Phosphatidylinositol 3-kinase signals activation of p70 S6 kinase in situ through site-specific p70 phosphorylation

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ABSTRACT The p70 S6 kinase is activated by insulin and mitogens through multisite phosphorylation of the enzyme. One set of activating phosphorylations occurs in a putative autoinhibitory domain in the noncatalytic carboxyl-terminal tail. Deletion of this tail yields a variant (p70ACT104) that nevertheless continues to be mitogen regulated. Coexpression with a recombinant constitutively active phosphatidylinositol (PI) 3-kinase (EC 2.7.1.137) gives substantial activation of both full-length p70 and p70ACT104 but not Rsk. Activation of p70ACT104 by PI 3-kinase and inhibition by wortmannin are each accompanied by parallel and selective changes in the phosphorylation of p70 Thr-252. A Thr or Ser at this site, in subdomain VIII of the catalytic domain just amino-terminal to the APE motif, is necessary for p70 40S kinase activity. The inactive ATP-binding site mutant K123M p70ACT104 undergoes phosphorylation of Thr-252 in situ but does not undergo direct phosphorylation by the active PI 3-kinase in vitro. PI 3-kinase provides a signal necessary for the mitogen activation of the p70 S6 kinase, which directs the site-specific phosphorylation of Thr-252 in the p70 catalytic domain, through a distinctive signal transduction pathway.

The advent of selective inhibitors of phosphatidylinositol (PI) 3-kinase (EC 2.7.1.137) (1) has begun to clarify the role of this enzyme in cell signaling. Wortmannin (2) and/or the structurally unrelated LY294002 (3) inhibits mitogenesis (2, 4), receptor-activated secretory (2) and transport responses—e.g., glucose transport and GLUT4 translocation (3, 5)—and receptor regulation of intracellular enzymes—e.g., insulin regulation of adipocyte lipolysis (5) and activation of p70 S6 kinase (4, 6). As to the biochemical mechanisms by which PI 3-kinase participates in these responses, the ability of PI 3-kinase to regulate membrane dynamics is best characterized in yeast, where VPS34, the major PI 3-kinase in Saccharomyces cerevisiae, is an essential protein required for the sorting of polypeptides to the regulated or constitutive secretory pathway (7). The immediate downstream target and effector pathway for PI 3-kinase activation in this system remains obscure.

The p70 S6 kinase is a ubiquitous mitogen-activated Ser/Thr kinase (8) that is necessary for cells to enter S phase after mitogen stimulation (9, 10). p70 and its closest homologs, the Rsk enzymes, are coordinately activated within minutes after mitogen addition through Ser/Thr phosphorylation of the enzyme (9). Abundant evidence indicates that the RskS are activated in a Ras-dependent way (11), entirely as a result of their phosphorylation by the mitogen-activated protein kinases, erk1 and erk2 (12–14). In contrast, p70 requires multiple independent inputs for activation. One input is directed at a pseudosubstrate autoinhibitory (SKAIPS) domain in the p70 noncatalytic carboxyl-terminal tail (15, 16). Concomitant with activation in situ, this segment undergoes Ser/Thr phosphorylation at multiple sites (17), catalyzed by an array of proline-directed protein kinases (18). Nevertheless, phosphorylation of these sites in vitro by such proline-directed kinases does not restore the activity of phosphatase 2A-deactivated p70 S6 kinase (18), suggesting the need for additional activating inputs. Direct evidence for such inputs is the observation that truncation of the entire carboxyl-terminal tail, including the SKAIPS domain, yields a recombinant p70 (called p70ACT104) that, when stably expressed in NIH 3T3 cells, continues to exhibit a low basal activity and is activated by serum to an extent comparable to the full-length p70 endogenous to these cells (19). Wortmannin inhibits the activity of both the p70ACT104 variant and the endogenous full-length p70 kinase (19), but it does not inhibit endogenous mitogen-activated protein kinases or Rsk activity in NIH 3T3 cells (K.A., Q.-P.W., M.T.K., and J.A., unpublished results).

The ability of wortmannin to inhibit the insulin/signal activation of both PI 3-kinase and p70 S6 kinase selectively and in parallel suggests that the increase in PI 3-kinase activity elicited by mitogen may provide an input necessary for the increase in p70 activity. If this is true, then increasing cellular PI 3-kinase activity independently of serum or insulin might also increase p70 S6 kinase activity. In this report, we show that coexpression with a recombinant constitutively active PI 3-kinase results in activation of p70 and p70ACT104 but not Rsk. PI 3-kinase-induced p70 activation in situ is accompanied by a selective increase in the phosphorylation of p70 Thr-252; reciprocally, wortmannin results in the selective dephosphorylation of p70 Thr-252 concomitant with inhibition of p70 activity in situ. A Ser or Thr at p70 position 252, located in catalytic subdomain VIII, is required for p70 activity in situ. On the basis of this evidence, we conclude that PI 3-kinase directs the activation of p70 in situ, and this activation results from the increase in phosphorylation of p70 Thr-252. In turn the ability of wortmannin to inhibit p70 in situ is attributable to the wortmannin inhibition of PI 3-kinase-directed phosphorylation of p70 Thr-252.

MATERIALS AND METHODS

The p70 cDNA constructions all employed the rat α1 p70 (20) with an influenza virus hemagglutinin (HA) epitope (YPY-DVDPYA) inserted after the initiator methionine, in the vector pMT2. The p70ACT104 variant contains a stop codon after residue 421. The S40A, K123M, T252A, T252D, T252E, T252S, T256A, T256D, T256E, and T256S mutations were generated by the Kunkel method (21). The HA-tagged rat Rsk cDNA was described in ref. 14. All mutants were verified by DNA sequence analysis. Myc-tagged constitutively active PI 3-kinase, p110+, and the catalytically inactive version of p110+ were described previously (22, 23). DNA transfections were

Abbreviations: PI, phosphatidylinositol; HA, hemagglutinin.
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carried out by calcium phosphate coprecipitation using 10 μg each of the cDNAs encoding p70 and PI 3-kinase. After extraction and centrifugation, aliquots matched for amount of extract protein were used for immunoprecipitation.

PI 3-kinase was assayed as in ref. 24; the products were separated by thin-layer chromatography and 32P incorporation into PI 3-phosphate was determined. Assay of 40S kinase was as in ref. 25; the 32P-labeled S6 product is shown. 32P labeling during transient expression was carried out by replacing the medium 24–28 hr after transfection with phosphate-free Dulbecco's modified Eagle's medium (DMEM) containing 10% dialyzed serum and 32P, at 0.15 mCi/ml (1 mCi = 37 MBq). At harvest, usually 24 hr later, 32P-labeled cells were rinsed in ice-cold phosphate-buffered (pH 7.4) 0.9 M NaCl and extracted, and aliquots containing equal amounts of protein were subjected to immunoprecipitation. The HA-tagged S6 kinases were immunoprecipitated with the anti-HA monoclonal antibody 12CA5 (26); the Myc-tagged p110 polypeptides were selectively immunoprecipitated with the anti-Myc monoclonal antibody 9B7.3 (27). The polyclonal anti-p110 antibody used reacts similarly with the active and inactive p110* polypeptides as well as with p110 polypeptides endogenous to 293 cells. Anti-phosphotyrosine immunoprecipitation employed the monoclonal antibody 4G10.

To generate polypeptide maps of recombinant 32P-labeled p70 polypeptides, the 12CA5 immunoprecipitates were separated by SDS/PAGE; 32P-labeled p70 was visualized by autoradiography, extracted from the wet gel, precipitated in chloroform/methanol, and digested sequentially with trypsin and chymotrypsin. The digests were dried and redissolved in water, and aliquots containing equal amounts of 32P were subjected to electrophoresis on cellulose-coated plates at pH 1.9 followed by chromatography.

Wortmannin was obtained from Sigma; radioisotopes, from New England Nuclear.

RESULTS AND DISCUSSION

The PI 3-kinase consists of a p85 regulatory subunit (28–30) and a p110 catalytic subunit (31). When expressed in COS-7 cells, the p110 subunit is active only when bound to the p85 subunit (31). The regions on p85 and p110 that mediate the interaction between these subunits have been mapped to a segment of the p85 polypeptide (<103 amino acids) located between the two SH2 domains (inter-SH2 region, refs. 32 and 33) and the amino-terminal 124 amino acids of the p110 subunit (22, 24, 33). Coexpression of intact p110 with an inter-SH2 fragment of p85 in COS cells is sufficient to reconstitute PI 3-kinase specific activity to approximately 50% of that recovered on coexpression of p110 with intact p85 (22). A constitutively active form of PI 3-kinase was constructed by fusion of the p85 inter-SH2 sequences directly to the amino terminus of the p110 subunit (23). Transfection of this fusion construct (p110*) into 293 cells increases total cellular PI 3-kinase activity 20- to 30-fold and modestly increases the PI 3-kinase activity recovered in anti-phosphotyrosine immunoprecipitates (Fig. 1A). A p110* polypeptide that contains a small deletion in the p110 ATP-binding site is expressed equally well, but it is devoid of PI 3-kinase activity and does not alter cellular PI 3-kinase activity on transfection (Fig. 1A); this inactive p110* construct served as a control.

Coexpression of active p110* with the full-length p70 S6 kinase or p70ΔCT104 results in an increase of the 40S kinase activity of recombinant p70 variants, whereas the activity of cotransfected Rsk is unchanged (Fig. 1B). The activity of full-length p70 kinase is increased 2- to 3-fold in serum-deprived cells by coexpression with active p110*, whereas the activity of the p70ΔCT104 variant is increased approximately 6-fold (Fig. 1B and C). The full-length p70 and p70ΔCT104, when expressed alone (19) or with the inactive p110* mutant (Fig. 1B), each exhibit a 2- to 3-fold increase in 40S kinase activity on readdition of serum to deprived 293 cells. Coexpression with active p110* alters the p70 response to serum. The recombinant full-length p70 exhibits a somewhat diminished although still significant (approximately 1.5-fold) stimulation by serum, whereas the p70ΔCT104 variant coexpressed with p110* consistently fails to show further stimulation by serum beyond the activity found in serum-deprived cells (Fig. 1B). This response is consistent with the idea that PI 3-kinase provides an activating input sufficient to fully replace the serum-generated signal required for activation of p70ΔCT104, whereas the full-length p70 requires, in addition to PI-3 kinase, a second serum-generated input to achieve full activation. This second input presumably leads to the phosphorylation of and disinhibition from the SKAIPS domain in the carboxyterminal tail. It appears that both of these two serum-generated signals, acting concurrently, are absolutely required for activation of the full-length p70, whereas only the PI 3-kinase input is required for full activation of p70ΔCT104.

The activation of endogenous p70 S6 kinase in situ by mitogens is accompanied by multiple Ser/Thr phosphorylation of the p70 polypeptide, and dephosphorylation of the active p70 kinase in vitro with phosphatase 2A deactivates the enzyme completely (25). Several of the major phosphorylation sites are located in the carboxy-terminal tail (15-18), whereas the Ser-40 is also phosphorylated in situ (26). The recombinant S40A p70ΔCT104 polypeptide nevertheless exhibits a 40S kinase activity in 293 cells that is stimulated by coexpression with recombinant p110* (Fig. 1C) and is associated with a 1.2-fold increase in overall 32P content of the S40A p70ΔCT104 polypeptide (Fig. 1C). This response suggests that the PI 3-kinase-sensitive component of p70ΔCT104 phosphorylation occurs on a subset of sites, among which are one or more whose phosphorylation is required for p70 kinase activity; 32P-labeled polypeptide mapping was therefore carried out to determine whether p70 activation and inhibition are accompanied by site-specific changes in phosphorylation. The p70 full-length and p70ΔCT104 polypeptides were 32P-labeled in situ during transient expression in 293 cells (Figs. 2B and 3B) and subjected to two-dimensional peptide mapping. Deletion of the p70 carboxy-terminal tail results in the loss of four major 32P-labeled peptides (spots 1-4 in Fig. 2A; see Fig. 2B), corresponding to sites in the SKAIPS domain (residues Ser-434, Ser-441, Ser-447, and Thr-444) as previously identified (15-18). Confining further analysis to the p70ΔCT104 polypeptide, 32P-labeled peptides were named from cathode to anode; spots a and a’, spots b and b’, the c cluster, and spot d were always evident. Spot e was faint in 293 cells (Figs. 2 and 3) but prominently labeled in the stably transformed NIH 3T3 cells (19). The spots a/a’ and b/b’, the c cluster, and spot d are evident in 32P-labeled peptide maps of the ATP site mutant, kinase-inactive K123M variants of full-length p70 and p70ΔCT104, 32P-labeled in 293 cells (Fig. 2B); thus phosphorylation of these sites in vivo is catalyzed by other protein kinases rather than by p70 itself. The 32P spot of p70/C' is generated entirely in Ser-40 (compare Fig. 2B with Fig. 2C), a classical kinase 2 site (SEDELEE); Ser-40 can be converted to Ala without any loss in the responsiveness of p70 to activation by serum (19) or PI 3-kinase (compare Fig. 1B with Fig. 1C).

To evaluate the changes in p70 site-specific phosphorylation in relation to the state of activation, the S40A variant of p70ΔCT104 was 32P-labeled during transient expression in 293 cells, exploiting the ability of coexpressed active p110* PI 3-kinase to strongly activate p70ΔCT104. Control transfections employed the inactive p110* variant, alone and with wortmannin treatment to inhibit endogenous PI-3-kinase. This provided a wide range of S6 kinase activity despite similar p70 polypeptide recoveries (Fig. 1C). Overall 32P incorporation into S40A p70ΔCT104 was unaffected by wortmannin but was increased slightly (1.2-fold) by active p110* (Fig. 1C). The 32P-labeled...
The 293 cells were mock transfected (left bars) or transfected with cDNAs encoding either a catalytically active (right bars) or an inactive (center bars) version of the Myc-tagged PI 3-kinase fusion protein derived from sequences of the p85 and p110 subunits. Forty-eight hours after transfection, serum-deprived cells were reexposed to basal medium (B, open bars) or 15% serum (S; hatched bars) for 15 min, rinsed, and extracted. Immunoprecipitates were carried out with the anti-Myc epitope monoclonal antibody 9B7.3 (Top), a polyclonal anti-p110 antibody (Middle), or the anti-phosphotyrosine antibody 4G10 (Bottom). The washed immunoprecipitates were assayed for PI 3-kinase; activity is expressed as pmol of 32P transferred to PI per min per mg of extract protein used for immunoprecipitation. The anti-Myc immunoprecipitates are not complete, precluding valid quantitative comparisons between the PI 3-kinase activities recovered in Top and Middle. (B) Effect of recombinant PI 3-kinase on recombinant S6 kinase activity during transient expression. The 293 cells were transfected with plasmids encoding full-length, influenza virus HA epitope-tagged p70 S6 kinase (Left), a carboxyl-terminally truncated HA-tagged p70 S6 kinase (p70\Delta CT104, Center), or HA-tagged rat Rsk-1 (Right); each S6 kinase construct was cotransfected with a cDNA encoding either the active (A) or inactive (I) version of PI 3-kinase fusion protein (p110*). After 48 hr, serum-deprived cells were treated with basal medium (B) or 15% serum (S) for a further 15 min, rinsed, and extracted. The S6 kinases were recovered by immunoprecipitation with the monoclonal anti-HA antibody 12CA5 and assayed for 40S kinase activity. The 32P-labeled S6 product is shown; actual values (pmol of 32P per min per mg of extract protein used for immunoprecipitation) in this experiment are as follows: p70 full-length + inactive p110*, B = 0.0192, S = 0.0432; p70 full-length + active p110*, B = 0.054, S = 0.0947; p70\Delta CT104 + inactive p110*, B = 0.0096, S = 0.0167; p70\Delta CT104 + active p110*, B = 0.002, S = 0.005; Rsk-1 + inactive p110*, B < 0.0003, S = 0.0125; Rsk-1 + active p110*, B < 0.0003, S = 0.0105. The S6 kinase polypeptide expression was assessed by immunoblotting, using a specific anti-peptide antibody for p70 (20) and polyclonal anti-mouse Rsk antibody for Rsk-1. The result shown is representative of three experiments. (C) Effect of PI 3-kinase and wortmannin on S40A p70\Delta CT104 kinase activity and phosphorylation. The 293 cells were transfected with cDNAs encoding inactive (lanes 1 and 2) or active (lane 3) PI 3-kinase fusion proteins (p110*) together with a cDNA encoding HA-tagged S40A p70\Delta CT104. After 24 hr cells were placed in phosphate-free DMEM containing 15% dialyzed serum and 32P, at 0.15 mCi/ml. After an additional 24 hr of incubation, a portion of the cells transfected with inactive PI 3-kinase were treated with wortmannin (lane 1) (0.5 μM, 15 min). The cells were rinsed and extracted, and immunoprecipitates isolated with anti-HA antibody 12CA5 were analyzed for 408 kinase activity (Left), p70 immunolabeling (Center), and 32P incorporation (Right). The S6 kinase activities (pmol of 32P per min per mg of extract protein) were as follows: lane 1 (inactive PI 3-kinase + wortmannin) < 0.002; lane 2 (inactive PI 3-kinase) = 0.007; and lane 3 (active PI 3-kinase) = 0.059. After SDS/PAGE and extraction of the 32P-labeled S40A p70\Delta CT104, the 32P recovered (cpm) were 7108 for inactive PI 3-kinase plus wortmannin, 7246 for the inactive PI 3-kinase, and 8446 for active PI 3-kinase.
peptides maps (Fig. 2C) revealed that coexpression of S40A p70ACT104 with active p110* resulted in a marked relative increase in the \(^{32}\)P content of spot a/a', whereas wortmannin diminished the \(^{32}\)P content of spot a/a' selectively. The c cluster, spot d, the faint spot e, and the other minor \(^{32}\)P-labeled peptides were not altered in relative \(^{32}\)P content by the active p110* or wortmannin; spot b/b' (i.e., Ser-40) was absent. These results indicate that the residues encompassed on peptides a/a' are specific targets for a PI 3-kinase-directed phosphorylation that correlates closely with, and thus may be required for, the activation of p70ACT104.

Phosphoamino acid analysis of spot a/a' reveals only \([^{32}\text{P}]\text{phosphothreonine} \) (Fig. 2C, Inset in Top). The p70 sequence contains Thr residues at positions 248, 250, 252, and 256 in subdomain VIII of the catalytic domain just upstream of the APE motif (amino acids 261–263; ref. 34); many protein kinases require a phosphorylation in this region for activity (35). We therefore examined the effects of mutation of Thr-252 and Thr-256 on the 40S kinase activity (Fig. 3A) and the \(^{32}\)P-labeled peptide map (Fig. 3B) of the p70ACT104 polypeptide. Conversion of either Thr-252 or Thr-256 to Ala was associated with a complete loss in 40S kinase activity; replacement of either Thr-252 or Thr-256 with Asp (Fig. 3A) or Glu (not shown) also resulted in polypeptides devoid of 40S kinase activity, whereas replacement with Ser resulted in 40S kinase activity near parental levels (Fig. 3A). The p70ACT104 and the T252A and T256A variants were \(^{32}\)P-labeled during transient expression in 293 cells and subjected to peptide mapping (Fig. 3B). Spots a/a', b/b', c, and d were evident in the p70ACT104 maps as before (Fig. 2B). The p70ACT104 T256A
FIG. 3. Thr-252 is a site of p70 phosphorylation in situ and is required for p70 activity. (A) Effect of mutation of p70 residues Thr-252 and Thr-256 on p70 40S kinase activity. The amino acid sequence of p70 56 kDa in subdomain VIII of the kinase catalytic domain (20, 34) is starting at Gly-247, ...TVTHHTFCGTIEY-MAP... Resiudues Thr-252 and Thr-256 in p70(ΔCT104) were replaced by Ala (A), Asp (D), Ser (S), or Glu (not shown): 32P incorporation into S70 catalyzed by these p70 variants, isolated after transient expression, is shown. (B) 32P-labeled peptide maps of wild-type p70(ΔCT104) (i.e., Thr at 252 and 256; Center), Thr-252 → Ala (Left), and Thr-252 → Ala (Right) expressed transiently in 293 cells. The arrow indicates the location of peptides a/a'.

variant exhibits a 32P-labeled peptide map that is identical to the parental pattern, whereas conversion of Thr-252 to Ala results in the selective and total loss of the a/a' 32P-labeled peptides.

These results demonstrate that although Ser or Thr is required at positions 252 and 256 for p70(ΔCT104) 40S kinase activity, only Thr-252 is a phosphorylation site in situ. The identification of the phosphorylated amino acid on a/a' as Thr-252, together with the observation that the phosphorylation of peptides a/a' is selectively increased during activation of p70(ΔCT104) by recombinant (p110) PI 3-kinase and is selectively reduced concomitantly with p70(ΔCT104) inhibition by wortmannin, constitutes strong evidence that regulation of the phosphorylation of Thr-252 is at least one of the mechanisms by which PI 3-kinase regulates the p70 56 kDa kinase.

The continued phosphorylation of the inactive K123M p70ΔCT104 ATP site mutant on peptides a/a' in situ indicates that this phosphorylation is catalyzed by another protein kinase. The PI 3-kinase exhibits an intrinsic Ser/Thr protein kinase activity, which catalyzes autophosphorylation of the p110 polypeptide and transphosphorylation of the p85 subunit (36, 37). Recombinant inter-SH2-p110 recovered from 293 cells by anti-Myc immunoprecipitation and incubated in vitro with [γ-32P]ATP catalyzes 32P incorporation into the p110* polypeptide; polykaryotic recombinant glutathione 3-transferase-p70ΔCT104 is not phosphorylated when incubated in vitro with active p110* and [γ-32P]ATP (data not shown). Thus the increased 32P incorporation into p70ΔCT104 induced by PI 3-kinase in situ is not attributable to a PI 3-kinase-catalyzed p70 Ser/Thr phosphorylation; PI 3-kinase-directed phosphorylation of Thr-252 is probably catalyzed by an intermediating protein kinase that is activated directly or indirectly by the PI 3-kinase.

On the basis of these findings, we propose that mitogen activation of p70 56 kDa kinase requires the phosphorylation of Thr-252, a reaction that is dependent on the activity of the PI 3-kinase. The present results thus identify p70 Thr-252 as a molecular target of PI 3-kinase-directed signal transduction.

The identification of the PI 3-kinase-responsive protein kinase(s) that mediates Thr-252 phosphorylation and the elucidation of their regulation will provide considerable insight into the mechanism by which PI 3-kinase controls signal transduction.

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