IRS-1 activates phosphatidylinositol 3'-kinase by associating with src homology 2 domains of p85

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ABSTRACT   IRS-1 is an insulin receptor substrate that undergoes tyrosine phosphorylation and associates with the phosphatidylinositol (PtdIns) 3'-kinase immediately after insulin stimulation. Recombinant IRS-1 protein was tyrosine phosphorylated by the insulin receptor in vitro and associated with the PtdIns 3'-kinase from lysates of quiescent 3T3 fibroblasts. Bacterial fusion proteins containing the src homology 2 domains (SH2 domains) of the 85-kDa subunit (p85) of the PtdIns 3'-kinase bound quantitatively to tyrosine phosphorylated, but not unphosphorylated, IRS-1, and this association was blocked by phosphotyrosine-containing synthetic peptides. Moreover, the phosphorylated peptides and the SH2 domains each inhibited binding of PtdIns 3'-kinase to IRS-1. Phosphorylated IRS-1 activated PtdIns 3'-kinase in anti-p85 immunoprecipitates in vitro, and this activation was blocked by SH2 domain fusion proteins. These data suggest that the interaction between PtdIns 3'-kinase and IRS-1 is mediated by tyrosine phosphorylated motifs on IRS-1 and the SH2 domains of p85, and IRS-1 activates PtdIns 3'-kinase by binding to the SH2 domains of p85. Thus, IRS-1 likely serves to transmit the insulin signal by binding and regulating intracellular enzymes containing SH2 domains.

Insulin binding to its receptor at the cell surface activates the tyrosine kinase of the insulin receptor β subunit (1). Although it is known that this tyrosine kinase is required for many insulin responses (2), the molecular link between the insulin receptor and the cellular enzymes that regulate cellular growth and metabolism has been difficult to establish. The identification of pp185, a band of proteins (170–185 kDa) that is tyrosine phosphorylated immediately after insulin stimulation of intact cells, provided evidence for the existence of cellular substrates for the insulin receptor (3). We recently purified and cloned IRS-1, a component of pp185 (4–6). IRS-1 is a hydrophilic phosphoprotein, which is tyrosine phosphorylated in response to insulin (5). IRS-1 contains 20 tyrosine phosphorylation consensus sequences, 6 of which appear in YMXM (Tyr-Met-Xaa-Met) motifs. YMXM and homologous YXXM motifs are found in other regulatory proteins, such as the platelet-derived growth factor receptor and polyoma middle-sized tumor antigen (7). Tyrosine phosphorylation of these motifs mediates the association of these regulatory molecules with the phosphatidylinositol 3'-kinase (PtdIns 3'-kinase) (8, 9).

The PtdIns 3'-kinase is composed of at least two subunits, including a 110-kDa catalytic subunit and an 85-kDa protein (p85), which contains two src homology 2 (SH2) domains (7, 10, 11). SH2 domains are noncatalytic domains that mediate protein–protein interactions by binding to phosphotyrosine residues in various proteins (12). The association of the

PtdIns 3'-kinase with tyrosine phosphorylated IRS-1 or peptides containing phosphorylated YMXM motifs activates the PtdIns 3'-kinase (13), which may mediate early biochemical events for the insulin receptor (5). To establish the molecular basis of the IRS-1–PtdIns 3'-kinase interaction, we studied the association between partially purified IRS-1 produced in Sf9 cells (IRS-1^{bac}; baculovirus-produced IRS-1), and the PtdIns 3'-kinase from cellular extracts, or glutathione S-transferase (GST) fusion proteins containing one or both of the SH2 domains of p85 (14).

MATERIALS AND METHODS

Production of IRS-1^{bac}. The cDNA for rat IRS-1 (5) was subcloned into pBluescript (Stratagene) using the 5' Spe I and 3' HindIII sites on IRS-1 and the complementary site in the polylinker of pBluescript. Most of the 3' untranslated region was removed by digestion with Aat II and BamHI. The vector was then religated with a linker containing Aat II and BamHI ends and an intervening Spe I cut site. All of the 5' untranslated sequences were then removed by digestion with Sac I, which cuts in the pBluescript vector, and BspEI, which cuts 12 nucleotides after the translation start site. The vector was religated with a linker containing a Sac I 5' end, a BspEI 3' end, and an Nhe I site just before the translation start site and coding sequences, which were removed. The entire coding sequence of IRS-1 was then excised by digestion with Nhe I and Spe I and ligated into the Nhe I cloning site of pBlueBac (Invitrogen). pBlueBac and wild-type AcNPV DNA (Invitrogen) were cotransfected into Sf9 cells (Invitrogen) and recombinant viruses were identified as described (15). For protein production, Sf9 cells were infected at high multiplicity of infection (15) and grown for 54–56 hr before lysis by Dounce homogenizing in buffer B containing 50 mM Tris·HCl (pH 7.8) and 1 M NaCl, and supplemented with aprotinin (1 µg/ml), leupeptin (1 µg/ml), phenylmethylsulfonyl fluoride (1 mM), and benzamidine (1 mM). Crude lysates contained ≈15% IRS-1^{bac}, and, for some experiments, IRS-1^{bac} was purified to 90% homogeneity by gel-filtration chromatography on SK 300 HR media (Pharmacia) (M.G.M., M.F.W., et al., unpublished results).

Phosphorylation of IRS-1^{bac} by the Partially Purified Insulin Receptor. Wheat germ agglutinin-purified glycoproteins (5–8 µg), prepared from Chinese hamster ovary (CHO) cells overexpressing the human insulin receptor (16), was incubated for 20 min in buffer B with 5 mM MnCl$_2$, 50 µM ATP (in some experiments [γ-^{32}P]ATP was added as tracer) in the

Abbreviations: IRS-1, insulin receptor substrate 1; PtdIns 3'-kinase, phosphatidylinositol 3'-kinase; IRS-1^{bac}, baculovirus-produced IRS-1; Tyr(P)-IRS-1^{bac}, IRS-1^{bac} tyrosine phosphorylated by insulin receptor in vitro; SH2, src homology domain 2; nSH2, N-terminal SH2 domain of p85; cSH2, C-terminal SH2 domain of p85; GST, glutathione S-transferase; GSH, glutathione.
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absence or presence of 100 nM insulin (Elanco). Approximately 2.5 μg of IRS-1bac was added, and the incubation was continued overnight unless stated otherwise.

Immunoblotting with Anti-Phosphotyrosine and Anti-IRS-1 Antibodies. Proteins were resolved by reducing SDS/PAGE and transferred to nitrocellulose in Towbin buffer containing 0.02% SDS and 20% (vol/vol) methanol for 2 hr at 100 V. Membranes were washed briefly with water and incubated overnight at 4°C in blocking buffer (20 mM Tris·HCl, pH 7.4/150 mM NaCl/0.01% Tween 20/3% bovine serum albumin). Membranes were then incubated with anti-phosphotyrosine antibody or anti-IRS-1 antibody (3 μg/ml) in blocking buffer. These antibodies were prepared from rabbit serum on peptide affinity columns as described (5, 17). The membranes were washed three times in blocking buffer without bovine serum albumin, reblocked, incubated with ^{125}I-labeled protein A (ICN) (0.2 μCi/ml; 1 Ci = 37 GBq), washed four times, dried, and exposed to Kodak X-Omat film.

Association of PtdIns 3'-Kinase Activity with IRS-1. NIH 3T3 cells expressing the transfected human insulin receptor (HIR-3.5) were the generous gift of J. Whittaker (18). PtdIns 3'-kinase activity in anti-IRS-1 immunoprecipitates from these cells was assayed as described (19). For in vitro PtdIns 3'-kinase association assays, ≈1.2 μg of IRS-1bac, which had been phosphorylated overnight in 500 μM ATP at 4°C, was added to 60 μl of protein A-Sepharose (1:1) slurry in phosphate-buffered saline) with 6 μg of anti-IRS-1 antibody. After 2 hr at 4°C, the immunoprecipitate was washed three times in cell lysis buffer containing 20 mM Tris·HCl (pH 7.4), 137 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 1 mM phenylmethylsulfonyl fluoride, 1% Nonidet P-40, and 100 μM sodium vanadate (19). Lysates of quiescent HIR-3.5 cells were prepared as described (19). Lysate from one 10-cm dish of cells was added to the immobilized IRS-1bac. After 30 min, the lysates were removed, the immunoprecipitates were washed, and the bound PtdIns 3'-kinase activity was measured (23).

Production of GST Fusion Proteins and Assay for Association with IRS-1bac. GST fusion proteins were produced as described (14). Glutathione (GSH) and dithiothreitol were removed by repeated ultrafiltration in a Centricon 30 microconcentration unit (Amicon). For association assays, 5 μg (0.15 nmol) of fusion protein was bound to 40 μl of GSH-Sepharose (Pharmacia) (1:1 suspension in phosphate-buffered saline with 10 mM dithiothreitol) for 30 min at 4°C.

Approximately 1 μg (7.5 pmol) of IRS-1bac from an in vitro phosphorylation reaction was bound for 30 min in 50 mM Tris·HCl (pH 7.8) with 250 mM NaCl/10 mM dithiothreitol/ 0.2% Triton X-100. Complexes were washed five times in the same buffer, boiled in Laemmli sample buffer for 3 min, and resolved by SDS/7.5% PAGE. For experiments with tyrosine phosphorylated IRS-1bac [Tyr(32P)-IRS-1bac], gels were dried and exposed to autoradiography. For experiments with unlabeled IRS-1bac or Tyr(P)-IRS-1bac, the proteins were transferred to nitrocellulose membranes and developed with anti-IRS-1 antibody. For assays with phosphopeptides, immobilized fusion proteins were preincubated with 100 μM peptide for 10 min before addition of Tyr(P)-IRS-1bac.

Inhibition of PtdIns 3'-Kinase Binding with Phosphopeptides or GST Fusion Proteins. Phosphorylated and unphosphorylated synthetic peptides were prepared as described (9, 20). Cell lysates and immunoprecipitates of Tyr(P)-IRS-1bac were prepared as described above. For experiments with synthetic peptides, various concentrations of peptides were preincubated with cell lysates for 10 min; lysates were then added to immunoprecipitates and the assay was continued as described above. For experiments with fusion proteins, immunoprecipitated Tyr(P)-IRS-1bac was preincubated with various concentrations of fusion protein for 10 min before addition of cell lysate. The PtdIns 3'-kinase assay then continued as described above.

Inhibition of PtdIns 3'-Kinase Activation by Blocking with GST Fusion Proteins. Quiescent CHO cells (13) were lysed as described above and PtdIns 3'-kinase was immunoprecipitated overnight with antibody directed against the p85 subunit of the enzyme (14). Immunoprecipitates were washed two times with cell lysis buffer and 30 μl of Tyr(P)-IRS-1bac (≈100 nM) was added for 30 min before washing the immunoprecipitate and assaying activity as described (13, 19). To block activation, 30 μl of 10 μM GST or GST–N-terminal SH2 domain of p85 (nSH2) fusion proteins was preincubated with the Tyr(P)-IRS-1bac for 10 min before addition to anti-p85 immunoprecipitates.

RESULTS

The PtdIns 3'-kinase associates with IRS-1 during insulin stimulation (5, 19). Before insulin stimulation of HIR-3.5 cells, anti-IRS-1 immunoprecipitates did not contain PtdIns 3'-kinase activity; however, after insulin stimulation, PtdIns
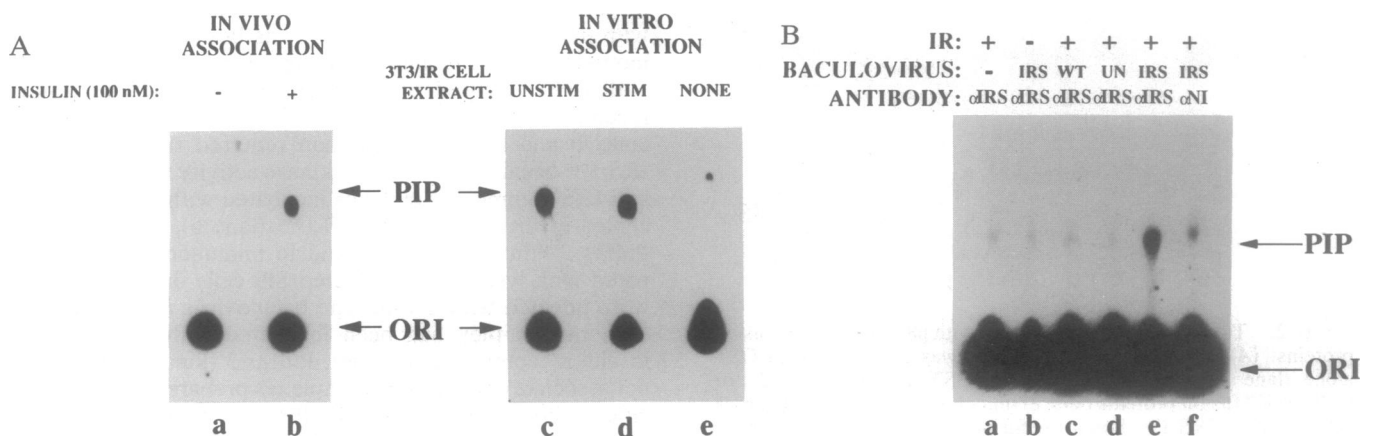

FIG. 1. Tyr(P)-IRS-1bac associates with PtdIns 3'-kinase from cell extracts. (A) NIH 3T3 cells were incubated in the absence (lanes a and c) or presence (lanes b and d) of 100 nM insulin and lysed. Lysates were either immunoprecipitated with anti-IRS-1 antibody (lanes a and b) or incubated with immunoprecipitates of Tyr(P)-IRS-1bac (lanes c and d). PtdIns 3'-kinase activity associated with each of the immunoprecipitates was assayed. The activity associated with Tyr(P)-IRS-1bac incubated with buffer alone is shown (lane e). (B) Immunocomplexes containing anti-IRS-1 (lanes a–e) or nonspecific antibody (lane f) were incubated with the wheat germ agglutinin-purified insulin receptor alone (lane a) or IRS-1bac alone, or with the insulin receptor and partially purified extracts from wild-type infected Sf9 cells (lane c), uninfected Sf9 cells (lane d), or IRS-1bac-producing Sf9 cells (lane e). The immunocomplexes were washed and incubated with lysates of unstimulated HIR-3.5 cells. Associated PtdIns 3'-kinase activity was assayed. The origin (ORI) and migration of the reaction product (PtdIns phosphate; PIP) on the TLC plate are indicated.
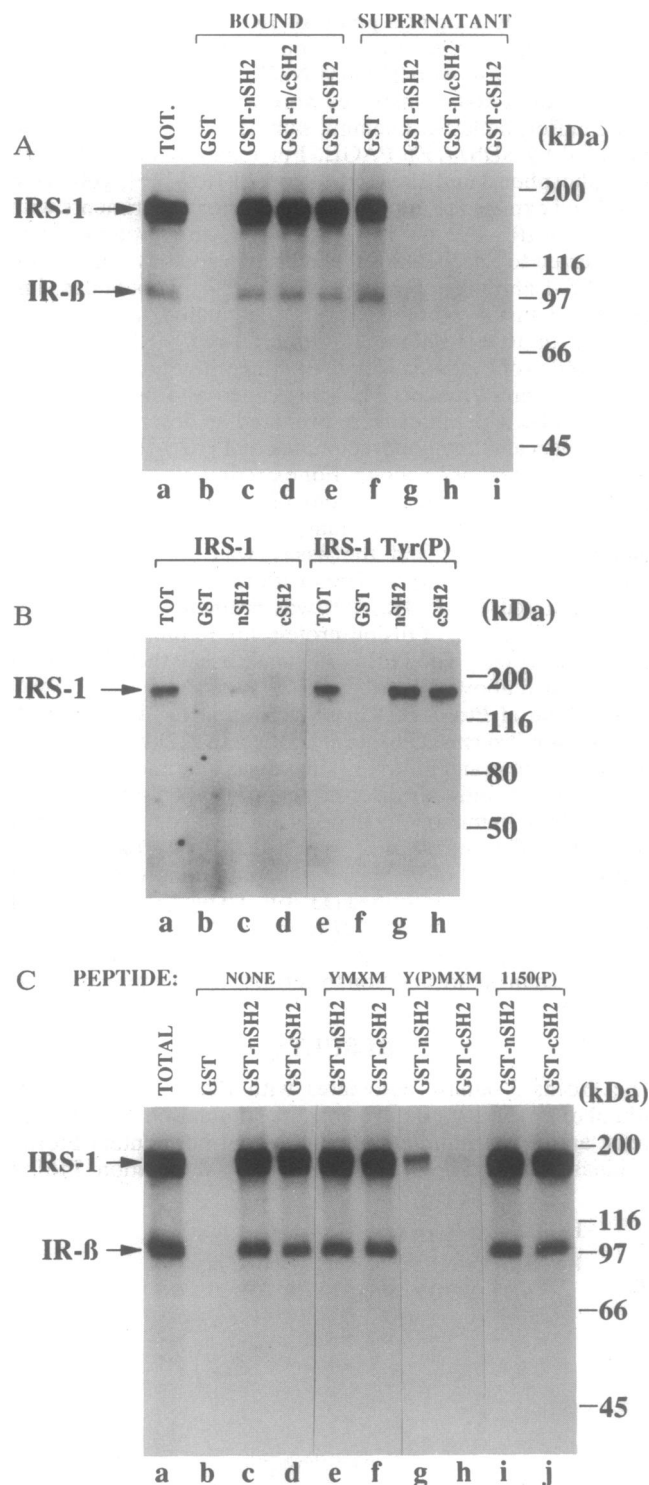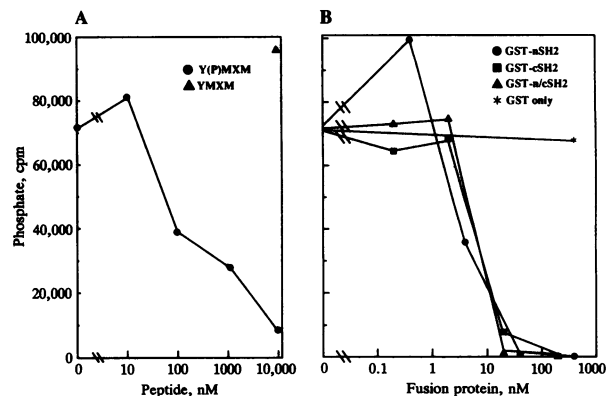
FIG. 3. Tyrosine phosphorylated YMXM peptides and SH2 domain fusion proteins block association of IRS-1 and PtdIns 3'-kinase. Antibody immobilized Tyr(P)-IRS-1^{bac} was incubated for 30 min with lysates from quiescent HIR-3.5 cells. (*A*) Various concentrations of Tyr-628 YMXM peptide or Y(P)MXM phosphopeptide were included during the incubations. (*B*) Various concentrations of GST-nSH2, GST-cSH2, or GST-n/cSH2 were included during the incubation. PtdIns 3'-kinase associated with the immunocomplexes was assayed as described (23). PtdIns 3-phosphate was quantified as Cerenkov radiation. Points represent average of two independent determinations.

3'-kinase was detected in the anti-IRS-1 immunoprecipitates, confirming our previous results (Fig. 1*A*, lanes a and b). To study the PtdIns 3'-kinase interaction *in vitro*, IRS-1 was produced in a baculovirus expression system (IRS-1^{bac}) (15) and tyrosine phosphorylated with the partially purified insulin receptor *in vitro* (21). This Tyr(P)-IRS-1^{bac} was immobilized on protein A-Sepharose with anti-IRS-1 antibody and used to bind PtdIns 3'-kinase from crude lysates of HIR-3.5. Before incubation of immobilized Tyr(P)-IRS-1^{bac} with the cell extracts, there was no PtdIns 3'-kinase activity in the immunocomplex (lane 3). However, PtdIns 3'-kinase bound to Tyr(P)-IRS-1^{bac} after incubation with cell extracts from quiescent or insulin-stimulated cells (lanes c and d). Similar amounts of PtdIns 3'-kinase activity bound to the immunocomplex from both stimulated and quiescent cells, suggesting that Tyr(P)-IRS-1^{bac} was sufficient for binding to PtdIns 3'-kinase in the absence of any other cellular effects of insulin. Furthermore, twice as much activity was recovered with immobilized Tyr(P)-IRS-1^{bac} as bound to endogenous mouse IRS-1 in the immunoprecipitates.

IRS-1^{bac} required prior phosphorylation by the insulin receptor for association with the PtdIns 3'-kinase (Fig. 1*B*, lanes b and e); this association required the presence of IRS-1^{bac} because no PtdIns 3'-kinase activity associated with anti-IRS-1 immunocomplexes incubated with the insulin receptor in the absence of IRS-1^{bac} (lane a). Moreover, no PtdIns 3'-kinase activity bound to immunocomplexes prepared with lysates of uninfected Sf9 cells or Sf9 cells that were infected with a wild-type baculovirus (lanes c and d) even though they had been incubated with the activated insulin receptor. Furthermore, PtdIns 3'-kinase activity did not associate with immunocomplexes prepared with nonspecific antibodies and Tyr(P)-IRS-1^{bac} (lane f). Taken together, these data demonstrate that Tyr(P)-IRS-1^{bac} binds to PtdIns 3'-kinase from HIR-3.5 cell lysates and that this binding requires tyrosine phosphorylation by the insulin receptor either *in vivo* or *in vitro*.

FIG. 2. Tyr(^{32}P)-IRS-1^{bac} associates with p85 SH2 domain fusion proteins. (*A*) Tyr(^{32}P)-IRS-1^{bac} (lane a) was incubated with GST alone (lane b), GST-nSH2 (lane c), GST-n/cSH2 (lane d), or GST-nSH2 fusion proteins (lane e) immobilized on GSH-Sepharose (14). Tyr(^{32}P)-IRS-1^{bac} bound to fusion proteins was resolved by SDS/PAGE and visualized by autoradiography. Lanes f–i contain unbound material from lanes b–e. (*B*) IRS-1^{bac} was incubated in the presence of ATP alone (lanes a–d) or with activated insulin receptor (lanes e–h). This material (lanes a and e) was assayed for its ability to bind immobilized GST alone (lanes b and f), GST-nSH2 (lanes c and g), or GST-cSH2 (lanes d and h). The bound proteins were separated by SDS/PAGE and transferred to nitrocellulose; IRS-1 was detected by immunoblotting with anti-IRS-1 antibody. (*C*) Ability of Tyr(^{32}P)-IRS-1^{bac} (lane a) to bind to GST-nSH2 or GST-cSH2 fusion proteins was assayed as described above (lanes b–d) or

in the presence of 100 μM Tyr-628 peptide YMXM peptide (lanes e and f), Y(P)MXM phosphopeptide (lanes g and h), or a tyrosine phosphorylated insulin receptor-derived (non-YMXM) control peptide (lanes i and j). Migration of molecular mass standards, IRS-1, and the β subunit of the insulin receptor (IR-P) are indicated.
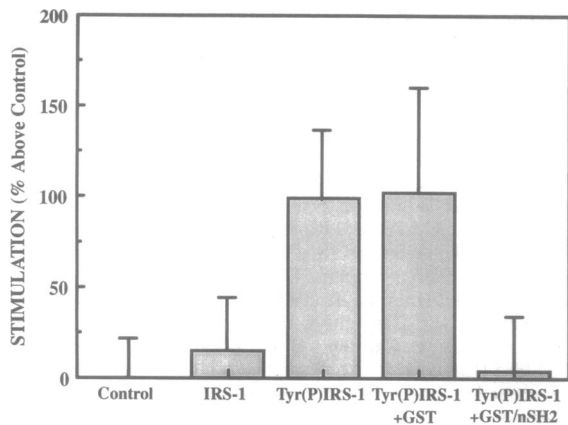
FIG. 4. SH2 domain fusion proteins block activation of PtdIns 3'-kinase. Antibody (anti-p85) immobilized PtdIns 3'-kinase was incubated with buffer alone, unphosphorylated IRS-1^{bac}, Tyr(P)-IRS-1^{bac} alone, or Tyr(P)-IRS-1^{bac} preincubated with GST or GST-nSH2. Immunoprecipitates were then washed and assayed for PtdIns 3'-kinase activity (23). PtdIns 3-phosphate was quantified as Cerenkov radiation. Values shown represent average of two experiments, each consisting of three independent determinations.

Since the association between IRS-1 and the PtdIns 3'-kinase was regulated by tyrosine phosphorylation of IRS-1, we inferred that the binding might involve the SH2 domains of the 85-kDa subunit of the PtdIns 3'-kinase (p85). To investigate this possibility, the N-terminal (nSH2) and C-terminal (cSH2) SH2 domains of p85 were expressed as GST fusion proteins alone (GST-nSH2, GST-cSH2) or in combination (GST-n/cSH2) (14). Approximately 150 pmol of these fusion proteins was immobilized on GSH-Sepharose and incubated with Tyr(^{32}P)-IRS-1^{bac} ($\approx$7.5 pmol) to determine their ability to associate with IRS-1. GST-nSH2, GST-cSH2, and GST-n/cSH2 fusion proteins, but not GST alone, bound Tyr(^{32}P)-IRS-1^{bac} (Fig. 2A, lanes a–d). Analysis of the supernatant demonstrated that all of the Tyr(^{32}P)-IRS-1^{bac} bound to the GST-SH2 fusion proteins (lanes f–i). The catalytic amount ($\approx$20 fmol) of insulin receptor used to phosphorylate IRS-1^{bac} also associated with the immobilized GST-SH2 fusion proteins.

To determine whether IRS-1^{bac} required tyrosine phosphorylation by the insulin receptor for binding to SH2 domain fusion proteins, we incubated tyrosine phosphorylated or unphosphorylated IRS-1^{bac} with GST-SH2 fusion proteins and detected bound IRS-1^{bac} by immunoblotting with anti-IRS-1 antibody. Before insulin-stimulated tyrosine phosphorylation, very little IRS-1^{bac} bound to immobilized GST-nSH2 or GST-cSH2 fusion proteins (Fig. 2B, lanes a–d). In contrast, Tyr(P)-IRS-1^{bac} bound quantitatively to both fusion proteins (lanes e–h). These results demonstrate that the interaction between IRS-1 and the SH2 domains of p85 is mediated by tyrosine phosphorylation of IRS-1.

Phosphorylated YMXM or related YVXM motifs of the platelet-derived growth factor receptor and polyoma middle-sized tumor antigen appear to be important for PtdIns 3'-kinase binding (9, 22). Although the exact tyrosine phosphorylation sites in IRS-1 remain unknown, the YMXM motifs are likely to be major sites (5); synthetic peptides based on these sequences are phosphorylated by the insulin receptor with low K_m values (20). Thus, the YMXM motifs in IRS-1 are likely to be important binding sites for the SH2 domains of the PtdIns 3'-kinase. Phosphorylated but not unphosphorylated YMXM peptide (based on the sequence surrounding Tyr-628 of IRS-1: GGYMPMSPKS) at a concentration of 100 μM inhibited the association between Tyr(^{32}P)-IRS-1^{bac} and immobilized GST-nSH2 and GST-cSH2 (Fig. 2C, lanes a–h), demonstrating the requirement for phosphotyrosine in this

interaction. Moreover, the surrounding amino acid sequence was important for this inhibition, as binding was not blocked by a phosphotyrosine-containing peptide (100 μM) based on the major autophosphorylation site of the insulin receptor [DIYETDY(P)YRKG] (lanes i and j). Tyr-628 phosphorylated YMXM also inhibited binding of the insulin receptor to the SH2 fusion proteins.

These results suggest that phosphotyrosine on IRS-1 and the SH2 domains of p85 may mediate binding of the PtdIns 3'-kinase to IRS-1. Therefore, we investigated the ability of Tyr-628 phosphorylated YMXM peptide or SH2 fusion proteins to inhibit the binding of PtdIns 3'-kinase to immobilized Tyr(P)-IRS-1^{bac}. Tyr-628 phosphorylated but not unphosphorylated YMXM peptide inhibited binding of PtdIns 3'-kinase activity to the immobilized IRS-1 with an approximate K_d of 100 nM (Fig. 3A). Moreover, GST-SH2 fusion proteins inhibited the association between Tyr(P)-IRS-1^{bac} and the PtdIns 3'-kinase with approximate K_d values of 5 nM, whereas GST alone had no inhibitory effect even at 500 nM (Fig. 3B).

The PtdIns 3'-kinase is activated during association with Tyr(P)-IRS-1^{bac} or phosphorylated YMXM peptides (13). To demonstrate that this activation depends on the binding of the Tyr(P)-IRS-1^{bac} to the p85 SH2 domains, we assessed the ability of GST alone or GST-nSH2 fusion proteins to inhibit this activation. Tyr(P)-IRS-1^{bac} but not unphosphorylated IRS-1^{bac} stimulated the activity of PtdIns 3'-kinase in anti-p85 immunoprecipitates 2-fold, confirming our previous results (13). Activation was inhibited by preincubating Tyr(P)-IRS-1^{bac} with GST-nSH2, but not with GST alone (Fig. 4). This result suggests that binding of Tyr(P)-IRS-1^{bac} to the SH2 domains of p85 is the critical step for activation of the PtdIns 3'-kinase *in vitro*.

DISCUSSION

Our results suggest that the molecular link between IRS-1 and the PtdIns 3'-kinase is due to the interaction between phosphorylated YMXM motifs in IRS-1 and the nSH2 and cSH2 domains in the 85-kDa subunit of the PtdIns 3'-kinase. The presence of two SH2 domains may act together to increase the valency of the interaction with tyrosine phosphorylated proteins, which could increase the overall affinity. Tyr(P)-IRS-1^{bac} and phosphorylated YMXM peptides activate the PtdIns 3'-kinase *in vitro* (13); our results here suggest that binding to the SH2 domains of p85 mediates this activation. Therefore, the SH2 domains of p85 appear to be allosteric regulatory sites.

It is unlikely that IRS-1 interacts with all SH2 isoforms found in various proteins. We have tried unsuccessfully to detect association between phosphorylated IRS-1 and phospholipase C$_\gamma$ and the ras-GAP (M.F.W. *et al.*, unpublished observations), suggesting that these proteins possess unique sequences in their SH2 domains that preferentially recognize different phosphotyrosine motifs (24). An analysis of amino acid sequences in the relevant proteins suggests that SH2 domains are composed of five conserved regions and four variable regions (24). It is thought that the conserved motifs may mediate binding to phosphotyrosine, whereas the interspersed variable regions act to recognize the surrounding sequences (24). The variable regions of the nSH2 and cSH2 within p85 are more homologous to one another than to other known SH2 domains, possibly explaining their similar affinity for phosphorylated YMXM motifs. Although our studies use the SH2 domains of the α isoform of p85, it is likely that the β isoform associates with IRS-1 as well, as its SH2 domains are highly conserved (25).

The p85 lacks an obvious catalytic domain (10, 11, 25) and appears to serve as a regulatory subunit that links the catalytic subunit of the PtdIns 3'-kinase to phosphotyrosine-

containing signaling proteins like IRS-1, some receptor tyrosine kinases, or the polyoma middle-sized tumor antigen (7, 9). Previous reports suggested that the PtdIns 3'-kinase may be activated by direct tyrosine phosphorylation (26); however, our data suggest that activation occurs during binding of phosphotyrosine residues to the SH2 domains, and we are unable to detect tyrosine phosphorylation of p85 during insulin stimulation (13). We do not know the functional significance of the involvement of IRS-1 in insulin signaling; however, IRS-1 may differentiate the effects of insulin from those of other tyrosine kinases or provide increased diversity or amplification in signal transmission by recruiting and regulating various cellular enzymes to a central molecule.

The phosphorylated insulin receptor also associates with the GST–SH2 fusion proteins. *In vitro*, this may occur through direct binding of p85 with a phosphorylated YXXM motif in the C terminus of the insulin receptor kinase; however, it is unlikely that the insulin receptor mediates association of IRS-1 with SH2 domains, as the insulin receptor is present in catalytic amounts during our *in vitro* assay. An IRS-1–insulin receptor complex may also be responsible for binding of the insulin receptor to GST–SH2. The PtdIns 3'-kinase is detected in anti-insulin receptor immunoprecipitates from insulin-stimulated cells, although this association is much weaker than that with IRS-1 (19, 23). Stable complexes have been detected between the insulin receptor and IRS-1 in the intact cell (21), so a tertiary complex involving the PtdIns 3'-kinase, IRS-1, and the insulin receptor may be relevant in the cells (13).

Based on the size of IRS-1 and the number of potential phosphorylation sites it contains, IRS-1 may transduce the insulin signal in many directions. Slight variations in the YMXM motifs of IRS-1 as well as other tyrosine phosphorylation sites may cause association with and regulation of other cellular enzymes containing distinct isoforms of the SH2 domain. It is also possible that p85 or other cellular proteins containing similar SH2 domains may link IRS-1 to other enzymes besides the PtdIns 3'-kinase catalytic subunit.

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