early (IE) gene response at both the transcriptional and the post-translational level. RSK1 was found to phosphorylate serum response factor (SRF) (70, 71) and cAMP response element-binding protein (CREB) (72-75). Two studies using human cells from CLS patients and primary fibroblast isolated from *rsk2*<sup>-/-</sup> mice showed that RSK2 mediates PDGF/IGF1 and EGF-induced *c-fos* transcription, respectively (70, 75). Two distinct mechanisms were proposed, including activation of the Elk1/SRF complex and CREB phosphorylation by RSK2, but differences between these two studies may be due to the use of human versus mouse cellular models. Interestingly, the related MSK1/2 protein kinases were also shown to phosphorylate CREB in cells (76), but deletion of both MSK1/2 isoforms in mouse embryonic fibroblasts did not completely abolish CREB phosphorylation in response to mitogens, suggesting that RSK2 or other RSK isoforms may cooperate with the MSK1/2 to phosphorylate CREB.

Histone H3 was also suggested to be regulated by RSK, but conclusive evidence demonstrated that MSK1/2 fulfill this function in response to both stress and mitogenic stimulations (77). At the post-translational level, RSK1 was found to phosphorylate the IE gene products c-Fos (68) and Nur77 (9, 78, 79). RSK1 and RSK2 were both shown to phosphorylate c-Fos at S362, resulting in protein stabilization which increases its growth-promoting effects (62, 80, 81). Hyperphosphorylation of c-Fos only occurs when ERK and RSK activation is sustained (82, 83), acting as a molecular sensor for ERK and RSK nuclear localization, signal duration, and signal strength (84).

Additional nuclear factors have been shown to be regulated by RSK, including the NF-kappaB transcription factor. RSK1 phosphorylates I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  on sites that promote their degradation, thereby stimulating NF-kappaB activity (85-87). While some NF-kappaB agonists also lead to the rapid activation of RSK, others such as TNF $\alpha$  do not, suggesting the presence of several I $\kappa$ B $\alpha/\beta$  kinases that may be activated under specific cellular circumstances. More recently, angiotensin II-mediated activation of NF-kappaB was shown to require RSK1/2-dependent p65 phosphorylation in vascular smooth muscle cells, suggesting that RSK may stimulate NF-kappaB activity using two independent mechanisms (88, 89). These findings indicate that RSK may be involved in NF-kappaB-dependent cell survival, but more experimentation will be required to fully understand the role of NF-kappaB activity in the biological function of RSK. RSK2 was also found to phosphorylate the CREB family member ATF4 (63), a transcription factor required for the timely onset of osteoblast differentiation. RSK2 was found to be required for osteoblast differentiation and function, suggesting a mechanism by which loss of RSK2 leads to CLS-associated skeletal abnormalities. The transcriptional coactivator CREB-binding protein (CBP) has been identified as a binding target of RSK1 (90). Stimulation of the Ras/MAPK pathway promotes the interaction between RSK1 and CBP (90, 91), but the exact outcome of this association remains to be determined. Interestingly, CBP also interacts with transcription factors that are phosphorylated by RSK1 and RSK2, such as CREB, c-Fos, ER81 and NF-kappaB, suggesting that RSK binding to CBP may be a prerequisite for regulating these transcription factors.

#### 7.2. Cell cycle progression and cell proliferation

Based on the nature of their substrates (Figure 5), among them IE gene products, the RSK isoforms have been thought to play roles in cell cycle progression and cell proliferation. A recently identified RSK inhibitor, termed SL0101 (24), has been tested for its anti-proliferative potency. Treatment of cells with SL0101 or RNA interference against RSK1 and RSK2 was found to inhibit proliferation of human prostate and breast cancer cell lines (P. Roux, unpublished observation) (22, 92). Accordingly, RSK1 and RSK2 were shown to be overexpressed in tumors of the breast and prostate (22, 92), indicating that these two isoforms positively regulate cancer cell proliferation (Figure 5).

## The RSK factors of activating the Ras/MAPK signaling cascade

**Table 1.** Phosphorylation substrates of the RSK isoforms

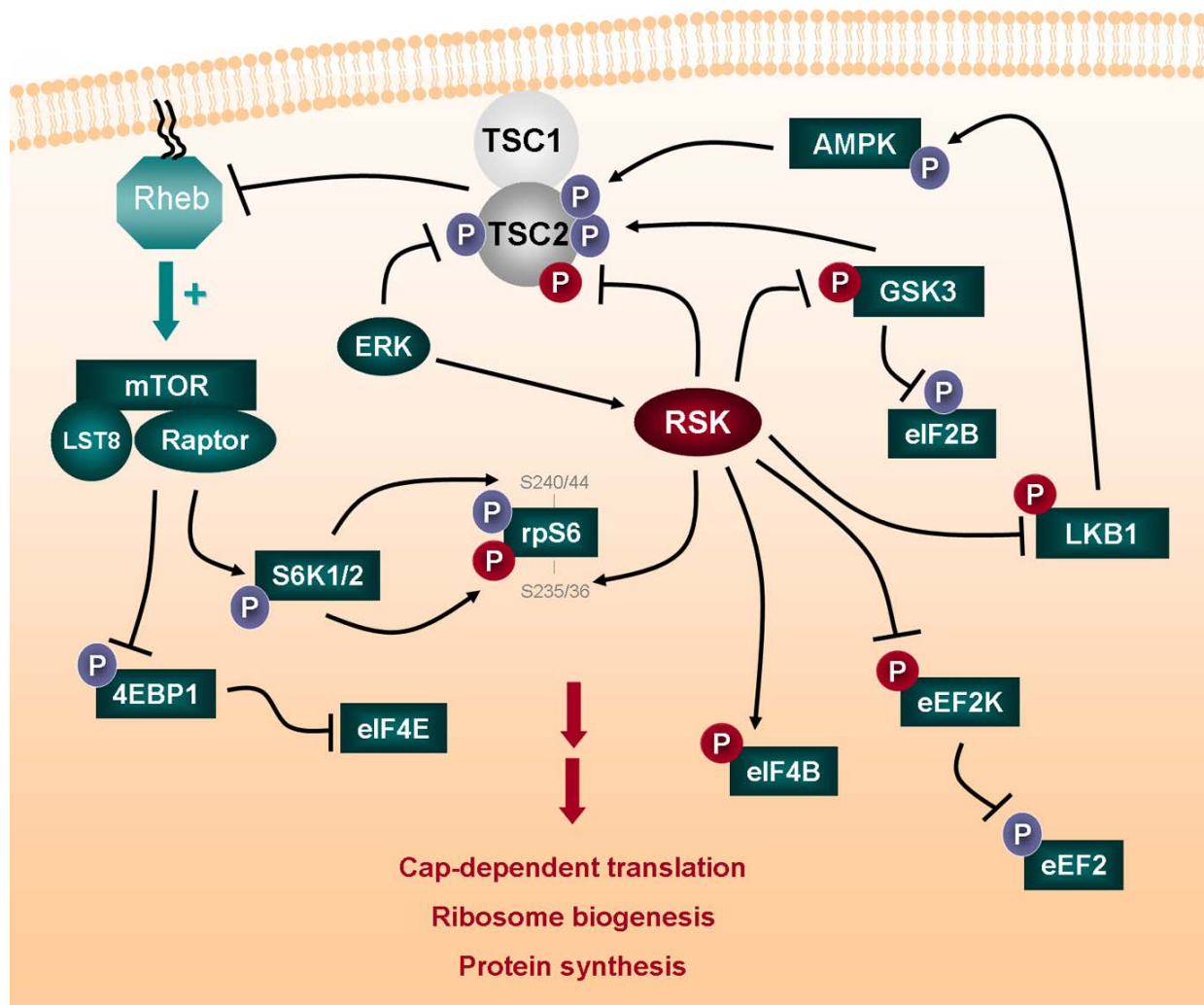
Main Functions	Substrates	Phosphorylation sites and biological functions	RSK isoforms	References
Proliferation	GSK3 $\beta$ (proliferation and metabolism)	(rat) Ser9 $\rightarrow$ inhibits its activity	RSK2	(125, 137)
	c-Fos (transcription factor)	(rat) Ser362 $\rightarrow$ stabilizes c-Fos and increases its growth-promoting effects	RSK1, RSK2	(62, 68, 80)
	NHE-1 (Na <sup>+</sup> /H <sup>+</sup> exchanger that regulates intracellular pH)	(human) Ser703 $\rightarrow$ promotes cell proliferation	RSK1	(138)
	MITF (transcription factor essential for melanocyte proliferation)	(human) Ser409 $\rightarrow$ increases transactivation potential	RSK1	(139)
	ER $\alpha$ (ligand-activated transcription factor)	(human)(rat) Ser167 $\rightarrow$ regulates AF1 transcriptional activation	RSK1, RSK2	(69)
	LKB1 (tumor suppressor that activates AMPK)	(mouse) Ser431 $\rightarrow$ necessary for LKB1-mediated growth suppression	RSK1	(94)
	p27 <sup>kip1</sup> (inhibitor of CDK-cyclin)	(human) Thr198 $\rightarrow$ promotes 14-3-3 binding and cytoplasmic retention	RSK1, RSK2	(93)
	SOS (guanine nucleotide exchange factors for Ras)	(human) $\rightarrow$ negative feedback on Ras/MAPK activation	RSK2	(42)
Oocyte maturation	Myt1 (kinase inhibitor of p34 <sup>cdc2</sup> /cyclin B)	(Xenopus) Ser residue $\rightarrow$ inactivates Myt1 function	RSK1	(103)
	Bub1 (mitotic checkpoint kinase)	(Xenopus) $\rightarrow$ promotes its kinase activity	RSK1	(109)
	Erp1/Emi2 (Anaphase Promoting Complex inhibitor)	(Xenopus) Ser335/Thr336, Ser342/4 $\rightarrow$ stabilizes protein and promotes PP2A binding	RSK2	(111, 113)
Translational regulation	eEF2 kinase (kinase that negatively regulates eEF2)	(human) Ser366 $\rightarrow$ inhibits kinase activity and promotes translation elongation	RSK1	(131)
	eIF4B (translation initiation factor)	(human) Ser422 $\rightarrow$ enhances interaction between eIF4B and eIF3	RSK1, RSK2	(132)
	rpS6 (component of the 40S ribosomal subunit)	(human) Ser235/6 $\rightarrow$ promotes rpS6 recruitment to the 7-methylguanosine cap	RSK1, RSK2	(119)
	TSC2 (tumor suppressor that controls the mTOR pathway)	(human) Ser1798 $\rightarrow$ inhibits TSC2 activity and promotes mTOR signaling	RSK1	(121, 140)
Survival and apoptosis	Bad (pro-apoptotic protein)	(human, rat) Ser112 $\rightarrow$ suppresses Bad-mediated apoptosis	RSK1	(72, 133)
	DAPK (tumor suppressor, proapoptotic protein)	(human) Ser289 $\rightarrow$ suppresses its proapoptotic function	RSK1, RSK2	(135)
	C/EBP $\beta$ (transcription factor involved in metabolism and differentiation)	(rat) Ser105 and (mouse) Thr217 $\rightarrow$ stimulates hepatocyte proliferation	RSK1	(134)
Transcriptional regulation	CREB (transcription factor that binds to CRE motif)	(human, mouse) Ser133 $\rightarrow$ stimulates its transcriptional activity and promotes cell survival	RSK1	(72-75)
	SRF (transcription factor that binds to SRE motif)	(human) Ser103 $\rightarrow$ enhances its affinity for SRE motif within the <i>c-fos</i> promoter	RSK1	(70, 71)
	ER81 (ETS transcription factor involved in homeostasis and development)	(human, mouse) Ser191/216 $\rightarrow$ enhances its transactivation potential	RSK1	(67)
	Nur77 (nuclear orphan receptor and transcription factor)	(human) Ser354 $\rightarrow$ no clear function	RSK1, RSK2	(9, 78, 79)
	I $\kappa$ B $\alpha$ (regulation of NF- $\kappa$ B)	(rat) Ser32 $\rightarrow$ induces its degradation thus promoting NF- $\kappa$ B activity	RSK1	(85, 86)
	I $\kappa$ B $\beta$ (regulation of NF- $\kappa$ B)	(rat) Ser19/23 $\rightarrow$ induces its degradation thus promoting NF- $\kappa$ B activity	RSK1	(87)
	p65 (subunit of NF- $\kappa$ B)	(rat, mouse) Ser536 $\rightarrow$ increases NF- $\kappa$ B activity	RSK1, RSK2	(88, 89)
	NFATc4 (transcription factor)	(human, mouse) Ser676 $\rightarrow$ promotes DNA binding	RSK1	(141)
	NFAT3 (transcription factor)	(human, mouse) multiple Ser sites $\rightarrow$ induces NFAT3 nuclear accumulation	RSK2	(142)
	TIF-1A (transcription initiation factor)	(human) Ser649 $\rightarrow$ enhances cellular pre-rRNA synthesis	RSK2	(143)
	ATF4 (osteoblast enriched-member of the CREB family)	(mouse) Ser251 and (human) Ser245 $\rightarrow$ promotes collagen synthesis	RSK2	(63, 144)
Others	nNOS (catalyses arginine conversion to citrulline and nitric acid)	(rat) Ser847 $\rightarrow$ (blocks the toxic accumulation of NO in the brain)	RSK1	(145)
	L1 CAM (neurite outgrowth)	(rat) Ser1152 $\rightarrow$ intracellular signaling cascade involved in neurite outgrowth	RSK1	(146)

Through the regulation of c-Fos, which promotes the expression of cyclin D1 during G<sub>0</sub>/G<sub>1</sub> transition to S phase, RSK1 and RSK2 are believed to stimulate cell cycle progression. RSK2 promotes the oncogenic properties of c-Fos, as development of osteosarcoma was shown to depend on RSK2-mediated c-Fos phosphorylation on Ser362 (62). RSK1 and RSK2 may also promote G<sub>1</sub> phase progression by phosphorylating the cyclin-dependent kinase (CDK) inhibitor p27<sup>kip1</sup> (93). Phosphorylation of p27<sup>kip1</sup> by RSK1 and RSK2 was found to promote its association with 14-3- $\beta$

and prevent its translocation to the nucleus. RSK2 has also been shown to phosphorylate the tumor suppressor LKB1, a kinase found mutated in the cancer prone Peutz-Jeghers syndrome (94). Phosphorylation of LKB1 on S431 was not found to affect its activity or membrane association, but was shown to be necessary for LKB1-mediated growth suppression through still currently unknown mechanisms. RSK2 appears to be a critical regulator of cell transformation, as ectopic expression of RSK2 was found to increase proliferation as well as



## The RSK factors of activating the Ras/MAPK signaling cascade



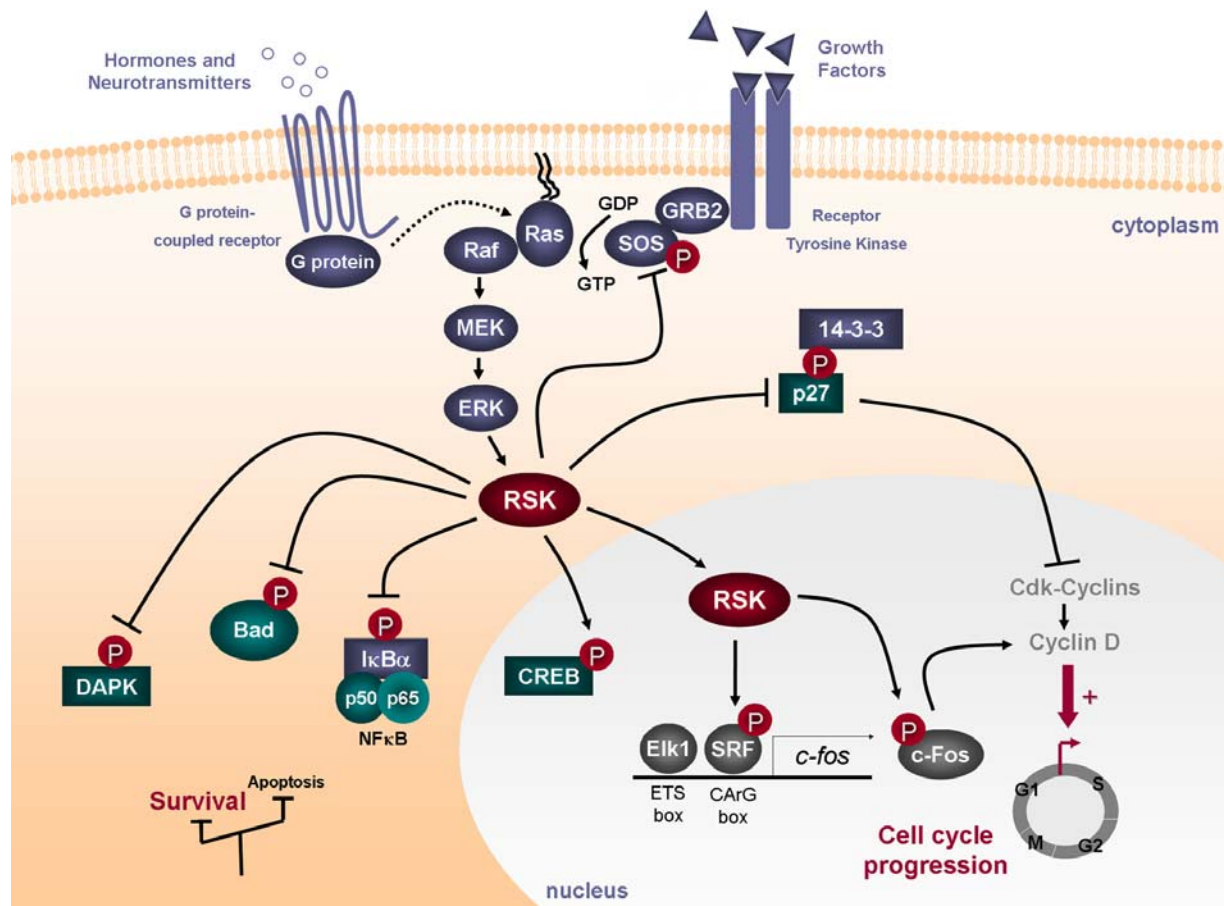
**Figure 4.** RSK regulates protein synthesis using mTOR-dependent and -independent mechanisms. This schematic representation demonstrates the reported protein synthesis by which RSK regulates mRNA translation and protein synthesis. RSK was found to phosphorylate diverse substrates that regulate the mTOR pathway or modulate the activity of components of the translational machinery.

anchorage-independent transformation (95). In accordance with a role for RSK1 and RSK2 in tumorigenesis, FGFR3 has been recently shown to promote hematopoietic transformation by activating RSK2 in a two-step fashion, by facilitating both ERK-RSK2 interaction and subsequent phosphorylation of RSK2 by ERK (40). RSK2 was recently shown to be mutated in breast cancer (96), but whether it is a gain or loss of function mutation remains to be determined.

Although RSK3 was initially suggested to play positive roles in cell proliferation (53), this isoform was recently shown to act as potential tumor suppressor in ovarian cancer (97). Overexpression of RSK3 was found to reduce proliferation by causing G1 arrest and increasing apoptosis, but the mechanisms by which RSK3 negatively regulate cell proliferation are currently unknown. One hypothesis

is that overexpression of RSK3 may promote the negative-feedback loop on the Ras/MAPK cascade, which would decrease ERK-dependent cell cycle progression and cell proliferation, but more investigation will be required to determine this. The role of RSK4 on proliferation appears to be even more enigmatic. Recent evidence suggested that RSK4 plays an inhibitory role during embryogenesis by negatively regulating receptor tyrosine kinase (RTK) signaling (47). RSK4 was also found to participate in p53-dependent cell growth arrest (98), indicating that this isoform potentially behaves as a tumor suppressor. However, aberrant expression of several X-linked genes, including *rsk4*, has been observed in breast cancer (99), suggesting an oncogenic role for RSK4. Clearly, a systematic analysis of the effects of each RSK isoforms on cell proliferation will be required to understand their respective function.

## The RSK factors of activating the Ras/MAPK signaling cascade



**Figure 5.** RSK is a central regulator of survival and proliferation. This diagram provides an overview of the cytoplasmic and nuclear targets of RSK involved in cell survival and proliferation. A more complete list of RSK substrates can be found in Table 1.

ERK1/2 and the RSK isoforms may also regulate progression through the G2 phase of the cell cycle. The role of these kinases in cell cycle regulation has been demonstrated by many groups using the preferred model of *Xenopus* oocyte maturation (100). Immature oocytes are arrested in the G2 phase of the first meiotic cell division. Addition of progesterone induces the synthesis of the MAPKKK c-Mos, which in turn activates the MEK1-ERK-RSK cascade leading to M phase entry and subsequent maturation to an unfertilized egg. M phase entry is controlled in part by Cdc2, which is a CDK normally kept in check by dual phosphorylation on both T14 and Y15 by the inhibitory kinase Myt1. RSK2 has been shown to be the prominent RSK isoform in *Xenopus* oocytes (101), and using this model system, RSK2 has been shown to contribute to the control of the meiotic cell cycle at several critical points (102). One mechanism by which RSK2 participates in the progression of oocytes through the G2/M phase of meiosis I is through phosphorylation and inhibition of the Myt1 kinase (103). The importance of RSK2 is such that constitutively-

activated RSK2 can mediate meiosis I entry even in the absence of progesterone or ERK activation (104). RSK2 regulates meiosis I entry by binding to and phosphorylating the C-terminus of Myt1, thereby reducing its ability to inhibit the kinase activity of Cdc2/cyclin B1 complexes (103). It remains unknown whether this mechanism is conserved in other species, but recent efforts demonstrated that Akt can also act as a Myt1 kinase in starfish oocytes (105). RSK2 may also be important for progression of mammalian somatic cells through the G2/M phase of mitosis because ERK1/2 activity was shown to be required in synchronized NIH-3T3 fibroblasts (106).

Another way by which RSK2 can modulate the meiotic cell cycle in *Xenopus* is through mediating ERK-mediated metaphase II arrest, an activity known as cytostatic factor (CSF). Two groups have found that *Xenopus* RSK1 and RSK2 are essential for CSF by showing that activated RSK2 causes CSF even when ERK is inactive, and that depletion of RSK2 from oocyte extracts removes CSF activity (107, 108). RSK1 was later shown to

## The RSK factors of activating the Ras/MAPK signaling cascade

phosphorylate and activate the kinase Bub1 *in vitro*, a mediator of anaphase-promoting complex (APC) inhibition (109), suggesting that RSK1-mediated Bub1 activation contributes, at least in part, to metaphase II arrest (110). Emi2 (also called Erp1) is another APC inhibitor that was initially thought to function independently from the ERK pathway. Three independent studies recently demonstrated that Emi2 is in fact a substrate for RSK. Phosphorylation of Emi2 by RSK promotes Emi2-PP2A association, facilitating Emi2 dephosphorylation at specific Cdc2 phosphorylation sites, which in turn enhances Emi2 stability and function (111-113). However, recent findings indicate that RSK1 and RSK2 are not involved in CSF arrest in mouse embryos, suggesting species-specific differences in RSK function (55).

### 7.3. Cell growth and protein synthesis

RSK1 was originally identified in *Xenopus laevis* oocytes as a serine kinase that phosphorylated the 40S ribosomal protein S6 (rpS6) *in vitro* (6, 114). S6K1 and S6K2 were later shown to be the predominant rpS6 kinases operating in somatic cells (115-118), but recent evidence in S6K1/2<sup>-/-</sup> cells (118) and in rapamycin treated cells (119) suggest that the RSK isoforms also contribute to rpS6 phosphorylation (Figure 4). In cells derived from S6K1/2<sup>-/-</sup> mice, phosphorylation of rpS6 at S240/44 was found to be completely abrogated, but persistent phosphorylation at S235/36 was still observed (118). In accordance with this finding, RSK1 and RSK2 were recently shown to phosphorylate rpS6 on S235/36 in response to Ras/MAPK pathway activation (119), indicating that rpS6 phosphorylation can occur in an mTOR-independent manner. This RSK mediated S235/36 phosphorylation was found to facilitate assembly of the translation preinitiation complex and to correlate with increased cap-dependent translation (119), suggesting that RSK provides an additional oncogene- and mitogen-regulated input linking the Ras/MAPK signaling pathway to the regulation of translation initiation. All four isoforms of RSK have been shown to stimulate translation initiation when overexpressed, suggesting the possibility that RSK may promote the translation of a specific group of transcripts upon stimulation of the Ras/MAPK signaling cascade.

Ras/MAPK signaling was also found to stimulate mTOR signaling through the regulation of the tuberous sclerosis complex (TSC) (Figure 4). RSK and ERK phosphorylate TSC2 at S1798 (and possibly S939) and S664, respectively, which negatively regulate the guanine nucleotide-activating protein (GAP) activity of TSC2 towards the small GTPase Rheb (120-122). Phosphorylation of TSC2 at S1798 was shown to regulate the subcellular localization of TSC2 (123), to promote mTOR signaling (121) and to mediate Ras/MAPK-dependent cell survival (124). RSK may also regulate mRNA translation through the phosphorylation of GSK3 $\beta$

(125, 126). RSK1-mediated phosphorylation of GSK3 $\beta$  on S9 was found to inhibit its kinase activity and thereby release the inhibition on the translation initiation factor eIF2B (127, 128). Interestingly, activated GSK3 $\beta$  and the LKB1-activated kinase AMPK were both shown to phosphorylate and activate TSC2 (129, 130), suggesting that RSK may inhibit TSC2 activity using direct and indirect mechanisms (Figure 4). Finally, RSK was shown to phosphorylate the eEF2 kinase (131) and the translation initiation factor eIF4B (132), underscoring the involvement of RSK at multiple levels of the pathway leading to protein synthesis.

### 7.4. Cell survival

RSK1 and RSK2 have also been shown to positively regulate cell survival in different cell types (Figure 5). Both RSK isoforms phosphorylate the pro-apoptotic protein Bad on S112 which inactivates its function and promotes survival of primary neurons (72) and a hematopoietic cell line (133). RSK1 was also found to promote survival of hepatic stellate cells by phosphorylating C/EBP $\beta$  T217 in response to the hepatotoxin CCl4 (134). Phosphorylation of T217 was suggested to create a functional XEVD caspase inhibitory box that binds and inhibits caspases 1 and 8. More recently, RSK1 and RSK2 were shown to phosphorylate and inactivate the death-associated protein kinase (DAPK). Phosphorylation of DAPK at S289 was found to inhibit its pro-apoptotic activity, resulting in increased HEK293 cell survival in response to mitogenic stimulation (135). DAPK has been shown to have tumor suppressor functions and its expression is commonly silenced in tumors through DNA methylation of its promoter (136). The ability of RSK1/2 to inactivate its activity suggests that these RSK isoforms promote metastasis by preventing DAPK-dependent cell death. RSK has also been shown to promote cell survival using transcription-dependent mechanisms. Indeed, RSK2-mediated phosphorylation of the transcription factor CREB was shown to promote survival of primary cortical neurons through increased transcription of survival-promoting genes (72-74). More recently, RSK1 was found to promote survival through the activation of the transcription factor NF-kappaB (85, 86, 88).

## 8. PERSPECTIVES

The last few years have seen major advances in our understanding of the biological function of the RSK isoforms. The RSK family has often been shadowed by the important role played by their upstream activators, ERK1 and ERK2, but a growing number of evidence now implicates the RSK isoforms as multifunctional effectors of Ras/MAPK signaling. These discoveries have been facilitated by the generation of RSK-specific reagents, such as small molecule inhibitors and RNA interference. The impact of the RSK isoforms in cell growth and proliferation is becoming more and more evident, but

more complete information on the roles of individual RSK isoforms remains to be demonstrated. These studies will be facilitated by the generation of knockout animals that will directly address the physiological roles played by each RSK isoforms. Finally, the roles played by RSK family members in human diseases will also require further analysis, especially with respect to the newly found link between RSK activity and cancer cell growth and proliferation. Research in this area will hopefully help understand the important molecular players involved in this condition and ultimately open up new therapeutic approaches for them.

### 9. ACKNOWLEDGMENTS

Research in the laboratory of P.P.R. is supported by a Terry Fox Foundation grant obtained through the National Cancer Institute of Canada (NCIC) and a Career Development Award from the Human Frontier Science Program Organization (HFSP). P.P.R. holds a Canada Research Chair in Signal Transduction and Proteomics. A.C. is recipient of a fellowship from the Fonds de la Recherche en Santé du Québec (FRSQ), and the IRIC is supported by a group grant from the FRSQ.

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## The RSK factors of activating the Ras/MAPK signaling cascade

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**Abbreviations:** Emi1, Early mitotic inhibitor 1; Erp1, Emi1 related protein; DAPK, Death associated protein kinase; eEF2, eukaryotic translation elongation factor 2; eIF4B, eukaryotic translation initiation factor 4B; C/EBP $\alpha$ , CAAT/enhancer-binding protein alpha; nNOS, Neuronal nitric oxide synthase; NFAT3, nuclear factor of activated T cells 3.

**Key Words:** RSK, MAPK, ERK, MK, protein kinases, Ras, Phosphorylation, Cell Proliferation, Review

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**Send correspondence to:** Philippe P. Roux, IRIC -  
Université de Montréal, P.O. Box 6128, Station Centre-  
ville, Montreal, Quebec H3C 3J7, Canada, Tel: 514 343-  
6111 x0308, Fax: 514 343-5839, E-mail:  
philippe.roux@umontreal.ca

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