Insulin Stimulates Tyrosine Phosphorylation of Multiple High Molecular Weight Substrates in Fao Hepatoma Cells†

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ABSTRACT: Insulin rapidly stimulates tyrosine phosphorylation of cellular proteins which migrate between 165 and 190 kDa during SDS–PAGE. These proteins, collectively called pp185, were originally found in anti-phosphotyrosine antibody (αPY) immunoprecipitates from insulin-stimulated Fao rat hepatoma cells. Recently, we purified and cloned IRS-1, one of the phosphoproteins that binds to αPY and migrates near 180 kDa following insulin stimulation of rat liver [Sun, X. J., et al. (1991) *Nature 352*, 73–77]. IRS-1 and pp185 undergo tyrosine phosphorylation immediately after insulin stimulation and show an insulin dose response similar to that of insulin receptor autophosphorylation. However, IRS-1 was consistently 10 kDa smaller than the apparent molecular mass of pp185. The pp185 contained some immunoblottable IRS-1; however, cell lysates depleted of IRS-1 with anti-IRS-1 antibody still contained the high molecular weight forms of pp185 (HMW-pp185). Furthermore, the tryptic phosphopeptide map of IRS-1 was distinct from that of HMW-pp185, suggesting that at least two substrates migrate in this region during SDS–PAGE. Moreover, the phosphatidylinositol 3′-kinase and its 85-kDa associated protein (p85) bound to IRS-1 in Fao cells, but weakly or not at all to HMW-pp185. Our results show that Fao cells contain at least two insulin receptor substrates, IRS-1 and HMW-pp185, which may play unique roles in insulin signal transmission.

The molecular link between the insulin receptor and cellular enzymes has been difficult to establish. As the insulin receptor is a protein tyrosine kinase that is stimulated during insulin binding, the initial step in signal transmission may involve tyrosine phosphorylation of cellular proteins (Rosen, 1987; Kahn & White, 1988). Furthermore, cell lines expressing kinase-deficient insulin receptors are biologically inactive, supporting this hypothesis (Chou et al., 1987; McClain et al., 1987). Three potential substrates for the insulin receptor were discovered during the past several years including pp15, pp120, and pp185 (Rees-Jones & Taylor, 1985; Bernier et al., 1987; White et al., 1985). The identities of pp15 and pp120 were soon established by comparison with known proteins: pp15 was shown to be the adipocyte fat-binding protein 422-(aP2), and pp120 was identified as an ecto-ATPase in the bile cannicular portion of the hepatocyte plasma membrane (Hresko et al., 1988; Margolis et al., 1988). However, the role that these proteins play in insulin signal transmission is unknown.

Immunoprecipitation of pp185 with anti-phosphotyrosine antibodies (αPY) provided the first evidence that the insulin receptor has cellular substrates (White et al., 1985). The pp185 was originally found in Fao hepatoma cells as a broad band during sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)[1] with an average molecular mass of 185 kDa; it was subsequently identified in many insulin-stimulated cells and tissues between 160 and 190 kDa, suggesting that it may undergo variable phosphorylation or be a mixture of several different phosphoproteins (Gibbs et al., 1986; Maegawa et al., 1988; White et al., 1987; Kadowaki et al., 1987; Shemer et al., 1987; Momomura et al., 1988; Tobe et al., 1990). The pp185 is immediately phosphorylated after insulin stimulation, suggesting that it may play a role in the initial steps of postreceptor insulin signal transduction. On the basis of work with mutant insulin receptors, the tyrosine phosphorylation of pp185 correlates with some of insulin's actions (White et al., 1988; Wilden et al., 1990; Thies et al., 1989; Backer et al., 1992a).

Recently, one of the phosphotyrosine-containing proteins that migrates near 180 kDa during SDS–PAGE was purified from insulin-stimulated rat liver by affinity chromatography on immobilized αPY (Rothenberg et al., 1991). The corresponding cDNA, isolated from rat liver cDNA libraries, encodes a phosphoprotein called IRS-1. IRS-1 has a calculated molecular mass of 131 kDa but migrates between 165 and 180 kDa during SDS–PAGE (Sun et al., 1991). IRS-1 shows no overall amino acid sequence identity with currently known proteins. It contains a potential ATP-binding site, over 30 potential sites of serine and threonine phosphorylation, and at least 10 putative tyrosine phosphorylation sites several of which reside in YMXM (TyrMetXaaMet) motifs (Sun et al., 1991). During insulin stimulation, IRS-1 associates with the phosphatidylinositol 3′-kinase in CHO cells (Sun et al., 1991). Therefore, IRS-1 may act as a "docking protein" that provides a molecular link between the insulin receptor and insulin-

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[1] Abbreviations: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; TPCK-trypsin, L-1-(tosylamino)-2-phenylethyl chloromethyl ketone-treated trypsin; EDTA, disodium ethylenediaminetetraacetate; PVP-40, poly(vinylpyrrolidone), average M_r 40 000.

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sensitive enzymes involved in the regulation of cellular growth and metabolism (Sun et al., 1991).

In this study, we used Fao hepatoma cells to determine whether pp185 is composed entirely of IRS-1 or also contains structurally and functionally unique insulin receptor substrates. Our results demonstrate that IRS-1 comigrated with pp185 from Fao cells. However, IRS-1 does not account for all of the phosphotyrosine-containing proteins which migrate in the pp185 band. The high molecular weight portion of pp185 (HMW-pp185) is immunologically, structurally, and functionally distinct from IRS-1. IRS-1 is the major protein that associates with the phosphatidylinositol 3'-kinase during insulin stimulation, whereas HMW-pp185 does not. Therefore, Fao hepatoma cells contain at least two and possibly more insulin receptor substrates with similar apparent molecular weight, which may mediate distinct signaling pathways in these cells.

MATERIALS AND METHODS

Cell Culture. The experiments were performed with a well-differentiated and insulin-sensitive hepatoma cell line (Fao) which possesses about 100 000 insulin receptors per cell (Crettaz & Kahn, 1984; Deschatrette et al., 1979). Fao cells were grown to confluence in 15-cm plastic tissue culture dishes (Nunclon or Costar) containing 20 mL of RPMI 1640 medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO). The cells were incubated at 37 °C in a humidified atmosphere composed of 5% CO_2 and 95% air. Twelve hours before each experiment, the medium was changed to serum-free RPMI 1640.

Preparation of Antibodies. Anti-phosphotyrosine polyclonal antibodies (αPY) were prepared as previously described (Pang et al., 1985; White & Backer, 1991). Polyclonal antibodies against a peptide from IRS-1 (Pep-80) were prepared in rabbits as described previously (Perlman et al., 1989). Pep-80 has the amino acid sequence Y-I-P-G-A-T-M-G-T-S-P-A-L-T-G-D-E-A-R, which was originally identified in the tryptic digest of the purified pp185 band from insulin-stimulated rat liver (Rothenberg et al., 1991) and later identified in the deduced amino acid sequence of the IRS-1 cDNA (Sun et al., 1991). A purified synthetic Pep-80 was prepared by The Protein/Peptide Core Facility at the Joslin Diabetes Center. Pep-80 was coupled to Keyhole limpet hemocyanin with bromoacetyl bromide, and the resulting conjugates were injected into female New Zealand White rabbits (White, 1990). The resulting antibody, αPep80, was purified from the sera by affinity chromatography on immobilized Pep-80. The affinity column was prepared by coupling the synthetic Pep-80 to Affi-Prep-10 in dimethyl sulfoxide according to the manufacturer's instructions (Bio-Rad). The IgG was eluted with 100 mM glycine, pH 2.9, and neutralized immediately with HEPES, pH 7.5. Polyclonal antibodies against IRS-1 expressed in Sf9 cells infected with a recombinant baculovirus containing the cDNA of IRS-1 (αbIRS-1) were prepared in female New Zealand White rabbits, and the serum was purified for use by affinity chromatography on protein A–Sepharose (M. G. Myers, Jr., M. F. White, et al., unpublished results). Antibodies against the 85-kDa subunit (p85) of the phosphatidylinositol 3'-kinase were purchased from UBI.

[^{32}P]Phosphate Labeling of Fao Cells. Confluent monolayer of Fao cells were labeled for 2 h in phosphate- and serum-free RPMI 1640 medium containing 0.5 mCi/mL [^{32}P]-orthophosphate (NEN) at 37 °C (White et al., 1987). Then the cells were incubated without or with 100 nM porcine insulin (Elanco) for 1 min at 37 °C. The incubations were stopped quickly by removing the medium and freezing the cell monolayers with liquid nitrogen. Cells were homogenized immediately with 2 mL of 50 mM HEPES (pH 7.5) containing 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 4 mM EDTA, 2 mM sodium vanadate, 1% Triton X-100, 1 mg/mL aprotinin (Sigma), and 2 mM phenylmethanesulfonyl fluoride (Sigma). The monolayers were scraped from the dishes, and the insoluble material was sedimented by centrifugation at 50 000 rpm in a Beckman 70.1 Ti rotor for 60 min at 4 °C. The supernatant was used for immunoprecipitation.

Immunoprecipitation from Fao Cell Extracts. For immunoprecipitation, cell extracts (1–2 mL) were incubated with 3 μg of either αPY or αPep80, or with 10 μg of αbIRS-1 overnight at 4 °C. The antibody complexes were precipitated with 100 μL of 10% Pansorbin (Kasuga et al., 1985) during 2-h incubation at 4 °C, and the precipitates were washed 3 times with 50 mM HEPES containing 150 mM NaCl, 100 mM NaF, 1% Triton X-100, and 0.1% SDS (White, 1990). Immunoprecipitated proteins were solubilized in Laemmli sample buffer, reduced with 100 mM dithiothreitol (Sigma), and separated by SDS–PAGE. The labeled proteins were identified by autoradiography at −70 °C of the corresponding electroblot or of the stained and dried gels using Kodak X-OMAT films. Molecular weights of proteins were calculated with standard proteins (Bio-Rad). The radioactivity in the protein bands was quantified by Cerenkov counting or scanning densitometry (Fast Scan, Molecular Dynamics, using Image Quant software).

Immunoblotting from Fao Cell Extracts. Immunoblotting was carried out after separation of immunoprecipitates by SDS–PAGE and electrotransfer onto nitrocellulose (0.45 μm, Schleicher & Schuell) for 1 h at 100 V. In order to increase the transfer of high molecular weight proteins, 0.02% SDS was included in the transfer buffer described by Towbin et al. (1979). Nonspecific antibody binding to the nitrocellulose was prevented by incubating the filter overnight at 4 °C with block solution (3% bovine serum albumin, 10 mM Tris, pH 7.4, 150 mM NaCl, and 0.01% Tween 20). The blot was incubated with the indicated antibody at dilution 1:100 (3 μg/mL) in blocking buffer for 2 h at 22 °C, and subsequently washed 4 times, for 10 min each, in a washing solution containing 10 mM Tris, pH 7.4, 150 mM NaCl, and 0.01% Tween 20. Then the nitrocellulose was incubated with 2 μCi of ^{125}I-labeled protein A (38.7 mCi/mg, ICN Biochemicals) in 10 mL of blocking solution for 1 h at 22 °C. The membrane was washed again, air-dried, and autoradiographed. Band intensities were quantified by densitometry.

HPLC Separation of the Tryptic Phosphopeptides. [^{32}P]-Phosphate-labeled proteins transferred to nitrocellulose were prepared for trypsin digestion as previously described (Aebersold, 1989). The nitrocellulose strips corresponding to the pp185, IRS-1, and the β-subunit were incubated with 0.5% PVP-40 (Sigma) in 100 mM acetic acid for 30 min at 37 °C to prevent adsorption of the protease to the nitrocellulose during digestion. Excess PVP-40 was removed by extensive washing with water, and the nitrocellulose strips were then cut into small pieces and incubated with 100 μg of TPCK-trypsin (Worthington) in 100 mM $NaHCO_3$ (pH 8.2)/acetonitrile, 95:5 (v/v), at 37 °C for 6 h. An additional 100 μg of trypsin was added, and the digestion was continued for 12 h (Shemer et al., 1987). The phosphopeptides eluted from the nitrocellulose were separated with a Waters high-performance liquid chromatography system equipped with a narrow-bore (2.1-mm i.d.) C_{18} column (VYDAC, 218TP52). Phosphopeptides acidified with 0.05% trifluoroacetic acid were applied to the column, which was washed at flow rate of 0.3 mL/min with

0.05% trifluoroacetic acid and 5% acetonitrile, and eluted with an acetonitrile gradient increasing linearly to 50% during 200 min. Fractions were collected at 1-min intervals, and the radioactivity in each tube was measured as Cerenkov radiation using a Beckman scintillation counter. All of the radioactivity in the trypsin digest was routinely recovered from the reverse-phase HPLC column.

Phosphoamino Acid Analysis. [^{32}P]Phosphate-labeled pp185 and IRS-1 from control and insulin-stimulated Fao cells were separated by SDS–PAGE. Phosphoproteins were eluted from the gel fragments by trypsin digestion and were hydrolyzed in 6 N HCl for 2 h at 110 °C as previously described (Haring et al., 1984). The phosphoamino acids from the whole pp185 and IRS-1 were separated by high-voltage electrophoresis on thin-layer plates (250 mm; Avicel, Analtech) saturated with pyridine/acetic acid/H_2O (1:10:89 v/v). Phosphoamino acid standards (Sigma) were visualized by reaction with ninhidrin, and the [^{32}P]amino acids were analyzed by a phosphoimager (Molecular Dynamics).

Assay of Phosphatidylinositol 3′-Kinase. In vitro phosphorylation of phosphatidylinositol was measured in αPY and αPep80 immunoprecipitates as previously described (Ruderman et al., 1990; Backer et al., 1992a). Subconfluent Fao cells grown in 100-mm dishes were made quiescent by an overnight incubation in RPMI 1640 medium containing 0.5% BSA. The cells were then incubated in the absence or presence of 100 nM insulin for 10 min, extracted, and incubated with antibody, and immunocomplexes were precipitated with protein A–Sepharose (Pharmacia). After extensive washing (Ruderman et al., 1990), the phosphorylation of phosphatidylinositol (2 μg/μL) (Avanti) was started by the addition of 5 μL of 440 μM ATP containing 30 μCi of [^{32}P]ATP (NEN). After 10 min at 22 °C, the reaction was stopped, and the phospholipids were separated on silica gel thin-layer chromatography plates (Merck) developed in $CHCl_3$/CH_3OH/H_2O/NH_4OH (60:47:11.3:2). The radioactivity that comigrated with phosphatidylinositol 4-phosphate standard (Sigma) was measured by Cerenkov counting, as previously described (Ruderman et al., 1990; Backer et al., 1992a).

RESULTS

Insulin Stimulates Tyrosine Phosphorylation of IRS-1 and pp185 in Fao Cells. The phosphorylation of IRS-1 was studied in Fao cells by immunoprecipitation with αPep80 (an anti-IRS-1 antibody), and pp185 phosphorylation was detected by immunoprecipitation with αPY. Insulin-stimulated phosphorylation of IRS-1 was initially compared to that of pp185 in confluent [^{32}P]orthophosphate-labeled Fao hepatoma cells incubated without or with 100 nM insulin for 1 min. Equal portions of cell extracts were incubated with either αPY or αPep80, and immunoprecipitated proteins were separated by SDS–PAGE (Figure 1A). In the basal state, αPY immunoprecipitated two major [^{32}P]phosphoproteins of 75 and 120 kDa as previously described (White et al., 1985), whereas αPep80 immunoprecipitated IRS-1 which migrated at 165 kDa as previous shown in CHO cells (Sun et al., 1991) (Figure 1A, lanes a and c).

After insulin stimulation of [^{32}P]phosphate-labeled Fao cells, αPY immunoprecipitated two additional phosphotyrosine proteins previously identified as the β-subunit of the insulin receptor and pp185 (Figure 1A, lane b) (White et al., 1985, 1987). Insulin also stimulated the phosphorylation of IRS-1 in Fao cells, which was immunoprecipitated with αPep80 (Figure 1A, lane d). Compared to the basal state, the phosphorylation of IRS-1 increased by 20%, and it migrated

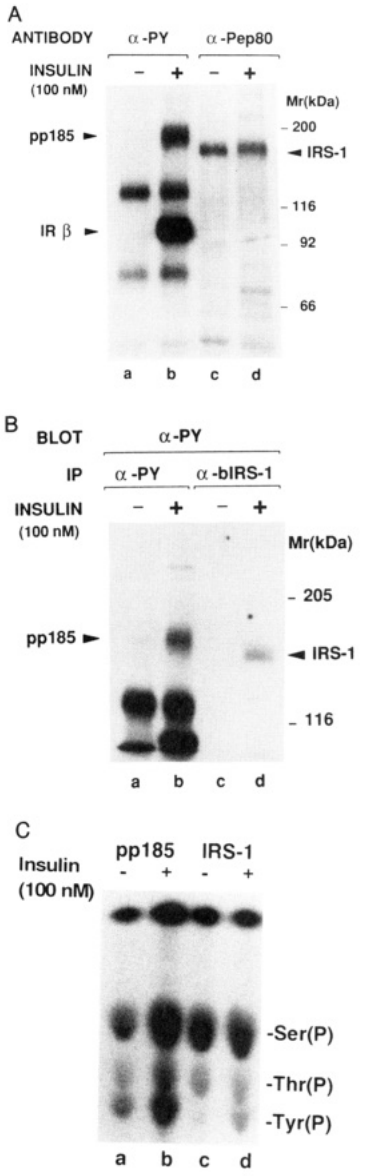

FIGURE 1: Insulin stimulates the phosphorylation of IRS-1 and pp185 in Fao cells. (Panel A) Fao cells were labeled for 2 h with [^{32}P]-orthophosphate and incubated without (–) or with 100 nM insulin (+) for 1 min. Each cell extract was divided equally and incubated either with αPY (lanes a and b) or with αPep80 (lanes c and d). Immunoprecipitated proteins were separated by SDS–PAGE on a 7.5% acrylamide gel and exposed to film for 2 h. (Panel B) Unlabeled Fao cells were incubated without (–) or with 100 nM insulin (+) for 1 min, and cell extracts were divided into two equal portions and incubated either with αPY (lanes a and b) or with αbIRS-1 (lanes c and d). Immunoprecipitated proteins (IP) were separated by SDS–PAGE on a 5% acrylamide gel, transferred to nitrocellulose, and immunoblotted (BLOT) with αPY/[^{125}I]protein A. (Panel C) The phosphoamino acid composition of pp185 (lanes a and b) and IRS-1 (lanes c and d) was determined as described under Materials and Methods.

at the slighter higher average molecular mass (175 versus 165 kDa) (Figure 1A, lane d). However, the apparent size of IRS-1 from insulin-stimulated Fao cells was about 10 kDa smaller than the average size of pp185 (185 kDa in these experiments).

Tyrosine phosphorylation of IRS-1 and pp185 was measured directly by Western analysis with αPY (Figure 1B). Fao cells were incubated without or with 100 nM insulin for 1 min, and proteins in equal portions of cell extracts were immunoprecipitated with either αPY (Figure 1B, lanes a and b) or αbIRS-1, a polyclonal antibody raised against recombinant

IRS-1 produced in baculovirus-infected Sf9 cells (Figure 1B, lanes c and d). As αbIRS-1 was raised against the entire IRS-1 protein, it is more likely than αPep80 to completely immunoprecipitate IRS-1. Therefore, we used αbIRS-1 to confirm that IRS-1 was immunologically distinct from the high molecular weight forms of pp185 (HMW-pp185). Immunoprecipitated proteins were separated by SDS–PAGE on 5% acrylamide gels to increase the separation in the 180-kDa region, transferred to nitrocellulose, and immunoblotted with αPY. In the absence of insulin, αPY immunoblots did not detect proteins in the αbIRS-1 immunoprecipitate (Figure 1B, lane c), whereas the 120-kDa constitutive phosphotyrosine-containing protein was detected in the αPY immunoprecipitate (Figure 1B, lane a). Consistent with the previous results, insulin stimulated tyrosine phosphorylation of pp185 and IRS-1 (Figure 1B, lanes b and d). On the basis of the intensity of the immunoblot, the content of Tyr(P) in IRS-1 was lower than that in pp185, and the apparent molecular mass of IRS-1 was clearly 10 kDa less than the average size of pp185. On the basis of the results of Figure 1A,B, IRS-1, recognized by two different antibodies (αPep80 and αbIRS-1), appears to compose the lower portion of the pp185 band. Thus, the high molecular weight portion of pp185 (HMW-pp185) was immunologically distinct from IRS-1.

Phosphoamino acid analysis was performed on pp185 and IRS-1 immunoprecipitated with αPY and αPep80, respectively, from control or insulin-stimulated ^{32}P-labeled Fao cells. Before insulin stimulation, IRS-1 contained predominantly Ser(P) with a small amount of Thr(P), and following insulin stimulation it became tyrosine-phosphorylated, and the phosphoserine/threonine levels did not change (Figure 1C, lanes c and d). In the basal state, pp185 immunoprecipitated with αPY from control cells revealed Ser(P), Thr(P), and a small amount of Tyr(P) (Figure 1C, lane a). Although a phosphotyrosine-containing protein is not detected in Figure 1A (lane a), longer exposure of the film revealed a faint band that can account for the amount of Ser(P), Tyr(P), and Thr(P) observed in the phosphoamino acid analysis. Insulin stimulation of Fao cells resulted in a significant increase of the Tyr(P), Ser(P), and Thr(P) content in the pp185 band (Figure 1C, lane b). Compared to the basal state, the Tyr(P), Ser(P), and Thr(P) levels of pp185 increased by 800%, 800%, and 600%, respectively, after insulin stimulation (Figure 1C, lanes a and b). Moreover, the insulin-stimulated phosphotyrosine content of IRS-1 was very low, representing only 10% of the insulin-stimulated Tyr(P) in pp185. These data suggest that pp185 may be composed of other insulin receptor substrates, as IRS-1 does not account for all the phosphotyrosine content of the pp185.

We carried out reciprocal immunoprecipitation/immunoblotting experiments to determine the relative amount of IRS-1 which was immunoprecipitated with αPY and comigrated in the pp185 band. An equal portion of Fao cell extract from control and insulin-stimulated cells was incubated with either αPY or αPep80, and the immunoprecipitated proteins were separated by SDS–PAGE and immunoblotted with αPep80 or αPY (Figure 2). Before insulin stimulation, αPep80 did not blot any proteins in the αPY immunoprecipitate (Figure 2, lane a), even though αPep80 strongly blotted IRS-1 in the αPep80 immunoprecipitate (Figure 2, lane c). After insulin stimulation, αPep80 weakly blotted IRS-1 in αPY immunoprecipitates (Figure 2, lane b), even though the αPY immunoprecipitate contained a strong insulin-stimulated pp185 signal by αPY blotting (Figure 2, lanes e and f). In contrast, IRS-1 immunoprecipitated from insulin-stimulated cells was

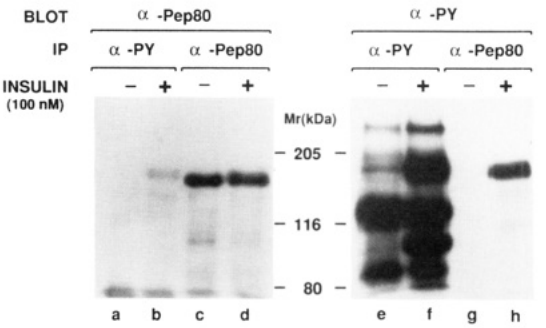

FIGURE 2: Detection of IRS-1 and pp185 by immunoblotting with αPY and αPep80. Fao cells were incubated without (–) or with 100 nM insulin (+) for 1 min. Each cell extract was divided into equal portions and immunoprecipitated either with αPY (lanes a, b, e, and f) or with αPep80 (lanes c, d, g, and h). Immunoprecipitated proteins (IP) were resolved by SDS–PAGE on 6% acrylamide gels, transferred to nitrocellulose, immunoblotted (BLOT) either with αPep80 (lanes a–d) or with αPY (lanes e–h), and detected with [^{125}I]protein A and autoradiography.

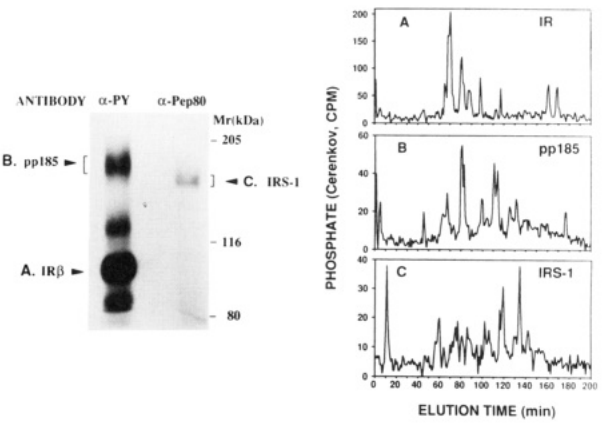

FIGURE 3: Separation of the tryptic phosphopeptides of the insulin receptor β-subunit (A), pp185 (B), and IRS-1 (C). Fao cells were labeled with [^{32}P]orthophosphate and stimulated with 100 nM insulin for 1 min. Cell extracts were divided into equal portions and incubated with αPY or αPep80. The phosphorylated proteins were resolved by SDS–PAGE on a 6% acrylamide gel and transferred to nitrocellulose. The bands corresponding to IRS-1 (C), HMW-pp185 (B), and the β-subunit of the insulin receptor (A) were excised and digested exhaustively with trypsin as described under Materials and Methods. The mixture of peptides corresponding to the insulin receptor β-subunit (panel A), HMW-pp185 (panel B), and IRS-1 (panel C) was separated by reverse-phase HPLC on a narrow-bore C18 column.

strongly blotted by both αPep80 and αPY, indicating that IRS-1 is recognized by both antibodies after insulin stimulation (Figure 2, lanes d and h). These results suggest that tyrosine-phosphorylated IRS-1 is poorly immunoprecipitated from Fao cell extracts by αPY and is a minor phosphotyrosine component of the pp185 band.

Finally, we compare the tryptic phosphopeptide map of IRS-1 to that of the pp185 to determine directly whether these proteins are distinct (Figure 3). The phosphoproteins were separated by SDS–PAGE and transferred to nitrocellulose, and the bands corresponding to IRS-1 and HMW-pp185 were cut out; to avoid including IRS-1 in HMW-pp185, the upper two-thirds of the pp185 band was excised as indicated (Figure 3). The phosphoproteins were digested with trypsin, and the phosphopeptides were separated by reverse-phase HPLC. The profiles obtained for IRS-1 and HMW-pp185 were distinct, and, as expected, both IRS-1 and HMW-pp185 were distinct from the β-subunit of the insulin receptor (Figure 3). Taken together, our results suggest that IRS-1 is one of the insulin receptor substrates that immunoprecipitates with αPY from insulin-stimulated cells but pp185 is composed

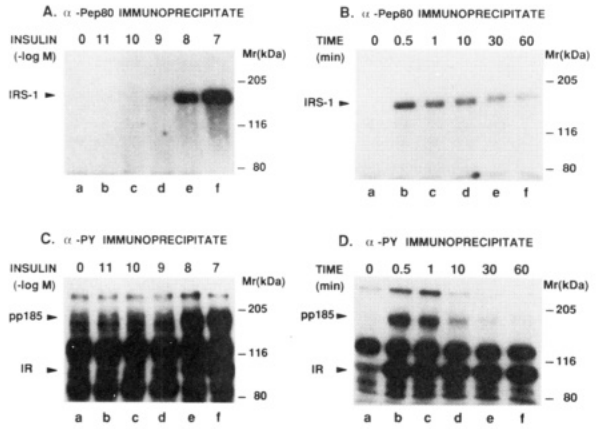

FIGURE 4: Insulin stimulates tyrosine phosphorylation of IRS-1 and pp185 in a time- and dose-dependent manner. Fao cells were incubated with various insulin concentrations for 1 min (panels A and C) or with 100 nM insulin for various time intervals (panels B and D). For each condition, the cell extracts were divided into equal portions, and incubated either with αPep80 (panels A and B) or with αPY (panels C and D). The immunoprecipitates were resolved by SDS–PAGE on 6% acrylamide gels, transferred to nitrocellulose, and incubated with αPY/[^{125}I]protein A, and the proteins were revealed by autoradiography.

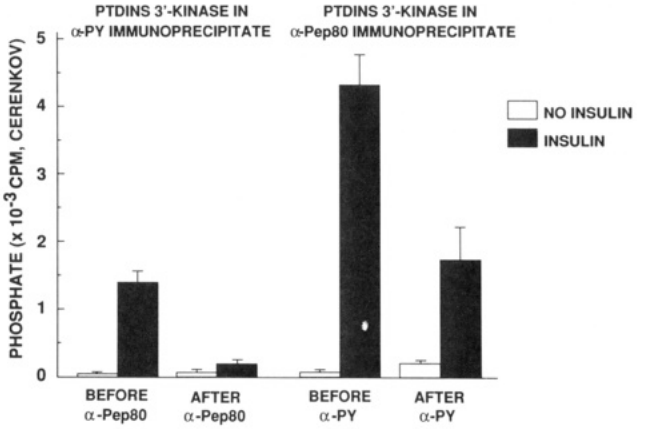

FIGURE 5: Immunoprecipitation of phosphatidylinositol 3′-kinase by αPY and αPep80. Fao cells were incubated in the absence or presence of 100 nM insulin for 10 min, solubilized, and incubated with αPY (left) or αPep80 (right). Then the supernatants from the αPY immunoprecipitates were treated with αPep80, whereas the supernatants from the αPep80 immunoprecipitates were treated with αPY antibody. PtdIns 3′-kinase in each protein A–Sepharose pellet was assayed. Each bar in the figure represents the mean ± SD of triplicate determinations. This figure represents the results of three separate experiments.

predominantly of a distinct substrate that migrates slightly higher than IRS-1 during SDS–PAGE (HMW-pp185).

Time Course and Insulin Dose Response of IRS-1 and pp185 Phosphorylation in Fao Cells. We measured the time course and dose response of IRS-1 and pp185 tyrosine phosphorylation by incubating Fao cells without or with 100 nM insulin for the indicated time intervals or for 1 min with different insulin concentrations (Figure 4). Each cell extract was divided equally and incubated with either αPY to immunoprecipitate pp185 (which also contained a small amount of IRS-1 from the results of Figure 2) or αPep80 to immunoprecipitate IRS-1. Immunoprecipitated proteins were separated by SDS–PAGE, transferred to nitrocellulose, and immunoblotted with αPY to detect the proteins that were phosphorylated on tyrosine residues. In the absence of insulin, αPY blotting did not detect any proteins in the αPep80 immunoprecipitates (Figure 4A, lane a; Figure 4B, lane a), whereas several constitutive phosphotyrosine-containing proteins were present in the αPY immunoprecipitates (Figure 4C, lane a; Figure 4D, lane a). With increasing insulin concentration, tyrosine phosphorylation of IRS-1 in aPep80 immunoprecipitates, and pp185 and the insulin receptor β-subunit in αPY immunoprecipitates increased in parallel, supporting the idea that pp185 and IRS-1 are substrates for tyrosine phosphorylation by the activated insulin receptor kinase (Figure 4A,C).

At 100 nM insulin, the insulin receptor β-subunit, pp185, and IRS-1 reached a maximum level of tyrosine phosphorylation after 30 s of insulin stimulation (Figure 4B, lane b; Figure 4D, lane b). However, the tyrosine phosphorylation of IRS-1 and pp185 decreased during 60 min of continued insulin exposure, whereas the phosphorylation of the β-subunit remained constant during this time interval (Figure 4B,D). After 10 min of insulin stimulation, the Tyr(P) content decreased by 50% for pp185 and by 20% for IRS-1. After 60 min, IRS-1 was still tyrosine-phosphorylated and migrated at a higher molecular mass (180 kDa), whereas pp185 was almost undetectable (Figure 4B, lane f; Figure 4D, lane f). These findings suggest that after long insulin treatment the patterns of tyrosine phosphorylation of pp185 and IRS-1 are distinct, in that pp185 appears to dephosphorylate more rapidly.

IRS-1 Associates with the Phosphatidylinositol (PtdIns) 3′-Kinase during Insulin Stimulation of Fao Cells. We demonstrated previously that PtdIns 3′-kinase activity associates with αPY and insulin receptor antibody following insulin stimulation of CHO cells (Ruderman et al., 1991; Backer et al., 1992a). Moreover, the PtdIns 3′-kinase is also strongly associated with IRS-1 in CHO cells (Sun et al., 1991; Backer et al., 1992a). As αPep80 immunoprecipitation from Fao cells separated IRS-1 from HMW-pp185, we used sequential immunodepletion to determine whether IRS-1, HMW-pp185, or the insulin receptor associated with the PtdIns 3′-kinase in Fao cells.

Before immunodepletion of IRS-1 from Fao cell extracts with αPep80, insulin-stimulated PtdIns 3′-kinase activity was detected in αPY immunoprecipitates as previously described (Ruderman et al., 1990; Endermann et al., 1990; Backer et al., 1992a); however, after removal of IRS-1 by two successive rounds of immunoprecipitation with αPep80, insulin-stimulated PtdIns 3′-kinase was significantly reduced in subsequent αPY immunoprecipitates (Figure 5). Immunoprecipitates of fresh Fao cell extracts with αPep80 contained the most insulin-stimulated PtdIns 3′-kinase activity; on average, it was 3-fold greater than αPY immunoprecipitates. Prior immunodepletion with αPY reduced by about 50% the amount of PtdIns 3′-kinase activity found in subsequent αPep80 immunoprecipitates; this decrease was almost equivalent to the PtdIns 3′-kinase immunoprecipitated by αPY from fresh cell extracts (Figure 5). Together, these results suggest that the PtdIns 3′-kinase binds predominantly to IRS-1 during insulin stimulation and insulin-stimulated PtdIns 3′-kinase detected by αPY is due to partial immunoprecipitation of the IRS-1/PtdIns 3′-kinase complex by αPY.

Similar results were obtained when αPep80 and αPY immunoprecipitates were immunoblotted with an antibody against the 85-kDa subunit of the PtdIns 3′-kinase (p85). Before insulin stimulation, p85 was not detected in αPY or αPep80 immunoprecipitates (Figure 6, lanes a, c, e, and g). The p85 was detected in αPY immunoprecipitates from insulin-stimulated cells (Figure 6, lane b); however, αPY did not immunoprecipitate p85 from insulin-stimulated extracts that did not contain IRS-1 due to immunodepletion by αPep80 (Figure 6, lane d). After insulin stimulation, immunopre-

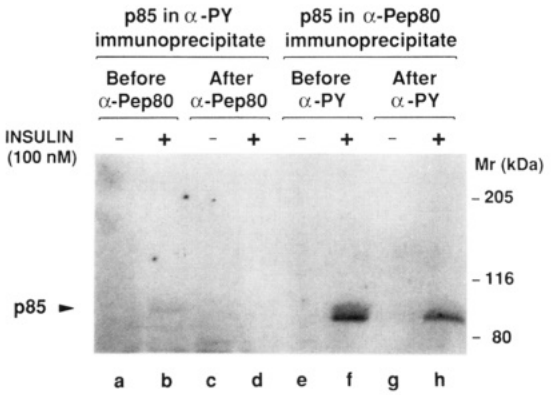

FIGURE 6: Immunoprecipitation by αPY and αPep80 of the 85-kDa subunit of the PtdIns 3′-kinase (p85). The experimental procedure was the same as described in Figure 5, but instead of enzymatic assay, the presence of the PtdIns 3′-kinase in the immunoprecipitate was determined by immunoblotting with αp85. Immunocomplexes were precipitated with protein A–Sepharose, separated by SDS–PAGE on a 6% acrylamide gel, transferred to nitrocellulose, and incubated with αp85 and then with [^{125}I]protein A for detection by autoradiography. The intensity of the p85 bands was quantified by densitometry: lane b, 178 units; lane f, 693 units; lane h, 485 units.

cipitation of p85 was 4-fold greater with αPep80 than with αPY (Figure 6, lanes b and f), and prior immunodepletion of the extracts with αPY reduced by 30% the recovery of p85 in αPep80 immunoprecipitates (Figure 6, lane h). Therefore, p85 was only detected in immunoprecipitates containing significant PtdIns 3′-kinase and appeared to associate preferentially with IRS-1.

To demonstrate that the β-subunit of the insulin receptor and the HMW-pp185 remained in αPY immunoprecipitates after removal of the PtdIns 3′-kinase and the associated p85 protein with αPep80, we carried out sequential immunodepletion experiments with ^{32}P-labeled Fao cells. Before insulin stimulation, pp185 was poorly detected with αPY (Figure 7A, lanes a and d), whereas IRS-1 was immunoprecipitated with αPep80 (Figure 7A, lanes b and c). After insulin stimulation, the HMW-pp185 and the insulin receptor β-subunit were strongly immunoprecipitated with αPY, both before (Figure 7A, lane e) and after immunodepletion with αPep80 (Figure 7A, lane h); however, the lower portion of the pp185 band (IRS-1) was removed by prior immunodepletion with αPep80. Scanning densitometry showed that the pp185 band is broader when the αPY immunoprecipitation is carried out before αPep80 immunodepletion (Figure 7B, compare lanes e and h). In contrast, IRS-1 immunoprecipitated with αPep80 resulted in a narrower peak that migrated slightly after that of pp185 (Figure 7B, compare lanes g and e); the intensity of the IRS-1 peak was reduced 50% by prior immunodepletion with αPY (Figure 7B, compare lanes g and f). This result is consistent with the partial removal of IRS-1 by αPY. Moreover, the difference profile between lanes e and h contains a peak that migrates with IRS-1, indicating that the IRS-1 was removed from αPY immunoprecipitates by prior immunodepletion with αPep80 [Figure 7B, Difference-(e–h)]. Therefore, PtdIns 3′-kinase and the associated p85 protein were recovered in immunoprecipitates which contained IRS-1, but not from IRS-1-depleted extracts containing only the β-subunit of the insulin receptor or HMW-pp185.

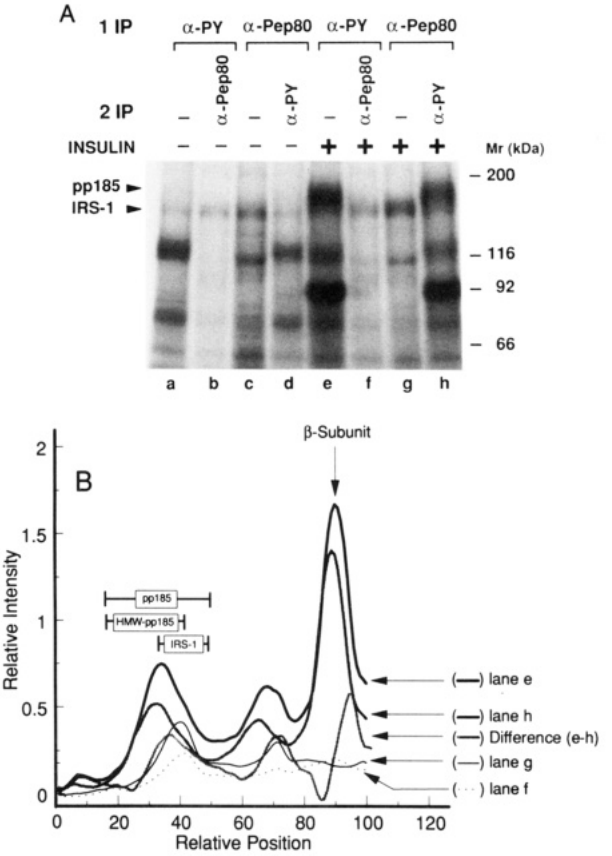

FIGURE 7: Sequential immunoprecipitation of pp185 and IRS-1 from [^{32}P]orthophosphate-labeled cells by αPY and αPep80. (Panel A) [^{32}P]Orthophosphate-labeled Fao cells were incubated in the absence (–) or in the presence (+) of 100 nM insulin for 1 min. Extracts from unstimulated and stimulated cells were divided into equal portions, and the phosphoproteins were initially immunoprecipitated (1 IP) overnight at 4 °C with either αPY (lanes a, b, e, and f) or αPep80 (lanes c, d, g, and h). Then a second immunoprecipitation (2 IP) was carried out: the supernatants of the αPY immunoprecipitates were treated with αPep80 (lanes b and f), and the supernatants from the αPep80 immunoprecipitates were treated with αPY (lanes d and h). Phosphoproteins were separated by SDS–PAGE on a 7.5% acrylamide gel. The autoradiogram represents an exposure of 3 h. 1 IP represents the first immunoprecipitation, and 2 IP represents the second immunoprecipitation. (Panel B) Graph that represents the relative intensity of the IRS-1, the pp185, and the β-subunit of the insulin receptor versus their relative positions in SDS–PAGE. This analysis was done by scanning densitometry of lanes e–h in the autoradiogram of panel A. The position of pp185 is indicated, as well as the positions of IRS-1 and HMW-pp185. The difference profile obtained by substracting lane e from lane h is shown [Difference (e–h)].

DISCUSSION

This study was undertaken to determine whether pp185 in Fao rat hepatoma cells is composed entirely of IRS-1, or whether pp185 is a mixture of several phosphotyrosine-containing insulin receptor substrates which migrate in the same region during SDS–PAGE. The cDNA of IRS-1 was isolated using oligonucleotide probes based on the partial amino acid sequence of purified rat liver pp185. Although pp185 purified from insulin-stimulated rat liver on immobilized αPY yields a relatively broad phosphoprotein band (Rothenberg et al., 1991), most of the tryptic peptides were located in the deduced amino acid sequence of IRS-1 (Sun et al., 1991). These results suggest that IRS-1 is the major constituent of rat liver pp185. However, the presence of two unidentified peptide sequences, albeit of low quality, in the purified pp185 is consistent with the existence of additional protein substrates. Furthermore, immunoprecipitation and immunoblotting revealed that IRS-1 may only represent the lower portion of the pp185 band in rat liver extracts (Rothenberg et al., 1991). The results of this report show that pp185 contains both IRS-1 and at least one other insulin receptor substrate (HMW-

pp185), which is immunologically, structurally, and functionally unique.

The pp185 was the first protein substrate for the insulin receptor found in many, and possibly all, insulin-stimulated cells and tissues (Gibbs et al., 1986; Maegawa et al., 1988; White et al., 1987; Kadowaki et al., 1987; Shemer et al., 1987; Momomura et al., 1988; Tobe et al., 1990). It was initially immunoprecipitated with anti-phosphotyrosine antibody (αPY) from insulin-stimulated Fao hepatoma cells, where it migrates during SDS–PAGE with an average molecular mass of 185 kDa (White et al., 1985). In other tissues and cells, its average molecular mass varies between 165 and 185 kDa, suggesting that pp185 may be a heterogeneous protein. The pp185 is not abundant, mainly cytoplasmic, and immunologically distinct from the insulin receptor (White et al., 1987). Partially purified pp185 is phosphorylated by the purified insulin receptor, suggesting that it is a direct substrate (Tashiro-Hashimoto et al., 1989). The pp185 is also phosphorylated during IGF-1 stimulation, but not during stimulation of cells with platelet-derived growth factor or the epidermal growth factor, suggesting that it is mainly related to signaling by insulin and related molecules (Izumi et al., 1987; Kadowaki et al., 1987). The phosphorylation of pp185 is barely detected in intact cells expressing few insulin receptors, strongly detected in cells expressing high levels of receptors, and decreased in cells expressing certain mutant receptors defective in biological response (White et al., 1987; Hofmann et al., 1989; Wilden et al., 1990; Backer et al., 1992a). Inactivation of the insulin receptor kinase by mutations in the ATP-binding site completely inhibits the autophosphorylation and kinase activity of the receptor, the phosphorylation of pp185, and the biological response (Chou et al., 1987).

IRS-1 is a unique hydrophilic protein containing several structural motifs which may provide clues regarding its role in insulin action. IRS-1 contains 8 potential nucleotide-binding motifs, a kinase-like ATP-binding site, over 30 serine/threonine phosphorylation sites, and possibly 14 tyrosine phosphorylation sites (Sun et al., 1991). However, IRS-1 contains only a few of the features expected for protein kinase catalytic domains (Sun et al., 1991; Hanks et al., 1990). Although insulin action is thought to be mediated through a cascade of protein phosphorylation and dephosphorylation, IRS-1 may not play a direct role in this mechanism as a protein kinase.

IRS-1 is expressed in Fao cells, and undergoes tyrosine phosphorylation and binds to αPY during insulin stimulation. However, the apparent molecular mass of IRS-1 after insulin stimulation is consistently 10 kDa lower than the average molecular mass of pp185. Immunoblotting with αPep80 demonstrates that IRS-1 composes only a small fraction of the pp185 band in αPY immunoprecipitated from insulin-stimulated Fao cells. Cell extracts contain the major high molecular weight portion of pp185 (HMW-pp185) even after immunodepletion of IRS-1. Moreover, tryptic peptide maps of IRS-1 and HMW-pp185 are distinct. Together, these results support the idea that pp185 is composed of at least two protein substrates, IRS-1 and HMW-pp185, which are immunologically and structurally distinct proteins.

The finding that IRS-1 is a relatively minor component of pp185 in Fao cells is surprising, especially as IRS-1 accounts for 9 out of 11 tryptic peptides derived from purified pp185 (Rothenberg et al., 1991). However, during insulin stimulation, IRS-1 contains a surprisingly small amount of phosphotyrosine relative to that of pp185. These results suggests that IRS-1 may contribute only slightly to the pool of phosphotyrosine, but significantly to the protein mass in the 180-kDa region. Consistent with this point, we found that IRS-1 was detectable in αPep80 immunoprecipitates from [^{35}S]methionine-labeled Fao cells whereas no labeled IRS-1 or pp185 was found in αPY immunoprecipitates (data not shown). Phosphorylation of only a few tyrosine residues in IRS-1 may not provide an adequate number of binding sites to allow efficient immunoprecipitation with αPY. Therefore, other substrates in the pp185 band may have a relatively higher stoichiometry of tyrosine phosphorylation than IRS-1.

Both IRS-1 and pp185 undergo tyrosine phosphorylation immediately after insulin stimulation, suggesting that they are involved in the initial response of cells to insulin. The phosphorylation of IRS-1 occurs rapidly at about the same rate and at the same concentrations of insulin as does the phosphorylation of the pp185 and the β-subunit of the insulin receptor. However, the tyrosine phosphorylation of pp185 decreases during prolonged insulin stimulation, until it is barely detectable by immunoblotting with αPY. In contrast, IRS-1 is dephosphorylated more slowly during 1 h of insulin stimulation, retaining about 50% of the initial phosphorylation after 30 min. These results suggest that insulin activates a phosphotyrosine phosphatase that dephosphorylates most of the components of the pp185 band including IRS-1; however, dephosphorylation of IRS-1 is slower possibly because it is a weaker substrate for the activated phosphatases, it is more rapidly phosphorylated by the insulin receptor, or some of its Tyr(P) residues are protected through association with cellular proteins like the PtdIns 3′-kinase.

Before insulin stimulation, IRS-1 immunoprecipitated from Fao cells migrates at 165 kDa during SDS–PAGE. This is 34 kDa larger than expected by the calculated molecular mass of 131 kDa (Sun et al., 1991). Dephosphorylated IRS-1 synthesized in a reticulocyte lysate migrates at 162 kDa during SDS–PAGE, suggesting that part of the dicrepancy is due to the anomalous behavior on SDS gels (Sun et al., 1992). However, immediately after insulin stimulation, the apparent molecular mass of IRS-1 increases to 170 kDa, which may be due to increased phosphorylation as shown for other proteins including the insulin receptor. Similar changes in the mobility of IRS-1 during SDS–PAGE have been demonstrated in CHO cell extracts (Sun et al., 1991). During 1 h of uninterrupted exposure to insulin, tyrosine-phosphorylated IRS-1 shifts gradually to an even higher molecular mass: 170 kDa after 1 min, and 173, 177, and 180 kDa after 10, 30, and 60 min of insulin stimulation, respectively. This result suggests that the overall phosphorylation of IRS-1 increases during prolonged insulin stimulation, even though the apparent phosphotyrosine content decreases. Thus, IRS-1 may also undergo a secondary insulin-stimulated serine/threonine phosphorylation, and these various forms of IRS-1 are recognized by the specific antibodies.

IRS-1 contains many potential phosphorylation sites. On the basis of typical motifs for cAMP-dependent protein kinase, protein kinase C, and casein kinase II, more than 30 putative Ser/Thr phosphorylation sites are distributed throughout the protein. We do not know which of the 34 tyrosine residues in IRS-1 are actually phosphorylated, although 14 have acidic residues N-terminal to the Tyr residue which categorizes them as conventional "consensus" sequences for tyrosine kinase recognition (Hunter & Cooper, 1986). Interestingly, six of these sites are within YMXM motifs, and three others are in YXXM motifs. Synthetic peptides corresponding to IRS-1 YMXM motifs are excellent substrates for the insulin receptor kinase (Shoelson et al., 1992). Synthetic peptides corresponding to these sites are phospho-

rylated by the insulin receptor more efficiently than previously studied peptides, with K_m values ranging from 24 to 92 μM and k_{cat}/K_m values between 6×10^5 and 2×10^4 M^{-1} s^{-1}.

Phosphorylated YMXM motifs form binding sites for certain proteins which contain SH2 (Src homology 2) domains (Koch et al., 1991; Cantley et al., 1991). The interaction between SH2 domains and Tyr(P) appears to play a regulatory role in some protein tyrosine kinases of the *src* family (Rousel et al., 1991). In addition, several signal transduction proteins also contain SH2 domains including the phosphoinositide-specific phospholipase C (PLCγ1), GTPase activating protein (ras-GAP), and the 85-kDa protein (p85) associated with the phosphatidylinositol 3′-kinase (PtdIns 3′-kinase) (Koch et al., 1991). These proteins have been shown to bind specifically to phosphotyrosine-containing proteins, probably through their SH2 domains (Koch et al., 1991). Moreover, the PtdIns 3′-kinase recognizes specifically phosphotyrosine in the YMXM motifs (Escobedo et al., 1991). Consistent with this model, the activated PtdIns 3′-kinase associates with IRS-1 in CHO cells (Sun et al., 1991), and this association activates the enzyme (Backer et al., 1992b). Thus, one function of IRS-1 may be to act as a multisite "docking" protein to link the insulin receptor kinase to specific cellular enzymes regulating cellular growth and metabolism (Sun et al., 1991).

We have previously shown that PtdIns 3′-kinase associates with proteins in αPY, αIR, and αIRS-1 immunoprecipitates from insulin-stimulated cells (Ruderman et al., 1990; Backer et al., 1992a). Thus, the PtdIns 3′-kinase may associate with IRS-1 and the insulin receptor, as well as other phosphoproteins in the αPY immunoprecipitates such as pp185. However, the immunodepletion experiments described in this report suggest that the PtdIns 3′-kinase associates mainly with IRS-1 after insulin stimulation of Fao cells. This conclusion is based on the finding that cell extracts from which IRS-1 was removed by αPep80 do not contain PtdIns 3′-kinase or p85 during subsequent immunoprecipitation with αPY. These results are consistent with the hypothesis that the PtdIns 3′-kinase associates after insulin stimulation with IRS-1 through the SH2 domains in the p85-associated protein. In contrast, pp185, from which IRS-1 was immunodepleted (HMW-pp185), does not bind a detectable amount of the PtdIns 3′-kinase, suggesting that HMW-pp185 is likely to be composed of proteins that are recognized as substrates by the insulin receptor, but after phosphorylation do not contain the appropriate phosphorylated YMXM motifs to allow high-affinity binding to the PtdIns 3′-kinase. The ability of αIR to immunoprecipitate the PtdIns 3′-kinase from CHO cells may be due to the presence of a YXXM motif in the carboxy terminus of the β-subunit, or to an association between the insulin receptor and IRS-1 (Sun et al., 1992). Thus, IRS-1 appears to be functionally distinct from other high molecular weight proteins that undergo tyrosine phosphorylation during insulin stimulation.

On the basis of our results, we conclude that IRS-1 migrates in the low molecular weight portion of most of the pp185 during SDS–PAGE but that most of the pp185 band is composed of HMW-pp185, a unique insulin receptor substrate. The HMW-pp185 has many different characteristics than IRS-1, such as an inability to react with anti-IRS-1 antibodies and weak or no binding to the PtdIns 3′-kinase. IRS-1 and HMW-pp185 may be isoforms of a family of insulin receptor substrates which comigrate as pp185. It is also possible that HMW-pp185 is a hyperphosphorylated form of IRS-1 that does not react with the antibody against IRS-1 (αPep80 or αbIRS-1). However, this conclusion is counterintuitive as hyperphosphorylated IRS-1 would also be expected to bind the PtdIns 3′-kinase. It is possible that hyperphosphorylation of tyrosine residues leads to the formation of a large molecular complex, which inhibits its immunoprecipitation by anti-IRS-1 antibodies; however, even after denaturing it in SDS–PAGE, αPep80 does not recognize the entire pp185 band. Together these results suggest that IRS-1 is one of the proteins that binds to αPY after insulin stimulation and migrates near 180 kDa but other high molecular weight substrates (HMW-pp185) also exist.

In summary, Fao hepatoma cells contain at least two cellular substrates for the insulin receptor which migrate around 180 kDa during SDS–PAGE. IRS-1 is the lower molecular weight protein that has been cloned from rat liver, and strongly associates to the PtdIns 3′-kinase. The higher molecular weight substrate, HMW-pp185, remains unidentified at the molecular level and may prove to be even more difficult to purify and clone than IRS-1. We are certain that rat liver contains IRS-1, as this was the source of the original purification and cloning; however, αPep80 appears to react with only a portion of the pp185 from rat liver extracts (Rothenberg et al., 1991; Sun et al., 1991). We are uncertain HMW-pp185 found in Fao hepatoma cells is also expressed in normal liver or other cells. Additional experiments including protein purification and cDNA cloning should provide additional information about HMW-pp185.

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Registry No. Insulin, 9004-10-8; tyrosine, 60-18-4; insulin receptor kinase, 88201-45-0; phosphatidylinositol 3′-kinase, 115926-52-8.