The Fyn Tyrosine Kinase Binds Irs-1 and Forms a Distinct Signaling Complex during Insulin Stimulation*

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Irs-proteins link the receptors for insulin/IGF-1, growth hormones, and several interleukins and interferons to signaling proteins that contain Src homology-2 (SH2). To identify new Irs-1-binding proteins, we screened a mouse embryo expression library with recombinant [^{32}P]Irs-1, which revealed a specific association between p59^{fyn} and Irs-1. The SH2 domain in p59^{fyn} bound to phosphorylated Tyr^{895} and Tyr^{1172}, which are located in YXX(L/I) motifs. Mutation of p59^{fyn} at the COOH-terminal tyrosine phosphorylation site (Tyr^{531}) enhanced its binding to Irs-1 during insulin stimulation. Binding experiments with various SH2 proteins revealed that Grb-2 was largely excluded from Irs-1 complexes containing p59^{fyn}, whereas Grb-2 and p85 occurred in the same Irs-1 complex. By comparison with the insulin receptor, p59^{fyn} kinase phosphorylated a unique cohort of tyrosine residues in Irs-1. These results outline a role for p59^{fyn} or other related Src-kinases during insulin and cytokine signaling.

Irs proteins are important elements in signal transduction by the receptors for insulin, IGF-1, various interleukins (interleukin-4, -9, -13, and -15), interferons (interferons a and b), and growth hormones (1–7). After tyrosine phosphorylation, Irs-1 provides a common interface between the activated receptor and various downstream signaling proteins containing Src homology-2 (SH2)[1] domains, including phosphatidylinositol 3-kinase, p55^{PIK}, Grb-2, SH-PTP2, Nck, and possibly Crk (8–14). Moreover, Irs-1 associates with the integrin a$_v$b$_3$ and SV40 T-antigen, which may play roles in growth regulation or transformation (15, 16). As a consequence of docking to these and possibly other proteins, Irs-1 mediates multiple downstream signals including the direct activation of phosphatidylinositol 3-kinase and SH-PTP2, the indirect stimulation of MAP kinase and p70^{s6k}, and other events that regulate gene expression, inhibit apoptosis, and stimulate mitogenesis, chemotaxis, and glucose transport (10, 17–20).

We utilized an expression screening approach based on the CORT technique to identify new proteins that bind to tyrosine phosphorylated Irs-1 (9, 21). The Src family tyrosine kinase, p59^{fyn}, was isolated from a mouse embryo library with [^{32}P]Irs-1. p59^{fyn} contains an SH3 and an SH2 domain at its NH$_2$ terminus and a catalytic domain at its COOH terminus (22). Both SH2 and SH3 domains are essential for regulation of the enzymatic activity, because deletion of either domain leads to constitutive activation of the kinase and an increased transformation potential (23–26). An important role for p59^{fyn} during signal transduction by tyrosine kinase receptors has been implicated in a number of systems: the p59^{fyn} associates with middle T-antigen (27, 28), the ligand-stimulated PDGF and colony-stimulating factor-1 receptors (29, 30), and B and T cell antigen receptors during antigen stimulation (31, 32). In these systems, p59^{fyn} may participate in the stimulation of cell proliferation. Inhibition of p59^{fyn} function by microinjection of antibodies against p59^{fyn} or DNA constructs encoding dominant negative proteins reduces the mitogenic response of fibroblasts to PDGF, suggesting that p59^{fyn} is an important signaling element (33). Moreover, disruption of the p59^{fyn} gene in mice causes a signaling defect in mature thymocytes (34, 35). The Fyn$^{(-/-)}$ mice also exhibit specific neurologic deficits such as impaired long term potentiation, spatial learning, hippocampal development (36), impaired ability to initiate suckling reflex (37), and defective myelination (33), suggesting a general role for p59^{fyn} during development.

In this paper, we demonstrate that Irs-1 binds p59^{fyn} *in vitro* and *in vivo*. The SH2 domain from p59^{fyn} specifically binds to the Tyr(P)^{895} and Tyr(P)^{1172} in Irs-1 located in YXX(L/I) motifs and may compete for with Grb-2 for association with Irs-1. Thus, p59^{fyn} may play an important role in Irs-1-mediated signaling during insulin/IGF-1 or cytokine signaling.

EXPERIMENTAL PROCEDURES

Expression Cloning with [^{32}P]Irs-1—To identify Irs-1-binding proteins, an oligo(dT) primed murine embryo (E15) cDNA library prepared in [lamdba]EXlox (Novagen) was screened with [^{32}P]Irs-1. Baculovirus-produced Irs-1 purified to 95% by gel filtration was labeled by incubation with purified insulin receptor in the presence of [γ-^{32}P]ATP and Mn^{2+}, as described previously (9). Phosphoproteins in the reaction mixture, predominantly (>95%) [^{32}P]Irs-1, were reduced with 100 mM dithiothreitol at 55 °C for 5 h in 50 mM Tris-HCl buffer (pH 7.4) containing 250 mM NaCl and 6 M guanidinium chloride and then carboxymethylated with iodoacetamide (14). The methylated and reduced [^{32}P]Irs-1 was washed several times in a Centricon-30 microconcentrator (Amicon) with 10 mM Tris-HCl (pH 7.4) containing 50 mM NaCl to remove contaminating [γ-^{32}P]ATP; this final step is essential to minimize background during the screening. [^{32}P]Irs-1 was resuspended in 10 mM Tris-HCl (pH 8.0) containing 150 mM NaCl and 0.05% Tween 20 to a concentration of 2–5 × 10^5 cpm/ml for use as the probe. The [^{32}P]Irs-1 in this reaction was immunoprecipitated completely with anti-phosphotyrosine antibody, indicating that each labeled molecule

[1] The abbreviations used are: SH2, Src homology-2; PDGF, platelet-derived growth factor; CHO, Chinese hamster ovary; CHO^{IR}, CHO insulin receptors; GST, glutathione S-transferase; SH3, Src homology-3; HPLC, high pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis.

contains phosphotyrosine.

Twenty 15-cm plates representing 500,000 plaques were overlaid with nitrocellulose filters (Millipore, HATF) that were impregnated with 10 mM isopropyl-*b*-D-thiogalactopyranoside (BRL) and incubated for 10 h at 37 °C. The filters were removed, briefly washed at room temperature with TNT buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20), and then incubated in TNT buffer containing 5% Carnation instant dry milk for 6 h. The filters were incubated overnight at 4 °C with [^{32}P]Irs-1 (50 mg/ml), and then washed 3 times at room temperature with 10 mM Tris-HCl (pH 8.0), containing 150 mM NaCl and 0.01% Tween 20. The dry filters were exposed at -70 °C for 24 h to Kodak XAR-5 film with an intensifying screen. Fifteen of the 30 primary positive plaques remained positive during two rounds of screening with [^{32}P]Irs-1. The cDNA inserts in pBluescript were prepared by *in vivo* excision according to the manufacturer's instructions (Stratagene).

Cell Culture—CHO cells and CHO cells expressing the human insulin receptors (CHO^{IR}) were grown in Ham's F-12 medium supplemented with 10% fetal bovine serum and fasted overnight in Ham's F-12 medium containing 0.5% bovine serum albumin before each experiment (19). CHO cells were transfected with Irs-1, murine p59^{fyn} containing a FLAG-tag (DYKDDDDK) at the COOH terminus (38). The cDNA for each molecule was subcloned into pCMV^{his} creating pCMV^{his}/Irs-1 or pCMV^{his}/Fyn^{flag}. Irs-1 expression was selected directly by resistance to histidinol as described previously (19); pCMV^{his}/Fyn^{flag} was co-transfected with pEBV^{his}/hyg (Invitrogene) and selected by resistance to 200 mg/ml hygromycin (ICN). Cells expressing equivalent amount of Irs-1 or p59^{fyn} were selected by immunoblotting with αFyn or αIrs-1 (19).

Immunocomplexes were prepared from cell extracts by incubation with various rabbit polyclonal antibodies and detected by immunoblotting as described previously (19). The antibody against Irs-1 (αIrs-1) was raised against the last 12 residues in the COOH terminus; αSH-PTP2 was raised against NH$_2$-SH2 domain of SH-PTP2; αGrb2 was purchased from Santa Cruz, and αp85 was from UBI. Normal rabbit IgG was used as a control antibody.

Preparation of GST Fusion Proteins—The SH2 domain (142–256 amino acids), Src homology-3 (SH3) domain (86–149 amino acids), and the entire p59^{fyn} was expressed as a GST fusion protein using the pGEX-2T vector (Pharmacia Biotech Inc.). The DNA fragment containing SH2 and SH3 domains of p59^{fyn} were synthesized by the polymerase chain reaction using a mouse brain cDNA clone of p59^{fyn} as a template and oligonucleotides that contain appropriate restriction sites bordering the domains of interest (14). The amplified cDNA was isolated, digested with *Bam*HI and *Eco*RI, and cloned into pGEX-2T, which was used to transform *Escherichia coli* JM109 (Promega). The new vectors (pGEX-Fyn, pGEX-SH2^{fyn}, and pGEX-SH3^{fyn}) were confirmed by sequencing. Fusion proteins were purified by affinity chromatography on glutathione-Sepharose (Pharmacia) (39). GST fusion protein containing the SH3 domain of p59^{fyn} was used to immunize rabbits (HRP Corp.), and the anti-serum was used for recognition of p59^{fyn} expressed in CHO cells and other cell lines.

Binding of GST Fusion Proteins with Irs-1—Recombinant Irs-1 was labeled with [^{32}P]ATP using the purified insulin receptor as the catalyst at 25 °C for 2 h (14). GST fusion proteins (GST, GST-SH2^{fyn}, GST-SH3^{fyn}, and GST-Fyn) were incubated with [^{32}P]Irs-1 at 4 °C for 1 h, precipitated with glutathione-Sepharose at 4 °C for 1 h, washed twice in 50 mM Tris-HCl (pH 7.4) containing 100 mM NaCl, 250 mg/ml bovine serum albumin, 0.2 mM vanadate, and 0.4 mM phenylmethylsulfonyl fluoride), and boiled for 5 min in 100 ml of Laemmli sample buffer containing 100 mM dithiothreitol. Samples were separated on 7.5% SDS-PAGE and analyzed by autoradiography (14).

In other experiments, cell lysates were prepared from insulin-stimulated or unstimulated CHO^{IR} and CHO^{IR}/Irs-1 cells in 20 mM Tris-HCl (pH 7.5) containing 137 mM NaCl, 100 mM NaF, 1 mM MgCl$_2$, 1 mM CaCl$_2$, 200 mM sodium orthovanadate, 0.4 mM phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, 50 μg/ml leupeptin, 10% glycerol, and 1% Nonidet P-40 (Calbiochem). The extracts were clarified by centrifugation at 100,000 $\times$ *g* for 1 h at 4 °C. The supernatants were incubated with the GSTSH2^{fyn} for 2 h, washed with extraction buffer, and analyzed by immunoblotting (14, 19).

In Vitro Kinase Assays—GST-Fyn or p59^{fyn} immunoprecipitated from cell lysates were incubated at 22 °C for 10 min with Irs-1 or denatured enolase in 50 mM HEPES (pH 7.4) containing 100 mM NaCl, 5 mM MnCl$_2$, and 50 mM [^{32}P]ATP. Reactions were terminated by boiling the sample in Laemmli sample buffer, and the phosphoproteins were separated on 10% SDS-PAGE gel and analyzed by autoradiography.

HPLC Analysis of Phosphopeptides by Association with GST-SH2 Fusion Protein—Tryptic peptides from recombinant [^{32}P]Irs-1 were pre-
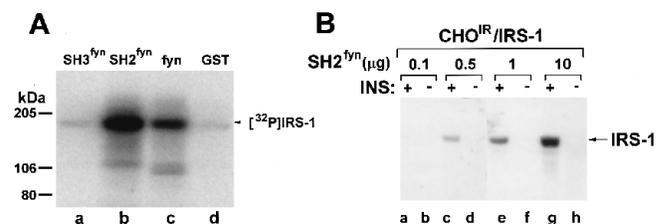
FIG. 1. **The association of p59^{fyn} with Irs-1 *in vitro*.** *A*, Irs-1 was labeled with [γ-^{32}P]ATP by insulin receptor and incubated with GST fusion proteins (1 mg of SH3^{Fyn}, SH2^{fyn} and GST, and 0.5 mg of GST-Fyn) as indicated. The complexes were precipitated with glutathione-Sepharose, washed, and analyzed by SDS-PAGE on a 7.5% SDS gel. *B*, cell lysates were prepared from insulin stimulated (+) and unstimulated (−) CHO^{IR}/Irs-1 cells and incubated with an increasing amount of GST-SH2^{fyn}. The GST fusion protein was then precipitated by glutathione-Sepharose, washed, and separated on 7.5% SDS-PAGE. Irs-1 was detected by immunoblotting with αIrs-1.

pared as described previously (14) and incubated with GST-SH2 domain proteins. The SH2 domain-associated peptides were resolved by reverse-phase HPLC analysis as described previously; the phosphoamino acid in each peptide was also identified (14, 40).

Mutagenesis of Tyr^{531} in p59^{fyn}—Tyr^{531} of p59^{fyn} was replaced with phenylalanine by polymerase chain reaction-mediated oligonucleotide-directed mutagenesis (Fyn^{F531}). Mouse p59^{fyn} cDNA was used as a template with the mutagenic primers 5'-GAGCCCCAGTTTCAGC-CCGGC-3' and 5'-ACCGGGCTGAAACTGGGGCTC-3' (18). The p59^{fyn} cDNA and mutant polymerase chain reaction product were digested with *Afl*II and *Eco*RV, and the mutant polymerase chain reaction fragment was inserted in place of the wild type sequence. Presence of the desired mutation was confirmed by sequencing the recombinant molecule. The cDNA for Fyn^{F531} was subcloned into the pCMV/his eukaryotic expression vector and expressed in the CHO cells as described previously.

RESULTS

In Vitro Association of p59^{fyn} with Irs-1—We used recombinant [^{32}P]Irs-1 to identify proteins in a mouse brain expression library that associated specifically with Irs-1. In addition to p85a and p85b, p59^{fyn} was isolated from 0.5 million plaques (data not shown). In order to test which portion of p59^{fyn} interacted with phosphorylated Irs-1, GST fusion proteins containing the SH3-domain, the SH2 domain, or the entire p59^{fyn} molecule were incubated with recombinant [^{32}P]Irs-1 (Fig. 1*A*). The GST fusion proteins containing the entire p59^{fyn} (GST-Fyn) or its SH2 domain (GST-SH2^{fyn}) associated with phosphorylated Irs-1; this binding was specific, because neither GST alone nor GST-SH3^{fyn} bound to [^{32}P]Irs-1 (Fig. 1*A*). The binding of Irs-1 to p59^{fyn} was slightly weaker than binding to the GST-SH2^{fyn} (Fig. 1*A*), possibly because the COOH-terminal phosphorylation site of p59^{fyn} occupies the SH2 domain, which competes with phosphorylated Irs-1 for binding (see below). Finally, GST-SH2^{fyn} also associated with Irs-1 in lysates from insulin-stimulated CHO^{IR}/Irs-1 cells (Fig. 1*B*).

In Vivo Association of p59^{fyn} with Irs-1—The association of p59^{fyn} with Irs-1 *in vivo* was studied in CHO, CHO^{IR}, CHO/Irs-1, and CHO^{IR}/Irs-1 cells, before and after expression of murine Fyn^{FLAG}(38). Endogenous p59^{fyn} was detected in αFyn immunoprecipitates from all four cell lines by blotting with αFyn. Cells transfected with murine Fyn^{FLAG} expressed 5–10-fold more p59^{fyn}; cell lines expressing approximately equal amounts of murine Fyn^{FLAG} were purposely selected for inclusion in this study (Fig. 2*C*).

Insulin stimulated tyrosine phosphorylation of Irs-1 in each cell line as measured in αIrs-1 immunoprecipitated by immunoblotting with aPY (Fig. 2*B*); the highest level of Irs-1 phosphorylation occurred in the CHO^{IR}/Irs-1 cells as described previously (Fig. 2*B*). The overexpression of p59^{fyn} had no reproducible effect on Irs-1 tyrosine phosphorylation.

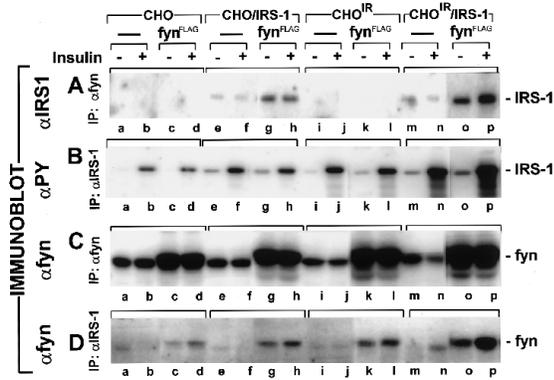Specific immunoprecipitates of Irs-1 were examined for

FIG. 2. **The association of p59^{fyn} with Irs-1 *in vivo*.** Cell extracts prepared from CHO, CHO^{IR}, CHO/Irs-1, or CHO^{IR}/Irs-1 cells before or after expression of murine Fyn^{FLAG} were incubated without or with 100 nM insulin for 5 min. Cell extracts were then incubated with various antibodies including αFyn (*A* and *C*) or αIrs-1 (*B* and *D*). The immuncomplexes were resolved by SDS-PAGE and immunoblotted with αIrs-1 (*A*), αPY (*B*), or αFyn (*C* and *D*) and detected with [^{125}I]protein A. These results are representative of two experiments.

p59^{fyn} association before and after insulin stimulation. Endogenous p59^{fyn} associated poorly with Irs-1 in CHO, CHO^{IR}, and CHO/Irs-1 (Fig. 2*D*); however, endogenous p59^{fyn} associated with Irs-1 in CHO^{IR}/Irs-1 cells during insulin stimulation (Fig. 2*D, lane n*). These results suggest that the association of Fyn with Irs-1 was relatively weak and required a high level of Irs-1 phosphorylation in order to be detected (Fig. 2*B*). Consistent with this hypothesis, overexpression of murine Fyn^{FLAG} increases the insulin-stimulated association of murine Fyn^{FLAG} with Irs-1 in all of the cell lines, as observed by aFyn immunoblots of αIrs-1 immunoprecipitates (Fig. 2*D, lanes d, h, l,* and *p*). Insulin-stimulated association of Fyn with Irs-1 was greatest in the CHO^{IR}/Irs-1 cells expressing the Fyn^{FLAG}, where it was also strong enough to be observed in αFyn immunoprecipitates by αIrs-1 blots (Fig. 2*A, lane p*). The basal association of Fyn^{FLAG} may reflect the basal phosphorylation of Irs-1 in these cells. Thus, insulin promoted the association of p59^{fyn} with Irs-1 in intact cells, consistent with our *in vitro* experiments.

Binding Site of p59^{fyn} on Irs-1—We determined the phosphorylation sites of Irs-1 that bind to p59^{fyn} kinase. The tryptic phosphopeptides of Irs-1 were precipitated with GST-SH2^{fyn} and analyzed by reverse-phase HPLC (14). Two phosphopeptides were depleted from the tryptic digests with GST-SH2^{fyn} in a concentration-dependent manner; the depleted peptides were bound to the GST-SH2^{fyn} (Fig. 3). Manual radio-sequencing of these two peptides identified Tyr^{895} and Tyr^{1172} as the major sites for binding of SH2^{fyn}. GST-SH2^{fyn} appeared to have a higher affinity for the peptide containing Tyr(P)^{895} than Tyr(P)^{1172}, because the former peptide bound more strongly at low concentrations of GST-SH2^{fyn} (Fig. 3).

The Binding of Fyn^{F531} to Irs-1—Our *in vitro* experiments (Fig. 1) suggested that GST-SH2^{fyn} associated more strongly with Irs-1 than GST-Fyn, which may be due to interference of the SH2 domain by the Tyr^{531} (22, 31, 32, 41). To test if the phosphorylation of Tyr^{531} in p59^{fyn} prevents it from binding to Irs-1, Tyr^{531} was replaced with phenylalanine (Fyn^{F531}), and Fyn^{F531} was expressed in CHO^{IR}/Irs-1 cells. During insulin stimulation, the binding of Fyn^{F531} to Irs-1 increased significantly compared with wild type p59^{fyn}, suggesting that phosphorylation of Tyr^{531} ordinarily inhibited the binding to Irs-1. This enhancement was observed by αFyn blotting of αIrs-1 immunoprecipiates (Fig. 4*B*) and by αPY blotting of αFyn immunoprecipiates (Fig. 4*A*). Consistent with these findings, αFyn also precipitated p85 more strongly from CHO^{IR}/Irs-1 cells expressing Fyn^{F531} than wild type p59^{fyn} (Fig. 4*D*).
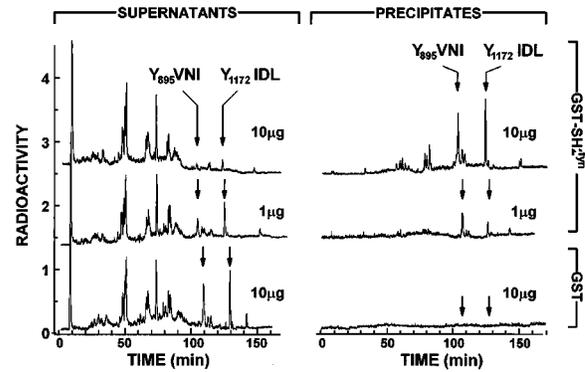

FIG. 3. **Binding of Irs-1 phosphopeptides to GST-SH2^{fyn}.** GST-SH2^{fyn} or GST alone were incubated with Irs-1 tryptic phosphopeptides and precipitated with glutathione-Sepharose. Supernatants and elutes from the precipitates were resolved by reverse-phase on the HPLC, and the radioactivity was measured with an on-line radiodetection system (Packard). These results are representative of two independent experiments.
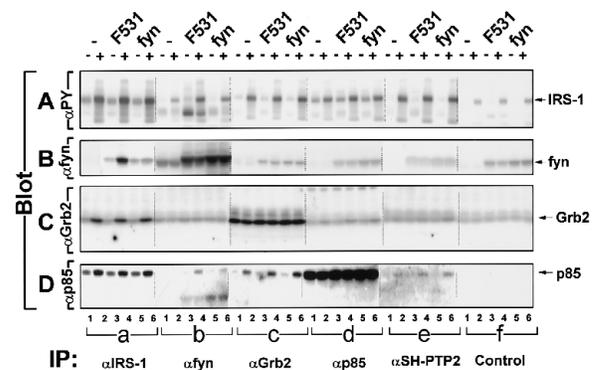

FIG. 4. **Formation of Irs-1 complexes.** Cell lysates prepared from insulin-stimulated or unstimulated CHO^{IR}/Irs-1 cells overexpressing either p59^{fyn} or the COOH-terminal mutant p59^{FynF531}. Before and after insulin stimulation (100 nM), cell extracts were incubated with the indicated antibodies, and proteins were immunoblotted with indicated antibodies. These results are representative of two independent experiments.

Heterogeneity of Irs-1 Complexes—Irs-1 associates with several SH2 proteins to form signaling complexes during insulin stimulation (42). Two SH2 proteins, Grb-2 and SH-PTP2, bind to Tyr^{895} and Tyr^{1172}, respectively, which are also the binding sites for p59^{fyn} in Irs-1. To see if these signaling molecules compete for Irs-1 binding, Irs-1 was immunoprecipitated with various antibodies from lysates of CHO^{IR}/Irs-1 cells or cells overexpressing Fyn^{FLAG} or Fyn^{F531} (Fig. 4). As expected, p85, SH-PTP2, Grb-2, and Fyn associated with Irs-1 during insulin stimulation, as specific antibodies against each SH2 protein immunoprecipitated Irs-1 from insulin-stimulated cells (Fig. 4*A*). Conversely, αIrs-1 immunoprecipitated p59^{fyn}, Grb-2, and p85 from insulin-stimulated cells (*sections a* in Fig. 4, *B*, *C*, and *D*). Interestingly, both Irs-1 and p85 were found in Grb-2 and p59^{fyn} immunocomplexes (Fig. 4*D, lanes b* and *c*), whereas no Grb-2 was observed in Fyn^{FLAG} or Fyn^{F531} complexes or *vice versa* (*section b* of Fig. 4*C*, and *section c* of Fig. 4*B*). These data suggest that p59^{fyn} and Grb-2 bind to the distinct Irs-1 complexes, both of which contain p85.

Phosphorylation of Irs-1 by Fyn Tyrosine Kinase—The association of p59^{fyn} could play several roles in an Irs-1 complex, including the direct activation of Fyn kinase, phosphorylation of Irs-1 or other associated molecule, or targeting Irs-1 to the membrane. The p59^{fyn} kinase activity was determined in αFyn immunocomplexes from CHO^{IR} and CHO^{IR}/Irs-1 cells without or with the expression of Fyn^{FLAG}. As expected, expression of
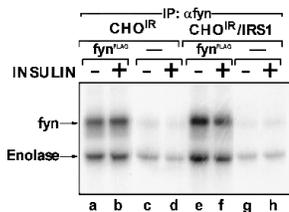
FIG. 5. **Insulin-stimulated p59^{fyn} tyrosine kinase activity.** Cell lysates from insulin-stimulated or unstimulated CHO cells overexpressing murine Fyn^{FLAG} were incubated with αFyn or αFLAG monoclonal antibody. The immuncomplexes were isolated on protein A-Sepharose, and kinase assays were performed in immuncomplexes with denatured enolase as a substrate. Autophosphorylated p59^{fyn} and phosphorylated enolase are indicated. These results are representative of two independent experiments.

FIG. 6. ***In vitro* phosphorylation of Irs-1 by p59^{fyn}.** *A*, Irs-1 or enolase was phosphorylated by GST, GST-Fyn, or GST-SH3^{fyn} for 1 h and separated on 7.5% SDS-PAGE, and phosphorylation of Irs-1 was measured by autoradiography. These results are the representative of three independent experiments. *B*, phosphorylated Irs-1 bands were excised, and amino acid analyses were performed as described under "Experimental Procedures." *C*, Irs-1 were phosphorylated by insulin receptor or GST-Fyn in the presence of [γ-^{32}P]ATP, separated on SDS-PAGE, and transferred to nitrocellulose membrane. Phosphorylated Irs-1 were excised and eluted from membrane by digestion with trypsin. Tryptic phosphopeptides were analyzed on HPLC, and radioactivity was detected with an on-line radiodetection system (Packard). This results are representative of two independent experiments.
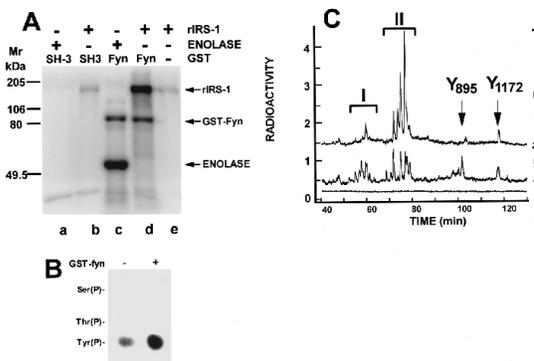
Fyn^{FLAG} significantly increased the amount of Fyn kinase measured *in vitro* (Fig. 5). However, insulin stimulation of the cells prior to immunoprecipitation had no effect on the kinase activity (Fig. 5). The activity of Fyn immunoprecipitated from the CHO^{IR}/Irs-1 was slightly greater than that from the CHO^{IR} cells, which may only reflect a small variation of expression among the cell lines.

In order to assess if Irs-1 is phosphorylated by p59^{fyn} kinase, an *in vitro* kinase assay was performed to examine the phosphorylation of recombinant Irs-1 by GST-Fyn (Fig. 6*A*). The phosphorylation of Irs-1 by GST-Fyn was comparable with the phosphorylation of an equimolar amount of enolase; phosphoamino acid analysis of phosphorylated Irs-1 showed that the phosphorylation was exclusive on tyrosine (Fig. 6*B*). The phosphorylation of Irs-1 was not due to kinase contamination during preparation of GST fusion protein, because neither substrate was phosphorylated by GST-SH3^{fyn} or GST alone (Fig. 6*A*, *lanes a*, *b*, and *e*). The pattern of tyrosine phosphorylation sites in Irs-1 was different during incubation with the insulin receptor or GST-Fyn (Fig. 6*C*). Based on our previous assignments (14), both the insulin receptor and Fyn phosphorylated Tyr^{1172}, whereas the phosphorylation of Tyr^{895} was much weaker by p59^{fyn} (Fig. 6*C*). Many other differences occurred in the phosphopeptides map, although the exact sites involved were not identified. Tyrosine phosphorylation of Irs-1 in the intact cells was not detectably increased by overexpression of Fyn^{FLAG}, probably owing to the low stoichiometry of the association, as described previously by the PDGF receptor (29).

DISCUSSION

The p59^{fyn} tyrosine kinase associates with Irs-1 *in vitro* and *in vivo* during insulin stimulation, suggesting that it may contribute to the signal mediated by Irs-1 in certain cellular backgrounds. The p59^{fyn} may also be involved in signaling from other receptors that engage Irs-1 if Tyr^{895} and Tyr^{1172} are phosphorylated by activated kinases in the receptor complex. Although p59^{fyn} is activated during association with several growth factor receptors (30, 43, 44), we did not detect activation during insulin stimulation. However, we cannot role out the possibility that the portion of p59^{fyn} associated with Irs-1 was activated but is impossible to detect in a large pool of unbound p59^{fyn} (44). The p59^{fyn} recruited into the Irs-1 signaling complex may mediate a second phase of tyrosine phosphorylation of Irs-1 or the phosphorylation of other proteins in the Irs-1 complex. Because other members of the Src kinase family have similar SH2 domains, these kinases may also associate with Irs-1 and contribute to the overall response in various cellular backgrounds.

The SH2 domain of p59^{fyn} selectively binds to two phosphorylation sites in Irs-1, EY^{895}VNI and NY^{1172}IDL, which are different from the consensus sequence YEEI for the SH2 domain of p59^{fyn} determined by phosphopeptide library screening (21). Both binding sites have hydrophilic residues at +1 and +3 position, which are consistent with the PDGF receptor binding sites for Src, including DY^{751}VPM (45), EY^{579}IYV, and IY^{581}VDP (29). Interestingly, Tyr^{895} and Tyr^{1172} also bind Grb-2 and SH-PTP2, respectively (14). In fact, p59^{fyn} and Grb-2 are not found in the same Irs-1 complex, indicating that a competition may exist in the cell for the binding of these two proteins to the activated Irs-1. The formation of unique Irs-1 signaling complexes may be a general mechanism to produce specific signals. This will become clearer as we identify more SH2 proteins that engage phosphorylated Irs proteins under various conditions.

In vitro kinase assays reveal that Irs-1 is a good substrate for p59^{fyn}. Some of the tyrosine phosphorylation sites on Irs-1 are shared by both the insulin receptor and Fyn, whereas others are different. However, overexpression of Fyn does not lead to a significant phosphorylation of Irs-1 in resting cells. Even during insulin stimulation, the phosphorylation of Irs-1 in CHO cells is not significantly enhanced by overexpressing murine p59^{fyn}, suggesting that the insulin receptor, not p59^{fyn}, is the major tyrosine kinase that phosphorylates Irs-1. However, it is possible that p59^{fyn} phosphorylates tyrosine residues in a small subset of Irs-1 molecules that are not detected by αPY immunoblotting.

The phosphorylation of Irs-1 by p59^{fyn} may play an important role in nontyrosine kinase receptors. Cross-linking of the B-cell antigen receptor in primary B cells and mature B cell lines causes the phosphorylation of Irs-1 (data not shown). Because p59^{fyn} is a known component of this signaling complex, it may be involved in the phosphorylation of Irs-1.

p60^{c-Src} from nontransformed fibroblasts is normally phosphorylated extensively at Tyr^{527}, which is highly conserved among the Src kinase family (Tyr^{505} in Lck and Tyr^{531} in p59^{fyn}) (22, 46). Phosphorylation of this tyrosine residue, mediated by p50^{csk}, down-regulates tyrosine kinase catalytic activity and biological functions of the Src-related kinases (31, 32, 46). This inhibition involves an intramolecular association between the SH2 domain and the COOH-terminal phosphotyrosine residue (22, 41). The removal of Tyr^{531} from p59^{fyn} enhances its binding to Irs-1 during insulin stimulation, suggesting that Tyr^{531} may compete with Irs-1 for binding to the

SH2 domain. Thus, the binding of wild type p59^{fyn} to Irs-1 should stimulate its tyrosine kinase by displacing the COOH terminus. Although we were unable to document this increase, future experiments along this line of investigation should be explored and could reveal a subset of Irs-1/Fyn complexes that mediate a specific biological response.

The association of p59^{fyn} with the PDGF or colony-stimulating factor-1 receptors may contribute to the mitogenic response (29, 30). Currently, we do not know the biological effect of p59^{fyn} during its association with Irs-1. Insulin-stimulated DNA synthesis appears normal in cells overexpressing p59^{fyn} and Irs-1 (data not shown). However, the endogenous level of p59^{fyn} in CHO cells is relatively high, and possibly sufficient for a maximal response. A reduction of the p59^{fyn} levels may help address this question. However, deletion of the COOH terminus of Irs-1, or substitution of Tyr^{895} and Tyr^{1172} with phenylalanine has no effect of Irs-mediated and insulin-stimulated mitogenesis in 32D myeloid cells, suggesting that Irs-1/Fyn complexes are not involved.[2]

In summary, p59^{fyn} takes a place next to other SH2 proteins as an Irs-1-associated enzyme that may contribute to the full Irs-1 signal. The differential association of p59^{fyn} with Irs-1 complexes supports the idea that Irs-1 signaling complexes are not homogenous, and different elements in the complex may influence the aggregate signal. Much work remains to identify other novel proteins that associate with Irs-1 and Irs-2 (47) and to determine the pattern of tyrosine phosphorylation that occurs during activation of various receptors, which will influence the ultimate biological signal.

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