Insulin-dependent Formation of a Complex Containing an 85-kDa Subunit of Phosphatidylinositol 3-kinase and Tyrosine-phosphorylated Insulin Receptor Substrate 1

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Monoclonal antibodies raised against the 85-kDa subunit (p85) of bovine phosphatidylinositol (PI) 3-kinase were found to recognize uncomplexed p85 or p85 in the active PI 3-kinase. Immunoprecipitation studies of Chinese hamster ovary cells, which overexpress the human insulin receptor when treated with insulin, showed increased amounts of p85 and PI 3-kinase activity immunoprecipitatable with monoclonal anti-p85 antibody and no increase in the tyrosine phosphorylation of p85. Insulin also induced an association of p85 with the tyrosine-phosphorylated insulin receptor substrate 1 (IRS-1) and other phosphorylated proteins ranging in size from 100 to 170 kDa but not with the activated insulin receptor. In vitro reconstitution studies were used to show p85 in the active PI 3-kinase complex with the tyrosine-phosphorylated IRS-1 but not with the activated insulin receptor. Competition studies using synthetic phosphopeptides corresponding to potential tyrosine phosphorylation sites of IRS-1 revealed that phosphopeptides containing YMXM motifs inhibited this association with different potencies, whereas nonphosphorylated analogues and a phosphopeptide containing the EYYE motif had no effect.

Phosphatidylinositol (PI) 3-kinase phosphorylates, at the D-3 position, the inositol ring of PI, PI 4-phosphate (PI-4-P), and PI 4,5-bisphosphate (PI-4,5-P_2) to produce PI 3-phosphate (PI-3-P), PI 3,4-bisphosphate (PI-3,4-P_2), and PI trisphosphate (PIP_3, probably PI 3,4,5-trisphosphate), respectively (Whitman et al., 1988; Auger et al., 1989). D-3-phosphorylated inositides may represent novel second messenger molecules distinct from the classical PI pathway (Auger et al., 1989). Purification of PI 3-kinase has demonstrated that the kinase is a heterodimer consisting of 85-kDa (p85) and 110-kDa (p110) subunits (Carpenter et al., 1990; Morgan et al., 1990; Shibasaki et al., 1991). Three groups, using different approaches, have recently cloned bovine, murine, and human p85 (Otsu et al., 1991; Escobedo et al., 1991b; Skolnik et al., 1991). The amino acid sequence of p85 showed that the protein contains two Src homology region 2 (SH-2) domains and one SH-3 domain with no apparent ATP binding site (Otsu et al., 1991; Escobedo et al., 1991b; Skolnik et al., 1991). Compelling evidence suggests that SH-2 domains are involved in interactions with tyrosine-phosphorylated proteins (Koch et al., 1991). When the p85 subunit is expressed alone, it binds to and is a substrate for tyrosine-phosphorylated PDGF and EGF receptor kinases and the poliovirus middle T antigen-pp60^src complex, but lacks PI 3-kinase activity (Escobedo et al., 1991b; Otsu et al., 1991). In addition, SH-2 domains of p85 expressed as fusion proteins in Escherichia coli were found to bind to receptors for the EGFR, PDGF-β, colony-stimulating factor 1, and Kit (Hu et al., 1992; McGlade et al., 1992; Klippel et al., 1992; Reedijk et al., 1992). These results suggest that p110 represents the catalytic subunit of PI 3-kinase, and p85 appears to be the subunit that links PI 3-kinase to the ligand-activated receptor. The YMXM motif was proposed to be a consensus sequence for tyrosine phosphorylation sites that bind to the SH-2 domains of p85 (Cantley et al., 1991). A recent report suggests that two PI 3-kinase recognition sites exist in the kinase insert region on the PDGF receptor which have phosphotyrosine residues...
located in a 5-amino acid motif with an essential methionine at the fourth position C-terminal to the tyrosine (Yu et al., 1989; Kashishian et al., 1992; Kazlauskas et al., 1992; Fantl et al., 1992). Mutation of both sites was found necessary to eliminate PI 3-kinase association with the receptor and the PDGF-mediated mitogenic response (Fantl et al., 1992).

The insulin receptor (IR) belongs to the family of structurally related transmembrane growth factor receptors with ligand-activated protein tyrosine kinase activity (Kasuga et al., 1983; Kahn and White, 1988). Several lines of evidence suggest that this kinase activity is essential in eliciting the complex cellular response to insulin (Kahn and White, 1988; Becker and Roth, 1990; Kasuga et al., 1959). Insulin treatment of cells has been found to increase PI 3-kinase activity in immunoprecipitates made using antibody to phosphorinsulin (Endemann et al., 1990; Ruderman et al., 1990). Unlike the observed responses of other tyrosine kinases, only a small fraction (1–3%) of PI 3-kinase activity which can be precipitated with anti-phosphophorinsulin antibody was co-precipitable with anti-insulin receptor antibodies (Endemann et al., 1990).

Thus, the association of PI 3-kinase to the insulin receptor appears to be too transient or the association is sufficiently weak to be detected by immunoprecipitation (Yonezawa et al., 1991). Treatment of insulin-stimulated intact cells with bifunctional cross-linkers has been found to cause a significant increase in PI 3-kinase association with insulin receptor (Yonezawa et al., 1991). Therefore, an association of PI 3-kinase with the receptor may be mediated by other tyrosine-phosphorylated proteins. Insulin or insulin-like growth factor I treatment of various intact cells causes rapid tyrosine phosphorylation of a high molecular weight protein (Mr = 160,000–185,000) termed pp185 (White et al., 1986, 1987; Iizumi et al., 1987; Kadokawa et al., 1987). Tyrosine phosphorylation of pp185 was shown to be catalyzed directly by the insulin receptor kinase and was not caused by auto-phosphorylation (Tashiro-Hashimoto et al., 1989). Insulin receptor substrate 1 (IRS-1) was recently purified and its sequence deduced by cDNA cloning (Rothenberg et al., 1991; Sun et al., 1991). The predicted protein encoded has characteristics similar to those of pp185 and contains at least 10 potential tyrosine phosphorylation sites which have an YMXM motif. In addition, immunoprecipitates made with anti-IRS-1 antibodies have been shown to contain significant levels of PI 3-kinase activity (Sun et al., 1991).

In the present study, we have examined the insulin-stimulated interaction of the insulin receptor, PI 3-kinase, and IRS-1 in cultured cells (in vivo) and through a cell free system for the reconstitution of enzyme complexes (in vitro). We found, using a CHO cell line which overexpresses the human insulin receptor, that insulin induced the association of pp85 in the active PI 3-kinase (presumably with the p110 catalytic subunit) with tyrosine-phosphorylated IRS-1 but not with the activated insulin receptor.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures and Antibodies—**CHO cells and CHO cells overexpressing wild-type human insulin receptor (Yonezawa et al., 1992) were routinely maintained in Ham’s F12 medium supplemented with 10% fetal calf serum. Spodoptera frugiperda (SF9) cells were maintained in IPL-41 medium supplemented with 10% fetal calf serum. The antibodies used were a monoclonal antibody against the human insulin receptor (2F3; Yonezawa et al., 1992); a polyclonal antipeptide antibody specific for amino acids 954–965 or 1317–1328 at the intracellular domain of the human insulin receptor 3 subunit (Ullrich et al., 1991); a monoclonal antibody (py20; ICM, Costa Mesa, CA) and a polyclonal antibody (URL, Lake Placid, NY) against phosphorinsulin residues; a polyclonal antibody against rat IRS-1 that were raised in rabbits immunized with a synthetic peptide (pep80) corresponding to residues 489–507 of the molecule (Sun et al., 1991); and three polyclonal antibodies against pp65: (i) rabbit antiserum against the human pp85α produced in E. coli (Yamanashi et al., 1992), (ii) rabbit antiserum against a synthetic C-terminal peptide of bovine pp85α (residues 713–724), and (iii) rabbit antiserum against a synthetic C-terminal peptide of bovine pp85α (residues 707–724). Rabbit antiserum against the human pp85α was used in the immunoblotting studies, unless otherwise specified.

**Production and Screening of Monoclonal Antibodies against Bovine pp85α—**Bovine pp85α (Otsu et al., 1991) was partially purified from SF9 cells infected with the recombinant virus, using Mono S anion exchange and Superose 12 gel filtration columns (Pharmacia, Uppsala, Sweden) chromatography. Female BALB/c mice were immunized by injection of partially purified pp85α mixed with Freund’s adjuvant into the hind pad. Animals received booster injections every 3 or 4 days for 2 weeks. Four days after final injection, lymphocytes from inguinal lymph nodes were fused with SP2/0 myeloma cells by the polyethylene glycol method (Roth et al., 1982). When the hybrid cells were semi-solvent, supernatants were screened by microtiter plate method (Morgan and Roth, 1985) for their ability to precipitate [32P]-labeled (Bolton-Hunter reagent) partially purified pp85α. Positive hybridomas were tested further for their ability to precipitate PI 3-kinase activity from cell lysates and pp85α or pp55 from SF9 cells infected with the recombinant virus. Positive hybrids were cloned by limiting dilution. PI 3-kinase activity was determined as described previously (Endemann et al., 1990).

**Generation of Glutathione S-Transferase Fusion Proteins—**For pp5 fusion proteins, the unique SalI site in the bovine p85α cDNA (1 bp upstream from the start codon) was filled with Klenow polymerase and modified by the addition of EcoRI linkers. Then a 2.5-kilobase EcoRI fragment (from the linker site to 324 bp downstream from the stop codon) including the entire coding region of pp55 was subcloned into the EcoRI site of the pGEX-2T plasmid (Pharmacia). For pp5 construct II, the unique SstI site in the bovine p85α cDNA (43 bp downstream from the stop codon) was modified by the addition of EcoRI linkers. A BgIII–EcoRI fragment (243 bp downstream from the start codon to the linker site, 44 bp downstream from the stop codon) was ligated into the BamHI–EcoRI site of the pGEX–3X plasmid. For pp85 constructs IV, V, or VII, an EcoRV–EcoRI fragment (1,029 bp downstream from the start codon to the linker site) of pp55α, an AccI–EcoRI fragment (1,331 bp downstream from the start codon to 324 bp downstream from the stop codon; the AccI site had been filled with Klenow polymerase) of pp85, or an HindIII–EcoRI (1,749 bp downstream from the start codon to the linker site, previously filled with Klenow polymerase, and the EcoRI site of the pGEX–3X plasmid, respectively. For pp85 construct VI, a BgIII–EcoRI fragment (1,453 bp downstream from the start codon to 324 bp downstream from the stop codon) was subcloned into the BamHI site, previously filled with Klenow polymerase, and the EcoRI site of the pGEX–3X plasmid. For pp5 construct V, a SalI–EcoRI fragment (1,453 bp downstream from the start codon to the linker site) of pp55α was subcloned into the BamHI–EcoRI site of pGEX–3X. For pp5 constructs III (encompassing nucleotides II through V and VIII (encompassing nucleotides 988–1300), oligonucleotides homologous to the boundaries of the desired sequence within the bovine pp55α cDNA were synthesized. These oligonucleotides contained BamHI and/or EcoRI sites. The required stretch of DNA was subsequently amplified by polymerase chain reaction using the bovine pp55α cDNA as a template. The DNA insert was then digested with BamHI and EcoRI and ligated into the pGEX–3X plasmid. The recombinant plasmids were introduced into E. coli (DH5α) and the bacterial transformants analyzed for the presence of inserts. The glutathione S-transferase–pp5 fusion proteins were then expressed by induction with 1 mM isopropyl-β-D-thiogalactopyranoside. Expressed glutathione S-transferase–pp5 fusion proteins were isolated from bacterial lysates by incubation with glutathione-Sepharose beads.

**Synthesis and Purification of Phosphorylated and Nonphosphorylated Peptides—**Five synthetic peptides (designated IRP-1, -2, -3, -4, and -5) containing putative tyrosine phosphorylation sites of IRS-1 are: TDDGYMPSPGV, GNGDYMPMSPKS, DPNGYMMMSPGV, ARLEYYENEKKW, corresponding to residues 604–615, 624–635, 654–665, 724–734, respectively (Sun et al., 1992). Mutation of both sites was found necessary to eliminate PI 3-kinase association with the receptor and the PDGF-mediated mitogenic response (Fantl et al., 1992).
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orotic acid, 5% phenol, 5% H2O, 5% thioanisole, 2.5% ethanedi-thiol) for 2 h at room temperature (King et al., 1990). Deprotected peptides were precipitated with cold anhydrous ether, solubilized in water, and then the aqueous phase was extracted five times with ethyl ether. The aqueous phase was rotary evaporated to remove the remaining water, and then freeze-dried. The crude peptides were purified by reverse phase HPLC on a delta-pack C18 column 300A (25 × 2.5 cm) in 0.1% trifluoroacetic acid using a linear gradient of acetonitrile (0–70%) over 70 min. Following purification, peptide composition after acid hydrolysis was confirmed by amino acid analysis performed on a 6300 Beckman apparatus. The peptides were hydrolyzed in 6 N NaOH at 115 °C for 24 h in evacuated sealed tubes. Gas phase sequencing of phosphorylated peptide showed no phenylthiohydantoin tyrosine in the appropriate cycles, indicating that all tyrosine residues in the corresponding positions were phosphorylated in the phosphopeptides.

Nonphosphorylated peptides were obtained by dephosphorylation with insoluble alkaline phosphatase (Sigma) as described previously (Zardena et al., 1990). The nonphosphorylated peptides were purified further by reverse phase HPLC as described above.

**Immunoprecipitation, Western Blotting, and Cell Labeling—Confluent 100-mm plates of CHO cells overexpressing wild-type human insulin receptor (CHO-IR) were starved in Ham's F-12 medium containing 20 mM Hepes (pH 7.6) for 12 h at 37 °C. Cells were then incubated with or without insulin at 37 °C, frozen with liquid nitrogen, and stored at −80 °C until lysis. Cells were then lysed in cold lysis buffer (137 mM NaCl, 20 mM Tris (pH 7.6), 1 mM MgCl2, 1 mM CaCl2, 10% glycerol, 1% Nonidet P-40, and 0.1% Triton X-100). The samples were electrophoresed in 10% SDS-polyacrylamide gels and visualized by fluorography.

**RESULTS**

Characterization of Monoclonal Antibodies against p85—The bovine p85α protein, expressed in insect cells using baculovirus vectors and purified by Mono Q and Superose 12 column chromatography, was used as antigen to produce monoclonal antibodies (mAbs). Three mAbs (P12, E10, and H1) were found to immunoprecipitate both forms of p85 (α and β) which were described by Otsu et al. (1991), whereas mAb 12G12 could only immunoprecipitate p85α (data not shown). All mAbs were able to recognize denatured p85 that was transferred to nitrocellulose paper. To determine the regions of p85 recognized by these mAbs, we constructed glutathione S-transferase-bovine p85 fusion proteins as depicted in Fig. 1A. An epitope mapping study showed that F12 and G12 recognized the hcr homology region (the region between SH-3 domain at the N terminus and the first SH-2 domain). E10 recognized an epitope corresponding to amino acids 330–337 of bovine p85α (homologous to residues 323–330 of bovine p85). H1 recognized an epitope corresponding to amino acids 452–492 of bovine p85α (homologous to residues 445–485 of bovine p85) (Fig. 1A). Furthermore, F12 and G12 but not E10 and H1 were also able to immunoprecipitate PI 3-kinase activity from bovine brain (Fig. 1B), whereas E10 and H1 immunoprecipitated free p85 but not the PI 3-kinase enzyme from bovine brain (data not shown), indicating that the F12 and G12 recognized PI 3-kinase as a heteromeric form of p85 presumably containing p110, whereas E10 and H1 recognized an uncomplexed form of p85. F12 but not G12 was able to immunoprecipitate PI 3-kinase activity from CHO cells (Fig. 1B) and hamster brain (data not shown). E10 but not H1 immunoprecipitated the uncomplexed form of p85 from hamster brain (data not shown). These results showed that F12 and E10 but not G12 or H1 were able to recognize hamster p85.

**Effect of Insulin on the Association of p85 in the Active PI 3-Kinase with Tyrosine-phosphorylated IRS-1 in Vivo—**Detergent lysates of CHO cells were immunoprecipitated with mAb F12 which recognized the active hamster PI 3-kinase. The immunoprecipitates were then immunoblotted with polyclonal anti-C-terminal peptide serum specific to either p85α or p85β, showing that in CHO cells the p85α form predominates rather than p85β (data not shown). Next, CHO-IR cell (CHO cell overexpressing the human insulin receptor) lysates from cells treated or untreated with insulin were immunoprecipitated with F12. The immunoprecipitates were then immunoblotted with polyclonal antiserum to p85α. F12 was able to precipitate p85 (Fig. 2A) and PI 3-kinase activity (Fig. 1B). In contrast, mAb E10, which has been shown to interact only with uncomplexed p85 and not PI 3-kinase, was unable to immunoprecipitate p85 protein from CHO lysates (data not shown), indicating that in CHO cells p85 exists predominantly as a heteromeric form presumably containing the p110 catalytic
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FIG. 1. Characterization of monoclonal anti-p85a antibodies. Panel A, epitope mapping of glutathione S-transferase-bovine p85α or p85β fusion proteins expressed in bacterial systems obtained by anti-p85α monoclonal antibodies. Each p85 construct was obtained as described under "Experimental Procedures." The presumed functional domains SH-3 and SH-2 are represented by hatched and black boxes, respectively. The amino acids of each fusion protein are numbered according to their relative positions within wild-type bovine p85α or p85β (first methionine is number 1). Fusion proteins were precipitated on glutathione-Sepharose beads, run on SDS-polyacrylamide gels, transferred to Immobilon-P membranes, and blotted by F12, G12, E10, and H1 antibodies. + and − represent fusion proteins recognized or not, respectively, by the antibody. Panel B, immunoprecipitation of PI 3-kinase activity with monoclonal anti-p85 antibodies. Detergent lysates of CHO cells or bovine brain were immunoprecipitated with each monoclonal anti-p85 antibody (F12, G12, E10, or H1) bound to protein G-agarose. The immunoprecipitates were washed, electrophoresed on SDS-polyacrylamide gels, transferred to Immobilon-P membranes, and then blotted with rabbit antiserum against p85α (panels A and B), a polyclonal anti-phosphotyrosine antibody (panel C), or a polyclonal anti-IRS-1 antibody (panel D) as described under "Experimental Procedures." Bound antibody was detected with horseradish peroxidase-conjugated anti-rabbit IgG followed by ECL reading. In panels A and B, p85 is indicated by an arrow. In panel C, the 160-kDa protein (pp185) and the position of the insulin receptor β-subunit are indicated by arrows. In panel D, IRS-1 is indicated by an arrow. The positions of prestained high and/or low molecular mass markers (in kDa) are indicated.

lytic subunit. There was a significant increase in the amount of p85 (Fig. 2A) precipitated with F12 after insulin treatment. The PI 3-kinase activity precipitated with F12 also showed an apparent 2-fold increase after insulin treatment (data not shown).

Treatment of cells with insulin increased the PI 3-kinase activity immunoprecipitable from cell extracts with anti-phosphotyrosine antibodies (Endemann et al., 1990; Ruderman et al., 1990). To examine whether immunoprecipitates made using a monoclonal anti-phosphotyrosine antibody (py20) from lysates of insulin-treated CHO-IR cells contain p85, Western blotting of these immunoprecipitates was performed with polyclonal antiserum against p85α. As shown in Fig. 2B, the monoclonal anti-phosphotyrosine antibody was found to precipitate p85 from insulin-treated cells (lane b) but not from untreated cells (lane a).

The presence of p85 in anti-phosphotyrosine immunoprecipitates would not seem to be a result of a physical association of PI 3-kinase with the autophosphorylated insulin receptor because immunoprecipitates with monoclonal antibodies against the insulin receptor have been shown previously not to contain significant amounts of PI 3-kinase activity (Endemann et al., 1990). Therefore, to test whether p85 or other associated proteins are tyrosine-phosphorylated in response to insulin, immunoprecipitates with anti-p85 F12 antibodies from nontreated and insulin-treated cells were analysed by Western blotting with a monoclonal anti-phosphotyrosine antibody. As illustrated in Fig. 2C, mAb F12 did not precipitate phosphoproteins such as the 95-kDa protein (pp95) and a doublet containing 110-kDa and 120-kDa proteins, but did precipitate IRS-1 from insulin-treated cells.

To detect other proteins associated with p85 and IRS-1 as a consequence of insulin stimulation, cells were labeled with 32P (Fig. 3A) or 35S (Fig. 3B), treated with insulin, lysed, and immunoprecipitation was performed with the monoclonal antiphosphotyrosine antibody py20 (lanes a and b in Fig. 3, A and B) or with the mAb F12 (lanes c and d in Figs. 3, A and B). As shown in Fig. 3A, lane c, the analysis of the immunoprecipitates from 35S-labeled cells revealed that, compared with controls in which mouse IgG was used (data not shown), F12 specifically immunoprecipitated an 85-kDa protein and a doublet containing 110-kDa and 120-kDa proteins, which may contain the PI 3-kinase subunits p85 and p110. In some cases a doublet at 110 kDa has been observed in purified PI 3-kinase (Carpenter et al., 1990). Insulin treatment of cells (Fig. 3A, lane d) caused detectable co-immunoprecipitation with p85 of a 160-kDa protein (identified as IRS-1) and a 170-kDa protein but not proteins of 210, 135, and 95 kDa (the insulin receptor precursor, α subunit, and β subunit of the insulin receptor, respectively), which were immunoprecipitated with py20 after insulin treatment (Fig. 3A, lane b). In py20 immunoprecipitates the 170-kDa protein was also detected (Fig. 3A, lane b). From insulin-treated 32P-labeled cells
**Fig. 3.** Immunoprecipitation of p85 from 32P-labeled (panel A) or 35S-labeled CHO-IR cells (panel B). CHO-IR cells were labeled with Trasn35S-label or 32Porthophosphate as shown under "Experimental Procedures." Cells without treatment (lanes a and c) or treated with insulin for 1 min at 37 °C were frozen with liquid nitrogen and then lysed. The lysates were immunoprecipitated with an anti-p85 antibody (F12) or a monoclonal anti-phosphotyrosine antibody (py20) and then immunoprecipitated with an anti-p85 antibody (F12). The immunoprecipitates were washed, electrophoresed on SDS-polyacrylamide gels, and autoradiographed. The 160-kDa protein (p185), p116, p85, and the insulin receptor are indicated by arrows. The positions of prestained high and low molecular mass markers (in kDa) are indicated.

**Insulin-dependent Association of p85 in the Active PI 3-Kinase with IRS-1 in Vitro—** As described above, p85 was detected in CHO cells only with antibodies which recognize p85 in the active enzyme complex. To define further the interaction of the p85 protein in the active PI 3-kinase with tyrosine-phosphorylated proteins after insulin treatment, the p85 protein in the active PI 3-kinase was immunoprecipitated from CHO cell lysates with the mAb F12. To avoid immunoprecipitating PI 3-kinase from CHO-IR cell lysates in the following incubation, the mAb-protein G-agarose complex was saturated with PI 3-kinase by incubating with an excess of CHO cell lysates, then washed and incubated with lysates from nontreated and insulin-treated CHO-IR cells. Proteins bound to p85 in the active PI 3-kinase were immunoblotted with either anti-insulin receptor or anti-IRS-1 antibodies. As shown in Fig. 4, A and B, IRS-1 bound to p85 in the active PI 3-kinase in an insulin-dependent fashion; however the insulin receptor did not. A 140-kDa band immunoblotted with anti-IRS-1 antibody was also detected in an insulin-dependent fashion (Fig. 4B).

**Inhibition of the Association of p85 in the Active PI 3-Kinase with IRS-1 by Phosphopeptides Corresponding to IRS-1 Putative Tyrosine Phosphorylation Sites—** IRS-1 has multiple putative tyrosine phosphorylation sites (Sun et al., 1991). To determine which tyrosine phosphorylation sites of IRS-1 are critical for the association with p85, tyrosine-phosphorylated synthetic peptides and nonphosphorylated analogues corresponding to putative tyrosine phosphorylation sites of IRS-1 were prepared as described under "Experimental Procedures." The p85 protein in the active PI 3-kinase immobilized on the

**Fig. 4.** In vitro association of the p85 protein in the active PI 3-kinase with IRS-1. Panels A and B. In vitro association of the PI 3-kinase form of p85 with IRS-1 but not with the activated insulin receptor. The PI 3-kinase form of p85 immunoprecipitated from CHO cells with a monoclonal anti-p85 antibody (F12) bound to protein G-agarose (anti-p85) was incubated with CHO-IR cell lysates treated with insulin for 1 min at 37 °C. The beads were washed and immunoblotted with polyclonal anti-insulin receptor (panel A) or anti-IRS-1 (panel B) antibody. In panel A, as control (Anti-P-Tyr), the insulin receptor was immunoprecipitated with a monoclonal anti-phosphotyrosine antibody, py20, from insulin-stimulated CHO cell lysates. The IRS-1 and β subunit of the insulin receptor are indicated by arrows. The positions of prestained high molecular mass markers (in kDa) are indicated. Panel C, the inhibitory effect of phosphorylated or nonphosphorylated peptides (IRP-1, 2, 3, 4, and 5) corresponding to putative tyrosine phosphorylation sites of IRS-1 on in vitro association of the PI 3-kinase form of p85 with IRS-1. The PI 3-kinase form of p85 from CHO cell lysates, immobilized on the protein G-agarose-bound monoclonal anti-p85 antibody (F12) was preincubated for 2 h at 4 °C with various concentrations of either phosphopeptides or nonphosphorylated peptide (NP) and then exposed for another 4 h at 4 °C to CHO-IR cell lysates without treatment (−) or treated with (+) 10−4 M insulin for 1 min at 37 °C in the absence or presence of the phosphopeptides (final concentrations, 0.5−100 μM) or nonphosphorylated peptides (final concentration, 100 μM). The beads were washed and then immunoblotted with anti-IRS-1 antibody.
Involvement of SH-2 Domains in the Association of p85 with IRS-1—To determine whether SH-2 domains of p85 are involved in the interaction of PI 3-kinase with IRS-1, we constructed three glutathione S-transferase fusion proteins expressing individual SH-2 domains (85N and 85C: VIII and VII in Fig. 1A, respectively) or both SH-2 domains together (85N+C: II in Fig. 1A). Binding of these immobilized fusion proteins to native IRS-1 was investigated by incubation with CHO-IR cell lysates from untreated cells or cells treated with insulin. As shown in Fig. 5A, all three fusion proteins were found to bind tyrosine-phosphorylated proteins from insulin-stimulated cell lysates, including IRS-1 and the insulin receptor β subunit. The identity of these proteins was confirmed by Western blotting with either anti-IRS-1 antibodies or anti-insulin receptor (data not shown). Binding was insulin-dependent, and glutathione S-transferase alone bound no tyrosine-phosphorylated proteins.

Association of the Uncomplexed Form of p85 with the Activated Insulin Receptor in Vitro—SH2 fragments of p85 bound to the activated insulin receptor but the p85 protein in the active PI 3-kinase did not. To determine whether the uncomplexed form of p85 binds to the insulin receptor in vitro, insulin receptor immunoprecipitated with monoclonal anti-insulin receptor antibodies from unstimulated CHO-IR cells were incubated in the presence or absence of $10^{-6}$ M insulin and 1 mM ATP, washed, and exposed to baculovirus-expressed p85α at 4 °C for 4 h. As shown in Fig. 5B, the uncomplexed form of p85α bound to the insulin receptor only when the receptor had been incubated with insulin and ATP.

**Discussion**

In this study, we prepared four mAbs (F12, G12, E10, and H1) against recombinant bovine p85α. F12 and G12 recognized active PI 3-kinase presumably in its heterodimeric form composed of p85 and p110 subunits as reported previously (Carpenter et al., 1990; Shibasaki et al., 1991; Otsu et al., 1991). E10 and H1 recognized the uncomplexed form of p85.

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mAb F12 immunoprecipitated only the p85 protein as part of the active PI 3-kinase in CHO cells. Immunoprecipitation studies with this antibody using CHO-IR cells revealed three main conclusions. (i) Insulin increased the amount of p85 and PI 3-kinase activity immunoprecipitable with monoclonal anti-p85 antibody. (ii) Insulin did not induce tyrosine phosphorylation of p85. (iii) Insulin induced an association of p85 as part of the PI 3-kinase complex with tyrosine-phosphorylated IRS-1 as well as with other phosphorylated proteins but not with the activated insulin receptor. Previous studies have demonstrated that insulin treatment of cells can cause a dramatic increase in PI 3-kinase activity detectable in anti-phosphotyrosine antibody immunoprecipitates of cell lysates (Endemann et al., 1990). The data presented here suggest that this is not because of an association of PI 3-kinase with the activated insulin receptor or increased phosphorylation of p85 on tyrosine. Instead it is likely to be a result of a tight association of PI 3-kinase with tyrosine phosphoproteins including IRS-1.

IRS-1 is one of the major tyrosine-phosphorylated proteins co-immunoprecipitated with p85. We could reconstitute the physical association of tyrosine-phosphorylated IRS-1 with the F12-immunoprecipitated p85 in the active PI 3-kinase complex in vitro assay system. This implies that not all tyrosine-phosphorylated IRS-1 is occupied by p85 in insulin-treated CHO-IR cell lysates. IRS-1 contains at least 10 potential tyrosine phosphorylation sites; six contain the YMXM motif, three others have the YXXM motif, and one site has the EYYE sequence (Sun et al., 1991). The YMXM motif has been proposed as a consensus sequence for tyrosine phosphorylation sites that bind to the SH-2 domain of p85 (Canley et al., 1991). Tyrosine phosphopeptides IRP-1, -2, -3, and -4 containing the motif YMXM all blocked the association of tyrosine phosphorylated IRS-1 with the p85 protein in the active PI 3-kinase. The simplest explanation is that these peptides are able to mimic IRS-1 sequences and thus act as competitive inhibitors. Since nonphosphorylated peptides were unable to inhibit the IRS-1-p85 association, phosphorylated tyrosine appears to be an essential structural recognition element. However, the phosphopeptide IRP-5 containing motif EYYE did not block this association, suggesting that p85 molecules recognize phosphotyrosine in a quite specific struc-
tural context. The relative potency of inhibition was IRP-1 > IRP-4 > IRP-3 > IRP-2. The IRP-1 peptide was the most efficient inhibitor and has been shown previously as the best exogenous substrate of the insulin receptor kinase (Shoelson et al., 1992). We specifically analyzed the YMMP motif since this sequence was also present in the major tyrosine phosphorylation site of murine and hamster polynoma virus middle T antigen induced by pp60c-src and was involved in pp60c-src-dependent binding of PI-3 kinase (Auger et al., 1992). Our results cannot entirely explain why peptide IRP-2 containing the same motif was relatively less efficient than IRP-4 and -3 with a slightly different YMNM motif. However, sequences surrounding this motif might possibly affect binding to p85. Nevertheless, these data suggest that tyrosine 608 in IRP-1 can be involved in the IRS-1-p85 association although further in vivo studies would be necessary to determine the relative contribution of IRS-1 phosphorylation sites in mediating the p85 association. It is possible that one IRS-1 molecule can associate with several molecules of p85. This could explain the increase in the amount of p85 and PI-3-kinase activity immunoprecipitated with monoclonal antibodies in CHO-IR cells after insulin stimulation.

In vitro assays were used to demonstrate the physical association of tyrosine-phosphorylated IRS-1 with SH-2 domains of p85 (85N, 85C, and 85N+C)-glutathione S-transferase fusion proteins expressed in E. coli. The present results indicate that SH-2 domains of p85 associate with IRS-1 when it is tyrosine-phosphorylated. Similar data were obtained with receptors for EGF, PDGF-β, colony-stimulating factor 1, and Kit (Hu et al., 1992; McClade et al., 1992; Klippel et al., 1992; Reedijk et al., 1992). Our data also showed an association of the activated insulin receptor with both SH-2 domains of p85-glutathione S-transferase fusion proteins. These data contrast with the present in vivo and in vitro findings showing that the p85 protein in the active PI-3 kinase associated with tyrosine-phosphorylated IRS-1 but not with the activated insulin receptor. The three-dimensional structure of SH-2 domains expressed as glutathione S-transferase fusion proteins may have differed from SH-2 domains of a free form of p85. However, this is probably not the case, since recombinant p85α expressed alone in insect cells bound to the activated receptor in vivo. These results suggest that in vitro studies on the association of tyrosine-phosphorylated proteins with SH-2 fragments may not reflect the in vivo situation, and these results should be interpreted with caution. There is a difference in specificity of binding between free p85 and PI-3-kinase form of p85 in the interaction of SH-2 domains with specific sequences containing phosphotyrosine. This is consistent with the previous observation showing that PI-3-kinase from bovine brain cannot interact with activated EGF receptors, but free p85α can (Otsu et al., 1991). Thus, the association of p85 with p110 might cause a conformational change of SH-2 domain(s) which would affect this interaction.

Several phosphoproteins, including tyrosine-phosphorylated IRS-1, were immunoprecipitated from CHO-IR cells by the monoclonal antibody to p85 after insulin treatment. A 170-kDa phosphoprotein was immunoprecipitated with anti-p85 antibody. This protein was poorly immunoblotted with polyclonal anti-phosphotyrosine antibody, suggesting that it mainly contained phosphoserine and phosphothreonine. However, it was immunoprecipitated with anti-phosphotyrosine antibody after insulin treatment. In addition, the 170-kDa protein was not immunoblotted with anti-IRS-1 antibody. These data suggest that p170 is not IRS-1-related and binds to tyrosine-phosphorylated proteins associated with the p85 protein in the active PI-3-kinase. Two other phosphoproteins of 120 and 110 kDa appeared to be tyrosine-phosphorylated in response to insulin. Proteins of the same molecular weights were also immunoprecipitated with the monoclonal anti-p85 antibody from 3H-labeled CHO-IR cells without insulin treatment. It has been reported previously that p110, one subunit of PI-3-kinase, was associated with an doublet of 110- and 120-kDa proteins as purified from rat liver (Carpenter et al., 1990). Studies with specific antibodies against p110 will be necessary to determine how tyrosine-phosphorylated 120- and 110-kDa proteins are related to the p110 subunit of PI-3-kinase. 140- and 100-kDa phosphoproteins were also co-immunoprecipitated together with the p85 in the active PI-3-kinase after insulin treatment. The former was tyrosine-phosphorylated, but the 100-kDa protein was not. A protein of a size similar to the 140 kDa was immunoblotted with anti-IRS-1 antibody in an in vitro assay for the association of IRS-1 (from insulin-stimulated CHO-IR cells) with the p85 in the active PI-3-kinase immobilized on beads. This could be an IRS-1-related protein or a proteolytic fragment of IRS-1. However, the 140-kDa protein was only poorly detected in the immunoblots of p85 immunoprecipitates with anti-IRS-1 antibody from insulin-stimulated CHO-IR cells because longer exposure of the immunoblots caused high background around the 140-kDa protein. In conclusion, we report that insulin can induce an association of the p85 in PI-3-kinase with the tyrosine-phosphorylated IRS-1, not with the activated insulin receptor, throughout the tyrosine-phosphorylated YMXM sequence of IRS-1 and the SH-2 domains of p85. Although p85 in PI-3-kinase form is not tyrosine-phosphorylated in response to insulin, the PI-3-kinase form of p85 displayed an insulin-dependent association with other phosphoproteins ranging from 100 to 170 kDa, resulting in the formation of a molecular complex. The signal transduction pathway of the insulin receptor tyrosine kinase differs from other receptor tyrosine kinases, such as those for PDGF and colony-stimulating factor 1, as well as Kit (Kazlauskas and Cooper, 1990; Escobedo et al., 1991a; Kashishian et al., 1992; Fantl et al., 1992; Reedijk et al., 1992; Lev et al., 1992), where p85 directly associates with receptor tyrosine kinases. The obvious questions to be answered are why signal transduction via the insulin receptor has this unique system and what is the role of IRS-1 in insulin signal transduction. Another question to be addressed is the mechanism of the increase of PI-3-phosphoinositide formation in insulin-treated cells (Ruderman et al., 1990). The cDNA cloning of p110 and the expression of the active protein would help to answer this question.

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