Positive and Negative Regulatory Role of Insulin Receptor Substrate 1 and 2 (IRS-1 and IRS-2) Serine/Threonine Phosphorylation

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ABSTRACT: Insulin receptor substrates (IRS) 1 and 2 are phosphorylated on serine/threonine (Ser/Thr) residues in quiescent cells (basal phosphorylation), and phosphorylation on both Ser/Thr and tyrosine residues is increased upon insulin stimulation. To determine whether basal Ser/Thr phosphorylation of IRS proteins influences insulin receptor catalyzed tyrosine phosphorylation, recombinant FLAG epitope-tagged IRS-1 (F-IRS-1) and IRS-2 (F-IRS-2) were expressed, purified, and subjected to both dephosphorylation and hyperphosphorylation prior to phosphorylation by the insulin receptor kinase. As expected, hyperphosphorylation of F-IRS-1 and F-IRS-2 by GSK3β decreased their subsequent phosphorylation on tyrosine residues by the insulin receptor. Surprisingly, however, dephosphorylation of the basal Ser/Thr phosphorylation sites impaired subsequent phosphorylation on tyrosine, suggesting that basal Ser/Thr phosphorylation of F-IRS-1 and F-IRS-2 plays a positive role in phosphorylation by the insulin receptor tyrosine kinase. Dephosphorylation of basal Ser/Thr sites on F-IRS-1 also significantly reduced tyrosine phosphorylation by the IGF-1 receptor. However, dephosphorylation of F-IRS-2 significantly increased phosphorylation by the IGF-1 receptor, suggesting that basal phosphorylation of IRS-2 has divergent effects on its interaction with the insulin and IGF-1 receptors. Phosphorylation of endogenous IRS-1 and IRS-2 from 3T3-L1 adipocytes was modulated in a similar manner. IRS-1 and IRS-2 from serum-fed cells were hyperphosphorylated, and dephosphorylation induced either by serum deprivation or by alkaline phosphatase treatment after immunoprecipitation led to an increase in tyrosine phosphorylation by the insulin receptor. Dephosphorylation of IRS-1 and IRS-2 immunoprecipitated from serum-deprived cells, however, resulted in inhibition of tyrosine phosphorylation by the insulin receptor. These data suggest that Ser/Thr phosphorylation can have both a positive and a negative regulatory role on tyrosine phosphorylation of IRS-1 and IRS-2 by insulin and IGF-1 receptors.

The first step in insulin action is ligand stimulation of the insulin receptor tyrosine kinase. A number of endogenous substrates, including insulin receptor substrates (IRS)[1] 1−4 (*1*−*4*), are phosphorylated on tyrosine residues. Tyrosine-phosphorylated IRS-1 and IRS-2 serve as the major docking proteins for Src homology 2 domain (SH2) containing proteins that mediate the biological actions of insulin (*5*, *6*). A central role for IRS-1 and IRS-2 in mediating the normal actions of insulin has been demonstrated by disruption of their respective genes in mice. Both IRS-1- and IRS-2-deficient mice are insulin resistant and hyperinsulinemic although only IRS-2-deficient mice develop diabetes (*7*−*11*).

Approximately 14% (>180) of the amino acids in IRS-1 and IRS-2 are serine residues while threonine and tyrosine residues comprise approximately 5% and 3%, respectively (*1*, *2*). Over 50 of these serine/threonine (Ser/Thr) residues are found in consensus phosphorylation sites for various Ser/Thr kinases including casein kinase 2 (CK2), protein kinase C (PKC), cAMP-dependent protein kinase (PKA), mitogen-activated protein (MAP) kinase, glycogen synthase kinase 3 (GSK3), and protein kinase B (PKB). Indeed, it has been reported that IRS-1 is a substrate in vitro for CK2, GSK3, MAP kinase, and c-Jun N-terminal kinase 1 (JNK1) (*12*−*15*). Hyperphosphorylation of IRS-1 on Ser/Thr decreases its electrophoretic mobility when analyzed by reducing SDS−PAGE and, more importantly, reduces its phosphorylation on tyrosine by the insulin receptor.

Hyperphosphorylation of IRS-1 on Ser/Thr residues has been shown in both cultured cells and in vivo to be associated with an insulin-resistant state (*16*, *17*). Examples of experimental induction of cellular insulin resistance include treatment with chronic insulin, platelet-derived growth factor, endothelin-1, tumor necrosis factor α, and okadaic acid (*17*−*21*). Thus, densensitization of the insulin signal via Ser/Thr hyperphosphorylation can result from counterregulatory hormonal activation, proinflammatory cytokine production/cellular stress, and inhibition of protein phosphatases 1 and 2A. The ability of hyper-Ser/Thr-phosphorylated IRS-1 to undergo tyrosine phosphorylation by the activated insulin receptor in vitro can be improved by incubation with alkaline

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[1] Abbrevations: IRS, insulin receptor substrate; F-IRS-1, FLAG-tagged IRS-1; F-IRS-2, FLAG-tagged IRS-2; hIR, human insulin receptor; IGF, insulin-like growth factor; Ser/Thr, serine/threonine; CK2, casein kinase 2; PKC, protein kinase C; PKA, cAMP-dependent protein kinase; MAPK, mitogen-activated protein kinase; GSK3, glycogen synthase kinase 3; PKB, protein kinase B; JNK1, c-Jun N-terminal kinase 1.

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phosphatase, which indicates that the effect of hyper-Ser/Thr-phosphorylation is reversible (*17*).

IRS-1 isolated from serum-deprived cells, prior to any treatments that induce insulin resistance, is already phosphorylated on Ser/Thr residues (*22*). The role of basal Ser/Thr phosphorylation in the function of IRS proteins is not known. To address this issue, purified recombinant IRS-1 and IRS-2 were used as substrates for the insulin receptor kinase following either dephosphorylation with alkaline phosphatase or hyperphosphorylation with GSK3β. In addition, we have tested the effect of alkaline phosphatase treatment on tyrosine phosphorylation of endogenous IRS-1 and IRS-2 immunoprecipitated from 3T3-L1 adipocytes under conditions in which IRS proteins exhibit either basal phosphorylation or hyperphosphorylation on Ser/Thr residues. Our results suggest that basal Ser/Thr phosphorylation plays a positive role in enhancing tyrosine phosphorylation by the insulin receptor, whereas hyper-Ser/Thr-phosphorylation is inhibitory. Therefore, Ser/Thr phosphorylation appears to play both a negative and a positive regulatory role in insulin receptor catalyzed IRS-1 and IRS-2 tyrosine phosphorylation.

MATERIALS AND METHODS

Materials. Polyclonal IRS-1 and IRS-2 and monoclonal anti-phosphotyrosine (4G10) antibodies were from Upstate Biotechnology (Lake Placid, NY). Monoclonal anti-phosphotyrosine (PY20) was from Oncogene Research Products (Cambridge, MA). Restriction enzymes, oligonucleotides, calf intestinal alkaline phosphatase, pFastBac, and Sf-9 serum-free medium II were from Life Technologies (Gaithersburg, MD). Turbo Pfu was from Stratagene (La Jolla, CA). Enhanced chemiluminescence detection reagents were from Pierce (Rockford, IL). GSK3β was obtained from Andrew Paterson and Philip Cohen (University of Dundee, Scotland). Porcine insulin, goat anti-mouse and anti-rabbit peroxidase conjugated antibodies, protein A−Sepharose, monoclonal anti-FLAG, anti-FLAG agarose, and other chemicals were from Sigma (St. Louis, MO). Tris−glycine gels and electrophoresis reagents were from Novex (San Diego, CA). Human IRS-1 and mouse IRS-2 cDNAs were kindly provided by Simeon Taylor (NIH, Bethesda, MD).

Recombinant IRS-1 and IRS-2 Expression and Purification. A FLAG epitope adapter complex was generated by annealing two single-stranded oligonucleotides, 5′ GTCGACGACTACAAGGACGACGATGACAAGTAGGCGGCCGC 3′ and 3′ CAGCTGCTGATGTTCCTGCTGCTACTGTTCATCCGCCGGCG 5′. The FLAG adapter complex was digested with *Sal*I and *Not*I, purified by spin columns, and ligated with *Sal*I- and *Not*I-digested pFastBac to generate pFastBac-FLAG. Human IRS-1 and mouse IRS-2 were subcloned from the pCIS2 vector by PCR amplification with Turbo Pfu using the following upstream/downstream oligonucleotide pairs: 5′ TTATGAATTCCCCCCACCCGGTTGTTTTTC 3′/5′ AAATCAGTCGACCTGACGGTCCTCTGGCTGCTTCTG 3′ and 5′ TTATGAATTCCTGACCAGTTATCGTGAGA 3′/5′ AGCGGTCGACCTCTTTCACGACTGT 3′, respectively. The PCR product was digested with *Eco*RI and *Sal*I and ligated into pFastBac-FLAG. The plasmid and insert were verified by restriction digestion and sequencing. Recombinant baculovirus particles were generated using the Bac-to-Bac expression system according to the manufacturer's instructions (Life Technologies, Bethesda, MD). Sf-9 cells ($\sim 1.5 \times 10^6$/mL) were infected with high titer FLAG-IRS-1 or FLAG-IRS-2 recombinant baculovirus particles and harvested after 40−48 h by centrifugation at 500*g* at 4 °C for 10 min, followed by storage at −20 °C. Cell pellets were resuspended in sonification buffer, 50 mM Tris, pH 7.4, 150 mM NaCl, and 1× protease inhibitor set I cocktail (Calbiochem, San Diego, CA), and sonicated on ice three times for 10 s at 50% power output. Cellular debris was removed by centrifugation at 15000 rpm for 15 min at 4 °C. Total lysate was applied, washed, and eluted from an anti-FLAG agarose column according to the manufacturer's instructions. Fractions were analyzed by reducing SDS−PAGE, then pooled, and dialyzed at 4 °C against buffer containing 50 mM Tris, pH 7.4, 50 mM NaCl, 0.05% Tween-20, and 1 mM DTT. The protein contentration was determined by the Bradford method (*23*), and aliquots were frozen at −80 °C. The purity of recombinant FLAG-tagged IRS-1 and FLAG-tagged IRS-2 was estimated to be 90% and 80%, respectively, by Coomassie staining and analysis using a Lumi-Imager Workstation and Lumi-Analyst software.

Cell Culture, Cell Lysis, and Immunoprecipitation. 3T3-L1 cells were obtained from ATCC and differentiated into adipocytes as previously described (*24*). 3T3-L1 adipocytes were maintained in 12 well dishes in DMEM containing 10% fetal bovine serum, at 37 °C and 10% CO_2, and then serum deprived for 5 h in DMEM containing 0.1% BSA. Lysis was performed by shaking on ice for 20 min with 50 mM Hepes, pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% deoxycholate, 0.1% SDS, and 1× protease inhibitor set I cocktail (Calbiochem, San Diego, CA). Cellular debris was removed by centrifugation at 15000 rpm for 15 min at 4 °C. Immunoprecipitation was performed with 4 μg of anti-IRS-1 or anti-IRS-2 antibodies at 4 °C overnight, followed by collection on protein A−Sepharose. Immunoprecipitated IRS proteins were washed twice with 50 mM Hepes, pH 7.4, 200 mM NaCl, and 0.1% Triton X-100 (buffer A), once with 50 mM Hepes, pH 7.4, and 500 mM NaCl (buffer B), once in buffer A, and once with 5 mM Tris, pH 7.6, and 75 mM NaCl (buffer C).

Alkaline Phosphatase Treatment. Recombinant protein ($\sim$750 ng) or immunoprecipitated IRS protein from 3T3-L1 adipocytes was incubated with or without 25 and 37.5 units, respectively, of calf intestinal alkaline phosphatase for 30 and 45 min, respectively, at 30 °C. As a control, calf intestinal alkaline phosphatase was heat inactivated by incubation at 100 °C for 10 min, prior to incubation with IRS proteins. Dephosphorylated recombinant protein was immunoprecipitated for 2 h at 4 °C using anti-FLAG agarose in 50 mM Tris, pH 7.4, 200 mM NaCl, and 10 mM EDTA. Immunoprecipitated, dephosphorylated recombinant proteins were washed twice with 50 mM Tris, pH 7.4, and 200 mM NaCl, once with 50 mM Tris, pH 7.4, and 300 mM NaCl, once with 50 mM Tris, pH 7.4, and 150 mM NaCl, and once with buffer C. Immunoprecipitated, dephosphorylated IRS proteins from 3T3-L1 adipocytes were washed twice with buffer A, once with buffer B, once in buffer A, and once with buffer C.

In Vitro Insulin and IGF Receptor Kinase Assays. Washed immunoprecipitates containing alkaline phosphatase treated or untreated recombinant protein or IRS protein from 3T3-L1 adipocytes were incubated with 30 μL of activated insulin

or IGF receptors for 30 min at 30 °C. The reaction was stopped by the addition of an equal volume of 2× Laemmli sample buffer (*25*) containing 200 mM DTT. Insulin receptors were activated at 4 °C for 1 h by incubation of 70 ng of solubilized hIR-293 cell membranes, prepared as previously described (*26*), in 20 mM Tris, pH 7.6, 10 mM MgOAc, 100 μM ATP, 1 mM DTT, 25 μg/mL BSA, and 100 nM insulin. IGF receptors were activated at 4 °C for 1 h by incubation of 0.3 μg of solubilized membranes from NIH-3T3-hIGFR cells (*27*), prepared as previously described (*26*), in 20 mM Tris, pH 7.6, 10 mM MgOAc, 100 μM ATP, 1 mM DTT, 25 μg/mL BSA, and 100 nM IGF-I. Control experiments demonstrated that tyrosine phosphorylation by solubilized hIR-293 and NIH-3T3-hIGFR cell membranes was linear with respect to enzyme and substrate (Figure 2 and data not shown).

GSK3β Hyper-Ser/Thr-Phosphorylation of Recombinant IRS Proteins and IR Kinase Assay. In vitro phosphorylation of F-IRS-1 and F-IRS-2 (~750 ng) by GSK3β was performed in the presence of 50 mM Tris, pH 7.4, 10 mM MgOAc, 50 μM ATP, 0.1% β-ME, and 10 μCi of [γ-^{32}P]-ATP with 50−350 milliunits of GSK3β for 30 min at 30 °C. Dual kinase assays were carried out according to Eldar-Finkelman and Krebs (*13*). Briefly, F-IRS-1 or F-IRS-2 was incubated with 350 milliunits of GSK-3β as described above except for the omission of [γ-^{32}P]ATP. Activated insulin receptor was then added, followed by incubation at 30 °C for 20 min. Activated insulin receptor (210 ng of solubilized membranes/reaction) was prepared as described above except that 20 mM $MnCl_2$ was used instead of 10 mM MgOAc. The final concentrations of the components in the IR kinase reaction were 50 mM Tris, pH 7.5, 7.5 mM MgOAc, 5 mM $MnCl_2$, 50 μM ATP, 0.05% β-mercaptoethanol, 25 μg/mL BSA, and 100 nM insulin.

Immunoblotting. Samples were boiled for 5 min and subjected to SDS−PAGE using 6% or 8% Tris−glycine gels and then transferred to nitrocellulose (S&S, Keene, NH). Membranes were incubated with anti-phosphotyrosine antibodies (PY20, 0.5 μg/mL, or 4G10, 1 μg/mL) in DPBS containing 0.1% Tween-20 and 2% BSA for 3 h at room temperature or overnight at 4 °C. Following washing, incubation with horseradish peroxidase-conjugated secondary antibody (1:5000) for 2 h at room temperature, and washing, detection was carried out with the enhanced chemiluminescent substrate. Nitrocellulose membranes were stripped in 0.1 M glycine, pH 3.5, and 0.2% SDS for 20 min with constant shaking. Protein content was then determined by immunoblotting with anti-IRS-1 (1 μg/mL) or anti-IRS-2 (1 μg/mL) antibodies in DPBS containing 3% nonfat dry milk or anti-FLAG (5 μg/mL) in TBS, overnight at 4 °C or at room temperature for 3 h.

Statistical Analysis. Chemiluminescent signals were directly quantitated using the Lumi-Imager Workstation and Lumi-Analyst software. The absolute integration value of the immunoreactive bands minus background was determined. Statistical significance was determined by Student's *t*-test ($\alpha = 0.05$).

RESULTS

Electrophoretic Mobility of Recombinant and Immunoprecipitated Endogenous IRS Proteins. The calculated molecular mass of rat IRS-1 (rIRS-1) is 131 kDa. Yet, from quiescent cells, rIRS-1 migrates at 165 kDa when analyzed by SDS−PAGE and upon insulin stimulation migrates at 180 kDa (*1*). The discrepancy between calculated molecular mass and relative molecular mass can be partially attributed to the fact that rIRS-1 is phosphorylated on Ser/Thr residues in quiescent cells and upon insulin stimulation there is an increase in serine phosphorylation in addition to tyrosine phosphorylation (*22*). To determine the relative Ser/Thr phosphorylation state of recombinant IRS proteins, epitope-tagged human IRS-1 (F-IRS-1) and mouse IRS-2 (F-IRS-2), affinity purified from Sf-9 cell lysates, were subjected to dephosphorylation with alkaline phosphatase and hyperphosphorylation with GSK3β and analyzed by SDS−PAGE and immunoblotting. In vitro phosphorylation of F-IRS-1 and F-IRS-2 by GSK3β retarded their migration (Figure 1A, lane 1, and Figure 1B, lane 1, respectively). Conversely, incubation with calf intestinal alkaline phosphatase increased the migration of F-IRS-1 and F-IRS-2 (Figure 1 A, lane 3, and Figure 1B, lane 3, respectively). Since purified F-IRS-1 and F-IRS-2 were not phosphorylated on tyrosine residues as determined by immunoblotting with two different anti-phosphotyrosine antibodies (Figure 3A,B and data not shown), the increased migration must be due to dephosphorylation of Ser/Thr residues. To distinguish these Ser/Thr phosphorylation sites from those in hyperphosphorylated F-IRS-1 and F-IRS-2, we refer to them as basal phosphorylation sites.

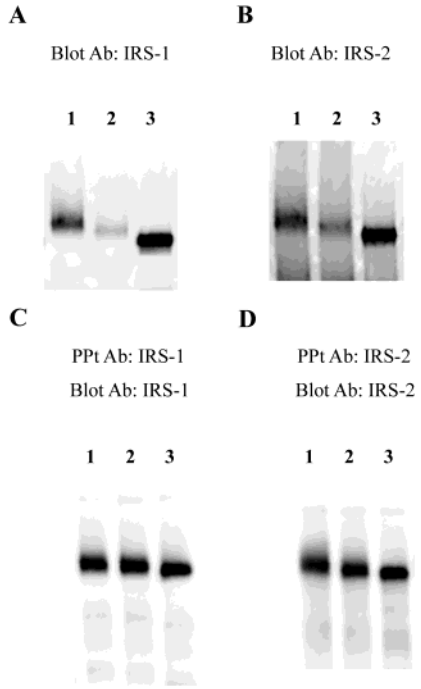

FIGURE 1: Electrophoretic mobility of recombinant and immunoprecipitated endogenous IRS proteins is altered by in vitro phosphorylation/dephosphorylation and serum deprivation, respectively. Recombinant FLAG-IRS-1 (A) and FLAG-IRS-2 (B) affinity purified from baculovirus-infected Sf-9 cells were hyperphosphorylated in vitro using GSK-3β (350 milliunits) (lane 1), untreated (lane 2), or dephosphorylated with 25 units of calf intestinal alkaline phosphatase (lane 3). IRS-1 (C) and IRS-2 (D) immunoprecipitated from 3T3-L1 adipocytes fed with DMEM containing 10% FBS (lane 1) or serum-free DMEM containing 0.1% BSA (lanes 2 and 3) and treated without (lanes 1 and 2) or with (lane 3) calf intestinal alkaline phosphatase (37.5 units).

The electrophoretic mobility of IRS-1 and IRS-2 from serum-fed and serum-deprived 3T3-L1 adipocytes was also assessed. Serum deprivation increased the migration of immunoprecipitated IRS-1 and IRS-2 (Figure 1C, lane 2, and Figure 1D, lane 2, respectively) as compared to IRS-1 and IRS-2 immunoprecipitated from serum-fed cells (Figure 1C, lane 1, and Figure 1D, lane 1, respectively), suggesting that serum deprivation induces Ser/Thr dephosphorylation. The electrophoretic mobility of immunoprecipated IRS-1 and IRS-2 from serum-deprived cells was increased further by incubation with calf intestinal alkaline phosphatase (Figure 1C, lane 3, and Figure 1D, lane 3, respectively).

Quantitation of Anti-phosphotyrosine Immunoreactive Bands. Quantitation of IRS-1 and IRS-2 tyrosine phosphorylation by the insulin and IGF-I receptor kinases utilized immunoblotting with anti-phosphotyrosine antibodies and direct quantition of chemiluminescent signals (see Materials and Methods). Reprobing immunoblots with antibodies that recognize IRS-1 or IRS-2 was used to normalize the tyrosine phosphorylation to IRS protein amount. Changes in IRS-1 and IRS-2 phosphorylation were linear over a wide range of IRS protein concentration and hIR kinase activity, as illustrated in Figure 2. A linear increase in IRS-1 tyrosine phosphorylation was observed with amounts of solublized hIR varied over an 8-fold range (Figure 2A). Similarly, using a constant amount of hIR, IRS-1 tyrosine phosphorylation increased linearly when the IRS-1 amount was varied over a 6-fold range (Figure 2B). Thus, these methods provide for accurate quantitation of both the extent and direction of changes in IRS-1 and 2 tyrosine phosphorylation in response to changes in Ser/Thr phosphorylation.

Dephosphorylation of Recombinant IRS Proteins Inhibits Human Insulin Receptor-Catalyzed IRS Tyrosine Phosphorylation. To determine the effect of dephosphorylation of basal Ser/Thr sites of IRS proteins on subsequent phosphorylation by the activated insulin receptor, recombinant F-IRS-1 and F-IRS-2 treated or untreated with calf intestinal alkaline phosphatase were immunoprecipitated with anti-FLAG agarose and washed, followed by incubation with activated insulin receptor as described in Materials and Methods. IRS protein tyrosine phosphorylation was subsequently analyzed by reducing SDS−PAGE and immunoblotting. Tyrosine phosphorylation was normalized to the amount of IRS-1 or IRS-2 present in each lane. Dephosphorylation of F-IRS-1 or F-IRS-2 increased their electrophoretic mobility (Figure 3A,B) and resulted in a reduction in insulin receptor catalyzed tyrosine phosphorylation of 27.3% ($p < 0.001$) and 52.6% ($p < 0.001$), respectively, whereas heat-inactivated calf intestinal alkaline phosphatase had no effect. Incubation of anti-FLAG agarose with or without calf intestinal alkaline phosphatase followed by washing and incubation with activated insulin receptor resulted in no difference in insulin receptor β-subunit autophosphorylation (Figure 3C). In addition, no phosphatase activity was detected using the exogenous substrate, *p*-nitrophenyl phosphate, in F-IRS-1 immunoprecipitates treated with calf intestinal alkaline phosphatase, followed by washing and incubation in kinase buffer (data not shown). These controls indicate that the observed decrease in IRS tyrosine phosphorylation was not due to inactivation of the insulin receptor or to residual alkaline phosphatase carried over into the kinase reaction.

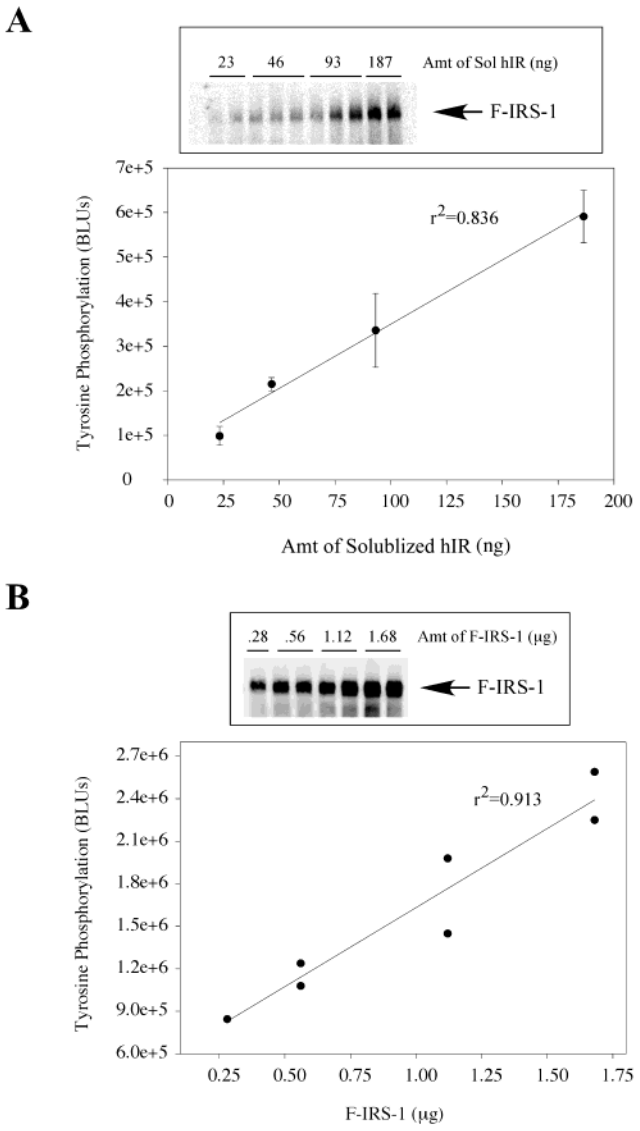

FIGURE 2: Quantitation of immunoreactive bands from (A) hIR enzyme titration and (B) F-IRS-1 substrate titration. Anti-phosphotyrosine (PY20) immunoblots were developed as described in Materials and Methods. Chemiluminescent signals were directly quantitated using the Lumi-Imager Workstation and Lumi-Analyst software. The absolute integration value of the immunoreactive bands minus background was calculated as Boehringer Light Units (BLUs). Simple linear regression was used to determine the relationship between BLUs and amount of hIR 293 cell soluble (Sol) membranes or recombinant F-IRS-1. (A) hIR in vitro kinase assay using various amounts of hIR 293 cell soluble membranes and a constant amount of recombinant F-IRS-1 (750 ng). (B) hIR in vitro kinase assay using a constant amount of hIR 293 cell soluble membranes (117 ng) and various amounts of recombinant F-IRS-1.

Dephosphorylation of Recombinant IRS-1 and IRS-2 Inhibits and Enhances, Respectively, Human IGF-I Receptor Catalyzed IRS Tyrosine Phosphorylation. To determine if dephosphorylation of basal Ser/Thr sites of IRS-1 and IRS-2 also impaired tyrosine phosphorylation by the IGF-I receptor, F-IRS-1 and F-IRS-2, treated or untreated with calf intestinal alkaline phosphatase, were immunoprecipitated with anti-FLAG agarose and washed, followed by incubation with activated human IGF-I receptor (hIGFR) as described in Materials and Methods. Similar to the observations with the insulin receptor, dephosphorylation of F-IRS-1 resulted in a reduction of 40.6% ($p < 0.001$) in hIGFR-stimulated tyrosine

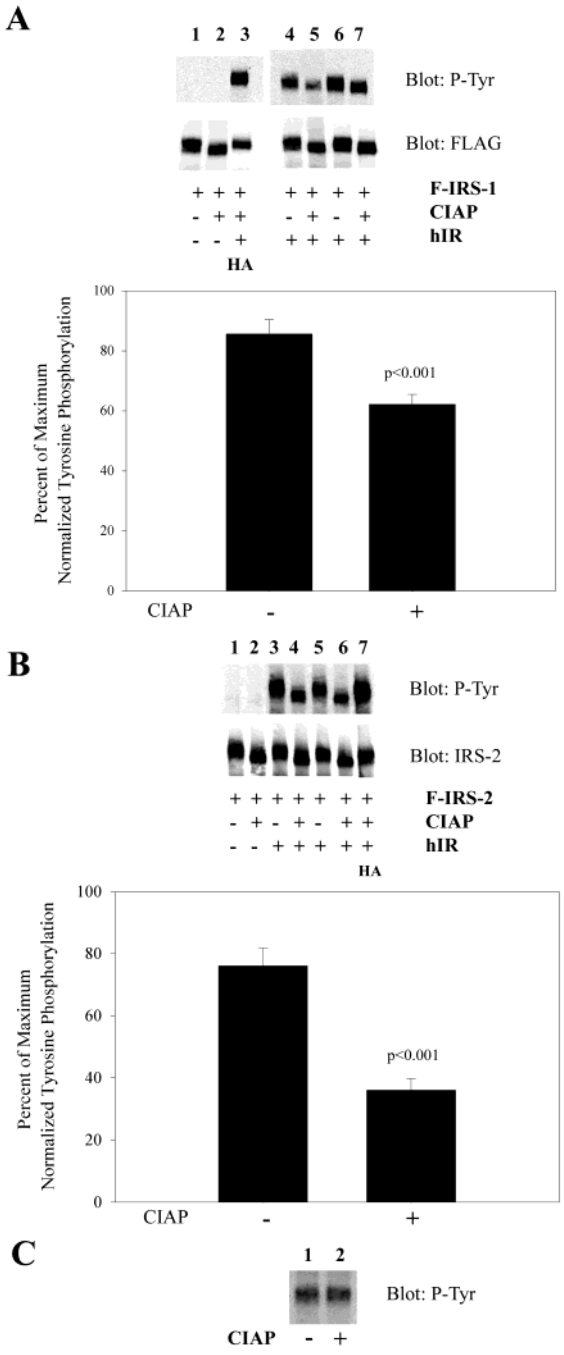

FIGURE 3: Dephosphorylation of recombinant IRS proteins inhibits human insulin receptor catalyzed IRS tyrosine phosphorylation. F-IRS-1 (A) or F-IRS-2 (B) was treated with (+) or without (−) calf intestinal alkaline phosphatase or with heat-inactivated (HA) calf intestinal alkaline phosphatase as described in Materials and Methods. Following immunoprecipitation with anti-FLAG agarose and washing, samples were incubated with activated human insulin receptor (hIR), as indicated. Samples were analyzed by SDS−PAGE using 6% Tris−glycine gels, transferred to nitrocellulose, and immunoblotted with anti-phosphotyrosine antibodies (P-Tyr). Nitrocellulose membranes were stripped with 0.1 M glycine, pH 3.5, and 0.2% SDS and reprobed with anti-FLAG (A) or anti-IRS-2 (B). Immunoreactive bands were directly quantitated using the Lumi-Imager Workstation and Lumi-Analyst software. Representative immunoblots are shown as well as the mean ± SE from at least three experiments each performed in quadruplicate. (C) Anti-FLAG agarose incubated with (+) or without (−) calf intestinal alkaline phosphatase, followed by washing and incubation with activated hIR, was analyzed by SDS−PAGE using 8% Tris−glycine gels, transferred to nitrocellulose, and immunoblotted with anti-phosphotyrosine antibodies (P-Tyr).

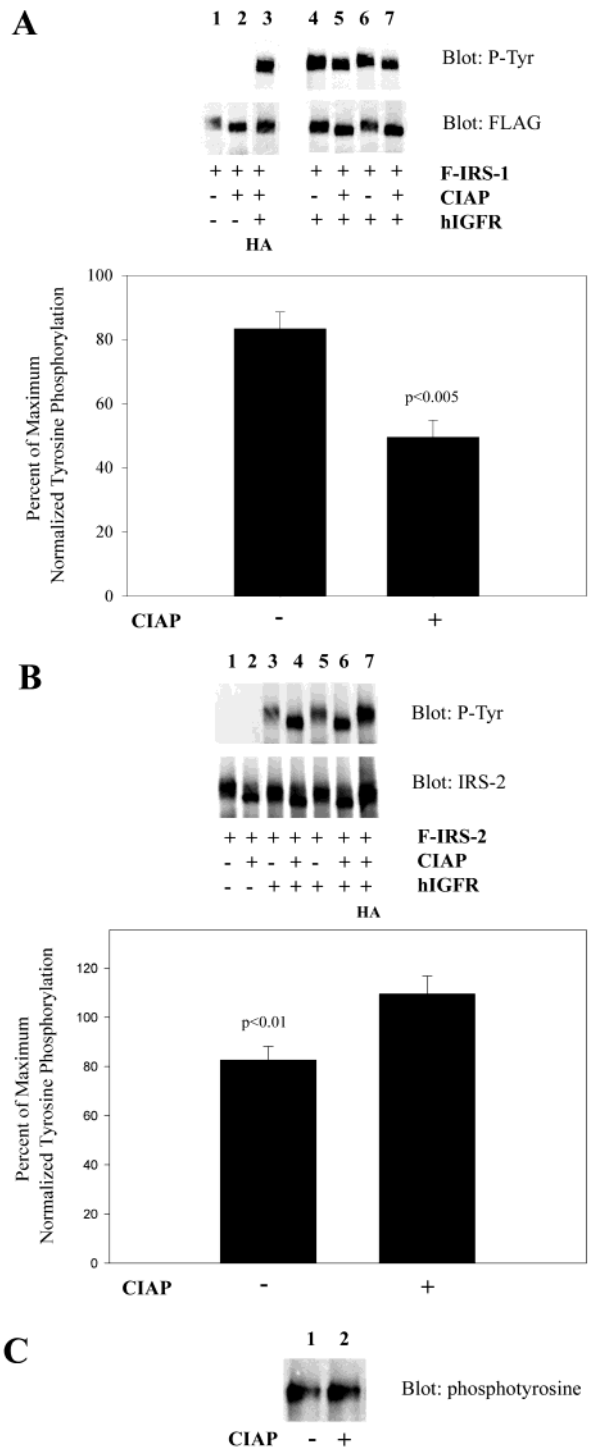

FIGURE 4: Dephosphorylation of recombinant IRS-1 and IRS-2 inhibits and enhances, respectively, human IGF-I receptor catalyzed IRS tyrosine phosphorylation. F-IRS-1 (A) or F-IRS-2 (B) was treated with (+) or without (−) calf intestinal alkaline phosphatase or with heat-inactivated (HA) calf intestinal alkaline phosphatase as described in Materials and Methods. Following immunoprecipitation with anti-FLAG agarose and washing, samples were incubated with activated human IGF-I receptor (hIGFR), as indicated. Samples were analyzed by SDS−PAGE using 6% Tris−glycine gels, then transferred to nitrocellulose, and immunoblotted with anti-phosphotyrosine antibodies (P-Tyr). Nitrocellulose membranes were stripped with 0.1 M glycine, pH 3.5, and 0.2% SDS and reprobed with anti-FLAG (A) or anti-IRS-2 (B). Immunoreactive bands were directly quantitated using the Lumi-Imager Workstation and Lumi-Analyst software. Representative immunoblots are shown as well as the mean ± SE from at least two experiments each performed in quadruplicate.

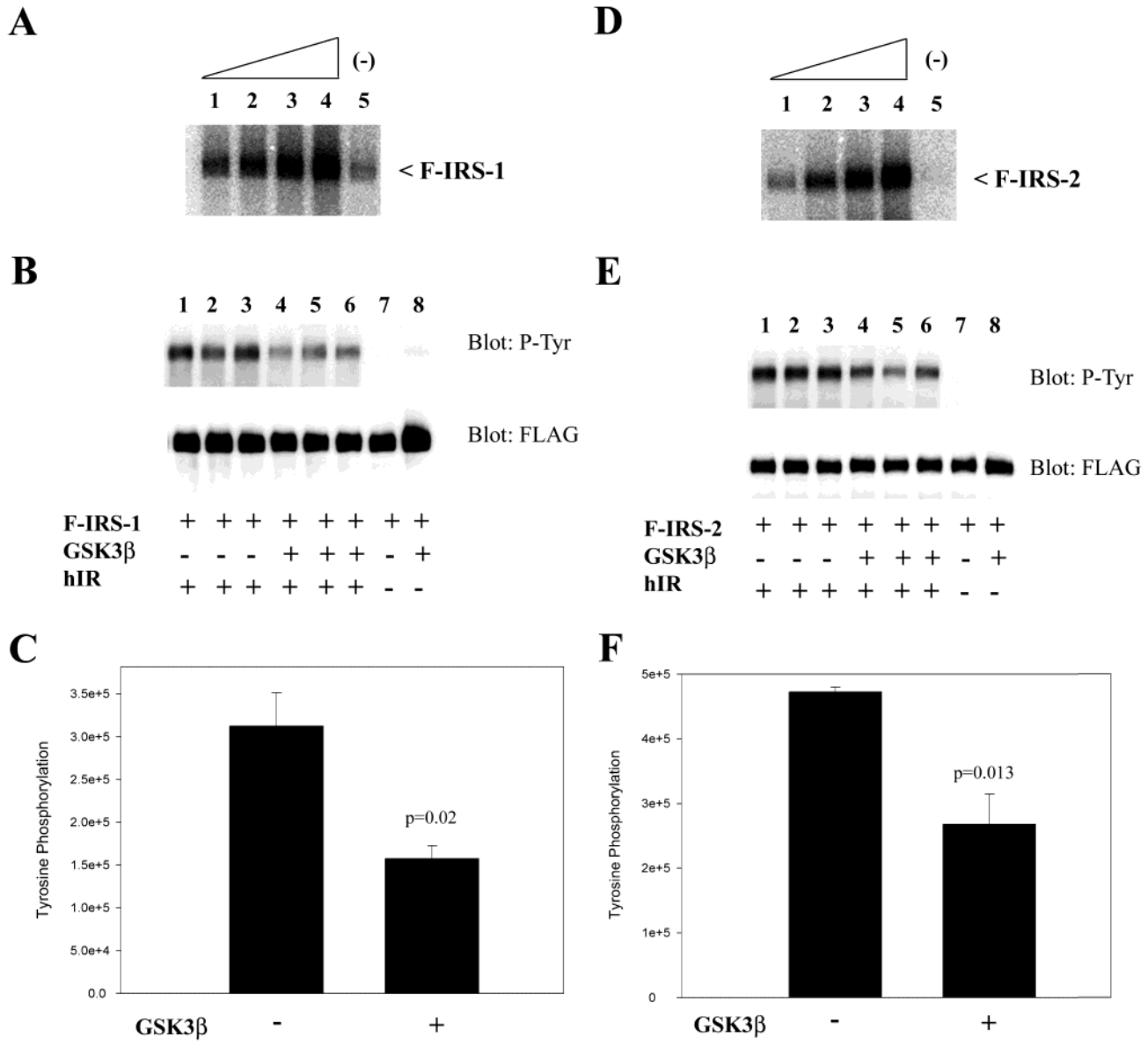

FIGURE 5: Hyperphosphorylation of recombinant IRS proteins inhibits human insulin receptor catalyzed IRS tyrosine phosphorylation. F-IRS-1 (A) or F-IRS-2 (D) was treated with 50−350 milliunits of GSK-3β (lanes 1−4) or without (−) in the presence of [γ-^{32}P]ATP. Dual kinase assays were performed with F-IRS-1 (B) or F-IRS-2 (E) treated with 350 milliunits of GSK-3β followed by incubation with activated human insulin receptor (hIR) as described in Materials and Methods. Samples were divided and analyzed in parallel by SDS−PAGE using 8% Tris−glycine gels, transferred to nitrocellulose, and immunoblotted with anti-phosphotyrosine (P-Tyr) and anti-FLAG antibodies. Immunoreactive bands in (B) and (E) were directly quantitated using the Lumi-Imager Workstation and Lumi-Analyst software, and the data are represented as the mean ± SE in (C) and (F), respectively. Similar results were observed in two independent experiments, each performed in triplicate.

phosphorylation as assessed by anti-phosphotyrosine immunoblotting (Figure 4A). In marked contrast to the reduction of F-IRS-1 tyrosine phosphorylation, hIGFR-stimulated tyrosine phosphorylation of F-IRS-2 increased 32.7% ($p < 0.01$) after dephosphorylation (Figure 4B), suggesting differential regulation of IRS-1 and IRS-2 tyrosine phosphorylation by the IGF-I receptor. No alteration in IGF receptor β-subunit autophosphorylation was observed following incubation of anti-FLAG agarose with or without calf intestinal alkaline phosphatase, followed by washing and incubation with activated hIGFR (Figure 4C).

Hyperphosphorylation of Recombinant IRS Proteins Inhibits Human Insulin Receptor Catalyzed IRS Tyrosine Phosphorylation. Recombinant F-IRS-1 and F-IRS-2 are in vitro substrates for GSK3β (Figure 5A,D), and both IRS proteins could be transformed into poorer substrates for the insulin receptor tyrosine kinase by hyperphosphorylation with this kinase (Figure 5). In agreement with Eldar-Finkelman and Krebs (*13*), prephosphorylation of IRS-1 resulted in a 50% reduction in insulin receptor catalyzed tyrosine phosphorylation (Figure 5B,C). A 44% reduction in insulin receptor catalyzed tyrosine phosphorylation of F-IRS-2 prephosphorylated with GSK3β was also observed (Figure 5E,F). No difference was observed in insulin receptor β-subunit autophosphorylation, when activated insulin receptors were incubated with or without GSK3β (data not shown). Thus, the effect of hyperphosphorylation of Ser/Thr sites of IRS-1 and IRS-2 on subsequent tyrosine phosphorylation by the insulin receptor is similar in magnitude to that observed in response to dephosphorylation of basal Ser/Thr sites.

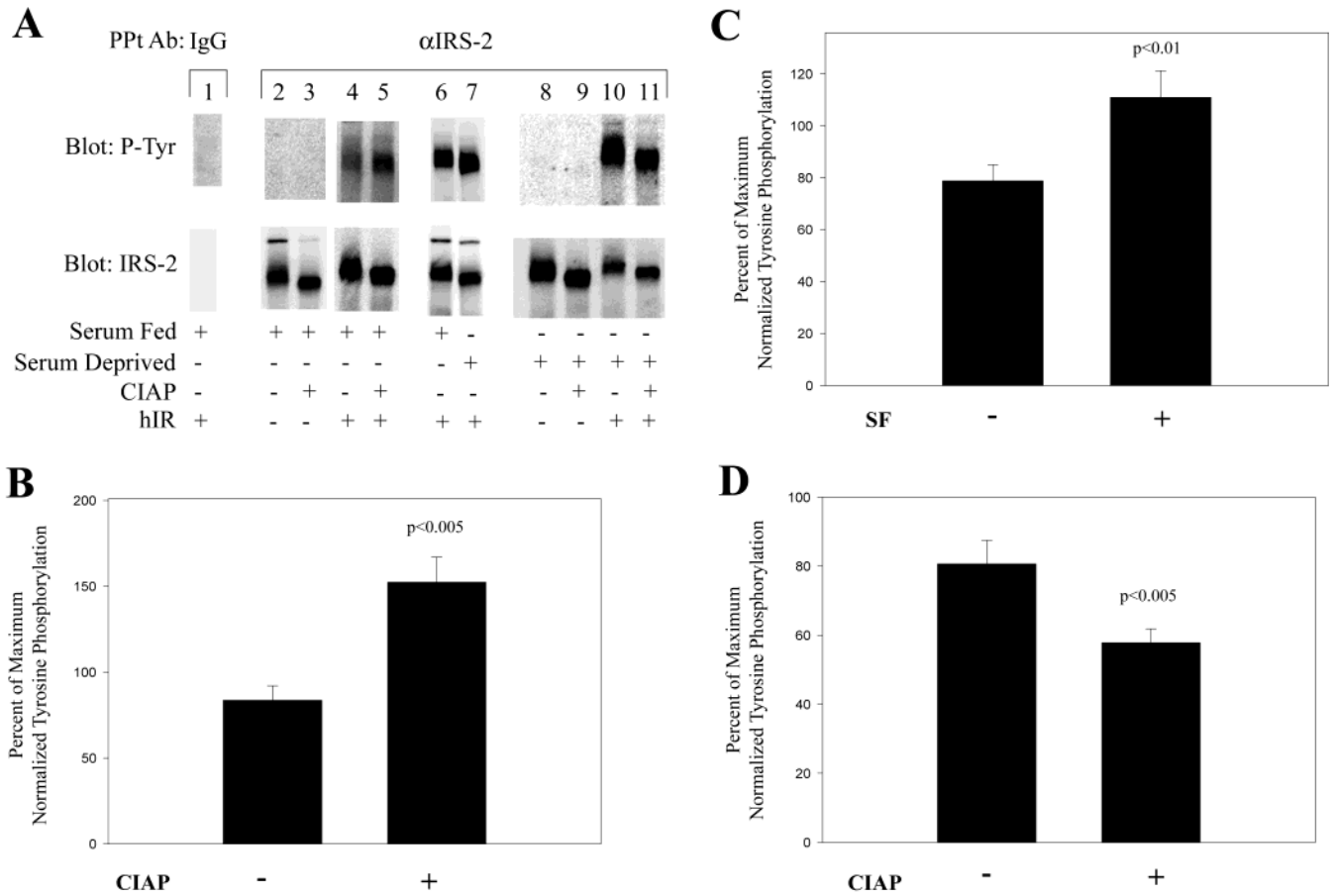

FIGURE 6: Effect of dephosphorylation of IRS-2 immunoprecipated from 3T3-L1 adipocytes on insulin receptor catalyzed tyrosine phosphorylation. (A) Anti-IRS-2 (lanes 2−11) or IgG (lane 1) immunoprecipitates from 3T3-L1 adipocytes incubated for 5 h in DMEM containing 10% FBS (lanes 1−6) or serum-free DMEM containing 0.1% BSA (lanes 7−11). Washed immunoprecipitates were incubated with (+) or without (−) 37.5 units of calf intestinal alkaline phosphatase as indicated. Following a second washing, the immunoprecipitates were incubated with (+) or without (−) activated human insulin receptor (hIR) as described in Materials and Methods. Samples were analyzed by SDS−PAGE using 6% Tris−glycine gels, transferred to nitrocellulose, and immunoblotted with anti-phosphotyrosine antibodies (P-Tyr). Nitrocellulose membranes were stripped with 0.1 M glycine, pH 3.5, and 0.2% SDS and reprobed with anti-IRS-2 antibodies. Representative immunoblots are shown in (A). Immunoreactive bands were quantitated using the Lumi-Imager Workstation and Lumi-Analyst software. (B) Mean ± SE of two experiments represented by the bands in lanes 2−5 of (A). (C) Mean ± SE of two experiments represented by the bands in lanes 6 and 7 of (A). (D) Mean ± SE of three experiments represented by the bands in lanes 8−11 of (A). Individual experiments were performed in quadruplicate.

Effect of Dephosphorylation of IRS Proteins Immunoprecipated from 3T3-L1 Adipocytes on Insulin Receptor Catalyzed Tyrosine Phosphorylation. To confirm that the modulation of insulin receptor catalyzed tyrosine phosphorylation observed with recombinant IRS-1 and IRS-2 was functionally relevant, the ability of endogenously Ser/Thr phosphorylated IRS-1 and IRS-2 immunoprecipitated from 3T3-L1 adipocytes to undergo tyrosine phosphorylation in vitro was examined. IRS-1 and IRS-2 were immunoprecipitated from serum-fed or serum-deprived 3T3-L1 adipocyte cell lysates, then incubated in the absence or presence of calf intestinal alkaline phosphatase, and washed, followed by incubation with activated insulin receptors as described in Materials and Methods. The electrophoretic mobility of IRS-2, immunoprecipitated from serum-fed cells, was increased by incubation with alkaline phosphatase (compare Figure 6A, lower panel, lane 2 with lane 3) or serum deprivation (compare Figure 6A, lower panel, lane 6 with lane 7), suggesting that both treatments dephosphorylate IRS-2 on Ser/Thr residues. Both alkaline phosphatase treatment (Figure 6B) and serum deprivation (Figure 6C) significantly improved the ability of IRS-2 from serum-fed cells to be phosphorylated on tyrosine by the insulin receptor kinase by 82.4% ($p < 0.005$) and 40.7% ($p < 0.005$), respectively. Conversely, further dephosphorylation of IRS-2 immunoprecipitated from serum-deprived 3T3-L1 adipocytes by alkaline phosphatase treatment resulted in a reduction of 28.4% ($p < 0.005$) in insulin receptor catalyzed tyrosine phosphorylation as assessed by anti-phosphotyrosine immunoblotting (Figure 6D). Similar to IRS-2, dephosphorylation of IRS-1 from serum-deprived 3T3-L1 adipocyte cell lysates resulted in an increase in electrophoretic mobility and a significant reduction in insulin receptor stimulated tyrosine phosphorylation as assessed by anti-phosphotyrosine immunoblotting (Figure 7).

DISCUSSION

IRS proteins play a central role in the propagation of the insulin signal (*5*, *6*). Ser/Thr phosphorylation of IRS proteins is a sensitive mechanism for downregulation of insulin signaling after insulin stimulation. Indeed, IRS-1 immunoprecipitated from insulin-stimulated cells exhibits increased Ser/Thr phosphorylation, which impairs its interaction with the juxtamembrane region of the insulin receptor (*19*). Treatment of cells with agents that induce insulin resistance,

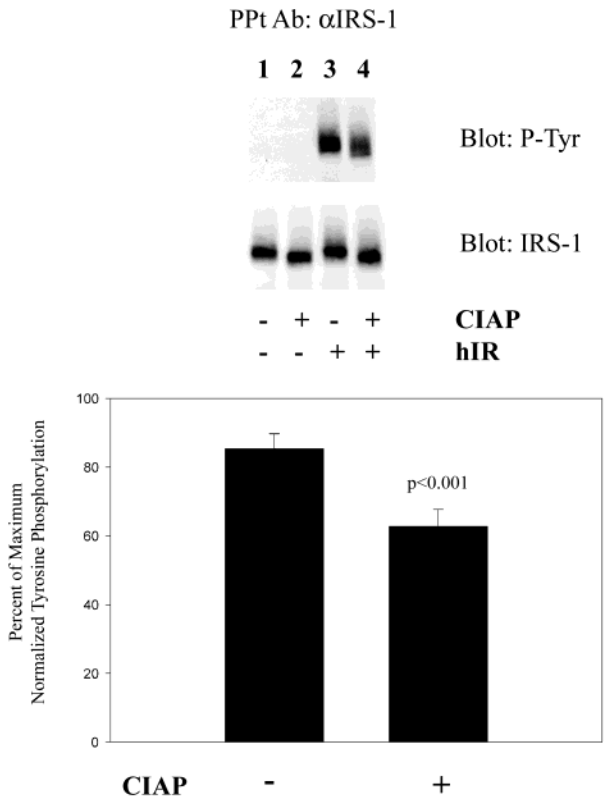

FIGURE 7: Dephosphorylation of IRS-1 immunoprecipated from serum-deprived 3T3-L1 adipocytes inhibits human insulin receptor catalyzed IRS-1 tyrosine phosphorylation. Anti-IRS-1 immunoprecipitates from serum-deprived 3T3-L1 adipocytes were washed and incubated with (+) or without (−) 37.5 units of calf intestinal alkaline phosphatase as indicated. Washed immunoprecipitates were incubated with (+) or without (−) 37.5 units of calf intestinal alkaline phosphatase as indicated. Following a second washing, the immunoprecipitates were incubated with (+) or without (−) activated human insulin receptor (hIR) as described in Materials and Methods. Samples were analyzed by SDS−PAGE using 6% Tris−glycine gels, transferred to nitrocellulose, and immunoblotted with anti-phosphotyrosine antibodies (P-Tyr). Nitrocellulose membranes were stripped with 0.1 M glycine, pH 3.5, and 0.2% SDS and reprobed with anti-IRS-1. Immunoreactive bands were directly quantitated using the Lumi-Imager Workstation and Lumi-Analyst software and are represented as the mean ± SE of the data. Similar results were observed in two independent experiments, each performed in quadruplicate.

including TNFα, PDGF, high glucose, okadaic acid, calyculin A, or TPA, results in hyper-Ser/Thr-phosphorylated IRS proteins that exhibit reduced insulin-stimulated tyrosine phosphorylation (*12*, *17*−*21*). Thus, evidence suggests that Ser/Thr phosphorylation of IRS proteins is an inhibitory event in insulin signaling that may be inappropriately elevated under conditions of pathological insulin resistance (*28*).

In addition to an inhibitory role, our data suggest that basal Ser/Thr phosphorylation of IRS proteins plays an important positive or permissive role in subsequent tyrosine phosphorylation by the insulin and IGF-I receptor kinases. Two experimental results support this conclusion. First, recombinant IRS-1 and IRS-2, affinity purified from Sf-9 cell lysates as Ser/Thr-phosphorylated but not tyrosine-phosphorylated proteins, are poorer substrates for the insulin receptor in vitro after dephosphorylation with alkaline phosphatase. Recombinant IRS-1 is also a poorer substrate for the IGF-I receptor in vitro after incubation with alkaline phosphatase. Second, although serum deprivation of 3T3-L1 adipocytes enhances tyrosine phosphorylation of IRS-1 and IRS-2, further dephosphorylation of IRS-1 and IRS-2, immunoprecipitated from serum-deprived cells, with alkaline phosphatase impairs subsequent tyrosine phosphorylation by the insulin receptor kinase. Alkaline phosphatase treatment of IRS-2 from serum-fed and serum-deprived cells increased their electrophoretic mobility in both cases, yet led to opposite effects on tyrosine phosphorylation. This suggests that not all Ser/Thr phosphorylation is equal and that the effects of phosphorylation of basal sites are distinct from those imparted by hyperphosphorylation. Notably, dephosphorylation of IRS-1 or IRS-2 from serum-fed adipocytes by either serum deprivation or alkaline phosphatase treatment resulted in a Ser/Thr-phosphorylated species with an intermediate electrophoretic mobility that exhibited enhanced tyrosine phosphorylation. The inability of enzymatic dephosphorylation to convert the hyperphosphorylated form of IRS-2 from serum-fed cells completely to the hypophosphorylated, suboptimal substrate suggests that basal phosphorylation sites are inaccessible in hyperphosphorylated IRS proteins due either to conformation or to masking by other bound proteins. Our data suggest a relationship between Ser/Thr and tyrosine phosphorylation of IRS proteins. Both hypo- and hyperphosphorylated IRS proteins are poor substrates for the insulin receptor, whereas IRS-1 or IRS-2 phosphorylated to an intermediate or basal level represents the optimal substrate. Modulation of Ser/Thr phosphorylation in either direction from this intermediate point, by dephosphorylation or masking of positive sites or phosphorylation at negative regulatory sites, may determine the extent of IRS protein tyrosine phosphorylation in response to insulin. Thus, our results suggest that some Ser/Thr phosphorylation of IRS-1 and IRS-2 is required for optimal tyrosine phosphorylation by the insulin receptor, although the specific Ser/Thr residues or the actual number of sites that are required for optimal tyrosine phosphorylation remains to be determined. In addition, it will be of interest to determine if the basal sites of Ser/Thr phosphorylation influence the metabolic and/or mitogenic signaling pathways of insulin and if this differs for IRS-1 and IRS-2. Further study is required to determine the sites of basal phosphorylation and whether phosphorylation of basal Ser/Thr sites is modulated in response to external stimuli or, conversely, is constitutive and part of IRS protein biosynthesis. Even if phosphorylation of basal sites is primarily permissive and not modulated in response to external signals, the finding that some level of Ser/Thr phosphorylation is required for IRS proteins to function as optimal insulin receptor substrates suggests that a defect in a positive regulatory IRS Ser/Thr kinase may be another mechanism contributing to insulin resistance.

Multiple Ser/Thr kinases, such as PI-3 kinase, GSK3, MAP kinase, and JNK1, have been proposed to be involved in transforming IRS-1 into a poorer substrate for the insulin receptor kinase (*12*−*14*, *29*). The data presented here indicate that, like IRS-1 (*13*), phosphorylation of IRS-2 by GSK3β inhibited subsequent tyrosine phosphorylation by the insulin receptor. However, the kinase(s) responsible for positive regulatory basal Ser/Thr phosphorylation of IRS proteins is (are) not known and may be distinct from those involved in negative regulation. The mechanism whereby basal Ser/Thr phosphorylation of IRS proteins enhances tyrosine phosphorylation may involve changes in IRS conformation or

association with other proteins. A recent report indicated that basal Ser/Thr-phosphorylated IRS-1 was associated with 14-3-3β proteins and that this association was reduced by alkaline phosphatase treatment (*30*), suggesting that IRS proteins in the basal state are already complexed with signaling proteins. PKB and PKC-ζ have been reported to associate with and phosphorylate IRS-1 in cells prior to insulin stimulation, although the amount of associated kinases increases in stimulated cells (*31*, *32*). PKB phosphorylation of IRS-1 exerted a positive influence on IRS-1 tyrosine phosphorylation but appeared to delay its dephosphorylation rather than increase the maximum extent of tyrosine phosphorylation (*32*). Notably, a Ser/Thr kinase activity was observed in our studies to be associated with purified, recombinant F-IRS-1 but not F-IRS-2 (Figure 5), suggesting differential complex formation by the two IRS proteins.

Contrary to IRS-1, dephosphorylation of basal Ser/Thr sites on F-IRS-2 enhanced its phosphorylation by the IGFR kinase (Figure 4). This suggests that IRS-2 in its basal phosphorylated state is optimized for phosphorylation by the insulin receptor rather than the IGF-I receptor. These differences in tyrosine phosphorylation of F-IRS-1 and F-IRS-2 by the IGF receptor kinase suggest functional distinctions between these two proteins in IGF signaling. Indeed, it has been suggested from studies in hepatocytes, where ablation of IR selectively reduces IRS-2 but not IRS-1 tyrosine phosphorylation and insulin action, that IRS-2 is coupled to the insulin receptor while IRS-1 is coupled to the IGF-I receptor (*33*). In addition, it has been suggested from gene ablation studies in mice that IRS-1 is the primary mediator of IGFR-dependent somatic growth while IRS-2 is the primary mediator of glucose homeostasis (*10*). It is interesting also to note that IRS-2 contains two domains, the PTB and SAIN/KRLB domains, that can strongly interact with the insulin and IGF receptors as compared to IRS-1, which has only the PTB domain (*34*−*38*). The SAIN/KRLB domain of IRS-2 contains at least 14 consensus Ser/Thr phosphorylation sites for multiple Ser/Thr kinases, suggesting that this domain may be involved in differential modulation.

The data presented here suggest that Ser/Thr phosphorylation of IRS proteins acts as a bidirectional sensor regulating the proximal steps of insulin and IGF-I signaling. Basal Ser/Thr phosphorylation of IRS proteins appears to be required for optimal phosphorylation by the insulin and IGF-I receptor kinases, whereas hyper-Ser/Thr-phosphorylation negatively modulates IRS tyrosine phosphorylation. Determination of the residues involved in both basal phosphorylation and hyperphosphorylation and the effects of their phosphorylation on IRS-1 and IRS-2 conformation and interactions with the receptors and other proteins are likely to be of critical importance to our full understanding of insulin action and insulin resistance.

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