Phosphoinositide 3-Kinase Is Activated by Phosphopeptides That Bind to the SH2 Domains of the 85-kDa Subunit*

(Received for publication, October 16, 1992, and in revised form, December 22, 1992)

Christopher L. Carpenter‡‡, Kurt R. Auger*, Manas Chanudhuri, Monique Yoakim**,
Brian Schaffhausen**, Steven Shoelson‡, and Lewis C. Cantley‡‡

From the ‡Hematology-Oncology Division, ‡‡Department of Medicine, Beth Israel Hospital, Boston, Massachusetts 02115,
*Division of Cellular and Molecular Biology, Dana-Farber Cancer Institute and Department of Pathology and ‡Research Division, Joslin Diabetes Center and Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115,
**Department of Biochemistry, Tufts University Medical School, Boston, Massachusetts 02111, and ‡‡Department of Physiology, Harvard Medical School, Boston, Massachusetts 02115

Tyrosine-phosphorylated peptides based on the regions of polyoma virus middle t antigen and the platelet-derived growth factor receptor that bind phosphoinositide 3-kinase are shown to activate this enzyme 2–3-fold in vitro. The concentrations of the peptides required to activate the enzyme are at least 10–1000-fold higher than the dissociation constants of these peptides for the individual SH2 domains of the 85-kDa subunit (K_D < 100 nM). Doubly phosphorylated peptides are more effective than singly phosphorylated peptides. The results suggest that a fraction of the cellular phosphoinositide 3-kinase has SH2 domains with relatively low affinity for phosphopeptides and that binding of phosphopeptides to these enzymes causes activation. Thus, SH2 domains may be involved not only in recruiting the enzyme but also in regulating activity.

Phosphoinositide (PtdIns) 3-kinase is the critical enzyme in a recently discovered intracellular signaling pathway that is activated by a wide range of growth factors, oncogenes, and nonmitogenic stimuli (1). PtdIns 3-kinase was discovered because of its association with the oncogene products pp60^c-src and middle T-pp60^c-src complex (2–5). Studies of polyoma virus middle t antigen (mT) mutants indicate that mT must associate with both pp60^c-src (or a close relative of pp60^c-src) and with PtdIns 3-kinase to transform cells (2, 4–12). The association of mT with pp60^c-src results in activation of the protein-tyrosine kinase activity of pp60^c-src and the phosphorylation of mT (11, 13). The lipid products of PtdIns 3-kinase are elevated in cells transformed by mT, indicating a correlation between the association of this enzyme with mT and increased activity in intact cells (14, 15). Similarly, PtdIns 3-kinase directly associates with and is activated by a variety of growth factor receptors. The mechanism of activation of PtdIns 3-kinase is not known.

PtdIns 3-kinase has been purified from rat liver, bovine brain, mouse fibroblasts, and bovine thymus (16–20). PtdIns 3-kinase purified from rat liver is a heterodimer of an 85-kDa protein (p85) and one of two 110-kDa proteins (p110) (16). The cDNA that encodes the 85-kDa subunit of PtdIns 3-kinase has been cloned (17, 18, 21). The protein encoded by this cDNA contains three regions of homology to pp60^c-src, two SH2 domains and an SH3 domain. This subunit also contains a region of homology to rho-GTPase-activating protein (rho-GAP) and the C terminus of the breakpoint cluster region gene product. The CDNA does not predict a nucleotide binding domain, and the expressed proteins do not have PtdIns kinase activity. The CDNA for the 110-kDa subunit of PtdIns 3-kinase has also been recently cloned, and the expressed protein has PtdIns kinase activity (22). The CDNA has homology only to a yeast gene VPS34. The SH2 domains of p85 bind to tyrosine kinases suggesting that p85 mediates the binding of PtdIns 3-kinase to tyrosine-phosphorylated proteins (18, 23–25).

Tyrosine 315 of mouse mT is implicated as the site which is important for PtdIns 3-kinase binding to mT. Tyrosine 315 has also been identified as a primary site of mT phosphorylation (26). A mutant of mT in which tyrosine 315 is replaced by phenylalanine has little associated PtdIns 3-kinase activity in vivo and is defective in cell transformation (4, 27). The SH2 domains of p85 bind in solution and on nitrocellulose blots to the region of mT that contains tyrosine 315, when that tyrosine is phosphorylated (28). Phosphorylation of mT is necessary for its association with PtdIns 3-kinase (29) and synthetic tyrosine-phosphorylated peptides, based on the region of tyrosine 315 of mT, block the association of baculovirus-expressed mT with purified PtdIns 3-kinase (30). The sequence surrounding tyrosine 315 of mT is similar to sequences of two regions of the kinase insert domain of the platelet-derived growth factor (PDGF) receptor, which also contain phosphotyrosine residues, that are implicated as PtdIns 3-kinase binding sites (31, 32). The sequences in the PDGF receptor are tyrosines 740 and 751 in the human β PDGF receptor. Similar sequences have also been identified in other proteins known to bind PtdIns 3-kinase (1).

Activation of PtdIns 3-kinase seems necessary for mitogenic responses to some growth factors and oncogene products (5, 33–35). Cells stimulated by growth factors or transformed by mT accumulate PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3, but PtdIns-3-P levels do not change significantly (14, 15, 36–39). Purified PtdIns 3-kinase will phosphorylate the D-3 position...
of PtdIns, PtdIns-4-P, and PtdIns-4,5-P₂ with reasonable specific activities, so lipids phosphorylated at the D-3 position are likely all products of the same enzyme (16).

Several possible mechanisms for generation of PtdIns-3,4-P₂ and PtdIns-3,4,5-P₃ in response to growth factors and oncogenes have been proposed (40-43). Recruitment of PtdIns 3-kinase from the cytosol to proteins at the plasma membrane, where the substrates PtdIns-4-P and PtdIns-4,5-P₂ are enriched, could partially explain the preferential elevation of PtdIns-3,4-P₂ and PtdIns-3,4,5-P₃ in stimulated cells. It is also possible that phosphorylation of PtdIns 3-kinase affects its activity. The effect of tyrosine phosphorylation on activity is not known.

PtdIns 3-kinase activity has usually been measured in immunoprecipitates from lysates of quiescent or stimulated cells using anti-receptor, anti-phosphotyrosine, or anti-p85 immunoprecipitates. These assays have not provided a reliable estimate of specific activity or been able to dissociate the effects of protein-protein interaction and phosphorylation of PtdIns 3-kinase. However, using purified PtdIns 3-kinase we have been able to address the regulation of this enzyme.

In this paper we have investigated the effects of association of PtdIns 3-kinase with mT and the PDGF receptor using phosphopeptides based on the binding sites of PtdIns 3-kinase. These proteolysis experiments show that doubly phosphorylated peptides that associate with the SH2 domains of p85 activate both purified PtdIns 3-kinase and the crude cytosolic enzyme. These results indicate that association with a tyrosine-phosphorylated protein alone can activate this enzyme and indicate that the SH2 domains regulate enzymatic activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—Phosphorylated peptides were synthesized using an N°Fmoc synthetic strategy as described elsewhere (30, 44). Phosphotyrosine residues were incorporated using N°Fmoc-L-phosphotyrosine and extended coupling times. Peptides were cleaved from the resin and the more labile side chain-protection groups were simultaneously removed using trifluoroacetic acid, thioanisole, ethanedithiol, and anisole (90:5:3:2). Following other precipitation, methyl ester protecting groups on the tyrosine phosphate side chain were removed during a second stage of deprotection with trimethylsilyl bromide (44). All phosphopeptides had the expected amino acid composition and were greater than 80% homogenous by reversed-phase high performance liquid chromatography analysis. The glutathione transferase fusion genes were expressed in *Escherichia coli* as described elsewhere (28). These genes were all derived from a p85a clone kindly provided by D. Pallas and T. Roberts of the Dana-Farber Cancer Institute. The antiserum to p85 was raised against glutathione-Transferrase fusions of the full length and C-terminal half of p85.

**Antibodies**—The mT antiserum is a polyclonal rabbit antiserum kindly provided by D. Pallas and T. Roberts of the Dana-Farber Cancer Institute. The antiserum to p85 was raised against glutathione-Transferrase fusions of the full length and C-terminal half of p85.

**Protein Purification**—PtdIns 3-kinase was purified from rat liver homogenate as previously described (16). The rat liver cytosol was prepared as previously described for the purification of PtdIns 3-kinase. pp60°c-src and polyoma mT genes were expressed in SF9 cells using baculovirus as previously described (30).

**PtdIns 3-Kinase Assays**—PtdIns 3-kinase assays were done as previously described (16) except that the assays contained 200 μM sodium vanadate. Peptides were preincubated with PtdIns 3-kinase for 20 min in a volume of 12 μl. Lipids and ATP/MgCl₂ were then added to start the reaction which had a final volume of 30 μl. The final concentration of PtdIns 3-kinase in these reactions was about 100 nM. The initial peptide concentrations during the preincubation were higher than the final concentration after adding the lipids and ATP/MgCl₂. The final peptide concentrations are shown in the figure legends. PtdIns 3-kinase activity was quantitated by liquid scintillation counting of the chloroform extract of the reaction. Rat liver cytosol was assayed as previously described (16) after preincubation with peptides for 20 min at room temperature. These assays, however, also contained 200 μM sodium vanadate. The products were separated by thin layer chromatography using 1-propanol and acetic acid as solvents (30).

**Association of Purified PtdIns 3-Kinase with Baculovirus-expressed mT-pp60°c-src Complex**—To determine the relative ability of the peptides to block the association of PtdIns 3-kinase and mT, we preincubated PtdIns 3-kinase with the peptides at 4°C for 1 h and then added lysates of SF9 cells which were doubly infected with baculoviruses containing mT and pp60°c-src. The incubation was then continued for another hour. Immunoprecipitates with antiserum raised against mT were done as previously described (30). PtdIns kinase assays were done on the washed immunoprecipitates, as previously described (30).

**Association of mT with the SH2 Domains of p85**—Constructs containing the individual SH2 domains of p85 were expressed in the pGEX3 vector. The peptides were purified from bacterial sonicates with glutathione-agarose beads. The proteins bound to the glutathione-agarose beads were washed extensively with a buffer of 150 mM NaCl, 10 mM Tris, pH 7.5, 1 mM EDTA, and 1% Nonidet P-40. The peptides were then added and incubated with the SH2 domains for 20 min. A mT lysate was then added and the incubation continued for another hour. The beads were then washed as for an immunoprecipitate, and the samples run on an 8.5% SDS-PAGE gel. The proteins were then transferred to nitrocellulose or Immobilon-P paper, blocked with milk, probed with the mT antiserum, and visualized by chemiluminescence.

**Cell Culture and Immunoprecipitates**—Rat 1 cells were grown and immunoprecipitated with the p85 antibody as described elsewhere (45). Immunoprecipitates were incubated at 4°C for 1 h with peptides before the addition of antibody. PtdIns 3-kinase activity was assayed on the immunoprecipitates as previously described (45). The products were separated by thin-layer chromatography, visualized by autoradiography, and quantitated by liquid scintillation counting.

**Succrose Gradients**—Purified PtdIns 3-kinase was incubated with 100 μM peptide G and then layered over a 5-25% sucrose gradient containing 100 μM peptide G. Protein standards were also included. PtdIns 3-kinase without phosphopeptide was run on a similar gradient as a control. The gradients were centrifuged for 4 h in a Beckman SW 55 rotor at 55,000 rpm. Fractions were removed and assayed for PtdIns kinase activity and Western blotted for p85. Samples were also separated by SDS-PAGE and Coomassie Blues stain to detect the standards (bovine serum albumin, yeast alcohol dehydrogenase, and horse apoferritin).

**RESULTS**

**Submicromolar Concentrations of Tyrosine-phosphorylated Peptides Block the Association of PtdIns 3-Kinase with mT-pp60°c-src**—Recombinant mT tightly associates with purified PtdIns 3-kinase and phosphopeptides by a submicromolar affinity on tyrosine (30). We wanted to further investigate the mechanism by which mT-pp60°c-src activates PtdIns 3-kinase. To test the possibility that association with mT activates PtdIns 3-kinase, we synthesized a series of tyrosine-phosphorylated peptides based on the regions of mT (30) and the PDGF receptor (31) that bind PtdIns 3-kinase (Table I).

To be certain that the peptides mediated their effect by binding to PtdIns 3-kinase we investigated the ability of the peptides to block association of purified PtdIns 3-kinase with baculovirus-expressed mT-pp60°c-src complex. Both a singly phosphorylated peptide (peptide C) and a doubly phosphorylated peptide (peptide G) block association of purified PtdIns 3-kinase with mT-pp60°c-src complex (Fig. 1). Peptide C inhibited 50% of maximal binding at a concentration of approximately 40 nM. Peptide G, which is phosphorylated at both tyrosine 315 and tyrosine 322, caused 50% maximal inhibition at a concentration of approximately 25 nM. Tyrosine 322 is phosphorylated in mT but not in the immunoprecipitates and may be phosphorylated in vivo (46, 47). Nonphosphorylated peptides had no effect in the concentration range investigated (not shown, see also Ref. 30).

**Phosphorylated Peptides Block Association of p85 with mT**
Phosphorylated peptides were used to study the interaction between PtdIns 3-kinase and mT. A mT immunoprecipitate was then done and assayed for phosphorylated peptides. To determine the binding of tyrosine-phosphorylated residues of mT with the SH2 domains of p85, we used constructs containing the individual SH2 domains of p85. The binding of a doubly phosphorylated 35S-mT to the SH2 domains was determined by Western blotting with an antiserum to mT. The experiments shown in Figs. 1 and 2 are competition experiments between the peptides and PtdIns 3-kinase (or its SH2 domains) for mT and so do not reflect true K\textsubscript{D} values.

**TABLE I**

| Peptide | Sequence | Based on |
|---------|----------|----------|
| A       | RENEYMPAQGHIH | Hamster mT (Tyr(P)-298) |
| B       | RENEYMPAQGHIH | Hamster mT (Tyr(P)-298) |
| C       | ESDNLEPEEEYMMPMDLYLDILPEE | Mouse mT (Tyr(P)-315) |
| D       | EEYMPMEDLY | Mouse mT (Tyr(P)-315) |
| E       | EEYMPMEDLY | Mouse mT (Tyr(P)-315) |
| F       | EEYMPMEDLYLDILPEE | Mouse mT (Tyr(P)-322) |
| G       | EEYMPMEDLYLDILPEE | Mouse mT (Tyr(P)-315, -322) |
| H       | EPQYQGENL | pp60*src(Tyr(P)-527) |
| I       | GGYMDMSKDESVDYPM | Human PDGF receptor (Tyr(P)-740, -751) |

**FIG. 2.** Phosphopeptides block the association of PtdIns 3-kinase with mT by binding to the SH2 domains of p85. Constructs containing the individual SH2 domains of p85 were expressed in pGEX3 and the proteins purified with glutathione-agarose beads. The beads containing the SH2 domains were incubated with peptide D at the indicated concentrations for 20 min at room temperature. An Sf9 cell lysate containing mT-pp60*src complex was then added and incubation continued for 1 h at 4°C. The beads were washed, and the amount of mT associated with the SH2 domains was determined by Western blotting with an antiserum to mT.

The experiments shown in Figs. 1 and 2 are competition experiments between the peptides and PtdIns 3-kinase (or its SH2 domains) for mT and so do not reflect true K\textsubscript{D} values.

**Tyrosine-phosphorylated Peptides Based on mT and PDGF Receptor Domains Sequence Activate Cytosolic PtdIns 3-Kinase**—We investigated the effect of these peptides on PtdIns 3-kinase activity in cell lysates. The enzymatic activity of PtdIns 3-kinase in cytosol when PtdIns-4,5-P\textsubscript{2} is used as a substrate appears to be suppressed compared to the activity of purified enzyme (16). We added tyrosine-phosphorylated peptide D or G or nonphosphorylated peptide E to the cytosolic fraction of liver in order to test the possibility that association with a phosphopeptide alone could activate the cytosolic enzyme. Since there are other phosphoinositide kinases in rat liver cytosol, we assayed PtdIns 3-kinase activity utilizing the substrate PtdIns-4,5-P\textsubscript{2}. No kinases other than PtdIns 3-kinase are known to phosphorylate this lipid. As shown in Fig. 3, both of the tyrosine-phosphorylated peptides stimulated the activity of the enzyme in crude cytosol. The nonphosphorylated peptide (E) had no significant effect on activity. The doubly phosphorylated peptide (G) activated the enzyme more than the singly phosphorylated peptide (D). Much higher concentrations of the peptides were required to elicit activation than were required to block binding of purified PtdIns 3-kinase to mT-pp60*src. Addition of higher concentrations of vanadate (up to 1 mM) to inhibit phosphatases did not significantly reduce the concentration of peptide necessary to activate the enzyme.

We have also examined the effect on PtdIns 3-kinase activity of a doubly phosphorylated peptide based on the human β PDGF receptor sequence (peptide I). This peptide is phos-
Phosphorylation at sites equivalent to tyrosines 740 and 751 in the PDGF receptor sequence. In rat liver cytosol this peptide results in a 1.7-fold increase in PtdIns 3-kinase activity at a concentration of 10 μM and a 2.4-fold increase in activity at a concentration of 100 μM (Fig. 4). Higher concentrations of the peptide do not result in further activation.

We also investigated the effect of the doubly phosphorylated peptide (G) on PtdIns 3-kinase activity in anti-p85 immunoprecipitates of fibroblast and rat liver cytosol. The antibodies we have raised against recombinant p85 quantitatively immunoprecipitate the purified enzyme and precipitate activity from cell lysates. However, immunoprecipitation of the purified enzyme results in loss of 80–90% of the activity. This may be due to steric hindrance of access of the substrate to the active site in the immune complex or to conformational effects of the antibodies. The phosphopeptides had no significant effect on the activity of PtdIns 3-kinase in anti-p85 immunoprecipitates from rat liver or fibroblasts (Fig. 4). However, if peptide G is preincubated with the cytosol or lysate before the addition of antibody, activation of PtdIns 3-kinase activity is seen after immunoprecipitation (Fig. 4). Precipitation with the singly phosphorylated peptide D resulted in a slight inhibition of activity (Fig. 5).

Since the antibody is partially inhibitory, the doubly phosphorylated peptide is likely relieving inhibition rather than activating in this experiment. A possible interpretation is that the doubly phosphorylated peptide locks the enzyme into an active conformation prior to immunoprecipitation so that some of the inhibitory antibodies in the polyclonal serum fail to bind. However, if the enzyme is precipitated with these antibodies first then the peptide is not effective at removing the inhibitory antibodies. The singly phosphorylated peptide was obviously not effective at blocking inhibition even when added prior to immunoprecipitation, perhaps do to a lower affinity for the SH2 domains.

Tyrosine-phosphorylated Peptides Based on mT and PDGF Receptor Domains Activate Purified PtdIns 3-Kinase—We also investigated the ability of the phosphopeptides to activate purified PtdIns 3-kinase. There was no loss of enzyme activity during incubation with buffer alone (data not shown). The unphosphorylated peptide E had no effect on the activity of purified PtdIns 3-kinase at a concentration of 300 μM (Fig. 5). A phosphopeptide based on a sequence from pp60src, which has a low affinity for the SH2 domains of PtdIns 3-kinase (51) also had no effect on activity at a concentration of 300 μM. However, incubation of purified PtdIns 3-kinase with the doubly phosphorylated peptide (peptide G) caused a 2-fold increase in enzymatic activity. The 50% maximal effect was approximately 1 μM. There is some variability among
different preparations of PtdIns 3-kinase. In some preparations of PtdIns 3-kinase, a 2-fold activation was seen at a concentration of peptide G of 50 nM and a 3-fold activation at 500 nM (data not shown). Considerable variation in response to the PDGF receptor phosphopeptide was also found: in some experiments a 3-fold activation was seen at 100 μM, whereas in other experiments no significant activation was seen (data not shown).

The singly phosphorylated peptides containing phosphotyrosine 315 (peptide D) and phosphotyrosine 322 (peptide F) were less effective in activating PtdIns 3-kinase. A 100-fold higher concentration was necessary to see any effect. The maximal activation by these peptides was also less than that caused by the doubly phosphorylated peptide. Similar activation curves were observed using all three substrates of the PtdIns 3-kinase (PtdIns, PtdIns-4-P, and PtdIns-4,5-P2, not shown). The concentration dependence for activation of the enzyme by both the doubly phosphorylated peptide and singly phosphorylated peptides was considerably higher than the concentration dependence for blocking binding to mT (Fig. 1). Singly phosphorylated peptides A and C activated PtdIns 3-kinase at similar concentrations and to the same degree as singly phosphorylated peptides D and F.

To determine whether doubly phosphorylated peptide G causes a change in the oligomerization state of PtdIns 3-kinase the rate of sedimentation in a sucrose gradient was investigated. Control enzyme and enzyme preincubated and sediment with 100 μM peptide G migrated identically, as determined by PtdIns 3-kinase activity and Western blotting for p85 with an apparent molecular mass of about 200 kDa (not shown). Thus, activation does not involve a change in the oligomerization of PtdIns 3-kinase.

**DISCUSSION**

Association of PtdIns 3-kinase with membrane-bound tyrosine-phosphorylated proteins is correlated with the appearance of PtdIns-3,4-P2 and PtdIns-3,4,5-P3 in vivo (14, 15, 36–39). The increase in activity of PtdIns 3-kinase likely involves several processes. Recruitment of the enzyme from the cytosol to the membrane provides proximity to the lipid substrates. Phosphorylation or dephosphorylation reactions involving tyrosine and/or serine residues of the 85-kDa subunit are also likely to be important. The fraction of PtdIns 3-kinase that is associated with mT is phosphorylated on both tyrosine and serine residues in vivo (48). Phosphorylation of p85 on serine by a tightly associated serine/threonine kinase inhibits the PtdIns 3-kinase activity (45), and dephosphorylation at these sites could cause activation in vivo.

Here we present an additional mechanism for regulation of PtdIns 3-kinase. We used tyrosine-phosphorylated peptides, containing either a single phosphotyrosine or two phosphotyrosines, based on mouse mT Tyr-315. This region of mT has been implicated in cell transformation and association with PtdIns 3-kinase. A mutant (d123) in which these residues are deleted is transformation defective and has dramatically reduced PtdIns 3-kinase activity (5). A point mutation, converting tyrosine 315 to phenylalanine, results in a reduced frequency and different spectrum of tumors (27) and loss of the majority of associated PtdIns 3-kinase activity (4). Antibodies against this region immunoprecipitate a fraction of mT that does not associate with PtdIns 3-kinase activity (27). In addition to tyrosine 315, tyrosine 322 is phosphorylated in immunoprecipitates (46, 47). The tyrosine 315 to phenylalanine mutant has some associated PtdIns 3-kinase activity which suggests that other tyrosine-phosphorylated sites might also be involved in this association. The ability of the doubly phosphorylated peptide (analogous to phosphotyrosine 315 and phosphotyrosine 322) to activate purified PtdIns 3-kinase and the lesser ability of singly phosphorylated peptides (analogous to phosphotyrosine 315 or phosphotyrosine 322) to activate in spite of comparable binding to PtdIns 3-kinase suggests that other phosphotyrosine residues downstream of tyrosine 315 could be important in regulation of activity. Besides tyrosine 322, other potential tyrosine-phosphorylation sites in this region might also be involved and need to be further investigated.

In the three conditions in which we have looked at the effect of phosphopeptides on PtdIns 3-kinase activity (purified enzyme, cell lysates, and immunoprecipitates) the doubly phosphorylated peptide is much more effective at activating PtdIns 3-kinase. The more marked effect of the doubly phosphorylated peptide in activating purified PtdIns 3-kinase suggests that the activation is a result of the peptide binding either to a region that best recognizes two phosphotyrosines or to two distinct sites that each recognize phosphotyrosine. The equal ability of both singly and doubly phosphorylated peptides to block association of PtdIns 3-kinase with mT at concentrations lower than those required for activation suggests that the activation is due to binding at a separate site or to the same sites on a pool of PtdIns 3-kinase with SH2 domains that have low affinities for the phosphopeptides. The doubly phosphorylated peptide appears to have a much higher affinity for the sites that activate. The PtdIns 3-kinase assays were done at substrate concentrations much above the K_m values, indicating the the activation is due to an increase the V_max of the reaction.

Backer et al. (49) have reported the activation of PtdIns 3-kinase by insulin receptor substrate (IRS-1) and tyrosine-phosphorylated peptides (phosphorylated at a single position) in immunoprecipitates of p85. Unlike Backer et al. we found no activation of PtdIns by phosphopeptides in immunoprecipitates. We did find greater activity in immunoprecipitates from cell lysates preincubated with phosphopeptides, suggesting that the activation in immunoprecipitates may be due to prevention of inhibition by the antibody. We used a different antibody than Backer et al. so that direct comparison of these results is not possible.

Several models would explain these data. Although the individually expressed SH2 domains have similar affinities for the phosphopeptide (see Fig. 2) the affinities of the SH2 domains in the native protein may differ markedly. In this model one SH2 domain may recognize singly and double phosphorylated peptide equally well, but binding to this SH2 domain would not cause activation. The other SH2 domain would have a much higher affinity for doubly phosphorylated peptide and binding to this SH2 domain would cause activation. At high concentrations the singly phosphorylated peptides would also bind to this SH2 domain and cause activation.

An alternative explanation is that two phosphotyrosine recognition sites must be occupied for activation: a high and a low affinity site. Only the high affinity site would be involved in binding to mT. Peptide competition for binding of PtdIns 3-kinase to mT then would occur at low peptide concentrations. The low affinity of the second site would be overcome by the doubly phosphorylated peptide because it would already be bound to the high affinity site and would have a significant entropic advantage in binding to the low affinity site. This second site could either be the other SH2 domain or a second phosphotyrosine recognition site within the same SH2 domain. At high concentrations the singly phosphorylated peptides would bind to both sites and cause activation.

A third possibility is that a fractions of the PtdIns 3-kinase...
is modified in such a way that the SH2 domains have low affinities for phosphopeptides. This fraction would not bind tightly to mT-pPp60ɛɛ and therefore would not be detected in the competition assay shown in Fig. 1. Binding of the phosphopeptides to the SH2 domains of this population of PtdIns 3-kinase could force the SH2 domains into a normal conformation and in so doing activate the catalytic subunit. We favor this model since there is already evidence that in PDGF-stimulated cells a significant fraction of p85 is modified so that association with another protein could alter the affinities. For this finding. Phosphatases may have dephosphorylated some of the peptide but activation is not seen at lower peptide concentrations even with very high concentrations of vandate. The peptide could be competing with another protein bound to p85 or the peptide could be binding to other proteins (lowering the effective concentration).

Many proteins that activate PtdIns 3-kinase have clusters of multiple tyrosine-phosphorylated peptides in close proximity. As discussed above tyrosines 708 and 719 of the mouse PDGF β receptor (and 740 and 751 of the human receptor) are known to be involved in PtdIns 3-kinase binding (31). IRS-1 also binds PtdIns 3-kinase. It is phosphorylated on tyrosine and has several closely spaced clusters of sequences that satisfy the requirements for being PtdIns 3-kinase binding sites (50). Interestingly, in addition to the 85-kDa subunit of PtdIns 3-kinase, several other enzymes are known to have two SH2 domains (PLCγ and ras-GAP); doubly phosphorylated peptides might also exert regulatory affects on these enzymes by a similar mechanism.

In summary, the results presented here suggest a new model for regulation of PtdIns 3-kinase that may be applicable for activation of this enzyme by a wide range of growth factors and oncogene products. This model also implies a new function for SH2 domains. These domains could act as direct switches to regulate enzymes by interacting with tyrosine phosphorylated proteins.

Acknowledgments—We thank Helen Piwnica-Worms and Peg Atkinson for help with baculovirus-produced proteins; Tom Roberts, Brian Drucker, and David Pallas for providing antibodies to mT; Songyang Zhou for help with the immunoprecipitates; Basim Khalife for help in cloning the cDNA-encoding p85; Rosana Kapeller for subcloning the p85 cDNA into the pCEX vector; and Alex Toker for help in purifying PtdIns 3-kinase.

REFERENCES

1. Cantley, L. C., Auger, K. R., Carpenter, L. C., Duckworth, B., Grazi, A., Kapeller, R., and Sollott, S. (1991) Cell 64, 291-302
2. Kaplan, D. R., Whitman, M., Schaffhausen, B., Pallas, D., White, M., Cantley, L., and Roberts, T. M. (1987) Cell 50, 1021-1029
3. Sugimoto, Y., Whitman, M., Cantley, L. C., and Erikson, R. L. (1986) Proc. Natl. Acad. Sci. U. S. A. 81, 2117-2121
4. Whitman, M., Kaplan, D. R., Schaffhausen, B., Cantley, L., and Roberts, T. M. (1985) Nature 315, 239-240
5. Kaplan, D. R., Whitman, M., Schaffhausen, B., Laptis, G., Gareca, R. L., Pallas, D., Roberts, T. M., and Cantley, L. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 3024-3028
6. Markland, W., Cheng, S. H., Ootra, B. A., and Smith, A. E. (1986) J. Virol. 59, 82-90
7. Kazlauskas, A., Hemmings, A., and Courtneidge, S. A. (1988) EMBO J. 7, 3837-3844
8. Kornbluth, S., Cheng, S. H., Markland, W., Fukui, Y., and Hanafusa, H. (1985) J. Virol. 64, 1584-1588
9. Courtneidge, S. A., and Smith, A. E. (1984) EMBO J. 3, 585-591
10. Cheng, S. H., Furino, K., Sambucetti, K., Yamamoto, T., Yonemoto, K., and Smith, A. E. (1988) EMBO J. 7, 3845-3853
11. Bolen, J. B., Thiele, C. J., Israel, M. A., Yonemoto, W., Lipschitz, L. A., and Drugg, J. S. (1987) EMBO J. 6, 1031-1037
12. Courtneidge, S. A., and Heber, A. (1987) Cell 50, 1031-1037
13. Courtneidge, S. A. (1986) EMBO J. 5, 471-7
14. Lyman, T., Hawkins, P., Hanley, M., and Courtneidge, S. A. (1990) J. Virol. 64, 3895-3904
15. Serunian, L. A., Auger, K. R., Roberts, T., and Cantley, L. C. (1990) J. Virol. 64, 4718-4727
16. Carpenter, L. C., Duckworth, B. C., Auger, K. R., Cohen, B., Schaffhausen, B. S., and Cantley, L. C. (1996) J. Biol. Chem. 271, 19704-19711
17. Escobedo, J. A., Navanavassattas, S., Kavanagh, W. M., Milay, D., Frew, V. A., and Williams, L. T. (1991) Cell 68, 75-82
18. Williams, L. T. (1991) Mol. Cell. Biol. 11, 1125-1132
19. Chou, W., Hiles, I., Gilboa, I., Ruiz-Larrea, F., Panayotou, G., Thompson, A., Dhand, R., Hsu, C., Wang, S. W., and Williams, L. T. (1989) Mol. Cell. Biol. 9, 819-816
20. Shiojani, F., Homma, Y., and Takenawa, T. (1992) J. Biol. Chem. 267, 8108-8114
21. Morgan, S. J., Smith, A. D., and Parier, P. J. (1990) Eur. J. Biochem. 191, 455-467
22. Skolnik, E. Y., Marpols, B., Mohammadi, M., Lowenstein, E., Fischer, R., Duggan, J. C., Ulrich, T., and Schlessinger, J. (1989) Nature 339, 560-160
23. Hiles, I. D., Osu, M., Volinia, S., Fry, M. J., Gout, I., Dhand, R., Panayotou, G., Ruiz-Larrea, F., Thompson, A., Totty, N. F., Hsu, C., and Courtneidge, S. A. (1991) Cell 65, 891-907
24. Yoakim, M., Hsu, E. W., Liu, Y., Carpenter, C. L., Cantley, L. C., and Schaffhausen, B. S. (1992) J. Virol. 66, 4541-4549
25. Chou, W., Yoakim, M., Piwnica-Worms, H., Roberts, T., and Schaffhausen, B. S. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 4458-4462
26. Auger, K. R., Carpenter, C., Shoezien, S., Piwnica-Worms, H., and Cantley, L. C. (1992) J. Biol. Chem. 267, 4504-1504
27. Escobedo, J. A., Kaplan, D. R., Kavanagh, W. M., Tureck, C. W., and Williams, L. T. (1990) Mol. Cell. Biol. 10, 5961-5970
28. McCadie, C. J., Ellis, C., Reddick, M., Anderson, D., and Schlessinger, J. (1987) J. Biol. Chem. 262, 5960-5968
29. Kazlauskas, A., and Cooper, J. A. (1989) Cell 58, 1211-1233
30. Escobedo, J. A., and Williams, L. T. (1988) Nature 335, 85-87
31. Fukui, Y., and Hanafusa, H. (1989) Mol. Cell. Biol. 9, 1651-1658
32. Shurtleff, S. A., Downing, J. R., Rock, C. O., Hawkins, S. A., Rousell, M. F., and Sherr, C. J. (1989) EMBO J. 8, 2451-2457
33. Auger, K. R., Serunian, L., Libby, P., and Cantley, L. C. (1989) Cell 57, 167-175
34. Reissman, N. B., Kapeller, R., White, M., and Cantley, L. C. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 1411-1415
35. Vartiovskio, L., Drucker, B., Morrison, D., Cantley, L., and Roberts, T. (1990) Cell 64, 55-65
36. Vartiovskio, L., Daley, G., Jackson, P., Baltimore, D., and Cantley, L. (1991) Mol. Cell. Biol. 11, 1107-1115
37. Stephens, L. R., Hughes, K. T., and Irvine, R. F. (1992) Nature 358, 13-13