THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 270, No. 8, Issue of February 24, pp. 3662–3666, 1995
Printed in U.S.A.

Regulation of Phosphatidylinositol 3′-Kinase by Tyrosyl Phosphoproteins

FULL ACTIVATION REQUIRES OCCUPANCY OF BOTH SH2 DOMAINS IN THE 85-kDa REGULATORY SUBUNIT*

(Received for publication, September 21, 1994, and in revised form, November 18, 1994)

Tamara Rordorf-Nikolic‡, Debra J. Van Horn‡, Daxin Chen‡, Morris F. White§, and Jonathan M. Backer‡¶

From the ‡Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, New York 10461 and the §Joslin Diabetes Center, Research Division, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02215

Phosphatidylinositol 3′-kinase (PI 3′-kinase) is activated in insulin-stimulated cells by the binding of the SH2 domains in its 85-kDa regulatory subunit to insulin receptor substrate-1 (IRS-1). We have previously shown that both tyrosyl-phosphorylated IRS-1 and mono-phosphopeptides containing a single YXXM motif activate PI 3′-kinase *in vitro*. However, activation by the mono-phosphopeptides was significantly less potent than activation by the multiply phosphorylated IRS-1. We now show that the increased potency of PI 3′-kinase activation by IRS-1 relative to phosphopeptide is not due to tertiary structural features IRS-1, as PI 3′-kinase is activated normally by denatured, reduced, and carboxymethylated IRS-1. Furthermore, activation of PI 3′-kinase by bis-phosphorylated peptides containing two YXXM motifs is 100-fold more potent than the corresponding mono-phosphopeptides and similar to activation by IRS-1. These data suggest that tyrosyl-phosphorylated IRS-1 or bis-phosphorylated peptides bind simultaneously to both SH2 domains of p85. However, these data cannot differentiate between an activation mechanism that requires two-site occupancy for maximal activity as opposed to one in which bivalent binding enhances the occupancy of a single activating site. To distinguish between these possibilities, we produced recombinant PI 3′-kinase containing either wild-type p85 or p85 mutated in its N-terminal, C-terminal, or both SH2 domains. We find that mutation of either SH2 domains significantly reduced phosphopeptide binding and decreased PI 3′-kinase activation by 50%, whereas mutation of both SH2 domains completely blocked binding and activation. These data provide the first direct evidence that full activation of PI 3′-kinase by tyrosyl-phosphorylated proteins requires occupancy of both SH2 domains in p85.

Phosphatidylinositol 3′-kinase (PI 3′-kinase)[1] is a lipid kinase that has been implicated in the regulation of cell growth by growth factor receptors and oncogene products (1). PI 3′-kinase phosphorylates phosphatidylinositol at the D-3 position of the inositol ring (2), and stimulation of cells with mitogens such as platelet-derived growth factor or transformation of cells with polyoma middle T antigen leads to increases in the levels of the lipid products PI 3,4-P_2 and PI 3,4,5-P_3 (3, 4). The function of these lipids has not yet been determined, but their low abundance and rapid appearance in mitogen-stimulated cells suggests that they may serve as intracellular second messengers.

PI 3′-kinase is a heterodimer composed of an 85-kDa regulatory subunit (p85) and a 110-kDa catalytic subunit (p110) (1). During activation of PI 3′-kinase by tyrosine kinase receptors such as the platelet-derived growth factor or colony stimulating factor-1 receptors, the SH2 domains of p85 bind directly to specific phosphorylated YXXM motifs present in the cytoplasmic domains of the receptors (reviewed in Ref. 5). In the case of the insulin receptor, PI 3′-kinase binds to phosphorylated YXXM motifs in the substrate IRS-1 to a greater extent than to the receptor itself (6, 7). The binding of receptors or substrates containing phosphorylated YXXM motifs activates the lipid kinase *in vitro* and in intact cells (6). Activation of PI 3′-kinase *in vitro* can be mimicked by synthetic phosphopeptides that contain the YXXM motif, and this peptide-mediated activation correlates with conformational changes in the p85 subunit (6, 8, 9). Thus, regulation of PI 3′-kinase catalytic subunit p110 appears to require conformation changes in the regulatory subunit p85, which are driven by SH2 domain binding to phosphorylated YXXM motifs. Recently, several additional mechanisms of activation have been described, including the binding of Src family SH3 domains to p85 (10) and the binding of $p21^{ras}$ to p110 (11). In contrast, PI 3′-kinase activity is reduced 80% by phosphorylation of Ser^{608} in p85 and can be restored by treatment with phosphatase 2A (12, 13).

In our previous study (6), we found that activation by mono-phosphorylated YXXM peptides is significantly less potent than activation by the multiply phosphorylated IRS-1; based on these data, we proposed that full activation of PI 3′-kinase requires occupancy of both SH2 domains. This hypothesis has been supported by studies showing that bis-phosphopeptides exhibit enhanced activation of PI 3′-kinase relative to mono-phosphopeptides (8, 14, 15). In the present study, we have directly tested our hypothesis using recombinant PI 3′-kinase containing disabling mutations in the N-terminal, C-terminal, or both SH2 domains. We find that mutation of either SH2 domain reduces phosphopeptide binding to p85 and reduces maximal PI 3′-kinase activation by 50%. Mutation of both SH2

* This work was supported by National Institutes of Health Grant DK-44541 and grants from the Juvenile Diabetes Foundation and the Alexandrine and Alexander L. Sinsheimer Fund (to J. M. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Dept. of Molecular Pharmacology, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461. Tel.: 718-430-2153; Fax: 718-829-8705.

[1] The abbreviations used are: PI 3′-kinase, phosphatidylinositol 3′-kinase; RCM, reduction and carboxymethylation of cysteine residues; IRS-1, insulin receptor substrate-1; P-IRS-1, phosphorylated IRS-1.

domains abolishes both binding and activation. These data demonstrate that full activation of PI 3'-kinase requires occupancy of both SH2 domains in the p85 regulatory subunit.

MATERIALS AND METHODS

Preparation of Recombinant Proteins and Antibodies—Recombinant IRS-1 was produced in Sf-9 cells as previously described (16). Polyclonal anti-p85 antibodies have been previously described (7, 17). Anti-p110 antibodies were raised in New Zealand White rabbits using a glutathione *S*-transferase fusion protein containing residues 1–126 of bovine p110 and purified by affinity chromatography. Monoclonal antibodies to the influenza hemaglutinin epitope tag (12CA5) were obtained from Babco (Berkeley, CA). Glutathione *S*-transferase fusion proteins containing residues 321–724 from the human p85 (18) were produced as previously described (17).

Synthesis of Peptides and Phosphopeptides—Peptides and phosphopeptides were synthesized on a Milligen/Biosearch 9600 synthesizer by Dr. Charles Dahl (Harvard Medical School). Sequences were derived from the cDNA sequence of rat IRS-1 (19) as follows: P-Y608, DDG-(P)YMPMSPGV; P-Y628, NGD(P)YMPMSPKS; and P-Y608/P-Y628, DD(P)YMPMSPGAGAGAGAGAGNGD-Y(P)MPMSPKS. A derivative of P-Y628, BPA-P-Y628, contained benzoylphenylalanine (provided by Dr. J. C. Kauer, Cepalon, West Chester, PA) in the (P)Y+1 position. Peptides were confirmed by amino acid analysis and reverse-phase high pressure liquid chromatography.

Denaturation, Reduction, and Carboxymethylation of IRS-1—IRS-1 from baculovirus-infected Sf-9 cell lysates was phosphorylated by incubation at 4 °C overnight with wheat germ agglutinin-purified human insulin receptors in the presence of 5 mM $MnCl_2$, 100 nM insulin, and 1 mM ATP. Phosphorylated IRS-1 was denatured by incubation with 5.3 M guanidine HCl (final) containing 10 mM dithiothreitol under argon at 50 °C for 4 h. Cysteine residues were carboxymethylated by incubation with 25 mM iodoacetamide for 20 min at 22 °C in the dark, followed by excess β-mercaptoethanol. The denatured, reduced, and carboxymethylated IRS-1 (RCM-IRS-1) was washed five times in a Centricon-30 filter (Amicon) in 50 mM Hepes containing 250 mM NaCl. Concentrations of phosphorylated IRS-1 and RCM-IRS-1 were normalized by blotting with anti-phosphotyrosine antibody.

In Vitro SH2 Domain Binding Assays—^{32}P-Labeled P-Y628 was prepared by incubating a slightly longer version of the Y628 peptide (RKGNDYMPMSPKSV, 1 mM) with insulin receptors in the presence of 50 μM ATP containing 300 μCi [^{32}P]ATP, 10 mM $MnCl_2$, and 1 μM insulin at 4 °C overnight. Receptors were precipitated by the addition of 30% trichloroacetic acid, and the supernatant was spotted onto phosphocellulose paper. After washes in 50 mM phosphoric acid to remove residual [^{32}P]ATP, the peptide was eluted in ammonium bicarbonate and lyophilized. SH2 fusion proteins (100 ng) were incubated for 30 min at 4 °C in the presence of ^{32}P-labeled phosphopeptide in 50 mM Tris, pH 7.4, containing 250 mM NaCl, 0.1% Triton X-100, and 10 mM dithiothreitol (glutathione *S*-transferase buffer). The fusion proteins were immobilized on glutathione-Sepharose at 4 °C and pelleted, and bound ^{32}P-labeled peptide was counted directly. Binding was expressed as a percentage of total radioactivity bound in the absence of unlabeled peptide or IRS-1.

In Vitro Activation of PI 3'-Kinase—Phosphorylated IRS-1 for activation experiments was prepared by incubating IRS-1 (10–20 μg of IRS-1) overnight at 4 °C in the presence of 100 μM ATP, 5 mM $MnCl_2$, 1 μM insulin, and wheat germ agglutinin-purified human insulin receptor (40 μg total protein); the overnight incubation maximized IRS-1 tyrosyl phosphorylation and rendered the receptors incompetent for subsequent activation experiments (6). Tyrosyl-phosphorylated IRS-1 was incubated at the indicated final concentrations with anti-p85/protein A-Sepharose immunoprecipitates from quiescent Chinese hamster ovary cells for 30 min at 4 °C. The anti-p85 immunoprecipitates were additionally washed and assayed for lipid kinase activity as previously described (6, 20). Peptide activation experiments were performed by incubating non-phosphorylated or phosphorylated peptides with washed ap85 immunoprecipitates for 30 min at 4 °C and then assaying for lipid kinase activity as previously described (6, 20).

Mutagenesis and Production of Recombinant Proteins in Sf-9 Cells—The bovine p85 or p110 cDNAs were provided by Dr. M. Waterfield (Ludwig Institute for Cancer Research). The p85 cDNA was subcloned into pGEM9zf(−), and an epitope tag from the influenza virus hemaglutinin was introduced at between amino acids 5–6 using a double-stranded oligonucleotide with *Kpn*I sticky ends. The epitope-tagged p85 was subsequently mutated by the method of Kunkel *et al.* (21), resulting in the substitution of alanine in place of Arg^{358}, Arg^{649}, or both Arg^{358} and Arg^{649}. All mutations were confirmed by sequencing. Wild-type p110 and wild-type and mutant p85 was subcloned into pBluebac III (Invitrogen). Sf-9 cells were co-transfected with the p85 or p110 constructs and Baculogold-linearized baculovirus DNA (Pharmigen). Recombinant virus was isolated as described by the manufacturer, and the production of p85 and p110 in baculovirus-infected Sf-9 cells was measured by immunoblotting with anti-p85, anti-p110, or anti-epitope tag antibody.

Preparation and Activation of Recombinant PI 3'-Kinase—Sf-9 cells, grown in 6-well dishes, were infected with recombinant baculovirus encoding wild-type p110 alone or p110 and wild-type or mutant p85. After 3 days in culture, the cells were washed in ice-cold phosphate-buffered saline and lysed by freeze thawing in 10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 100 μg/ml aprotinin, 1 μg/ml leupeptin, and 350 μg/ml phenylmethylsulfonyl fluoride. After removal of particulate material by centrifugation at 12,000 × *g*, the lysates were assayed directly for PI 3'-kinase activity as described above. Control experiments showed that the lipid activity in lysates from infected Sf-9 cells was completely inhibited by 10 nM wortmannin. Activation experiments were performed as described above, except that IRS-1 was tyrosyl phosphorylated with the baculovirus insulin receptor construct BIRK (22), which eliminates problems due to the presence of detergent in partially purified insulin receptor preparations.

Labeling of Recombinant p85 with ^{125}I-Labeled BPA-P-Y628—BPA-P-Y628 peptide was ^{125}I iodinated using Bolton-Hunter reagent. Recombinant p85/p110 dimers were incubated in the presence of 1.5 μM ^{125}I-labeled BPA-P-Y628 for 30 min at 4 °C, followed by irradiation at 350 nm for 60 min on ice. Proteins were separated by SDS-polyacrylamide gel electrophoresis, and labeled p85 was visualized by autoradiography. Labeling was quantitated using a phosphorimager (Molecular Dynamics) and normalized to the amount of wild-type or mutant p85 in each sample as determined by Western blotting with anti-p85 or anti-epitope tag antibody.

RESULTS

PI 3'-Kinase Binding and Activation by IRS-1—We have previously shown that both phosphorylated IRS-1 and peptides containing phosphorylated YXXM motifs activate PI 3'-kinase *in vitro* (6). Activation of immunopurified PI 3'-kinase by IRS-1 was maximal between 100-1000 nM and half-maximal at approximately 10 nM IRS-1. In contrast, maximal activation by phosphopeptides containing one phosphorylated YXXM motif was not observed until 100 μM peptide. The potent activation of PI 3'-kinase by IRS-1 was likely due to the fact that it contains nine potential YXXM phosphorylation sites, at least four of which have so far been shown to be phosphorylated by the insulin receptor (23). Consistent with this, we have previously shown that peptides derived from six of these nine potential YXXM phosphorylation sites activate PI 3'-kinase *in vitro* (6). Alternatively, conformational features in IRS-1 might be responsible for its enhanced ability to activate PI 3'-kinase relative to mono-phosphorylated peptides. We tested this possibility by comparing PI 3'-kinase activation by native phosphorylated IRS-1 or by IRS-1 that had been phosphorylated followed by denaturation in guanidine HCl and reduction and carboxymethylation of cysteine residues (RCM-IRS-1). Interestingly, activation of PI 3'-kinase by P-IRS-1 and RCM-P-IRS-1 were similar (Fig. 1), suggesting that tertiary structural features of IRS-1 were not required for its interaction with PI 3'-kinase.

PI 3'-Kinase Binding and Activation by Mono- and Bis-phosphorylated YXXM Peptides—Our data suggested that activation of PI 3'-kinase by phosphorylated IRS-1 involved the binding of phosphorylated YXXM motifs to both SH2 domains of the p85 regulatory subunit. This hypothesis predicted that a peptide containing two phosphorylated YXXM motifs would mimic IRS-1 with regard to PI 3'-kinase binding and activation. We therefore synthesized a phosphopeptide of the sequence DD(P)YMPMSP-GAGAGAGAGAGNGDY(P)MPMSPKS (P-Y608/P-Y628), corresponding to the P-Y608 and P-Y628 peptides linked by a 9-amino acid spacer. The resulting peptide positions the phosphotyrosine residues 20 amino acids apart, corresponding to their position in

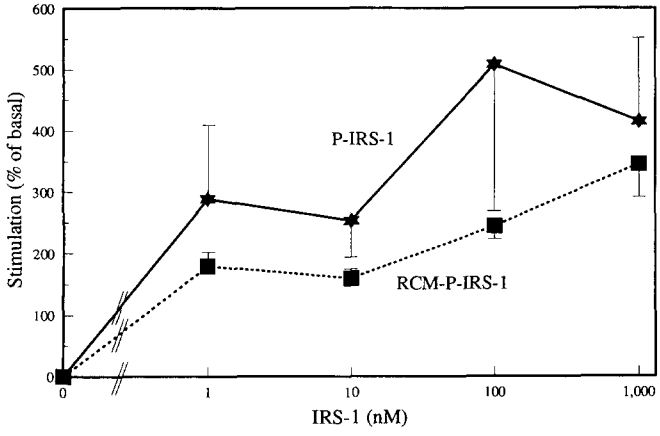


FIG. 1. **PI 3′-kinase activation by wild-type and denatured IRS-1.** Native P-IRS-1 or denatured RCM-P-IRS-1 was incubated with immunopurified PI 3′-kinase for 30 min at 4 °C. The immunoprecipitates were washed and assayed for PI 3′-kinase activity as described. All determinations were done in triplicate, and the data are the mean ± S.E. from three experiments.

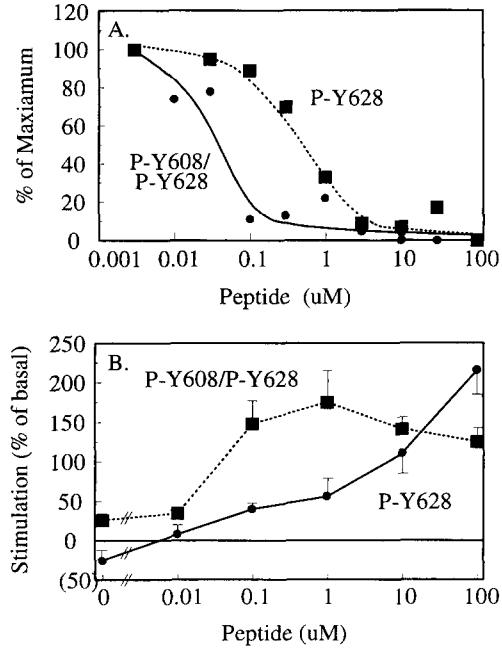


FIG. 2. **PI 3′-kinase binding and activation by mono- and bis-phosphorylated *YXXM* peptides.** *A*, a glutathione *S*-transferase fusion protein containing both SH2 domains from p85 was incubated with ^{32}P-labeled P-Y628 in the presence of the indicated concentrations of unlabeled phosphorylated mono-phosphorylated (P-Y628) or bis-phosphorylated (P-Y608/P-Y628) peptide for 30 min at 4 °C. The fusion protein was immobilized on glutathione-Sepharose, the beads were pelleted, and bound radioactivity was measured by Cerenkov counting. The data are representative of two separate experiments. *B*, PI 3′-kinase, immunopurified from quiescent Chinese hamster ovary cells with anti-p85 antibody and protein A-Sepharose, was incubated with mono-phosphorylated (P-Y628) or bis-phosphorylated (P-Y608/P-Y628) YXXM peptide at the indicated concentrations for 30 min at 4 °C. PI 3′-kinase was assayed as described in the continued presence of the peptide. All determinations were done in triplicate, and the data are the mean ± S.E. from three experiments.

the primary structure of IRS-1 (19). The relative binding affinities of P-Y628 and P-Y608/P-Y628 for p85 SH2 domains was determined by incubating varying concentrations of unlabeled phosphopeptides with ^{32}P-labeled P-Y628 and a p85 SH2 fusion protein. Half-maximal displacement of ^{32}P-labeled P-Y628 by P-Y608/P-Y628 was observed at approximately 50 nM peptide, as compared with approximately 500 nM for P-Y628 (Fig. 2*A*). Moreover, *in vitro* activation of PI 3′-kinase by P-Y608/P-Y628 was half-maximal at approximately 30 nM peptide, whereas comparable activation by P-Y628 was not seen until 10–100 μM peptide (Fig. 2*B*); activation by a mono-phosphorylated peptide containing the P-Y608 sequence was identical to that obtained with the P-Y628 peptide (data not shown).

Phosphopeptide Binding and PI 3′-Kinase Activity of Mutant p85/p110 Dimers—Bis-phosphopeptides exhibit increased binding to a glutathione *S*-transferase fusion protein containing the N- and C-terminal SH2 domains of p85 and show enhanced activation of PI 3′-kinase relative to mono-phosphopeptides (Fig. 2). These data confirm our earlier hypothesis that the potent activation of PI 3′-kinase by IRS-1 was due to its multiple tyrosine phosphorylation sites. However, these data alone do not prove that full activation of PI 3′-kinase requires the occupancy of both SH2 domains, as they can be explained by two distinct models (Fig. 3). In the first model (Fig. 3*A*), activation of PI 3′-kinase is mediated solely by occupancy of a single SH2 domain. Although the model is depicted with a single defined activating site, the same analysis could be applied to the hypothesis that occupancy of either SH2 domain fully activates PI 3′-kinase. In contrast, the second model (Fig. 3*B*) requires that simultaneous occupancy of both SH2 domains is required for full activation of PI 3′-kinase. In either case, bis-phosphopeptides bind with increased affinity as compared with mono-phosphopeptides, due to their bivalent interactions with the two SH2 domains of p85. This increased binding affinity would lead to an increased occupancy of a single activating site, as shown in Fig. 3*A*, or a pair of activating sites, as shown in Fig. 3*B*. Thus, both models predict that bis-phosphopeptides will activate with enhanced potency relative to mono-phosphopeptides and cannot be distinguished on this basis.

To differentiate these two models, we produced recombinant PI 3′-kinase by co-infection of Sf-9 cells with baculovirus constructs coding for p110 plus wild-type p85, or p85 containing disabling mutations in the N-terminal, C-terminal, or both

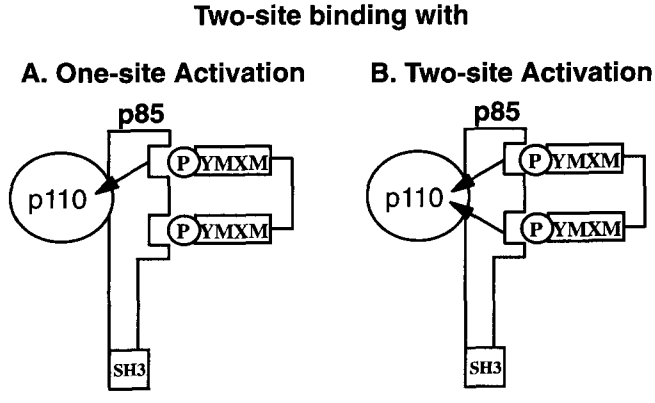
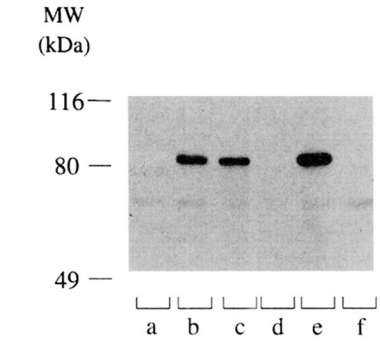


FIG. 3. **Model of enhanced PI 3′-kinase activation by bis-phosphopeptides.** *A*, activation of PI 3′-kinase by tyrosyl phosphoproteins requires the occupancy of only the upper SH2 domain. However, the interaction of the bis-phosphopeptide with the two SH2 domains increases its net binding affinity and leads to an increased occupancy of the single activating site. Occupancy of this second site does not itself add to the magnitude of activation. *B*, activation of PI 3′-kinase requires occupancy of both SH2 domains. Partial activation occurs when either SH2 domain is occupied, and full activation occurs only when both sites are occupied.

SH2 domains. In the mutant p85 constructs, the arginine residue in the invariant FLVRES motif was mutated to alanine (Arg^{358} and Arg^{649} in the N- and C-terminal SH2 domains, respectively). This substitution has been shown to inhibit phosphopeptide binding to the Abl SH2 domains without causing



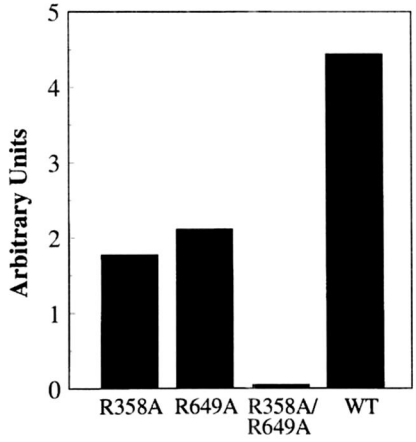


FIG. 4. **Labeling of recombinant p85 with ^{125}I-labeled BPA-P-Y628.** Lysates from control or baculovirus-infected SF-9 cells were incubated with ^{125}I-labeled BPA-P-Y628 for 30 min at 4 °C, followed by irradiation at 350 nm for 60 min on ice. The proteins were separated by SDS-polyacrylamide gel electrophoresis (12.5% resolving) and visualized by autoradiography. Labeling of p85 was quantitated using a Molecular Dynamics phosphorimager. *Top panel*: *lane a*, control cells; *lane b*, p110 plus mutant p85 (R358A); *lane c*, p110 plus mutant p85 (R649A); *lane d*, p110 plus mutant p85 (R358A/R649A); *lane e*, p110 plus wild-type p85; *lane f*, p110 alone. *Bottom panel*, labeling of p85 in Sf-9 lysates was normalized for the expression of p85, as determined by immunoblotting with anti-p85 antibody. *WT*, wild type.

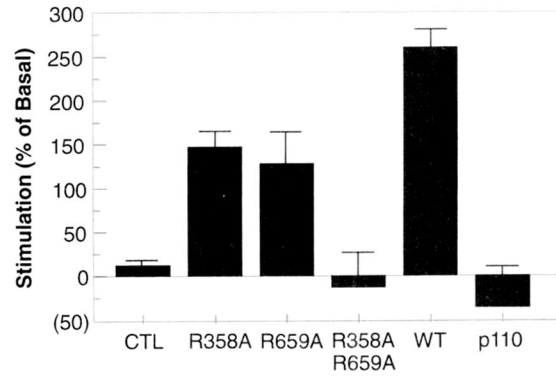


FIG. 5. **Activation of recombinant PI 3′-kinase by IRS-1 and phosphopeptide.** Sf-9 cells were infected with baculovirus encoding p110 alone or in combination with wild-type or mutant p85. Lysates were prepared from control cells (*CTL*), cells infected with p110 alone (p110), or cells co-infected with p110 and the N-terminal SH2 p85 mutant (R358A), the C-SH2 mutant p85 (R659A), the double-SH2 p85 mutant (R358A/R659A), or wild-type p85 (*WT*). Lysates were incubated with tyrosyl-phosphorylated IRS-1 (100 nM) for 30 min at 4 °C and then assayed for PI 3′-kinase activity. All determinations were done in duplicate, and the data are the mean ± S.E. from four experiments.

global structural changes (24). If the model in Fig. 3*A* is correct, then maximal activation of recombinant PI 3′-kinase containing one or two functional SH2 domains should be equivalent. If the model in Fig. 3*B* is correct, then the magnitude of PI 3′-kinase activation at saturating levels of P-IRS-1 or phosphopeptide should be reduced in the constructs containing a single functional SH2 domain.

Wild-type and mutant p85 was co-expressed in Sf-9 cells with wild-type p110. All of the mutants were expressed at similar levels and were shown by co-immunoprecipitation experiments to form stable dimers with p110. The ability of recombinant wild-type and mutant p85 to bind to tyrosyl phosphopeptides was determined using an ^{125}I-labeled analog of P-Y628 that contains the photo-activable amino acid benzoylphenylalanine in the Y+1 position (Fig. 4). Cross-linking of p85 SH2 domains by ^{125}I-labeled BPA-P-Y628 was completely inhibited by 100 μM unlabeled P-Y628 and was therefore specific (data not shown). Incubation of ^{125}I-labeled BPA-P-Y628 with SF-9 cell lysates containing wild-type p85, followed by irradiation at 350 nm for 60 min at 4 °C, lead to the labeling of a single 85-kDa band (Fig. 4, *lane e*). No labeling was observed in lysates from control Sf-9 cells or cells expressing p110 alone (Fig. 4, *lanes a* and *f*). Cross-linking of p85 was significantly reduced in lysates from Sf-9 cells producing N- or C-terminal SH2 p85 mutants (Fig. 4, *lanes b* and *c*, respectively) and was barely detectable in lysates containing p85 mutated at both SH2 domains (Fig. 4, *lane d*). When the data were normalized to the amount of p85 in each lysate by immunoblotting with anti-p85 antibody, mutation of either the N- or C-terminal SH2 domains reduced phosphopeptide cross-linking by 50%. However, these data do not take into account possible differences in the binding affinity of the N- and C-terminal SH2 domains in intact p85 nor the possible differential yields of covalent labeling of each SH2 domain upon photolysis, and a comparison of the relative decrease in binding to the different single mutants is therefore only approximate.

We then examined PI 3′-kinase activation in lysates from cells infected with baculovirus constructs producing wild-type p110 and wild-type or mutant p85 (Fig. 5). Incubation of lysates containing wild-type p85/p110 dimers with saturating concentrations of tyrosyl-phosphorylated IRS-1 (100 nM) lead to a 3.5-fold activation of PI 3′-kinase. In contrast, no activation was detected in control Sf-9 lysates or in lysates containing only p110. Mutation of either the N- or C-terminal SH2 domain of p85 reduced activation by 50%, whereas mutation of both domains completely eliminated activation by P-IRS-1.

DISCUSSION

SH2 domains have been implicated in the regulation of an increasing number of proteins involved in signal transduction by growth factors and oncogene products (25). For the PI 3′-kinase, the binding of its SH2 domains to phosphorylated IRS-1 or peptides containing phosphorylated Y*XX*M motifs can directly regulate its activity (6). We and others (6, 8, 14, 15) have shown that peptides or proteins containing multiple phosphotyrosine residues bind and activate PI 3′-kinase with enhanced potency relative to mono-phosphopeptides. However, these studies cannot differentiate between a mechanism that requires two-site occupancy for maximal activity as opposed to one in which bivalent binding enhances the occupancy of a single activating site. In the present study, we show that point mutations in either the N- or C-terminal SH2 domains of p85 inhibit phosphopeptide binding to p85 and reduce the activation of PI 3′-kinase by IRS-1 by 50%. These data are the first direct demonstration that full PI 3′-kinase activation by tyrosyl phosphoproteins requires occupancy of both SH2 domains.

As we have previously noted, a number of the receptors that activate PI 3′-kinase have pairs of tyrosine phosphorylation

sites that may be involved in the bivalent binding of p85 SH2 domains (6). Thus, the kinase insert region of the platelet-derived growth factor β-receptor contains two tyrosine phosphorylation sites, both of which are important for PI 3′-kinase binding (26–29). Recent studies (8, 30) have also suggested that two phosphotyrosine residues are required for activation of PI 3′-kinase by the hepatocyte growth factor receptor and polyoma middle T antigen. Mutation of Tyr^{721} but not Tyr^{697} or Tyr^{706} within the colony stimulating factor-1 receptor kinase insert domain blocks PI 3′-kinase binding (30); however, activation of PI 3′-kinase was not examined and may require an additional phosphotyrosine residue. In contrast, *in vitro* activation of PI 3′-kinase by a peptide containing the motif Y^{1322}THM from the insulin receptor C terminus is unaffected by the phosphorylation of the neighboring Tyr^{1316} (31). The ability of a second phosphotyrosine residue to augment the activation by a phosphorylated YXXM motif presumably depends on the primary sequence surrounding the second phosphotyrosine residue or perhaps the distance between the residues. In this regard, the optimal spacing between phosphotyrosine residues for PI 3′-kinase activation has not been determined. The two phosphotyrosine residues in the IRS-1-derived peptide examined here are 20 amino acids apart, whereas the phosphotyrosine residues in activating peptides derived from polyoma middle T and the hepatocyte growth factor receptor are 7 residues apart; those in the insulin receptor bis-phosphopeptide, which does not show enhanced activation relative to monophosphopeptides, are only 6 residues apart.

Little is known about the native tertiary structure of p85, but these observations suggest that the phosphopeptide binding sites of the p85 SH2 domains must lie quite close to each other. The demonstration that p110 binds to a region between the two SH2 domains of p85 (32–34) suggests an intriguing model in which occupancy of both SH2 domains causes a conformational change in the intervening region and activates p110. In contrast, a recent report showed that a construct containing either SH2 domain plus the intervening region could bind to the p110 catalytic subunit and mediate phosphopeptide-stimulated activation (35); it is not clear how the structure of this truncated construct relates to that of the intact p85 molecule. The resolution of these questions must await a better structural characterization of the intact regulatory and catalytic subunits of PI 3′-kinase.

Acknowledgments—We thank Dr. Charles Dahl (Harvard Medical School) for his assistance with phosphopeptide synthesis, Dr. James C. Kauer for his gift of benzoylphenylalanine, Dr. Michael Waterfield for providing the p85 and p110 constructs, and Drs. Joseph Schlessinger and Patrick Hu for providing the p85 glutathione *S*-transferase fusion proteins and the anti-p85 antibody used in the activation experiments.

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