MOLECULAR AND CELLULAR BIOLOGY, Dec. 1993, p. 7418–7428
0270-7306/93/127418-11$02.00/0

Vol. 13, No. 12

Pleiotropic Insulin Signals Are Engaged by Multisite Phosphorylation of IRS-1

XIAO JIAN SUN,[1] DAN L. CRIMMINS,[2] MARTIN G. MYERS, JR.,[1] MONTSERRAT MIRALPEIX,[1] AND MORRIS F. WHITE[1]*

Research Division, Joslin Diabetes Center, and Department of Medicine and Program in Cellular and Development Biology, Harvard Medical School, Boston, Massachusetts 02215,[1] and Howard Hughes Medical Institute, Washington University School of Medicine, St. Louis, Missouri 63110[2]

Received 28 June 1993/Returned for modification 23 July 1993/Accepted 23 August 1993

IRS-1 (insulin receptor substrate 1) is a principal insulin receptor substrate that undergoes tyrosine phosphorylation during insulin stimulation. It contains over 20 potential tyrosine phosphorylation sites, and we suspect that multiple insulin signals are enabled when the activated insulin receptor kinase phosphorylates several of them. Tyrosine-phosphorylated IRS-1 binds specifically to various cellular proteins containing Src homology 2 (SH2) domains (SH2 proteins). We identified some of the tyrosine residues of IRS-1 that undergo insulin-stimulated phosphorylation by the purified insulin receptor and in intact cells during insulin stimulation. Automated sequencing and manual radiosequencing revealed the phosphorylation of tyrosine residues 460, 608, 628, 895, 939, 987, 1172, and 1222; additional sites remain to be identified. Immobilized SH2 domains from the 85-kDa regulatory subunit (p85α) of the phosphatidylinositol 3′-kinase bind preferentially to tryptic phosphopeptides containing Tyr(P)-608 and Tyr(P)-939. By contrast, the SH2 domain in GRB2 and the amino-terminal SH2 domain in SHPTP2 (Syp) specifically bind to Tyr(P)-895 and Tyr(P)-1172, respectively. These results confirm that p85α recognizes YMXM motifs and suggest that GRB2 prefers a phosphorylated YVNI motif, whereas SHPTP2 (Syp) binds to a phosphorylated YIDL motif. These results extend the notion that IRS-1 is a multisite docking protein that engages various downstream regulatory elements during insulin signal transmission.

IRS-1 (insulin receptor substrate 1) is a principal insulin receptor substrate that undergoes tyrosine phosphorylation during insulin stimulation. It was originally identified as a 185-kDa phosphoprotein in antiphosphotyrosine antibody (αPY) immunoprecipitates from insulin-stimulated Fao hepatoma cells (42). IRS-1 was subsequently purified and cloned from rat liver and found to be expressed in most cells and tissues (2, 28, 37). Several observations suggest that IRS-1 plays a central role in signal transduction by the insulin receptor. During differentiation of 3T3-L1 fibroblasts into insulin-sensitive adipocytes, IRS-1 expression increases coordinately with that of the insulin receptor and GLUT-4 (27). Overexpression of IRS-1 in Chinese hamster ovary (CHO) cells increases insulin-stimulated mitogenesis (36), and microinjection of recombinant IRS-1 into *Xenopus* oocytes significantly increases their sensitivity to insulin and IGF-1 (8). Moreover, a myeloid progenitor cell (32D) which contains few insulin receptors and no detectable IRS-1 is completely insensitive to insulin even after expression of the insulin receptor; however, expression of IRS-1 together with the insulin receptor restores a normal insulin mitogenic response (41). Thus, IRS-1 may be an essential element for certain insulin responses.

During ligand binding, insulin and other growth factor receptors undergo autophosphorylation on specific tyrosine residues (16). Most evidence suggests that the tyrosine kinase is essential for biological activity. In the case of the epidermal growth factor (EGF) and the platelet-derived growth factor (PDGF) receptors, the autophosphorylation sites bind directly to the Src homology 2 (SH2) domains in certain signaling proteins (SH2 proteins), and this appears to be essential for normal biological sensitivity (26). The selection of specific SH2 proteins depends on the amino acid sequence surrounding the tyrosine autophosphorylation site (6, 34, 39). Thus, the EGF and PDGF receptors directly bind the phosphatidylinositol (PI) 3′-kinase, p21^{ras}–GTPase-activating protein, phospholipase Cγ, and GRB2 through distinct autophosphorylation sites (1, 26, 34).

A variation of this model is employed by the insulin receptor. Insulin receptor autophosphorylation stimulates kinase activity, but unlike the case for EGF and PDGF receptors, this does not appear to induce a strong association with SH2 proteins. However, IRS-1 contains over 20 potential tyrosine phosphorylation sites (23, 37). Six of them are in YMXM motifs, three others are in YXXM motifs, and the remainder are found in various hydrophobic motifs (23). These sites play a dual role during insulin stimulation by linking the upstream tyrosine kinase to the downstream regulatory enzymes and adapter molecules that contain SH2 domains. Many synthetic peptides based on amino acid sequences in IRS-1 are efficient substrates for the insulin receptor, suggesting that multiple tyrosine-containing motifs in IRS-1 may be recognized by the catalytic domain of the insulin receptor (31). Phosphorylation of these Tyr residues enables a specific and high-affinity association between IRS-1 and SH2 domains in various signaling molecules. Thus, IRS-1 is in the middle of a two-step recognition process which establishes an upstream link to the insulin receptor kinase and multiple downstream links to various regulatory elements that contain recognizable SH2 domains.

We have only begun to identify the family of IRS-1-associated SH2 proteins. Phosphorylated IRS-1 associates with PI 3′-kinase, which is implicated in the control of cell growth and metabolism in mammals and protein sorting in yeast species (6, 30). In addition to the 110-kDa catalytic

* Corresponding author.

subunit, PI 3'-kinase is composed of an 85-kDa regulatory subunit (p85) that contains two SH2 domains which associate specifically to phosphorylated IRS-1 (3, 22). Occupancy of the SH2 domains with tyrosine-phosphorylated IRS-1 or phosphorylated YMXM peptides based on IRS-1 sequences activates PI 3'-kinase (3). Recently, several other SH2 proteins, including GRB2 (also known as SEM-5 or drk) (21), SHPTP2 (also known as Syp and phosphotyrosine phosphatase 1D) (13), and Nck (19), were found to associate with phosphorylated IRS-1. These regulatory proteins associate to IRS-1 or directly to other tyrosine-phosphorylated growth factor receptors through their SH2 domains (21).

In this report, some of the tyrosine phosphorylation sites in IRS-1 are identified and SH2 domains with which they interact are demonstrated. Phosphotyrosine residues were found in the rat IRS-1 sequence at positions 460, 608, 628, 895, 939, 987, 1172, and 1222. The amino-terminal SH2 (nSH2) domain of p85α binds mainly to Tyr-939 and Tyr-608 and weakly to Tyr-460, Tyr-895 and Tyr-987. In contrast, the SH2 domains of GRB2 and SHPTP2 bind preferentially to Tyr-895 and Tyr-1172, respectively. These results support the hypothesis that IRS-1 is a multisite docking protein that mediates various insulin responses through distinct phosphorylation sites.

MATERIALS AND METHODS

Preparation of recombinant IRS-1. Sf9 cells grown in TNM-FH medium (Sigma) supplemented with 10% fetal bovine serum were infected at high multiplicity with a recombinant baculovirus containing IRS-1 cDNA (3, 22, 36). After 50 to 54 h, the cells were harvested by low-speed centrifugation and lysed by 20 strokes in a Dounce homogenizer containing 50 mM Tris-HCl (pH 7.8), 1 M NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, 10 μg of aprotinin per ml, and 10 μg of leupeptin per ml. Insoluble material was removed by centrifugation at 100,000 × *g* for 1 h, and the supernatant containing IRS-1 protein ($IRS\text{-}1^{bac}$) was purified partially on SK 300 HR gel filtration medium (Pharmacia). The peak fraction typically contained approximately 0.5 mg of IRS-1 per ml with a purity of ~90%, as determined by scanning densitometry of Coomassie blue-stained polyacrylamide gels.

Preparation of GST-SH2 fusion proteins. The nSH2 domain of murine p85α (residues 321 to 440) was expressed as a glutathione *S*-transferase (GST) fusion protein by using the pGEX-2T vector (Pharmacia). The DNA fragment was synthesized by the polymerase chain reaction, using a mouse brain cDNA clone of p85α as a template and oligonucleotides that contain appropriate restriction sites bordering the domain of interest. The amplified DNA 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 vector ($pGEX\text{-}nSH2^{p85\alpha}$) was confirmed by sequencing. GST fusion proteins which contained the SH2 domain (residues 50 to 161) of GRB2 (provided by J. Schlessinger, New York University) or the nSH2 domain (residues 1 to 105) of SHPTP2 (provided by B. Neel, Harvard Medical School) were also expressed. Transformed cells were grown to an A_{600} of 0.6 in LB medium supplemented with 0.1 mg of ampicillin per ml and stimulated for 12 h with 1.0 mM isopropylthiogalactopyranoside (IPTG). Fusion proteins were isolated and purified by affinity chromatography on glutathione-Sepharose (Pharmacia) as described by Smith and Johnson (33). Glutathione was removed by dialysis against phosphate-buffered saline containing 10 mM dithiothreitol.

Phosphorylation and tryptic digestion of recombinant IRS-1. Insulin receptor (2 to 5 mg of total protein) purified from insulin receptor-expressing CHO (CHO/IR) cells on immobilized wheat germ agglutinin (Vector Laboratories) was activated by autophosphorylation at 22°C for 15 min in 50 mM HEPES (pH 7.4) containing 5 mM $MnCl_2$, 100 μM ATP, and 100 nM insulin. IRS-1 (500 μg) was then phosphorylated with the activated insulin receptor in 50 mM Tris-HCl (pH 7.8) containing 0.5 M NaCl, 5 mM $MnCl_2$, 100 μM ATP, and 1 mCi of [γ-^{32}P]ATP (3,000 Ci/mmol) for 1.5 h. [^{32}P]IRS-1 was denatured, reduced in Laemmli sample buffer containing 40 mM dithiothreitol at 100°C for 5 min, and carboxymethylated in 100 mM iodoacetamide at 22°C for 20 min. Next, it was separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (7.5% gel), transferred (4 h, 4°C, and 100 V) to nitrocellulose paper, localized by brief autoradiography, excised, and incubated with 0.5% PVP-40 in 0.1 M acetic acid at 37°C for 1 h. The nitrocellulose fragment was washed 10 times with H_2O, and the [^{32}P]IRS-1 was digested with 20 μg of sequencing-grade trypsin (Promega) in 50 mM $(NH_4)HCO_3$ containing 5% acetonitrile for 15 h at 37°C and then incubated for 10 h with 10 μg of additional trypsin.

Precipitation of IRS-1 from intact cells by GST-SH2 fusion proteins. Confluent monolayers of CHO cells expressing the human insulin receptor and rat IRS-1 (CHO/IR/IRS-1 cells) were grown in 15-cm dishes (Nunc) as previously described; in some experiments, the cells were labeled for 2 h at 37°C in 10 ml of 0.4 mCi of $^{32}P_i$ (NEN) per ml (36). The cells were triggered with 100 nM insulin for 1 min and then solubilized in 2 ml of 20 mM Tris-HCl (pH 7.5) containing 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 100 μM sodium orthovanadate, 0.34 mg of PMSF per ml, 100 μg of aprotinin per ml, 10% glycerol, and 1% Nonidet P-40 (Calbiochem) (36). Lysates were clarified by centrifugation at 100,000 × *g* for 1 h at 4°C. GST-SH2 fusion proteins (1 μg) were incubated with each lysate at 4°C for 30 min. The GST-SH2 fusion protein–IRS-1 complexes were precipitated from lysates on 20 μl of glutathione-Sepharose 4B (Pharmacia) at 4°C for 30 min. The precipitates were washed twice with binding buffer (50 mM Tris-HCl [pH 7.4], 100 mM NaCl, 250 μg of bovine serum albumin [BSA] per ml, 100 μg of aprotinin per ml, 1 mM vanadate, 1 mM PMSF) and boiled in Laemmli sample buffer containing 20 mM dithiothreitol. In some cases, 50 μg of $IRS\text{-}1^{bac}$ was added as a carrier. The denatured proteins were carboxymethylated with 50 mM iodoacetamide for 20 min at 22°C, separated by 7.5% SDS-PAGE (7.5% gel), and transferred to nitrocellulose (Schleicher & Schuell) at 100 V (constant) for 2 h at 5 to 15°C in a Bio-Rad Mini-Protean apparatus containing Towbin buffer supplemented with 0.02% SDS. [^{32}P]IRS-1 was identified directly by autoradiography, and unlabeled IRS-1 was detected by immunoblotting with an anti-IRS-1 antibody (αIRS-1) or αPY as previously described (36).

Analysis of phosphopeptides by association with GST-SH2 fusion proteins. Tryptic peptides obtained from IRS-1 labeled in vitro or in vivo were dissolved in 20 μl of 0.05% trifluoroacetic acid (TFA)–50% acetonitrile and clarified by centrifugation. A portion of the tryptic peptides was incubated at 22°C for 10 min with 25 to 2,500 nM various GST-SH2 fusion proteins or GST alone in 100 μl of binding buffer (50 mM Tris-HCl [pH 7.4], 100 mM NaCl, 250 μg of BSA per ml, 100 μg of aprotinin per ml, 1 mM vanadate, 1 mM PMSF). Glutathione-Sepharose 4B (20 μl, packed volume; Pharma-

cia) was added at 22°C, suspended for 20 min by continuous rotation, sedimented by centrifugation, and quickly washed at 22°C with 500 μl of binding buffer. The unbound ^{32}P-labeled phosphopeptides were collected from the supernatant, and the bound ^{32}P-labeled phosphopeptides were eluted from the glutathione-Sepharose 4B with two 100-μl portions of 0.5% TFA at 22°C for 20 min. The ^{32}P-labeled phosphopeptides in the supernatant and the pellet were analyzed by reverse-phase high-pressure liquid chromatography (HPLC).

Reverse-phase HPLC separation of tryptic phosphopeptides. The tryptic phosphopeptides derived from in vitro- and in vivo-^{32}P-labeled IRS-1 were separated in a Waters HPLC system equipped with a Hi-Pore RP-318 reverse-phase column (Bio-Rad) eluted at a flow of 0.5 ml/min with 0.055% TFA (solution A) modified with 75% acetonitrile–0.05% TFA (solution B). The mobile phase began with 5% solution B (5 min) and increased linearly to 25% solution B at 70 min, 35% solution B at 140 min, and 90% solution B at 145 min. The mobile phase remained at 90% for 10 min and then returned to 5% in 10 min. Peptides were detected at 214 nm, and ^{32}P-labeled phosphopeptides were detected with an on-line radioactivity detector (Radiomatic) or by measuring Cerenkov radiation in 0.2-ml fractions in a Beckman LS 1801 scintillation counter.

The phosphoamino acids in each peptide were identified as previously described (11). ^{32}P-labeled phosphopeptides purified from HPLC were hydrolyzed in 6 N HCl at 110°C for 120 min. The amino acid mixture was separated by thin-layer electrophoresis on microcellulose plates developed at 1,000 V (constant) in H_2O-acetic acid-pyridine (89:10:1). The phosphoamino acid standards (Sigma) were detected by reaction with ninhydrin, and the radioactive amino acids were detected by autoradiography.

Identification of phosphorylation sites in IRS-1. The phosphorylation sites in various tryptic phosphopeptides were determined by automated and manual radiosequencing as previously described (11, 35, 38). Manual radiosequencing was performed as described previously (35) except that the coupling and cleavage temperatures were 55°C. Automated sequence analysis of the immobilized radiolabeled sample was performed essentially as described previously (38). Prior to phenylthiohydantoin conversion, the membrane was incubated for 40 s with TFA (three times), and the effluent was collected in a fraction collector for radioactivity analysis.

Most peptide fractions recovered from the HPLC contained mixtures of peptides which required further purification before automated sequencing. Various approaches were used to isolate the interesting tryptic peptides containing the phosphorylation sites, including dephosphorylation with alkaline phosphatase, secondary digestion of the tryptic fragments with protease V8 (endoproteinase Glu-C from Boehringer Mannheim), endoproteinase Asp-N (Boehringer Mannheim), or chymotrypsin (Sigma), and affinity purification with GST-SH2 fusion proteins. All sequences obtained were consistent with the amino acid sequence of rat IRS-1 (37), and the first residue of each peptide was adjacent to a recognition site for trypsin, protease V8, or chymotrypsin as indicated.

The identification of Tyr-895 and Tyr-1172 phosphopeptides was straightforward because the HPLC fractions (C2 and C5, respectively) containing these peptides were relatively pure according to the HPLC profile at an optical density of 214 nm (OD_{214}). These sites were directly identified by automated sequencing, and the location of the phosphotyrosine residue was verified by manual radiosequencing.

Tyr-987 was identified by dephosphorylation of the original fraction (B3) and rechromatography under identical conditions to localize the newly dephosphorylated peptide. The HPLC fraction (B3) was dried in vacuo and incubated at 22°C for 10 min in 200 μl of 20 mM Tris-HCl (pH 8.0) containing 1 mM $MgCl_2$, 1 mM $ZnCl_2$, and 1 U of alkaline phosphatase (Boehringer Mannheim). During HPLC analysis, all of the [^{32}P]phosphate eluted at the breakthrough, indicating that it was removed from the peptide. Compared with the original HPLC profile at OD_{214}, a single new nonphosphopeptide eluted at a higher concentration of acetonitrile, whereas the contaminating nonphosphopeptides eluted at the same position in the gradient. This new peptide was isolated and subjected to automated sequencing. The Tyr-987 phosphopeptide in the original HPLC fraction (B3) was confirmed by manual radiosequencing and automated sequencing. Phosphorylation of Tyr-987 was also identified in HPLC fraction A3 by manual radiosequencing of the tryptic peptide A3 or A3 subdigested with endoproteinase Asp-N and protease V8.

Tyr-939 and Tyr-460 were identified by secondary digestion of the tryptic peptide fractions (B1 and B6, respectively) with protease V8 followed by HPLC separation of the products. Tryptic peptides were digested in 200 μl of 50 mM $(NH_4)HCO_3$ (pH 8.0) containing 10 μg of protease V8 for 24 h at 22°C prior to quenching the reaction with 20 μl of 10% TFA. After protease V8 treatment, the peptides shifted away from original position on the HPLC profile and eluted at lower concentrations of acetonitrile. The peptides were isolated and subjected to automated sequencing and manual radiosequencing.

The identification of Tyr-608 was especially difficult, as the original tryptic peptide (B5) was difficult to separate from peptide B4 and was unreactive during manual radiosequencing. Protease V8 fragment of B5 was also unreactive; however, chymotrypsin digestion produced a reactive peptide. ^{32}P-labeled IRS-1 tryptic peptides (500 μg) were incubated with 10 μg of GST-nSH2$^{p85\alpha}$ in 400 μl of binding buffer and precipitated on 50 μl of glutathione-Sepharose 4B. The ^{32}P-labeled phosphopeptide in the pellet was eluted with 200 μl of 0.5% TFA and separated on HPLC. The peptide migrated as B5 was collected, dried in vacuo, and dissolved in 10 μl of 0.05% TFA–50% acetonitrile and 200 μl of digestion buffer (80 mM Tris-HCl [pH 7.8], 100 mM $CaCl_2$) containing 20 μg of chymotrypsin to start the digestion. After 15 h at 22°C, 20 μl of 10% TFA was added to quench the reaction, and the products were separated by HPLC under the same condition. The chymotryptic ^{32}P-labeled peptides of B5 eluted from the reverse-phase column at an earlier position and were isolated for automated and manual radiosequencing.

Identification of Tyr-628 and Tyr-1222 was difficult because sufficient material was not available for automated sequencing as a result of losses during purification. However, the phosphorylation of Tyr-628 and Tyr-1222 in tryptic peaks A2 and B4, respectively, was deduced by manual radiosequencing of the tryptic peptides and their endoproteinase Asp-N and protease V8 subdigestion products.

RESULTS

Phosphorylation of recombinant IRS-1. IRS-1 is phosphorylated exclusively on tyrosine by the purified insulin receptor; in the intact cell, IRS-1 is phosphorylated on tyrosine,

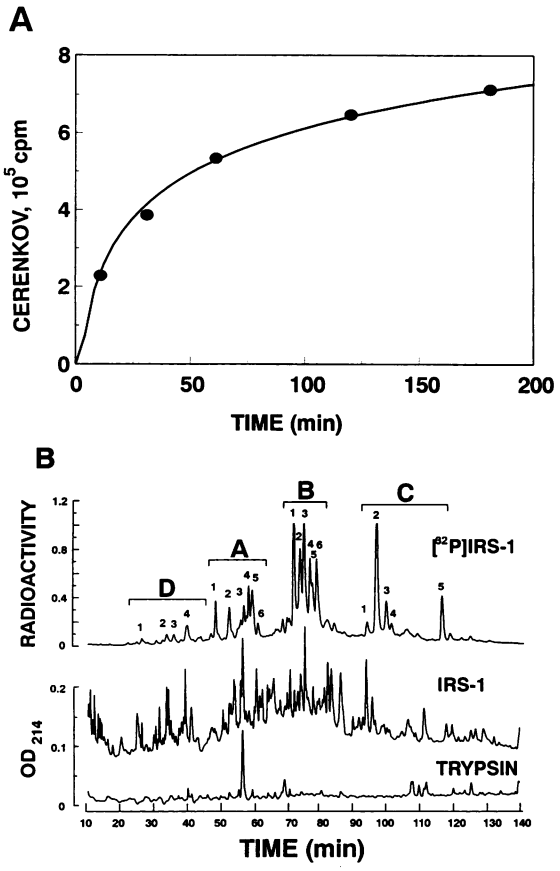

FIG. 1. HPLC analysis of tryptic phosphopeptides derived from in vitro-phosphorylated IRS-1^{bac}. (A) Recombinant IRS-1 was incubated with the activated wheat germ agglutinin-purified insulin receptor in the presence of [^{32}P]ATP and 5 mM $MnCl_2$ for the indicated time intervals. Phosphorylated IRS-1 was separated by SDS-PAGE, and radioactivity in the IRS-1 band was measured by Cerenkov counting. (B) Tryptic peptides were obtained from 500 μg of recombinant IRS-1 phosphorylated for 1.5 h with the activated wheat germ agglutinin-purified insulin receptor containing [^{32}P]ATP and 5 mM Mn^{2+}. The tryptic peptides were separated by reverse-phase HPLC and detected by A_{214}; ^{32}P-labeled peptides were identified by an on-line radiodetector. Fragments due to autodigestion of trypsin were detected at OD_{214}.

threonine, and serine residues during insulin stimulation (36). The tyrosine phosphorylation sites were identified initially in recombinant IRS-1 phosphorylated in vitro by the partially purified insulin receptor. Under our assay conditions, a steady state of approximately 3 mol of phosphate per mol of recombinant IRS-1 was reached after 1.5 h (Fig. 1A). Therefore, a 1.5-h phosphorylation was used throughout this study.

Tryptic peptides obtained from recombinant [^{32}P]IRS-1 were separated by HPLC on a Bio-Rad RP-318 reverse-phase column (Fig. 1B). The radioactivity (Cerenkov) profile is subdivided arbitrarily into four regions designated A, B, C, and D (Fig. 1B). About 95% of the radioactivity was recovered from nitrocellulose after trypsin digestion, and between 80 and 90% of the radioactive peptides were eluted from the HPLC column. Phosphoamino acid analysis confirmed that each peak contained only phosphotyrosine (data not shown). Six major peaks (B1, B3, B4, B6, C2, and C5) were reproducibly detected. B5 sometimes comigrated with B4. Several ^{32}P-labeled peptides of intermediate intensity (A1, A2, A4, A5, B2, and C3) were observed in a variable manner. In some cases, peak A4 was more intense, and peaks B2 and C3 were smaller.

The identification of tyrosine phosphorylation sites in IRS-1. IRS-1 can theoretically generates 106 complete tryptic peptides. Table 1 lists the subset of peptides that contain one or several tyrosine residues. Most of the HPLC fractions containing ^{32}P-labeled phosphopeptides were difficult to sequence directly because they contained other nonphosphopeptides. Different approaches were used after the initial HPLC separation to isolate the ^{32}P-labeled phosphopeptides that contained the major in vitro tyrosine phosphorylation sites. This strategy included dephosphorylation (alkaline phosphatase), secondary protease subdigestion (protease V8, chymotrypsin, or endoproteinase Asp-N), or affinity purification by GST-SH2 fusion proteins (see Materials and Methods).

Tryptic peptides C2 and C5 (Fig. 1B) were relatively pure after HPLC separation, as judged from the elution profile at OD_{214}, and were directly sequenced as Tyr-895 and Tyr-1172 peptides, respectively; phosphorylation of the tyrosine residues was confirmed in each case by manual radiosequencing (Table 2). Tryptic peak B3 (Fig. 1B) contained Tyr-987. This assignment was made by automated sequencing of the dephosphorylated peptide and manual radiosequencing of the phosphopeptide (Table 2). Tryptic peaks B1 and B6 (Fig. 1B) contained Tyr-939 and Tyr-460, respectively; each assignment was made by automated sequencing of the corresponding protease V8 fragments, and the location of the phosphotyrosine residues was confirmed by manual radiosequencing (Table 2). Tryptic peak B5 (Fig. 1B), which was purified on immobilized GST-$nSH2^{p85\alpha}$ before analysis, contained Tyr-608. This assignment was made by automated sequencing and manual radiosequencing of the corresponding chymotryptic fragment (Table 2).

The amino acid sequences of three other tryptic peptides (A2, A3, and B4) were not obtained by automated sequencing because the yields of pure peptides were too low; however, manual radiosequencing of the tryptic peptides and secondary digests with protease V8 and endoproteinase Asp-N revealed the likely tyrosine phosphorylation sites. Tryptic peptide B4 (Fig. 1B) released radioactivity at cycle 8 (Fig. 2, B4), suggesting four possible sites, Tyr-460, Tyr-727, Tyr-939, and Tyr-1222 (Table 1). After endoproteinase Asp-N treatment, radioactivity was released at cycle 5 (Fig. 2, $B4^{Asp\text{-}N}$), restricting attention to two candidate tyrosines, Tyr-658 and Tyr-1222 (Table 1). Protease V8 treatment of B4 resulted in the release of radioactivity at cycle 5 (Fig. 2, $B4^{V8}$), indicating two candidate sites, Tyr-107 and Tyr-1222 (Table 1). Thus, the most likely assignment for the B4 peptide is Tyr-1222, as it is the only residue that appears in all three cases (Fig. 2).

Similar analysis revealed the phosphorylation of Tyr-987 in tryptic peptide A3. Manual radiosequencing released radioactivity from the tryptic peptide at cycle 3 (Fig. 2, A3). After secondary digestion with endoproteinase Asp-N, radioactivity was released at cycle 2, restricting attention to Tyr-987 (Fig. 2, $A3^{Asp\text{-}N}$); radioactivity in cycle 3 is probably due to incomplete digestion. Since protease V8 did not change the original pattern of manual radiosequencing in A3, glutamic acid was probably absent from the N-terminal side, suggesting that Tyr-987 is the phosphorylation site in peak A3. Peak A3 had the same assignment as peak B3, suggesting that it is the incomplete digestion product.

The assignment of tryptic peptide A2 was ambiguous even

TABLE 1. Sequences of tyrosine-containing tryptic peptides of IRS-1[a]

Cleavage site	Tyrosine position	Sequence
16	18	VGYLR
44	46,[b] 47[b]	LEYYENEK
82	87	HLVALYTR
90	107[b]	DEHFAIAADSEAEQDSWYQALLQLHNR
119	147[b]	AHHDGAGGGCGGSCSGSSGVGEAGEDLSYDTGPGPAFK
173	178	NLIGIYR
407	426[b]	SSASVSGSPSDGGFISSDEYGSSPCDFR
453	460[b]	GEEELSNYICMGGK
467	478	GASTLTAPNGHYILSR
489	489	YIPGATMGTSPALTGDEAAGAADLDNR
534	546,[b] 554	TPSQSSVVSIEEYTEMMPAAYPPGGGSGGR
564	567	LPGYR
569	578[b]	HSAFVPTHSYPEEGLEMHHLER
597	608[b]	GGHHRPDSSNLHTDDGYMPMSPGVAPVPSNR
(623) 624	628[b]	(K)GNGDYMPMSPK[c]
653	658,[b] 690	VDPNGYMMMSPSGSCSPDIGGGSCSSSSISAAPS--GSSYGKPWTNGVGGHHTHALPHAKPPVESGGGK
720	727,[b] 745,[b] 746,[b] 759, 760	LLPCTGDYMNMSPVGDSNTSSPSECYYGPEDPQHKPVLSYYSLPR
795	795, 815	YTATAEDSSSSTSSDSLGGGYCGARPESSVTHPHHHALQPHLPR
891	895,[b] 907	SPGEYVNIEFGSGQPGYLAGPATSR
932	939[b]	EETGSEEYMNMDLGPGR
985	987[b]	GDYMTMQIGCPR
997	999,[b] 1010[b]	QSYVDTSPVAPVSYADMR
1169	1172[b]	SLNYIDLDLVK
1215	1222[b]	SSEDLSTYASINFQK

[a] Tyrosine-containing complete tryptic peptides from the deduced amino acid sequence of rat IRS-1 (37) are shown along with their expected N-terminal tryptic cleavage sites. Lys-Pro and Arg-Pro are assumed to be unreactive to trypsin (5).
[b] Putative tyrosine phosphorylation site.
[c] Possible partial cleavage tryptic product (5).

after secondary digestion with protease V8 and endoproteinase Asp-N. Radioactivity was released from A2 at cycle 6 (Fig. 2, A2), suggesting four possible assignments, Tyr-87, Tyr-178, Tyr-628, and Tyr-658; Tyr-628 is possible since trypsin frequently generates incomplete digestion products (5) (Table 1). A2 was unaffected by subdigestion with protease V8 (Fig. 2, $A2^{V8}$). Radioactivity was released at cycle 2 after endoproteinase Asp-N, suggesting the phosphorylation of Tyr-628, Tyr-727, and Tyr-987 (Fig. 2, $A2^{Asp\text{-}N}$; Table 1). The best common assignment is Tyr-628, assuming that peptide A2 was generated by tryptic cleavage between Arg-622 and Lys-623.

SH2 domains in p85α, GRB2, and SHPTP2 bind to distinct phosphorylation sites in IRS-1. To identify some SH2 domain

TABLE 2. Microsequencing of IRS-1 phosphopeptides[a]

Cycle no.	B1 (Y-939)			B3 (Y-987)				B5 (Y-608)			B6 (Y-460)			C2 (Y-895)			C5 (Y-1172)		
	TPep	AS	% cpm	TPep	AS	% cpm	AlkP	TPep	AS	% cpm	TPep	AS	% cpm	TPep	AS	% cpm	TPep	AS	% cpm
1	E			G		6.2	G	P			G			S	s	16	S	s	24
2	E			D	D	11	D	D			E			P	P	8	L	L	13
3	T			Y	—	100	Y	S			E			G	G	8	N		11
4	G			M	M	12	M	S			E			E	E	14	Y	—	100
5	S			T	t	11	T	N			L	L	4.7	Y	—	100	I	I	29
6	E			M	M	10		L			S	S	2.6	V	V	33	D	D	19
7	E			Q	Q	9		H		1.3	N	N	2	N	n	18	L	L	14
8	Y	—	100	I	i	10		T	T	6.4	Y	—	100	I	I	15	D	D	19
9	M	M	12	G	g	12		D		13	I	I	5.2	E		13	L	L	16
10	N	N	4.1	C		11		D	d	8.0	C		2.1	F	F	11	V	V	13
11	M	M	3.7	P	P	7.4		G	G	4.8	M	M	1.3	G	G	12	K		
12	D	D	3	R				Y	—	100	G	G	1.5	S		8.7			
13	L	L	3.3					M	M	51	G	G	1.6	G	g	8			
14	G	G	2.5					P	P	25									
15	P	P	2					M	M	19									
16	G	G	2.3					S											

[a] Six tryptic peptides (B1, B3, B5, B6, C2, and C5) were analyzed by automated amino acid sequencing (AS). Uppercase letters indicate determinations with high certainty, and lowercase letters indicate those with less certainty; a dash indicates the expected absence of a predicted tyrosine residue; tryptic peptide B3 was also analyzed after dephosphorylation with alkaline phosphatase, revealing the presence of tyrosine (AlkP). The assigned phosphorylation sites are indicated in parentheses, and the amino-terminal portion of the corresponding complete tryptic peptide is shown (TPep). Manual radiosequencing reveals the Edman cycle of ^{32}P release and is reported relative to the radioactivity in the major fraction (% cpm). Tryptic peaks B1 and B6 were subdigested with protease V8, and B5 was analyzed after secondary digestion with chymotrypsin; tryptic peptides C2 and C5 were analyzed directly.

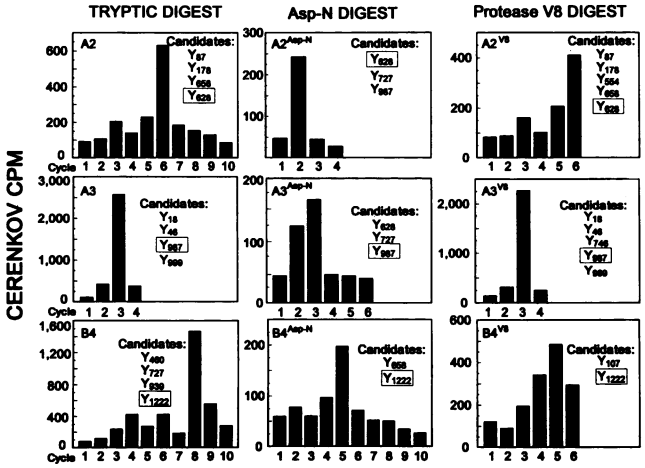

FIG. 2. Manual radiosequencing of phosphopeptides A2, A3, and B4 before or after secondary digestion. The tryptic peptides were purified by HPLC and directly subjected to manual radiosequencing as described in Materials and Methods. A portion of each tryptic peptide was treated with endoproteinase Asp-N ($A2^{Asp\text{-}N}$, $A3^{Asp\text{-}N}$, and $B4^{Asp\text{-}N}$) or protease V8 ($A2^{V8}$, $A3^{V8}$, and $B4^{V8}$) and subjected to manual radiosequencing without further purification.

binding sites in IRS-1, GST fusion proteins containing $nSH2^{p85\alpha}$, $nSH2^{GRB2}$, and $nSH2^{SHPTP2}$ were used to characterize the tryptic phosphopeptide map. IRS-1 labeled in vitro bound to the immobilized GST-SH2 domains, whereas GST alone does not, confirming that IRS-1 contains binding sites to these SH2-proteins (Fig. 3). Similar results were obtained with tyrosine-phosphorylated IRS-1 extracted from insulin-stimulated cells (Fig. 4). Following insulin stimulation for 1 min, cell extracts from CHO/IR/IRS-1 cells were incubated with the same GST-SH2 fusion proteins. Bound IRS-1 was separated by SDS-PAGE, transferred to nitrocellulose paper, and detected by immunoblotting with αIRS-1 or αPY (Fig. 4). $nSH2^{p85\alpha}$, $SH2^{GRB2}$, and $nSH2^{SHPTP2}$ bound only to IRS-1 from insulin-stimulated cells (Fig. 4, lane d, f, and h), confirming that the binding was specific to phosphorylated IRS-1; αPY immunoblotting showed that the SH2 fusion proteins bound to tyrosine-phosphorylated IRS-1 (Fig. 4, lane l, n, and p).

The principal binding sites in IRS-1 that interact with $nSH2^{p85\alpha}$, $SH2^{GRB2}$, and $nSH2^{SHPTP2}$ were determined by incubating the tryptic phosphopeptides derived from in

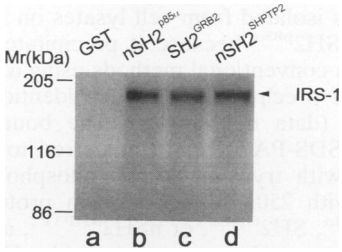

FIG. 3. Binding of SH2 fusion proteins to [^{32}P]IRS-1 in vitro. IRS-1 was labeled with [γ-^{32}P]ATP by activated wheat germ agglutinin-purified insulin receptor. Phosphorylated IRS-1 was then precipitated by GST, GST-$nSH2^{p85\alpha}$, GST-$SH2^{GRB2}$, and GST-$nSH2^{SHPTP2}$ fusion proteins. Phosphorylated IRS-1 in each precipitate was separated by SDS-PAGE (7.5% gel) and detected by autoradiography.

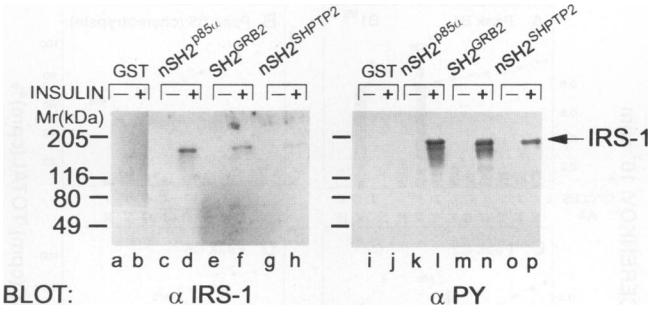

FIG. 4. Binding of SH2 fusion proteins to in vivo-phosphorylated IRS-1. Cell lysates derived from insulin-stimulated or unstimulated CHO/IR/IRS-1 (36) cells were incubated with GST, GST-$nSH2^{p85\alpha}$, GST-$SH2^{GRB2}$, and GST-$nSH2^{SHPTP2}$ fusion proteins and immobilized on glutathione-Sepharose. The proteins in the precipitates were separated by SDS-PAGE (7.5% gel) and transferred to nitrocellulose membranes. IRS-1 and other phosphotyrosine proteins were detected by αIRS-1 (lanes a to h) or αPY (lanes i to p) immunoblots.

vitro-labeled [^{32}P]IRS-1 with the corresponding GST-SH2 fusion proteins immobilized on glutathione-Sepharose. ^{32}P-labeled phosphopeptides associated with the pellet and remaining in the supernatant were analyzed by HPLC. None of the phosphopeptides bound to GST alone (Fig. 5A); however, the 250 nM GST-$nSH2^{p85\alpha}$ selectively bound two phosphopeptides, one containing Y-608MPM and the other containing Y-939MNM (Fig. 5B). More than 85% of the Tyr-939 peptide bound to GST-$nSH2^{p85\alpha}$; however, it was difficult to quantitate Tyr-608 binding because several phosphopeptides migrated in this fraction in the FREE profile (Fig. 5B). To confirm these assignments, the phosphopeptides precipitated by GST-$nSH2^{p85\alpha}$ were purified by HPLC and sequenced by manual radiosequencing. Radioactivity was released from the putative Tyr-939 peptide at cycle 8 before protease V8 treatment and at cycle 1 after protease

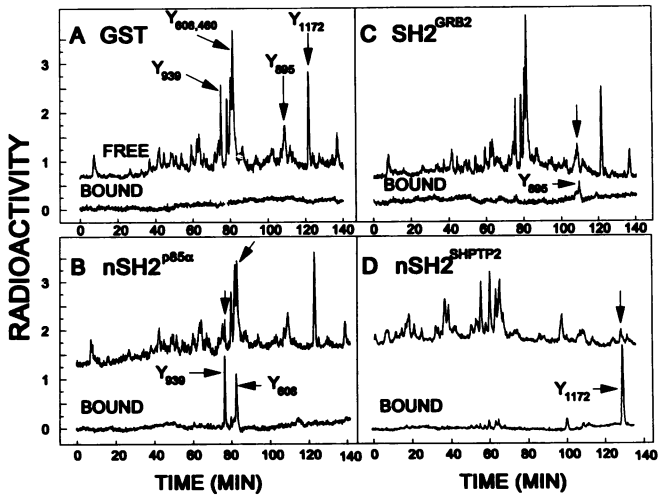

FIG. 5. HPLC analysis of SH2 binding peptides. Tryptic digests of [^{32}P]IRS-1 were incubated with GST alone (A) or with GST-$nSH2^{p85\alpha}$, GST-$SH2^{GRB2}$, and GST-$nSH2^{SHPTP2}$ (B to D). The SH2 fusion proteins and bound phosphopeptides were precipitated with glutathione-Sepharose. ^{32}P-labeled peptides in the supernatants (FREE) and the ^{32}P-labeled peptides eluted from pellets (BOUND) were analyzed by HPLC as described in the text.

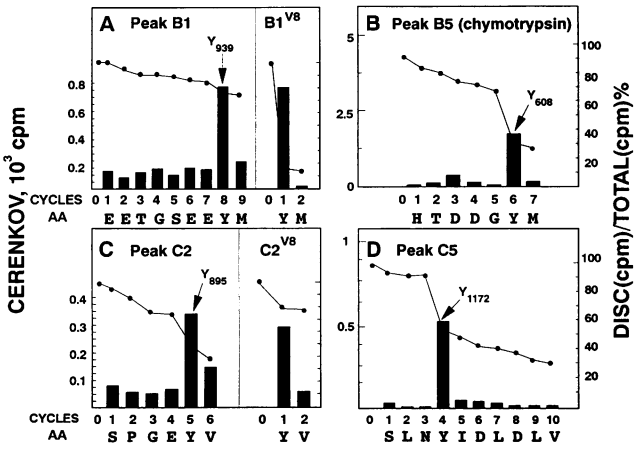

FIG. 6. Manual radiosequencing of SH2 binding peptides. The SH2 domain-bound tryptic peptides chosen in Fig. 5 were analyzed by manual radiosequencing before or after secondary digestion with protease V8 (V8) or chymotrypsin. The bars represent the radioactivity released from the disk; solid circles represent the radioactivity left on the disk expressed as a percentage of the original radioactivity.

V8 (Fig. 6A), confirming its identity (Table 2). The putative Tyr-608 peptide was unreactive during manual radiosequencing; however, chymotrypsin produced a single reactive peptide that released radioactivity at the sixth cycle during manual radiosequencing (Fig. 6B), and automated amino acid sequencing of this chymotryptic peptide confirmed the Tyr-608 identity. A 10-fold-lower concentration of GST-nSH2$^{p85\alpha}$ (25 nM) mainly bound the Tyr-608 peptide, whereas 2,500 nM GST-nSH2$^{p85\alpha}$ bound four phosphopeptides (Fig. 7). Thus, the binding of GST-nSH2$^{p85\alpha}$ may have the following preference for sites in IRS-1: Y-608MPM > Y-939MNM > Y-460ICM and Y-987MTM.

Similar experiments were conducted with GST fusion proteins of SH2^{GRB2} and nSH2^{SHPTP2}. SH2^{GRB2} fusion protein preferentially bound to a single phosphopeptide which was eluted at the same position as the Tyr-895 peptide (Fig. 5C). Only 40% of the Tyr-895 peptide was found in the

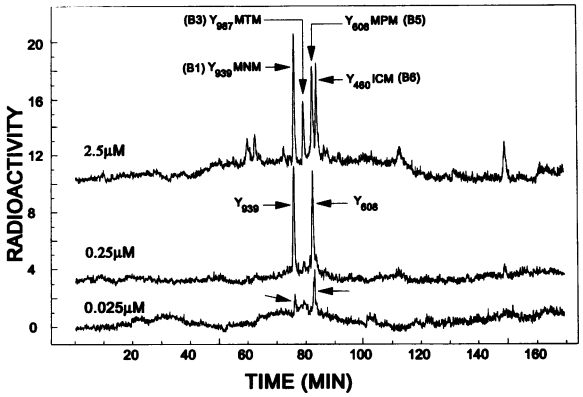

FIG. 7. HPLC analysis of phosphopeptides binding to the different concentration of nSH2$^{p85\alpha}$. Different amounts of GST-nSH2$^{p85\alpha}$ fusion proteins were incubated with tryptic phosphopeptides of IRS-1 and immobilized on glutathione-Sepharose. Bound phosphopeptides were eluted and separated by HPLC. The assignments were made on the basis of relative migration and confirmed by manual radiosequencing.

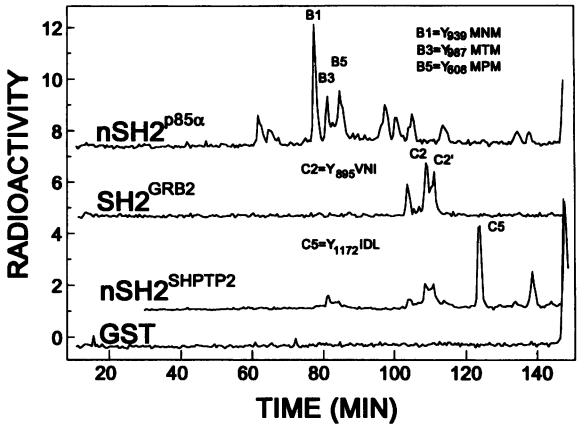

FIG. 8. HPLC analysis of SH2 binding peptides derived from in vivo-labeled IRS-1. CHO/IR/IRS-1 cells were labeled with [^{32}P]phosphate and stimulated with 100 nM insulin for 1 min. [^{32}P]IRS-1 was precipitated from the whole-cell extract on immobilized GST-nSH2$^{p85\alpha}$, separated by SDS-PAGE, and transferred to nitrocellulose paper. The band containing [^{32}P]IRS-1 was excised and digested with trypsin. The tryptic phosphopeptides were incubated with 250 nM GST alone or GST fusion proteins containing nSH2$^{p85\alpha}$, SH2^{GRB2}, and nSH2^{SHPTP2}. The complexes were immobilized on glutathione-Sepharose and analyzed by HPLC. The assignments were made by relative migration and manual radiosequencing.

pellet, indicating that the binding of the Tyr-895 peptide to 250 nM GST-SH2^{GRB2} was weaker than the binding of Tyr-939 or Tyr-608 to nSH2$^{p85\alpha}$. The putative Tyr-895 peptide eluted from the SH2^{GRB2} was confirmed by manual radiosequencing of the tryptic peptide and the corresponding protease V8 subdigest (Fig. 6C). GST-SH2^{SHPTP2} bound 95% of the Y-1172IDL peptide (Fig. 5D); this assignment was confirmed by manual radiosequencing of the purified peptide, which released radioactivity at cycle 4 (Fig. 6D).

In vivo phosphorylation sites on IRS-1. IRS-1 is phosphorylated on multiple sites in vivo during insulin stimulation (36). Previous studies revealed over 20 tryptic phosphopeptides separated by HPLC which are phosphorylated on serine and threonine residues, in addition to phosphotyrosines. We took the advantage of the binding specificity of several GST-SH2 fusion proteins to simplify the in vivo tryptic peptide map of IRS-1 and focus specifically on phosphotyrosine peptides. SH2 domain binding sites on IRS-1 were studied with CHO/IR/IRS-1 cells metabolically labeled with $^{32}P_i$ and stimulated with insulin for 1 min. [^{32}P]IRS-1 was isolated from cell lysates on 1 μg of immobilized GST-nSH2$^{p85\alpha}$ because it precipitated IRS-1 more efficiently than conventional methods using αPY or αIRS-1; all methods of precipitation produce identical radioactive peptide maps (data not shown). The bound IRS-1 was separated by SDS-PAGE and transferred to nitrocellulose and digested with trypsin. Tryptic phosphopeptides were precipitated with 250 nM GST fusion protein containing either nSH2$^{p85\alpha}$, SH2^{GRB2}, or nSH2^{SHPTP2}, and bound ^{32}P-labeled phosphopeptides were analyzed by HPLC (Fig. 8).

GST alone did not bind to any phosphopeptide, whereas the major phosphopeptides binding to the SH2 domains were similar to the binding phosphopeptides derived from the in vitro-phosphorylated IRS-1 (Fig. 5). GST-nSH2$^{p85\alpha}$ bound several peptides, with the major one migrating at the position expected for the Y-939MNM peptide (Fig. 8, B1). In addi-

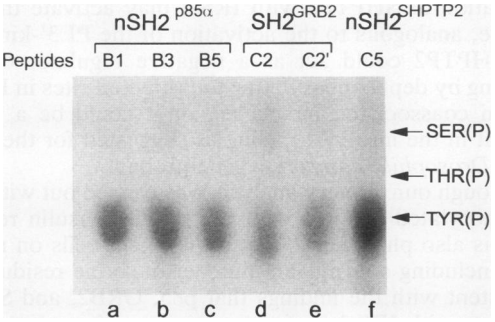

FIG. 9. Phosphoamino acid analysis of in vivo-labeled IRS-1 phosphopeptides that bind to SH2 domains. The phosphopeptides were isolated, dried, and resuspended in 100 μl of 6 N HCl. The phosphoamino acid analysis was performed as described in Materials and Methods.

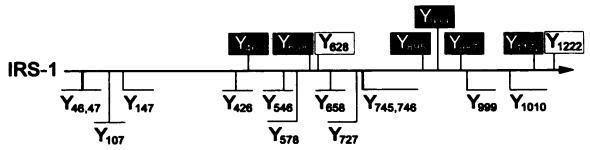

FIG. 10. Potential phosphorylation sites of IRS-1. Phosphorylation sites identified in this report with high certainty (automated sequencing and manual radiosequencing) are in black boxes with white letters, whereas likely phosphorylation sites predicted from manual radiosequencing alone are in white boxes. Other putative phosphorylation sites that have not been identified are listed below the line.

tion, the Y-608MPM and Y-987MTM peptides were detected in the GST-nSH2$^{p85\alpha}$-bound fractions (Fig. 8, B5 and B3). These peptides (B1, B3, and B5) contained phosphotyrosine exclusively (Fig. 9, lane a to c), and manual radiosequencing confirmed these assignments (data not shown). GST-SH2^{GRB2} precipitated three peptides, one of which migrated at the position expected for the Y-895 peptide (Fig. 8, C2). This peptide contained phosphotyrosine exclusively (Fig. 9, lane d), and manual radiosequencing confirmed the assignment of Tyr-895 (data not shown). The nSH2^{SHPTP2} fusion protein precipitated a predominant phosphopeptide that eluted at the position expected for the Y-1172IDL peptide (Fig. 8, C5). This peptide contained only phosphotyrosine (Fig. 9, lane f), and manual radiosequencing confirmed the assignment (data not shown). Some other in vivo phosphopeptides were also precipitated by the SH2 fusion proteins (Fig. 8), but they were generally minor peaks and are likely to be the low-affinity binding peptides. It is also possible that they are distinct in vivo phosphopeptides which remain to be identified.

DISCUSSION

Insulin stimulates many biological responses in cells and tissues, including glucose uptake in adipocyte and muscle (20), glycogen synthesis in liver and muscle, and gene transcription in most cells (24). Insulin increases the expression of various metabolic enzymes, including pyruvate kinase, malic enzyme, and glucokinase, whereas others, such as phosphoenolpyruvate carboxykinase, carbamoyl phosphate synthetase I, and fructose 1,6-bisphosphatase, are inhibited (15, 24). Moreover, insulin activates several pathways with potent mitogenic activity, including p21^{ras}, Raf-1 kinase, the mitogen-activated protein kinases, the S6 kinases, and protein phosphatases (4). The molecular mechanisms linking the insulin receptor to these and other processes have been elusive; however, the structure of IRS-1 suggests a simple molecular mechanism for the pleiotropic insulin response.

IRS-1 is a molecular adapter or docking protein that links the insulin receptor to various downstream regulatory elements that contain SH2 domains (SH2 proteins) (23). IRS-1 distinguishes insulin receptor signaling from that of other receptor tyrosine kinases which interact directly with SH2 proteins through their autophosphorylation sites (26). Before tyrosine phosphorylation, IRS-1 does not associate significantly with SH2 domains (22). Insulin immediately stimulates tyrosine phosphorylation of IRS-1, enabling its interaction with various SH2 proteins, including PI 3′-kinase, GRB2, and SHPTP2 (Syp) (3, 17, 32). Although tyrosine phosphorylation enables the binding of IRS-1 to the SH2 domains in these proteins, the selection of specific SH2 proteins depends on the amino acid sequence motif around the phosphotyrosine residues and the isoform of the SH2 domain. Our finding that the insulin receptor kinase phosphorylates multiple sites in IRS-1 in vivo and in vitro suggests that IRS-1 regulates a wide array of SH2 proteins which together contribute to the full cellular insulin response.

IRS-1 contains 21 potential tyrosine phosphorylation sites. Using a combination of manual radiosequencing and automated sequencing, we identified eight tyrosine residues that are phosphorylated by the activated insulin receptor (Fig. 10). Tyrosine residues 608, 628, 939, and 987 are in YMXM motifs, which are predicted to bind to the SH2 domains in the 85-kDa regulatory subunit (p85) of the PI 3′-kinase (6, 34). In contrast, the other phosphorylation sites, including Y-460ICM, Y-895VNI, Y-1172IDL, and Y-1222ASI, are located in distinct hydrophobic motifs. Thirteen additional tyrosine residues may be phosphorylated by the insulin receptor, given the proximity of an aspartate or glutamate residue (Fig. 10). It is likely that several of these are phosphorylated, as many minor unassigned peaks remain in the tryptic peptide map of IRS-1. Thus, IRS-1 has a significant potential to interact simultaneously with multiple downstream elements containing distinct SH2 domains.

The phosphorylation sites in IRS-1 serve a dual function by linking the insulin receptor to downstream signaling elements. First, IRS-1 is an ideal substrate for the insulin receptor catalytic domain, and second, the phosphorylated IRS-1 binds specifically to SH2 proteins. We have previously investigated the catalytic efficiency of various peptides based on sequences in IRS-1 for phosphorylation by the insulin receptor kinase (31). Although, in addition to proximal acidic residues, methionine residues at the Y-+1 and Y-+3 positions are particularly important for efficient phosphorylation of the synthetic peptides by purified insulin receptor; however, they are not essential for the phosphorylation of tyrosine residues in the intact IRS-1 molecule. Thus, the structure of intact IRS-1 may contribute significantly to orient the phosphorylation sites at the insulin receptor catalytic domain for an efficient interaction.

There are a growing number of SH2 domain-containing proteins, and we suspect that IRS-1 has the potential to simultaneously regulate several of them. We have only begun to identify the specific proteins that associate with phosphorylated IRS-1 (23). One of them, the PI 3′-kinase, associates with IRS-1 through the SH2 domains in its 85-kDa

subunit, p85 (22). Both the amino- and carboxy-terminal SH2 domains associate specifically with phosphorylated IRS-1 in the intact cell and with recombinant IRS-1 in vitro (3, 18, 22). This association is blocked with phosphorylated peptides containing the YMXM motif but not with unphosphorylated YMXM peptides or the phosphorylated peptide containing a scrambled amino acid sequence (3). The association of IRS-1 with p85 activates the PI 3′-kinase in the 110-kDa catalytic subunit, suggesting an important mechanism by which tyrosine-phosphorylated IRS-1 can mediate the insulin signal through downstream elements.

The three-dimensional crystal structure of various SH2 domains show that the phosphotyrosine residue and the next three amino acids are recognized at the binding pocket (9). Our analysis of the tryptic phosphopeptide maps of IRS-1 suggest that Tyr-608 may be the principal site of interaction between IRS-1 and p85. GST-nSH2$^{p85\alpha}$ at a concentration of 25 nM preferentially binds to Tyr-608 which resides in a YMPM motif and on the basis of published predictions is expected to associate with nSH2^{p85} (34). However, at 250 nM, GST-nSH2$^{p85\alpha}$ also associates with the tryptic peptide containing Tyr-939, and at 2,500 nM, peptides containing Tyr-987 and Tyr-460 also associate. Thus, the nSH2 domain of p85α shows the following binding preference: Y-608MPM > Y-939MNM > Y-987MTM and Y-460ICM. Tyr-628 also resides in a YMXM motif, but it is not detected as a strong binding site in our assays. Perhaps the N-terminal portion of the motif is involved in preferential binding of TDDGY-608MPMSP compared with GNGDY-628MPMSP. Alternatively, the phosphorylation of Tyr-628 is routinely low in our preparations, so this peptide may compete poorly with the other peptides even though it would be expected to associate at low levels of GST-nSH2.

GRB2 and SHPTP2 (Syp) also associate with IRS-1 (17, 32). GRB2 is a small adapter molecule that contains one SH2 domain and two SH3 domains. Genetic and biochemical evidence suggests that GRB2 is an upstream regulator of a guanine nucleotide exchange protein, son-of-sevenless, which stimulates the formation of active p21^{ras}-GTP complexes (10, 14, 25, 29). On the basis of amino acid sequence homologies in other proteins known to associate with GRB2, including the EGF receptor (Y-1068VNI) and Shc (Y-317VNV), Tyr-895 in IRS-1 was predicted to be the binding site for GRB2 (32). This prediction is supported by our finding that Tyr-895 is phosphorylated in vitro and in vivo, and the corresponding tryptic peptide associates with GST-SH2^{GRB2}. Thus, Tyr-895 is likely to be the primary binding site for GRB2 in IRS-1 and may be the principal molecular link between insulin and p21^{ras}. However, Shc is tyrosine phosphorylated during insulin stimulation, providing an alternate path for communication between insulin and GRB2 (32). Whether IRS-1 and Shc provide redundant or functionally distinct pathways for GRB2 signaling during insulin stimulation is not known.

The SHPTP2 (also known as Syp and phosphotyrosine phosphatase 1D) is a protein tyrosine phosphatase that contains two SH2 domains in the amino-terminal third of the molecule (12, 13). It associates with a number of activated tyrosine kinase receptors and with IRS-1 (17). Our analysis indicates that the nSH2 domain of SHPTP2 preferentially binds to Tyr-1172, which is found in a YIDL motif. This inference is consistent with reports that a Y[I/V]X[VI] motif is selected from a partially random peptide library by GST-nSH2^{SHPTP2} (34). The binding of GST-nSH2^{SHPTP2} to the Tyr-1172 tryptic peptide was relatively strong, suggesting that this may be the initial site of interaction with IRS-1. The association of SHPTP2 with IRS-1 may activate the phosphatase, analogous to the activation of the PI 3′-kinase (3). The SHPTP2 could act as a negative regulator of IRS-1 signaling by dephosphorylating unprotected sites in IRS-1 or sites in coassociated molecules, or it could be a positive element in the insulin signaling as suggested for the homologous *Drosophila corkscrew* gene product.

Although our primary analysis was carried out with IRS-1 phosphorylated in vitro with the purified insulin receptor, IRS-1 is also phosphorylated in the intact cells on multiple sites, including a significant number of serine residues (38). Consistent with the findings that p85, GRB2, and SHPTP2 associate with IRS-1 immunoprecipitated from intact cells, the expected binding sites occur in vivo as well. The phosphorylation of IRS-1 in cells is specific for the insulin receptor family, including the IGF-1 receptor and insulin receptor-related receptor. The receptors for PDGF and EGF do not phosphorylate IRS-1. Interestingly, the interleukin-4 receptor, which does not contain intrinsic tyrosine kinase activity, mediates tyrosine phosphorylation of IRS-1 (40). Whether identical sites are phosphorylated by the tyrosine kinase that is activated by the interleukin-4 receptor is currently unknown.

IRS-1 appears to mediate some insulin responses, but whether it is essential for all insulin effects is unknown. CHO cells, which contain endogenous IRS-1, display a modest increase of insulin-stimulated mitogenesis following overexpression of IRS-1 (36). *Xenopus* oocytes, which ordinarily respond poorly to insulin and IGF-1, display significantly increased sensitivity to insulin and IGF-1 following microinjection of IRS-1 (8). The response to injected IRS-1 is inhibited by coinjection of GST-nSH2$^{p85\alpha}$ and GST-SH2^{GRB2}, suggesting that the interaction between IRS-1 and SH2 proteins is essential for the insulin response in this system (7). Recently, Wang et al. demonstrated that mitogenesis in a myeloid progenitor cell, 32D, is insensitive to insulin even after overexpression of the insulin receptor; however, the insulin response is completely restored in these cells by expression of both insulin receptor and IRS-1 (41). Thus, IRS-1 appears to provide an essential link between the insulin receptor and downstream pathways essential for DNA synthesis. Whether IRS-1 is important for other biological responses such as glycogen synthesis and glucose uptake remains unknown.

The finding that IRS-1 undergoes multisite tyrosine phosphorylation during insulin stimulation supports our original hypothesis that IRS-1 may function as a docking protein that mediates the insulin responses by associating with multiple SH2 proteins. We have identified at least eight tyrosine phosphorylation sites in IRS-1; others remain to be found, and the correspondence between various phosphorylation sites and insulin action must be established. As more SH2 proteins are discovered, it is likely that more signaling molecules which associate with IRS-1 will be found. Each IRS-1-associated protein may initiate a unique signaling pathway which mediates a subset of cellular insulin actions. IRS-1 is essential for insulin-stimulated cellular proliferation (8, 36, 41), and we suspect that IRS-1 plays a role in metabolic signaling as well. Moreover, we expect the regulation of IRS-1 to be complicated by serine and threonine phosphorylation, which could also influence its interaction with downstream mediators.

ACKNOWLEDGMENTS

We thank J. Schlessinger for GST-SH2^{GRB2} and B. Neel for GST-nSH2^{SHPTP2}.

This work was supported by NIH grants DK38721 and DK43808 to M.F.W. X.J.S. was supported by the Juvenile Diabetes Foundation, and M.G.M. is a fellow of the Markey Program in Developmental Biology at the Division of Medical Sciences, Harvard Medical School, Boston, Mass.

REFERENCES

1. **Anderson, D., C. A. Koch, L. Grey, C. Ellis, M. F. Moran, and T. Pawson.** 1990. Binding of SH2 domains of phospholipase Cγ1, GAP and Src to activated growth factor receptors. Nature (London) **250**:979–982.
2. **Araki, E., X. J. Sun, B. L. Haag, L. M. Chuang, T. Yang-Feng, M. F. White, and C. R. Kahn.** 1993. Human skeletal muscle insulin receptor substrate-1: characterization of the cDNA, gene and chromosomal localization. Diabetes **42**:1041–1054.
3. **Backer, J. M., M. G. Myers, Jr., S. E. Shoelson, D. J. Chin, X. J. Sun, M. Miralpeix, P. Hu, B. Margolis, E. Y. Skolnik, J. Schlessinger, and M. F. White.** 1992. The phosphatidylinositol 3′-kinase is activated by association with IRS-1 during insulin stimulation. EMBO J. **11**:3469–3479.
4. **Blenis, J.** 1991. Growth-regulated signal transduction by MAP kinases and RSKs. Cancer Cells **3**:445–449.
5. **Boyle, W. J., P. van der Geer, and T. Hunter.** 1991. Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. Methods Enzymol. **201**:110–149.
6. **Cantley, L. C., K. R. Auger, C. Carpenter, B. Duckworth, A. Graziani, R. Kapeller, and S. Soltoff.** 1991. Oncogenes and signal transduction. Cell **64**:281–302.
7. **Chuang, L.-M., M. G. Myers, Jr., J. M. Backer, S. E. Shoelson, M. F. White, M. J. Birnbaum, and C. R. Kahn.** 1993. Insulin-stimulated oocyte maturation requires IRS-1 and interaction with the SH2 domains of phosphatidylinositol 3-kinase. Mol. Cell. Biol. **13**:6653–6660.
8. **Chuang, L. M., M. G. Myers, Jr., G. A. Seidner, M. J. Birnbaum, M. F. White, and C. R. Kahn.** 1993. Insulin receptor substrate-1 (IRS-1) mediates insulin and IGF-1 stimulated maturation of Xenopus oocytes. Proc. Natl. Acad. Sci. USA **90**:5172–5175.
9. **Eck, M. J., S. E. Shoelson, and S. C. Harrison.** 1993. Recognition of a high-affinity phosphotyrosyl peptide by the Src homology-2 domain of p56*lck*. Nature (London) **362**:87–91.
10. **Egan, S. E., B. W. Giddings, M. W. Brooks, L. Buday, A. M. Sizeland, and R. A. Weinberg.** 1993. Association of Sos ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. Nature (London) **363**: 45–51.
11. **Feener, E. P., J. M. Backer, G. L. King, P. A. Wilden, X. J. Sun, C. R. Kahn, and M. F. White.** 1993. Insulin stimulates serine and tyrosine phosphorylation in the juxtamembrane region of the insulin receptor. J. Biol. Chem. **268**:11256–11264.
12. **Feng, G.-S., C.-C. Hui, and T. Pawson.** 1993. SH-2 containing phosphotyrosine phosphatase as a target of protein-tyrosine kinase. Science **259**:1607–1614.
13. **Freeman, R. M., J. Plutzky, and B. G. Neel.** 1992. Identification of a human src homology 2-containing protein-tyrosine-phosphatase: a putative homolog of *Drosophila* corkscrew. Proc. Natl. Acad. Sci. USA **89**:11239–11243.
14. **Gale, N. W., S. Kaplan, E. J. Lowenstein, J. Schlessinger, and D. Bar-Sagi.** 1993. Grb2 mediates the EGF-dependent activation of guanine nucleotide exchange on Ras. Nature (London) **363**:88–92.
15. **Granner, D. K., and R. M. O'Brien.** 1992. Molecular physiology and genetics of NIDDM. Diabetes Care **15**:369–395.
16. **Hanks, S. K., A. M. Quinn, and T. Hunter.** 1990. The protein kinase family: conserved features and deduced phylogeny of the catalytic domain. Science **241**:42–52.
17. **Kuhne, M. R., T. Pawson, G. E. Lienhard, and G.-S. Feng.** 1993. The insulin receptor substrate 1 associates with the SH2-containing phosphotyrosine phosphatase Syp. J. Biol. Chem. **268**:11479–11481.
18. **Lavan, B. E., M. R. Kuhne, C. W. Garner, D. Anderson, M. Reedijk, T. Pawson, and G. E. Lienhard.** 1992. The association of insulin-elicited phosphotyrosine proteins with src homology 2 domains. J. Biol. Chem. **267**:11631–11636.
19. **Li, C.-H., R. Nishimura, M. Zhou, A. Batzer, M. G. Myers, Jr., M. F. White, J. Schlessinger, and E. Y. Skolnik.** Nck associates with the SH2 domain docking protein IRS-1 in insulin stimulated cell. Proc. Natl. Acad. Sci. USA, in press.
20. **Lienhard, G. E., J. W. Slot, D. E. James, and M. M. Mueckler.** 1992. How cells absorb glucose. Sci. Am. **266**:86–91.
21. **Lowenstein, E. J., R. J. Daly, A. G. Batzer, W. Li, B. Margolis, R. Lammers, A. Ullrich, E. Y. Skolnik, D. Bar-Sagi, and J. Schlessinger.** 1992. The SH2 and SH3 domain-containing proteins GRB2 links receptor tyrosine kinases to ras signaling. Cell **70**:431–442.
22. **Myers, M. G., Jr., J. M. Backer, X. J. Sun, S. E. Shoelson, P. Hu, J. Schlessinger, M. Yoakim, B. Schaffhausen, and M. F. White.** 1992. IRS-1 activates the phosphatidylinositol 3′-kinase by associating with the src homology 2 domains of p85. Proc. Natl. Acad. Sci. USA **89**:10350–10354.
23. **Myers, M. G., Jr., and M. F. White.** 1993. The new elements of insulin signaling: insulin receptor substrate-1 and proteins with SH2 domains. Diabetes **42**:643–650.
24. **O'Brien, R. M., and D. K. Granner.** 1991. Regulation of gene expression by insulin. Biochem. J. **278**:609–619.
25. **Olivier, J. P., T. Raabe, M. Henkemeyer, B. Dickson, G. Mbamalu, B. Margolis, J. Schlessinger, E. Hafen, and T. Pawson.** 1993. A drosophila SH2-SH3 adaptor protein implicated in coupling the sevenless tyrosine kinase to an activator of Ras guanine exchange, Sos. Cell **73**:179–191.
26. **Pawson, T., and G. D. Gish.** 1992. SH2 and SH3 domains: from structure to function. Cell **71**:359–362.
27. **Rice, K. M., G. E. Lienhard, and C. W. Garner.** 1992. Regulation of the expression of pp160, a putative insulin receptor signal protein, by insulin, dexamethasone, and 1-methyl-3-isobutylxanthine in 3T3-L1 adipocytes. J. Biol. Chem. **267**:10163–10167.
28. **Rothenberg, P. L., W. S. Lane, J. M. Backer, M. F. White, and C. R. Kahn.** 1991. Purification and partial sequence analysis of pp185, the major cellular substrate of the insulin receptor tyrosine kinase. J. Biol. Chem. **266**:8302–8311.
29. **Rozakis-Adcock, M., R. Fernley, J. Wade, T. Pawson, and D. Bowtell.** 1993. The SH2 and SH3 domains of mammalian Grb2 couple the EGF receptor to the Ras activator mSos1. Nature (London) **363**:83–84.
30. **Schu, P. V., T. Kaoru, M. J. Fry, J. H. Stack, M. D. Waterfield, and S. D. Emr.** 1993. Phosphatidylinositol 3-kinase encoded by yeast *VPS*34 gene essential for protein sorting. Science **260**:88–91.
31. **Shoelson, S. E., S. Chatterjee, M. Chaudhuri, and M. F. White.** 1992. YMXM motifs of IRS-1 define the substrate specificity of the insulin receptor kinase. Proc. Natl. Acad. Sci. USA **89**: 2027–2031.
32. **Skolnik, E. Y., C. H. Lee, A. G. Batzer, L. M. Vicentini, M. Zhou, R. J. Daly, M. G. Myers, Jr., J. M. Backer, A. Ullrich, M. F. White, and J. Schlessinger.** 1993. The SH2/SH3 domain containing protein GRB2 interacts with tyrosine phosphorylated IRS1 and Shc: implications for insulin control of *ras* signaling. EMBO J. **12**:1929–1936.
33. **Smith, D. B., and K. S. Johnson.** 1988. Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase. Gene **67**:31–40.
34. **Songyang, Z., S. E. Shoelson, M. Chaudhuri, G. Gish, T. Roberts, S. Ratnofsky, R. J. Lechleider, B. G. Neel, R. B. Birge, J. E. Fajardo, M. M. Chou, H. Hanafusa, B. Schaffhausen, and J. C. Cantley.** 1993. SH2 domains recognized specific phosphopeptide sequences. Cell **72**:767–778.
35. **Sullivan, S., and T. W. Wong.** 1991. A manual sequencing method for identification of phosphorylated amino acids in phosphopeptides. Anal. Biochem. **197**:65–68.
36. **Sun, X. J., M. Miralpeix, M. G. Myers, Jr., E. M. Glasheen, J. M. Backer, C. R. Kahn, and M. F. White.** 1992. The expression and function of IRS-1 in insulin signal transmission. J. Biol. Chem. **267**:22662–22672.
37. **Sun, X. J., P. Rothenberg, C. R. Kahn, J. M. Backer, E. Araki, P. A. Wilden, D. A. Cahill, B. J. Goldstein, and M. F. White.**

1991. The structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein. Nature (London) **352:**73–77.

38. **Tranisijevik, M., M. G. Myers, Jr., R. S. Thomas, D. Crimmons, M. F. White, and D. Sacks.** 1993. Phosphorylation of the insulin receptor substrate IRS-1 by casein kinase II. J. Biol. Chem. **286:**18157–18166.
39. **Waksman, G., S. E. Shoelson, N. Pant, D. Cowburn, and J. Kuriyan.** 1993. Binding of a high affinity phosphotyrosyl peptide to the Src SH2 domain: crystal structures of the complexed and peptide-free forms. Cell **72:**779–790.
40. **Wang, L.-M., A. D. Keegan, W. Li, G. E. Lienhard, S. Pacini, J. S. Gutkind, M. G. Myers, X. J. Sun, M. F. White, S. A. Aaronson, W. E. Paul, and J. H. Pierce.** 1993. Common elements in IL4 and insulin signaling pathways in factor dependent hematopoietic cells. Proc. Natl. Acad. Sci. USA **90:**4032–4036.
41. **Wang, L. M., M. G. Myers, X. J. Sun, S. A. Aaronson, M. F. White, and J. H. Pierce.** 1993. IRS-1: essential for insulin- and IL-4-stimulated mitogenesis in hematopoietic cells. Science **261:**1591–1594.
42. **White, M. F., R. Maron, and C. R. Kahn.** 1985. Insulin rapidly stimulates tyrosine phosphorylation of a Mr 185,000 protein in intact cells. Nature (London) **318:**183–186.