14-3-3β Protein Associates with Insulin Substrate 1 and Decreases Insulin-stimulated Phosphatidylinositol 3′-Kinase Activity in 3T3L1 Adipocytes*

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The 14-3-3 protein family has been implicated in growth factor signaling. We investigated whether 14-3-3 protein is involved in insulin signaling in 3T3L1 adipocytes. A significant amount of insulin receptor substrate 1 (IRS-1) was immunodetected in the immunoprecipitate with anti-14-3-3β antibody at the basal condition. 100 nM insulin increased the amount of IRS-1 in the immunoprecipitate 2.5-fold. The effect of insulin was abolished by 100 nM wortmannin. An in vitro binding study revealed that glutathione S-transferase-14-3-3 fusion protein directly associates with recombinant IRS-1. Pretreatment of recombinant IRS-1 with alkaline phosphatase clearly decreased this association. Because the recombinant IRS-1 was not phosphorylated on its tyrosine residues, the results suggest that serine/threonine phosphorylation of IRS-1 is responsible for the association. When the cells are treated with insulin, phosphatidylinositol 3′-kinase (PI3K) is supposed to complex either 14-3-3β-IRS-1 or IRS-1. The 14-3-3β-IRS-1-PI3K and IRS-1-PI3K complexes were separately prepared by a sequential immunoprecipitation, first with anti-14-3-3β and then with anti-IRS-1 antibodies. The specific activity of the PI3K in the former was approximately half of that in the latter, suggesting that 14-3-3β protein bound to IRS-1 inhibits insulin-stimulated lipid kinase activity of PI3K in 3T3L1 adipocytes.

Insulin promotes the rapid autophosphorylation of its receptor β-subunits and tyrosine phosphorylation of several cytoplasmic proteins such as Shc, pp60, Gab-1, and insulin receptor substrate (IRS)1 and 2. IRS-1 plays a central role in insulin signaling. The protein contains a pleckstrin homology domain, a phosphotyrosine-binding domain that binds to NPXY motif of the insulin receptor β-subunit and multiple tyrosine residues that are potential phosphorylation sites. The tyrosine-phosphorylated IRS-1 associates with several Src homology 2 domain-containing proteins including Grb-2, SHP-2, Nck, Fyn, and the 85-kDa subunit (p85) of phosphatidylinositol 3′-kinase (PI3K).

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Associations of these molecules with IRS-1 are believed to further activate downstream signaling systems, including mitogen-activated protein kinase and PI3K cascades, that promote mitogenic and metabolic effects of insulin (for review see Refs. 1 and 2).

14-3-3 protein family, first discovered as acidic proteins in the brain, is highly conserved in animals and plants. At least nine mammalian isoforms have been identified, and six are expressed ubiquitously (3). Originally, 14-3-3 proteins have been shown to be functional as regulators of tryptophan and tyrosine hydroxylases as well as protein kinase C (for review see Ref. 4). However, recently 14-3-3 proteins were found to associate oncogene products, including Raf-1 (5–11), Bcr-Abl, Bcr (12, 13), polyoma middle T antigen (14), and cell cycle control proteins such as Cdc25 phosphatases (15). The interaction of Raf with 14-3-3 leads to Raf activation in several in vitro systems (5, 7, 8). Moreover, the importance of 14-3-3 proteins in signal transduction is suggested by the reports that 14-3-3 protein interacts with PI3K (16) and glycoprotein Ib-IX (17). However, both the mechanism and the physiological role of protein-protein interactions mediated by 14-3-3 proteins are still unclear.

The aim of the present study is to investigate whether the 14-3-3 protein is involved in the insulin signaling pathway. We showed that 14-3-3β protein directly binds to IRS-1 and that insulin increases this binding via a wortmannin-sensitive pathway in 3T3L1 adipocytes. We also demonstrated that the 14-3-3β protein negatively regulates insulin-stimulated activity of PI3K.

EXPERIMENTAL PROCEDURES

Materials—Anti-IRS-1 (SC559), anti-phosphotyrosine (SC508), anti-14-3-3β (SC628), and anti-GST (SC459) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rat recombinant IRS-1 (number 12-128) and anti-p85PI3K antibodies (numbers 05-217 and 06-195) were from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-IRS-1 (SC559) and anti-phosphotyrosine (SC508) antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rat recombinant IRS-1 (number 12-128) and anti-p85PI3K antibodies (numbers 05-217 and 06-195) were from Upstate Biotechnology, Inc. (Lake Placid, NY). 14-3-3β protein directly binds to IRS-1 and that insulin increases this binding via a wortmannin-sensitive pathway in 3T3L1 adipocytes. We also demonstrated that the 14-3-3β protein negatively regulates insulin-stimulated activity of PI3K.

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with insulin for 3 min at 37 °C and then immediately frozen with liquid nitrogen. For some experiments, cells were preincubated with 100 nM wortmannin for 20 min. Total cell lysates were prepared by a solubilization in 0.5 mL/10-cm dish of ice-cold lysis buffer (50 mM Heps, pH 7.5, 10 mM EDTA, 100 mM NaF, 10 mM sodium pyrophosphate, 1% Nonidet P-40, 0.1 mg/ml aprotinin, 2 mM sodium orthovanadate) for 30 min at 4 °C. The insoluble materials were removed by centrifugation (20,000 × g for 20 min at 4 °C). Cytosolic fractions were prepared by suspending the cells in ice-cold lysis buffer without Nonidet P-40 and homogenizing with a Teflon homogenizer for several strokes. The membrane fractions were removed by ultracentrifugation (100,000 × g for 1 h at 4 °C).

Construction of Fusion Protein—To prepare GST fusion protein, full-length rat 14-3-3β cDNA was amplified by polymerase chain reaction using sense primer added to the BamHI site and antisense primer added to the EcoRI site and cloned into pCRII vector. The insertion was restricted with both BamHI and EcoRI and ligated into pGEX-2T vector in-frame. The plasmid was subcloned into DH5α cells. Fusion proteins were affinity purified using glutathione-Sepharose 4B beads according to the manufacturer’s protocol. The protein is designated GST-14-3-3β. GST protein alone was also prepared for a negative control.

Peptide Synthesis—YMMX-containing peptide, YPZPSGSYYPZPS (nonphosphorylated form), was synthesized by an Fmoc-based strategy. Norleucines (Z) were used instead of methionines (M) as described previously (19). Fmoc-based techniques were utilized for the purification of the phosphopeptide possessing substitution of phosphothreonine (Y) or difluoropmp for tyrosine in the two YZPZ motifs (20, 21). The peptides were purified by preparative reverse phase HPLC. Analysis of the purified products by mass spectroscopy and analytical HPLC demonstrated homogeneity and sequence accuracy of the synthesized peptides.

In Vivo Association Study—2 mg of total cell lysates were incubated with 2 μg of the antibody indicated for 16 h at 4 °C in an end-over-end mixer, followed by the addition of protein A/G-Sepharose for the last hour. The Sepharose-bound immune complexes were then collected by centrifugation (10,000 × g for 1 min) and washed four times in ice-cold washing buffer (50 mM Heps, pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40) and once in phosphate-buffered saline. The immune complexes were denatured in Laemmli’s sample buffer by boiling for 5 min and then subjected to immunoblotting.

In Vitro Association Study—To see whether 14-3-3β protein associates with IRS-1 in vitro, either 2 mg of total cell lysate of 3T3-L1 adipocytes or 400 ng of rat recombinant IRS-1 protein was incubated with 50 pmol of either GST alone or GST-14-3-3β fusion protein in 1.0 mL of PBS buffer for 1 h and then with glutathione-Sepharose 4B beads for another hour at 4 °C. The Sepharose-bound complexes were collected by centrifugation (10,000 × g for 1 min) and washed four times in ice-cold washing buffer and once in phosphate-buffered saline. The complexes were denatured in Laemmli’s sample buffer by boiling for 5 min and then subjected to immunoblotting with anti-IRS-1 antibody.

To study whether PI3K associates with 14-3-3β protein in vitro, 1.5 mg of cytosolic fraction of untreated cells was incubated with 10 μL of YMMX-, pYMMX-, or difluoropmpMMX-containing peptide for 20 min at 25 °C, and the total mixture was immunoprecipitated with 3 μg of anti-p85 antibody followed by the addition of protein A/G-Sepharose. The immunoprecipitated PI3K was further phosphorylated in vitro in 50 μL of buffer A (20 mM Heps, pH 7.5, 10 mM MgCl₂, 3 mM MnCl₂, and 10 μM ATP) for 10 min at 25 °C and then incubated with GST-14-3-3β fusion protein in 250 μL of lysis buffer for 1 h at 4 °C. The Sepharose-bound immune complexes were collected by centrifugation (10,000 × g for 1 min) and washed four times in ice-cold washing buffer and once in phosphate-buffered saline. The immune complexes were denatured in Laemmli’s sample buffer by boiling for 5 min and then subjected to immunoblotting with anti-GST antibody.

Alkaline Phosphatase Treatment—400 μg of rat recombinant 14-3-3β protein was incubated with 15 units of active or heat-inactivated calf intestine alkaline phosphatase in 20 μL of 50 mM Tris-HCl, pH 8.0, for 30 min at 25 °C and then heat-inactivated for 10 min at 70 °C. The recombinant IRS-1 proteins were then subjected to in vitro association study.

Immunoblotting—Samples were separated by electrophoresis on 7.5–10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked with 3% bovine serum albumin in TBS (10 mM Tris-HCl, pH 7.5, 150 mM NaCl) and then incubated with the antibody indicated for 3 h at room temperature. The membranes were washed in T-TBS (TBS containing 0.1% Tween-20) for 30 min, incubated with donkey anti-rabbit or anti-mouse horseradish peroxidase-conjugated antibody in T-TBS for 60 min, and washed for 60 min in T-TBS, and then the proteins were visualized using an ECL chemiluminescent kit. The band densities were quantified using an image analyzer Quantity One System (PDI, Sunnyvale, CA).

PI3K Assay—PI3K activity was measured as described previously with slight modifications (22, 23). Briefly, the Sepharose-bound immune complexes with the indicated antibody were resuspended in 50 μL of 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA. The reaction was then initiated by the addition of 10 μL of 100 mM MgCl₂, 10 μL of 1 mg/ml of phosphatidylinositol, and 10 μL of 100 μM (γ32P)ATP (~20 μCi). After 10 min of incubation at 25 °C, the reaction products were analyzed by thin layer chromatography on silica gel plate followed by a BAS-2000 (Fujifilm, Tokyo, Japan) detection. To measure the PI3K activity in Fig. 4, 2 mg of total cell lysate was first immunoprecipitated with 2 μg of anti-14-3-3β antibody, and then half of the supernatant was immunoprecipitated with 2 μg of anti-IRS-1 antibody. To eliminate the influence of each antibody on the activity of PI3K, 2 μg of anti-IRS-1 and anti-14-3-3β antibody were added to the first and the second immunoprecipitate, respectively, before the assay.

Statistical Analysis—Results are expressed as the means ± S.E. All comparisons were made using a two-tailed t test, p < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

To investigate whether 14-3-3β protein is involved in the insulin signaling pathway, total cell lysates of 3T3-L1 adipocytes were immunoprecipitated with anti-14-3-3β antibody, and the immune complexes were subjected to immunoblots with antibodies against molecules implicated in insulin signaling. We found that a significant amount of IRS-1 was immunodetected in the immunoprecipitate at the basal condition. 100 nM insulin increased 2.5-fold (basal versus insulin-stimulated condition; 5.30 ± 0.41 versus 13.57 ± 1.16 arbitrary units, n = 6, p < 0.01) the amount of IRS-1 in the immunoprecipitate (Fig. 1A, lanes 1 and 2). The increase was detected very rapidly after insulin treatment, reaching to the plateau at 1 min (data not shown). The effect of insulin was abolished when the cells were pretreated with 100 nM wortmannin (Fig. 1A, lane 3). However, wortmannin did not affect the amount of immunoprecipitated IRS-1 at the basal condition (data not shown). To confirm these results, the total cell lysates were immunoprecipitated with anti-IRS-1 antibody, and the immune complexes were subjected to immunoblotting with anti-14-3-3β antibody (Fig. 1B). Consistent with the former results, 14-3-3β proteins were immunodetected in the immunoprecipitate at the basal condition. Insulin increased the amount of immunoprecipitated 14-3-3β proteins. The effect of insulin was reduced by wortmannin pretreatment.

To ensure that 14-3-3β protein associates with IRS-1, in vitro association experiments were performed using a GST-14-3-3β fusion protein. GST-14-3-3β protein was incubated with total cell lysates of 3T3-L1 adipocytes treated with or without insulin, followed by glutathione-Sepharose 4B beads precipitation. The precipitates were subjected to immunoblotting using anti-IRS-1 antibody (Fig. 2A). The GST-14-3-3β protein (but not GST alone) associated with endogenous IRS-1 in the cell lysates at the basal condition. Insulin increased the amount of immunoprecipitated 14-3-3β proteins. The effect of insulin was reduced by wortmannin pretreatment.

To further address whether the direct association of 14-3-3β protein with IRS-1 can occur, in vitro reconstitution experiments were performed using recombinant IRS-1 and GST-14-3-3β fusion protein. We found that the GST-14-3-3β protein directly associates with recombinant IRS-1 (Fig. 2B). Recently, it has been determined that 14-3-3 protein binds to phosphoserine (pS)-containing motifs by the peptide binding and peptide mapping analysis (24, 25). Therefore, to test whether phosphorylated residues of recombinant IRS-1 are involved in the association with 14-3-3β protein, effects of alkaline phosphatase on the association were determined. Pretreatment of
The Association of 14-3-3β Protein with IRS-1

An alternative possibility is that insulin-stimulated association of 14-3-3β proteins with IRS-1 is mediated by PI3K. It is well known that tyrosine-phosphorylated IRS-1 upon insulin stimulation associates directly with the p85 subunit of PI3K through pYMXM and pYXXM motifs, resulting in the activation of PI3K (2). It has been reported that insulin induces serine-phosphorylation of p85 by PI3K itself (27, 29). As expected, pS8 was detected in the immunoprecipitates with anti-14-3-3β antibody mainly upon insulin treatment and little in the basal condition. This band was decreased in the presence of wortmannin (Fig. 3A). If 14-3-3β protein associates with PI3K directly, insulin apparently increases the amount of IRS-1 immunoprecipitated with 14-3-3β antibody via PI3K. Therefore, to explore this possibility, we activated PI3K in vitro without the insulin receptor-IRS-1 system. pYMXM- or difluoroPmp-MXM-containing peptide was incubated with the cytosolic frac-

**Fig. 1.** Insulin-induced association of 14-3-3β protein with IRS-1 in 3T3L1 adipocytes. 3T3L1 adipocytes were pretreated with (lanes 3 and 8) or without (lanes 1, 2, 4–7, and 9) 100 nm wortmannin for 20 min at 37 °C. The cells were then treated with (lanes 1, 2, 3, 4, and 8) or without (lanes 1, 4, 5, 6, and 7) 100 nm insulin for 3 min at 37 °C. 2 mg of the total cell lysates were immunoprecipitated with anti-14-3-3β antibody (lanes 1–3 and 9), anti-IRS-1 antibody (lanes 5–7), or control IgG (lane 4). The immunoprecipitates were subjected to immunoblots with anti-IRS-1 (A) or anti-14-3-3β (B) antibody. Each representative photograph is shown. Blot, immunoblot; α-IRS-1, anti-IRS-1 antibody; α-14-3-3β, anti-14-3-3β antibody; CO, control IgG.

The recombinant IRS-1 with alkaline phosphatase clearly decreased this association (Fig. 2C). The results indicate that 14-3-3β protein directly binds to IRS-1 via its phosphorylated residues, probably phosphorylated serine/threonine residues, because the recombinant IRS-1 that was used for the experiment was not phosphorylated on tyrosine residues. These in vitro reconstitution experiments could explain the association of 14-3-3β protein with IRS-1 at the basal condition. However, mechanisms of the insulin-induced increase in the association of 14-3-3β protein with IRS-1 remains unknown. It was shown that RSXpSXP, RXRXpS and a cluster of serine residues in the α-helix are the binding motifs for 14-3-3 proteins (24–26). Interestingly, IRS-1 contains six RXRXpS motifs (but no RXSXP motifs) in its amino acid sequence. Four of them are in the phosphotyrosine-binding domain, and the others are located in the region containing the YXXM motifs. Moreover, it was reported that IRS-1 is a substrate for serine kinase of PI3K activated by insulin (27). Taken together with our results, it is tempting to speculate that IRS-1 binds to 14-3-3β protein via its phosphorylated serine residues such as RXRXpS motif, and insulin treatment results in the activation of serine kinase of PI3K, which in turn further phosphorylates the serine residues of IRS-1, leading to more association with 14-3-3β protein.

This could be a reason why wortmannin inhibits insulin-induced increase of the IRS-1–14-3-3β complex (Fig. 1). Of course, other molecules downstream from PI3K, for example, protein kinase B, which is another serine/threonine kinase, may be involved in the insulin-induced increase of the IRS-1–14-3-3β complex (28). Further study will be necessary to clarify this point.

**Fig. 2.** In vitro association of 14-3-3β protein with IRS-1. A, 3T3L1 adipocytes were pretreated with (lanes 1 and 2) or without (lanes 3 and 4) 100 nm insulin for 3 min at 37 °C. 2 mg of the total cell lysates were incubated with 50 pmol of GST alone (lanes 1 and 2) or GST-14-3-3β fusion protein (lanes 3 and 4) for 1 h, followed by the precipitation with glutathione-Sepharose 4B beads for another hour at 4 °C. The precipitates were washed and subjected to an immunoblot with anti-IRS-1 antibody. B, 400 ng of rat recombinant IRS-1 protein were incubated with 50 pmol of either GST alone (lane 6) or GST-14-3-3β fusion protein (lane 7) in lysis buffer for 1 h and then with glutathione-Sepharase 4B beads for another hour at 4 °C. The precipitates were washed and subjected to an immunoblot with anti-IRS-1 antibody. One-quarter the amount of input recombinant IRS-1 was subjected to the immunoblot as a positive control (lane 5). C, 15 units of each active (lanes 9 and 11) or heat-inactivated (lanes 8 and 10) calf intestine alkaline phosphatase were incubated with 400 ng of rat recombinant IRS-1 protein for 30 min at 25 °C and then heat-inactivated for 10 min at 70 °C. The recombinant IRS-1 proteins were then incubated with 50 pmol of either GST alone (lanes 10 and 11) or GST-14-3-3β fusion protein (lanes 8 and 9) in lysis buffer for 1 h and then with glutathione-Sepharase 4B beads for another hour at 4 °C. The precipitates were washed and subjected to an immunoblot with anti-IRS-1 antibody. The results show one representative out of three independent experiments. GS4B, glutathione-Sepharase 4B beads; rIRS-1, recombinant IRS-1; ALP, calf intestine alkaline phosphatase; prep., precipitation.
The cytosol fraction of untreated 3T3L1 adipocytes was incubated with 10 pmol of GST-14-3-3 protein. Although both pYM- and difluoroPmpXM-containing peptide increased the association of GST-14-3-3β protein with PI3K in the absence of IRS-1 molecule (Fig. 3D, upper panel). It is thus unlikely that 14-3-3β protein directly associates with PI3K in 3T3L1 adipocytes, accounting for the insulin-stimulated increase of IRS-1 in the immunoprecipitates with anti-14-3-3β antibody. Recently, it was reported that 14-3-3 protein directly binds to PI3K in human T lymphocytes (16). The difference from our results may be due to the tissue, 14-3-3 isoform, or species specificity. It is of interest to note that human but not mouse p85 of PI3K contains RS motif in the breakpoint cluster region homologue domain.

Because less than half the amount of total IRS-1 associates with 14-3-3β protein when the cells are treated with insulin (Fig. 1A), PI3K may complex either 14-3-3β-IRS-1 or IRS-1. To address the physiological significance of the 14-3-3β-IRS-1 complex, the 14-3-3β-IRS-1-PI3K and IRS-1-PI3K complexes were separately prepared by a sequential immunoprecipitation (Fig. 4). The total cell lysates were first immunoprecipitated with anti-14-3-3β antibody (Fig. 4, lanes 1 and 2), and then half of the supernatant (1/2 Sup.) was then with anti-IRS-1 antibody (α-IRS-1) (lanes 3 and 4). The immunoprecipitates were subjected to lipid kinase assay (A). A part of the immunoprecipitate in A was immunoblotted with anti-IRS-1 (B), anti-phosphotyrosine (C), anti-p85PI3K (D), or anti-14-3-3β (E) antibody. Representative photographs are shown. PI3P, phosphatidylinositol 3-phosphate.
The means interaction between IRS-1 and Raf-1 via 14-3-3
3T3L1 adipocytes treated with or without insulin were immu-
phasized that PI3K activity is reduced in the 14-3-3
further confirmed using different antibodies that work well in
the p110
an adapter molecule (13, 32). Therefore, it
more, an importance of 14-3-3 protein as a chaperone as well as
PI3K complex when normalized by the amount of p85 subunit.
After insulin stimulation, the amounts of IRS-1, tyrosine-phos-
phorylated p110, and p85
the ratio of p110α
2.8 100
7.4 100
14-3-3β
14-3-3
24.9 ± 7.4a
56.6 ± 4.7
50.1 ± 2.8
51.4 ± 3.1
1.4 ± 0.3

* p < 0.02 versus the values of IRS-1, pTyr, and p85 in the first IP.
2. Attribution: The values of the second IP for lipid kinase, IRS-1, pTyr, and p85 and as the percentages of the value of the first IP for 14-3-3β. Data are the means ± S.E. of three or four experiments.

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** Table I
Lipid kinase activity of PI3K bound to the IRS-1-14-3-3β complex

| Lipid kinase or immunoblot | First IP anti-14-3-3β | Second IP anti-IRS-1 |
|---------------------------|----------------------|---------------------|
| Lipid kinase              | 24.9 ± 7.4a          | 100                 |
| IRS-1                     | 56.6 ± 4.7           | 100                 |
| pTyr                      | 50.1 ± 2.8           | 100                 |
| p85                       | 51.4 ± 3.1           | 100                 |
| 14-3-3β                   | 1.4 ± 0.3            | 0.3                 |

** a p < 0.02 versus the values of IRS-1, pTyr, and p85 in the first IP.