Ribosomal S6 kinase 1 (RSK1) activation requires signals dependent on and independent of the MAP kinase ERK

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Background: The *rsk1* gene encodes the 90 kDa ribosomal S6 kinase 1 (RSK1) protein, which contains two kinase domains. RSK1, which is involved in regulating cell survival and proliferation, lies at the end of the signaling cascade mediated by the extracellular signal-regulated kinase (ERK) subfamily of mitogen-activated protein (MAP) kinases. ERK activation and subsequent phosphorylation of the RSK1 carboxy-terminal catalytic loop stimulates phosphotransferase activity in the RSK1 amino-terminal kinase domain. When activated, RSK1 phosphorylates both nuclear and cytoplasmic substrates through this amino-terminal catalytic domain. It is thought that stimulation of the ERK/MAP kinase pathway is sufficient for RSK1 activation, but how ERK phosphorylation activates the RSK1 amino-terminal kinase domain is not known.

Results: The individual isolated RSK1 kinase domains were found to be under regulatory control. *In vitro* kinase assays established that ERK phosphorylates RSK1 within the carboxy-terminal kinase domain, and the phosphoinositide-dependent kinase 1 (PDK1) phosphorylates RSK1 within the amino-terminal kinase domain. In transiently transfected HEK 293E cells, PDK1 alone stimulated phosphotransferase activity of an isolated RSK1 amino-terminal kinase domain. Nevertheless, activation of full-length RSK1 in the absence of serum required activation by both PDK1 and ERK.

Conclusions: RSK1 is phosphorylated by PDK1 in the amino-terminal kinase-activation loop, and by ERK in the carboxy-terminal kinase-activation loop. Activation of phosphotransferase activity of full-length RSK1 *in vivo* requires both PDK1 and ERK. RSK1 activation is therefore regulated by both the mitogen-stimulated ERK/MAP kinase pathway and a PDK1-dependent pathway.

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Received: 10 May 1999
Revised: 28 June 1999
Accepted: 7 July 1999

Published: 22 July 1999

Current Biology 1999, 9:810–820
http://biomednet.com/elecref/0960982200900810

 ISSN 0960-9822

Background

Mitogenic stimuli activate the ribosomal S6 kinase 1 (RSK1) through the pathway involving the extracellular signal-regulated kinase (ERK) subfamily of mitogen-activated protein (MAP) kinases. Generation of the GTP-bound form of Ras initiates a kinase cascade whereby Raf kinases activates MAP kinase kinase (MEK), which activates ERK1/ERK2, which in turn phosphorylates RSK1 (reviewed in [1]). RSK1 is a serine/threonine kinase with two distinct kinase domains, both of which are catalytically functional [2]. The carboxy-terminal kinase domain is believed to be involved in autophosphorylation, a critical feature of RSK1 activation, whereas the amino-terminal kinase domain is responsible for the phosphorylation of all exogenous substrates tested.

RSK is cytoplasmic, but it can translocate to the nucleus upon mitogenic stimulation [3]. Activated RSK has both cytoplasmic and nuclear substrates. Phosphorylation of the apoptotic protein BAD by RSK can protect cells from BAD-mediated cell death (A.S., unpublished observations). RSK also phosphorylates IκBα, the inhibitor of the transcription factor NF-κB, inducing degradation of IκBα and allowing NF-κB to translocate to the nucleus [4]. RSK plays an important role in nuclear signaling by phosphorylating various transcription factors, including the cyclic AMP (cAMP) response element binding protein (CREB) [5,6] and c-Fos [7,8]; RSK therefore has a role in transcriptional regulation. RSK has also been implicated in cell-cycle regulation. In *Xenopus* extracts RSK phosphorylates and downregulates Myt1, a $p34^{cdc2}$ inhibitory kinase [9]. Downregulation of Myt1 leads to activation of cyclin B–$p34^{cdc2}$ kinase and cell-cycle progression. Therefore, RSK has been implicated in a myriad of cellular processes, but its cellular role and substrate(s) remain unclear. The diversity of its substrates suggests that RSK may participate in cell proliferation and differentiation through multiple pathways.

Other than the requirements for ERK and autophosphorylation, relatively little is known about the mechanism of RSK regulation. The presence of two active kinase domains

within the enzyme undoubtedly increases the complexity of studying this regulation. Six phosphorylation sites have been identified in RSK1, two of which are basally phosphorylated, and four of which are phosphorylated upon mitogen stimulation [10]. Among the mitogen-responsive sites are a putative ERK phosphorylation site in the RSK1 carboxyl terminus [11] and a putative RSK1 autophosphorylation site in the linker region between the two RSK1 kinase domains [12]. While sequence comparison suggests that the remaining sites may be ERK or RSK1 phosphorylation sites, it is possible that they are phosphorylated by other kinases. ERK alone is not capable of fully activating RSK1 *in vitro* [13], suggesting that additional input(s) may be required for full RSK1 activation.

The RSK amino-terminal kinase domain has homology to other kinase domains, including those in cAMP-dependent protein kinase A (PKA) and p70 S6 kinase 1 ($p70^{S6K1}$) [14]. An important regulatory enzyme for PKA and $p70^{S6K1}$ is phosphoinositide-dependent kinase 1 (PDK1). PDK1 phosphorylates a site in the activation loops of protein kinase Bα (PKBα, also known as c-Akt) [15,16], various protein kinase Cs (PKCs) [17–19], PKA [20] and $p70^{S6K1}$ [21,22]. PDK1 is a protein of 556 amino acids that contains a kinase domain and a pleckstrin homology (PH) domain [15,16]. The PH domain binds phosphatidylinositol 3,4,5-trisphosphate ($PtdIns(3,4,5)P_3$) generated by phosphoinositide (PI) 3-kinases [23]. PDK1 is found in the cell cytosol and at the plasma membrane [23,24].

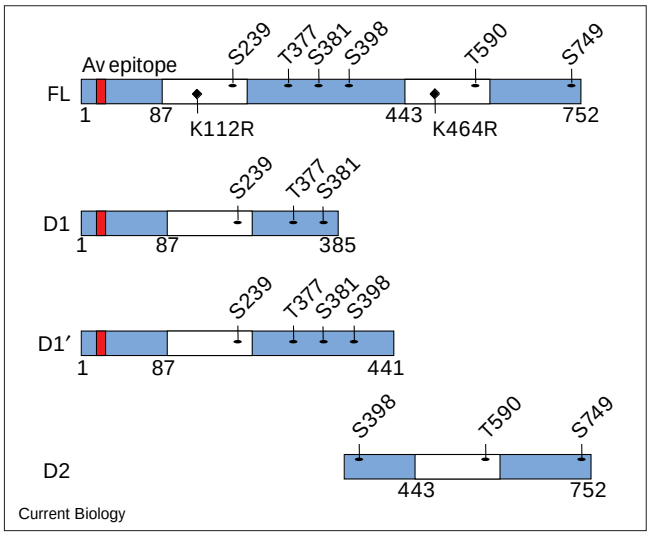
We are interested in the regulation of the individual RSK1 kinase domains. Here, we report that each isolated RSK1 kinase domain can be activated. Surprisingly, PDK1 could activate an isolated RSK1 amino-terminal kinase domain *in vivo*, but activation of the full-length protein required cooperation with the ERK/MAP-kinase pathway. We propose a model for RSK1 activation that incorporates regulatory inputs from the ERK/MAP-kinase pathway and additional phosphorylation events.

Results

Activation of isolated RSK1 kinase domains

Studies *in vitro* have indicated that both RSK1 kinase domains are active [2]. It has not, however, been determined whether or how each isolated kinase domain is activated *in vivo*. Because the regulation of each kinase domain could be different, knowing whether the two domains are independently activated would be useful in identifying the requirements for activation of full-length RSK1. Therefore, the RSK1 constructs D1, D1′ and D2 were generated (Figure 1). D1 and D1′ encode the amino-terminal kinase domain, with the only difference being an additional 56 amino acids in D1′. This additional sequence includes Ser398, which is analogous to the Ser380 residue in *Xenopus* RSK1 that has been found to be an autophosphorylation site [12] and is phosphorylated upon mitogen stimulation in rat RSK1 [10]. D2 encodes the carboxy-terminal kinase domain (Figure 1).

Figure 1



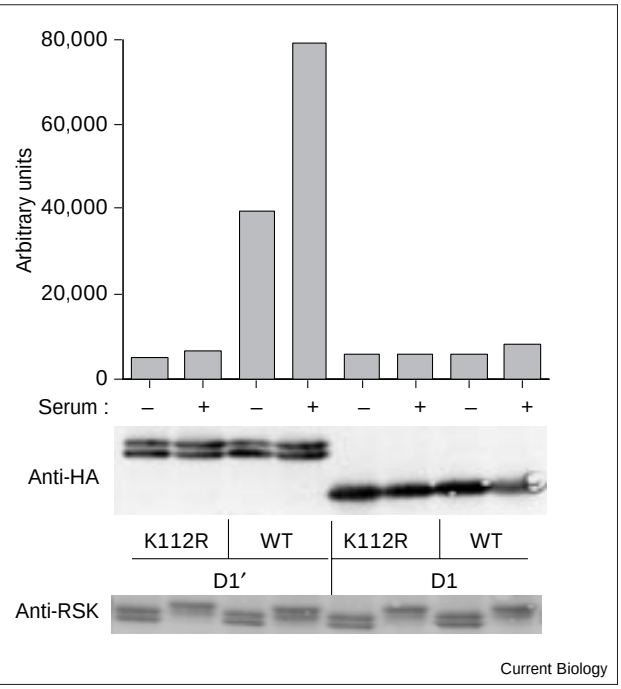
Constructs encoding full-length (FL) RSK, D1, D1′ and D2 were generated. Each construct was fused at the 5′ end to sequences encoding a triple hemagglutinin (HA) epitope tag. White boxes, kinase domains; numbers, amino-acid positions; identified phosphorylation sites are S239, T377, S381, S398, T590 and S749; diamonds, inactivating mutations in each kinase domain. Amino-acid residues are indicated in the single-letter amino-acid code. The RSK clone used was avian RSK1, which encodes an avian-specific epitope between amino-acid residues 37 and 54 (Av epitope).

When expressed in HEK 293E cells and stimulated with serum, D1′ was active towards the substrate, a fusion protein between glutathione-S-transferase and a ribosomal S6 protein peptide (GST–S6), whereas D1 was not (Figure 2). Even in the absence of serum, D1′ exhibited a significant level of basal activity. The K112R mutation abolished D1′ phosphotransferase activity. Mobility shifting of endogenous RSK indicated that the cells were serum-stimulated; therefore the lack of phosphotransferase activity in D1 was not due to lack of stimulation. These data suggest that the additional amino acids are critical for the activation of the RSK1 amino-terminal kinase domain.

The isolated D2 kinase domain was also expressed in cells (Figure 3a,b). In serum-stimulated cells, D2 was active towards exogenous GST–D2 (K464R) and, interestingly, D2 also underwent a significant amount of autophosphorylation (Figure 3c,d). Again, a K464R mutation reduced kinase activity of this domain.

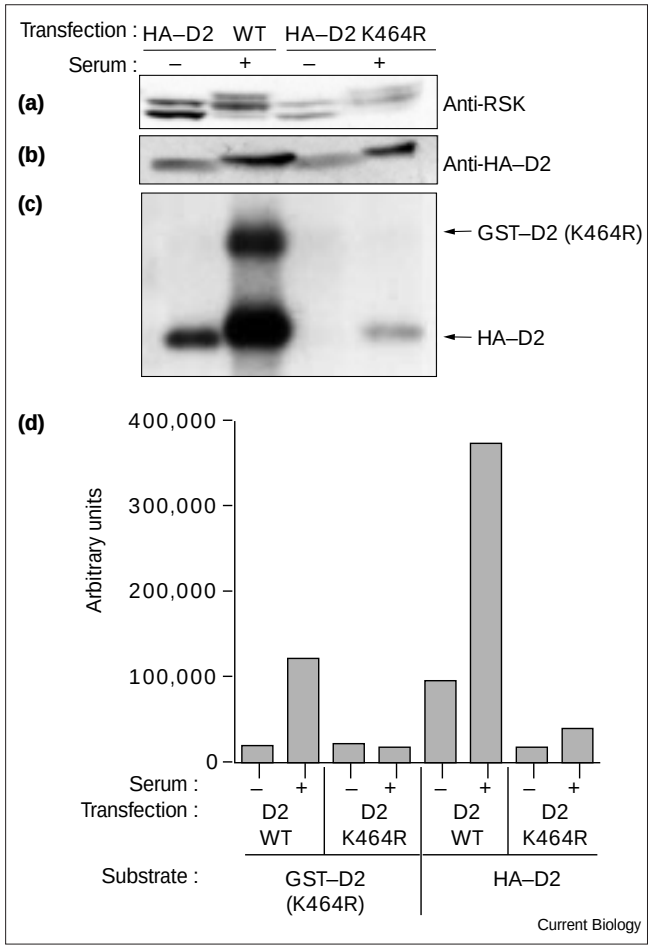
Because of the known contribution of ERK to RSK activation, we examined the effect of blocking ERK activation on the kinase activity of each isolated kinase domain (Figure 4). Cells transfected with either HA–D1′ or HA–D2

Figure 2



Activation of an isolated amino-terminal RSK kinase domain. HEK 293E cells were transfected with constructs expressing either wild-type (WT) or K112R mutant versions of HA–D1 or HA–D1′ as indicated. Starved cells were stimulated for 10 min and lysed as described in Materials and methods. Lysates were blotted for D1 and D1′ expression with an anti-HA antibody. Mobility shifting of endogenous RSK was visualized by blotting with an anti-RSK antibody. HA-tagged proteins were immunoprecipitated with an anti-HA antibody, and immunoprecipitation kinase assays were performed using GST–S6 as substrate. The graph shows quantitation of S6 phosphorylation using a phosphorimager. The results are representative of three separate experiments.

Figure 3



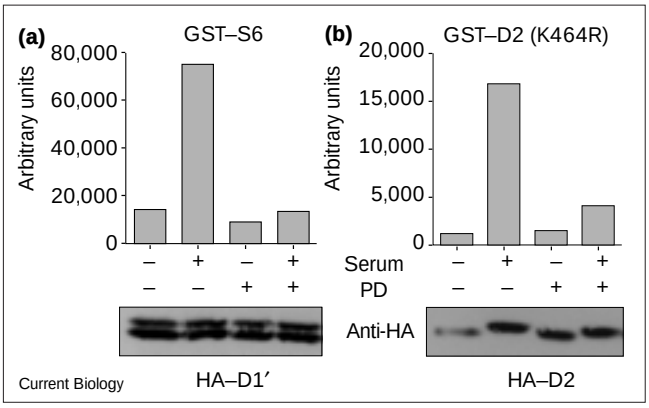
Activation of an isolated carboxy-terminal RSK kinase domain. HEK 293E cells were transfected with constructs expressing wild-type (WT) or the K464R mutant of HA–D2 as indicated. After stimulating the starved cells for 10 min with serum, cells were lysed and subjected to SDS–PAGE and western blotting. **(a)** Western blot with an anti-RSK antibody showing shifting of endogenous RSK due to *in vivo* phosphorylation upon serum stimulation. **(b)** Western blot with an anti-HA antibody to show expression of HA–D2. **(c)** Kinase assay of HA–D2 phosphotransferase activity. Transfected HA–D2 (WT) or HA–D2 (K464R) was immunoprecipitated with an anti-HA antibody and incubated with GST–D2 (K464R) and [γ-^{32}P]ATP. Upper band, phosphorylated exogenous substrate GST–D2 (K464R); lower band, autophosphorylation of transfected HA–D2. **(d)** Quantitation of HA–D2 phosphotransferase activity. Phosphotransferase activity towards GST–D2 (K464R) and autophosphorylation of HA–D2 were quantitated on a phosphorimager. The results are representative of three separate experiments.

were treated with the MEK inhibitor PD098059 before serum stimulation, and phosphotransferase activity was measured. For HA–D1′, PD098059 treatment severely curtailed serum-stimulated kinase activity towards GST–S6, but had no effect on the electrophoretic mobility of HA–D1′ (Figure 4a). For HA–D2, PD098059 treatment also reduced its phosphotransferase activity, both towards an exogenous substrate and its autophosphorylation (Figure 4b). Interestingly, HA–D2 still underwent a serum-stimulated mobility shift, suggesting that HA–D2 may be phosphorylated by a mitogen-dependent MEK-independent kinase.

RSK1 is phosphorylated by PDK1 and ERK *in vitro*

We have thus far demonstrated that isolated RSK1 kinase domains can be activated *in vivo*. The signals responsible for that activation have not been completely elucidated, however. It is well established that ERK phosphorylation is a critical event for RSK1 activation. ERK binds the RSK1 carboxyl terminus [2,25,26] and phosphorylates a site within the activation loop of the carboxy-terminal kinase domain [11]. Activated ERK also activates the RSK1 carboxy-terminal autophosphorylation activity *in vitro* [2]. Mutation of the putative ERK phosphorylation site in full-length RSK1 reduces RSK1 amino-terminal kinase activity towards exogenous substrate ([10]; data not shown), reinforcing the importance of this phosphorylation event. In avian RSK1, this phosphorylation site is Thr590. When

Figure 4

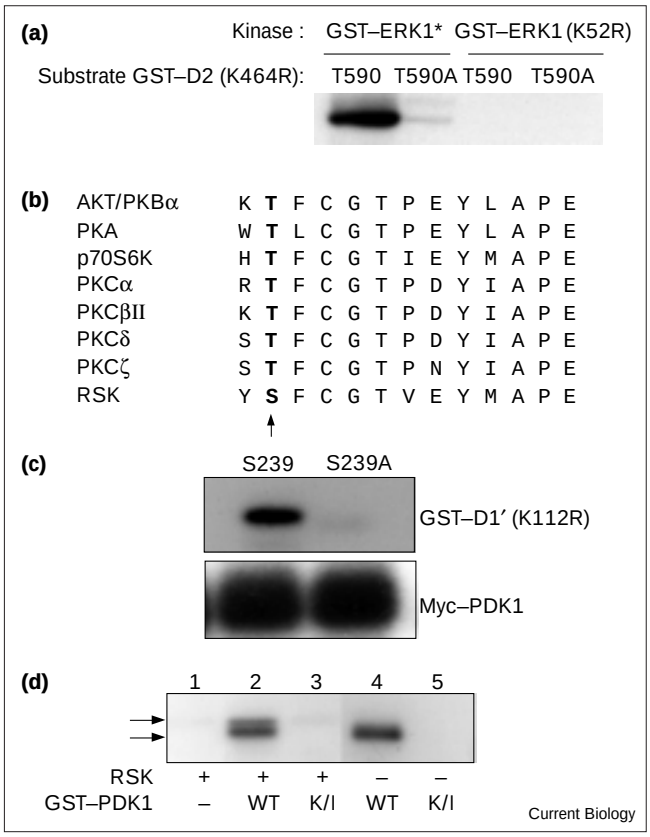


The MEK inhibitor PD098059 affects HA–D1′ and HA–D2 activation. HEK 293E cells were transfected with (a) HA–D1′ or (b) HA–D2 and starved of serum. After a 30 min pretreatment with PD098059, transfected cells were stimulated with serum where indicated. Cell lysates were subjected to SDS–PAGE and quantitative western blotting with an anti-HA antibody (lower panels). Volumes corrected for equal HA–D1′ or HA–D2 expression were used in immunocomplex kinase assays. (a) GST–S6 was used to assay HA–D1′ activity, and (b) GST–D2 (K464R) was used to assay HA–D2 activity. Quantitation was on a phosphorimager. The results are representative of four separate experiments.

GST–D2 (K464R) was used as a substrate for activated ERK1 in an *in vitro* kinase reaction, it was found to be phosphorylated (Figure 5a), but the phosphorylation was eliminated when the substrate contained a T590A mutation. We conclude that Thr590 in avian RSK1 is an ERK phosphorylation site.

Signals required for the activation of the amino-terminal kinase domain are less well understood, and are of particular interest as all exogenous RSK substrates are phosphorylated by this kinase domain. Four phosphorylation sites have been identified within this region in rat RSK1 [10]. In avian RSK1, these sites are Ser239, Thr377, Ser381 and Ser398 (in rat RSK1, the corresponding sites are Ser222, Thr360, Ser364 and Ser381). Sequence comparison suggested that the Ser239 residue of avian RSK1 was either an autophosphorylation site or a site phosphorylated by PDK1 ([10]; Figure 5b). To test the hypothesis that PDK1 phosphorylates the RSK1 amino terminus, a GST–D1′ fusion protein was made; a second GST–D1′ fusion protein carrying a point mutation (S239A) at the putative PDK1 site was also generated. When purified GST–D1′ (K112R) and GST–D1′ (K112R, S239A) were used as substrates in an *in vitro* kinase assay, only GST–D1′ (K112R) was phosphorylated by PDK1 (Figure 5c, upper panel); the S239A mutant was not phosphorylated. The lack of phosphorylation in the S239A mutant also indicated that the GST moiety was not phosphorylated by PDK1. PDK1 was active in both kinase reactions, as indicated by its autophosphorylation

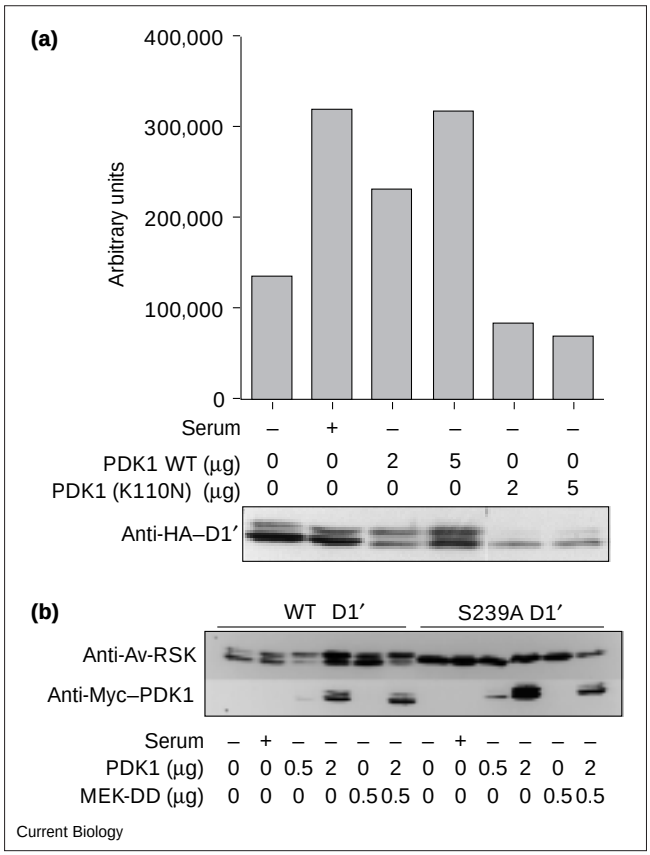
Figure 5



RSK1 is phosphorylated by ERK and PDK1. (a) Phosphorylation of GST–D2 by ERK. *In vitro* kinase assays were performed using a bacterially expressed MEK-activated ERK1 (GST–ERK1*), or GST–ERK1 (K52R). The mutated ERK residue is K52R, which inactivates the ERK catalytic activity. The ERK fusion proteins were incubated with [γ-^{32}P]ATP and 1 μg of either GST–D2 (K464R, T590) or GST–D2 (K464R, T590A). Autoradiography indicates that activated ERK phosphorylated the T590 substrate but not the T590A mutant. (b) Alignment of sequences phosphorylated by PDK1 with the putative PDK1 phosphorylation site in RSK1 (adapted from [35]). Arrow indicates the phosphorylated residue. (c) Phosphorylation of GST–D1′ by a fusion protein between PDK1 and the Myc epitope tag (Myc–PDK1). Myc–PDK1 was immunoprecipitated from transfected HEK 293E cells and used in an *in vitro* kinase assay with 1 μg GST–D1′ (K112R) as substrate. Upper panel, phosphorylation of GST–D1′ (K112R) and GST–D1′ (K112R, S239A) by Myc–PDK1; lower panel, PDK1 autophosphorylation. (d) Phosphorylation of full-length RSK by PDK1. Transfected HA–RSK (K112R, K464R) was immunoprecipitated from quiescent 293 cells and used in an *in vitro* kinase assay with 0.2 μg GST–PDK1 (WT) or GST–PDK1 (K110I) as indicated, or 50% glycerol (lane 1). The mutated residue in GST–PDK1 (K/I) is K110I. Autoradiography indicated that full-length RSK (upper arrow) was phosphorylated in the presence of active PDK1 (lower arrow), which autophosphorylates itself.

(Figure 5c, lower panel). The kinase-inactive PDK1 (K110N) mutant did not phosphorylate D1′ (data not shown). To compare the efficiency of RSK1 phosphorylation by Myc–PDK1 with a characterized PDK1 substrate, *in vitro* kinase assays were performed using GST–D1 RSK

Figure 6



PDK1 activates an isolated RSK1 amino-terminal kinase domain. (a) Activation of HA–D1′ by PDK1. The construct encoding HA–D1′ (1 μg) was cotransfected with the indicated amounts of construct encoding wild-type or K110N mutant of PDK1. Transfected cells were starved or stimulated with serum as indicated. Cell lysates were subjected to SDS–PAGE and transferred to nitrocellulose. Quantitative western blots were performed using an anti-HA antibody, which detected HA–D1′. Upper panel, volumes corrected for HA–D1′ expression levels were used in anti-HA antibody immunocomplex assays, using GST–S6 as substrate (see Materials and methods; the autoradiogram was quantitated by phosphorimager analysis); lower panel, HA–D1′ expression before correction for equivalent protein expression. The results are representative of three separate experiments. (b) Effect of PDK1 and MEK-DD on HA–D1′ (S239A). Constructs (1 μg) encoding wild-type HA–D1′ or the S239A mutant were cotransfected with the indicated amounts of PDK1 or MEK-DD, starved or stimulated with serum as indicated, and subjected to SDS–PAGE. Western blots were performed with antibodies against the avian RSK epitope or Myc to detect transfected D1′ and PDK1, respectively.

and GST–PKCζ as substrates (see Supplementary material). With equimolar amounts of substrate under linear reaction conditions, Myc–PDK1 phosphorylated both GST–D1 and GST–PKCζ, with PKCζ phosphorylation slightly higher (1.5–2-fold) than GST–D1 RSK.

Finally, we examined the ability of PDK1 to phosphorylate full-length RSK *in vitro*. A kinase-deficient RSK was immunoprecipitated from quiescent cells and incubated with GST–PDK1. As shown in Figure 5d, the full-length protein was phosphorylated by PDK1 (lane 2) but not by a kinase-deficient PDK1 (lane 3). Because this RSK fusion protein was catalytically inactive, the RSK phosphorylation is not due to autophosphorylation activity.

The data presented above suggest that RSK is phosphorylated by PDK1, and that S239 in RSK1 is a PDK1 phosphorylation site. This is the first indication that a regulatory input other than from ERK plays a role in RSK1 activation.

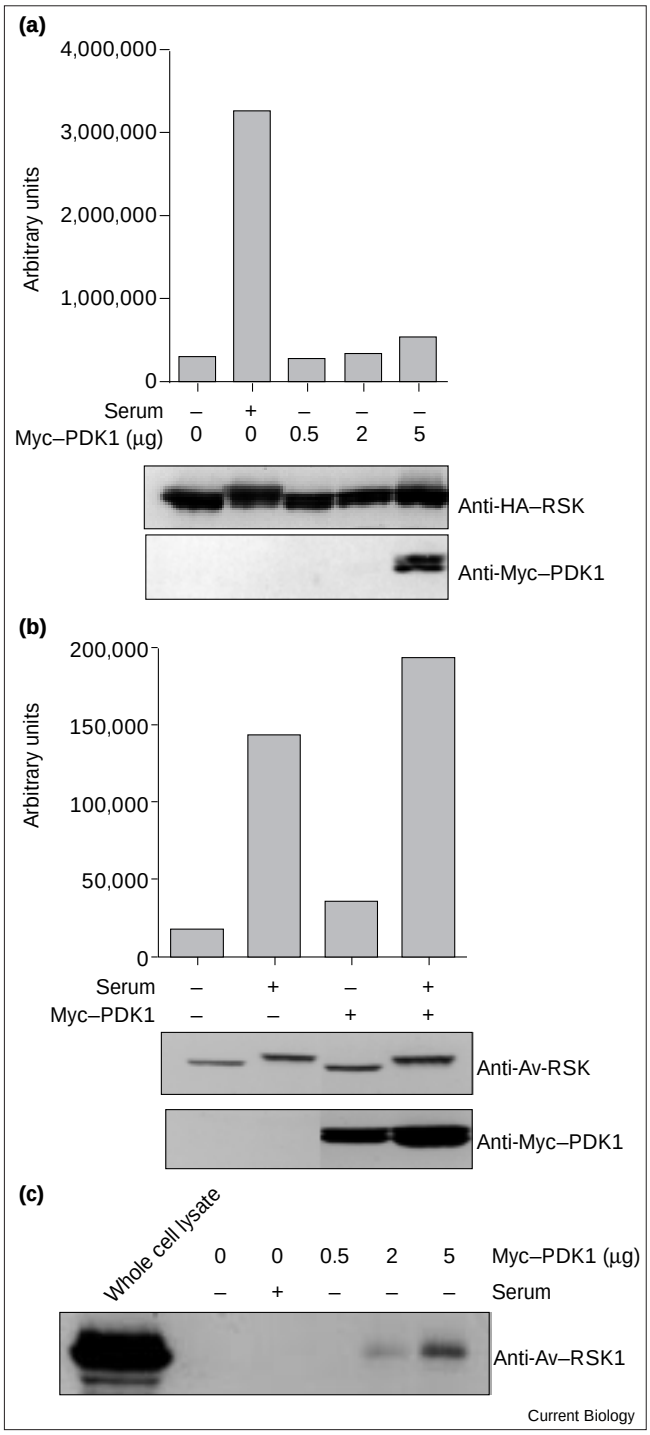
PDK1 activates an isolated RSK1 amino-terminal kinase domain

Because PDK1 phosphorylates the RSK1 amino terminus *in vitro*, we tested the effect of PDK1 on D1′ activation *in vivo*. HEK 293E cells were cotransfected with D1′ and increasing amounts of either wild-type PDK1 or the kinase-inactive PDK1 (K110N). Figure 6a shows that increasing amounts of PDK1 increased D1′ kinase activity towards the exogenous substrate. Surprisingly, D1′ activation by PDK1 alone approached the activation level induced by serum. Coexpression of the kinase-inactive PDK1 (K110N) mutant actually decreased basal D1′ kinase activity, a result consistent with the finding that the S239 site is basally phosphorylated [10]. PDK1 (K110N) may reduce basal phosphorylation at S239, thereby decreasing basal phosphotransferase activity. These results suggest that PDK1 alone can activate the RSK1 amino-terminal kinase domain.

Although the *in vitro* phosphorylation of D1′ by PDK1 suggests that the phosphorylation is direct, it is possible that the overexpression of PDK1 could induce activation of various PKCs, which could indirectly activate D1′ through the ERK/MAP kinase pathway. To address this possibility, we expressed an S239A D1′ mutant in the presence of PDK1 and a constitutively active MEK-1 protein in which serine residues 218 and 222 were substituted by aspartic acid residues (MEK-DD) and examined mobility shifts; because a S239A mutation renders the amino-terminal kinase domain inactive ([10]; data not shown), kinase assays to examine the effect on activity are not possible. Figure 6b shows that when S239 is mutated to alanine in D1′, the doublet seen in the wild-type protein is replaced by a single band of faster mobility. No mobility shift was seen in D1′ (S239A) upon serum stimulation or cotransfection with PDK1 or MEK-DD. For wild-type D1′, intensification of the upper band was seen upon coexpression with PDK1 but not with MEK-DD. While this result does not eliminate the possible indirect contribution of other kinases activated by PDK1, it does suggest that the slower-migrating form of D1′ is the result of basal phosphorylation, and that this phosphorylation is not a direct result of activation of the ERK/MAP kinase pathway.

Figure 7

PDK1 alone does not activate full-length RSK1. (a) Effect of PDK1 on RSK1 activation *in vivo*. The construct encoding full-length HA–RSK1 (1 μg) was cotransfected with the indicated amounts of construct encoding Myc–PDK1. Transfected cells were starved or stimulated with serum as indicated. Cell lysates were subjected to SDS–PAGE and transferred to nitrocellulose. Quantitative western blots were performed using an anti-HA antibody to detect full-length HA–RSK1. Samples with volumes adjusted to obtain equal HA–RSK1 expression were used in anti-HA antibody immunocomplex assays with GST–S6 as substrate. Upper panel, HA–RSK1 kinase activity measured by immunocomplex assay; lower panels, western blots showing expression of HA–RSK1 and Myc–PDK1 as indicated. The results are representative of three separate experiments. (b) Effect of PDK1 on serum-stimulated RSK1 activity. The construct encoding full-length HA–RSK (1 μg) was transfected alone or with 2 μg plasmid expressing Myc–PDK1 as indicated. Transfected cells were starved or stimulated with serum as indicated. HA–RSK was immunoprecipitated with an anti-HA antibody for immunocomplex assays using GST–S6 as substrate (upper panel). Western blots (lower panels) were performed with antibodies against the avian RSK epitope or Myc to detect transfected full-length RSK or PDK1, respectively. The results are representative of two separate experiments. (c) RSK1 and PDK1 interact *in vivo*. HA–RSK1 and Myc–PDK1 were cotransfected as in (a). Myc–PDK1 was immunoprecipitated from cell lysates [38] and subjected to SDS–PAGE. Western blotting with antibodies against the avian RSK epitope detected RSK1 associated with Myc–PDK1.



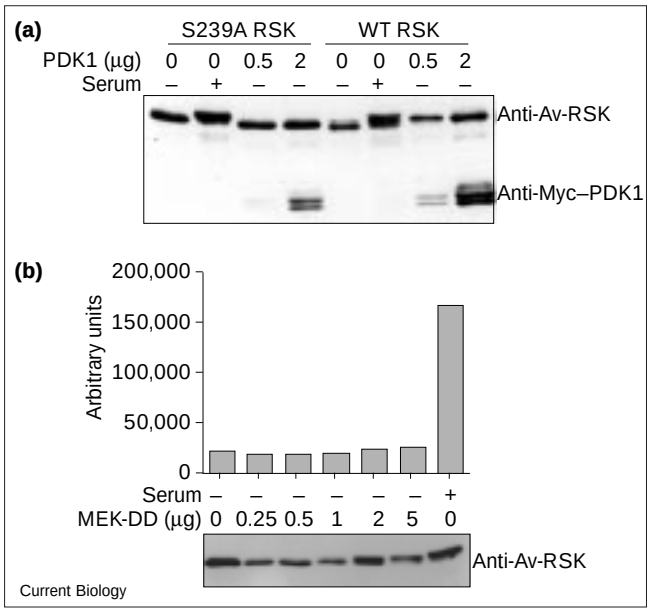
PDK1 is not sufficient to activate full-length RSK1

We have established that PDK1 alone can activate phosphotransferase activity of the isolated amino-terminal kinase domain of RSK1, and we next investigated the effect of PDK1 on full-length RSK1, which carries both kinase domains. HEK 293E cells were cotransfected with full-length RSK1 and PDK1 (Figure 7a), and RSK1 activity was assayed. At higher expression levels, PDK1 induced a small activation of RSK1 kinase activity, but did not approach the level induced by serum. Under these conditions, immunoprecipitated Myc–PDK1 coprecipitated HA–RSK1, as detected by western blotting, indicating that the two proteins are interacting (Figure 7c). RSK activation was slightly enhanced upon coexpression of PDK1 with RSK in serum-stimulated cells, although fold-activation was slightly lower, possibly as a result of higher basal activity (Figure 7b).

Although PDK1 could phosphorylate full-length RSK1 *in vitro* (Figure 5d), it was possible that PDK1 did not regulate the phosphorylation of full-length RSK1 *in vivo*. To examine this possibility we took advantage of the observation that phosphorylation of wild-type full-length RSK affects its mobility in SDS–polyacrylamide gels [27]. In quiescent cells, RSK1 existed primarily as a faster-migrating species but, upon activation by serum, slower-migrating middle and upper bands were observed (Figure 8a). Coexpression with PDK1 in the absence of serum induced a shift in wild-type RSK1 to the middle band, implying that full-length RSK1 can be phosphorylated upon coexpression with PDK1. A shift to the uppermost band was never observed, however.

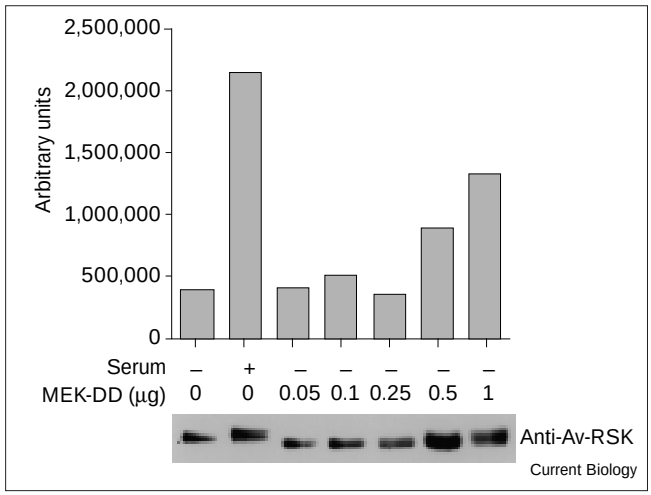
To confirm that PDK1 induced a mobility shift in full-length RSK1 *in vivo*, an S239A mutation was introduced into full-length RSK1. This mutation renders the phosphotransferase activity of the RSK1 amino-terminal kinase domain inactive ([10]; data not shown). When RSK1 (S239A) was expressed in cells, serum stimulation was still able to induce a shift to the slower-migrating band (Figure 8a); in the absence of serum, however, PDK1 did not induce a shift to a slower-migrating species. This is in

Figure 8



Serum-stimulated activation of the RSK carboxyl terminus is independent of phosphorylation at Ser239. (a) Western blot showing mobility shift of RSK1 when co-expressed with PDK1. Plasmids encoding wild-type RSK1 or the S239A mutant (1 μg of either) were cotransfected with increasing amounts of the construct for Myc–PDK1. Transfected cells were starved or stimulated with serum as indicated. Cell lysates were probed for expression of RSK1 (using the antibody against the avian-specific epitope) and PDK1 (using anti-Myc antibody). (b) The carboxy-terminal kinase domain of RSK1 (S239A) is activated by serum. The construct for the S239A mutant of RSK1 (1 μg) was cotransfected with increasing amounts of plasmid encoding MEK-DD. The western blot (lower panel) shows that serum and MEK-DD induce a shift in mobility of RSK1 (S239A). An immunocomplex kinase assay performed using the antibody against the HA epitope, and GST–D2 (K464R) as substrate, showed activation of phosphotransferase activity of the carboxy-terminal kinase domain (upper panel). The results are representative of two separate experiments.

Figure 9



RSK1 activation by MEK-DD. Plasmid (1 μg) encoding HA–RSK1 was transfected with increasing amounts of plasmid encoding MEK-DD. Transfected cells were starved or stimulated with serum as indicated. Cell lysates were subjected to SDS–PAGE and transferred to nitrocellulose. RSK1 expression was detected using the antibody against the avian-specific RSK epitope. The graph shows RSK1 immunocomplex assays using GST–S6 as substrate, performed as described in Materials and methods. The results are representative of two separate experiments.

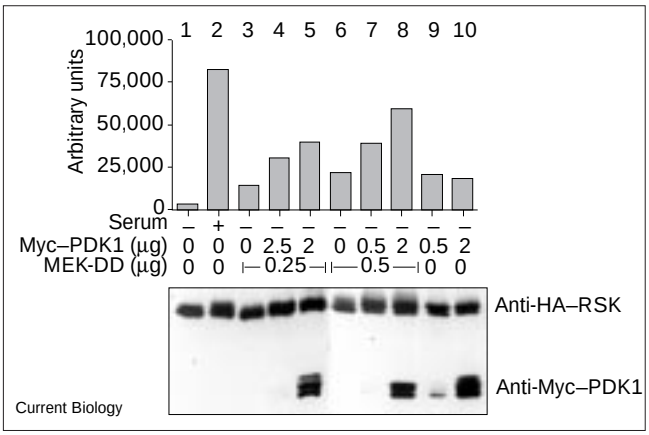
contrast to the shift induced in wild-type RSK1 under these conditions. These data suggest that, although PDK1 does not activate full-length wild-type RSK1, it is capable of inducing RSK1 phosphorylation *in vivo*.

The serum-induced shift in RSK1 (S239A) could be the result of phosphorylation of the carboxyl terminus and autophosphorylation by this activated carboxy-terminal kinase domain. The amino-terminal kinase domain is inactive [10], but to test whether the carboxy-terminal kinase domain of this mutant was active, kinase assays were performed with immunoprecipitated RSK1 (S239A), using GST–D2 (K464R) as substrate. As seen again in Figure 8b, RSK1 (S239A) underwent a shift upon serum stimulation (lower panel). This shift correlated with both the ability of the carboxyl terminus to phosphorylate the exogenous substrate (upper panel) and the ability to autophosphorylate (data not shown). To examine whether the activation of the carboxy-terminal kinase domain was solely due to ERK phosphorylation, RSK1 (S239A) was coexpressed with increasing amounts of MEK-DD. While coexpression with MEK-DD induced a mobility shift in the S239A mutant of RSK1 (lower panel), it was not sufficient to activate carboxy-terminal-domain kinase activity towards GST–D2 (K464R) — the exogenous substrate (upper panel) — or for autophosphorylation (data not shown). These results suggest that, for RSK1 (S239A), inactivation of the amino-terminal kinase domain does not affect the ability of the carboxy-terminal kinase domain to be activated by mitogenic stimuli such as serum, nor does it block presumed autophosphorylation. Nevertheless, full activation of the RSK1 carboxyl terminus requires inputs in addition to ERK, either by autophosphorylation by the amino-terminal kinase domain or by other mitogen-activated kinases.

MEK-DD and PDK1 cooperate to activate full-length RSK1

RSK1 activation requires interaction between the RSK1 carboxyl terminus and ERK [25], and blocking MEK activation with PD098059 decreases RSK1 activation [28]. We therefore examined the possibility that both ERK and PDK1 are required for RSK1 activation. To do this, RSK1 activity was first assayed upon coexpression with increasing amounts of MEK-DD in HEK 293E cells; MEK-DD causes constitutive activation of ERK [29]. As seen in Figure 9, higher doses of MEK-DD could significantly activate RSK1 phosphotransferase activity. To investigate

Figure 10



PDK1 and MEK-DD cooperate to activate RSK1. The construct encoding HA–RSK1 (1 μg plasmid) was transfected with the indicated amounts of plasmids for Myc–PDK1 and MEK-DD. Transfected cells were starved or stimulated with serum as indicated. Western blots were probed with an antibody against the HA epitope tag and subjected to quantitative enhanced chemiluminescence (ECL) and corrected levels were used for RSK1 anti-HA antibody immunocomplex assays, using GST–S6 as a substrate (upper panel). The lower panel shows expression of HA–RSK1 and Myc–PDK1. The results are representative of four separate experiments.

whether there was a co-operative effect with PDK1, subactivating levels of MEK-DD were chosen: 0.25 and 0.5 μg. We then expressed full-length RSK1 with both MEK-DD and PDK1. As seen above and in Figure 10, expression of wild-type RSK1 with either MEK-DD (lanes 3,6) or PDK1 alone (lanes 9,10) did not significantly activate RSK1 kinase activity. When MEK-DD and PDK1 were co-expressed, however, RSK1 phosphorylation of GST–S6 was activated at a level higher than with either PDK1 or MEK-DD alone (lanes 4,5 and 7,8), and this activation was dose dependent. Therefore, MEK-DD and PDK1 cooperatively activate RSK1.

Discussion

Activation of RSK1 has been thought to be dependent solely on phosphorylation by activated ERK and subsequent RSK1 autophosphorylation. We have shown here that PDK1 phosphorylates wild-type avian RSK1 and is even capable of activating the isolated RSK1 amino-terminal kinase domain. Furthermore, cooperation between ERK and PDK1 can produce an active RSK1 enzyme. The contribution of PDK1 means that we have uncovered an additional signaling pathway involved in the regulation of RSK1.

That phosphorylation by ERK is critical for RSK1 activation is indisputable. The observation that mutation of the ERK phosphorylation site in the RSK1 carboxyl terminus severely compromises mitogen-stimulated RSK1 kinase activity ([10]; data not shown) reinforces this supposition. Preventing ERK activation by inhibiting upstream MEK with PD098059 blocks RSK1 activation [28]. There have been indications, however, that ERK is not sufficient for full activation of RSK1; activation of quiescent RSK *in vitro* with activated ERK restores only a portion of RSK1 phosphotransferase activity [30]. Although we have shown here that an isolated amino-terminal RSK1 kinase domain is readily activated by PDK1, this contribution would be masked in the full-length protein because of the requirement for ERK in RSK1 activation. The role of PDK1 would be concealed under conditions where ERK is quiescent. We propose a model for RSK1 activation that incorporates the requirements for phosphorylation by ERK and PDK1, as well as other activating phosphorylation events (Figure 11).

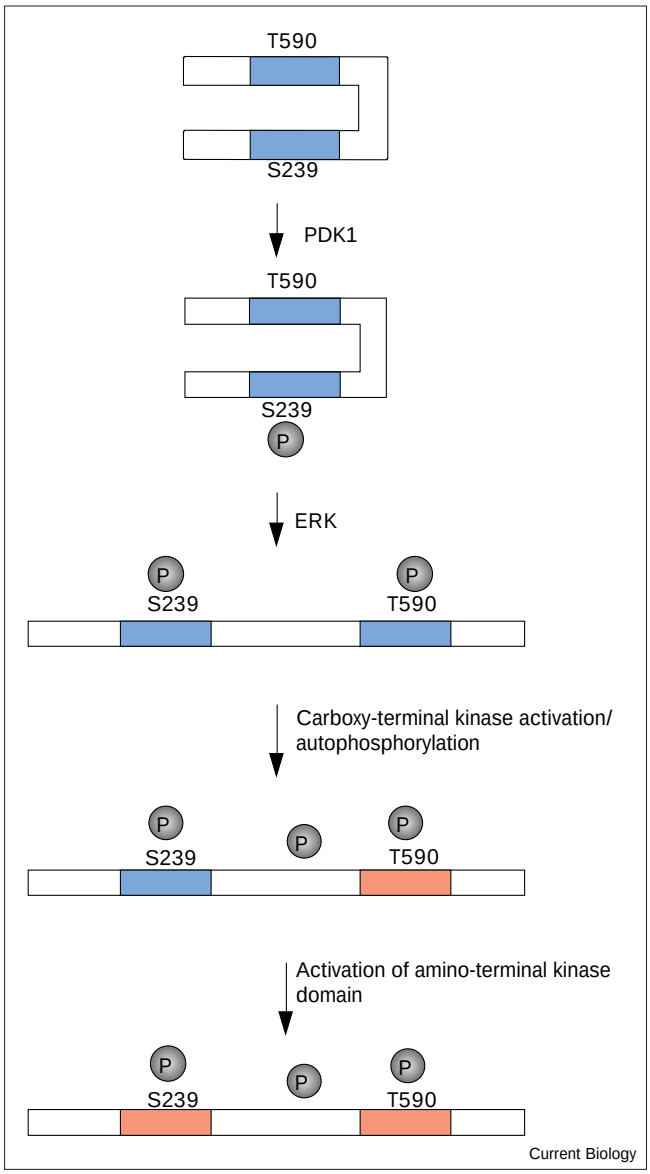
The PDK1 phosphorylation site identified here in RSK1 is preserved in all three human RSK isoforms: RSK1, RSK2, and RSK3 [31]. In a patient diagnosed with Coffin–Lowry syndrome, the PDK1 phosphorylation site is mutated in RSK2, and several patients lack active RSK2 [32]. Coffin–Lowry syndrome is characterized by mental retardation, various musculoskeletal defects and motor difficulties. This suggests that RSK enzyme activity could be involved in normal development.

The RSK carboxy-terminal kinase domain is homologous to the mitogen- and stress-activated protein kinases MNK1 and MNK2, which are also phosphorylated by ERK [33,34]. We have previously shown that the RSK carboxy-terminal kinase domain is active *in vitro* [2]. Here, we have extended the characterization to demonstrate that the catalytic activity exhibited by this kinase domain is regulated *in vivo* in response to growth stimuli.

The RSK amino-terminal kinase domain is homologous to the kinase domains in other members of the AGC family, which includes PKA, various PKCs, and $p70^{S6K1}$ (Figure 5b; reviewed in [35]). The PDK1 phosphorylation site in RSK1 is similar to PDK1 sites in these other enzymes, suggesting that this a general regulatory site for these kinases. Using immunoprecipitated PDK1, similar levels of *in vitro* phosphorylation were seen with equimolar amounts of GST–D1 (K112R) and GST–PKCζ, a known PDK1 substrate (data not shown). This site was found to be basally phosphorylated in RSK1, with a further increase in phosphorylation upon stimulation with phorbol myristoyl acetate [10]. This basal phosphorylation is consistent with our observations that D1′ has high basal activity relative to the serum-stimulated activity level, and that expression of a kinase-inactive PDK1 can decrease D1′ basal phosphotransferase activity.

Although PDK1 does phosphorylate Akt/PKBα, $p70^{S6K1}$ and RSK1, there are differences in the phospholipid requirement for this phosphorylation. For Akt/PKBα, this

Figure 11



Model of RSK1 activation by PDK1 and ERK. We propose that PDK1 phosphorylates RSK1, even when RSK1 is quiescent. The RSK1 carboxyl terminus acts as a negative regulator, blocking activation of the amino-terminal kinase domain. Upon mitogen stimulation, ERK becomes activated and phosphorylates the RSK1 carboxyl terminus. This phosphorylation activates the RSK1 carboxy-terminal kinase domain and stimulates RSK1 autophosphorylation by this domain. Activation of the carboxy-terminal kinase domain induces a conformational change, abrogating the negative regulatory effect of this domain. The RSK1 amino-terminal kinase domain is then freed to phosphorylate exogenous substrates. P, phosphate groups.

reaction is dependent on phospholipids [15,16,36,37]. Binding of phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P_2) or PtdIns(3,4,5)P_3 to the PH domain of Akt/PKBα, targets the enzyme to the membrane and brings it into proximity with membrane-associated PDK1. Phosphorylation of p70^{S6K1} by PDK1 is independent of phospholipids [21,22], and the ability of PDK1 to phosphorylate RSK1 *in vitro* suggests that this reaction is also phospholipid independent.

There are also differences in how PDK1 collaborates with different inputs to fully activate a kinase. With Akt/PKBα, it has been suggested that accessibility of the PDK1 phosphorylation site is regulated by binding of PtdIns(3,4)P_2/PtdIns(3,4,5)P_3 to the Akt/PKBα PH domain [23]. This induces a conformational change, rendering the PDK1 site on Akt/PKBα accessible. With p70^{S6K1}, PDK1 cooperates with Rho family GTPases, PI 3-kinase, and inputs regulated by mTOR or FK506-binding protein (FKBP)–rapamycin associated protein (FRAP) ([38]; data not shown). PDK1 phosphorylation and activation of p70^{S6K1} requires prior phosphorylation of residues in the p70^{S6K1} regulatory domain [22]. In this case, phosphorylation is used instead of phospholipid binding to achieve the same goal: a conformational change exposing a PDK1 phosphorylation site.

Here, we have shown that RSK1 activation requires cooperation between the Ras–ERK/MAP kinase pathway and PDK1. RSK1 is unique in that activation of one kinase domain requires activation of another. Nature has solved the problem of bringing these two kinases together by fusing the RSK carboxy-terminal kinase domain (homologous to MNK1/MNK2) to the amino-terminal kinase domain (homologous to the AGC superfamily of kinases). Activation of RSK1 phosphotransferase activity towards exogenous substrates necessitates the convergence of the two signaling pathways, with each kinase domain requiring a different signal: the carboxy-terminal kinase domain requires phosphorylation by ERK, and the amino-terminal kinase domain requires phosphorylation by PDK1.

Full-length RSK1 and PDK1 can be coimmunoprecipitated from quiescent cells, suggesting that they interact *in vivo*. The phosphorylation of quiescent full-length RSK1 by PDK1 without activating the amino-terminal kinase domain suggests that the PDK1 site in RSK1 is accessible. This phosphorylation is quite different from PDK1 phosphorylation of Akt/PKBα and p70^{S6K1}. In this regard, RSK1 may be similar to PKA, whose basal phosphorylation at the PDK1 site is co-translational [20,39,40]. If this were also the case for RSK1, it may explain the inability of a dominant-negative PDK1 to antagonize serum-stimulated RSK1 activation (data not shown). We also suggest that, when quiescent, the RSK1 carboxyl terminus acts as a negative regulator of the amino-terminal kinase domain. We have shown here that phosphotransferase activity of the isolated RSK1 amino-terminal kinase domain can be activated by PDK1 alone, but the full-length RSK1 cannot; activation of full-length RSK1 requires activated ERK.

Although we have demonstrated that phosphorylation by PDK1 and ERK are necessary and sufficient for RSK1 activation, we have not eliminated the possibility of additional activating signals. Activation by MEK-DD and PDK1 does not activate RSK1 to levels attained by serum stimulation, and full activation of the RSK1 carboxyl terminus requires signals in addition to phosphorylation by ERK. The amino-terminal kinase domain is likely to be important, as are the additional RSK1 phosphorylation sites. The inhibition of D1′ kinase activity by PD098059 indicates that this kinase domain is regulated by the ERK/MAP kinase pathway, either directly or indirectly. Additional sites for phosphorylation by ERK have been suggested within this domain [10]. In addition, S398, which has been identified as a RSK autophosphorylation site [12] is a consensus sequence for PDK2 phosphorylation [41].

Further studies addressing the autoregulation of the individual kinase domains and the interactions between RSK1 and regulatory kinases will be important for understanding the regulatory role of other RSK1 phosphorylation sites. The data presented here support a role for an ERK-independent event in regulating RSK1 kinase activity. This finding introduces both new clarity and increased complexity in understanding the role of RSK1 in cell proliferation and survival signaling.

Conclusions

PDK1 and ERK act cooperatively to activate phosphotransferase activity of the serine/threonine kinase RSK1. Our results show that, in addition to having an important role in signaling to kinases regulated by PI 3-kinase, PDK1 also participates in Ras–ERK/MAP kinase signaling through RSK1.

Materials and methods

Plasmid constructs

Avian RSK1 was subcloned into pKH3, which encodes a triple HA tag at the amino terminus [42]. Avian RSK1 contains an avian-specific epitope between amino acids 37 and 54. The S239A mutant was generated using Stratagene's QuikChange kit. D1, D1′ and D2 in pKH3 were generated by PCR. D1′ (K112R) in pGEX-2T was generated by PCR using avian RSK1 (K112R) as target, and this was then used as target DNA for PCR-based site-directed mutagenesis to generate the D1′ (K112R, S239A) in pGEX-2T. A similar strategy was used to generate GST–D2 (K464R) with and without the T590A mutation. MEK-DD in pCDNA3 was a gift from R. Erikson. Wild-type and K110N versions of Myc–PDK1 in pCDNA3 were previously described [17].

Cell culture, transfection and lysis

HEK 293E cells were grown in DME with 10% FBS in 6% CO_2. Cells were transfected with $CaPO_4$ for 5 h with 1 μg RSK with a total of 5 μg DNA per 60 mm dish unless otherwise indicated; DNA quantities were equalized with empty vector pRK7. Cells were rinsed with PBS and starved in DME without serum for 16 h. Where indicated, cells were stimulated with 10% calf serum at 37°C, 6% CO_2 for 10 min. Cells were rinsed with PBS on ice before lysis in cell-lysis buffer (10 mM KPO_4, 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 50 mM β-glycerophosphate, 0.5% NP-40, 0.1% Brij-35, 0.1% deoxycholic acid, 1 mM sodium orthovanadate, 2 mM DTT, 1 mM PMSF, 5 μg/ml pepstatin and 10 μg/ml leupeptin). Lysates were centrifuged at 14,000 rpm at 4°C for 10 min before separating into aliquots and storage at –80°C.

Western blotting

Lysates were subjected to SDS–PAGE and transferred to nitrocellulose. The filter was blocked with 3% milk/TBS-Tween for 1 h at room temperature. Primary antibody treatment was for 1 h at room temperature. Polyclonal antibody against the RSK1 avian-specific epitope (1:5,000), and polyclonal anti-RSK1 (1:500) and anti-MAP kinase (1:2,000) antibodies were generated as described [2]. Anti-HA (1:60,000) and anti-Myc (1:2,000) antibodies were used where indicated. Blots were washed with TBS-T before treatment with either goat anti-mouse HRP (1:30000 for quantitative ECL) and anti-rabbit HRP (1:5,000) for 1 h at room temperature. Blots were visualized by ECL (NEN) or quantitated by ECL on a BioRad FluorS with BLAZE ECL reagents (Pierce).

Immunoprecipitation kinase assays

For RSK1 kinase assays, antibody to the HA epitope tag was added to cell lysates and incubated at 4°C for 2 h. Immunoprecipitation kinase assays with GST–S6 were performed as described [43]. Because the RSK1 carboxy-terminal kinase domain does not phosphorylate GST–S6, GST–D2 (K464R) was used as a substrate to assay carboxy-terminal kinase activity of RSK1. For MAP kinase kinase assays, anti-MAP kinase antibody was added to lysates and the assay was performed as for anti-RSK1 antibody assays except using GST–D2 (K464R) as substrate [2]. Reactions were subjected to SDS–PAGE and quantitated on a BioRad Phosphorimager.

In vitro *kinase assays*

Substrate GST fusion proteins were purified with glutathione–sepharose beads (Pharmacia). For *in vitro* ERK phosphorylation, GST–ERK* and GST–ERK K52R* were activated with MEK1 and purified [2]. For *in vitro* PDK1 phosphorylation, Myc–PDK1 was immunoprecipitated from transiently transfected HEK 293E cells and washed once each with buffer A (10 mM Tris pH 7.2, 1% NP-40, 0.5% sodium deoxycholate, 100 mM NaCl, 1 mM EDTA, 0.5% sodium orthovanadate, 2 mM DTT, 100 mM PMSF), buffer B (same as buffer A except 0.1% NP-40 and 1 M NaCl), and ST (50 mM Tris-HCl, 5 mM Tris-Base, 150 mM NaCl) before resuspension in kinase buffer [43]. All reactions were carried out with 10 μCi [γ-^{32}P]ATP (NEN) per reaction at 30°C for 15 min. Before stopping the Myc–PDK1 reactions, samples were placed on ice and centrifuged 10 min at 14,000 × *g* at 4°C to precipitate Myc–PDK1. Supernatant and pellet fractions were run separately. Reactions were resolved by SDS–PAGE and exposed to film.

Supplementary material

Supplementary material, including *in vitro* kinase assays performed using GST–D1 RSK and GST–PKCζ as substrates, is available at http://current-biology.com/supmat/supmatin.htm.

Acknowledgements

This work was supported by NIH grant RO1 CA46595 (J.B.), the Postdoctoral Research Fellowship for Physicians from the Howard Hughes Medical Institute and the V Foundation Scholar Award (A.S.), the Juvenile Diabetes Foundation International (A.R.), and the Leukamia Society of America (S.A.R.).

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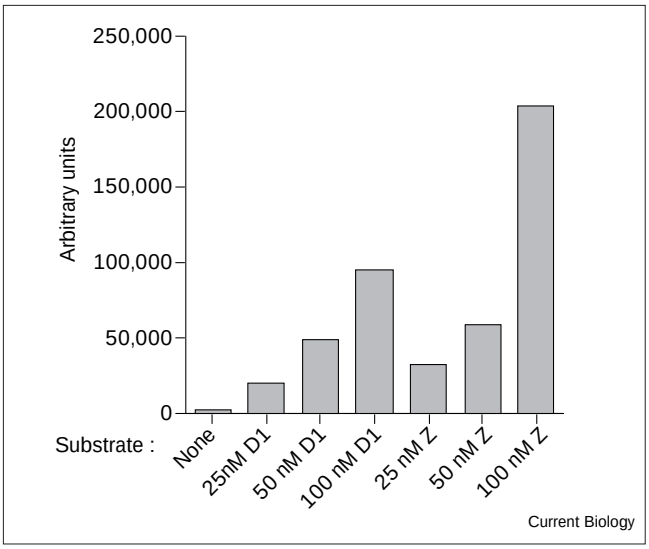
Supplementary material

Ribosomal S6 kinase 1 (RSK1) activation requires signals dependent on and independent of the MAP kinase ERK

Stephanie A. Richards, Joyce Fu, Angela Romanelli, Akiko Shimamura and John Blenis

Current Biology 22 July 1999, 9:810–820

Figure S1



In vitro phosphorylation of GST–D1 K112R with GST–PKCζ (Z) by Myc–PDK1. HEK 293E cells were transfected for 5 h with 1 μg Myc–PDK1, rinsed and recovered in DMEM containing 10% foetal bovine serum. After 16 h, cells were lysed and treated with an anti-Myc antibody to precipitate Myc–PDK1. The indicated amounts of GST–substrate were added to PDK1 immunoprecipitates in an *in vitro* kinase reaction that contained [γ-^{32}P]ATP. After 20 min at 30°C, reactions were placed on ice and spun at 14,000 x *g* for 10 min at 4°C. Sample buffer was added to the supernatant, which was then separated by SDS–PAGE. Phosphate incorporation was quantitated on a phosphorimager. The data show that MycPDK1 phosphorylates GST–D1 K112R and GST–PKCζ at comparable levels *in vitro*. Results are representative of two separate experiments.