14-3-3 Protein Binds to Insulin Receptor Substrate-1, One of the Binding Sites of Which Is in the Phosphotyrosine Binding Domain*

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Insulin binding to its receptor induces the phosphorylation of cytosolic substrates, insulin receptor substrate (IRS)-1 and IRS-2, which associate with several Src homology-2 domain-containing proteins. To identify unique IRS-1-binding proteins, we screened a human heart cDNA library with 32P-labeled recombinant IRS-1 and obtained two isoforms (ε and ζ) of the 14-3-3 protein family. 14-3-3 protein has been shown to associate with IRS-1 in L6 myotubes, HepG2 hepatoma cells, Chinese hamster ovary cells, and bovine brain tissue. IRS-2, a protein structurally similar to IRS-1, was also shown to form a complex with 14-3-3 protein using a baculovirus expression system. The amount of 14-3-3 protein associated with IRS-1 was not affected by insulin stimulation but was increased significantly by treatment with okadaic acid, a potent serine/threonine phosphatase inhibitor. Peptide inhibition experiments using phosphoserine-containing peptides of IRS-1 revealed that IRS-1 contains three putative binding sites for 14-3-3 protein (Ser-270, Ser-374, and Ser-641). Among these three, the motif around Ser-270 is located in the phosphotyrosine binding domain of IRS-1, which is responsible for the interaction with the insulin receptor. Indeed, a truncated mutant of IRS-1 consisting of only the phosphotyrosine binding domain retained the capacity to bind to 14-3-3 protein in vivo. Finally, the effect of 14-3-3 protein binding on the insulin-induced phosphorylation of IRS-1 was investigated. Phosphoamino acid analysis revealed that IRS-1 coimmunoprecipitated with anti-14-3-3 antibody to be weakly phosphorylated after insulin stimulation, on tyrosine as well as serine residues, compared with IRS-1 immunoprecipitated with anti-IRS-1 antibody. Thus, the association with 14-3-3 protein may play a role in the regulation of insulin sensitivity by interrupting the association between the insulin receptor and IRS-1.

Protein tyrosine kinases play key roles in transmitting extracellular signals that induce specific cellular events such as proliferation, differentiation, gene expression, and metabolism. These signals are propagated by sequential protein-protein interactions and the resulting protein phosphorylation cascade. Thus, to identify the molecule(s) associated with the same key molecules mediating these events constitutes a strategy for elucidating the signal transduction network. The CORT (cloning of receptor target) method is a modification of the expression cloning method, using tyrosine-phosphorylated growth factor receptor as a probe, which was originally established by Skolnik et al. (1) for the purpose of isolating cDNAs coding for proteins bound to the tyrosine-phosphorylated epidermal growth factor receptor.

In the case of insulin signaling, the first action exerted by insulin is activation of insulin receptor tyrosine kinase, which leads to the phosphorylation of several cytosolic substrates including insulin receptor substrate (IRS)-1/2 and IRS-2/3. IRS-1 possesses 21 potential tyrosine phosphorylation sites and functions as a "docking protein," transmitting insulin signals to several proteins containing Src-homology 2 (SH2) domains (4). Thus, similar methods have been employed successfully to clone cDNAs coding for the proteins that bind to phosphorylated IRS-1. To date, three novel regulatory subunits for phosphatidylinositol 3-kinase (p55γ, p56N, and p55γ) have been isolated by this method and reported. In addition, Fyn tyrosine kinase (9) was demonstrated to bind phosphorylated IRS-1.

In this study, to identify additional IRS-1-binding proteins, we screened a human heart cDNA library and obtained cDNAs coding for the 14-3-3 family proteins, which function as IRS-1-binding proteins. Recently, the roles of 14-3-3 proteins have been clarified with respect to the regulation of various signal transductions and enzyme activities (10–19). These proteins are known to bind to the phosphoserine-containing motifs in several proteins (20) but not to the phosphotyrosine-containing motifs in IRS-1.

**Experimental Procedures**

Expression Screening with Human [32P]IRS-1 Protein—Baculovirus-produced IRS-1 was labeled by means of incubation with activated insulin receptor in the presence of Mn2+ and γ-32P]ATP, as described previously (6). An oligo(dT)‐primed human heart cDNA library was prepared in Amelox (Novagen) according to the manufacturer’s instructions. A Amelox library was plated at 50,000 phages/plate in Escherichia coli. The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EMBL Data Bank with accession number(s) AB000729 (human IRS-2).

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The abbreviations used are: IRS, insulin receptor substrate; GST, glutathione S-transferase; CHO, Chinese hamster ovary; PH, pleckstrin homology; PTB, phosphotyrosine binding; PAGE, polyacrylamide gel electrophoresis; SH2, Src-homology 2.
coli strain BL21(DE3)pLysE. The 15-cm plates containing 2,000,000 plaques were incubated for 8 h at 37 °C and then overlaid with nitrocellulose filters (Millipore) that had been impregnated with isopropyl-
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\beta$-thiogalactopyranoside and incubated for 10 h at 37 °C. Hybridization of the filters with the 32P-labeled probe and washing were performed as described previously (21). The Southern blots were exposed to x-ray film and analyzed as described (21).

Preparation of Glutathione S-transferase (GST)-14-3-3 Fusion Protein—The cDNA encoding full-length human 14-3-3ε, cloned in our laboratory, was subcloned into a pGEX-4X-5 vector (Pharmacia Biotech Inc.) which was used to transform E. coli JM105 (Promega). The colony plasmids were grown on LB medium supplemented with 0.1 mg/ml ampicillin and 0.1% glucose and 37 °C. The plasmids were isolated from the cultures and transformed into E. coli JM105 (Promega). The transformed colonies were grown to an A600 of 0.6 in LB medium supplemented with 0.1 mg/ml ampicillin and then incubated in 3 ml of culture at 37 °C for 3 h. The culture supernatants were collected and the supernatants were dialyzed against 10 mM Tris, pH 7.6, 150 mM NaCl, and 1 mM dithiothreitol.

Antibodies–The antibodies against the COOH-terminal portions of IRS-1 and IRS-2 were prepared as described previously (21). Two kinds of polyclonal anti-14-3-3 antibody were generated. GST-14-3-3 or a 16 times tandemly linked anti-14-3-3 box 1 region, a highly conserved COOH-terminal acidic region derived from 14-3-3ε (amino acids 171–213) (22), was expressed in Escherichia coli and the affinity-purified GST fusion protein GST-14-3-3ε or GST-14-3-3β, respectively. The 14-3-3 box 1 antibody and GST-14-3-3ε antibody were used for immunoprecipitation and immunoblotting, respectively. The anti-14-3-3 box 3 antibody immunoprecipitated 1–2% of endogenous 14-3-3 protein with the procedure used in this study, as assessed by comparing the band intensities obtained by immunoblotting with the anti-14-3-3 antibody from anti-14-3-3 box 3 blotting with anti-IRS-1 antibody. The immunoprecipitates were dissolved in 1 ml of Laemmli buffer, and the bound proteins were analyzed by SDS-PAGE and subsequent immunoblotting with anti-IRS-1 antibody and subsequently by enhanced chemiluminescence.

Preparation of Bovine Brain Tissue—Bovine brain tissue was homogenized in 5 volumes of 25 mM Tris, pH 7.5, and the extract was obtained by centrifugation at 35,000 × g for 30 min at 4 °C. The supernatant was incubated with 20 μl of Protein A Sepharose (Pharmacia) for 1 h. The immunocomplexes were washed five times with the same lysis buffer and boiled in 3 ml of Laemmli sample buffer containing 10% glycine.

Phosphoamino Acid Analysis of 32P-Labeled IRS-1—The 32P-labeled IRS-1 was isolated by affinity chromatography on glutathione-Sepharose 4B (Pharmacia). Glutathione was removed by dialysis against phosphate-buffered saline containing 10 mM dithiothreitol.

Preparation of Glutathione-Sepharose 4B beads was incubated with 500 ng of GST-14-3-3 protein for 1 h. Then, the Glutathione-Sepharose 4B beads were added, and the cells were washed five times with the solubilizing buffer. The beads were boiled in Laemmli buffer, and the bound proteins were analyzed by SDS-PAGE and subsequent immunoblotting with anti-IRS-1 antibody.

Peptide Competition Experiments—Phosphorylated and nonphosphorylated serine-containing peptides were purchased from Research Genetics, Inc. The peptide sequences were LPRKINR[Ser]PESPLLHR ([ps] corresponds to Ser-635 in human IRS-1) used by Muslin et al.

Phosphoamino Acid Analysis of 32P-labeled IRS-2—The 32P-labeled IRS-2 was isolated by affinity chromatography. The 32P-labeled IRS-2 was isolated by affinity chromatography on glutathione-Sepharose 4B (Pharmacia). Glutathione was removed by dialysis against phosphate-buffered saline containing 10 mM dithiothreitol.
were hydrolyzed in 6 N HCl at 110 °C for 90 min. The hydrolyzed proteins were dried under a vacuum and resolved in 5 μl of H2O. Cold phosphoserine, phosphothreonine, and phosphotyrosine were added to 1-μl samples that were applied together onto a cellulose-precoated thin layer chromatography plate (Merek) and separated by electrophoresis utilizing an NA-4000 apparatus (Nihon-Eido, Tokyo) with pH 3.5 buffer containing 5% acetic acid and 0.5% pyridine. The location of the phosphoamino acids was determined by ninhydrin staining and autoradiography. The radioactivities were quantified using Bioimage analyzer BAS2000 (Fuji).

RESULTS

Expression Cloning of 14-3-3 Protein and Its Association with IRS-1—We have employed an expression cloning method using 32P-labeled recombinant IRS-1 as a probe to isolate cDNAs coding for the proteins that bind to IRS-1. In this study, 21 positive clones were isolated after three or four rounds of screening from a human heart cDNA library. Sequence analysis revealed that 7 of the 21 cDNA clones encode 14-3-3 protein ε or ζ isoforms. To confirm the in vivo binding of 14-3-3 protein with IRS-1, IRS-1 was overexpressed in L6 myotubes, HepG2 hepatoma cells, and CHO cells using an adeno-virus expression system (Fig. 1A, upper panel). The cell lysates of these cells overexpressing either IRS-1 or LacZ (control), were immunoprecipitated with anti-IRS-1 antibody and immunoblotted with anti-14-3-3 antibody. As shown in Fig. 1A, lower panel, the amounts of immunoprecipitated endogenous 14-3-3 proteins were increased markedly in the IRS-1-overexpressing cells compared with the control cells expressing lacZ, in all cell lines. In L6 and HepG2 cells, two bands were observed and were thought to represent different isoforms of 14-3-3 protein. Based upon the 14-3-3 protein band intensities in the lysates and the anti-IRS-1 immunoprecipitates and the relative amounts of each loaded, approximately 1% of endogenous 14-3-3 protein was immunoprecipitated with the IRS-1.

In addition, 14-3-3 protein either alone or in combination with IRS-1 or IRS-2 was overexpressed in SF9 cells using a baculovirus expression system (Fig. 1B, upper and middle panels). The 14-3-3 protein overexpressed was shown to be present in the anti-IRS-1 and anti-IRS-2 antibody immunoprecipitates from the cell lysates overexpressing both 14-3-3 protein and either IRS-1 or IRS-2, but not from those overexpressing only 14-3-3 protein (Fig. 1B, lower panel). IRS-1 and IRS-2 share a similar structure (3), and our results indicate that both IRS-1 and IRS-2 can form a complex with 14-3-3 protein in a variety of cultured cells.

To demonstrate that the association of 14-3-3 protein with IRS-1 is not limited to cultured cells but rather is also present in animal tissue, the bovine brain lysate was immunoprecipitated with the antibody against the 14-3-3 box I region (see “Experimental Procedures”). The immunoprecipitates were separated by micro two-dimensional PAGE and visualized by immunoblotting with anti-IRS-1 antibody. As shown in Fig. 2, the anti-14-3-3 box I antibody immunoprecipitate contained IRS-1 (left panel, indicated by an arrow), whereas the control IgG immunoprecipitate did not (right panel). In addition, in bovine brain tissue, 14-3-3 protein was shown to associate with IRS-1. These results suggest that IRS-1 exists as a complex form with 14-3-3 protein in all and most cell lines or tissues.

The Association of 14-3-3 Protein with IRS-1 Was Unaffected by Insulin Stimulation but Increased by Okadaic Acid Treatment—Next, we investigated which factors regulate the association of 14-3-3 protein with IRS-1. Because 14-3-3 protein contains no SH2 domain (10), we speculated that the tyrosine phosphorylation of IRS-1 is not necessary for the association with these 14-3-3 proteins. Indeed, recent reports have shown that members of the 14-3-3 protein family bind to the specific phosphoserine-containing motif (20, 24). It has been shown that IRS-1 is heavily phosphorylated on serine and threonine residues even in the basal condition (26), as demonstrated by treatment with alkaline phosphatase, which reduced the molecular mass of IRS-1 on the SDS-PAGE by 30–40 kDa (data not shown). In addition to basal phosphorylation, the increase in serine phosphorylation of IRS-1 which is induced by insulin stimulation occurs more slowly and is less marked than the increase in tyrosine phosphorylation (26). First, we investigated the effect of insulin stimulation on the association of 14-3-3 protein with IRS-1 at various periods after the addition of insulin.
Fig. 2. The association of 14-3-3 protein with IRS-1 in bovine brain. Bovine brain tissue was homogenized in 5 volumes of 25 mM Tris-HCl, pH 7.5, and the extract was obtained by centrifugation for 30 min at 15,000 × g. The extract (250 μl, approximately 2 mg of protein) was immunoprecipitated with anti-14-3-3 box I antibody (20 μg) or with control rabbit IgG and then separated by micro two-dimensional polyacrylamide gel electrophoresis as described under "Experimental Procedures." The proteins were visualized by immunoblotting with anti-IRS-1 antibody and then by enhanced chemiluminescence. The profiles of the anti-14-3-3 box I antibody immunoprecipitates (left panel) and of control IgG (right panel) are shown. IRS-1 was detected only in the immunoprecipitate with anti-14-3-3 box I antibody at a position corresponding to pI = 4.8 and an apparent molecular mass of 180 kDa (indicated by the arrow). The data are representative of three independent experiments.

of insulin. As shown in Fig. 3A, overexpression levels of IRS-1 were not changed before and after the addition of insulin (upper panel). Tyrosine phosphorylation of overexpressed IRS-1 increased at 10 and 30 min after the addition of insulin (middle panel). However, no significant difference was observed in the amount of 14-3-3 protein associated with IRS-1 for the initial 30 min after the addition of insulin. These results suggest that the tyrosine phosphorylation, as well as the serine/threonine phosphorylation, of IRS-1 induced by insulin stimulation did not affect the association of 14-3-3 protein with IRS-1.

Treatment with okadaic acid, a potent cell-permeable serine/threonine phosphatase inhibitor, also increases the serine phosphorylation of IRS-1 and reportedly induces the suppression of tyrosine phosphorylation of IRS-1 by 100 nM okadaic acid. In contrast to the case of insulin stimulation, treatment with okadaic acid was shown to increase the association of 14-3-3 protein with IRS-1 by 80% (Fig. 3B, lower panel). The lysates from the cells treated with okadaic acid were revealed to contain larger amounts of IRS-1 capable of binding to GST-14-3-3 compared with control cell lysates (Fig. 3B, middle panel), with no alteration of the total IRS-1 content in the cells (Fig. 3B, upper panel). These results indicate that the serine residues in IRS-1, the phosphorylation level of which is increased by okadaic acid but not by insulin stimulation, play a critical role in the association with 14-3-3 protein.

Binding Sites of IRS-1 with 14-3-3 Protein—A previous study showed that 14-3-3 proteins bind to phosphorylated serine residues within the consensus Arg-Ser-Xaa-pSer-Xaa-Pro (where Xaa is any amino acid) motif (24). Although this motif has been shown to be present in various proteins bound to 14-3-3 proteins (24), human IRS-1 does not contain this motif, instead having three similar amino acid sequences. These are the sequences including Ser-270 (DEFRPRSK[Q]SSSNC, Ser-374 (HPPLNHSR[Q]IPMPAS), and Ser-641 (MPMSPKSV[Q]APQQL) (SI) (S) is the serine residue corresponding to phosphoserine in the 14-3-3 binding motif). To investigate whether these sequences are potential 14-3-3 binding sites, we prepared phosphorylated or nonphosphorylated serine-containing peptides corresponding to the sequences around Ser-270, Ser-374, and Ser-641 in IRS-1, as described under "Experimental Procedures." We utilized the peptide around Ser-621 in Raf-1, which was shown to inhibit 14-3-3 binding (24), as a positive control.

We examined whether these peptides inhibit the binding of IRS-1 and GST-14-3-3. As shown in Fig. 4A, all three phosphoserine-containing peptides inhibited the in vitro binding of GST-14-3-3 with IRS-1 to an extent similar to that observed for Raf-1 phosphopeptide (upper panel), whereas none of the corresponding nonphosphorylated peptides affected this association (lower panel). The results of the peptide competition experiments suggested that these three phosphoserine-containing motifs in IRS-1 can bind to the same COOH-terminal acidic portion of 14-3-3 protein to which Raf-1 binds (22, 28, 29). The inhibition produced by the phosphoserine peptide corresponding to Ser-374 was weaker than those of the other phosphoserine peptides, suggesting that Ser-374 may have a lower affinity for 14-3-3 than Ser-270 and Ser-641. Recently, Zha et al. (20) suggested that RSXSXP and overlapping RXRXXS motifs found in Ser-259 in Raf-1 and Ser-112 and Ser-136 in BAD exhibit a high capacity for binding with 14-3-3 proteins. Among the three 14-3-3 binding motifs of IRS-1, only the motif around Ser-270 possesses the overlapping 14-3-3 binding motifs, although the two proline residues upstream from phosphoserine are replaced with serine.

Ser-270 is within the PTB domain of IRS-1, which plays an important role in the association with the insulin receptor. Reportedly, the NPXY motif including Tyr-960 in the juxtamembrane region of the insulin receptor couples with the PTB domain of IRS-1, resulting in the tyrosine phosphorylation of IRS-1 (30). Thus, binding of the PTB domain of IRS-1 with another molecule may interrupt the association of IRS-1 with the insulin receptor, possibly functioning as a negative regulation mechanism for insulin signaling by suppressing the tyrosine phosphorylation of IRS-1. To confirm the association of the PTB domain of IRS-1 with 14-3-3 in vitro, we overexpressed full-length IRS-1 and its truncated mutants containing the PH and the PTB domains (IRS-1<sup>PH-PTB</sup>) or only the PTB domain (IRS-1<sup>PTB</sup>) in HepG2 cells, using the adenovirus expression system. Immunoblotting of the cell lysates with the antiserum against the whole IRS-1 molecule showed bands of 180, 55,
and ~35 kDa corresponding to whole IRS-1, IRS-1PH-PTB, and IRS1PTB, respectively. Fig. 4B shows that GST-14-3-3 binds to not only IRS-1 but also IRS-1PH-PTB and IRS1PTB, whereas GST alone binds neither IRS-1 nor its truncated mutants. Based upon the band intensities in the lysates and the GST-14-3-3 precipitates and the relative amount of each loaded, approximately 6% of IRS-1 and IRS-1PH-PTB and 30% of IRS1PTB in cell lysate (2% of a 10-cm plate) (lanes 1–3), bound to GST-14-3-3 (derived from 30% of a 10-cm plate) (lanes 4–6), and bound to GST alone (lanes 7–9). The data are representative of three or five independent experiments.

Phosphorylation State of IRS-1 Associated with 14-3-3 Protein—Finally, we performed an experiment to investigate the effect of the association of 14-3-3 proteins on the function of IRS-1. To date, numerous biological activities have been attributed to 14-3-3 proteins. They were first suggested to be activators of tyrosine/tryptophan hydroxylase (10, 11) and then identified as regulators of protein kinase C (12). In addition, 14-3-3 proteins are required cofactors for the bacterial toxin of Pseudomonas, an ADP-ribosylase known as exoenzyme S (14), and have also been implicated in cell cycle control (15). More recently, 14-3-3 proteins were found to interact with several important molecules that modulate signal transduction pathways, including Raf-1 (16, 17), polyoma middle T antigen (18), Bcr (19), and the cell death agonist BAD (20). In these cases, 14-3-3 proteins do not transmit the signal directly, but rather they modulate the functions of associated proteins. The roles of IRS-1 and IRS-2 are thought to involve signal transmission from the insulin receptor downstream to several SH2 proteins via their tyrosine-phosphorylated binding sites. Thus, we investigated the effect of 14-3-3 protein association with respect to the insulin-induced phosphorylation of IRS-1. To do so, we compared the tyrosine and serine phosphorylation levels of IRS-1 immunoprecipitated with anti-IRS-1 antibody with those coimmunoprecipitated with the anti-14-3-3 box I antibody. First, tyrosine phosphorylation levels of the IRS-1 were compared (Fig. 5A). Judging from quantitation of the band inten-
sities and the ratios of phosphotyrosine to IRS-1 on the blots, insulin-induced tyrosine phosphorylation of IRS-1 was increased approximately 4.5-fold in anti-IRS-1 immunoprecipitates but was increased by only 12% in that with anti-14-3-3 antibody. These results suggest that the IRS-1 coimmunoprecipitated with the anti-14-3-3 box I antibody was more weakly tyrosine phosphorylated with insulin stimulation than the IRS-1 immunoprecipitated with anti-IRS-1 antibody. Based upon the IRS-1 band intensities in the immunoprecipitates with anti-IRS-1 and anti-14-3-3 antibody and the relative amounts of each loaded, the IRS-1 coimmunoprecipitated with anti-14-3-3 antibody amounted to about 5% of the IRS-1 immunoprecipitated with anti-IRS-1 antibody. Because anti-IRS-1 antibody immunoprecipitates about 80% of IRS-1, as assessed by immunoblotting the lysate before and after immunoprecipitation (data not shown), IRS-1 coimmunoprecipitated with anti-14-3-3 antibody amounted to approximately 4% of total IRS-1.

In addition, employing phosphoamino acid analysis, the incorporations of $^{32}$P into serine and tyrosine residues were compared between the IRS-1 in the anti-IRS-1 antibody immunoprecipitate and the IRS-1 in the anti-14-3-3 box I antibody immunoprecipitate. HepG2 cells overexpressing IRS-1 were labeled with $[^{32}P]$orthophosphate as described under “Experimental Procedures.” The cells were solubilized, and the lysate was immunoprecipitated using anti-IRS-1 or anti-14-3-3 box I antibody.

**Fig. 5.** Effect of the association with 14-3-3 protein on insulin-induced IRS-1 phosphorylation. Panel A, HepG2 cells overexpressing IRS-1 were incubated with or without $10^{-7}$ M insulin for 30 min. The cells were solubilized with 1 ml of lysis buffer, and the lysate was immunoprecipitated with anti-IRS-1 antibody (upper panel) or anti-phosphotyrosine antibody (lower panel). In each lane, 5 µl (anti-IRS-1 immunoprecipitates) and 20 µl (anti-14-3-3 immunoprecipitates) were loaded. Panel B, HepG2 cells overexpressing IRS-1 were labeled with $[^{32}P]$orthophosphate as described under “Experimental Procedures.” The cells were solubilized, and the lysate was immunoprecipitated using anti-IRS-1 or anti-14-3-3 box I antibodies. The immunoprecipitates were subjected to SDS-PAGE and transferred to polyvinylidine difluoride membranes. The $^{32}$P-labeled IRS-1 were detected by autoradiography (upper panel), and the bands corresponding to IRS-1 were excised. The incorporated $^{32}$P was determined by phosphoamino acid analysis as described under “Experimental Procedures” (lower panel). The data are representative of three independent experiments. Panel C, insulin-induced tyrosine and serine phosphorylation of IRS-1 in the anti-IRS-1 and anti-14-3-3 antibody immunoprecipitates. After the phosphoamino acid analysis, the radioactivities of $^{32}$P incorporated into tyrosine and serine residues were determined using Bioimage analyzer BAS2000 (Fuji). Fold increases after insulin stimulation are shown. Bars indicate standard errors from three independent experiments.

**Fig. 6.** Sequential alignments of PTB domains identified in various proteins. PTB domains in IRS-1 and IRS-2 are aligned with the putative PTB domains of various proteins (3, 37). Gaps are indicated by dashed lines. Numbers in parentheses indicate the numbers of omitted amino acids. The α-helices and β-sheet strands are boxed (36). The putative 14-3-3 binding motif is also boxed.
antibodies. As shown in the upper panel of Fig. 5B, the bands corresponding to $^{32}$P-labeled IRS-1 were clearly observed in the immunoprecipitates obtained with the anti-IRS-1 antibodies and with the anti-14-3-3 box I antibody. The IRS-1 proteins in these bands were hydrolyzed and subjected to phosphoamino acid analysis (Fig. 5B, lower panel). The quantification of the incorporated $^{32}$P revealed insulin-induced tyrosine and serine phosphorylation of IRS-1 immunoprecipitated with anti-IRS-1 antibody and with anti-14-3-3 box I antibody (Fig. 5C). The incorporations of $^{32}$P into the tyrosine and serine residues of IRS-1 in the anti-IRS-1 antibody immunoprecipitate were increased to 2.59- and 1.95-fold with insulin stimulation, respectively, whereas those in the anti-14-3-3 box I antibody immunoprecipitates were increased by only 14 and 2%, respectively. In these experiments, the incorporation of $^{32}$P into the threonine residue was below detectable limits. These results show that the small percentage of IRS-1 (5%) associated with the small percentage of 14-3-3 protein immunoprecipitated (1–2%) is less highly phosphorylated, suggesting that 14-3-3 protein has a higher affinity for less highly phosphorylated IRS-1. If such is the case, then association of 14-3-3 protein with IRS-1 may inhibit its phosphorylation on tyrosine and serine in response to insulin. We speculate that the most plausible explanation for reduced phosphorylation of IRS-1 is that the 14-3-3 protein binds to the PTB domain, thereby interrupting the optimal association between IRS-1 and the insulin receptor. Although the amount of 14-3-3 protein which associated with IRS-1 was unchanged after compared with before insulin stimulation (Fig. 3A), IRS-1 that was not associated with 14-3-3 protein was more highly phosphorylated than that which was associated with 14-3-3 protein, and total IRS-1 phosphorylation was increased, described in Fig. 5C.

In the insulin signaling, 14-3-3 protein may function as a negative regulator. Another reported example of the negative regulatory function of 14-3-3 protein is its effect on spinach leaf nitrate reductase (31). In this case, 14-3-3 proteins bind to a regulatory phosphoserine of nitrate reductase and thereby inactivate the enzyme.

**DISCUSSION**

Recently, extensive studies have been done to clarify the mechanism of insulin resistance, which is involved in the pathogenesis of non-insulin-dependent diabetes mellitus, as well as the factors determining insulin sensitivity. A number of reports have demonstrated that serine phosphorylation of IRS-1 affects its tyrosine phosphorylation (27, 32, 33). When the serine phosphorylation of IRS-1 is augmented by tumor necrosis factor-$\alpha$, which has been suggested to be a mediator of insulin resistance in obesity, insulin-induced tyrosine phosphorylation of IRS-1 is impaired (32). Tumor necrosis factor-$\alpha$ was also shown to induce serine phosphorylation of IRS-1 and to convert IRS-1 into an inhibitor of the insulin receptor tyrosine kinase activity in vitro (33). The 14-3-3 protein association with the serine-phosphorylated IRS-1, and possibly IRS-2, may contribute to the regulation of insulin sensitivity.

In this study, we have not determined whether IRS-1 is actually phosphorylated on the serine residues in the 14-3-3 protein binding motifs. However, if it is assumed that phosphorylation of these motifs is required for the observed association, then the amount of 14-3-3 protein associated with IRS-1 would be determined by the activity of an as yet unknown serine kinase(s) that phosphorylates the serine residue in the 14-3-3 binding motif of the target protein, rather than the level of 14-3-3 protein expression, because these proteins are extremely abundant (reportedly approximately 1% of total brain tissue-soluble proteins (34), probably more than 0.1% of cytosolic protein in most cells). Thus, to identify the serine kinase involved in the 14-3-3 protein association is essential for clarifying the regulatory mechanism of the 14-3-3 protein association with IRS-1.

In the case of tyrosine/tryptophan hydroxylase, it has been demonstrated that calmodulin kinase II phosphorylates the 14-3-3 binding motif of hydroxylase (10, 11). 14-3-3 proteins bind to phosphorylated hydroxylase, thereby activating the enzyme (35). In the case of BAD, heart muscle kinase (a form of protein kinase A) was shown to phosphorylate the serine residue in the 14-3-3 binding motif of BAD in vitro, but the kinase acting in vivo remains unknown (20).

We investigated whether PTB domains found in other proteins share a 14-3-3 protein binding motif similar to that of IRS-1 and IRS-2. Sequential alignments of PTB domains identified in various proteins are shown in Fig. 6. The $\alpha$-helices and $\beta$-sheet strands based on a structural study (36) and the putative 14-3-3 binding motif (around Ser-270) are boxed. Based on crystallographic study, $\beta_5, \beta_6, \beta_7$, and $\alpha_2$ of IRS-1 are important for recognizing phosphotyrosine in the insulin receptor NPXY motif (36). Because the putative 14-3-3 binding motif is next to the $\alpha_2$-helix, 14-3-3 protein binding to IRS-1 would presumably influence the binding of its PTB domain to the insulin receptor. Another interesting point is that IRS-1 and IRS-2 contain the insertion in the corresponding regions of other proteins and that the putative 14-3-3 binding motif is in this insertion sequence. Thus, the binding of 14-3-3 protein to the PTB domain may be specific to IRS-1 and IRS-2.

Further study is necessary to elucidate the physiological role of the 14-3-3 protein association with IRS-1 and IRS-2 in insulin signaling and the regulation of insulin sensitivity, as well as its possible involvement in the insulin resistance observed in non-insulin-dependent diabetes mellitus.

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**REFERENCES**

1. Skolnik, E. Y., Margolis, B., Mohammadi, M., Lowenstein, E., Fischer, R., Drepps, A., Ulrich, A., and Schlessinger, J. (1991) Cell 65, 83–90
2. Sun, X. J., Rothenberg, P., Kahn, C. R., Backer, J. M., Araki, E., Wilde, P. A., Cahill, D. A., Goldstein, J. B., and White, M. F. (1991) Nature 353, 73–77
3. Sun, X. J., Wang, L.-M., Zheng, Y., Yenush, L., Myers, M. G., Jr., Glashieen, E., Lane, W. S., Pierce, J. H., and White, M. F. (1995) Nature 377, 173–177
4. Sun, X. J., Crimmings, D. L., Myers, M. G., Jr., Miralpeix, M., and White, M. F. (1995) Mol. Cell. Biol. 15, 7418–7428
5. Ports, S., Asano, T., Glashieen, E., Miralpeix, M., Zhang, Y., Fisher, T. L., Myers, M. G., Jr., Sun, X. J., and White, M. F. (1995) Mol. Cell. Biol. 15, 4455–4465
6. Inukai, K., Anai, M., Van Breda, E., Hosaka, T., Katagiri, H., Funakaki, M., Fukushiyama, Y., Ogihara, T., Yazaki, Y., Kikuchi, M., Oka, Y., and Asano, T. (1996) J. Biol. Chem. 271, 5317–5320
7. Antonetti, D. A., Algenstaedt, F., and Kahn, C. R. (1996) Mol. Cell. Biol. 16, 2185–2203
8. Inukai, K., Funaki, M., Ogihara, T., Katagiri, H., Kanda, A., Anai, M., Fukushiyama, Y., Hosaka, T., Suzuki, M., Shin, B.-C., Takata, K., Yazaki, Y., Kikuchi, M., Oka, Y., and Asano, T. (1997) J. Biol. Chem. 272, 7873–7882
9. Sun, X. J., Ports, S., Asano, T., Myers, M. G., Jr., Glashieen, E., and White, M. F. (1996) J. Biol. Chem. 271, 10583–10587
10. Ichimura, T., Isobe, T., Nakajia, Y., Takahashi, N., Araki, K., Kawanor, R., and Takahashi, Y. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 7084–7088
11. Isobe, T., Ichimura, T., Sunaya, T., Okuyama, T., Takahashi, N., Kawanor, R., and Takahashi, Y. (1991) J. Mol. Biol. 217, 125–132
12. Aitken, A., Ellis, C. A., Sellers, L. A., and Toker, A. (1990) Nature 344, 594
13. Isobe, T., Hyiane, Y., Ichimura, T., Okuyama, T., Takahashi, N., Nakajo, S., and Nakaya, R. (1992) FEBS Lett. 308, 121–124
14. Fu, H., Coburn, J., and Collier, R. J. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 2320–2324
15. Ford, J. C., Al-Khodairy, F., Fletcher, E., Sheldrick, K. S., Griffiths, D. J., and Carr, A. M. (1994) Science 265, 533–535
16. Irie, K., Gotoh, Y., Yashar, B. M., Errede, B., Nishida, E., and Matsumoto, K. (1994) Science 265, 1716–1719
17. Freed, E., Symons, M., MacDonald, S. G., McCormick, F., and Ruggeri, R. (1994) Science 265, 1713–1716
18. Pallas, D. C., Fu, H., Haehnel, L. C., Wellar, M., Collier, R. J., and Roberts, T. M. (1994) Science 265, 535–537
19. Reutter, G. W., Fu, H., Cripe, L. D., Collier, R. J., and Pendergast, A. M. (1994) Science 266, 129–133
20. Zha, J., Harida, H., Yang, E., Jockel, J., and Korsemeyer, S. J. (1996) Cell 87, 619–628
14-3-3 Association with IRS-1 and IRS-2

21. Ogihara, T., Shin, B.-C., Anai, M., Katagiri, H., Inukai, K., Funaki, M., Fukushima, Y., Ishihara, H., Takata, K., Kikuchi, M., Yazaki, Y., Oka, Y., and Asano, T. (1997) J. Biol. Chem. 272, 12868–12873
22. Ichimura, T., Uchiyama, J., Kunihiro, O., Ito, M., Horigome, T., Omata, S., Shinkai, F., Kaji, H., and Isobe, T. (1995) J. Biol. Chem. 270, 28515–28518
23. Miyake, S., Makimura, M., Kanegae, Y., Harada, S., Sato, Y., Takamori, K., Tokuda, C., and Sato, I. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 1320–1324
24. Muslin, A. J., Tanner, J. W., Allen, P. M., and Shaw, A. S. (1996) Cell 84, 889–897
25. Boyle, W. J., Van Der Geer, P., and Hunter, T. (1991) Methods Enzymol. 201, 110–148
26. Sun, X. J., Miralpeix, M., Myers, M. G., Jr., Glasheen, E. M., Backer, J. M., Kahn, C. R., and White, M. F. (1992) J. Biol. Chem. 267, 22662–22672
27. Tanti, J.-F., Gremeaux, T., Van Obberghen, E., and Le Marchand-Brustel, Y. (1994) J. Biol. Chem. 269, 6051–6057
28. Xiao, B., Smerdon, S. J., Jones, D. H., Bedson, G. G., Soneji, Y., Atitken, A., and Gamblin, S. J. (1995) Nature 376, 188–191
29. Liu, D., Bienkowski, J., Petosa, C., Collier, R. J., Pu, H., and Liddington, R. (1995) Nature 376, 191–194
30. Wolf, G., Trub, T., Ottinger, E., Groninga, L., Lynch, A., White, M. F., Miyazaki, M., Lee, J., and Shoelson, S. E. (1995) J. Biol. Chem. 270, 27407–27410
31. Bachmann, M., Huber, J. L., Athwal, G. S., Wu, K., Ferl, R. J., and Huber, S. C. (1996) FEBS Lett. 388, 26–30
32. Kanety, H., Feinstein, R., Papa, M. Z., Hemi, R., and Karasik, A. (1995) J. Biol. Chem. 270, 23780–23784
33. Hotamisligil, G. S., Peraldi, P., Budavari, A., Ellis, R., White, M. F., and Spiegelman, B. M. (1996) Science 271, 665–668
34. Bostom, P. F., Jackson, P., Kynsch, P. A. M., and Thompson, R. J. (1992) J. Neurochem. 58, 1466–1474
35. Furukawa, Y., Ikuta, N., Omata, S., Yamauchi, T., Isobe, T., and Ichimura, T. (1993) Biochem. Biophys. Res. Commun. 194, 144–149
36. Eck, M. J., Dhe-Paganon, S., Trub, T., Nolte, R. T., and Shoelson, S. E. (1996) Cell 85, 695–705
37. Bork, P., and Margolis, B. (1995) Cell 80, 693–694