THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 268, No. 11, Issue of April 15, pp. 8204-8212, 1993
Printed in U.S.A.

Association of IRS-1 with the Insulin Receptor and the Phosphatidylinositol 3′-Kinase

FORMATION OF BINARY AND TERNARY SIGNALING COMPLEXES IN INTACT CELLS*

(Received for publication, September 9, 1992)

Jonathan M. Backer‡, Martin G. Myers, Jr., Xiao-Jian Sun, Debra J. Chin, Steven E. Shoelson, Montserrat Miralpeix, and Morris F. White

From the Research Division, Joslin Diabetes Center, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston Massachusetts 02215

Insulin stimulates the formation of binary and ternary signaling complexes between the phosphatidylinositol (PtdIns) 3′-kinase, IRS-1, and the insulin receptor *in vivo*. Binary complex formation between IRS-1 and the PtdIns 3′-kinase occurs in intact cells and requires the tyrosyl phosphorylation IRS-1, as mutant insulin receptors which weakly phosphorylate IRS-1 *in vivo* do not mediate formation of IRS-1/PtdIns 3′-kinase complexes in transfected CHO cells. Association with IRS-1 involves as much as 70% of total cellular PtdIns 3′-kinase activity. Insulin also stimulates the formation of ternary signaling complexes, as both IRS-1 and the PtdIns 3′-kinase are present in anti-insulin receptor immunoprecipitates from insulin-stimulated cells. Overexpression of IRS-1 in CHO cells increases the amount of PtdIns 3′-kinase activity in αIR immunoprecipitates, and IRS-1 markedly increases the *in vitro* binding of p85α and PtdIns 3-kinase activity to anti-receptor immunoprecipitates. The mechanism for this association is unknown, but appears to involve the binding of IRS-1/PtdIns 3′-kinase complexes to the insulin receptor. The formation of binary and ternary complexes between the insulin receptor, IRS-1 and the PtdIns 3′-kinase may play a critical role in transmission of the insulin signal.

The insulin receptor is the principal mediator of insulin action on cellular mitogenic and metabolic processes. The insulin receptor β-subunit, which contains an intrinsic tyrosine kinase, undergoes tyrosyl autophosphorylation and is activated in response to insulin binding to the extracellular α-subunit (Kahn and White, 1988). Although the discovery of the insulin receptor's tyrosine kinase activity suggested that the mechanism of insulin action involved the tyrosyl phosphorylation of intracellular substrates, these substrates have in fact been difficult to identify. Using anti-phosphotyrosine antibodies we identified a phosphoprotein called pp185 in many cell and tissues after insulin stimulation (White *et al.*, 1985). A major protein constituent of the pp185 band was recently purified and its cDNA sequence determined (Rothenberg *et al.*, 1990; Sun *et al.*, 1991). The cloned protein, called IRS-1, contains 20 potential tyrosyl phosphorylation sites. Nine of these tyrosine residues are present within the motif Y(M/*X*)*X*M. Moreover, synthetic peptides containing these motifs are efficiently phosphorylated by the purified insulin receptor kinase, suggesting that these are phosphorylation sites in IRS-1 (Shoelson *et al.*, 1992).

Phosphorylated Y(M/*X*)*X*M motifs in IRS-1, in the kinase insert region of the platelet-derived growth factor (PDGF)[1] and colony stimulating factor 1 receptors, as well as in the epidermal growth factor receptor and in the polyoma middle T antigen serve as binding sites for the phosphatidylinositol 3′-kinase (PtdIns 3′-kinase), a lipid kinase which appears to play an important role in the regulation of cell growth (reviewed in Cantley *et al.*, 1991). The PtdIns 3′-kinase consists of a 110-kDa catalytic subunit (p110) which is homologous to a *Saccharomyces cerevisiae* protein involved in vacuolar sorting, and a 85-kDa regulatory subunit (p85α) which contains two SH2 and one SH3 domain (Carpenter *et al.*, 1990; Shibasaki *et al.*, 1991; Escobedo *et al.*, 1991b; Skolnik *et al.*, 1991; Otsu *et al.*, 1991; Hiles *et al.*, 1992). SH2 domains bind to phosphotyrosine residues in Y(M/*X*)*X*M motifs with high affinity, and the SH2 domains of p85α mediate the binding of the PtdIns 3′-kinase to tyrosine kinase growth factor receptors (McGlade *et al.*, 1992; Hu *et al.*, 1992; Klippel *et al.*, 1992; Reedjik *et al.*, 1992).

Insulin stimulates the PtdIns 3′-kinase (Endemann *et al.*, 1990; Ruderman *et al.*, 1990; Kelly *et al.*, 1992) and a major mechanism of activation of the PtdIns 3′-kinase appears to involve the association of phosphorylated IRS-1 with p85α (Backer *et al.*, 1992b). Phosphorylated Y(M/*X*)*X*M phosphopeptides activate the PtdIns 3′-kinase *in vitro*, suggesting that the binding of tyrosyl-phosphorylated Y(M/*X*)*X*M motifs in IRS-1 to SH2 domains in p85α may be the critical step in the activation of the PtdIns 3′-kinase by insulin (Backer *et al.*, 1992b; Myers *et al.*, 1992). Insulin also stimulates the appearance of PtdIns 3′-kinase activity in anti-insulin-receptor immunoprecipitates (Ruderman *et al.*, 1990; Backer *et al.*, 1992a). Interactions between the PtdIns 3′-kinase and the insulin receptor may occur directly through the SH2 domains of p85α and phosphorylated Y(M/*X*)*X*M motifs in the C

* This work was supported in part by grants from the Juvenile Diabetes Association (to J. M. B and S. E. S.), a postdoctoral fellowship from the Juvenile Diabetes Foundation (to X.-J. S.), National Institutes of Health Grants DK-38712 and DK-43808 (to M. F. W.), and Joslin's Diabetes Endocrinology Research Center Grant DK 36836. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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

[1] The abbreviations used are: PDGF, platelet-derived growth factor; PtdIns, phosphatidylinositol; CHO, Chinese hamster ovary; HPLC, high-performance liquid chromatography; IR, insulin receptor; PAGE, polyacrylamide gel electrophoresis; TLC, thin-layer chromatography.

terminus of the insulin receptor, similar to the mechanism proposed for the PDGF and colony stimulating factor 1 receptors (Cantley *et al.*, 1991). Alternatively, IRS-1 associates with the insulin receptor in insulin-stimulated cells (Sun *et al.*, 1992) and may provide a bridge between the insulin receptor and p85α.

This study addresses the *in vivo* association of the PtdIns 3'-kinase with tyrosyl-phosphorylated IRS-1 and the insulin receptor in Chinese hamster ovary (CHO) cells. The PtdIns 3'-kinase 85-kDa subunit can be detected immunologically in both anti-IRS-1 and anti-insulin receptor immunoprecipitates from insulin-stimulated cells. The association with IRS-1 occurs in intact cells and is dependent upon the *in vivo* tyrosyl phosphorylation of IRS-1. Association with tyrosyl-phosphorylated IRS-1 accounts for as much as 70% of total cellular PtdIns 3-kinase. In addition to forming binary complexes with IRS-1, the PtdIns 3'-kinase forms ternary complexes with IRS-1 and the insulin receptor; these ternary complexes may account at least in part for the presence of PtdIns 3'-kinase in insulin receptor immunoprecipitates from insulin-stimulated cells (Backer *et al.*, 1992a). Thus, overexpression of IRS-1 increases the amount of insulin receptor-associated PtdIns 3'-kinase in insulin-stimulated cells, and IRS-1 increases the ability of immunopurified insulin receptors to bind the PtdIns 3'-kinase *in vitro*. These data suggest that the binding of p85α to tyrosyl-phosphorylated IRS-1 and IRS-1/insulin receptor complexes plays a role in the regulation of the PtdIns 3'-kinase by insulin.

EXPERIMENTAL PROCEDURES

Antibodies to IRS-1, the Insulin Receptor, and the PtdIns 3'-Kinase—A polyclonal rabbit antibody (αpep80) against the peptide YIPGATMGTSPALTGD (Peptide 80) from the cDNA sequence of rat IRS-1 (Sun *et al.*, 1991), was prepared by coupling to Keyhole limpet hemocyanin and injection into New Zealand White rabbits as previously described (Perlman *et al.*, 1989). Antibodies were affinity-purified by chromatography over a column of peptide 80 coupled to Affi-Gel 10 (Bio-Rad). Polyclonal antibodies to baculovirus-produced rat IRS-1 (αIRS-1^{bac}) and to a glutathione *S*-transferase-p85α bacterial fusion protein containing amino acids 321–724 (Skolnik *et al.*, 1991; Hu *et al.*, 1992) of the human p85α (αp85) were produced by injection into New Zealand White rabbits and were purified by protein A-Sepharose chromatography. For p85α Western blots, αp85 antibody (UBI) was used without further purification. A mouse monoclonal antibody to the human insulin receptor (83–14) was provided by Dr. Kenneth Siddle, University of Cambridge. Polycolonal rabbit anti-phosphotyrosine antibodies (αPY) have been previously described (White and Backer, 1991).

Synthesis of Peptides and Phosphopeptides—Peptides and phosphopeptides were synthesized using a Milligen/Biosearch 9600 synthesizer using FmocTyr(OP(OCH$_2$)$_2$) as previously described (Backer *et al.*, 1992b). IRS-1 peptides were derived from the region surrounding Y^{628} (Sun *et al.*, 1991), include Y-628 (NDGYMPMSPKS), P-Y628 (NDG(P)YMPMSPKS) and the scrambled peptide P-Y628$_{scr}$ (PMPNSKMD(P)YGG). All peptides were purified by gel exclusion chromatography and were > 80% pure by analysis on reversed-phase HPLC.

Cell Culture and Transfection of CHO Cells—CHO cells expressing only endogenous insulin receptor and IRS-1 (CHO/neo), or expressing 1.2×10^6 wild-type human insulin receptors (CHO/IR) have been previously described (White *et al.*, 1988). Several previously described insulin receptor mutants were used: IR$_{A1018}$ contains a mutation of Lys^{1018} to Ala^{1018} in the putative ATP binding site of the receptor, and is an inactive kinase (Chou *et al.*, 1987); IR$_{\Delta960}$ lacks 12 amino acids in the cytoplasmic juxtamembrane region and is biologically inactive, although it undergoes significant insulin-stimulated autophosphorylation *in vivo* and is a normal kinase *in vitro* once autophosphorylated (Backer *et al.*, 1991); IR$_{F1146}$ contains phenylalanine in place of one of the first sites of insulin receptor tyrosyl autophosphorylation, and is a partially impaired kinase (Wilden *et al.*, 1990). The corresponding cells lines (CHO/IR$_{A1018}$, CHO/IR$_{\Delta960}$, and CHO/IR$_{F1146}$) express similar numbers of these mutant receptors, but are defective for phosphorylation of pp185 and activation of the PtdIns 3'-kinase *in vivo* (Backer *et al.*, 1992a).

CHO/IRS-1 cells, which overexpress the rat IRS-1 cDNA, were prepared by calcium phosphate cotransfection of the IRS-1 cDNA in the expression vector pCMVhis (M. Birmbaum, Harvard Medical School) and the pSVEneo plasmid. CHO/IR/IRS-1 cells, which overexpress insulin receptors and IRS-1, were prepared by transfection of CHO/IR cells with the IRS-1/pCMVhis vector. CHO/his cells, which were transfected with pCMV/his alone, were used as control cells in all experiments involving the CHO/IRS-1 or CHO/IR/IRS-1 lines. Cells were selected by culture in 800 μg/ml geneticin (GIBCO) and 10 mM histidinol (Sigma) as previously described (Sun *et al.*, 1991).

Preparation of IRS-1 in Baculovirus-infected Sf-9 Cells—The rat IRS-1 cDNA, with the 5'- and 3'-untranslated regions removed, was subcloned into the vector pBlueBac (Invitrogen) and was recombined into the baculovirus genome using standard techniques (Meyers *et al.*, 1992). Sf9 cells were infected with 10–20 plaque-forming units/cell of recombinant baculovirus as described (Herrera *et al.*, 1988). Cells were lysed after 48–52 h of infection in a dounce homogenizer in 50 mM Tris, pH 7.8, containing 1 M NaCl, 10 μg/ml aprotinin, 10 mM benzamidine, 10 μg/ml leupeptin, 350 μg/ml phenylmethylsufonyl fluoride. Insoluble material was removed by sedimentation at 100,000 $\times g$ for 1 h, and IRS-1 was purified to 90% purity by chromatography on Sephacryl S-300 HR (Pharmacia LKB Biotechnology Inc.). Baculovirus-produced IRS-1 (IRS-1^{bac}) migrated identically to immunopurified IRS-1 from CHO/IRS cells in SDS-PAGE gels.

In vitro lipid kinase assays and analysis of products by TLC and HPLC. Quiescent CHO/neo, CHO/his, CHO/IRS-1, CHO/IR/IRS-1, or CHO/IR cells were incubated in the absence or presence of insulin (100 nM) for 10 min, washed with ice-cold phosphate-buffered saline/100 μM Na_3VO_4 and twice with 20 mM Tris (pH 7.5) containing 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 100 μM Na_3VO_4 (Buffer A), and solubilized in Buffer A containing 1% Nonidet P-40 (Sigma), 10% glycerol, and 350 μg/ml phenylmethylsufonyl fluoride (lysis buffer). A post-13,000 $\times g$ supernatant was immunoprecipitated with anti-phosphotyrosine (αPY), anti-IRS-1 (αIRS-1), anti-insulin receptor (αIR), or anti-PtdIns 3'-kinase (αp85) antibody and protein A-Sepharose (Pharmacia). The pellets were washed and *in vitro* lipid kinase activity was measured as previously described (Ruderman *et al.*, 1990) using 10 μl of a sonicated dispersion of PtdIns (2 μg/μl) or PtdIns, PtdIns(4)P, PtdIns(4,5)P$_2$, and phosphatidylserine (2:1:1:0.4, 2.2 μg/μl) in 10 mM Tris (pH 7.5)/1 mM EGTA as substrates. The phosphorylated lipids were analyzed by thin-layer chromatography on silica gel TLC plates (Merck) coated with 1% potassium oxalate. TLC plates were developed in $CHCl_3$:CH_3OH:H_2O:NH_4OH (60:47:11.3:2) or propanol/2 M acetic acid (65:35), dried, and visualized by autoradiography. The radioactivity in spots which comigrated with PtdIns(4)P and PtdIns (4,5)P$_2$ standards (Sigma) or a [^{32}P]InsP$_3$ standard (from αPY immunoprecipitates of insulin-stimulated cells) was measured by Cerenkov counting as previously described (Ruderman *et al.*, 1990).

HPLC analysis was performed as described by Auger *et al.* (1989). Samples were deacylated by incubation in 25% methlyamine:methanol:n-butanol (57.7:61.6:15.6) for 50 min at 53 °C, dried and extracted twice in n-butanol:petroleum ether:ethyl formate (20:4:1). Water-soluble products were dried, resuspended in water, and analyzed by anion-exchange HPLC on a Partisil SAX-5 column (Whatman) as described (Auger *et al.*, 1989), using a 0–1 M $(NH_2)_2HPO_4$ (pH 3.8) gradient. [^{3}H]IP$_3$ and [^{3}H]IP$_4$ (Du Pont-New England Nuclear) and deacylated [^{3}H]PtdIns(4)P and [^{3}H]PtdIns(4,5)P$_2$ (Du Pont-New England Nuclear) were used as standards.

Western Blotting—Immunoprecipitations for Western blot analysis were performed exactly as described above for analysis of lipid kinase activity. Proteins were separated on 7.5% resolving SDS-PAGE minigels (Bio-Rad), transferred to nitrocellulose in Towbin buffer containing 0.02% SDS (Towbin *et al.*, 1979), and blotted with αp85, αIRS-1, or αPY antibodies. Proteins were visualized using [^{125}I] protein A (ICN) and autoradiography.

In vitro tyrosyl phosphorylation of IRS-1. IRS-1 from baculovirus-infected Sf-9 cell lysates (IRS-1^{bac}; approximately 1.9 μg of IRS-1/point) was incubated for 20 min at 22 °C with wheat germ agglutinin-purified human insulin receptors (4–6 μg of protein) in the presence of 5 mM $MnCl_2$, 100 nM insulin, and 100 μM ATP containing 30 μCi of [^{32}P]ATP (Du Pont-New England Nuclear). Samples were boiled in Laemmli sample buffer (Laemmli, 1970), boiled, and analyzed by SDS-PAGE (7.5% resolving) and autoradiography. IRS-1 samples for PtdIns 3'-kinase binding assays were incubated in the absence or presence of wheat germ agglutinin-purified insulin receptors overnight at 4 °C in the presence of 5 mM $MnCl_2$, 100 nM insulin, and 100 μM ATP, immobilized on αIRS-1/protein A-Sepharose beads, and washed three times in lysis buffer prior to use.

In Vitro IRS-1/PtdIns 3′-Kinase Binding Assays and Peptide Competition Studies—Quiescent CHO/neo or CHO/IR cells were incubated in the absence or presence of 100 nM insulin for 10 min, washed, and lysed as described above. The cell lysates were incubated for an additional 15 min at 4 °C in the absence or presence of the indicated peptides derived from sequences in IRS-1 (concentrations as described in the text). The lysates were added to immobilized nonphosphorylated or tyrosyl-phosphorylated IRS-1 (approximately 1.9 μg/point) for 15 min at 4 °C, the pellets were washed as described for analysis of lipid kinase activity, and IRS-1-associated PtdIns 3′-kinase activity was determined as described above.

In Vitro Insulin Receptor/IRS-1/PtdIns 3′-Kinase Binding Assays—Partially purified insulin receptors (2 nM final) were incubated at 22 °C for 20 min in 50 mM HEPES, pH 7.4, containing 0.1% Triton X-100, 50 μM ATP, 5 mM $MnCl_2$, and the absence or presence of IRS-1^{bac} (75 nM final) and insulin (100 nM). The reactions were stopped by the addition of 500 μl of ice-cold Buffer A containing 1% Nonidet P-40 (Sigma), 10% glycerol, 100 μM vanadate, and 350 μg/ml phenylmethylsufonyl fluoride (stop buffer). Anti-insulin receptor (83-14) or anti-IRS-1 (αIRS-1^{bac}) immunoprecipitates where prepared using protein A-Sepharose (Pharmacia), washed three times in stop buffer, and incubated with for 30 min at 4 °C with lysates from quiescent CHO/neo cells, prepared as described above. The protein A-Sepharose/antibody pellets were additionally washed as described for the lipid kinase assay, and either assayed for PtdIns 3′-kinase activity or boiled in Laemmli sample buffer (Laemmli, 1970) and analyzed by immunoblotting.

RESULTS

PtdIns 3′-Kinase Binds to Tyrosyl-phosphorylated IRS-1 in Vivo—PtdIns 3′-kinase activity was measured in immunoprecipitates from CHO cells expressing the wild-type human insulin receptor (CHO/IR cells). PtdIns 3′-kinase activity was barely detected in anti-phosphotyrosine (αPY) and anti-IRS-1 (αIRS-1) immunoprecipitates from unstimulated CHO/IR cells (Fig. 1*A*). However, a significant basal activity was detected using an antibody (αp85) to the PtdIns 3′-kinase-associated protein p85α (Skolnik *et al.*, 1991; Hu *et al.*, 1992). After insulin stimulation, a marked increase in PtdIns 3′-kinase activity was found with all three antibodies (Fig. 1*A*). Although a precise comparison of the amount of activity immunoprecipitated with each antibody was difficult due to uncertainty as to their concentration and affinity, the IRS-1-associated PtdIns 3′-kinase activity was approximately 50% of the insulin-stimulated activity detected with αp85. The lipid kinase activity in both αPY and αIRS-1 immunoprecipitates from insulin-stimulated cells utilized PtdIns, PtdIns(4)P, and PtdIns(4,5)P_2 as substrates, yielding phosphatidylinositol phosphate, phosphatidylinositol P_2, and phosphatidylinositol P_3, respectively (data not shown). The deacylated glycerophosphoinositol polyphosphate products from the αIRS-1 lipid kinase reactions eluted identically to those from the αPY immunoprecipitates (Fig. 2, *A–C*), which have previously been shown to be derived from phosphatidylinositol 3-P, phosphatidylinositol 3,4-P_2, and phosphatidylinositol 3,4,5-P_3, respectively (Ruderman *et al.*, 1990).

The presence of the 85-kDa subunit of the PtdIns 3′-kinase (p85α) in αIRS-1 immunoprecipitates was confirmed by immunoblotting with an αp85 antibody. Similar amounts of p85α were detected in αp85 immunoprecipitates from unstimulated or insulin-stimulated control (CHO/his) and CHO/IR cells (Fig. 1*B*). In contrast, insulin slightly increased the amount of p85α in αIRS-1 immunoprecipitates from CHO/his cells, and caused a marked increase in the amount of p85α in αIRS-1 immunoprecipitates from CHO/IR cells; IRS-1-associated p85 was approximately 75% of that detected with αp85 antibody. These data are consistent with the increased tyrosyl phosphorylation of IRS-1 and increased IRS-1-associated PtdIns 3′-kinase activity in CHO/IR as opposed to CHO/his cells (Fig. 3A, 3*B*). Interestingly, a small amount of p85α was also observed in αIR immunoprecipitates from insulin-stimulated CHO/IR cells, consistent with the presence of PtdIns 3′-kinase activity in αIR immunoprecipitates (Ruderman *et al.*, 1990; Backer *et al.*, 1992b). p85α in αIR-immunoprecipitates was approximately 10% of that detected in αIRS-1 immunoprecipitates. Together with the previous results, the association of PtdIns 3′-kinase activity with IRS-1 correlated with the increased immunoprecipitation of p85α by αIRS-1. In contrast, the increased PtdIns 3′-kinase activity in αp85 immunoprecipitates from insulin-stimulated CHO/IR cells (Fig. 1*A*) occurred without a change in the amount of p85α in these immunoprecipitates (Fig. 1*B*). Insulin-stimulated association of p85α with IRS-1 is therefore coincident with activation of the PtdIns 3′-kinase in intact CHO/IR cells (Backer *et al.*, 1992b).

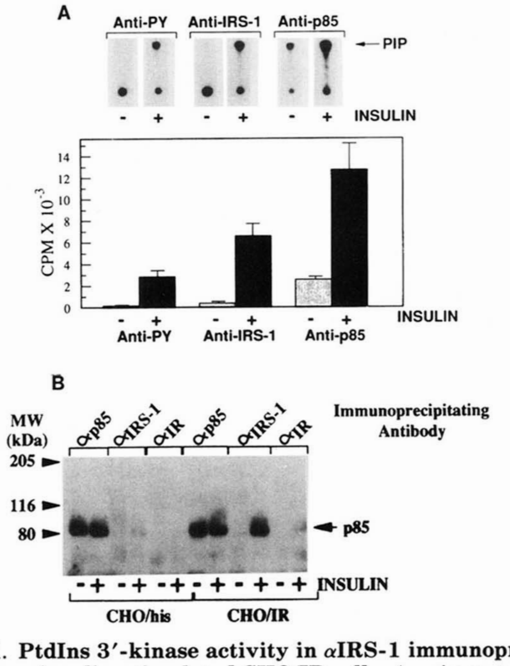

FIG. 1. **PtdIns 3′-kinase activity in αIRS-1 immunoprecipitates from insulin-stimulated CHO/IR cells.** *A*, quiescent CHO/IR cells were incubated in the absence or presence of 100 nM insulin for 10 min at 37 °C, solubilized, and incubated at 4 °C with antibody and protein A-Sepharose. PtdIns 3′-kinase activity in the washed αPY, αIRS-1, and αp85 immunoprecipitates was assayed as described, and the lipids were extracted and separated by TLC in $CHCl_3$:CH_3OH:H_2O:NH_4OH (60:47:11.3:2). The TLC plates were visualized by autoradiography and [^{32}P]phosphate incorporation into PtdIns(3)P was quantitated by Cerenkov counting. *B*, quiescent CHO/his or CHO/IR cells were incubated in the absence or presence of 100 nM insulin for 10 min at 37 °C. The cells were lysed and incubated with αIR, αIRS-1, or αp85 antibodies followed by protein A-Sepharose. Washed immunoprecipitates were analyzed by Western blotting with αp85 (*UBI*). Blots were visualized by autoradiography after incubation with [^{125}I]protein A.

Tyrosyl phosphorylation of IRS-1 is required for PtdIns 3′-kinase binding *in vitro* (Backer *et al.*, 1992b; Myers *et al.*, 1992). To examine this requirement *in vivo*, we studied IRS-1/PtdIns 3′-kinase binding in CHO cells expressing mutant insulin receptors defective for the phosphorylation of IRS-1. Although overexpression of wild-type receptor increased the tyrosyl phosphorylation of IRS-1 in CHO/IR cells as compared to control CHO/neo cells, equivalent overexpression of IR_{A1018}, $IR_{\Delta960}$, or IR_{F1146} did not (Fig. 3*A*); each of these mutants has been previously shown to be deficient for the insulin-stimulated phosphorylation of pp185 (Backer *et al.*,

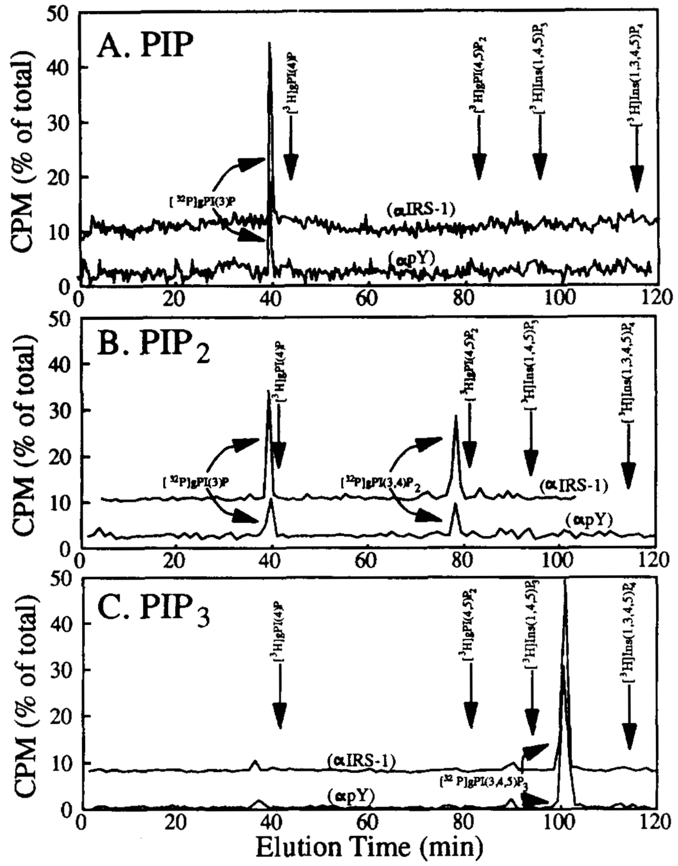

FIG. 2. **Analysis of *in vitro* lipid products from αPY and αIRS-1 immunoprecipitates by TLC and HPLC.** αPY and αIRS-1 immunoprecipitates from insulin-stimulated CHO/IR cells were prepared as described and assayed for lipid kinase activity toward a mixture of PtdIns, PtdIns(4)P, PtdIns(4,5)P_2, and phosphatidylserine (2:1:1:0.4). The lipid products were extracted and separated by TLC in propanol:2 M acetic acid (65:35), and visualized by autoradiography. The lipids from the putative PtdIns(3)P (*A*), PtdIns(3, 4)P_2 (*B*), and PtdIns(3,4,5)P_3 (*C*) spots were deacylated as described and analyzed by anion exchange HPLC against a 0–1 M $(NH_2)_2HPO_4$ (pH 3.8) gradient. The elution of [^{3}H]IP_3, [^{3}H]IP_4 and deacylated [^{3}H] PtdIns(4)P and [^{3}H]PtdIns(4,5)P_2 standards is indicated.

1992a). Insulin stimulation of IRS-1/PtdIns 3′-kinase association paralleled IRS-1 phosphorylation in all the cell lines. The insulin-stimulated appearance of PtdIns 3′-kinase activity in αIRS-1 immunoprecipitates from the CHO/IR cells was 5-fold greater than in the control CHO/neo cells, whereas in CHO/IR_{A1018} cells it was slightly lower than in control cells (Fig. 3*B*). IRS-1-associated PtdIns 3′-kinase activity was similar to control in the CHO/$IR_{\Delta960}$ and CHO/IR_{F1146} cells (Fig. 3, *C* and *D*). In all three mutant cell lines, the insulin-stimulated appearance of PtdIns 3′-kinase activity in αIRS-1 immunoprecipitates was reduced in a manner consistent with our previous results obtained with αPY antibody (Backer *et al.*, 1992a). Thus, mutations in the insulin receptor have similar effects on insulin-stimulated IRS-1 tyrosyl phosphorylation and αIRS-1-precipitable PtdIns 3′-kinase activity.

Association of IRS-1 and the PtdIns 3′-kinase occurs in intact insulin-stimulated cells. We have previously shown that PtdIns 3-kinase kinase present in CHO cells lysates will bind to *in vitro*-tyrosyl-phosphorylated IRS-1, and that this binding is inhibited by tyrosyl phosphopeptides containing Y*XX*M motifs (Backer *et al.*, 1992b). Thus, when tyrosyl-phosphorylated IRS-1^{bac} was immobilized on αIRS-1/protein A-Sepharose beads and incubated for 15 min at 4 °C with lysates from quiescent CHO/neo cells, PtdIns 3′-kinase binding was inhibited strongly by a tyrosyl-phosphorylated peptide derived from the Y^{628}MPM region of IRS-1 (P-Y628) (Fig. 4*A*). Nonphosphorylated peptide (Y-628) had no effect on the binding of the PtdIns 3′-kinase to tyrosyl-phosphorylated IRS-1 (data not shown), and a scrambled version of P-Y628 (P-$Y628_{scr}$) was a weak inhibitor of IRS-1^{bac}/PtdIns 3′-kinase binding (Fig. 4*A*).

Dissociation of preformed IRS-1/PtdIns 3′-kinase complexes could only be observed at peptide concentrations >1 mM (data not shown). We used this characteristic to demonstrate that the presence of PtdIns 3′-kinase in anti-IRS-1 immunoprecipitates (Fig. 1) was due to association *in vivo*. Insulin-stimulated CHO/IR cells were solubilized in the absence or presence of 100 μM nonphosphorylated or phosphorylated YM*X*M peptide (Y628 and P-Y628, respectively), and the cell lysates were immunoprecipitated with αIRS-1 antibody (Fig. 4*B*). Neither the Y628 nor the P-Y628 had any effect on the amount of IRS-1-associated PtdIns 3′-kinase activity, despite the fact that 100 μm of P-Y628 blocks greater than 90% of IRS-1/PtdIns 3′-kinase binding *in vitro* (Fig. 4*A*). Thus, the insulin-stimulated association of IRS-1 and the PtdIns 3′-kinase occurred in the intact cell prior to solubilization.

Quantitation of in Vivo IRS-1/PtdIns 3′-Kinase Association—Previous studies have shown cytoplasmic PtdIns 3′-kinase associates quantitatively with the PDGF receptor in PDGF-stimulated cells (Kazlauskas and Cooper, 1989, 1990). Similarly, the activity and immunoblotting data in Fig. 1 suggest that as much as 50–75% of cellular PtdIns 3-kinase associates with IRS-1 after insulin stimulation of CHO/IR cells. To estimate the fraction of cellular PtdIns 3′-kinase which associates with endogenous CHO cell IRS-1 after insulin stimulation, lysates from quiescent or insulin-stimulated CHO/IR cells were analyzed using the *in vitro* IRS-1 binding assay. The binding of PtdIns 3′-kinase activity from both quiescent and insulin-stimulated cell lysates was markedly increased by tyrosyl phosphorylation of IRS-1 (Fig. 5, compare *lanes a* with *b*, *c* with *d*; the apparent binding of PtdIns 3′-kinase to nonphosphorylated IRS-1 in *lane c* reflects the binding of endogenous IRS-1/PtdIns 3′-kinase complexes to excess αIRS-1 antibody on the Protein-A beads). Interestingly, the immobilized tyrosyl-phosphorylated IRS-1^{bac} bound 70% less PtdIns 3′-kinase activity in lysates from insulin-stimulated as compared to unstimulated cells (Fig. 5, compare *lanes b* and *d*). This decrease in bound PtdIns 3′-kinase presumably reflects the formation of complexes between the PtdIns 3′-kinase and endogenous IRS-1 or other cellular proteins in the insulin-stimulated cells, rendering a significant portion of the cellular PtdIns 3′-kinase pool unavailable for *in vitro* interactions with immobilized phosphorylated IRS-1. Thus, during insulin stimulation at least 70% of cellular PtdIns 3′-kinase associates with endogenous proteins, including IRS-1.

An alternative estimate of the extent of IRS-1/PtdIns 3′kinase binding *in vivo* was derived from our previously published data on the activation of PtdIns 3′-kinase by association with IRS-1 (Backer *et al.*, 1992b). We previously showed that the specific activity of PtdIns 3′-kinase in anti-p85 immunoprecipitates from insulin-stimulated cells was increased approximately 3-fold over basal activity, whereas that in αIRS-1 immunoprecipitates was increased approximately 8-fold. If one assumes that the entire stimulation of PtdIns 3′-kinase in the αp85 immunoprecipitates was due to association with IRS-1, then the binding of 30% of the total p85 pool to IRS-1 would account for the observed change in

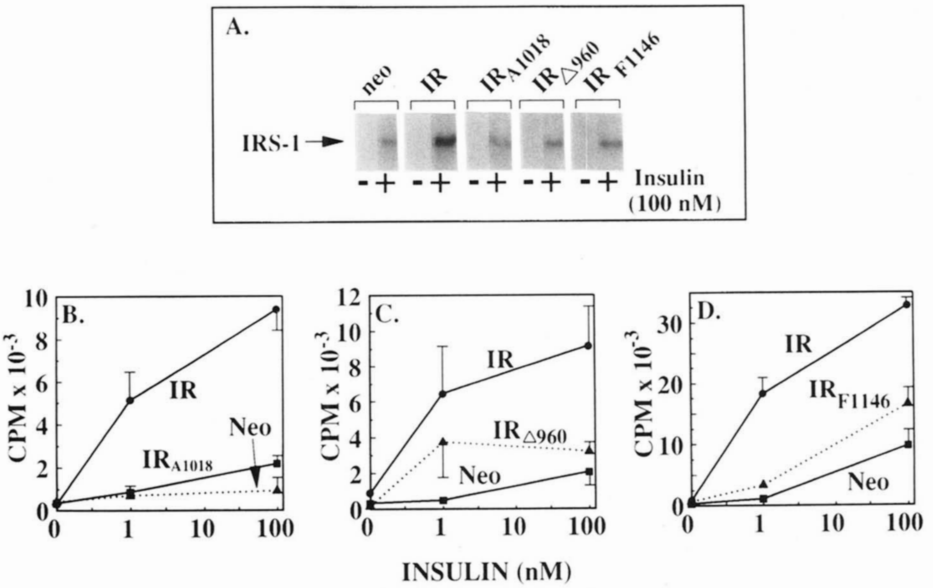

FIG. 3. **Stimulation of IRS-1 phosphorylation and IRS-1/PtdIns 3′-kinase association in CHO cells expressing mutant insulin receptors.** Control CHO cells or CHO cells expressing 10^6 wild-type or mutant insulin receptors were stimulated with varying concentrations of insulin for 10 min at 37 °C. The cells were solubilized and αIRS-1 (αpep80) immunoprecipitates were prepared. *A*, immunoprecipitated proteins were eluted in Laemmli sample buffer (Laemmli, 1970), separated by SDS-PAGE (7.5% resolving), and immunoblotted with αPY. *B*, αIRS-1 immunoprecipitates from CHO/IR, CHO/IR_{A1018}, and CHO/neo cells were assayed for PtdIns 3′-kinase activity. *C*, αIRS-1 immunoprecipitates from CHO/IR, CHO/$IR_{\Delta 960}$, and CHO/neo cells were assayed for PtdIns 3′-kinase activity. *D*, αIRS-1 immunoprecipitates from CHO/IR, CHO/IR_{F1146}, and CHO/neo cells were assayed for PtdIns 3′-kinase activity.

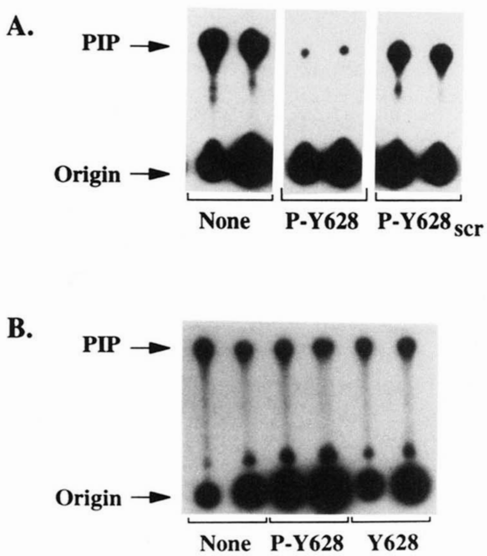

FIG. 4. **αIRS-1-precipitable PtdIns 3′-kinase from insulin-stimulated CHO/IR cells: effect of YMXM peptides.** *A*, IRS-1[bac] was immobilized on αIRS-1/protein A-Sepharose beads and tyrosyl-phosphorylated by wheat germ agglutinin-purified insulin receptors as described. The pellets were washed and incubated for 15 min at 4 °C with detergent lysates from quiescent CHO/neo cells in the absence or presence of 100 μM P-Y628 or scrambled P-Y628 (P-$Y628_{scr}$) peptide. The pellets were then washed and assayed for associated PtdIns 3′-kinase activity as described. Lipid products were separated by TLC, visualized by autoradiography, and quantitated by Cerenkov counting. *B*, CHO/IR cells were incubated in the presence of 100 nM insulin for 10 min at 37 °C. The cells were lysed as described, or in the presence of 100 μM Y628 or P-Y628 peptide. αIRS-1 immunoprecipitates were prepared and assayed for PtdIns 3′-kinase activity as described.

specific activity. Although somewhat lower than the estimates derived from the data in Figs. 1 and 5, this calculation still suggests that a significant portion of the total intracellular PtdIns 3′-kinase pool, at least one-third and as much as two-thirds, binds to IRS-1 following insulin stimulation.

Formation of Ternary IRS-1/Insulin Receptor/PtdIns 3′-Kinase Complexes in Vivo and in Vitro—We have previously shown that the PtdIns 3′-kinase can be specifically immunoprecipitated by anti-insulin receptor antibodies (αIR) (Backer *et al.*, 1992a), and p85α can be detected in αIR immunoprecipitates from insulin-stimulated CHO/IR cells by Western blotting (Fig. 1*B*). While this may be due to a direct interaction between the insulin receptor and the PtdIns 3′-kinase, we have recently found that the insulin receptor and IRS-1 form a complex in insulin-stimulated cells (Sun *et al.*, 1992). Moreover, we have found that both the insulin receptor and IRS-1 can be detected in αp85 immunoprecipitates, raising the possibility of ternary receptor/IRS-1/PtdIns 3′-kinase complexes in insulin-stimulated cells (Backer *et al.*, 1992b).

To examine whether insulin receptor/IRS-1 complex formation mediates the immunoprecipitation of the PtdIns 3′-kinase by anti-insulin receptor antibodies, we compared the amount of αIR-precipitable PtdIns 3′-kinase in CHO/IR and CHO/IR/IRS-1 cells (Fig. 6). An insulin-stimulated increase in αIR-immunoprecipitable PtdIns 3′-kinase activity was seen in both cell lines. Interestingly, increasing the cellular expression of IRS-1 markedly increased the amount of αIR-precipitable PtdIns 3′-kinase, suggesting that αIR-precipitable PtdIns 3′-kinase may be due to the association of receptors with IRS-1/PtdIns 3′-kinase complexes.

The role of IRS-1 in the association of the PtdIns 3′-kinase with the insulin receptor was examined using an *in vitro* binding assay similar to that used in Fig. 4. Partially purified insulin receptors were incubated in the presence of insulin, $MnCl_2$, and ATP and the absence or presence of IRS-1[bac]. The mixtures were immunoprecipitated with either αIR or αIRS-1 antibody, immobilized on protein A-Sepharose beads, and washed. The beads were then incubated with lysates from quiescent CHO/neo cells, washed, and assayed for associated for PtdIns 3′-kinase activity. Unstimulated insulin receptors, immunoprecipitated with αIR, bound a small amount of PtdIns 3′-kinase from CHO lysates, and insulin-stimulated receptors bound approximately 4-fold more activity (Fig. 7*A*). However, αIR immunoprecipitates of insulin receptor/IRS-1 mixtures bound an additional 3–4-fold more PtdIns 3′-kinase activity than immunoprecipitates containing only insulin-stimulated receptors; αIR immunoprecipitates from the receptor/IRS-1 mixtures bound nearly 35% of the PtdIns 3′-kinase activity which bound to αIRS-1 immunoprecipitates from the same mixtures. Thus, IRS-1 significantly increased the amount of receptor-associated PtdIns 3′-kinase activity.

Similar results were obtained using αp85 immunoblots to measure the amount of p85α from CHO lysates which asso-

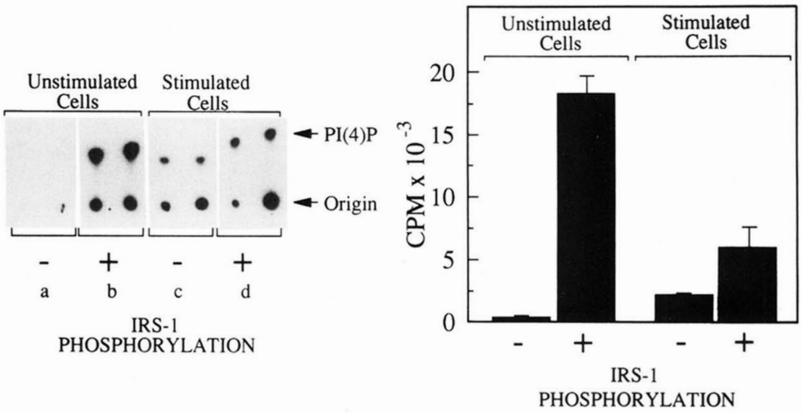

FIG. 5. **Availability of PtdIns 3'-kinase from insulin-stimulated cells for *in vitro* binding to IRS-1.** *A*, IRS-1^{bac} (*a* and *c*) or tyrosyl-phosphorylated IRS-1^{bac} (*b* and *d*), immobilized on αIRS-1/protein A beads, was incubated for 15 min at 4 °C with lysates from quiescent (*a* and *b*) or insulin-stimulated (*c* and *d*) CHO/IR cells. The beads were washed and assayed for associated PtdIns 3'-kinase activity. Lipid products were separated by TLC, visualized by autoradiography and quantitated by Cerenkov counting.

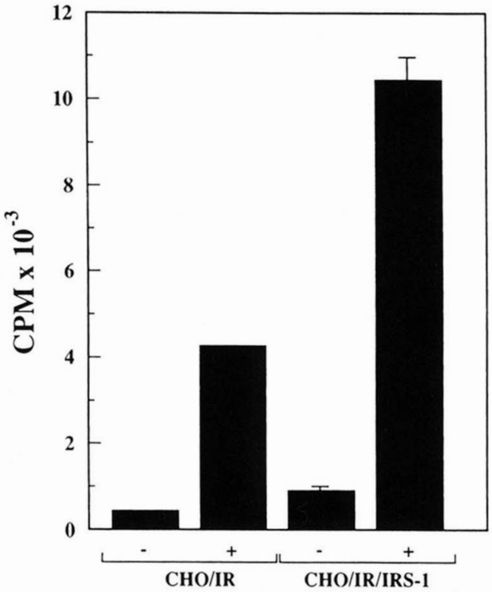

FIG. 6. **αIR-precipitable PtdIns 3'-kinase activity in cells overexpressing IRS-1.** CHO/IR and CHO/IR/IRS-1 cells were incubated in the absence or presence of insulin for 10 min at 37 °C. The cells were solubilized and αIR immunoprecipitates were prepared and assayed for PtdIns 3-kinase activity.

ciated with the anti-receptor and anti-IRS-1 immunoprecipitates (Fig. 7*B*). The binding of p85α to unstimulated αIR immunoprecipitates was undetectable, and binding to insulin-stimulated immunoprecipitates was barely detectable (Fig. 7*B*, *lanes a* and *b*). In contrast, the binding of p85α to αIR immunoprecipitates from insulin stimulated receptor/IRS-1 mixtures was easily detected (Fig. 7*B*, *lane d*). Control αIRS-1 immunoprecipitates from unstimulated and stimulated IR/IRS-1 mixtures bound even more p85 (Fig. 7*B*, *lanes e* and *f*), due to basal (Fig. 7*C*, *lane c*) and insulin-stimulated (Fig 7*C*, *lane d*) receptor and IRS-1 tyrosyl phosphorylation. The increased binding of p85α to αIR immunoprecipitates from IR/IRS-1 mixtures was likely due to association between the insulin receptor and IRS-1, as αIRS-1 immunoblots revealed the presence of IRS-1 in αIR immunoprecipitates from IR/IRS-1^{bac} mixtures (Fig. 7*B*, *lanes g* and *h*). No immunoprecipitation of IRS-1 by αIR was seen in the absence of added receptors (Fig. 7*B*, *lanes i* and *j*). These data strongly support the formation of ternary receptor/IRS-1/PtdIns 3'-kinase complexes, and suggest that IRS-1 forms a stable link between the insulin receptor and the PtdIns 3'-kinase.

The blots in Fig. 7*B*, *lanes a–d* were reblotted with anti-phosphotyrosine antibody to confirm the presence of IRS-1 in the αIR immunoprecipitates (Fig. 7*C*). The 95-kDa insulin receptor β-subunit was apparent in all lanes; there was significant basal receptor phosphorylation as well as a marked insulin-stimulated increase in tyrosyl phosphorylation (Fig 7*C*, compare *lanes a* and *b*, *lanes c* and *d*). An uncharacterized 205-kDa tyrosyl-phosphorylated band may represent uncleaved receptor precursors. αIR immunoprecipitates from unstimulated receptor/IRS-1 mixtures contained a small amount of tyrosyl-phosphorylated IRS-1, presumably due to basal receptor kinase activity, but tyrosyl-phosphorylated IRS-1 increased markedly in αIR immunoprecipitates from insulin-stimulated receptor/IRS-1 mixtures (Fig. 7, *lanes c* and *d*). Interestingly, tyrosyl-phosphorylated IRS-1 was also clearly seen in the αIR immunoprecipitates from insulin-stimulated mixtures which contained no added IRS-1^{bac} (Fig. 7, *lanes b*). IRS-1 is not present in lectin-purified insulin receptor preparations, and its tyrosyl phosphorylation is undetectable in unstimulated CHO cell lysates (data not shown). Thus, the only likely source of this tyrosyl-phosphorylated IRS-1 is the phosphorylation by the immobilized insulin receptors of endogenous IRS-1 present in the CHO cell lysates, with subsequent formation of receptor/IRS-1 complexes. Given the ability of tyrosyl-phosphorylated IRS-1 to bind the PtdIns 3'-kinase *in vitro* (Backer *et al.*, 1992b), the formation of ternary receptor/IRS-1/PtdIns 3'-kinase complexes may account for much of the apparent *in vitro* association of the insulin receptor with the PtdIns 3'-kinase.

DISCUSSION

Binding of insulin to its receptor leads to the rapid autophosphorylation and activation of the insulin receptor tyrosyl kinase, which in turn phosphorylates the cytoplasmic protein IRS-1 (Sun *et al.*, 1991). Tyrosyl-phosphorylated insulin receptors and tyrosyl-phosphorylated IRS-1 both appear to associate with the Type I or PtdIns 3'-kinase (Backer *et al.*, 1992a). We have recently shown that the PtdIns 3'-kinase is activated by its association with IRS-1, which in the absence of detectable p85 tyrosyl phosphorylation suggests that IRS-1 plays a key role in the regulation of the PtdIns 3'-kinase by insulin (Backer *et al.*, 1992b). This report describes the physical associations which link the insulin receptor, its substrate IRS-1, and its downstream effector PtdIns 3'-kinase in intact cells.

The association between IRS-1 and the PtdIns 3'-kinase occurs in intact cells and is modulated by the extent of *in vivo* IRS-1 tyrosyl phosphorylation. Consistent with our previous data, the IRS-1-associated enzyme appears to be the activated form of the enzyme which can utilize PtdIns, PtdIns(4)P, and PtdIns(4,5)P_2 as substrates (Carpenter *et al.*, 1991). The lack of quantitative anti-IRS-1 and anti-p85 antibodies has so far prevented a direct measurement of the percentage of cellular

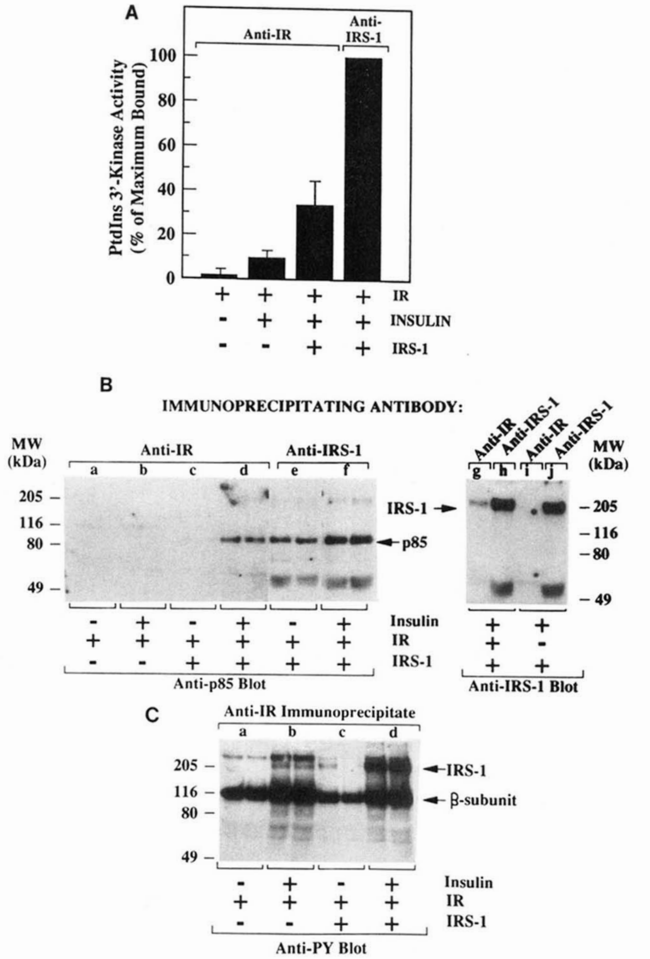

FIG. 7. ***In vitro* association of the PtdIns 3'-kinase with the insulin receptor.** *A*, partially purified insulin receptors (2 nM final) were incubated in the absence or presence of 100 nM insulin and IRS-1^{bac} (75 nM final) for 30 min at 22 °C. αIR (83-14) or αIRS-1 (αIRS-1^{bac}) immunoprecipitates were prepared and incubated for 30 min at 4 °C with lysates from quiescent CHO/neo cells. The immunoprecipitates were washed again and assayed for PtdIns 3'-kinase activity. The data is pooled from three separate experiments; bound PtdIns 3'-kinase activity is expressed as a percentage of that present in anti-IRS-1 immunoprecipitates. *B*, left panel: αIR (*lanes a–d*) or αIRS-1 (*lanes e* and *f*) immunoprecipitates were prepared from unstimulated (*lanes a, c,* and *e*) or insulin-stimulated (*lanes b, d,* and *f*) mixtures containing insulin receptors and the absence (*lanes a* and *b*) or presence (*lanes c–f*) of IRS-1^{bac}. The washed immunoprecipitates were incubated with lysates from quiescent CHO cells, washed again, and boiled in Laemmli sample buffer (Laemmli, 1970). Eluted proteins were separated by SDS-PAGE (7.5% reducing) and blotted with αp85 antibody (*UBI*). The position of p85α is indicated. Right panel: αIR (*lanes g* and *i*) or αIRS-1 (*lanes h* and *j*) immunoprecipitates were prepared from IRS-1^{bac}/insulin receptor mixtures (*lanes g* and *h*) or IRS-1^{bac} alone (*lanes i* and *j*) and analyzed by blotting with αIRS-1 antibody as described above. The position of IRS-1 is indicated. *C*, the blot in Fig. 8*B*, *lanes a–d*, was reblotted with αPY antibody. The positions of IRS-1 and the insulin receptor β-subunit are indicated.

PtdIns 3'-kinase which associates with IRS-1 after insulin stimulation. However, in lysates from insulin-stimulated cells, 70% less PtdIns 3'-kinase is available for *in vitro* binding to phosphorylated IRS-1. It seems likely that these data reflect the association of the PtdIns 3'-kinase with endogenous IRS-1 in the insulin-stimulated cells, as the insulin receptor and IRS-1 are the major tyrosyl-phosphorylated species detected in αp85 immunoprecipitates (Backer *et al.*, 1992b). Furthermore, this approximation of IRS-1-associated PtdIns 3'-kinase is similar in magnitude to estimates based on immunoprecipitations or insulin-stimulated changes in the specific activity of total *versus* IRS-1-associated PtdIns 3'-kinase. Association with IRS-1 thus seems to account for between one and two thirds of endogenous PtdIns 3'-kinase (Fig. 8), somewhat lower than the near-quantitative association of the PtdIns 3-kinase with the PDGF receptor in PDGF-stimulated fibroblasts (Kazlauskas and Cooper, 1989, 1990). However, this difference may depend on the relative intracellular concentrations of the interacting molecules.

PtdIns 3'-kinase activity has also been detected in αIR immunoprecipitates from insulin-stimulated cells (Ruderman *et al.*, 1990; Backer *et al.*, 1992a). αp85 immunoblots of αIR immunoprecipitates detect approximately 10% of the p85α seen in αIRS-1 immunoprecipitates, suggesting that, at steady state the bulk of IRS-1/PtdIns 3'-kinase complexes are binary and do not involve the insulin receptor (Fig. 8). Our current estimate of IR-associated PtdIns 3-kinase is lower than our previous finding that 50% of αPY-precipitable activity was associated with the insulin receptor. However, our previous estimate was generated using αPY as opposed to αIRS-1 antibody; αPY is less efficient than αIRS-1 for immunoprecipitation of IRS-1, and probably underestimated the amount of IRS-1-associated PtdIns 3'-kinase.

The association between the insulin receptor and the PtdIns 3'-kinase observed in intact cells may be mediated at least in part by the formation of ternary complexes with IRS-1. The formation of such complexes in insulin-stimulated cells has been suggested previously, based on the presence of IRS-1 and insulin receptors in αp85 immunoprecipitates, and the presence of p85α and the insulin receptor in αIRS-1 immunoprecipitates (Backer *et al.*, 1992b; Sun *et al.*, 1992). We have now shown that overexpression of IRS-1 increases the amount of αIR-precipitable PtdIns 3'-kinase activity *in vivo*. Furthermore, addition of IRS-1 increases the binding of PtdIns 3'-kinase activity and p85α to insulin receptors *in vitro*. These data strongly suggest that both the insulin receptor and the PtdIns 3'-kinase associate simultaneously with IRS-1. The formation of ternary complexes or even higher

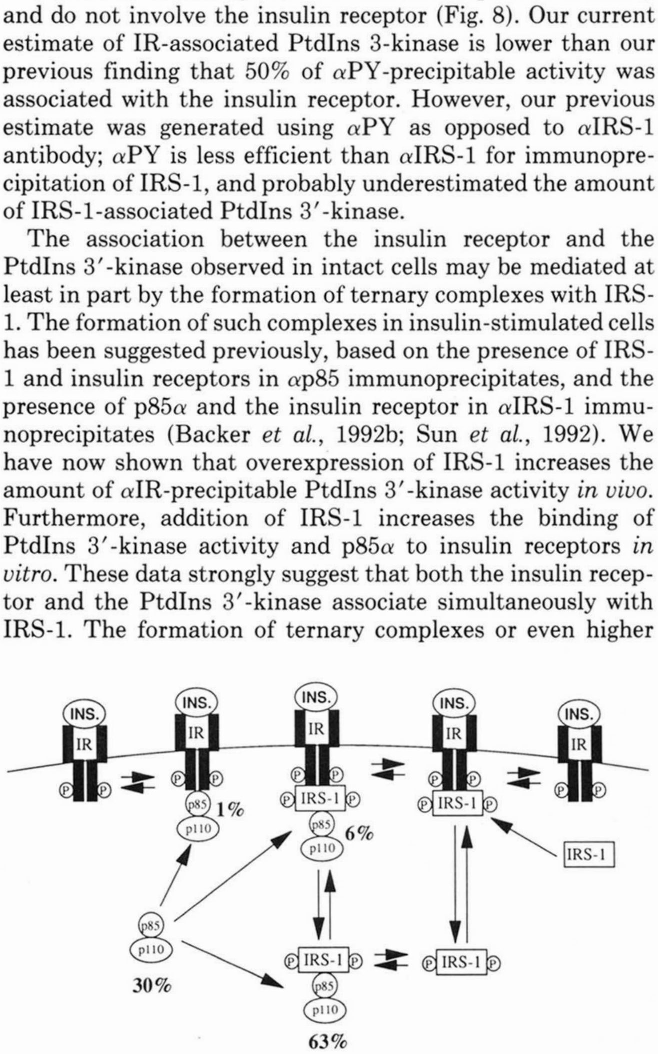

FIG. 8. **Formation of binary and ternary signaling complexes in insulin-stimulated cells.** The formation of binary and ternary complexes involving the insulin receptor, IRS-1, and the PtdIns 3'-kinase in insulin-stimulated cells is shown. The approximate percentages of total cellular PtdIns 3'-kinase present in each complex is indicated. Based on data in Figs. 1 and 5 and calculations based on previous data (Backer *et al.*, 1992b), 30–70% of total PtdIns 3'-kinase activity is complexed with other intracellular proteins after insulin stimulation; the higher estimate is illustrated in the figure. Within this pool, data from Fig. 1 suggests that 90% of the PtdIns 3'-kinase (or 63% of total) is present in binary IRS-1/PtdIns 3'-kinase complexes, and roughly 10% (or 6% of total PtdIns 3'-kinase) is present in ternary receptor/IRS-1/PtdIns 3'-kinase complexes. Although the amount of binary receptor/PtdIns 3'-kinase complexes cannot be accurately determined, it is likely to be small (1% of total) in cells where IRS-1 tyrosyl phosphorylation is robust.

order complexes between IRS-1 and other signal transducing molecules may have important implications for the mechanism of insulin signaling.

We observe some *in vitro* binding of the PtdIns 3'-kinase to immunopurified insulin receptors, but it is weak relative to that seen with IRS-1. Furthermore, the incubations of immunopurified receptors with lysates from quiescent CHO cells lead to the tyrosyl phosphorylation of endogenous IRS-1 and its association with the immobilized receptors. The receptor-associated PtdIns 3'-kinase observed under these conditions may therefore be due in part to IRS-1. Others have reported the *in vitro* association of the insulin receptor and the insulin-like growth factor I receptor with the PtdIns 3'-kinase (Yonezawa *et al.*, 1992; Yamamoto *et al.*, 1992). If a direct association between the insulin receptor and the SH2 domains in p85α does occur, it is likely to be mediated by the phosphorylation of the Y^{1322}THM (Y(M/*X*)*X*M) motif in the insulin receptor C terminus. In contrast, recent work by Yonezawa *et al.* (1992) have suggested that *in vitro* binding of immunopurified insulin receptors to the PtdIns 3'-kinase in CHO cells lysates does not require the C terminus, and we observed normal levels of receptor-associated PtdIns 3'-kinase activity in CHO cells expressing a 43-amino acid C-terminal truncation mutant (Backer *et al.*, 1992a). However, as we demonstrate here, the levels of ATP present in Nonidet P-40 lysates from CHO cells are sufficient to support the insulin-stimulated phosphorylation of IRS-1 and its association with the insulin receptor; insulin-stimulated increases in αIRS-1-immunoprecipitable PtdIns 3'-kinase also occur under these assay conditions.[2] Thus, anti-receptor immunoprecipitations from intact cells or incubations involving immunopurified insulin receptors and CHO lysates are likely to overestimate the extent of direct receptor/PtdIns 3'-kinase binding, as they reflect the phosphorylation of endogenous CHO cell IRS-1 and its subsequent binding to both the PtdIns 3'-kinase and the insulin receptor. Consistent with this explanation, mutant receptors which are deficient for IRS-1 phosphorylation were also reported to be deficient for *in vitro* association with the PtdIns 3'-kinase (Yonezawa *et al.*, 1992).

Preliminary experiments with partially purified receptors and immunopurified p85α or GST-fusion proteins containing p85α SH2 domains do detect *in vitro* insulin receptor/p85α binding in the absence of IRS-1.[2] However, the formation of ternary IRS-1/receptor/PtdIns 3'-kinase complexes *in vivo* and *in vitro* makes it difficult to evaluate whether the extent to which this occurs in intact cells. It is possible that direct association between the insulin receptor and the PtdIns 3'-kinase may be proportionally more significant in cells with little IRS-1 or in which IRS-1 phosphorylation is low relative to insulin receptor tyrosyl phosphorylation. Alternatively, receptor/PtdIns 3'-kinase complexes may become predominant after 30–60 min of insulin stimulation, when receptor tyrosyl autophosphorylation is still elevated, whereas IRS-1 tyrosyl phosphorylation is significantly attenuated (Miralpeix *et al.*, 1992; Sun *et al.*, 1992).

If interactions with IRS-1 alone can activate the PtdIns 3'-kinase, what cellular function is served by the formation of ternary insulin receptor/IRS-1/PtdIns 3'-kinase complexes? The estimate that 10% of cellular PtdIns 3'-kinase is involved in ternary complexes reflects the steady state distribution, and does not rule out transient interactions between the insulin receptor and the PtdIns 3'-kinase or PtdIns 3'-kinase/IRS-1 complexes. IRS-1 is primarily cytosolic (Sun *et al.*, 1992), although it must come close to the plasma membrane during its phosphorylation by the insulin receptor. Furthermore, activated PtdIns 3'-kinase is detected in membrane fractions following insulin stimulation (Kelly *et al.*, 1992). Thus, a possible role for the formation of ternary complexes is the translocation of the PtdIns 3'-kinase to the plasma membrane; insulin receptor/IRS-1 complex formation might create a signaling unit analogous to the PDGF receptor, which contains an intrinsic PtdIns 3'-kinase binding domain.

The regions of the insulin receptor which bind to IRS-1 are not yet known, the binding affinities between the two molecules have not been measured, and the role of receptor and IRS-1 tyrosyl phosphorylation in these interactions are not yet understood. The use of insulin receptor mutants should provide a means to address this question in the future. Nonetheless, the potential importance of insulin receptor/IRS-1 complex formation during signaling is underscored by the recent finding that the drosophila insulin receptor contains a C-terminal extension which is highly homologous to IRS-1.[3] Complex formation between the insulin receptor and IRS-1 may reflect the fact that these molecules were physically linked in evolutionarily distant time.

In summary, insulin stimulation of CHO/IR cells leads to the association of PtdIns 3'-kinase with IRS-1 and the insulin receptor, accounting for between one and two thirds of total cellular PtdIns 3'-kinase. Association with IRS-1 appears to account for much of the previously observed presence of PtdIns 3'-kinase in αIR immunoprecipitates from insulin-stimulated cells, as insulin stimulates the formation of ternary receptor/IRS-1/PtdIns 3'-kinase complexes. In light of our recent observation that IRS-1 activates the PtdIns 3'-kinase *in vitro* and in intact cells (Backer *et al.*, 1992b), the binding of tyrosyl-phosphorylated IRS-1 to the insulin receptor, p85α and other signal transducing proteins is likely to play a pivotal role in insulin action.

Acknowledgments—We thank Dr. Ken Siddle (University of Cambridge) for the gift of monoclonal antibodies to the human insulin receptor, and Drs. Joseph Schlessinger and Patrick Hu (New York University School of Medicine) for p85-SH2/GST fusion protein constructs.

REFERENCES

Auger, K. R., Serunian, L. A., Soltoff, S. P., Libby, P., and Cantley, L. C. (1989) *Cell* **57,** 167–175
Backer, J. M., Schroeder, G. G., Cahill, D. A., Ullrich, A., Siddle, K., and White, M. F. (1991a) *Biochemistry* **300,** 6366–6372
Backer, J. M., Schroeder, G. G., Kahn, C. R., Myers, M. G., Jr., Wilden, P. A., Cahill, D. A., and WHite, M. F. (1992a) *J. Biol. Chem.* **267,** 1367–1374
Backer, J. M., Myers, M. G., Jr., Shoelson, S. E., Chin, D. J., Sun, X.-J., Miralpeix, M., Hu, P., Margolis, B., Skolnik, E. Y., Schlessinger, J., and White, M. F. (1992b) *EMBO J.* **11,** 3469–3479
Cantley, L. C., Auger, K. R., Carpenter, C., Duckworth, B., Kapeller, R., and Soltoff, S. (1991) *Cell* **64,** 281–302
Carpenter, C. L., Duckworth, B. C., Auger, K. R., Cohen, B., Schaffhausen, B. S., and Cantley, L. C. (1990) *J. Biol. Chem.* **265,** 19704–19711
Chou, C. K., Dull, T. J., Russell, D. S., Gherzi, R., Lebwohl, D., Ullrich, A., and Rosen, O. M. (1987) *J. Biol. Chem.* **262,** 1842–1847
Endemann, G., Yonezawa, K., and Roth, R. A. (1990) *J. Biol. Chem.* **265,** 396–400
Escobedo, J. A., Kaplan, D. R., Kavanaugh, W. M., Turck, C. W., and Williams, L. T. (1991a) *Mol. Cell. Biol.* **11,** 1125–1132
Escobedo, J. A., Navankasattusas, S., Kavanaugh, W. M., Milfay, D., Fried, V. A., and Williams, L. T. (1991b) *Cell* **65,** 75–82
Fantl, W. J., Escobedo, J. A., Martin, G. A., Turck, C. W., del Rosario, M., McCormick, F., and Williams, L. T. (1992) *Cell* **69,** 413–423
Herrera, R., Lebwohl, D., Garcia de Herreros, A., Kallen, R. G., and Rosen, O. M. (1988) *J. Biol. Chem.* **263,** 5560–5568
Hiles, I. D., Otsu, M., Volinia, S., Fry, M. J., Gout, I., Dhand, R., Panayouou, G., Ruiz-Larrea, F., Thompson, A., Totty, N. F., Hsuan, J. J., Courtneidge, S. A., Parker, P. J., and Wterfield, M. D. (1992) *Cell* **70,** 419–429
Hu, P., Skolnik, E. Y., Lammers, R., Ullrich, A., and Schlessinger, J. (1992) *Mol. Cell Biol.* **12,** 981–990
Kahn, C. R., and White, M. F. (1988) *J. Clin. Invest.* **82,** 1151–1156
Kazlauskas, A., and Cooper, J. A. (1989) *Cell* **58,** 1121–1133
Kazlauskas, A., and Cooper, J. A. (1990) *EMBO J.* **9,** 3279–3286
Kelly, K. L., Ruderman, N. B., and Chen, K. S. (1992) *J. Biol. Chem.* **267,** 3423–3428
Klippel, A., Escobedo, J. A., Fantl, W. J., and Williams, L. T. (1992) *Mol. Cell. Biol.* **12,** 1451–1459

[2] J. M. Backer, unpublished observations.

[3] J. Schlessinger, personal communication.

Laemmli, U. K. (1970) *Nature* **227,** 680–685
McGlade, C. J., Ellis, C., Reedijk, M., Anderson, D., Mbamalu, G., Reith, A. D., Panayotou, G., End, P., Bernstein, A., Kazlauskas, A., Waterfield, M. D., and Pawson, T. (1992) *Mol. Cell. Biol.* **12,** 991–997
Miralpeix, M., Sun, X.-J., Backer, J. M., Myers, M. G., Jr., Araki, E.', and White, M. F. (1992) *Biochemistry* **31,** 9031–9039
Myers, M. G., Jr., Backer, J. M., Sun, X.-J., Shoelson, S. E., Hu, P., Schlessinger, J., Yoakim, M., Schaffhausen, B., and White, M. F. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 10350–10354
Otsu, M., Hiles, I., Gout, I., Fry, M. J., Ruis-Larrea, F., Panayotou, G., Thompson, A., Dhand, R., Hsuan, J., Totty, N., Smith, A. D., Morgan, S. J., Courtneidge, S. A., Parker, P. J., and Waterfield, M. D. (1991) *Cell* **65,** 91–104
Perlman, R., Bottaro, D., White, M. F., and Kahn, C. R. (1989) *J. Biol. Chem.* **264,** 8946–8950
Reedijk, M., Liu, X., van der Geer, P., Letwin, K., Waterfield, M. D., Hunter, T., and Pawson, T. (1992) *EMBO J.* **11,** 1365–1372
Rothenberg, P. L., Lane, W. S., Karasik, A., Backer, J., White, M., and Kahn, C. R. (1991) *J. Biol. Chem.* **266,** 8302–8311
Ruderman, N., Kapeller, R., White, M. F., and Cantley, L. C. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 1411–1415
Shoelson, S. E., Chatterjee, S., Chaudhuri, M., and White, M. F. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 2027–2031
Shibasaki, F., Homma, Y., and Takenawa, T. (1991) *J. Biol. Chem.* **266,** 8108–8114
Skolnik, E. Y., Margolis, B., Mohammadi, M., Lowenstein, E., Fischer, R., Drepps, A., Ullrich, A., and Schlessinger, J. (1991) *Cell* **65,** 83–90
Sun, X. J., Rothenberg, P., Kahn, C. R., Backer, J. M., Araki, E., Wilden, P. A., Cahill, D. A., Goldstein, B. J., and White, M. F. (1991) *Nature* **352,** 73–77
Sun, X.-J., Miralpeix, M., Myers, M. G., Jr., Glasheen, E., Backer, J. M., Kahn, C. R., and White, M. F. (1992) *J. Biol. Chem.* **267,** 22662–22672
Towbin, H., Staehelin, T., and Gordon, J. (1970) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 4350–4354
White, M. F., and Backer, J. M. (1991) *The Enzymes* **201,** 65–79
White, M. F., Maron, R., and Kahn, C. R. (1985) *Nature* **318,** 183–186
White, M. F., Livingston, J. N., Backer, J. M., Lauris, V., Dull, T. J., Ullrich, A., and Kahn, C. R. (1988) *Cell* **54,** 641–649
Wilden, P. A., Backer, J. M., Kahn, C. R., Cahill, D. A., Schroeder, G. J., and White, M. F. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 3358–3362
Yamamoto, K., Altshuler, D., Wood, E., Horlick, K., Jacobs, S., and Lapetina, E. G. (1992) *J. Biol. Chem.* **267,** 11337–11343
Yonezawa, K., Yokono, K., Shii, K., Ogawa, W., Ando, A., Hara, K., Baba, S., Kaburagi, Y., Yamamoto-Honda, R., Momomura, K., Kadowaki, T., and Kasuga, M. (1992) *J. Biol. Chem.* **267,** 440–446