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Phosphatidylinositol 3-Kinase Activation Is Mediated by High-Affinity Interactions between Distinct Domains within the p110 and p85 Subunits

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Domains of interaction between the p85 and p110 subunits of phosphatidylinositol 3-kinase (PI 3-kinase) were studied with the yeast two-hybrid expression system. A gene fusion between the GAL4 transactivation domain and p85 activated transcription from a *GAL1-lacZ* reporter gene when complemented with a gene fusion between the GAL4 DNA binding domain and p110. To define subdomains responsible for this interaction, a series of p85 deletion mutants were analyzed. A 192-amino-acid inter-SH2 (IS) fragment (residues 429 to 621) was the smallest determinant identified that specifically associated with p110. In analogous experiments, the subdomain within p110 responsible for interaction with p85 was localized to an *Eco*RI fragment encoding the amino-terminal 127 residues. Expression of these two subdomains [p85(IS) with p110RI] resulted in 100-fold greater reporter activity than that obtained with full-length p85 and p110. Although the p85(IS) domain conferred a strong interaction with the p110 catalytic subunit, this region was not sufficient to impart phosphotyrosine peptide stimulation of PI 3-kinase activity. In contrast, coexpression of the p110 subunit with full-length p85 or with constructs containing the IS sequences flanked by both SH2 domains of p85 [p85(n/cSH2)] or either of the individual SH2 domains [p85(nSH2+IS) or p85(IS+cSH2)] resulted in PI 3-kinase activity that was activated by a phosphotyrosine peptide. These data suggest that phosphotyrosine peptide binding to either SH2 domain generates an intramolecular signal propagated through the IS region to allosterically activate p110.

Several growth factors mediate their pleiotropic responses by activation of the intrinsic tyrosine-specific protein kinase activities of cell surface receptors (3, 41). Tyrosine autophosphorylation of these receptors at specific sites generates recognition signals for interaction with other downstream effector molecules, such as GTPase-activating protein for Ras (17, 19), Src family protein-tyrosine kinases (23), phospholipase Cγ (26, 27), phosphotyrosine protein phosphatases (13), and phosphatidylinositol 3-kinase (PI 3-kinase) (1, 9, 18). These effector molecules have in common the presence of *src* homology 2 (SH2) domains, which are specific for high-affinity binding to phosphorylated tyrosine residues within distinct peptide sequences. In addition, many SH2 domain proteins also contain *src* homology 3 (SH3) domains, which are specific for interactions with other signaling and/or structural molecules (21, 22).

PI 3-kinase appears to be a downstream effector of numerous tyrosine kinase growth factor receptors (3). The PI 3-kinase holoenzyme is composed of an 85-kDa regulatory subunit (p85) and a 110-kDa catalytic subunit (p110) (5, 12, 16, 29, 32, 39). Sequence analysis of the p85 subunit has revealed several recognition sequence motifs, including an SH3 domain, a region related to the breakpoint cluster region gene (*bcr*), and two SH2 domains. The interaction of the p85 SH2 domains with tyrosine-phosphorylated proteins and/or peptides was recently shown to stimulate the PI 3-kinase activity of the p110 subunit catalytic subunit (2, 4, 30, 38).

SH3 motifs have been suggested to mediate interactions with cytoskeleton components and with proline-rich regions (7, 21, 35), such as those found in guanylnucleotide exchange factors (11, 15, 24, 25, 34). It has also been suggested that the *bcr* domain may be involved in interactions with small GTP binding proteins (7). However, the specific sites within p85 and p110 that are necessary for a physical interaction between the two subunits have not been identified. In this study, we used the yeast two-hybrid system to identify the specific sites of physical interaction between the p85 and p110 subunits of bovine PI 3-kinase. In addition, we tested the ability of the full-length p85 subunit and a series of p85 deletion mutants to transmit the allosteric phosphotyrosine peptide binding signal to the p110 catalytic subunit.

MATERIALS AND METHODS

Materials. Yeast strain Y526 (*MATα ura3-52 his3-Δ200 ade2-101 lys2-801 trp1-901 leu2-3,112 can^r gal4-542 gal80-538 URA3::GAL1-lacZ*) and the yeast two-hybrid expression vectors pGBT9 and pGAD424 were provided by Paul Bartel and Stanley Fields (State University of New York at Stony Brook). The full-length p85 and p110 subunit cDNAs of bovine PI 3-kinase [in Bluescript(I) SK−] were generous gifts from Michael Waterfield (Ludwig Institute for Cancer Research, London, England). A rabbit polyclonal antibody directed against the transactivation domain of GAL4 was provided by James Hopper (Hershey Medical School, Hershey, Pa.). The tyrosine-phosphorylated peptide corresponding to the 12 residues surrounding y628 of the IRS-1 protein [GNGDY(PO_4)MPMSPKS] was synthesized by Chiron Mimotopes (Emeryville, Calif.). Oligonucleotides were

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TABLE 1. Various GAD-p85 fusion proteins[a]

Subdomain	Sequence (amino acids)	5′ Primer	3′ Primer
p85(SH3)	9–87	A	B
p85(*bcr*)	77–353	C	D
p85(nSH2)	330–434	E	F
p85(n/cSH2)	330–720	E	G
p85(cSH2)	621–720	H	G
p85(IS)	429–621	I	J
p85(nSH2+IS)	330–621	E	J
p85(IS+cSH2)	429–720	I	G
GCN4 bZIP	225–281	K	L

[a] The fusion proteins were generated by polymerase chain reaction amplification by use of the designated primers and then in-frame insertion of the subdomain into the polylinker of the GAD expression vector. The native amino acid residues which correspond to each subdomain are listed. The primers (5′ to 3′) were as follows: A, gccgaggggtacgaattccgggcgctg; B, tcga gggatccggggctatggtgt; C, ggaacttacgtagaattcattgg; D, cctcgggatccctcaccagta cca; E, aatccgcggatggaattcgatgctgaatggtactgg; F, aactggatcctgttggtatttggatac tgg; G, atcgcctcggatccgcgtacactgggtagg; H, aatccgcggatggaattcgagaagacttg gaatgttgg; I, ctttatccagaattcaaataccaacag; J, caacattccggatcctctcatcgtggtg; K, cccgaattcagtgatcctgctgctcta; and L, cgggtcgacctagttaacgcgttcgccaactaatttctt.

prepared by the Diabetes & Endocrinology Research Center (The University of Iowa, Iowa City).

Yeast methods. Yeast strain Y526 has deletions of GAL4 and GAL80 and carries the *GAL1-lacZ* reporter gene integrated at the *URA3* locus. This strain was used to assay the constructs described here. The cells were transformed by the high-efficiency method of Schiestl and Gietz (36), and transformants were selected on 2% sucrose minimal plates lacking the appropriate amino acids (tryptophan and leucine).

Expression plasmids. The full-length p110 cDNA was subcloned into the yeast two-hybrid expression vector pGBT9, encoding the GAL4 DNA binding domain (GBT). The full-length p85 cDNA was subcloned into the yeast two-hybrid expression vector pGAD424, encoding the GAL4 transcriptional activation domain (GAD). These fusion proteins (GBT-p110 and GAD-p85) were generated by use of convenient restriction sites which maintained the appropriate open reading frames. The GBT-p110BX and GBT-p110RI constructs were prepared by isolation of the amino-terminal *Bam*HI-*Xba*I fragment of p110 (corresponding to amino acid residues 1 to 576) and the *Eco*RI fragment of p110 (corresponding to amino acids 1 to 127), respectively, and then in-frame insertion into the polylinker of GBT. The GBT-p110ΔRI clone was an amino-terminal deletion of 127 amino acids of p110 obtained by digestion of the GBT-p110 construct with *Eco*RI and then in-frame religation.

The various subdomain clones of GAD-p85 were prepared by polymerase chain reaction amplification of the domains of interest by use of specific primers (Table 1) designed with *Eco*RI and *Bam*HI sites in the 5′ and 3′ primers, respectively. The fragments were then inserted in frame into the polylinker of GAD. The bZIP (basic leucine zipper) dimerization domain of GCN4 was also prepared by polymerase chain reaction (Table 1) and inserted in frame into the *Eco*RI-*Sal*I sites of the polylinkers of GBT and GAD. The fusion junctions were verified by sequencing with the primers 5′ ctattcgatgatgaagatacc 3′ and 5′ tcatcggaagagagtag 3′, which were complementary for GAD and GBT, respectively.

Reporter activity. β-Galactosidase reporter activity was determined by plate or liquid culture assays. Plate assays were performed as previously described (10), except that individual colonies of transformed cells were streaked onto fresh plates, allowed to grow for 2 days, and then replicated onto Whatman filter paper circles. The filters were immersed in liquid nitrogen for 10 s, allowed to thaw briefly, and then immersed again. Each filter was then placed onto a filter circle saturated with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) solution in a petri dish (permeabilized cells up) and incubated overnight at 30°C. Liquid culture assays were performed as previously described (14) but with the following modifications. Individual colonies of transformed cells were grown overnight in 3% raffinose minimal medium lacking the appropriate amino acids (tryptophan and leucine). The optical density at 600 nm was determined, and 1.5 ml of the cell culture was permeabilized with sodium dodecyl sulfate (SDS)-chloroform and assayed for the optical density at 574 nm with the chromogenic substrate CPRG (chlorophenol red-β-D-galactopyranoside). One unit of β-galactosidase activity was defined as $(A_{574} \times 1{,}000)/[A_{600} \times$ volume (in milliliters) $\times$ time (in minutes)] as described by Miller (28).

GAL4 immunoblotting. Transformed yeast cells from 50-ml cultures (1.0 optical density unit at 600 nm) grown in 3% raffinose minimal medium lacking the appropriate amino acids were pelleted, washed, resuspended in 20% trichloroacetic acid, and vortexed with acid-washed glass beads at 4°C (five times at 30 s each time). The trichloroacetic acid precipitates were neutralized with acetone-ammonium hydroxide (5:0.3), and the pellets were boiled in 1% SDS. Protein concentrations were quantified with the bicinchoninic acid protein assay reagent (Pierce), and 100 μg of protein per sample was fractionated by SDS–8% polyacrylamide gel electrophoresis. The fusion proteins were visualized by immunoblotting with a 1:1,000 dilution of a rabbit GAL4 antibody and then a goat anti-rabbit polyclonal antibody conjugated to alkaline phosphatase (37).

PI 3-kinase assays. PI 3-kinase activity was determined as previously reported (43) but with the following modifications. Overnight cultures of transformed yeast cells were pelleted, washed in sterile distilled H_2O, and resuspended in 1% Triton X-100 lysis buffer with protease and phosphatase inhibitors. The resuspended cells were vortexed with 0.3 g of acid-washed glass beads at 4°C (five times at 30 s each time). The insoluble material was pelleted at 100,000 $\times$ *g* for 60 min, and the supernatant was mixed overnight with the GAL4 antibody. Protein A-Sepharose (50 μl per sample) was added, the mixture was allowed to mix for 2 h, and the resultant immune complexes were washed and assayed for PI 3-kinase activity as described previously (40). The reaction mixture was separated by one-dimensional thin-layer chromatography and then autoradiography. The effect of a phosphotyrosine peptide (P-Y628) on PI 3-kinase activity was determined by the addition of the [phosphopeptide (150 μM) 30 min prior to the addition of [γ-^{32}P]ATP (2). As a control, immune complexes were incubated with a nonphosphorylated peptide (Y628) under identical assay conditions.

RESULTS

Specificity of the yeast two-hybrid expression system. To identify the specific protein-protein interactions responsible for the high-affinity binding of the p85 regulatory subunit to the p110 catalytic subunit of PI 3-kinase, we prepared several fusion constructs with GAD and GBT. In this system, protein domains that specifically interact will reconstitute GAL4 transcriptional activity, which is readily de-

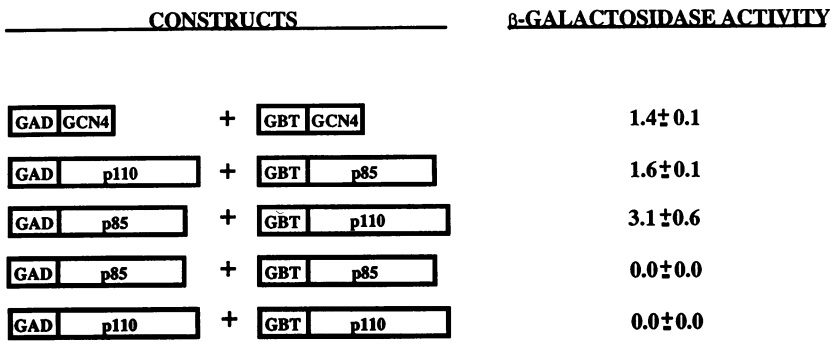

FIG. 1. Specificity of the yeast two-hybrid system for detecting interactions between the p85 and the p110 subunits of PI 3-kinase. The top constructs represent fusion genes between the bZIP dimerization domain of GCN4 and GAD (GAD-GCN4) or GBT (GBT-GCN4). The p85 and p110 fusion genes were prepared by use of convenient restriction sites and insertion of the full-length genes in frame with the GAL4 coding sequences. Transformed cells were grown in liquid minimal medium culture and assayed for β-galactosidase expression as described in Materials and Methods. β-Galactosidase activity was expressed in units according to Miller (28). All data represent the mean ± standard deviation for at least three independent assays performed on individual colonies.

tectable in a yeast reporter strain containing *lacZ* under the control of the *GAL1* upstream activation sequence (6, 14). Coexpression of the full-length p85 subunit fused to GAD (GAD-p85) with the full-length p110 subunit fused to GBT (GBT-p110) resulted in 3.1 U of β-galactosidase activity (Fig. 1). As a positive control, the coexpression of GAD and GBT as fusion genes with the bZIP dimerization domain of the yeast transcriptional activator GCN4 also resulted in the induction of β-galactosidase activity. In contrast, the coexpression of GAD-p85 with GBT-p85 or GAD-p110 with GBT-p110 did not produce detectable levels of reporter activity (Fig. 1). Thus, a specific interaction between the PI 3-kinase p85 and p110 subunits reconstituted GAL4 transcriptional activity. Since the combination of the GAD-p85 and GBT-p110 fusion constructs consistently produced a slightly higher level of induction of β-galactosidase activity than the combination of GAD-p110 and GBT-p85 (3.1 versus 1.6 U of activity), all subsequent cloning strategies with the p85 and p110 deletion mutants were designed to place the p85 subdomains into the GAD vector and the p110 subdomains into the GBT vector.

Identification of the p85 domain responsible for the p110 interaction. To define the region(s) within the p85 subunit responsible for binding to the p110 subunit, a series of GAD-p85 subdomains were coexpressed with the full-length GBT-p110 fusion (Fig. 2). As observed in Fig. 1, the induction of β-galactosidase activity was observed when GAD-p85 and GBT-p110 were coexpressed. In contrast, the coexpression of GBT-p110 with the GAD-p85(SH3), GAD-p85(*bcr*), GAD-p85(nSH2), or GAD-p85(cSH2) domain had no effect on β-galactosidase activity. However, cotransformation of the yeast reporter strain with GBT-p110 and GAD-p85(n/cSH2), which consisted of the inter-SH2 (IS) region plus the two flanking SH2 domains, resulted in the induction of β-galactosidase activity. Since the coexpression of each individual p85 SH2 domain with GBT-p110 was

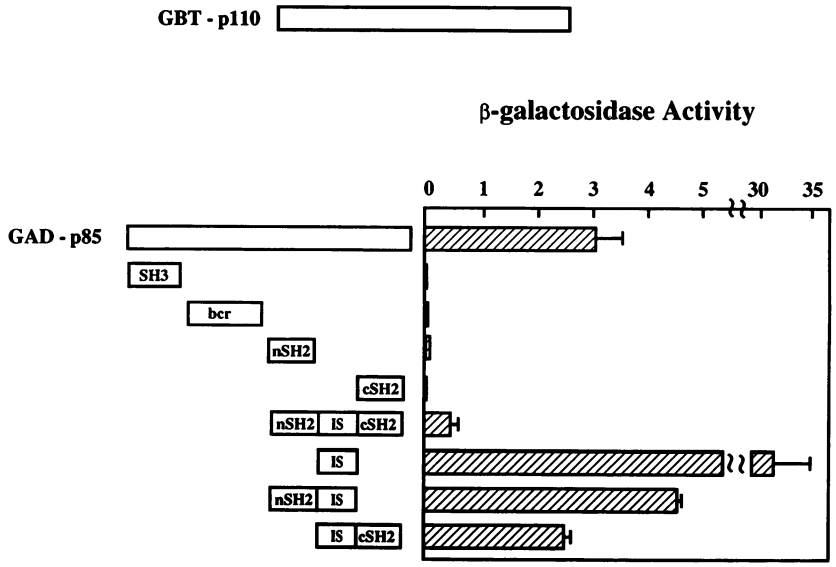

FIG. 2. Identification of p85 subdomains that interact with the full-length p110 protein. The GBT-p110 construct was coexpressed with the full-length GAD-p85 construct and a series of GAD-p85 deletion mutant expression plasmids as described in Table 1. Transformed cells were grown in liquid culture, and β-galactosidase activity units were determined as described in Materials and Methods. All data represent the mean ± standard deviation for at least three independent assays performed on individual colonies.

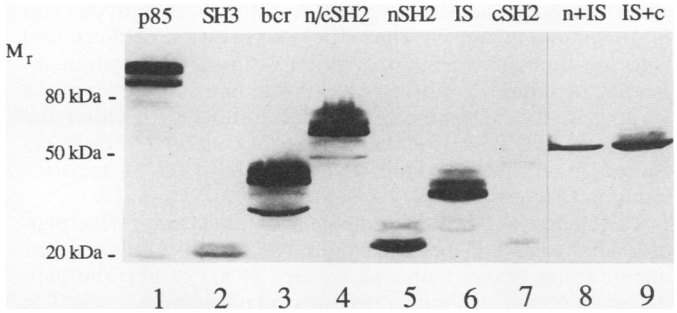

FIG. 3. Immunoblot detection and relative levels of expression of GAD-p85 fusion proteins. Transformed cells were grown in liquid minimal medium culture and lysed by disruption with glass beads, and total cellular proteins were precipitated with trichloroacetic acid. The precipitated proteins were denatured, resolved on an SDS–8% polyacrylamide gel, and transferred to nitrocellulose. The GAD-p85 fusion proteins were immunoblotted with a GAL4 polyclonal antibody and incubated with an alkaline phosphatase-conjugated goat anti-rabbit polyclonal antibody as described in Materials and Methods. All lanes were loaded with 100 μg of total cellular protein.

unable to induce β-galactosidase activity, we next examined the ability of the p85 IS domain-GAD fusion to interact with the p110 subunit. The GAD-p85(IS) construct induced approximately 10-fold-higher levels of β-galactosidase activity than the full-length GAD-p85 construct when coexpressed with the full-length GBT-p110 fusion (Fig. 2). A single SH2 domain, either the nSH2 or cSH2 domain, in the presence of the IS region markedly attenuated the high level of β-galactosidase activity observed with the IS region alone [compare the GAD-p85(nSH2+IS) and GAD-p85(IS+cSH2) constructs with the GAD-p85(IS) construct in Fig. 2].

Levels of expression of the GAD-p85 fusion constructs. To ascertain whether the different levels of expressed β-galactosidase activity with the various GAD-p85 fusion constructs were due to discrete alterations in transcriptional activity or due to dissimilar levels of protein expression, we next performed a Western blot (immunoblot) analysis of total yeast cell lysates (Fig. 3). The GAD-specific antibody identified two major proteins in extracts from cells expressing the full-length GAD-p85 fusion protein (lane 1). The size of the larger protein (M_r, ~100,000) is consistent with the GAD moiety of 15 kDa plus the p85 subunit of 85 kDa, and that of the smaller protein (M_r, ~90,000) is consistent with a partial degradation product. Similarly, a Western blot analysis of extracts from all the GAD-p85 fusion proteins (lanes 2 to 9) demonstrated multiple species consistent with the presence of the appropriately sized fusion protein in addition to smaller partial degradation products. Although most of the GAD-p85 fusion proteins were expressed at levels similar to those of the full-length GAD-p85 construct, the GAD-p85(SH3) and GAD-p85(cSH2) proteins (lanes 2 and 7) were consistently observed to be expressed at somewhat reduced levels compared with the other constructs. Nevertheless, all the constructs which specifically interacted with GBT-p110 were expressed at essentially identical levels. Thus, these data directly demonstrate that the 10-fold-higher level of activation of the β-galactosidase reporter gene by the IS domain (lane 6) of the p85 subunit compared with the full-length p85 protein (lane 1) cannot be ascribed to differences in protein expression. Therefore, the differences in reporter activity most likely reflect a specific increase in the level of binding of the IS domain to the full-length p110 subunit.

Identification of the p110 domain responsible for the p85 interaction. By analogy to the GAD-p85 deletion constructs, we also defined the specific p110 subunit domain responsible for binding to the full-length p85 subunit (Fig. 4). In this analysis, the full-length GAD-p85 fusion was coexpressed with various fragments of the p110 subunit fused to the GBT expression plasmid. A p110 carboxy-terminal deletion mutant which consisted of residues 1 to 576 (GBT-p110BX) induced β-galactosidase activity to levels similar to those induced by the full-length GBT-p110 construct when coexpressed with GAD-p85 (Fig. 4). A smaller p110 deletion

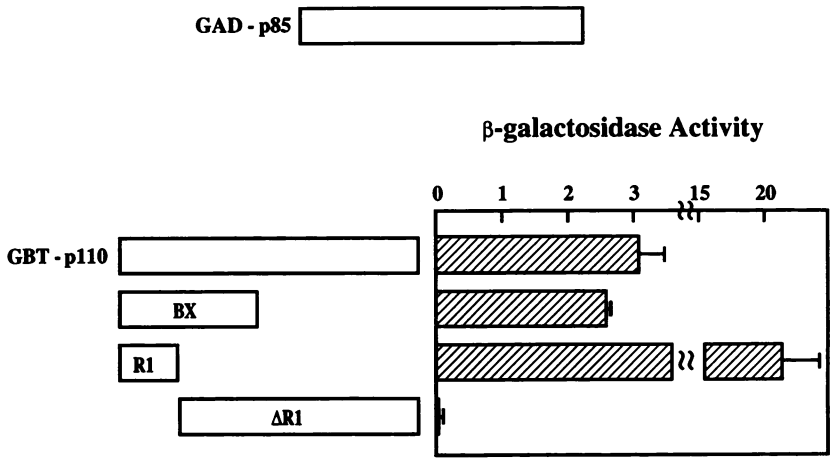

FIG. 4. Identification of p110 subdomains that interact with the full-length p85 protein. The GAD-p85 construct was coexpressed with the full-length GBT-p110 construct and a series of GBT-p110 deletion mutants. The GBT-p110 deletion mutant constructs were prepared by use of convenient restriction sites and insertion of the fragments in frame with the GBT sequence as described in Materials and Methods. The GBT-p110BX construct was an amino-terminal *Bam*HI-*Xba*I fragment of p110 corresponding to residues 1 to 576. The GBT-p110RI construct was an amino-terminal *Eco*RI fragment of p110 corresponding to residues 1 to 127. The GBT-p110ΔRI construct was the full-length p110 protein minus the amino-terminal *Eco*RI fragment. Cotransformed cells were grown in liquid minimal medium culture and assayed for β-galactosidase activity. All data represent the mean ± standard deviation for at least three independent assays performed on individual colonies.

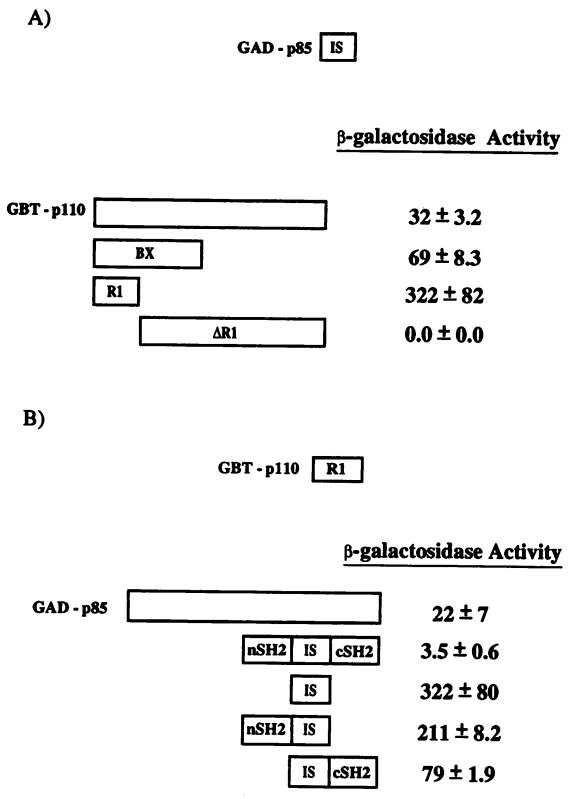

FIG. 5. Synergistic activation of β-galactosidase activity by coexpression of GAD-p85(IS) with GBT-p110RI. (A) The GAD-p85(IS) construct was coexpressed with full-length GBT-p110 and three p110 deletion mutants as indicated. (B) The GBT-p110RI construct was coexpressed with full-length GAD-p85 and the p85 deletion mutants as indicated. Transformed cells were assayed for β-galactosidase activity as described in Materials and Methods. Data represent the mean ± standard deviation for three independent assays.

mutant which contained residues 1 to 127 (GBT-p110RI) was constructed, and this subdomain was observed to activate the reporter gene to a substantially higher level (~sevenfold) than the full-length GBT-p110 construct when coexpressed with GAD-p85. As a control, the GBT-p110ΔRI construct, which lacked the amino-terminal 127 residues, was unable to induce β-galactosidase activity when coexpressed with GBT-p110 (Fig. 4).

Expression of the p85 IS domain with the amino-terminal p110 domain synergistically activates β-galactosidase activity. Since the p85 IS domain [GAD-p85(IS)] and the amino-terminal p110 domain (GBT-p110RI) were the functional units responsible for the physical interaction, we next examined the effect of coexpression of the GAD-p85(IS) fusion with the GBT-p110RI fusion (Fig. 5). As previously observed, the GAD-p85(IS) fusion coexpressed with full-length GBT-p110 resulted in an approximately 10-fold-higher level of induction of β-galactosidase activity than that obtained with the full-length GAD-p85 construct (Fig. 2). Similarly, the GBT-p110RI fusion coexpressed with full-length GAD-p85 resulted in a 6-fold-higher level of induction of the reporter gene than that obtained with the full-length GBT-p110 construct (Fig. 4). However, the coexpression of both subdomains together [GAD-p85(IS) with GBT-p110RI] induced a 100-fold-higher level of transactivation of β-galactosidase activity than that obtained with the full-length p85 and p110 subunits (Fig. 5). Thus, the coexpression of these two specific domains resulted in synergistic transactivation directly attributed to an increase in binding interactions. As expected, the coexpression of GBT-p110RI with either the GAD-p85(nSH2+IS) or the GAD-p85(IS+cSH2) fusion resulted in an intermediate level of activation of reporter activity (Fig. 5B).

Regulation of PI 3-kinase activity by phosphotyrosine peptide binding to p85 deletion mutants. Previous studies documented that tyrosine-phosphorylated IRS-1 or phosphopeptides which correspond to residues surrounding tyrosine 628 of the IRS-1 protein can function as an activation signal for PI 3-kinase (2). This signal is thought to be transmitted allosterically through a conformational change within the p85 regulatory subunit upon binding of the phosphotyrosine peptide to the SH2 domains (33, 38). We therefore examined the PI 3-kinase activities of the various p85 deletion mutants to determine whether they were able to transmit the phosphotyrosine peptide activation signal to the p110 catalytic subunit. In Fig. 6, 1% Triton X-100 extracts of cotransformed yeast cells were immunoprecipitated overnight with the GAD-specific antibody. The washed precipitates were incubated with and without the tyrosine-phosphorylated peptide (P-Y628) prior to the determination of PI 3-kinase activity. As controls, extracts from cells transfected with GAD-p85 and the parental GBT vector displayed undetectable PI 3-kinase activity. In contrast, coexpression of full-length GBT-p110 with GAD-p85 activated PI 3-kinase activity approximately 2-fold in the presence of the phosphotyrosine peptide (Fig. 6A). The degree of activation observed with the P-Y628 phosphotyrosine peptide in this system was identical to that previously reported for p85 immunoprecipitates of CHO cells (2). Furthermore, this activation was specific for the phosphotyrosine peptide, as the same nonphosphorylated peptide had no effect on PI 3-kinase activity (Fig. 6A). Although the GAD-p85(IS) fusion exhibited the strongest interaction with GBT-p110, the phosphotyrosine peptide had no effect on PI 3-kinase activity, presumably because of the absence of the SH2 domains.

To determine whether a single and/or both SH2 domains in conjunction with the IS domain would be sufficient to activate the p110 catalytic subunit, we next examined the three p85 deletion mutants which tested positive for an interaction with p110 (Fig. 6B). As observed for the full-length p85 subunit, the GAD-p85(n/cSH2) construct displayed a 2.6-fold level of activation of PI 3-kinase activity in the presence of the phosphotyrosine peptide. Similarly, the GAD-p85(nSH2+IS) and GAD-p85(IS+cSH2) constructs demonstrated an approximately 2-fold level of activation in the presence of the P-Y628 phosphotyrosine peptide. These data demonstrated that a single SH2 domain in conjunction with the IS domain of p85 was sufficient to mediate the allosteric regulation of PI 3-kinase activity by phosphotyrosine peptide binding.

DISCUSSION

PI 3-kinase plays a key role as a molecular switch in many diverse signal transduction pathways, acting downstream of the tyrosine-specific protein kinase growth factor receptors (3, 41). PI 3-kinase is a heterodimeric enzyme consisting of an 85-kDa regulatory subunit and a 110-kDa catalytic subunit. The function of this enzyme is to transfer a phosphate group to the 3′-hydroxyl group of the inositol ring of phosphatidylinositol and its phosphorylated derivatives to

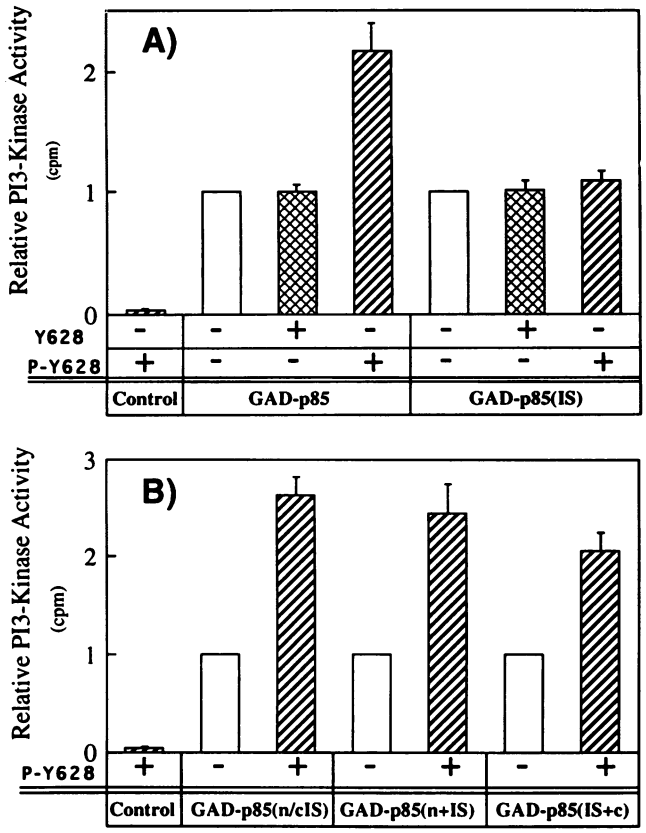

FIG. 6. Phosphotyrosine peptide regulation of p110 PI 3-kinase activity with various p85 mutants. Cells were cotransformed with the designated constructs, and total cellular proteins were extracted with 1% Triton X-100. The GAD fusion proteins were immunoprecipitated by use of a GAL4 polyclonal antibody, and immunoprecipitates were assayed under the following conditions: no peptide (□), Y628 nonphosphorylated peptide (▩), or P-Y628 phosphotyrosine peptide (▨) as described in Materials and Methods. (A) The GAD-p85 fusion was coexpressed with the GBT parental vector (Control) or with the full-length GBT-p110 expression plasmid (GAD-p85). The GAD-p85(IS) fusion was coexpressed with the full-length GBT-p110 fusion [GAD-p85(IS)]. PI 3-kinase activity was expressed relative to PI 3-kinase activity in the absence of the phosphopeptide and represents the mean ± standard deviation from three independent experiments. (B) The full-length GBT-p110 fusion construct was coexpressed with GAD-p85(n/cSH2+1S), GAD-p85(nSH2+IS), and GAD-p85(IS+cSH2). PI 3-kinase activity was expressed relative to PI 3-kinase activity in the absence of the P-Y628 phosphotyrosine peptide and represents the mean ± standard deviation for three independent experiments.

generate the phospholipid species phosphatidylinositol-3-phosphate, phosphaditylinositol-3,4-bisphosphate, and phosphaditylinositol-3,4,5-trisphosphate (3, 44). While it is unclear how the phospholipid products participate in the signaling event, on the basis of their early accumulation in the plasma membrane and the fact that they are not cleaved by any of the known phospholipases, they may function directly as second messengers.

An emerging theme of intracellular signal transduction is that effector molecules physically associate as complexes upon ligand activation of transmembrane tyrosine kinase receptors. Many of the signaling molecules possess SH2 domains, which bind specifically to phosphorylated tyrosine residues in activated receptors or their substrates (21). For PI 3-kinase, tight binding to autophosphorylated growth factor receptors or, in the case of insulin signaling, the tyrosine-phosphorylated IRS-1 protein, is mediated by the SH2 domains of the 85-kDa regulatory subunit (31). In addition to providing a docking site, SH2 domain binding to phosphotyrosine residues also results in conformational changes that activate the catalytic activity of the p110 subunit of PI 3-kinase (2, 4, 30, 38, 42).

To define the molecular determinants involved in this allosteric interaction between the p110 and p85 subunits of PI 3-kinase, we took advantage of the yeast two-hybrid system. This yeast genetic system is based on the modular nature of the yeast transcriptional activator GAL4 and its ability to activate transcription upon binding to its DNA regulatory element (6, 14). In this system, proteins that physically interact are able to reconstitute GAL4 transcriptional activity, and the strength of the protein-protein interaction is proportional to the level of *lacZ* reporter gene expression. This approach has a distinct advantage over alternative in vitro methods in that the protein interactions are studied in intact cells and therefore are not subject to artifactual results due to assay conditions.

We used the yeast two-hybrid system to define the subdomains which confer a physical interaction between the PI 3-kinase subunits. These data demonstrated that a 192-amino-acid region located between the amino- and carboxy-terminal SH2 domains of the p85 subunit (IS region) specifically interacted with the amino-terminal 127-amino-acid region of the p110 subunit. Surprisingly, the presence of the p85 SH2 domains markedly inhibited the interaction of the p85(IS) domain with p110, and a single SH2 domain alone did not physically associate with full-length p110 or any of the p110 deletion constructs.

Our findings are in contrast to a previous report indicating that either p85 SH2 domain was sufficient for coimmunoprecipitation of the p110 subunit following transient transfection (8). It should be noted that in their study, binding interactions were indirectly assayed by measuring coprecipitating PI 3-kinase activity from p85-overexpressing cells. However, since submission of the manuscript, epitope-tagged mouse p85α deletion constructs stably transfected in CHO cells were also used to map the region of p85 which binds full-length p110 (20). In that study, a 249-amino-acid domain which included flanking residues from both SH2 domains was identified. This latter finding is in excellent agreement with our data demonstrating that the 192-amino-acid IS domain of the p85 subunit is sufficient to confer a high-affinity interaction with p110.

Like the p85 deletions, the GBT-p110RI fusion construct, containing the amino-terminal 127 amino acid residues, was observed to display a substantially higher level of activation of reporter β-galactosidase activity than the full-length GBT-p110 or GBT-p110BX fusion constructs. It is likely that the increased reporter activity resulted from the deletion of a negative effector domain(s) residing between amino acid residues 128 and 1069 of the p110 subunit. However, since our antibody does not detect GBT, we were unable to directly assess the levels of GBT-p110 protein expression. Nevertheless, coexpression of GAD-p85(IS) with GBT-p110RI resulted in a 100-fold increase in the level of β-galactosidase reporter activity, compared with that obtained with the full-length constructs. Thus, we speculate that the sequences surrounding the physical contact points between the two subunits attenuate the interactions observed between the GAD-p85(IS) and GBT-p110RI constructs.

Recent studies have provided important insight into the

regulation of PI 3-kinase activity (2, 30, 33, 38). Initial tyrosine phosphorylation within distinct sequence motifs (YXXM and YMXM) creates a recognition target signal for the p85 subunit of PI 3-kinase (40). Binding is mediated through the SH2 domains of the p85 subunit and results in a conformational change that is thought to be transmitted to the p110 subunit. Consistent with this model, we have observed that the p85(IS) domain was insufficient to mediate phosphotyrosine peptide activation of PI 3-kinase activity. However, the IS domain in conjunction with both SH2 domains or with either the amino-terminal or the carboxy-terminal SH2 domain was fully capable of transmitting the phosphotyrosine peptide binding signal to the p110 catalytic subunit.

In summary, we have defined the subdomains of the p85 and p110 subunits of bovine PI 3-kinase which are responsible for mediating their high-affinity association. The domain located between the two SH2 domains of the p85 regulatory subunit, corresponding to amino acid residues 429 to 621, mediates the binding interaction with the amino-terminal 127-amino-acid domain of the p110 catalytic subunit. Although the p85 IS domain is sufficient for the association of the p85 subunit with the p110 subunit, at least one SH2 domain in addition to the IS domain is required for allosteric activation of p110 catalytic activity through phosphotyrosine peptide binding to the p85 regulatory subunit.

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