Association of Phosphorylated Insulin-like Growth Factor-I Receptor with the SH2 Domains of Phosphatidylinositol 3-Kinase p85*

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Insulin-like growth factor-I (IGF-I) stimulates the production of 3-inositides and markedly increases the phosphatidylinositol 3-kinase activity that is immunoprecipitated by anti-phosphotyrosine antibodies, a portion of which is also associated with the IGF-I receptor. In this study, recombinant p85, the regulatory subunit of phosphatidylinositol 3-kinase, and fusion proteins containing various subdomains were used to investigate the association of p85 with the IGF-I receptor and to demonstrate that p85 is a direct in vitro substrate of the IGF-I receptor kinase.

Solubilized IGF-I receptor was immobilized on an antibody-agarose beads. Following in vitro receptor phosphorylation and incubation with cell lysate, immobilized receptor became associated with phosphatidylinositol 3-kinase activity and with protein bands with molecular masses of 85 and 110 kDa, which correspond to the known molecular masses of the subunits of phosphatidylinositol 3-kinase. These associations were inhibited by the addition of recombinant intact p85 or SH2-containing fusion proteins, but not by fusion proteins containing its SH3 domain or breakpoint cluster homology region. A fusion protein containing the SH2 domains of Ras GTPase-activating protein also inhibited the association of phosphatidylinositol 3-kinase activity with immobilized IGF-I receptor, although less effectively than p85, whereas a similar construct containing the SH2 domain of pp60^c-src was without effect. When immobilized phosphorylated IGF-I receptor was incubated with intact p85 or the SH2-containing fusion proteins, it became associated with and phosphorylated these proteins.

These results demonstrate that at least in vitro, a tight association occurs between phosphorylated IGF-I receptor and phosphatidylinositol 3-kinase, that the region of phosphatidylinositol 3-kinase that contains its SH2 domains is directly involved in this association, and that this region is a direct substrate for IGF-I receptor tyrosine kinase. Furthermore, these results suggest that Ras GTPase-activating protein can also interact with the IGF-I receptor and that different SH2 domain-containing proteins interact with the IGF-I receptor with widely differing affinities.

Phosphatidylinositol 3-kinase (PtdIns 3-kinase) is thought to be an important component of the signaling pathway of several receptor and nonreceptor tyrosine kinases (1). The evidence for this, however, is circumstantial, and the functions of its products are unknown. PtdIns 3-kinase is a heterodimer composed of 85- and 110-kDa subunits (2-4). The 85-kDa subunit, p85, has been cloned (5-7). Its amino-terminal domain bears considerable homology with the SH2 domain of pp60^c-src. This region has weak homology with BCR, the breakpoint cluster involved in c-abl translocations (7, 8). Its carboxy-terminal half contains two regions with high homology to the SH2 domain of pp60^c-src (See Fig. 1A). SH2 domains are found in a variety of proteins including the viral oncogene, v-crk, nonreceptor tyrosine kinases, and proteins important in signaling pathways including phospholipase C-γ and Ras GTPase-activating protein (GAP). SH2 domains are known to bind to phosphotyrosine-containing proteins and therefore may play a role in the formation of multisubunit signaling complexes which form in response to tyrosine kinase activation and regulate intracellular signaling pathways (1, 9-14).

Expression of recombinant p85 in different systems shows that this protein is not the PtdIns 3-kinase catalytic subunit (7). Instead, it appears to be a regulatory subunit and/or acts as a bridge between the tyrosine kinase and the catalytic subunit (2, 6). Recombinant p85 is able to bind to middle t-pp60^c-src complex and to platelet-derived growth factor (PDGF) receptor and serves as a substrate for tyrosine phosphorylation (6, 7). PDGF receptor mutant in the kinase insert domain is no longer able to associate to recombinant p85, and a tyrosine-phosphorylated peptide from the same kinase insert region is able to inhibit the binding of p85 to the receptor (6). Polyclonal antibodies against recombinant p85 immunoprecipitated PtdIns 3-kinase activity from bovine brain and p85 and p110 were found in this immunoprecipitate (7). These data are consistent with the hypothesis that p85 functions as a regulatory subunit through which the catalytic subunit of PtdIns 3-kinase is modulated by tyrosine kinases.

IGF-I and insulin, which have as receptors closely related tyrosine kinases, stimulate the production of 3-inosides and cause a marked increase in the amount of PtdIns 3-kinase activity that can be immunoprecipitated by anti-phosphotyrosine antibodies (15-20). There are several possible explanations for the latter finding: PtdIns 3-kinase may be precipitated by anti-phosphotyrosine antibodies because it is di-

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rectly or indirectly tyrosine-phosphorylated in response to IGF-I or insulin. Currently, there is no direct evidence for this. PtdIns 3-kinase may be precipitated by anti-phosphotyrosine antibodies because it is associated with tyrosine phosphorylated IGF-I receptors. Following IGF-I stimulation, PtdIns 3-kinase activity is present in IGF-I receptor immunoprecipitates (17, 18). However, this accounts for only a small percent of the PtdIns 3-kinase activity that is precipitated by anti-phosphotyrosine antibody (17, 18). PtdIns 3-kinase may be precipitated by anti-phosphotyrosine antibodies, because it is associated with other proteins that are phosphorylated in response to IGF-I. Following insulin stimulation, PtdIns 3-kinase activity is present in immunoprecipitates of IRS-1, a major substrate of insulin receptor kinase in most cells (21). IRS-1 is also a major substrate of IGF-I receptor kinase. Both IGF-I receptors and IRS-1 have tyrosine phosphorylation sites that are homologous to the site on middle T which PtdIns 3-kinase is known to bind (1, 21).

In order to further investigate the role of PtdIns 3-kinase in the IGF-I receptor-signaling pathway, we cloned and expressed full-length p85 and different portions of this molecule. We describe here that exogenous p85 inhibited the association of PtdIns 3-kinase activity with IGF-I receptor and that the carboxyl-terminal portion containing both SH2 domains was responsible for this inhibition. We also showed that an SH2-containing fragment of GAP partially inhibited this association, whereas an SH2-containing fragment of pp60^c-src did not.

**EXPERIMENTAL PROCEDURES**

**Materials**—LISN C4 cells, a mouse fibroblast cell line that overexpresses human IGF-I receptors (22), were kindly provided by Michael Kaleko. The anti-phosphotyrosine monoclonal antibody (a-Ptyr) was purchased from Upstate Biotechnology, Inc. a-IR-3 is an anti-IGF-I receptor monoclonal antibody (23). Phosphatidylinositol phosphate and reduced glutathione were from Sigma. [-p^32P]ATP (30 Ci/mmol) was purchased from Du Pont-New England Nuclear. Alkaline phosphatase-linked goat anti-rabbit IgG was purchased from Jackson Immunoresearch. The pGEX2T vector and glutathione-Sepharose 4B columns were purchased from Pharmacia LKB Biotechnology Inc. 4B columns were purchased from Pharmacia LKB Biotechnology Inc. Elmer Cetus following the manufacturer's recommended procedure. 

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**Expression and Purification of Glutathione S-Transferase Fusion Protein**—Glutathione S-transferase fusion protein expression was induced as described previously (24) with minor modifications. Overnight cultures grown in Luria broth containing ampicillin (0.1 mg/ml) were diluted 1:10 in 500 ml of fresh medium and grown for 2 h at 37°C. Isopropyl-1-thio-β-D-galactopyranoside was then added to 0.5 mM, and cultures were grown for approximately 1 h. Cells were pelleted and resuspended with 10 ml of cold phosphate-buffered saline containing 50 mM EDTA, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1% aprotinin. The cell suspension was lysed by sonication, and Triton X-100 was added to 1%. The lysate was centrifuged at 10,000 x g for 10 min, and the supernatant was applied on a glutathione-Sepharose 4B column. After adsorption, the column was washed with 10 column volumes of phosphate-buffered saline. The glutathione S-transferase fusion proteins were eluted with 50 mM Tris-HCl (pH 8.0) plus 10 mM reduced glutathione.

**Cell Culture**—LISN C4 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5% bovine calf serum. The cells were plated once with 10 ml of Dulbecco's modified Eagle's medium and then detached by incubation in 10 ml of phosphate-buffered saline, 1 mM EDTA. The detached cells were collected by centrifugation and lysed in lysis buffer composed of 20 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% (v/v) Nonidet P-40, 2 mM Na3VO4, 0.1% phenylmethylsulfonyl fluoride, 50 μg/ml leupeptin, and 50 μg/ml aprotinin. The lysed cells were centrifuged at 10,000 x g for 10 min, and supernatants (lyastes) were removed and saved.

**Preparation of a-IR-3-3-Agarose Beads**—a-IR-3 was coupled to Affigel 15 at a concentration of 1 mg of antibody/ml of gel for 4 h at 4°C. The resin was washed extensively with buffer, blocked with 0.1 M ethanalamine for 2 h at 22°C, and extensively washed with 0.1 M Heps (pH 7.5) before final suspension in 0.1 M Heps (pH 7.5), 0.025% NaN3. The efficiency of immunoprecipitation of labeled IGF-I receptors by this gel-coupled antibody was judged to be identical to that of an equivalent amount of uncoupled a-IR-3 (data not shown).

**Adsortion of IGF-IReceptor to a-IR-3-3-Agarose Beads and in Vitro Phosphorylation—Lyastes (0.5 ml) were incubated with a-IR-3 coupled to Affigel 15 for 2 h at 4°C. The beads with adsorbed receptor were collected by centrifugation, and the supernatant was saved for further experiments. The beads were washed three times with lysis buffer and twice with 10 mM Tris (pH 7.4), 0.1 M NaCl, 1 mM EDTA (TNE). The beads were resuspended with TNE containing 50 mM ATP and 20 mM MgCl2 and incubated at 25°C for 15 min. They were then washed three times with TNE and resuspended with TNE. This suspension was used for further incubation with the saved beads for SDS-gel electrophoresis as described.

**Binding of PtdIns 3-Kinase Activity to IGF-I Receptor**—The protein concentration of the saved lysate (IGF-I receptor-depleted lyaste) was determined by the method of Bradford (25), and lysates (0.5 ml) containing approximately 700 μg of protein were incubated with immobilized phosphorylated IGF-I receptor in the presence or absence of the fusion protein of the different part of p85 or a SH2-containing fusion protein from GAP or p85 plus for 2 h at 4°C. After incubation, the immunoprecipitated IGF-I receptors were washed three times with lysis buffer, twice with 0.5 M LiCl, 0.1 M Tris (pH 7.6), and then twice with TNE and resuspended with TNE. The final immuno-precipitates were used for PtdIns kinase assay.

**PtdIns Kinase Assay**—After indicated incubations, washed a-IR-3-agarose beads containing adsorbed proteins were incubated with 400 μg PtdIns liposome/ml, 20 mM MgCl2, 10 μg/ml of [γ-^32P]ATP at a final concentration of 50 μM ATP in a volume of 0.025 ml for 15 min at 25°C. The reaction was terminated by the addition of 0.1 ml cold TNE, and half of the solution was transferred to an Eppendorf tube containing 0.02 ml of 8 N HCl and 0.16 ml chloroform/methanol (1:1). Phospholipids were extracted, washed once with 0.1 ml of 1 M HCl/methanol (1:1) and separated by thin layer chromatography (TLC) (26). The band corresponding to the PtdIns phosphate detected on TLC was confirmed by lipid extraction, desalyciation, and analysis on high performance liquid chromatography (HPLC) as described previously (27).

**Gel Electrophoresis**—Purified p85 proteins, phosphorylated or un-
phosphorylated IGF-I receptor immunoprecipitates, or the half of the incubation from the PtdIns kinase assay were mixed with SDS solubilizing solution consisting of 0.125 M Tris (pH 6.8), 0.1% SDS, 10% glycerol, 100 mM dithiothreitol and heated at 95 °C for 5 min. The solubilized proteins were subjected to gel electrophoresis in the presence of SDS on 10-20% gradient or 7.5% homogeneous polyacrylamide gels as described previously (28).

**Immunoblotting**—In some experiments, separated proteins were transferred electrophoretically to nitrocellulose blots as described previously (29). After blocking the nitrocellulose for 4 h, the blot was incubated with α-Ptyr at a 1:1000 dilution for 4-24 h at 4 °C. The blot was washed extensively, and immunoreactive proteins were visualized by incubation with goat anti-mouse antibody coupled to alkaline phosphatase as described previously (29).

**RESULTS**

**Expression and Purification of p85 Fusion Proteins**—To investigate the association between the IGF-I receptor and PtdIns 3-kinase activity, different regions of p85, a subunit of PtdIns 3-kinase, were expressed as fusion proteins in E. coli (Fig. 1A). These regions were: an amino-terminal fragment containing the SH3 region, a carboxyl-terminal fragment containing two SH2 regions, and a middle fragment shown previously to have homology with BCR (7, 8). All sequences were fused downstream of the glutathione S-transferase gene in the pGEX2T expression vector. The glutathione S-transferase fusion proteins were expressed to high levels in bacteria and were rapidly purified in a single step by affinity chromatography on glutathione-Sepharose (Fig. 1B).

**PtdIns 3-Kinase Activity Is Associated with the Autophosphorylated IGF-I Receptor**—IGF-I receptor was adsorbed to α-IR-3-agarose beads by incubating the beads with LISN C4 cell lysate. The washed beads were then incubated in the absence or presence of ATP, and phosphorylated receptor was detected by anti-phosphotyrosine Western blots. As shown in Fig. 2A, following incubation with ATP there are prominent phosphotyrosine-containing bands with molecular weights of 95,000 and 180,000, which correspond to the autophosphorylated β subunit of the IGF-I receptor and its unprocessed precursor. Thus IGF-I receptor was adsorbed to α-IR-3-agarose beads, and in the basal state the majority of the receptor was unphosphorylated, but became autophosphorylated following incubation with ATP.

An in vitro reconstitution system was used to study the association between PtdIns 3-kinase activity and the IGF-I receptor. Adsorbed IGF-I receptor, treated as described above, was incubated with the IGF-I receptor-depleted LISN C4 cell lysate, and the resulting immunocomplex was assayed for PtdIns kinase activity. PtdIns kinase activity was associated with IGF-I receptor which had been phosphorylated in vitro but not with unphosphorylated receptor (Fig. 2B). The iden-
Activity with IGF-I Receptor: Involvement was confirmed as PtdIns 3-monophosphate (PtdIns(3)P) tity of the lipid moiety produced in these PtdIns kinase assays was measured. The P85 fusion protein (100 nM) totally was dose-dependent with an IDso through the analysis of the deacylated lipid products on HPLC (data not shown). Thus, the PtdIns kinase that was associated with IGF-I receptor immunoprecipitates possessed PtdIns 3-kinase activity.

Recombinant p85 Inhibits the Association of PtdIns 3-Kinase Activity with IGF-I Receptor: Involvement of the SH2 Domain—To test if p85 has an effect on the association between IGF-I receptor and PtdIns 3-kinase activity, the p85 fusion protein was incubated with LISN C4 cell lysates and the immunoprecipitated IGF-I receptor, and PtdIns 3-kinase activity was measured. The P85 fusion protein (100 nM) totally inhibited the association of PtdIns 3-kinase activity with the immunoprecipitated IGF-I receptor (Fig. 3A). This inhibition was dose-dependent with an IDso <10 nM (Fig. 3A). To identify the region in the p85 molecule responsible for the inhibition, the constructs shown in Fig. 1 were tested in the same assay. Only the SH2-containing fusion protein was able to inhibit the association of PtdIns 3-kinase activity with IGF-I receptor, whereas fusion proteins of p85 containing SH3 domain, BCR homologous domain, and glutathione S-transferase had no effect (Fig. 3B). No PtdIns 3-kinase activity was detected when the immunoprecipitated IGF-I receptor was incubated with buffer alone (Fig. 3B).

Beads, treated exactly as described in Fig. 3B, were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography to identify 32P-phosphorylated proteins (Fig. 3C). A prominent phosphorylated IGF-I receptor β subunit is present in all lanes. When the SH2-containing fusion protein (lane 5) or intact p85 fusion protein (lane 6) was included in the incubation, prominent phosphorylated bands corresponding to these proteins were present in addition. Two faint bands with molecular weights of 110,000 and 85,000, which correspond to the known molecular weights of the subunits of PtdIns 3-kinase, were also present when IGF-I receptor adsorbed beads were incubated with LISN C4 cell lysate (lane 2), but not with buffer alone (lane 1). Inclusion in the incubation of the SH3 fusion protein (lane 3), the BCR fusion protein (lane 4), or glutathione S-transferase (lane 7) did not diminish the intensity of these bands, whereas inclusion of the SH2-containing fusion protein (lane 5) did. Inclusion of the intact p85 fusion protein (lane 6) also diminished the intensity of the 85-kDa band. The presence of the 110-kDa band was obscured by the phosphorylated fusion protein.

The prominent bands corresponding to the SH2-containing and p85 fusion proteins suggest that these proteins bind directly to IGF-I receptor, most probably through SH2-phosphotyrosine interaction. To confirm this direct association, exogenous SH2-containing and p85 fusion proteins were incubated with immunoprecipitated IGF-I receptor. After an in vitro kinase reaction, the sample was subjected to SDS-polyacrylamide gel electrophoresis, and phosphorylated bands were analyzed by autoradiography. Fig. 4 clearly shows that the SH2-containing fusion protein was bound to IGF-I receptor and phosphorylated by the receptor tyrosine kinase. This occurred only if the receptor was activated by in vitro autophosphorylation prior to incubation with the SH2-containing fusion protein. The same result was obtained when p85 fusion protein was used (data not shown).

To further establish that the SH2-containing fusion protein was inhibiting PtdIns 3-kinase activity by competing for binding to the IGF-I receptor and not by directly inhibiting PtdIns 3-kinase catalytic activity, IGF-I receptor was incubated with the fusion protein, washed, and then incubated with cell lysate before measuring PtdIns 3-kinase activity or first incubated with cell lysate and then the fusion protein. Preincubation of the immunoprecipitated IGF-I receptor with the SH2-containing fusion protein inhibited the association of PtdIns 3-kinase activity with the immunoprecipitated IGF-I receptor (Fig. 5, lanes 1 and 2). However the SH2-containing

FIG. 3. Inhibition of the association between PtdIns 3-kinase and phosphorylated IGF-I receptor by the SH2-containing and p85 fusion proteins. A, IGF-I receptor was immunoprecipitated from unstimulated LISN C4 cells and phosphorylated in vitro. After washing, the immunocomplexes were incubated with lysis buffer (lane 1) or with IGF-I receptor-depleted LISN C4 cell lysate (lanes 2–10). Intact p85 fusion protein or glutathione S-transferase were added to these incubations at the concentrations indicated in the figure. After washing, the associated PtdIns kinase activity was assayed, and resultant PtdIns(3)P was separated by TLC and visualized by autoradiography. These results are representative of two different experiments. B, IGF-I receptor was immunoprecipitated from unstimulated LISN C4 cells and phosphorylated in vitro. After washing, the immunocomplexes were incubated with lysis buffer (lane 1) or with IGF-I receptor-depleted LISN C4 cell lysate (lanes 2–7). Different portions of the p85 molecule at the concentration of 200 nM were included in these incubations as indicated in the figure. After washing, the associated PtdIns kinase activity was assayed, and resultant PtdIns(3)P was separated by TLC and visualized by autoradiography. These results are representative of three different experiments. The radioactivity contained in the area comigrating with PIP was 31 cpm (lane 1), 28,600 cpm (lane 2), 27,500 cpm (lane 3), 24,600 cpm (lane 4), 210 cpm (lane 5), 160 cpm (lane 6), and 26,500 cpm (lane 7). C, half of the incubation of the assay in B was mixed with SDS sample buffer and proteins were separated on a 10–20% gradient gel. Phosphorylated bands were visualized by autoradiography. The positions of the exogenously added SH2-containing and p85 fusion proteins are indicated by the white arrow and arrowhead, respectively. The positions of the LISN C4 cells p110 and p85 are indicated by the black arrow and arrowhead, respectively. The position of the β subunit of IGF-I receptor is also indicated.
FIG. 4. Binding of the SH2-containing fusion protein to IGF-I receptor and phosphorylation of the protein by the receptor. IGF-I receptor that was immunoprecipitated from unstimulated LISN C4 cells was incubated with (lane 2) or without (lane 1) 50 μM ATP. The immunocomplexes were washed and incubated with the SH2-containing fusion protein (200 nM) in lysis buffer (0.5 ml) for 2 h at 4°C. After washing, the immunocomplexes were phosphorylated in the presence of [γ-32P]ATP as described under “PtdIns Kinase Assay” and separated on a 7.5% SDS gel. Phosphorylated bands were visualized by autoradiography. The position of the exogenously added SH2-containing fusion protein is indicated by the black arrow. The position of the β subunit of IGF-I receptor is also indicated.

FIG. 5. Sequential incubation of the phosphorylated IGF-I receptor with LISN C4 cell lysates and the SH2-containing fusion protein. IGF-I receptor was immunoprecipitated from unstimulated LISN C4 cells, phosphorylated in vitro, and washed. In the first incubation, the immunocomplexes were incubated with lysis buffer (lane 1), lysis buffer plus the SH2-containing fusion protein (200 nM) (lane 2), or IGF-I receptor-depleted LISN C4 cell lysate (lanes 3 and 4) for 1 h at 4°C. The immunocomplexes were washed three times with lysis buffer and subsequently incubated with IGF-I receptor-depleted LISN C4 cell lysate (lanes 1 and 2), lysis buffer plus the SH2-containing fusion protein (lane 3), or lysis buffer alone (lane 4) for 1 h at 4°C. After washing, the associated PtdIns kinase activity was assayed, and resultant PtdIns(3)P was separated by TLC and visualized by autoradiography. These results are representative of two different experiments. The radioactivity contained in the area comigrating with PIP was 5200 cpm (lane 1), 460 cpm (lane 2), 3600 cpm (lane 3), and 2500 cpm (lane 4).

FIG. 6. The effect of SH2-containing fusion proteins from p85, GAP, and pp60^c^ on the binding of PtdIns 3-kinase activity to phosphorylated IGF-I receptor. IGF-I receptors were immunoprecipitated from unstimulated LISN C4 cells and phosphorylated in vitro. After washing, the immunocomplexes were incubated with IGF-I receptor-depleted LISN C4 cell lysate in the absence (lanes 1 and 2) or in the presence of exogenous proteins (lanes 3–10). The proteins were added as follows: lanes 3 and 4, glutathione S-transferase (300 nM); lanes 5 and 6, the SH2-containing protein from p85 (100 or 300 nM, respectively); lanes 7 and 8, the SH2-containing fusion protein from GAP (100 or 300 nM, respectively); lanes 9 and 10, the SH2-containing fusion protein from pp60^c^ (100 or 300 nM, respectively). After washing, the associated PtdIns kinase activity was assayed, and resultant PtdIns(3)P was separated by TLC and visualized by autoradiography. These results are representative of two different experiments. The radioactivity contained in the area comigrating with PIP was 7100 cpm (lane 1), 8100 cpm (lane 2), 9200 cpm (lane 3), 7400 cpm (lane 4), 260 cpm (lane 5), 150 cpm (lane 6), 1500 cpm (lane 7), 770 cpm (lane 8), 7600 cpm (lane 9), and 7300 cpm (lane 10).
the possibility that a tightly associated protein kinase other than IGF-I receptor is actually responsible for the observed phosphorylation cannot be excluded.

How does the association of IGF-I receptor with Ptdlns 3-kinase and its phosphorylation observed in these in vitro studies relate to what happens in intact cells, and what role does it play in Ptdlns 3-kinase activation? When intact cells are treated with IGF-I there is a large increase in the amount of Ptdlns 3-kinase activity that is immunoprecipitated by anti-phosphotyrosine antibody (17, 18). This result is consistent with a direct phosphorylation of Ptdlns 3-kinase by IGF-I receptor tyrosine kinase. However, another possible explanation for these findings is that Ptdlns 3-kinase becomes associated with a protein which is tyrosine phosphorylated in response to IGF-I and is co-immunoprecipitated. Indeed, Ptdlns 3-kinase is co-precipitated with IRS-1, a major substrate for both insulin and IGF-I receptors, when cells are treated with insulin (21). The availability of anti-Ptdlns 3-kinase antibodies should make it possible to determine whether Ptdlns 3-kinase is directly phosphorylated in intact cells exposed to IGF-I.

When intact cells are incubated with IGF-I, some Ptdlns 3-kinase activity appears to associate with the IGF-I receptor (17, 18). However, this represents only a small fraction of the Ptdlns 3-kinase activity that can be precipitated with antiphosphotyrosine antibodies, even in LISN C4 cells which express a very large number of IGF-I receptors (17, 18). This is surprising in view of the very high affinity of p85 for phosphorylated IGF-I receptor, and the very much greater amount of IGF-I receptor that associates with PtdIns 3-kinase (12). The tyrosine-containing IGF-I receptor sequence (1, 32), but only a minor phosphorylation site in intact cells (1, 32), but extrapolated from the case of the insulin receptor this site is more extensively phosphorylated in broken cells. Interestingly, a mutant insulin receptor in which this region was deleted was able to increase the activity of Ptdlns 3-kinase in anti-phosphotyrosine immunoprecipitates (33). It will be interesting to determine if this mutation or a comparable mutation in the IGF-I receptor affects its ability to bind Ptdlns 3-kinase or directly phosphorylate p85.

A fusion protein containing the SH2 domains of GAP, but not a fusion protein containing the SH2 domain of pp60src inhibited the association of Ptdlns 3-kinase activity with phosphorylated IGF-I receptor. The GAP fusion protein was less effective than the p85 fusion protein. It is not clear if this is because GAP has a lower affinity for IGF-I receptor or because it binds to a different site on the receptor and only partially inhibits p85 binding. Although GAP is known to associate with several tyrosine kinases (34), this is the first report of a direct association of GAP with IGF-I receptors. Whether the interaction is sufficiently strong to have any physiological significance is unclear.

It is possible that the binding of endogenous p85 to its target protein is regulated not only by tyrosine phosphorylation of the protein but also by competition with other SH2-containing molecules. It is also likely that the SH2 domain of p85 possesses specificity for each phosphotyrosine containing motif and that it directs Ptdlns 3-kinase activity to its target. Experiments are currently underway to define which part of the SH2 domain of p85 is responsible for the inhibitory effect on the association between IGF-I receptor and Ptdlns 3-kinase activity. Although the role of Ptdlns 3-kinase in signal transduction is unknown, further studies on its association with other molecules will help to increase our understanding of the importance of this enzyme.

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