The Src-family tyrosine kinase inhibitor PP1 interferes with the activation of ribosomal protein S6 kinases

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Considerable biochemical and pharmacological evidence suggests that the activation of ribosomal protein S6 kinases (S6Ks) by activated receptor tyrosine kinases involves multiple co-ordinated input signals. However, the identities of many of these inputs remain poorly described, and their precise involvement in S6K activation has been the subject of great investigative effort. In the present study, we have shown that 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP1), a selective inhibitor of the Src family of non-receptor tyrosine kinases, interferes with the activation of 70 and 85 kDa S6K gene products (p70S6K1 and p85S6K1) by insulin, insulin-like growth factor 1, sodium orthovanadate and activated alleles of phosphoinositide 3-kinase and H-Ras. PP1 also impedes the activation of AKT/protein kinase B and the extracellular signal-regulated protein kinases

1 and 2 by these various stimuli. Insulin-like growth factor 1 was observed to induce a sustained increase in c-Src autophosphorylation as revealed using anti-phospho-Y416 antisera, but this effect was absent from the cells treated with PP1. To conclude, an activated allele of p70S6K1 is compared with the wild-type allele, resistant to inhibition by PP1 when co-expressed with phosphoinositide-dependent kinase 1 (PDK1), suggesting that PP1 affects p70S6K1 via a PDK1-independent pathway. Thus activation of Src may supply a necessary signal for the activation of p70S6K1 and possibly other S6Ks.

Key words: AKT/protein kinase B, ERK1/ERK2, insulin-like growth factor I (IGF1), phosphatidylinositol 3-kinase (PI3K), phosphoinositide-dependent kinase 1 (PDK 1).

INTRODUCTION

The initial growth phase of cell cycle, G₁ phase, in metazoans is supported by an increase in the activity of the 70 kDa cytoplasmic S6 kinase 1 (S6K1) gene product (p70S6K1) [1,2]. This enzyme, and perhaps its nuclear counterpart p85S6K1, phosphorylates the S6 protein of the 40 S ribosomal subunit during entry into G₁ phase (either from G₀ phase or M phase) or in response to cellular stimulation by mitogens (reviewed in [3,4]). The phosphorylation of S6 facilitates the incorporation of an otherwise poorly translated class of terminal oligo(pyrimidine)-bearing mRNAs into polyribosomes. Collectively, these mRNAs encode proteins involved in translation itself, such as ribosomal proteins and translation factors. Therefore activation of p70S6K1 drives ribosome biogenesis, which in turn supports cell growth [5].

The activation of p70S6K1 in response to growth factors relies primarily on the activation of phosphoinositide 3-kinase (PI 3-kinase) [6], which phosphorylates membrane phosphoinositides at the D3 position of the inositol ring, thereby generating lipid second messengers with a distinct bioactivity. These modified lipids draw several potential p70S6K1 activators to the membrane. Among these, the phosphoinositide-dependent kinase 1 (PDK1) phosphorylates p70S6K1 directly at T229 within the activation loop [7,8], and indeed does so at similar sites within the activation loops of other candidate p70S6K1 activators such as AKT and the atypical protein kinase C (aPKC), PKC λ [9] and PKC ζ [10]. Activated alleles of other signalling molecules such as H-Ras (in the present study), Rac and Cdc42 [11] have also been shown to stimulate the activity of p70S6K1; however, it is unclear whether or not this derives from the tendency of these signalling effectors to activate PI 3-kinase [12,13]. Finally, the mammalian

target of rapamycin (mTOR) proteins of *Saccharomyces cerevisiae* appear to supply a necessary signal to p70S6K1, as perturbation of mTOR function inhibits the phosphorylation and activity of p70S6K1.

Several lines of evidence suggest that the Src family of non-receptor tyrosine kinases may play an important role in the activation of p70S6K1 by growth factors. First, in v-Src-transformed cells, the predominant lipid products of PI 3-kinase are elevated [14], which correlates positively with an interaction between PI 3-kinase and v-Src [15]. In the case of Fyn, this interaction involves the Src homology 3 domain of Fyn and a proline-rich region within the p85 regulatory subunits of PI 3-kinase [16]. AKT activation, which relies strictly on PI 3-kinase activation, is also induced by the activated alleles of Src (v-Src and c-Src) [17,18]. In PC12 cells, Src has been observed to associate with PKC ζ in a nerve growth factor-dependent manner, and expression of dominant-negative Src impairs the activation of PKC ζ by nerve growth factor [19]. Furthermore, the growth characteristics of chicken embryo fibroblasts expressing v-Src are suppressed by rapamycin, indicating that a part of the transformation pathway initiated by v-Src involves signalling at the level of mTOR [20].

MATERIALS AND METHODS

Antibodies and reagents

Anti-S6K1 (C-18; cat no. sc-230) recognizes an epitope within the C-terminus of both p70S6K1 and p85S6K1 and was purchased from Santa Cruz Biotechnology (New York, NY, U.S.A.).

Abbreviations used: CHO-IR, Chinese hamster-ovary cells overexpressing the human insulin receptor; ERK-1, extracellular signal-regulated protein kinase 1; G12V, Gly¹² → Val; HA, haemagglutinin; IGF 1, insulin-like growth factor 1; K227E, Lys²²⁷ → Glu; MAP kinase, mitogen-activated protein kinase; MEK-1, MAP kinase/ERK kinase 1; mTOR, mammalian target of rapamycin; p70S6K1, 70 kDa gene product of S6K1; PDK1, phosphoinositide-dependent kinase 1; PI 3-kinase, phosphoinositide 3-kinase; PKC, protein kinase C; PP1, 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]-pyrimidine; S6K, S6 kinase.

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Anti-phospho-Thr⁴²¹/Ser⁴²⁴ (cat no. 9204) recognizes S6K1 when doubly phosphorylated on Thr⁴²¹ and Ser⁴²⁴, anti-phospho-Thr³⁸⁹ (cat no. 9205) recognizes S6K1 when phosphorylated on Thr³⁸⁹, anti-Ser⁴⁷³ (cat no. 9271) recognizes AKT when phosphorylated on Ser⁴⁷³, anti-phospho-Tyr⁴¹⁶ (cat no. 2101) recognizes Src when phosphorylated on Tyr⁴¹⁶ and anti-phospho-Thr²⁰²/Tyr²⁰⁴ (cat no. 9101) recognizes extracellular signal-regulated protein kinases 1 and 2 (ERK-1 and ERK-2) when doubly phosphorylated on Thr²⁰² and Tyr²⁰⁴. These anti-phosphopeptide antibodies were purchased from Cell Signaling Technology (Lexington, KY, U.S.A.). Anti-AKT antibody (cat no. 9272) was also from Cell Signaling Technology. Anti-haemagglutinin (anti-HA; cat no. sc-805) antibody and anti-Src antibody (cat no. sc-19) were purchased from Santa Cruz Biotechnology. Anti-Myc antibody (cat no. 9E10) was purchased from Calbiochem (La Jolla, CA, U.S.A.). Insulin-like growth factor 1 (IGF 1), insulin and sodium orthovanadate were purchased from Sigma. Wortmannin and PD098059 were purchased from Calbiochem. 4-Amino-5-(4-methylphenyl)-7-(t-butyl)-pyrazolo[3,4-d]pyrimidine (PP1) was purchased from Biomol (Plymouth Meeting, PA, U.S.A.).

Plasmids and cDNAs

All p70S6K1 constructs are cloned into *Xba*I and *Eco*RI sites in the pRK7 expression vector and are 5'-HA-tagged. Wild-type and E389D₃E p70S6K1 constructs and the empty pRK7 plasmid were kindly provided by Dr John Blenis (Harvard Medical School). E389 was generated using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.). Δ CT was created by the amplification of an HA-S6K1 fragment comprised of amino acids 1–398. This fragment was mutagenized so as to include an *Xba*I site 5' of the HA epitope and a stop codon followed by an *Eco*RI site, just 3' to S398. The fragment was subsequently cloned into pRK7 to generate Δ CT, in which the putative autoinhibitory domain, which extends from amino acids 399–502, has been deleted. Δ NT Δ CT was constructed by PCR amplification of an HA-tagged fragment comprising amino acids 31–398 of the full-length parent. This fragment included *Xba*I and *Eco*RI sites at the 5'-end and 3'-end respectively and was cloned into the corresponding sites in pRK7. p110 α Lys²²⁷ → Glu (K227E) was kindly provided by Dr Hava Avraham and H-Ras Gly¹² → Val (G12V) by Dr Julian Downward (Imperial Cancer Research Fund). Myc-tagged PDK1 was from Dr Phillip T. Hawkins (Babraham Institute).

Cell culture and transient transfection

L6 skeletal myoblasts (A.T.C.C.) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) foetal calf serum and 1% (v/v) penicillin/streptomycin. Cells were seeded in 6-well plates or 60 mm dishes and cultured in the presence of serum for 24 h. The culture medium was then replaced with serum-free Dulbecco's modified Eagle's medium for an additional 24 h and the cells were subsequently assayed.

Chinese hamster-ovary cells stably overexpressing the human insulin receptor (CHO-IR; a gift from Joseph Avruch, Harvard Medical School) were cultured in F12 Ham's medium supplemented with 10% (v/v) foetal calf serum and 1% (v/v) penicillin-streptomycin. CHO-IR were transiently transfected with a total volume of 3 μ g of plasmid DNA using LIPOFECTAMINETM 2000 (Gibco BRL, Gaithersburg, MD, U.S.A.) in accordance with the manufacturer's instructions (see the legends of Figures 4 and 5). After transfection for 24 h, cells were rinsed with PBS and cultured in serum-free F12 Ham's medium for an additional 24 h as indicated.

Western-blot analysis

Cellular protein was prepared initially by harvesting cells in buffer A [50 mM Tris/HCl (pH 7.4), 1% (v/v) Nonidet P40, 0.25% deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 100 μ M PMSF, 200 μ M benzamide, 800 nM leupeptin, 1 mg/ml pepstatin and 1 mM Na₃VO₄]. Solubilization of cell membranes was facilitated by rocking extracts on an orbital rocker for an additional 20 min. Cell lysates were then clarified by centrifugation at 14000 *g* for 20 min at 4 °C and subjected to Western-blot analysis as described previously [21,22]. For each immunoblot displayed, samples from each condition were pooled from either duplicate or triplicate assays before electrophoresis. Thus the Western blots accurately reflected mean immunoreactivity for a given experiment.

S6K1 activity

The procedure followed has been described previously [23]. Briefly, the activity of endogenous or ectopically expressed S6K1 was determined in anti-S6K1 or anti-HA immune complexes respectively by measuring ³²P incorporation into a synthetic peptide (AKRRRLSSLRA) derived from the region of naturally phosphorylated S6. In the cell system employed here, expression levels of different S6K1 constructs were somewhat variable between treatment conditions, when S6K1 was ectopically expressed, kinase activity determinations are normalized for protein expression levels.

RESULTS AND DISCUSSION

To explore the possibility that the Src-family tyrosine kinases are involved in the regulation of S6Ks, we assessed the effect of PP1, a widely applied inhibitor of Src-family kinases, on the growth factor-stimulated activity of p70S6K1. Serum-deprived L6 myoblasts were treated with varying doses of PP1 before stimulation with IGF 1. In cells pretreated with PP1, the S6K activity measured in anti-S6K1 immunoprecipitates displayed a concentration-related inhibition (Figure 1A). The inhibition of S6K activity was associated with decreased phosphorylation of sites in the putative autoregulatory tail (i.e. Thr⁴²¹/Ser⁴²⁴ of p70S6K1 and Thr⁴⁴⁴/Ser⁴⁴⁷ of p85S6K1) as well as within a conserved hydrophobic motif (i.e. Thr³⁸⁹ of p70S6K1 and Thr⁴¹² of p85S6K1; Figure 1B) suggesting that PP1 may interfere with one or more signals required for p70S6K1 activation. Consistent with this possibility, the IGF 1-induced phosphorylation of AKT within its own hydrophobic motif, Ser⁴⁷³, was decreased in cells treated with PP1 (Figure 1B). Additionally, the mitogen-activated protein kinases (MAP kinases) ERK-1 and ERK-2 displayed a similar inactivation when L6 cells were incubated with PP1 (Figure 2A). The phosphorylation of Src at Tyr⁴¹⁶, a known site of autophosphorylation [24], was decreased by PP1 treatment (Figure 2B), suggesting that the inhibitor was indeed inactivating Src. It was interesting to note that in L6 cells, IGF 1 induced a sustained increase in Tyr⁴¹⁶ phosphorylation (Figure 2B), which to our knowledge has not been reported previously.

Incubation of cultured cells with the tyrosine phosphatase inhibitor, sodium orthovanadate, by favouring the phosphorylated states of tyrosine phosphophorylated molecules, simulates the activation of growth factor receptor-utilized pathways. As shown in Figure 3, sodium orthovanadate treatment of L6 cells induced the activation of p70S6K1 (as shown by the increased phosphorylation of Thr³⁸⁹), of AKT (as indicated by phosphorylation of Ser⁴⁷³) and of ERK-1 and ERK-2 (as indicated by dual phosphorylation of threonine and tyrosine). Again,

Figure 1 Mitogen-stimulated S6K1 activity is decreased in cells exposed to the Src-family tyrosine kinase inhibitor PP1

L6 cells were cultured in a medium devoid of serum for 24 h. The Src-family tyrosine kinase inhibitor PP1 was added at the indicated concentrations for 20 min before the addition of IGF 1 (20 ng/ml) for an additional 30 min. Cells were lysed and clarified by centrifugation. (A) Anti-S6K1 immune complexes were prepared from the resulting supernatant and subjected to an immune complex S6K assay as described in the Materials and methods section. The data are representative of two independent experiments, each of which was performed in triplicate. (B) Equal amounts of cell-extracted protein were separated by SDS/PAGE and immunoblotted either with S6K1-specific [α -(P)Thr⁴²¹/Ser⁴²⁴, α -(P)Thr³⁸⁹, α -S6K1] or with AKT-specific [α -(P)Ser⁴⁷³, α -AKT] antibodies. In each experiment, three samples for each condition were pooled before electrophoresis. Thus immunoblots display the mean immunoreactivity of three samples. The data are representative of at least three independent experiments.

pretreatment of these cells with PP1 inhibited the activation of each of these signalling molecules.

The activation of p70S6K1 is mostly dependent on the signals generated by PI 3-kinase, presumably phosphatidylinositol 4,5-bisphosphate- and phosphatidylinositol 1,4,5-trisphosphate-dependent pathways. Because of the important role PI 3-kinase plays in regulating p70S6K1 activation, we determined whether artificial activation of PI 3-kinase would prevent or interfere with the inhibition of p70S6K1 by PP1. Therefore CHO-IR were transiently transfected with a wild-type, HA-tagged p70S6K1 and one of two additional constructs, p110 α K227E or H-Ras

Figure 2 In PP1-treated L6 cells, both ERK-1/ERK-2 and Src undergo regulatory dephosphorylation

L6 cells were cultured in medium devoid of serum for 24 h. PP1 (50 μ M) was added 20 min before the addition of IGF 1 (20 ng/ml) for the indicated time periods. Cells were lysed and clarified by centrifugation. Equal amounts of cell-extracted protein were separated by SDS/PAGE and immunoblotted either with ERK-specific [A; α -(P)ERK-1/ERK-2, α -ERK-1/ERK-2] or with Src-specific [B; α -(P)Tyr⁴¹⁶, α -Src] antibodies. In each experiment, three samples for each condition were pooled before electrophoresis. Thus immunoblots display the mean immunoreactivity of three samples. The data are representative of at least three independent experiments.

G12V, the expression in cells of either of which leads to an increase in the abundance of membrane phosphoinositides [25]. Whereas expression of either p110 α K227E or H-Ras G12V elevated the activity of co-expressed p70S6K1 to that achieved in insulin-treated cells (Figure 4A), neither construct protected p70S6K1 from inhibition by PP1 (Figure 4B). This effect of PP1 was similar to that of the PI 3-kinase inhibitor wortmannin, whereas PD098059, an inhibitor of MEK-1 (MAP kinase/ERK kinase 1) and MEK-2, failed to inhibit p70S6K1 activity in cells expressing the activated forms of PI 3-kinase or H-Ras alone or in conjunction (Figure 4A). These observations suggest that activation of p70S6K1 by H-Ras G12V does not involve the MEK/ERK pathway, but instead relies on the activation of PI 3-kinase. Although p110 α K227E is an activated allele of the catalytic subunit of PI 3-kinase, it is unclear whether its kinase activity would remain regulated by a PP1-inhibitory pathway.

Since PP1 and wortmannin were both sufficient inhibitors of p70S6K1, we determined whether a panel of p70S6K1 variants, which exhibit varying degrees of sensitivity to rapamycin and wortmannin, would display a similar or dissimilar profile of inactivation in response to PP1. Relative to wild-type p70S6K1, the sensitivities of the deletion and phospho-mimic variants tested to inhibition by rapamycin are Δ CT > E389 $\geq$ E389D₃E > Δ NTACT, where Δ CT is almost completely inhibited by rapamycin and Δ NTACT is completely resistant [3,6,26]. Relative to wild-type p70S6K1, the sensitivities to inhibition by wortmannin are Δ CT > Δ NTACT > E389 $\geq$ E389D₃E [3,27].

Figure 3 PP1 inhibits the activation of p70S6K1, AKT and ERK-1/ERK-2 in cells treated with sodium orthovanadate

L6 cells were cultured in medium devoid of serum for 24 h. PP1 (50 μ M) was added 20 min before the addition of sodium orthovanadate (1 μ M) for an additional 30 min. Cells were lysed and clarified by centrifugation. Equal amounts of cell-extracted protein were separated by SDS/PAGE and immunoblotted with S6K1-specific [α -(P)Thr³⁸⁹, α -S6K1], AKT-specific [α -(P)Ser⁴⁷³, α -AKT] and ERK-specific [α -(P)ERK-1/ERK-2, α -ERK-1/ERK-2] antibodies. In each experiment, three samples for each condition were pooled before electrophoresis. Thus immunoblots display the mean immunoreactivity of three samples. The data are representative of at least three independent experiments.

The insulin-stimulated value of wild-type p70S6K1 was decreased to approx. 30% in cells treated with PP1 (Figure 5A). A similar reduction in activity was observed for each of the variants examined. Thus because the rapamycin-resistant construct (Δ NTACT) was inhibited to an extent similar to the wild-type, the regulation of p70S6K1 by rapamycin and PP1 must involve different mechanisms. Again, this is strikingly similar to what has been shown for wortmannin and more recently, for *N*- α -tosyl-L-phenylalanyl chloromethane [28]. Given their different wortmannin sensitivities, the similar response of the p70S6K1 variants was somewhat unexpected. It is conceivable that PP1 may impede both rapamycin- and wortmannin-sensitive regulatory mechanisms, which would render the activities of these variants indistinguishable.

To probe further the regulatory properties of PP1 and wortmannin, E389D₃E was co-expressed with PDK1, and the resulting activity of this p70S6K1 mutant was determined in cells treated with wortmannin and PP1. PDK1 has been identified as a critical S6K, which is responsible for the phosphorylation of a conserved threonine in the activation loop of S6Ks [7,8]. Phosphorylation of S6Ks by PDK1 requires first a battery of phosphorylations within the C-terminal tail, postulated to relax or unfold the kinase, thereby making the internally situated activation loop site accessible to PDK1. Moreover, the phosphorylation of the PDK1 site (Thr²²⁹ in p70S6K1) and of the hydrophobic site (Thr³⁸⁹ in p70S6K1), which is also internally localized, is highly co-operative, where phosphorylation at either site favours or promotes phosphorylation of the other. Hence, the high basal activity of E389D₃E is attributable to both a

Figure 4 Activated alleles of PI 3-kinase and H-Ras fail to restore p70S6K1 activity in PP1-treated cells

(A) CHO-IR were transfected with HA-p70S6K1 and then deprived of serum overnight. Subsequently, cells were pretreated with or without wortmannin (50 nM) or PD098059 (dose) for 30 min, before the addition of insulin (10 nM) for an additional 30 min. Cells were lysed and clarified by centrifugation. Anti-HA immune complexes were prepared from the resulting supernatant and subjected to immune complex S6K assay as described in the Materials and methods section. The data are representative of two independent experiments, each of which was performed in triplicate. (B) CHO-IR were transfected with HA-p70S6K1 and either p110 α K227E or H-Ras G12V, and then deprived of serum for 24 h. Subsequently, cells were pretreated with or without PP1 (50 μ M) for 20 min. Cells were lysed and clarified by centrifugation. Anti-HA immune complexes were prepared from the resulting supernatant and subjected to an immune complex S6K assay as described above. The data represent the means of two independent experiments, each of which was performed in duplicate, i.e. $n = 4$ per condition.

Figure 5 The effects of PP1 and wortmannin on p70S6K1 share similar regulatory characteristics

(A) CHO-IR were transfected with HA-p70S6K1 variants as indicated and then deprived of serum overnight. Cells were subsequently treated for 20 min with PP1 (50 μ M) before the addition of insulin (10 nM) for 30 min. Cells were lysed and clarified by centrifugation. Anti-HA immune complexes were prepared from the resulting supernatant and subjected to immune complex S6K assay as described in the Materials and methods section. The data are representative of at least two independent experiments, each of which was performed in triplicate. (B) CHO-IR were transfected with HA-p70S6K1 alone or with Myc-PDK1 and then deprived of serum for 24 h. Subsequently, cells were pretreated with or without wortmannin (50 nM) for 30 min or with PP1 (50 μ M) for 20 min before the addition of insulin (10 nM) for 30 min. Cells were lysed and clarified by centrifugation. Anti-HA immune complexes were prepared from the resulting supernatant and subjected to an immune complex S6K assay as described above.

‘relaxed’, disinhibited conformation as well as simulated phosphorylation of hydrophobic motif within, i.e. Thr³⁸⁹. This variant exhibits a heightened resistance to inhibition by wortmannin when PDK1 is also expressed [7] (Figure 5B). Importantly, PDK1 also protects E389D₃E from inhibition by PP1, again similar in characteristic to wortmannin. Collectively, these findings are consistent with the observation that PP1 hinders the activation of p70S6K1 by various stimuli and in different cell

types via pathways, which would otherwise be permissive for phosphorylation of p70S6K1 by PDK1. This may explain why co-expression of PDK1 with E389D₃E circumvents inhibition of this p70S6K1 variant by PP1. Conceivably, this could occur via the regulation of PI 3-kinase by Src-family kinases, a hypothesis for which there is precedent [15,16,29,30].

Of the Src family of protein tyrosine kinases, PP1 has been shown to inhibit Lck and Lyn with an IC₅₀ approx. 30-fold lower than that of Src [31]. Whereas the *in vitro* concentration needed to inhibit these Src kinases is in the nanomolar range, micromolar concentrations are needed to bring about the same changes in intact cells. This difference may be related to permeability, but it should be noted that PP1 inhibition of Lck displays complex kinetics, which may, at particular concentrations, directly compete with ATP binding to the kinase [31]. Thus it is difficult to relate concentration-dependent changes in p70S6K1 to one or more Src-family kinases. Nevertheless, PP1 concentrations of the order of 50 μ M are required to elicit dramatic reductions in the activity of p70S6K1 (see Figure 1A).

Although PP1 concentrations of 50 μ M are often used to ascribe the function of Src-family kinases to a particular physiological event, there is also the possibility that in addition to inhibition of Src and its relatives, PP1 may directly inhibit other tyrosine kinases at these high concentrations. In fact, *in vitro* PP1 has been shown to inhibit the tyrosine kinase activity of the epidermal growth factor receptor with an IC₅₀ of 250 nM (compared with 170 nM for Src [31]). Whether or not inhibition of p70S6K1 by PP1 involves inhibition of the epidermal growth factor receptor or additional tyrosine kinases is not clear. However, in C-terminal Src kinase-deleted mouse embryo fibroblasts, which exhibit elevated Src kinase activity [32,33], we have observed elevated basal and IGF 1-stimulated p70S6K1 activity compared with embryo fibroblasts from control mouse, consistent with a role for Src to positively regulate p70S6K1 (O. J. Shah, S. R. Kimball and L. S. Jefferson, unpublished work). Further work is needed to clarify precisely the role played by Src-family tyrosine kinases in the activation of S6Ks.

We thank John Blenis, Phillip Hawkins, Julian Downward and Joseph Avruch for reagents. L.S.J. and O.J.S. were supported by National Institutes of Health grants (DK-15658 and T32 GM 08619 respectively).

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Received 30 January 2002/26 April 2002; accepted 15 May 2002

Published as BJ Immediate Publication 15 May 2002, DOI 10.1042/BJ20020198