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IRS-1 Is a Common Element in Insulin and Insulin-Like Growth Factor-I Signaling to the Phosphatidylinositol 3′-Kinase*

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ABSTRACT

IRS-1 is a unique cytosolic protein that becomes tyrosine phosphorylated during insulin stimulation of intact cells and immediately associates with the phosphatidylinositol 3′-kinase (PtdIns 3′-kinase). The insulin-like growth factor-I (IGF-I) receptor also mediated the tyrosine phosphorylation of IRS-1 and increased the amount of PtdIns 3′-kinase activity bound to IRS-1 in Chinese hamster ovary cells. Purified insulin receptor and IGF-I receptor phosphorylated recombinant baculovirus-produced IRS-1 on similar sites *in vitro*, and phosphorylated baculovirus-produced IRS-1 bound PtdIns 3′-kinase activity from lysates of quiescent cells. Treatment of cells with IGF-I activated the PtdIns 3′-kinase, suggesting that IGF-I activates the PtdIns 3′-kinase through IRS-1 binding to p85 in a manner similar to insulin. Chinese hamster ovary cells overexpressing IRS-1 demonstrated increased tyrosine phosphorylation of IRS-1, and more PtdIns 3′-kinase activity associated with IRS-1 in these cells. These data demonstrate that IRS-1 is a common element for signal transmission by the IGF-I and insulin receptors. (*Endocrinology* **132:** 1421–1430, 1993)

INSULIN and Insulin-like growth factor-I (IGF-I) are homologous growth factors which control cellular growth and metabolism. The cell surface receptors for insulin (IR) and IGF-I (IGF-Ir) are highly homologous transmembrane glycoproteins which are composed of two α- and two β-subunits (1, 2). Whereas the α-subunits contain distinct ligand-binding domains, the cytoplasmic domain of the β-subunits contains homologous tyrosine kinases (1, 3, 4). Binding of insulin and IGF-I to their cognate receptors activates the tyrosine kinases, causing a cascade of tyrosine autophosphorylation in the β-subunit and further stimulation of the receptor kinase activity (5). Both IR and IGF-Ir activation result in the tyrosine phosphoryation of pp185, thought to be the major receptor kinase substrate (6–8).

Recently, we purified pp185 from insulin-stimulated rat liver by affinity chromatography on immobilized antiphosphotyrosine antibody (9) and isolated a complementary DNA (cDNA) molecule encoding this protein, which we call IRS-1 (10, 11). IRS-1 is a unique hydrophilic phosphoprotein with a calculated molecular mass of 131 kilodaltons (kDa); however, IRS-1 migrates between 170–185 kDa when expressed in Chinese hamster ovary (CHO) cells (10, 12). IRS-1 contains more than 30 potential serine and threonine phosphorylation sites and 15 potential tyrosine phosphorylation sites (10, 13). Six of the tyrosine residues are found in YMXM (TyrMetXaaMet) motifs, three in YXXM motifs, and several in other related motifs (*e.g.* YVNI) which are thought to associate with proteins containing certain isoforms of the *src* homology-2 (SH2) domain (10, 14). There is a 90.5% amino acid sequence identity between rat and human IRS-1, and all of the potential tyrosine phosphorylation sites are conserved, suggesting that they may be important in signaling (10, 15).

IRS-1 undergoes tyrosine phosphorylation immediately after insulin stimulation and associates with the phosphatidylinositol 3′-kinase (PtdIns 3′-kinase) (10). The PtdIns 3′-kinase phosphorylates the D-3 position of phosphatidylinositol, forming PtdIns(3)P, PtdIns(3,4)P_2, and PtdIns(3,4,5)P_3 in the intact cell, which may control certain aspects of cellular growth and metabolism (14, 16–18). The PtdIns 3′-kinase is activated when the SH2 domains in its 85-kDa subunit (p85) bind to phosphotyrosine residues in IRS-1 (19–23). Together these results suggest that IRS-1 is a regulatory docking protein that is activated by tyrosine phosphorylation. The association of IRS-1 with cellular enzymes or adapter molecules that contain the correct SH2 domain isoform may play an important role in the molecular link between receptor tyrosine kinases and enzymes controlling cellular growth and metabolism.

As the receptors for insulin and IGF-I are highly homologous, we examined whether IRS-1 was involved in IGF-I signaling. In this report we show that IRS-1 is a substrate for the IGF-Ir and that IRS-1 is a common element in the IGF-I and insulin signaling pathways involving the PtdIns 3′-kinase.

Materials and Methods

Cell lines and growth factors

Untransfected CHO cells expressing approximately 30,000 endogenous IRs and 200,000 endogenous IGF-Irs or CHO cells expressing 10^6

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copies of the human IR (CHO/IR) were previously described (24). CHO cells overexpressing rat IRS-1 (rIRS-1) (CHO/IRS-1) and control cells transfected with the histidinol resistance plasmid pCMVHis (CHO/HIS) have also been described (10, 12). Cells were grown in Ham's F-12 media supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO) (24). For insulin stimulation, recombinant human insulin (ELANCO, Indianapolis, IN) was added to a final concentration of 100 nM, and recombinant human IGF-I (Calbiochem, La Jolla, CA) was used at 130 nM, final concentration, unless otherwise noted.

Antibodies

Anti-IRS-1 antibodies were protein A-purified polyclonal antibodies from rabbits immunized with recombinant baculovirus-produced IRS-1 protein (αIRS-1^{bac}) or affinity-purified antipeptide antibodies (αpep80) (10). Monoclonal anti-IRS-1 antibodies (1M92–7) were used for immunoblotting; these are mouse monoclonal immunoglobulin Gs raised against recombinant IRS-1 protein (22). Supernatants from cultured 1M92–7 hybridoma cells were used for immunoblotting. Affinity-purified polyclonal antiphosphotyrosine (αPY) antibodies were described previously (25). Immunoprecipitating antibodies specific for the p85 subunit of the PtdIns 3′-kinase were protein A-purified polyclonal antibodies against the C-terminal two-thirds of p85 expressed as a GST fusion protein (26) (Backer J. M., M. F. White, unpublished data).

Immunoprecipitation

Cells were grown to 80% confluence on 15-cm dishes (Costar, Cambridge, MA) and made quiescent overnight in serum-free media containing 0.5% BSA (Fluka, Ronkonkoma, NY). Cells were stimulated with growth factor for 1 min, frozen in liquid nitrogen, and extracted in 100 mM Tris, pH 8.0, containing 100 mM NaF, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonylfluoride, 10 μg/ml Aprotinin, and 10 mM NaP_2O_4. Insoluble material was pelleted at 100,000 × *g* for 1 h, and supernatants were incubated with αPY (3 μg/ml) or αIRS-1^{bac} (10 μg/ml) antibodies. Immunecomplexes were collected with Pansorbin cells (Calbiochem), and complexes were washed three times in 50 mM HEPES, pH 7.4, containing 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl, and 2 mM Na_3VO_4 before being denatured in Laemmli Sample buffer containing 10 mM dithiothreitol (DTT) and resolved by SDS-polyacrylamide gel electrophoresis (PAGE).

Immunoblotting

Proteins were resolved by 7.5% SDS-PAGE on a generic apparatus at 5 mA or in Bio-Rad (Richmond, CA) miniprotean apparatuses at 100 V. Gels were transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH) for αPY and αp85 immunoblotting and to polyvinyldifluoridene membranes (Millipore, Milford, MA) for αIRS-1 (1M92–7) immunoblotting. Proteins were transferred for 1.5 h at 100 V in Towbin buffer containing 0.02% SDS and 20% methanol (27). Membranes were blocked overnight at 4 C in wash buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.01% Tween-20) supplemented with 3% BSA (Fluka). Membranes were then incubated for 2 h at room temperature in wash buffer containing 3% BSA and either αPY (3 μg/ml) or αIRS-1 (1M92–7, 1:500 dilution of tissue culture supernatant) antibodies. The membranes were subsequently washed three times in wash buffer, and reblocked for 1 h at room temperature in wash buffer containing 3% BSA. For blots with 1M92–7, membranes were subsequently incubated for 1 h with wash buffer containing 3% BSA and 2.5 μg/ml rabbit antimouse immunoglobulin G (H + L; Pierce, Rockford, IL) and washed twice in wash buffer with 3% BSA. Blots were then incubated with [^{125}I] protein A (ICN, Costa Mesa, CA; 0.2 μCi/ml) for 1 h in wash buffer containing 3% BSA. Blots were washed four or five times in wash buffer, dried, and exposed to autoradiography with Kodak X-AR film (Eastman Kodak Co., Rochester, NY) or phosphorimager screens (Molecular Dynamics, Sunnyvale, CA).

Production of recombinant IRS-1 protein

The rIRS-1 cDNA, with the 5′- and 3′-untranslated regions removed, was subcloned into the pBlueBac transfer vector (Invitrogen, San Diego, CA) using standard techniques. pBlueBac containing IRS-1 cDNA was then recombined with the wild type AcNPV baculovirus genome in Sf9 insect cells and recombinant baculoviruses were selected (23, 28, 29). Sf9 cells were infected for 54–56 h at high multiplicity of infection of recombinant virus as described (28, 29). Cells were collected by centrifugation and lysed by douncing in B buffer (50 mM Tris-HCl, pH 7.8, 1 M NaCl) supplemented with 10 μg/ml Aprotinin, 10 mM benzamidine, 10 μg/ml leupeptin, 350 μg/ml phenylmethylsulfonylfluoride, and 10 mM DTT. Insoluble material was removed by sedimentation at 100,000 × *g* for 1 h. Recombinant IRS-1 protein (IRS-1^{bac}) accounted for approximately 15% of total protein in clarified lysates and was subsequently purified to approximately 90% homogeneity by gel filtration chromatography on SK 300 HR media (Pharmacia, Piscataway, NJ) in B buffer (Myers, Jr., M. G., M. F. White, unpublished observations).

In vitro tyrosine phosphorylation of IRS-1^{bac}

Wheat germ agglutinin (WGA)-purified IR (WGA-IR) was prepared from CHO/IR cells (24, 30), and WGA-purified IGF-Ir (WGA-IGF-Ir) was prepared from CHO cells overexpressing the human IGF-Ir (generously provided by Drs R. J. Smith and G. Condorelli, Joslin Diabetes Center) as described (31). IRS-1^{bac} (~1 μM) was incubated for the indicated time in the presence of 4 μg WGA-IR or WGA-IGF-Ir in 60 μl B Buffer containing 50 mM ATP, 5 mM $MnCl_2$, and 100 nM insulin or IGF-I. For some experiments, 60 μCi [γ-^{32}P]ATP (Du Pont-New England Nuclear, Boston, MA) was added as tracer.

Trypsin digestion and HPLC analysis of IRS-1 phosphopeptides

IRS-1^{bac} from 3-h *in vitro* phosphorylation reactions was boiled in Laemmli sample buffer containing 10 mM DTT and separated by SDS-PAGE. Gels were exposed to autoradiography, and bands corresponding to IRS-1 were excised and washed in 20% (vol/vol) methanol overnight at 37 C. Gel slices were dried and incubated in 50 mM NH_4HCO_3 containing 10 mM DTT and 6 M guanidine-HCl for 5 h at 55 C. Iodoacetamide (25 mg; Pierce) was added to slices, and incubation was continued in the dark for 30 min at room temperature before neutralizing the iodoacetamide with 10 μl 2-mercaptoethanol for 1 h at room temperature. Slices were then washed five times in 50 mM NH_4HCO_3, dried, and incubated overnight at 37 C in 1 ml 50 mM NH_4HCO_3 containing 0.1 mg/ml tosylphenylalanine chloromethylketone trypsin (Worthington Enzymes, Freehold, NJ). An additional 100 μl 50 mM NH_4HCO_3 containing 1.0 mg/ml tosylphenylalanine chloromethylketone trypsin were added, and the incubation was continued overnight. Supernatants were dried, resuspended in 0.055% trifluoroacetic acid (TFA) and analyzed by reversed-phase HPLC on a Beckman System Gold (Beckman Instruments, Palo Alto, CA) equipped with a Hi-Pore reversed phase RP-318 column (Beckman) eluted at a flow rate of 1 ml/min with 0.055% TFA modified with 75% acetonitrile-0.05% TFA solution as described (12). Eluted radioactivity was collected in 0.5-ml fractions and quantitated (Cerenkov counting) in a Beckman LS 1801 scintillation counter.

PtdIns 3′-kinase activity

In vitro phosphorylation of PtdIns was carried out in the immune complexes as described previously (32). Subconfluent CHO cells grown in 100-mm dishes were made quiescent by an overnight incubation in F-12 medium containing 0.5% BSA. The quiescent cells were incubated with insulin or IGF-I for 10 min and washed once with ice-cold PBS and twice with 20 mM Tris-HCl, pH 7.5, containing 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 100 μM Na_3VO_4 (buffer A). The cells were solubilized in 1 ml buffer A containing 1% NP-40 (Sigma) and 10% glycerol, and insoluble material was removed by centrifugation at 13,000 × *g* for 10 min. Supernatant was incubated with antibody overnight at 4 C, and immune complexes were precipitated from the supernatant with Protein A-Sepharose (Pharmacia) and washed successively in PBS containing 1% NP-40 and 100 μM Na_3VO_4 (three times), 100 mM Tris-HCl, pH 7.5, containing 500 mM LiCl and 100 μM Na_3VO_4 (three times), and 10 mM Tris-HCl, pH 7.5, containing 100 mM NaCl, 1 mM EDTA, and 100 μM Na_3VO_4 (two times). The pellets were resuspended in 50 μl

10 mM Tris-HCl, pH 7.5, containing 100 mM NaCl and 1 mM EDTA, and combined with 10 μl 100 mM $MnCl_2$ and 10 μl 2 μg/μl PtdIns (Avanti, Pelham, AL) sonicated in 10 mM Tris-HCl (pH 7.5) containing 1 mM EGTA. The phosphorylation reaction was started by adding 10 μl 440 μM ATP containing 30 μCi [γ-^{32}P]ATP. After 10 min at 22 C, the reaction was stopped with 20 μl 8 N HCl and 160 μl $CHCl_3$:methanol (1:1). The samples were centrifuged, and the lower organic phase was removed and applied to a silica gel TLC plate (Merck, Rahway, NJ) which had been coated with 1% potassium oxalate. TLC plates were developed in $CHCl_3$:CH_3OH:H_2O:NH_4OH (60:47:11.3:2), dried, and visualized by autoradiography. The radioactivity in spots which comigrated with PtdIns-4P standard (Sigma) was measured by Cerenkov counting as previously described (32).

In vitro association of PtdIns 3′-kinase with IRS-1^{bac}

IRS-1^{bac} incubated overnight with activated WGA-IR, WGA-IGF-Ir, or buffer alone was immunoprecipitated for 2 h at 4 C with αpep80 immobilized on Protein A-Sepharose (Pharmacia). Immunoprecipitates were washed twice with PtdIns 3′-kinase cell lysis buffer and incubated with lysates from unstimulated CHO cells for 30 min. Immunoprecipitates were then washed and assayed for associated PtdIns 3′-kinase activity as described above.

Results

IRS-1 is tyrosine phosphorylated after stimulation of CHO cells with IGF-I or insulin

We assessed the tyrosine phosphorylation state of cellular proteins in CHO and CHO/IR cells which had been incubated in the absence or presence of insulin, IGF-I, or both. Cell extracts were incubated with antiphosphotyrosine antibody (αPY), and immunoprecipitated phosphotyrosine-containing proteins were separated by SDS-PAGE and detected by immunoblotting with αPY antibodies (Fig. 1, A, lanes a–d and B, lanes a–d). The β-subunits of the insulin and IGF-I receptors were tyrosine phosphorylated immediately after insulin or IGF-I stimulation, respectively (Fig. 1, A and B, lanes a–d). Moreover, pp185 was detected with αPY in CHO and CHO/IR cells only after stimulation with insulin, IGF-I, or both. There are significantly more receptors for IGF-I than insulin in CHO cells, and there was correspondingly greater stimulation of pp185 phosphorylation by IGF-I than insulin (Fig. 1A, compare lanes b and c). In contrast, the insulin stimulation of pp185 phosphorylation was greater in CHO/IR cells due to the overexpression of insulin receptors (Fig. 1, A and B, lanes a and b).

In order to directly assess whether IRS-1 is phosphorylated by the IGF-Ir, we used anti-IRS-1 (αIRS-1^{bac}) antibodies to immunoprecipitate the endogenous hamster IRS-1 from CHO or CHO/IR cells incubated in the absence or presence of insulin, IGF-I, or both. Immunoblots with αPY indicated that IRS-1 was tyrosine phosphorylated during stimulation with insulin and IGF-I, alone or in combination (Fig. 1, A and B, lanes e–h). Tyrosine phosphorylation of IRS-1 in CHO cells was greater with IGF-I than insulin, consistent with the greater number of IGF-I receptors than insulin receptors in CHO cells (Fig. 1A, lanes e–h). In contrast, insulin stimulated more phosphorylation of IRS-1 in CHO/IR cells (Fig. 1B, lanes e–h). These data suggest that IGF-I stimulates the tyrosine phosphorylation of IRS-1 in CHO cells and that this effect is predominantly mediated via IGF-I receptors.

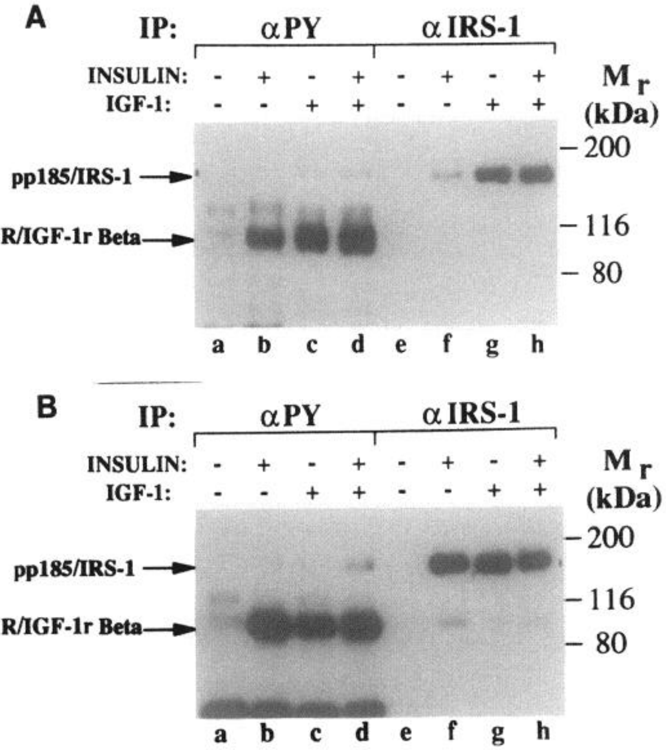

FIG. 1. Tyrosine-phosphorylated proteins in αPY and αIRS-1 immunoprecipitates of cells stimulated with insulin and/or IGF-I. CHO (A) and CHO/IR (B) cells were incubated in the absence of insulin and IGF-I (lanes a and e) or with insulin (lanes b and f), IGF-I (lanes c and g), or both insulin and IGF-I (lanes d and h). Cell lysates were immunoprecipitated with αPY (lanes a–d) or αIRS-1 (lanes e–h) antibodies. Immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with αPY. Migration of IRS-1/pp185, the β-subunits of IR and IGF-Ir, and molecular mass standards are indicated.

Recombinant IRS-1 protein is phosphorylated by the IR and IGF-Ir in vitro

Recombinant IRS-1 protein (IRS-1^{bac}) produced in a baculovirus expression system (23) was incubated *in vitro* with WGA-purified insulin or IGF-I receptors. Phosphorylation of the WGA-purified receptors and IRS-1 was analyzed by SDS-PAGE and autoradiography. Insulin and IGF-I stimulated *in vitro* phosphorylation of the β-subunit of the IR and IGF-Ir, respectively (Fig. 2, lanes a and b). Reduction and seperation of the receptors by SDS-PAGE confirmed that the β-subunit of the IGF-Ir migrated more slowly than the IR (31). Incubation of IRS-1^{bac} alone resulted in little phosphate incorporation into IRS-1 (Fig. 2, lane e). However, after incubation of IRS-1bac with the IR (lane c) or the IGF-Ir (lane d), both IRS-1bac and the receptor β-subunit were phosphorylated (Fig. 2, lanes c and d). Phosphoamino acid analysis demonstrated that IRS-1 was phosphorylated on tyrosine residues (data not shown). The tyrosine phosphorylation sites of IRS-1 were identical during phosphorylation by insulin and IGF-I receptors, as a similar pattern of tryptic fragments was

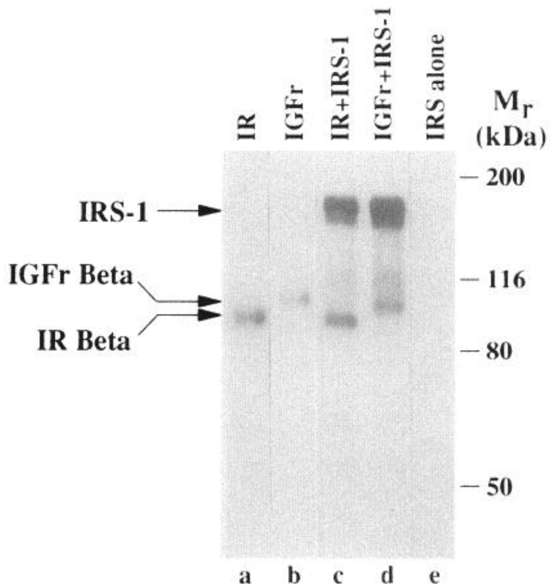

FIG. 2. *In vitro* tyrosine phosphorylation of IRS-1^{bac} by the IR and IGF-Ir. WGA-purified proteins containing ligand-activated IR (lanes a and c) or IGF-Ir (lanes b and d) were incubated with [γ^{32}P]ATP in the absence (lanes a and b) or presence of IRS-1^{bac} (lanes c and d), or IRS-1^{bac} was incubated alone (lane e) in the presence of [γ^{32}P]ATP. Proteins were resolved by SDS-PAGE and detected by autoradiography. Migration of IRS-1^{bac}, the β-subunits of IR and IGF-Ir, and molecular mass standards are indicated.

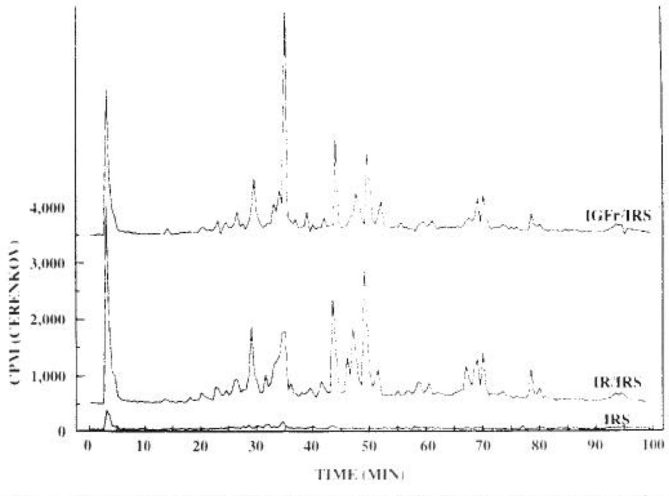

FIG. 3. Reversed-phase HPLC analysis of IRS-1*bac* phosphopeptides after incubation alone (IRS) or with the IR (IR/IRS) or IGF-Ir (IGFr/IRS) *in vitro*. Bands containing labeled IRS-1^{bac} were excised from gels such as that in Fig. 2 and subjected to reduction carboxymethylation and tryptic digestion. The resulting phosphopeptides were analyzed by reversed-phase HPLC, and radiation in each 0.5-min fraction was determined by Cerenkov counting.

obtained in each case when analyzed by reversed-phase HPLC (Fig. 3); the minor differences in the phosphopeptide maps shown are not reproducible and are primarily due to the splitting of peaks into two fractions during the collection process. We conclude that IRS-1 is phosphorylated on identical sites by each receptor. No significant peaks were seen in the absence of WGA receptors.

Insulin and IGF-I stimulate the association of the PtdIns 3-kinase with IRS-1

We measured the amount of PtdIns 3′-kinase activity detectable in αPY or αIRS-1^{bac} immunoprecipitates from CHO and CHO/IR cells after stimulation with insulin or IGF-I (Fig. 4). Both insulin and IGF-I increased the amount of PtdIns 3′-kinase activity in αPY and αIRS-1^{bac} immunoprecipitates. The amount of PtdIns 3′-kinase in αIRS-1 immunoprecipitates was 10-fold greater than the activity found in αPY immunoprecipitates, reflecting the greater ability of αIRS-1^{bac} to immunoprecipitate IRS-1, as seen by immunoblotting (Fig. 1). The ratios of αPY/αIRS-1^{bac} associated PtdIns 3′-kinase activity were nearly identical after stimulation by insulin or IGF-I, suggesting that the PtdIns 3′-kinase binds IRS-1 similarly after stimulation with IGF-I or insulin. In CHO cells, more PtdIns 3′-kinase activity associated with IRS-1 after IGF-I stimulation than with insulin stimulation (Fig. 4C), which reflects the lesser number of IRs and the lower incorporation of phosphate into IRS-1 in CHO cells after insulin treatment (Fig. 1). The amount of PtdIns 3′-kinase activity associated with IRS-1 from CHO/IR cells after insulin stimulation was greater than that after IGF-I stimulation, which reflected the higher phosphorylation of IRS-1 in cells overexpressing the IR (Fig. 4D). These data suggest that the stimulation of IRS-1 association with the PtdIns 3′-kinase after IGF-I or insulin stimulation is predominantly mediated by the IGF-Ir and IR, respectively.

The association of the PtdIns 3′-kinase with IRS-1 was reconstituted *in vitro* using IRS-1^{bac} that was phosphorylated by WGA-IR or WGA-IGF-Ir (Fig. 5). Phosphorylated IRS-1^{bac} was immobilized on αIRS-1 (αpep80) immunoprecipitates and incubated with lysates of quiescent CHO cells. No PtdIns 3′-kinase activity associated with the unphosphorylated IRS-1^{bac}; however, large amounts of activity associated with IRS-1^{bac} which had been phosphorylated by the IR or IGF-Ir (Fig. 5).

Overexpression of IRS-1 in CHO cells enhances tyrosine phosphorylation of IRS-1 and association of the PtdIns 3′-kinase with IRS-1 after stimulation with IGF-I

CHO cells overexpressing rIRS-1 (CHO/IRS-1) and control CHO cells (CHO/HIS) (12) were stimulated with IGF-I or insulin, and lysates were analyzed by immunoblotting with αIRS-1 (1M92–7) (Fig. 6A) or αPY antibodies (Fig. 6B). This analysis demonstrated that CHO/IRS-1 cells express at least 20-fold more IRS-1 than control CHO/HIS cells (Fig. 6A). The amount of tyrosine-phosphorylated IRS-1 was increased approximately 2-fold in CHO/IRS-1 cells (Fig. 6B, lanes d–f) compared to control CHO/HIS cells (Fig. 6B, lanes a–c) after growth factor stimulation. Thus, this overexpressed IRS-1 acts as a substrate for the IGF-Ir and the IR after stimulation of cells with growth factor. The amount of tyrosine-phosphorylated IRS-1 was greater after IGF-I stimulation than insulin stimulation (Fig. 6B, lanes e and f), reflecting the greater amount of IGF-Ir than IR in these cells. Thus, both the amount of IRS-1 and amount of receptor expressed are important determinants of IRS-1 tyrosine phosphorylation.

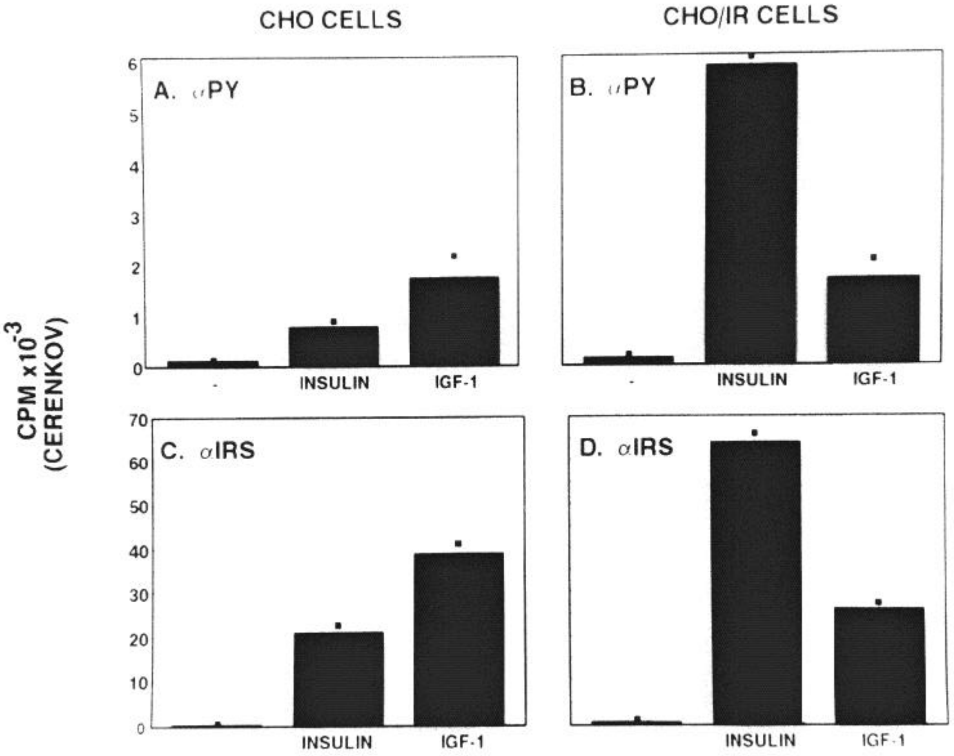

FIG. 4. Association of PtdIns 3′-kinase activity with αPY and αIRS-1 immunoprecipitates of CHO and CHO/IR cells after stimulation with IGF-I or insulin. CHO (A and C) or CHO/IR (B and D) cells were incubated in the absence or presence of insulin or IGF-I. αPY (A and B) or αIRS-1^{bac} (C and D) immunoprecipitates were prepared, and associated PtdIns 3′-kinase activity was assayed. Reaction products were quantitated by Cerenkov counting, and values representing the average of three independent determinations are shown ± SEM.

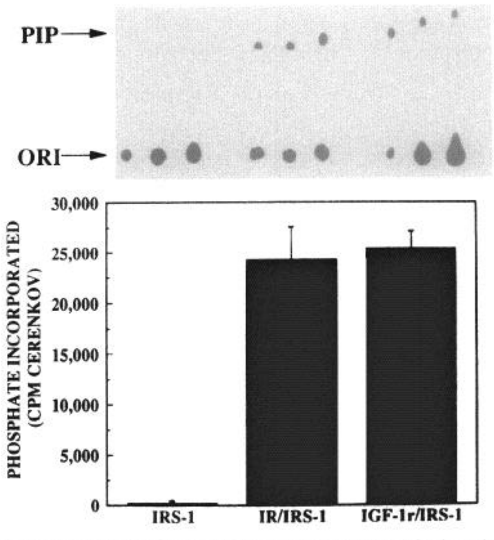

FIG. 5. Association of PtdIns 3′-kinase activity with *in vitro*-phosphorylated IRS-1^{bac}. IRS-1 was incubated alone (IRS-1) or in the presence of WGA-IR (IR/IRS-1) or WGA-IGF-Ir (IGF-Ir/IRS-1) and immunoprecipitated with αIRS-1 (αpep80) antibodies. These immunoprecipitates were washed and exposed to lysates of quiescent CHO cells for 30 min before being washed and assayed for associated PtdIns 3′-kinase activity. Reaction products were quantitated by Cerenkov counting, and values representing the average of three independent determinations are shown ± SEM.

The increased fold-stimulation of IRS-1 tyrosine phosphorylation in CHO/IRS-1 cells compared to CHO/HIS is significantly less than the increased fold-expression of IRS-1 in these cells, suggesting that the relatively few insulin and IGF-I receptors are inadequate to phosphorylate all of the overexpressed IRS-1 in the CHO/IRS-1 cells.

We assayed the amount of PtdIns 3′-kinase activity associated with IRS-1 in CHO/HIS and CHO/IRS-1 cells incubated in the absence or presence of 130 nM IGF-I (Fig. 7). The PtdIns 3′-kinase activity recovered in αIRS-1^{bac} immunoprecipitates was increased by overexpression of IRS-1. This is consistent with an increased amount of tyrosine-phosphorylated IRS-1 in CHO/IRS-1 cells.

Insulin stimulates the association of IRS-1 and the PtdIns 3′-kinase through specific binding of the SH2 domains in the p85 subunit of PtdIns 3′-kinase to tyrosine-phosphorylated motifs on IRS-1 (22, 23). The mechanism of PtdIns 3′-kinase binding to IRS-1 after IGF-I stimulation is likely the same, as IRS-1 was detected in αp85 immunoprecipitates by immunoblotting with αPY antibodies after stimulation of CHO cells with insulin, IGF-I, or both (Fig. 8, lanes b–d), and the receptors for insulin and IGF-I phosphorylate identical sites on IRS-1 *in vitro* (Fig. 3). More IRS-1 was associated with the p85 after stimulation with IGF-I than with insulin, as the levels of IGF-I receptor and IRS-1 tyrosine phosphorylation are higher (Fig. 1). Additional tyrosyl phosphoproteins were also detected in αp85 immunoprecipitates with αPY immunoblotting after insulin or IGF-I stimulation (Fig. 8). This suggests that the activation of the IR or the IGF-Ir stimulates the formation of a complex of proteins containing the PtdIns 3′-kinase, IRS-1, the insulin or IGF-I receptor (12), and other tyrosine-phosphorylated proteins, which may contribute to the full insulin/IGF-I response. Interestingly, the p85 does not appear to be tyrosine phosphorylated after stimulation with insulin or IGF-I, in agreement with previously reported data (22, 26).

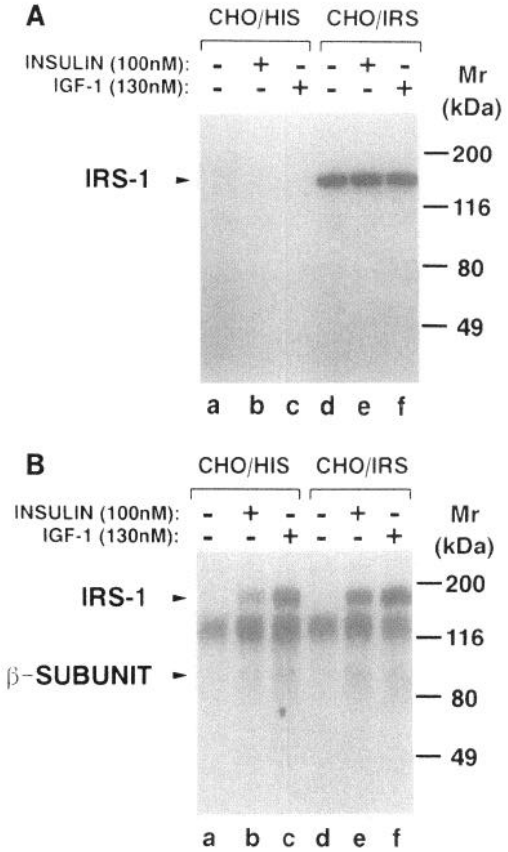

FIG. 6. Tyrosine phosphorylation of proteins in CHO/HIS and CHO/IRS-1 cells after stimulation with IGF-I or insulin. CHO/HIS (lanes a–c) or CHO/IRS-1 (lanes d–f) cells which had been incubated in the absence (lanes a and d) or presence of insulin (lanes b and e) or IGF-I (lanes c and f) for 1 min were lysed in Laemmli sample buffer. Lysates were resolved by SDS-PAGE, transfered to nitrocellulose membranes, and analyzed by immunoblotting with αIRS-1 (1M92–7) (A) or αPY antibodies and [^{125}I]protein A (B). Membranes were dried and exposed to autoradiography or phosphorimager screens. Positions of IRS-1, receptor β-subunits, and molecular mass standards are shown.

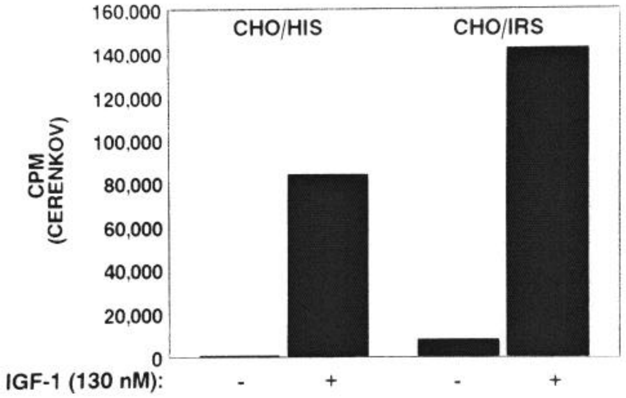

FIG. 7. Association of PtdIns 3′-kinase activity with IRS-1 in CHO/HIS and CHO/IRS-1 cells. CHO/HIS and CHO/IRS-1 cells were incubated in the absence (−) or presence (+) of 130 nM IGF-I. PtdIns 3′-kinase activity in αIRS-1^{bac} immunoprecipitates was assayed and quantitated (Cerenkov counting).

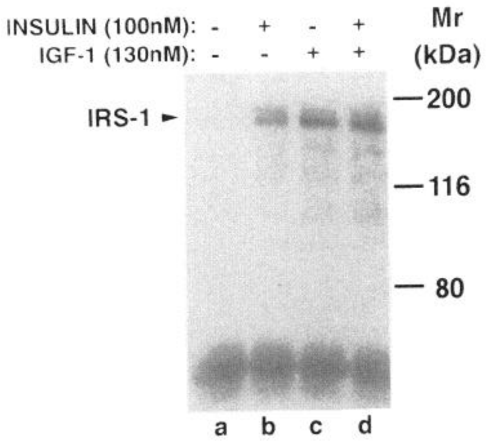

FIG. 8. IRS-1 is found in αp85 immunoprecipitates from CHO cells stimulated with IGF-I or insulin. Quiescent CHO cells (lane a) or CHO cells stimulated with insulin (lane b), IGF-I (lane c), or both (lane d) were lysed, and p85 was immunoprecipitated as for a PtdIns 3′-kinase assay. Immunoprecipitated proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblotting with αPY antibodies and [^{125}I]protein A. Membranes were dried and exposed to phosphorimager screens. Positions of IRS-1 and molecular mass standards are shown.

IGF-I activates the PtdIns 3′-kinase

The PtdIns 3′-kinase is activated by interacting with IRS-1 after insulin stimulation (22, 23). Stimulation of cells with growth factors was initially thought to cause the tyrosine phosphorylation and subsequent activation of the PtdIns 3′-kinase (16). However, we and others have been unable to detect tyrosine phosphorylation of p85 after stimulation with a variety of growth factors (Fig. 8) (12, 26). Both IGF-I and insulin stimulate the PtdIns 3′-kinase in αp85 immunoprecipitates (Fig. 9). The activity of the PtdIns 3′-kinase was stimulated approximately 2.5-fold in CHO cells after stimulation with insulin and 3.5-fold after stimulation with IGF-I alone or with both insulin and IGF-I. Thus, activation of the PtdIns 3′-kinase by insulin and IGF-I was not additive, suggesting that a similar mechanism was involved. Activation of the PtdIns 3′-kinase correlated with increased amounts of tyrosine-phosphorylated IRS-1 in CHO cells.

Discussion

The receptors for IGF-I and insulin possess a homologous tyrosine kinase that is required for cellular signaling (1, 3, 4). However, the common and distinct signaling pathways used by the insulin and IGF-I receptors have remained elusive. The discovery that pp185 is a substrate for the insulin and IGF-I receptors provided the first evidence for the existence of a common cellular substrate for these receptors (6–8). Recently, we cloned IRS-1 from the partial amino acid sequence of pp185 (9–11). IRS-1 is a substrate for the insulin receptor tyrosine kinase and acts as a docking protein which binds and regulates the activity of cellular enzymes such as the PtdIns 3′-kinase (10, 12, 22, 23). Our current study suggests that IRS-1 is a common element in signal transmission by both the insulin and IGF-I receptors.

IRS-1 is tyrosine phosphorylated in CHO cells after IGF-I

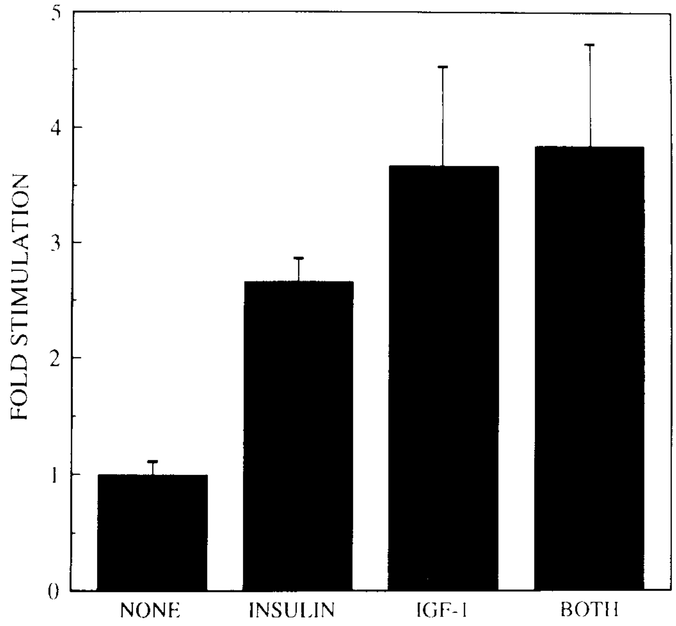

FIG. 9. The PtdIns 3′-kinase is activated in CHO cells after stimulation with insulin or IGF-I. αP85 immunoprecipitates were prepared from unstimulated CHO cells or CHO cells stimulated with insulin, IGF-I, or both, and assayed for associated PtdIns 3′-kinase activity. Reaction products were quantitated by Cerenkov counting, and values representing the average of three independent determinations are shown ± SEM.

and insulin stimulation. However, the phosphorylation is significantly stronger during IGF-I stimulation, reflecting the relatively greater number of IGF-I receptors in these cells; this pattern is reversed in CHO/IR cells which overexpress the human insulin receptor. Similarly, the activated IGF-I receptor tyrosine kinase phosphorylates recombinant IRS-1^{bac} protein *in vitro* on sites identical to those phosphorylated by the IR. As tyrosine-phosphorylated sites on IRS-1 create binding sites for certain cellular proteins containing SH2 domains (23), these results suggest that IRS-1 interacts with similar downstream elements after IGF-I or insulin stimulation. Indeed, insulin or IGF-I treatment of CHO cells recruit similar proteins into a complex or complexes including IRS-1, p85, and other unidentified phosphotyrosine-containing proteins, probably including the insulin or IGF-I receptor (12). A molecular complex containing several distinct components, including tyrosyl-phosphorylated IRS-1 at the core, may provide a molecular explanation for the pleotropic effects of insulin and IGF-I.

PtdIns 3′-kinase is a heterodimeric enzyme consisting of a 110-kDa catalytic subunit which is regulated by an 85-kDa subunit containing two SH2 domains and an SH3 domain (19–23, 33). IGF-I stimulates the association of the PtdIns 3′-kinase with IRS-1 in CHO cells. This association is greater in IGF-I-treated CHO cells than in insulin-treated CHO cells, which reflects the stronger phosphorylation of IRS-1 due to the higher level of IGF-I receptors than insulin receptors in these cells. Overexpression of IRS-1 in CHO cells also increases the amount of tyrosine-phosphorylated IRS-1 during IGF-I stimulation, suggesting that there is a direct kinase-substrate relationship between the IGF-Ir and IRS-1. This increase in tyrosine-phosphorylated IRS-1 correlates with the increase in PtdIns 3-kinase activity associated with IRS-1 after stimulation with IGF-I. Also, tyrosyl-phosphorylated IRS-1^{bac} binds to PtdIns 3′-kinase activity from extracts of quiescent CHO cells. Both insulin and IGF-I receptor-catalyzed phosphorylation of IRS-1 produce an identical result, suggesting that the mechanism of association between IRS-1 and the PtdIns 3′-kinase is similar after insulin or IGF-I stimulation. As PtdIns 3′-kinase binds to tyrosyl-phosphorylated sequences (YMXM motifs) in IRS-1 through the SH2 domains of the p85 subunit during insulin stimulation (22, 23), it is likely that this mechanism accounts for the association during IGF-I stimulation as well.

It has been reported that the PtdIns 3-kinase associates with the receptors for insulin and IGF-I after ligand stimulation (32, 34, 35). The association between IGF-Ir and the PtdIns 3′-kinase has been studied *in vitro* using tyrosine-phosphorylated, immunoprecipitated IGF-I receptors and fusion proteins containing the SH2 domains of p85 (35). Immobilized IGF-Ir binds PtdIns 3′-kinase activity from cell lysates. Furthermore, this *in vitro* association is blocked by incubation with SH2 domain fusion proteins from the PtdIns 3-kinase p85 subunit (35). We have observed similar results with the insulin receptor; however, the amount of PtdIns 3′-kinase associated with IRS-1 is much greater than with the insulin receptor *in vitro* as well as *in vivo* (10, 49). Furthermore, the receptors for insulin and IGF-I stably associate with IRS-1 after ligand binding and receptor activation in intact cells, suggesting that the PtdIns 3′-kinase associated with the IR and IGF-Ir *in vivo* may actually represent PtdIns 3′-kinase bound to receptor-associated IRS-1 (12). We have also observed the association of IRS-1 from cell lysates with the activated insulin receptor *in vitro* (49), suggesting that IRS-1 can account for receptor-associated PtdIns 3′-kinase activity *in vitro* as well as *in vivo*.

Treatment of cells with growth factors such as insulin and IGF-I increases the amount of PtdIns 3-phosphate [PtdIns(3)P], PtdIns(3,4)P_2, and PtdIns(3,4,5)P_3 in cells, suggesting that the PtdIns 3′-kinase is activated during activation of receptor tyrosine kinases (14, 16–18). This activation was originally thought to be secondary to tyrosine phosphorylation of the p85 subunit of the PtdIns 3′-kinase (16, 36–38), but tyrosine phosphorylation of the p85 is not detected after stimulation by a variety of growth factors (22, 26). We have observed activation of the PtdIns 3′-kinase in ap85 immunoprecipitates from insulin-stimulated cells (22); in these immunoprecipitates, p85 is not tyrosine phosphorylated but is associated with tyrosine-phosphorylated IRS-1. Furthermore, tyrosine-phosphorylated IRS-1 activates the PtdIns 3′-kinase in immunoprecipitates from unstimulated cells (22), an effect which appears to be mediated by the binding of tyrosine-phosphorylated IRS-1 to the SH2 domains of the PtdIns 3′-kinase (22, 23). Similarly, PtdIns 3′-kinase is activated in immunoprecipitates from IGF-I-treated CHO cells, even though p85 is not tyrosine phosphorylated. The presence of tyrosine-phosphorylated IRS-1 in αp85 immunoprecipitates suggests that the PtdIns 3′-kinase is similarly regulated after stimulation with insulin or IGF-I.

IRS-1 may bind several distinct SH2 domain-containing proteins in addition to p85, contributing to the transmission of the full insulin signal. SH2 domains are thought to mediate protein-protein interactions by binding to tyrosine-phosphorylated motifs; different isoforms of the SH2 domain found in different proteins are thought to mediate binding to distinct tyrosine-phosphorylated motifs. The platelet-derived growth factor (PDGF) receptor and similar receptors, such as the fibroblast growth factor receptor, bind to phospholipase C_γ and *ras*-GTPase activating protein (as well as the PtdIns 3′-kinase) after ligand-stimulated tyrosine phosphorylation (39–42). We have been unable to detect an association between either of these enzymes and IRS-1 (Myers, Jr., M. G., B. C. Cheatham, and M. F. White, unpublished observations). The tyrosine-phosphorylated motifs YLDL (in the fibroblast growth factor receptor) and YMAP (in the PDGF receptor) are thought to be the preferred binding sites for the SH2 domains of phospholipase C_γ and *ras*-GTPase activating protein, respectively (43, 44). Phosphorylation sites in these motifs are not found in IRS-1 (10); thus, it is not surprising that these molecules do not associate with IRS-1. In contrast, IRS-1 contains 15 potential sites of tyrosine phosphorylation. In addition to the consensus PtdIns 3′-kinase binding sites in YMXM and YXXM motifs, IRS-1 also contains consensus tyrosine phosphorylation sites in the motifs YQAL, YDTG, YVNI, and YPEE (10). Phosphorylation of these or other sites could create binding sites on IRS-1 for proteins containing distinct isoforms of the SH2 domain, contributing to the full insulin/IGF-I signal.

Insulin and IGF-I perform different functions in the intact organism, and studies with chimeric receptors have shown that the IR and IGF-Ir β-subunits have different signaling functions in cultured cells (45). We have not observed differences in signaling by the IGF-Ir *vs.* the insulin receptor at the level of IRS-1. It is therefore likely that differences in signaling by the IR and the IGF-Ir are not mediated by IRS-1 but are mediated by other cellular proteins. As IRS-1 appears to be expressed in most tissues (Araki, E., X. J. Sun, M. F. White, submitted), the divergence between insulin and IGF-I actions may arise through differential expression of the receptors for insulin and IGF-I and through unique downstream elements which are selectively expressed in various tissues. For instance, although the PtdIns 3′-kinase appears to be ubiquitously expressed, other SH2 domain-containing signaling enzymes which associate with IRS-1 could be expressed in concert with either the insulin or IGF-I receptor. Also, the possibility that IRS-1 is differentially phosphorylated by the insulin and IGF-I receptors *in vivo* is not entirely ruled out by our *in vitro* observations. Thus, differential tyrosine phosphorylation of IRS-1 by these receptors could also account for the differences in signal transmission by these receptors observed in intact cells.

IRS-1 may not be the sole substrate and mediator of IGF-I/insulin receptor signaling; careful analysis of pp185 has shown that this band of protein likely represents several proteins of similar molecular weights (11). The lower portion of this band is IRS-1, whereas the upper portion is a distinct substrate for the insulin (and possibly IGF-I) receptors, which does not associate with the PtdIns 3′-kinase (11). Thus, tyrosine kinase substrate signaling pathways divergent from IRS-1 may exist downstream of the insulin and IGF-I receptors and could account for the differences in insulin and IGF-I signaling.

Our data show that IRS-1 is a substrate and mediator for both the IGF-I and insulin receptor systems, which are highly homologous. Similarly, IRS-1 may be involved in signaling by other systems. Likely tyrosine kinases include the Insulin receptor-related receptor, *ros*, and *trk*, all of which possess degrees of homology to the receptors for insulin and IGF-I (46–48). IRS-1 may also mediate signaling by some less-related systems, although it does not function downstream of the PDGFr (Myers, Jr., M. G., B. Cheatham, and M. F. White, in preparation). Thus, IRS-1 may act to distinguish between the signals generated by different classes of tyrosine kinases. For the insulin and IGF-I receptors, IRS-1 acts similarly as a docking protein which binds and regulates the PtdIns 3′-kinase and potentially other SH2 domain-containing molecules. IRS-1 also appears to be a common element controlling cell proliferation in the IR and IGF-Ir signaling cascades.

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