Evidence That 3-Phosphoinositide-dependent Protein Kinase-1 Mediates Phosphorylation of p70 S6 Kinase in Vivo at Thr-412 as well as Thr-252*

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Protein kinase B and p70 S6 kinase are members of the cyclic AMP-dependent/cyclic GMP-dependent/protein kinase C subfamily of protein kinases and are activated by a phosphatidylinositol 3-kinase-dependent pathway when cells are stimulated with insulin or growth factors. Both of these kinases are activated in cells by phosphorylation of a conserved residue in the kinase domain (Thr-308 of protein kinase B (PKB) and Thr-252 of p70 S6 kinase) and another conserved residue located C-terminal to the kinase domain (Ser-473 of PKB and Thr-412 of p70 S6 kinase). Thr-308 of PKB and Thr-252 of p70 S6 kinase are phosphorylated by 3-phosphoinositide-dependent protein kinase-1 (PDK1) in vitro. Recent work has shown that PDK1 interacts with a region of protein kinase C-related kinase-2, termed the PDK1 interacting fragment (PIF). Interaction with PIF converts PDK1 from a form that phosphorylates PKB at Thr-308 alone to a species capable of phosphorylating Ser-473 as well as Thr-308. This suggests that PDK1 may be the enzyme that phosphorylates both residues in vivo. Here we demonstrate that PDK1 is capable of phosphorylating p70 S6 kinase at Thr-412 in vitro. We study the effect of PIF on the ability of PDK1 to phosphorylate p70 S6 kinase. Surprisingly, we find that PDK1 bound to PIF is no longer able to interact with or phosphorylate p70 S6 kinase in vitro at either Thr-252 or Thr-412. The expression of PIF in cells prevents insulin-like growth factor 1 from inducing the activation of the p70 S6 kinase and its phosphorylation at Thr-412. Overexpression of PDK1 in cells induces the phosphorylation of p70 S6 kinase at Thr-412 in unstimulated cells, and a catalytically inactive mutant of PDK1 prevents the phosphorylation of p70 S6K at Thr-412 in insulin-like growth factor 1-stimulated cells. These observations indicate that PDK1 regulates the activation of p70 S6 kinase and provides evidence that PDK1 mediates the phosphorylation of p70 S6 kinase at Thr-412.

p70 S6 kinase (p70 S6K)1 is activated by insulin and growth factors and mediates the phosphorylation of the 40 S ribosomal protein S6 (1). This enables efficient translation of mRNA molecules containing a polyuridylic tract at their 5′-transcriptional start sites (2). p70 S6K also phosphorylates unknown proteins to permit progression through the G1 phase of the cell cycle (3).

p70 S6K is activated by insulin and growth factors, through a phosphoinositide 3-kinase-dependent pathway and becomes phosphorylated on at least 7 Ser/Thr residues in response to these agonists. The phosphorylation of two of these residues namely Thr-252 and Thr-412 on the longer splice variant of the α-isofrom (Thr-229 and Thr-389 on the shorter splice variant) appears to make the most important contribution to the activation of p70 S6K (4–6). Phosphorylation of Thr-252 alone or mutation of Thr-412 to glutamic acid to mimic phosphorylation of this residue results in a small activation of p70 S6K. In contrast, phosphorylation of both residues or phosphorylation of Thr-252 in the T412E mutant of p70 S6K results in a large activation of expressed p70 S6K, showing that phosphorylation of Thr-252 and Thr-412 leads to a synergistic activation p70 S6K (7, 8).

p70 S6K is a member of the AGC subfamily of protein kinases, which include protein kinase B (PKB) (9), protein kinase C (PKC) isoforms (10), and serum- and glucocorticoid-regulated protein kinase (11). The residues surrounding Thr-252 and Thr-412 of p70 S6K are highly conserved in all AGC family members, and phosphorylation of the residues equivalent to Thr-252 and Thr-412 of p70 S6K is necessary for activation and/or stability of these kinases in vivo (12). Thr-252 is located between subdomains VII and VIII of the kinase domain, a region whose phosphorylation activates many kinases. The residue equivalent to Thr-252 lies in a Thr-Phe-Cys-Gly-Thr-9-Glu-Tyr consensus motif (where the underlined Thr corresponds to Thr-252 and Xaa is a variable residue). Thr-412 is located C-terminal to the catalytic domain, and the residues surrounding Thr-412 lie in a Phe-Xaa-Xaa-Phe-Ser/Thr-Phe/Tyr consensus motif.

We (7) and others (8) have demonstrated that 3-phosphoinositide-dependent protein kinase-1 (PDK1) (13) can phosphorylate p70 S6K at Thr-252 in vitro and in transfection experiments. Phosphorylation of p70 S6K by PDK1 in vitro is independent of the presence of inositol 3, 4, 5 trisphosphate, kinase B; PKC, protein kinase C; PDK1, 3-phosphoinositide-dependent protein kinase-1; PKR3, protein kinase C-related kinase-2; PIF, PDK1 interacting fragment; HA, hemagglutinin; GST, glutathione S-transferase; HPLC, high pressure liquid chromatography; IGF, insulin-like growth factor.
and activation is increased greatly if the noncatalytic C-termini
tail of p70 S6K is deleted and if Thr-412 is mutated to an
acidic residue. Other studies have shown that PDK1 phospho-
ylates the residue equivalent to Thr-252 of p70 S6K in PKB
isoforms (14, 15), PKC isoforms (16–18), serum- and glucocor-
ticoid-activated protein kinase (19, 20), and cAMP-dependent
protein kinase (21).

Recently, we made the surprising observation that PDK1 can
be converted from a form that phosphorylates Thr-308 of PKB
alone (the residue equivalent to Thr-252 in p70 S6K) to a form
that phosphorylates both Thr-308 and Ser-473 (the residue
equivalent to Thr-412 in p70 S6K) through interaction with a
region of protein kinase C-related kinase-2 (PRK2), termed the
PDK1 interacting fragment (PIF) (22). In this study, we sought
to identify the effect that PIF had on the ability of PDK1 to
phosphorylate and activate p70 S6K. Surprisingly, we find that,
in contrast to PKB, PDK1 complexed to PIF can no longer
phosphorylate p70 S6K in vitro. We also report that expression
of PIF or a catalytically inactive mutant of PDK1 in cells
prevents the activation of p70 S6K and its phosphorylation at
Thr-412 as well as Thr-252, suggesting that PDK1 may medi-
ate the phosphorylation of both residues in vivo.

**EXPERIMENTAL PROCEDURES**

**Materials**—The peptides used to assay PKBs (RPRAATP) (23) and
p70 S6K (GRPRTPSFAEG) (24) and the peptides used to raise and to
purify the T412-P antibody were synthesized by Dr. G. Blomberg (Uni-
versity of Bristol, United Kingdom). Protein G-Sepharose, glutathione-
Sepharose, and CH-Sepharose were purchased from Amersham Phar-
macia Biotech; prestatic inhibitor tablets were from Roche Molecular
Biochemicals; tissue culture reagents, IGF1, and microcin-LR were
from Life Technologies, Inc.; sensorsChips CM5 and SA were from Bia-
Core AB; biotinylated recombinant and secondary antibodies coupled to
horse radish peroxidase were from Pierce.

**Antibodies**—The phospho-specific antibody recognizing p70 S6K
phosphorylated at Thr-412 was raised in sheep against the peptide
SESANQVFLGFTYVAPSV (corresponding to residues 401–418 of the
region of protein kinase C-related kinase-2 (PRK2), termed the
PDK1 binding site on PIF, was unable to phosphorylate
nonphosphorylated peptide, and the antibodies that did not bind to
this column were selected. Monoclonal antibodies recognizing the HA
or Myc epitope were purchased from Roche Molecular Biochemicals; the
monoclonal antibody recognizing GST was purchased from Sigma and
used to verify the level of expression of GST-PIF in cells, whereas rabbit
polyclonal antibodies recognizing the 18 C-terminal residues of PRK2/
PIF were purchased from Santa Cruz Biotechnology.

**Preparation of Insect Cell His-p70 S6K—**p70 S6K with a His epitope
tag at its N terminus lacking the C-terminal 104 residues is termed p70
S6K-T2. To prepare wild type and the mutant 412E-p70 S6K-T2 the
cDNA for these constructs was amplified by polymerase chain reaction
from the pMT2 vector encoding these forms of p70 S6K (6) using the
following oligonucleotides: 5′-TGC ACC CAC CAT GCA CCA TCA TCA
TCA TAT GAG GGC AGC AAG GAG GGC G-3′ and 5′-GCG GCC
GCT CAA CTT TCA AGT ACA GAT GGA GCC-3′. The polymerase
chain reaction products were then subcloned into the BamHI/NotI sites of
the pFASTBAC 1 vector, and this vector was used to generate recombinant baculoviruses using the Bac-to-Bac system (Life Technolo-
gies, Inc.). The resulting viruses encoded p70 S6K-T2 or 412E-p70 S6K-T2 and
an N-terminal hexahistidine tag and was used to infect SF21 cells (1.5 × 10^6/ml) at a multiplicity of infection of 5. The infected cells were harvested 72 h post-infection, and the His-p70 S6K proteins purified by Ni^2+/-imidicarboxylic acid-agarose chromatography as described previously for PKBβ (25). They were then dialyzed against
50 mM Tris/HisCl, pH 7.5, 0.1 mM EGTA, 0.27 M sucrose, 0.03% (by
volume) Brij-35, 0.1% (by volume) 2-mercaptoethanol, 1 mM benzamid-
ine, and 0.2 mM 1,10-phenanthroline, snap frozen in aliquots,
and stored at −80 °C. p70 S6K-T2 or 412E-p70 S6K-T2 were both
recovered with a yield of 60 mg/liter infected SF21 cells and were >90%
homogeneous as judged by polyacrylamide gel electrophoresis followed
by Coomassie Blue staining.

**Phosphorylation of GST-p70 S6K by PDK1—**GST-PIF and GST-
D975A-PIF were expressed in human embryonic kidney 293 cells and
puriﬁed on glutathione-Sepharose, and the very small amount of en-
dogenous PDK1 associated with GST-PIF was removed by immuno-
precipitation with a PDK1 antibody (22). Phosphorylation of GST-p70
S6K-T2 by PDK1 was carried out as described previously (7) except that
PDK1 was incubated with the indicated concentration of GST-PIF or PIF
peptide for 10 min on ice prior to initiation of the assay with
magnesium [γ-^32P]ATP. The wild type GST-p70 S6K-T2, and the mu-
ant T252A-GST-p70 S6K-T2, T412A-GST-p70 S6K-T2 proteins were
expressed in 293 cells and puriﬁed as described previously (7). Wild
type and catalytically inactive GST-PDK1 were expressed either in 293
cells (26) or in Escherichia coli (27).

**Surface Plasmon Resonance Measurements of PDK1 Binding to p70 S6 Kinase**—Cell lysates were made with 1% SDS, and the indicated
amounts of protein were subjected to SDS-polyacrylamide gel elec-
trophoresis, subsequently transferred to nitrocellulose, and immuno-
blobbed using the indicated monoclonal antibody or the T412-P phospho-
speciﬁc antibody (0.4 μg/ml in 50 mM Tris/HisCl, pH 7.5, 0.15 mM
NaCl, 0.5% (by volume) Tween, and 10% (by mass) skimmed milk. Detection
was performed using the enhanced chemiluminescence reagent (Amer-
sham Pharmacia Biotech).

**Surface Plasmon Resonance Measurements of PDK1 Binding to p70 S6 Kinase—**p70 S6K-T2 and T412E p70 S6K-T2 mutant were amine-
coupled to a CM5 sensor chip (BiaCore AB) according to the manufac-
turer’s instructions. The indicated concentrations of His-PDK1 were
injected over the chip at a flow rate of 30 μl/min, and the steady-state
binding was determined in the presence or absence of PIF peptide.
The apparent equilibrium dissociation constant (Kd) for the binding of His-
PDK1 to p70 S6 kinase was determined by fitting the increase in
steady-state binding upon increasing PDK1 concentration to a rectan-
gular hyperbola using SigmaPlot 4 (SPSS Inc.). The measure of re-
sponse in our experiments is termed RU; 1000 RU = 1 ng/mmol of
protein bound to the surface.

**RESULTS**

**Phosphorylation of p70 S6K by PDK1 Is Inhibited by PIF—**PDK1
binds with submicromolar affinity to a region of PRK2 termed PIF (22). PIF is situated C-terminal to the kinase
region of PRK2, and the binding of this region of PRK2 to
PDK1 is mediated by a consensus motif similar to that encom-
passing Thr-412 of p70 S6K, except that the residue at this
position is Asp (Asp-978), rather than Thr or Ser. In Fig. 1, we
demonstrate that PDK1, when complexed to either GST-PIF or
a 24 residue synthetic peptide whose sequence encompasses the
PDK1 binding site on PIF, was phosphorylate
PIF Inhibits IGF1-induced Activation of p70 S6K—To determine whether expression of PIF in cells could prevent the endogenous PDK1 from phosphorylating and activating p70 S6K, HA-tagged full-length p70 S6K (HA-p70 S6K) was transfected into 293 cells together with constructs encoding either GST-PIF, a mutant form of GST-PIF that interacts with PDK1 (GST-F977A-PIF), or GST itself. The wild type or mutant GST-PIF and GST itself were all expressed at a similar level and were present at a much higher concentration than the endogenous PDK1 or PRK2 (data not shown). The cells were subsequently stimulated with IGF1 for 40 min (the time at which HA-p70 S6K is maximally activated) (data not shown); the cells were lysed, and the HA-p70 S6K was immunoprecipitated and assayed. Cells expressing HA-p70 S6K and GST exhibited a readily measurable basal p70 S6K activity in unstimulated cells, which was increased 10-fold in response to IGF1 (Fig. 3A). In contrast, cells expressing HA-p70 S6K and GST-PIF possessed a basal HA-p70 S6K activity that was virtually undetectable, and IGF1-stimulation caused only a very slight increase in the HA p70 S6K activity (Fig. 3A). In cells expressing HA-p70 S6K and GST-F977A-PIF, HA-p70 S6K was substantially activated by IGF1, although not to the same extent as in cells expressing HA-p70 S6K and GST (Fig. 3A). This is probably explained by a weak interaction of GST-F977A-PIF with PDK1.

PIF Inhibits IGF1-induced Phosphorylation of p70 S6K at Thr-412—As PIF inhibited p70 S6K activation in cells, we sought to determine the effect of PIF expression on the phosphorylation of p70 S6K at Thr-412 and Thr-252. We used the T412-P antibody to measure the phosphorylation of p70 S6K at Thr-412. These experiments showed that IGF1 triggered the phosphorylation of Thr-412 (Fig. 3A). This was abolished by incubation of the T412-P antibody with the phosphorylated

GST-p70 S6K-T2 (a deletion mutant of p70 S6K that lacks the C-terminal 104 residues) in vitro. In a parallel experiment it was verified that PDK1 complexed to GST-PIF or the PIF peptide was able to phosphorylate PKB at both Thr-308 and Ser-473 to near stoichiometric levels (data not shown) as reported previously (22). GST-p70 S6K-T2 was used as a PDK1 substrate (Fig. 1) rather than the full-length p70 S6K, which is very poorly phosphorylated by PDK1 in vitro (7, 8). Truncation of the C-terminal 104 residues of p70 S6K is likely to be benign, as p70 S6K-T2-2 when expressed in cells possesses indistinguishable properties to the full-length protein as it is still activated by insulin and growth factors in a rapamycin- and wortmannin-sensitive manner (5, 6).

A mutant form of GST-PIF or the 24-residue PIF peptide in which the amino acid equivalent to Asp-978 in PRK2 is mutated to Ala (GST-D978A-PIF) possesses markedly reduced affinity for PDK1 (22). Consistent with this, GST-D978A-PIF or the mutant D978A-PIF peptide poorly inhibited the phosphorylation of GST-p70 S6K-T2 by PDK1 (Fig. 1). If Asp-978 in the PIF peptide is mutated to a phosphoserine residue instead of an Ala, to restore the negative charge, the resulting peptide interacted with PDK1 with the same affinity as the wild type PIF peptide (22) and prevented PDK1 from phosphorylating GST-p70 S6K-T2 (Fig. 1).

PIF Inhibits IGF1-induced Phosphorylation of p70 S6K at Thr-412—As PIF inhibited p70 S6K activation in cells, we sought to determine the effect of PIF expression on the phosphorylation of p70 S6K at Thr-412 and Thr-252. We used the T412-P antibody to measure the phosphorylation of p70 S6K at Thr-412. These experiments showed that IGF1 triggered the phosphorylation of Thr-412 (Fig. 3A). This was abolished by incubation of the T412-P antibody with the phosphorylated...
PIF peptides (4 μM) were added to cells, then stimulated for 40 min with 100 nM IGF1. The cells were lysed and assayed for p70 S6K activity. 24 h posttransfection the cells were serum-starved for 16 h and then stimulated for 40 min with 100 nM IGF1. The cells were lysed and either GST or GST-F977A-PIF was added. Previous work has shown that PIF does not inhibit the activation of PKB or its phosphorylation at Ser-473—

![Figure 2](image2.png)

**FIG. 2.** PDK1 phosphorylates p70 S6K at Thr-412 in vitro and this is inhibited by PIF. 0.5 μg of wild type GST-p70 S6K-T2 (wt) or T252A-GST-p70 S6K-T2 (252A), or T412A-GST-p70 S6K-T2 (412A) were incubated for 90 min at 30 °C with magnesium ATP in the presence or absence of wild type (wt) or kinase-dead (kd) GST-PDK1 expressed in either 293 cells or bacteria in the presence (+) or absence (−) of the wild type PIF peptides (4 μM) in a final volume of 20 μl. The reactions were terminated by making the solutions 1% in SDS; the samples were subjected to SDS-polyacrylamide gel electrophoresis, and the phosphorylation of p70 S6K at Thr-412 was assayed by immunoblotting with the T-412P phospho-specific antibodies recognizing p70 S6K phosphorylated at Thr-412 and this is inhibited by PIF. Similar results were obtained in three separate experiments.

![Figure 3](image3.png)

**FIG. 3.** PIF inhibits p70 S6K activation and phosphorylation at Thr-252 and Thr-412. 293 cells were co-transfected with constructs expressing the wild type (wt) full-length HA-p70 S6K (A) or the full-length HA-T412E p70 S6K (B) with either GST-PDF, GST-F977A-PDF, or GST. 24 h posttransfection the cells were serum-starved for 16 h and then stimulated for 40 min with 100 nM IGF1. The cells were lysed and HA-p70 S6K was immunoprecipitated and assayed as described under “Experimental Procedures.” Protein from each lysate (10 μg for the HA blots or 20 μg for the T412-P blot) was electrophoresed on a 10% SDS/polyacrylamide gel and immunoblotted using HA-antibody or the T412-P antibody. The T412-P antibody was incubated with either the synthetic peptide (10 μg/ml) corresponding to residues 401 to 418 of p70 S6K phosphorylated at Thr-412 (phospho-T412 peptide) or the unphosphorylated peptide (dephospho-T412 peptide). The T412-P antibody consistently recognizes a protein termed “nonspecific band” in cell lysates, which migrates at (75 kDa) derived from nontransfected and transfected cells. The intensity of this band does not change with IGF1. It is not co-immunoprecipitated with HA-p70 S6K (data not shown). The HA-p70 S6K activities shown are the average ± S.E. for a single experiment carried out in triplicate. Similar result were obtained in eight separate experiments (A) and two experiments (B).

Thr-412 peptide immunogen used to raise the antibody but not with the dephosphorylated peptide (Fig. 3A) or a phosphopeptide corresponding to the sequence surrounding Thr-252 (data not shown). Furthermore, a mutant form of HA-p70 S6K in which Thr-412 was changed to an Ala was not recognized by the T412-P antibody following IGF1 stimulation (Fig. 5C).

When HA-p70 S6K was coexpressed in cells with GST-PDF, IGF1 failed to induce the phosphorylation of HA-p70 S6K at Thr-412 (Fig. 3A). In contrast, in cells expressing HA-p70 S6K and the mutant GST-F977A-PDF, the phosphorylation of HA-p70 S6K still occurred but at a lower level than that observed in cells expressing HA-p70 S6K and GST. The decrease in Thr-412 phosphorylation is consistent with the reduced activation of HA-p70 S6K in these cells compared with those expressing GST alone (Fig. 3A). It should be noted, however, that cotransfection of HA p70 S6K with the GST-F977A-PDF mutant induced a 50% maximal activation of P70 S6K, despite inducing a significantly greater reduction in the level of phosphorylation of Thr-412 (Fig. 3A). This finding demonstrates that the relationship between p70 phosphorylation at Thr-412 and level of p70 S6K activity does not appear to be linear. One explanation for this observation is that the F977A-PDF mutant may inhibit more potently p70 S6K phosphorylation at Thr-412 than Thr-252; however, thus far we have not been able to raise phospho-specific antibodies recognizing p70 S6K phosphorylated at Thr-252 to explore this possibility. The overexpression of GST-PDF in cells also abolished the IGF1-induced activation and phosphorylation at Thr-412 of the p70 S6K-T2 mutant, which lacks the C-terminal 104 residues (data not shown).

**PIF Inhibits IGF1-induced Phosphorylation of p70 S6K at Thr-252**—A mutant form of HA-p70 S6K in which Thr-412 was altered to glutamic acid to mimic the presence of a phosphorylated residue at this position possessed an elevated basal activity that was further activated by IGF1 when co-expressed with GST or the mutant GST-F977A-PDF (Fig. 3B). Previous work has established that the basal and IGF1-stimulated activity of this mutant are mediated through phosphorylation of Thr-252 (6). In Fig. 3B, we demonstrate that co-expression of HA-412E p70 S6K with PIF greatly reduced the basal activity of this mutant and largely prevented its activation by IGF1. This suggests that PIF also inhibits the phosphorylation of p70 S6K at Thr-252. The overexpression of PIF in cells also greatly reduced the basal and IGF1-stimulated activity of T412E p70 S6K-T2 mutant in cells (data not shown).

**PIF Does Not Inhibit the Activation of PKB or Its Phosphorylation at Ser-473**—Previous work has shown that PIF does not prevent PDK1 from phosphorylating PKB in the presence of 3-phosphoinositide lipids but instead enables PDK1 to phosphorylate PKB at both Thr-308 and Ser-473 (see the introduction). Here we show that in marked contrast to the effect of GST-PDF on p70 S6K activation, expression of GST-PDF in 293 cells does not prevent IGF1 from inducing a ~20-fold activation of HA-PKB. This activation is similar to that observed when HA-PKBα is coexpressed with GST (Fig. 4). Expression of GST-PDF did not inhibit or potentiate the IGF1-induced phosphorylation of HA-PKBα at Ser-473 (the residue equivalent to Thr-412 in p70 S6K) (Fig. 4). GST-PDF is expressed at a similar level when cotransfected with PKB and HA-p70 S6K (data not shown), indicating that the inability of PIF to affect the activation of PKB in cells is not because of it being expressed at a low level.

**A Catalytically Inactive Mutant of PDK1 Prevents the Activation and Phosphorylation of p70 S6K**—Consistent with earlier findings (7, 8), co-expression of HA-p70 S6K with wild type PDK1 induced a large activation of HA p70 S6K, which was not
PIF Prevents the Interaction of PDK1 with p70 S6 Kinase—A recent study by Blenis and colleagues (29) reported that, when PDK1 and p70 S6K were cotransfected into cells, a small amount of PDK1 was immunoprecipitated with p70 S6K, suggesting that these proteins may interact directly. Using surface plasmon resonance measurements, we were able to detect a significant interaction (apparent $K_d$ 8 $\mu M$) between PDK1 and p412E-p70 S6K-T2 (Fig. 6). This interaction was abolished in the presence of the 24-residue wild type PIF peptide but not the D978A mutant of the PIF peptide (Fig. 6), suggesting that both the PIF and 412E-p70 S6K-T2 mutant compete for the same binding site on PDK1. In parallel experiments, a significantly weaker interaction between PDK1 and wild type p70 S6K-T2 kinase was detected (Fig. 6).

**DISCUSSION**

Recent work has shown a high affinity-interaction between PIF and the kinase domain of PDK1, which enhances the rate at which PDK1 phosphorylates PKB$\alpha$ and allows it to phosphorylate Ser-473 as well as Thr-308. In this study we have made the surprising observation that PIF prevents PDK1 from phosphorylating p70 S6K (Figs. 1 and 2), and expression of PIF in 293 cells prevents the IGF1-induced activation of p70 S6K (Fig. 3) without affecting the activation of PKB$\alpha$ (Fig. 4). Mutant forms of PIF, which interact weakly with PDK1, were much less effective at inhibiting the phosphorylation of p70 S6K by PDK1 in vitro or at inhibiting the IGF1-induced activation of the p70 S6K. These observations could be explained if p70 S6K, but not PKB$\alpha$, needed to interact with PDK1 at a site that overlaps with the PIF binding site before p70 S6K can become phosphorylated by PDK1. This conclusion is supported by the findings in Fig. 6 that p70 S6K does interact with PDK1, and this interaction is abolished in the presence of the PIF peptide. The finding that the T412E mutant form of p70 S6K interacts with PDK1 with higher affinity than the wild type enzyme may...
Peptide PIF prevents IGF1-induced phosphorylation of p70 S6K in cells. These observations were interpreted as indicating that PKCα/PKCζ may have a role in activating p70 S6K in cells. However, PKCα and PKCζ are both AGC kinase family members, which are likely to be activated by PDK1 in vivo, and possess an acidic residue rather than Ser/Thr in their PKD2 consensus motif. Furthermore, PKCζ, like PIF has been shown to interact directly with the kinase domain of PKD1 (16, 18). It is therefore possible that both PKCα and PKCζ interact with PDK1 in the same way as PIF and so prevent PDK1 from inducing the activation of p70 S6K. Recent work also implicated PKCζ in mediating a rapamycin-sensitive phosphorylation of the novel PKC isofrom (PKCδ) at the residue equivalent to Thr-412 of p70 S6K (31). This study did not, however, rule out the possibility that PDK1 complexed to PKCζ acquires the ability to phosphorylate PKCδ at this residue, rather than PKCζ itself directly phosphorylating this residue. To complicate matters further, it has also recently been shown that conventional PKCα is capable of auto-phosphorylation itself at the residue equivalent to Thr-412 of p70 S6K (18, reviewed in Ref. 33). Sabatini and colleagues (34, 35) have reported that mTOR phosphorylates p70 S6K directly at Thr-412. However, much recent evidence suggests that the ability of rapamycin, an inhibitor of the mTOR kinase, to suppress the activity of p70 S6K is mediated primarily through the rapamycin-induced activation of an mTOR-regulated protein phosphatase, which dephosphorylates p70 S6K (32, 36, 37). It will be important to establish whether mTOR or any other insulin-stimulated kinase, which can phosphorylate p70 S6K at Thr-412, is inhibited by PIF.

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PDK1 Regulates p70 S6K Phosphorylation at Thr-412

Fig. 6. Quantitative analysis of the binding of PDK1 to p70 S6K. Surface plasmon resonance measurements were carried out on a BiaCore instrument as described under “Experimental Procedures.” His-PDK1 was injected at the indicated concentrations over (A) 2000 RU of p70 S6K-T2 (closed squares) or 412E-p70 S6K-T2 (closed circles), which was immobilized by amine coupling to a CM5 Sensorchip. Experiments carried out in the presence of either 10 μM wild type PIF peptide (octagons) or 10 μM D978A mutant PIF peptide (triangles) are indicated. The responses at steady state binding were recorded. All data are single determinations from a representative experiment that was repeated at least three times with similar results. The data on the binding of wild type p70 S6K to PDK1 at concentrations above 2 μM are not shown, as our analysis of the data suggested that nonspecific protein binding was contributing to part of the observed binding response under these conditions.

also explain why the T412E mutant of p70 S6K was observed in previous studies to be a better substrate for PDK1 than the wild type or T412A mutant of p70 S6K (7, 8). Phosphorylation of PBKα by PDK1 is not inhibited by the presence of PIF, and we could not detect any significant interaction between PBKα and PDK1 in vitro by surface plasmon resonance (data not shown). As PBKα and PDK1 both interact with 3-phosphoinositides through their pleckstrin homology domains, it is possible that this is the primary determinant for co-localizing these molecules at the plasma membrane and hence allowing PDK1 to phosphorylate PBKα. In contrast, substrates for PDK1 such as p70 S6K, which do not interact with 3-phosphoinositides, may actually need to interact with PDK1 with relatively high affinity before they can become phosphorylated. Previous evidence that PDK1 is an activator of p70 S6K rested largely on the demonstration that PDK1 phosphorylates and activates p70 S6K in vitro and in cotransfection experiments. The finding in this study that expression of PIF can prevent the activation of p70 S6K in vivo, presumably by binding to PDK1, provides further evidence that PDK1 is required for the activation of p70 S6K in cells.

Interaction with PIF converts PDK1 into a kinase that is capable of phosphorylating both Thr-308 and Ser-473 sites of PKB. This demonstrates that PDK1 has the intrinsic ability to phosphorylate the residue in the T-loop as well as the PKD2 motif of at least one AGC kinase family member. As the residues surrounding Thr-412 of p70 S6K are highly homologous to those surrounding Ser-473 of PKB, it could be argued that PDK1, perhaps in complex with another protein(s), would also possess the intrinsic ability to phosphorylate p70 S6K at Thr-252 and Thr-412. The present study supports this hypothesis because first, overexpression of wild type PDK1 triggers the phosphorylation of p70 S6K at Thr-412 (Fig. 5A). Second, expression with PIF prevents the IGF1-induced phosphorylation of p70 S6K at Thr-412 in cells (Fig. 3A). Third, the PDK1-catalyzed phosphorylation of p70 S6K at Thr-412 in vitro was prevented by PIF (Fig. 2). Finally, the overexpression of a kinase-dead mutant of PDK1 in cells not only prevented the activation of p70 S6K, as reported by others (8), but also prevented the phosphorylation of p70 S6K at Thr-412 (Fig. 5). Taken together, the data suggest that PDK1 could phosphorylate p70 S6K at Thr-412 in vivo. As PDK1 phosphorylation of Thr-412 of p70 S6K in vitro is not dependent upon 3-phosphoinositides, it is possible that the sensitivity of PDK1 to these lipids in cells is conferred by the interaction of PDK1 with another protein. In this respect it should be recalled that the interaction of PDK1 with PIF enables PDK1 to be directly activated by 3-phosphoinositides (22). It is also possible that a PDK1-interacting protein(s) could increase the rate at which PDK1 phosphorylates both Thr-252 and Thr-412 of p70 S6K.
