Phosphorylation of Insulin Receptor Substrate-1 (IRS-1) by Protein Kinase B Positively Regulates IRS-1 Function*

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Keren Paz, Yan-Fang Liu, Hagai Shorer, Rina Hemi,‡ Derek LeRoith,§ Michael Quan,¶ Hannah Kanety,‡ Rony Seger, and Yehiel Zick**

From the Departments of Molecular Cell Biology and Biological Regulation, the Weizmann Institute of Science, Rehovot 76100, Israel, the Institute of Endocrinology, Chaim Sheba Medical Center, Tel-Hashomer 52621, Israel, the Molecular and Cellular Endocrinology Branch, NIDDK, and the Hypertension-Endocrine Branch, NHLBI, National Institutes of Health, Bethesda, Maryland 20892

Incubation of cells with insulin leads to a transient rise in Tyr phosphorylation of insulin receptor substrate (IRS) proteins, accompanied by elevation in their Ser(P)/Thr(P) content and their dissociation from the insulin receptor (IR). Wortmannin, a phosphatidylinositol 3-kinase inhibitor, selectively prevented the increase in Ser(P)/Thr(P) content of IRS-1, its dissociation from IR, and the decrease in its Tyr(P) content following 60 min of insulin treatment. Four conserved phosphorylation sites within the phosphotyrosine binding/SAIN domains of IRS-1 and IRS-2 served as in vitro substrates for protein kinase B (PKB), a Ser/Thr kinase downstream of phosphatidylinositol 3-kinase. Furthermore, PKB and IRS-1 formed stable complexes in vivo, and overexpression of PKB enhanced Ser phosphorylation of IRS-1. Overexpression of PKB did not affect the acute Tyr phosphorylation of IRS-1; however, it significantly attenuated its rate of Tyr dephosphorylation following 60 min of treatment with insulin. Accordingly, overexpression of IRS-1AA, lacking the four potential PKB phosphorylation sites, markedly enhanced the rate of Tyr dephosphorylation of IRS-1, while inclusion of vanadate reversed this effect. These results implicate a wortmannin-sensitive Thr/Thr kinase, different from PKB, as the kinase that phosphorylates IRS-1 and acts as the feedback control regulator that turns off insulin signals by inducting the dissociation of IRS proteins from IR. In contrast, insulin-stimulated PKB-mediated phosphorylation of Ser residues within the phosphotyrosine binding/SAIN domain of IRS-1 protects IRS-1 from the rapid action of protein-tyrosine phosphatases and enables it to maintain its Tyr-phosphorylated active conformation. These findings implicate PKB as a positive regulator of IRS-1 functions.

The insulin receptor (IR)1 is a heterotetrameric transmembrane glycoprotein composed of two extracellular α subunits and two transmembrane β subunits linked by disulfide bonds. The α subunits contain the insulin-binding domain, while the transmembrane β subunits function as Tyr-specific kinases (insulin receptor kinases). Insulin signaling utilizes the Tyr kinase activity of the receptor to phosphorylate docking proteins on multiple Tyr residues and further propagate insulin action (1).

The major substrates of insulin receptor kinase are Shc (2) and the IRS proteins, IRS-1 (3), IRS-2 (4), IRS-3 (5), and IRS-4 (6). IRS proteins contain a conserved pleckstrin homology domain (7, 8) located at the amino terminus, adjacent to a phosphotyrosine binding (PTB) domain. The PTB domain is present in a number of signaling molecules (9) and shares 75% sequence identity between IRS-1 and IRS-2 (10). This domain interacts with the NPXY motif of the juxtamembrane (JM) region of IR and promotes IR/IRS-1 interactions (11, 12). The C-terminal region of IRS proteins is poorly conserved. It contains multiple Tyr phosphorylation motifs that serve as docking sites for SH2 domain-containing proteins like the p85α regulatory subunit of PI3K, Grb2, Nck, Crk, Fyn, SHP-2, and others, which mediate the metabolic and growth-promoting functions of insulin (1, 13).

The signaling pathways regulated by IRS proteins control glucose uptake and lipogenesis, protein synthesis, and cell survival (1, 13). The relative roles of the different IRS proteins in mediating insulin action are still unclear; however, studies of gene disruption revealed that IRS-2 compensates for the absence of IRS-1 in hepatocytes of IRS-1 null mice, while IRS-3 provides the major alternative pathway to PI3K activation in skeletal muscle and adipocytes of these animals (14–17). In contrast, IRS-2 null mice develop both insulin resistance and beta cell failure, which leads to their death (18). These data implicate different IRS proteins as mediators of insulin action in different tissues.

IRS proteins contain over 30 potential Ser/Thr phosphorylation sites for kinases like protein kinase A, PKC, and mitogen-activated protein kinase (3, 4, 19). In previous studies, we have demonstrated that Ser/Thr phosphorylation of IRS-1 and IRS-2 significantly reduces their ability to interact with the JM region of IR. Such impaired interactions abolish the ability of IRS-1 and IRS-2 to undergo insulin-induced Tyr phosphorylation and further propagate insulin signaling, thus providing a possible molecular mechanism for the induction of an insulin-resistant state (20, 21). Ser/Thr phosphorylation of IRS pro-

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** An incumbent of the Philip Harris and Gerald Ronson Career Development Chair in Diabetes Research. To whom correspondence should be addressed. Tel.: 972-8-9342-380; Fax: 972-8-9344-125; E-mail: Lizick@weizmann.weizmann.ac.il.

1 The abbreviations used are: IR, insulin receptor; IRS, insulin receptor substrate; IRS-1, insulin receptor substrate-1; IRS-2, insulin receptor substrate-2; IRS-1AA, IRS-1 whose Ser residues 265, 302, 325, and 358 were mutated to Ala; JM, juxtamembrane; PTB, phosphotyrosine binding; PKB, protein kinase B, PI3K, phosphatidylinositol 3-kinase; PTP, protein Tyr phosphatase; PKC, protein kinase C; CHO, Chinese hamster ovary; PAGE, polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography.

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teinseems to be a key mechanism to regulate their function and raises several questions: which residues within IRS proteins undergo Ser phosphorylation, which are the kinases involved, and how such phosphorylations affect insulin signal transduction. The PTB domain of IRS proteins, which interacts with the JM region of IR, is a likely candidate to undergo Ser/Thr phosphorylation. Indeed, alignment of the PTB domains of IRS proteins reveals the presence of 16 conserved Ser/Thr residues, four in consensus PKB phosphorylation sites, that could be targets for different Ser/Thr kinases, including PKB.

PKB (Akt), a Ser/Thr kinase, has been shown to function in the IR signaling cascade downstream of PI3K (22). PKB is activated by insulin in isolated adipocytes and plays a role in glucose metabolism (23). Furthermore, expression of a constitutively active PKB in 3T3-L1 cells or primary adipocytes stimulates glucose uptake and Glut4 translocation (23, 24). PKB is phosphorylated and activated by phosphatidylinositol 3-kinase-dependent kinases (25–27), but the detailed mechanism of this activation process is presently unknown.

In the present study, we show that phosphorylation of Ser/Thr residues of IRS proteins has a dual function and serves either as a positive or as a negative modulator of insulin signal transduction. Our results implicate a wortmannin-sensitive Ser/Thr kinase, different from PKB, as the kinase that phosphorylates IRS-1 and acts as the negative feedback control regulator that turns off insulin signals by inducing the dissociation of IRS proteins from IR. In contrast, phosphorylation of Ser residues within the PTB domain of IRS-1 by insulin-stimulated PKB protects IRS proteins from the rapid action of protein Tyr phosphatases and enables the Ser-phosphorylated PKB to protect IRS proteins from IR. In contrast, phosphorylation of IRS proteins by PKB regulates glucose metabolism (23). Furthermore, expression of a constitutively active PKB in 3T3-L1 cells or primary adipocytes stimulates glucose uptake and Glut4 translocation (23, 24). PKB is activated by insulin in isolated adipocytes and plays a role in glucose metabolism (23). Furthermore, expression of a constitutively active PKB in 3T3-L1 cells or primary adipocytes stimulates glucose uptake and Glut4 translocation (23, 24). PKB is phosphorylated and activated by phosphatidylinositol 3-kinase-dependent kinases (25–27), but the detailed mechanism of this regulation was performed using a QuikChange kit (Stratagene) according to the manufacturer's instructions. pcDNA3-IRS-1 encoding mouse IRS-1 (29) served as a template. pcDNA3-IRS-1A encoding for IRS-1A (whose Ser residues 265, 302, 325, and 358 were mutated to Ala), was generated sequentially using four sets of overlapping primers: (a) S285A, 5'-GAGGTTCGCCCAGCGAAGAAAGCCCAATCTTCATCCAG-3' and 5'-GGGATGAAGATTGGGCTTTCGTCCGCGGGCGAAACTC-3' (an additional restriction site for SacI is underlined); (b) S302A, 5'-CTGACTCAGAGATCACTGACCCCTCAGATCCTCGATCAG-3' (a restriction site for BglII that is eliminated was underlined); (c) S265A, 5'-GGGTCGCTGCTCCGGCTCGGATCCGAAGGCACC-3' and 5'-GGTGGTCGATCTCCGGATCCGACGATC-3' (an additional restriction site for SacI is underlined); and (d) S358A, 5'-GGCATCGAGGCAGGCTTAGGTCGACCCCTC-3' and 5'-GGGGGTGTCGACCTAGGCTGCCGCTCTGCC-3' (an additional restriction site for EcoRIFIII is underlined). The mutations were confirmed by restriction digestion and by DNA sequencing.

 Generation of Myc-tagged IRS-1—pcDNA3-IRS-1 was digested with HindIII, and BspE1 and a 9-base pair piece of IRS-1 DNA was deleted and replaced by double-stranded matching overhangs of synthetic oligonucleotide, containing Myc and the remaining IRS-1 sequence.

**Experimental Procedures**

**Materials**—Recombinant human insulin was a gift from Novo-Nordisk (Copenhagen, Denmark). Wortmannin and wheat germ agglutinin were purchased from Calbiochem. LipofectAMINE was obtained from Life Technologies, Inc. Monoclonal FY-20 antibodies were obtained from Transduction Laboratories (Lexington, KY). Polyclonal IRS-1 antibodies (anti-YR-1) were prepared as described (28). Polyclonal antibodies against native PKB and its Ser473-phosphorylated form were obtained from Sigma and New England Biolabs, respectively.

**Treatment of Cells with Kinase Inhibitors**—Rat hepatoma Fao cells or CHO cells overexpressing the insulin receptor (CHO-T cells) were grown in RPMI or F-12 medium, respectively, supplemented with 10% fetal calf serum as described (21, 29). Confluent monolayers, grown in 60-mm dishes, were deprived of serum for 16 h prior to each experiment. The medium was aspirated, and the cells were incubated with the indicated inhibitors in serum-free medium for different time periods at 37 °C. Cells were then incubated with or without 100 nM insulin for 1 or 60 min at 37 °C. Cells were washed three times with phosphate-buffered saline and harvested in 300 μl (Fao cells) or 100 μl (CHO-T) of buffer A. Following three cycles of freezing and thawing, the cell extracts were centrifuged at 12,000 × g for 20 min at 4 °C, and the supernatants were collected. Samples (100 μl) were resolved by SDS-PAGE and immunoblotted with the indicated antibodies.

**Binding of IRS-1 to Immobilized IRS-1—Insulin receptors were purified from liver plasma membranes of 10-week-old rats. The preparation of membranes, solubilization in Trition X-100, and immobilization of IR on wheat germ agglutinin-coupled beads were carried out as described previously (30). The immobilized IR was washed with buffer A prior to use. Confluent monolayers of Fao or CHO-T cells, grown in 60-mm dishes, were incubated with 100 nM insulin for 1 or 60 min at 37 °C. Cells were washed three times with phosphate-buffered saline and harvested in 300 μl (Fao) or 100 μl (CHO-T) of buffer A. Following three cycles of freezing and thawing, the cell extracts were centrifuged at 12,000 × g for 30 min at 4 °C, and the supernatants were collected. Aliquots (300 μl) were incubated for 1 h with 30 μl of immobilized IR, with shaking, at 4 °C. Beads were washed four times with buffer A and boiled in 50 μl of Laemmli “sample buffer” (57). Samples were resolved by means of SDS-PAGE and immobilized with IRS-1 antibodies.

Generation of pcDNA3-IRS-1—Site-directed mutagenesis was performed using a QuikChange kit (Stratagene) according to the manufacturer's instructions. pcDNA3-IRS-1 encoding mouse IRS-1 (29) served as a template. pcDNA3-IRS-1A encoding for IRS-1A (whose Ser residues 265, 302, 325, and 358 were mutated to Ala), was generated sequentially using four sets of overlapping primers: (a) S285A, 5'-GAGGTTCGCCCAGCGAAGAAAGCCCAATCTTCATCCAG-3' and 5'-GGGATGAAGATTGGGCTTTCGTCCGCGGGCGAAACTC-3' (an additional restriction site for SacI is underlined); (b) S302A, 5'-CTGACTCAGAGATCACTGACCCCTCAGATCCTCGATCAG-3' (a restriction site for BglII that is eliminated was underlined); (c) S265A, 5'-GGGTCGCTGCTCCGGCTCGGATCCGAAGGCACC-3' and 5'-GGTGGTCGATCTCCGGATCCGACGATC-3' (an additional restriction site for SacI is underlined); and (d) S358A, 5'-GGCATCGAGGCAGGCTTAGGTCGACCCCTC-3' and 5'-GGGGGTGTCGACCTAGGCTGCCGCTCTGCC-3' (an additional restriction site for EcoRIFIII is underlined). The mutations were confirmed by restriction digestion and by DNA sequencing.

**Generation of Myc-tagged IRS-1—pcDNA3-IRS-1 was digested with HindIII, and BspE1 site, respectively, are indicated in boldface type. The correctness of the construct (pcDNA3-Myc-IRS1) was verified by restriction mapping.

**Overexpression of PKB or IRS-1—CHO-T cells (31) were transiently transfected using LipofectAMINE as described (32). The constructs used to overexpress PKB were pCI792-Akt-WT, pCI792-Akt-K179A, or pCI792-Akt-Myr (33).

**Immunoprecipitation—Cells were solubilized at 4 °C in buffer B (25 mM Tris-HCl, 2 mM sodium orthovanadate, 0.5 mM EDTA, 10 mM NaF, 10 mM sodium pyrophosphate, 80 mM β-glycerophosphate, 25 mM NaCl, 1% Triton X-100, protease inhibitor mixture (Sigma), 1:1000, pH 7.4). Following three cycles of freezing and thawing, the cell extracts were centrifuged at 12,000 × g for 20 min at 4 °C, and the supernatants were collected. Samples (100 μl) were resolved by means of SDS-PAGE and immunoblotted with the indicated antibodies.

**Chromatography on Mono-Q FPLC—**Chromatographic fractionation were carried out at 4 °C using an Amerham Pharmacon Biotech Mono-Q FPLC system, as described previously (34). Fao cells from two confluent 15-cm plates were extracted in 1 ml of buffer A, disrupted on ice by 2 × 10 s sonication (30 watts), and centrifuged at 100,000 × g for
30 min at 4 °C. Supernatants containing the cytosolic extracts were brought to a 10-ml volume with buffer C (50 mM β-glycerophosphate, 1.5 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM sodium vanadate, pH 7.3) and were loaded at 0.5 ml/min on a Mono-Q column equilibrated with buffer C. Following a brief wash, 1-ml fractions were eluted at 1 ml/min with a 100-ml gradient from 0 to 0.4M NaCl in buffer C. Fractions were stored at 4 °C, retaining activity for at least 3 weeks.

In Vitro Kinase Assay—Fao cells were treated for 20 min with a combination of 3 mM H₂O₂ and 1 mM sodium orthovanadate. Cell extracts (1 mg for CHO-T and 0.5 mg for Fao cells) were bound to immobilized IR as described under “Experimental Procedures.” Results are mean ± S.D. of two independent experiments.

RESULTS

IRS-1 Serves as a Substrate for an Insulin-stimulated and Wortmannin-sensitive Ser/Thr Kinase—We have previously shown that IRS-1 and IRS-2 selectively interact with immobilized peptides comprising the JM region of the IR (20, 21). Moreover, prolonged treatment of Fao cells with insulin or insulin; however, complex formation was enhanced 2-fold as a result of association between IRS-1 and PKB co-precipitated with IRS-1 even in the absence of activated myristoylated form of this enzyme (33). The cells were then treated with insulin, and the extent of association between IRS-1 and PKB was assessed following immunoprecipitation with IRS-1-specific antibodies. As shown in Fig. 3, wild-type PKB co-precipitated with IRS-1 even in the absence of insulin; however, complex formation was enhanced 2-fold as a result of insulin treatment. In contrast, the constitutively active form of PKB remained maximally associated with IRS-1 even in the absence of insulin, and insulin treatment had no effect on these interactions. These findings suggest that PKB forms stable complex with IRS-1 in vivo. Formation of these complexes is enhanced by insulin and could therefore serve to further propagate insulin signaling.

IRS-1 Serves as a Substrate for an Insulin-stimulated and Wortmannin-sensitive Ser/Thr Kinase—We have previously shown that IRS-1 and IRS-2 selectively interact with immobilized peptides comprising the JM region of the IR (20, 21). Moreover, prolonged treatment of Fao cells with insulin or PD-98059, a specific mitogen-activated protein kinase/extracellular signal-regulated kinase kinase inhibitor (36), SB-202190, a specific inhibitor of p38 mitogen-activated protein kinase, and the PKC inhibitors Go-6976 and GF-109203X had no such protective effect (Fig. 1, top). Furthermore, IRS-1, derived from wortmannin- and insulin-treated cells, interacted with IR to a higher extent when compared with IRS-1 derived from cells that were incubated with insulin only for 60 min (Fig. 1, bottom). These findings suggest that IRS-1 serves as a substrate for an insulin-stimulated and wortmannin-sensitive Ser/Thr kinase, whose activity reduces the ability of IRS-1 to interact with the IR.

Ser Residues within the PTB/SAIN Domain of IRS-1 Are Potential Substrates of PKB—Several studies have indicated that the PTB domain of IRS proteins interacts with an NPEY motif within the JM region of IR (11, 12). Alignment of the PTB domains of mouse IRS-1 (amino acids 155–350) and mouse IRS-2 (amino acids 191–350) (4) (Table I) revealed the presence of two conserved Ser residues (Ser²⁶⁵ and Ser³⁰² in mouse IRS-1) that conform to a consensus PKB phosphorylation site (RXRXX(S/T)) (37). Two additional Ser residues (Ser²⁵⁵ and Ser³⁵⁸ in mouse IRS-1) were found in a consensus PKB phosphorylation sequence within a conserved region of IRS-1 and IRS-2, named SAIN, located —50 amino acids C-terminal to the PTB domain (4). Since PKB is a wortmannin-sensitive Ser/Thr kinase present downstream of PI3K (22, 38), we wished to determine whether PKB might phosphorylate these Ser residues. For this purpose, the above mentioned Ser residues of IRS-1 (serines 265, 302, 325, 358) were mutated to Ala, and the wild-type IRS-1 as well as its mutated form (IRS-1₄₄A) were transiently overexpressed in CHO-T cells. Cell extracts were immunoprecipitated with IRS-1 antibodies, and equal amounts of the precipitated IRS-1 proteins were subjected to in vitro phosphorylation by an activated PKB, derived from Fao extracts, fractionated over Mono-Q FPLC. As shown in Fig. 2, immunoprecipitated wild-type IRS-1 served as an in vitro substrate for the activated PKB and underwent phosphorylation in a time-dependent manner. Phosphorylation was markedly inhibited when IRS-1₄₄A was used as a substrate. These results suggest that Ser residues, located within the PTB/SAIN domain of IRS-1, are in vitro phosphorylation sites for PKB, that might regulate IRS-1 function in vivo.

Insulin Induces Formation of Complexes between IRS-1 and PKB in Vivo—To determine whether IRS-1 might interact with PKB in vivo, CHO-T cells were transiently transfected with either a wild-type PKB or with PKB-Myc, the constitutively active myristoylated form of this enzyme (38). The cells were then treated with insulin, and the extent of association between IRS-1 and PKB was assessed following immunoprecipitation with IRS-1-specific antibodies. As shown in Fig. 3, wild-type PKB co-precipitated with IRS-1 even in the absence of insulin; however, complex formation was enhanced 2-fold as a result of insulin treatment. In contrast, the constitutively active form of PKB remained maximally associated with IRS-1 even in the absence of insulin, and insulin treatment had no effect on these interactions. These findings suggest that PKB forms stable complex with IRS-1 in vivo. Formation of these complexes is enhanced by insulin and could therefore serve to further propagate insulin signaling.

IRS-1 Is an in Vivo Substrate of PKB—To determine whether PKB serves as an IRS kinase in vivo, CHO-T cells were transiently transfected with Myc-tagged IRS-1 (Myc-IRS-1) in the
The PTB domains of mouse IRS-1 (amino acids 155–309) and mouse IRS-2 (amino acids 191–350) were aligned. Shown are four conserved Ser/Thr residues, found within a consensus PKB phosphorylation site (RXRXX(S/T)) (37). Four additional residues Ser325 and Ser358 (IRS-1) and Ser325 and Ser342 (IRS-2) are also within a consensus PKB phosphorylation sequence in a conserved region located ~50 amino acids C-terminal to the PTB domain.

**TABLE I**

Alignment of the PTB domains of mouse IRS-1 and mouse IRS-2 (4)

| Substrate | Sequence  | Substrate | Sequence  | Substrate | Sequence  | Substrate | Sequence  |
|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| IRS-1 265 | RVRSKSQ   | IRS-1 302 | RVRTEI    | IRS-1 325 | RVRASQD   | IRS-1 358 | RVRSSSR   |
| IRS-2 301 | RVRSKSQ   | IRS-2 342 | RVRTEI    | IRS-2 362 | RVRTEAE   | IRS-2 397 | RVRAPLSR   |

**Fig. 2.** In vitro phosphorylation of wild-type and mutated form of IRS-1 by PKB. CHO-T cells were transiently transfected with either pcDNA3-IRS-1 or pcDNA3-IRS-1 4A, as described under “Experimental Procedures.” Cellular extracts were prepared, samples (1 mg) were subjected to immunoprecipitation with IRS-1 antibody, and equal amounts of precipitated IRS-1 proteins were subjected to in vitro kinase assay by activated, partially purified PKB, derived from Fao extracts, fractionated over Mono-Q FPLC as described under “Experimental Procedures.”

**Fig. 3.** Co-immunoprecipitation of IRS-1 and PKB. CHO-T cells were transiently transfected with pcIS2-PKB-WT or pcIS2-PKB-Myr as described under “Experimental Procedures.” Cells were incubated with or without 100 nM insulin for 15 min at 37 °C. Cellular extracts were prepared, and samples (1 mg) were subjected to immunoprecipitation (IP) with PKB antibodies, or with normal serum (NS) as a control. Immunoprecipitates were resolved by means of 7.5% SDS-PAGE and immunoblotted with IRS-1 or PKB antibodies absent or in the presence of a constitutively active form of PKB. As shown in Fig. 4, incubation of CHO-T cells (transfected with Myc-IRS-1), with 100 nM insulin resulted in Tyr phosphorylation of the Myc-IRS-1. Tyr phosphorylation was maximal following 2-min insulin treatment and was reduced by ~50% following 60-min incubation with the hormone. This was accompanied by a decrease in the electrophoretic mobility of IRS-1. Overexpression of a constitutively active form of PKB (PKB-Myr) resulted in an insulin-independent reduction in the electrophoretic mobility of Myc-IRS-1, suggesting that IRS-1 underwent a PKB-mediated Ser/Thr phosphorylation in vivo. Furthermore, overexpression of PKB, did not affect the acute (2-min) insulin-induced Tyr phosphorylation of Myc-IRS-1, but it significantly attenuated the extent of dephosphorylation of the Myc-IRS-1 protein, observed following 60-min insulin treatment. These findings suggest that IRS-1 is an in vivo substrate for a PKB-mediated phosphorylation, which attenuates its rate of Tyr dephosphorylation. Similar results were obtained when PKB-Myr was transiently transfected into CHO cells that stably co-express both IR and IRS-1 (29) (Fig. 5).

**Fig. 4.** Effect of overexpression of PKB on Myc-tagged IRS-1 phosphorylation. CHO-T cells were transiently transfected with both pcDNA3-Myc-IRS-1 and pCIS2-PKB-Myr as described under “Experimental Procedures.” Cells were incubated with 100 nM insulin for increasing time periods at 37 °C, and cellular extracts were prepared. Samples (500 µg) were subjected to immunoprecipitation (IP) with Myc antibody. Immunocomplexes were resolved by means of 7.5% SDS-PAGE and immunoblotted with IRS-1 or Tyr(P) antibodies. The ratio between the bands corresponding to Tyr(P) and IRS-1 was quantitated by densitometry. Results are mean ± S.D. of two independent experiments.

Phosphorylation of Ser Residues within the PTB/SAIN Domain Protects IRS-1 from Tyr Dephosphorylation—Since the above findings suggested that IRS-1 could form complexes with PKB and could serve as an in vivo substrate for PKB-mediated phosphorylation, we wished to determine the consequences of mutations of the potential PKB phosphorylation sites on IRS-1 function. For this purpose, CHO-T cells were transfected with either wild-type IRS-1 or with the mutant IRS-1 4A. As shown in Fig. 6, 1-min incubation of the cells with 100 nM insulin enhanced the Tyr phosphorylation of the overexpressed wild-type and mutated IRS-1 proteins to comparable levels, which were significantly higher than the levels of IRS-1 phosphorylation in the nontransfected cells. These results already indicated that the mutation of the four Ser residues did not grossly alter IRS-1 conformation. Consistent with our previous findings (21), when the incubation with insulin was prolonged to 60 min, the extent of Tyr phosphorylation of the overexpressed wild-type IRS-1 declined and was accompanied by a decrease in its ability to interact with the receptor (not shown). When CHO-T cells, transfected with the mutated form of IRS-1, were incubated with insulin for 60 min, the rate of Tyr dephosphorylation of IRS-1 4A was enhanced compared with the wild-type protein, suggesting that the mutated IRS-1 served as a better substrate for protein Tyr phosphatases (PTPs). The enhanced dephospho-
oroverexpressing IRS-14A with vanadate, prior to a 60-min incubation, the effects of vanadate, a potent inhibitor of PTPs (39) were studied. As shown in Fig. 7, pretreatment of CHO-T cells with 100 nM insulin for increasing time periods at 37 °C, and cellular extracts were prepared. Samples (100 μg) were resolved by means of 7.5% SDS-PAGE and immunoblotted with IRS-1, PKB, or Tyr(P) antibodies. The ratio between the bands corresponding to Tyr(P) and IRS-1 was quantitated by densitometry. Results are mean ± S.D. of three independent experiments.

Fig. 6. Effects of insulin on Tyr phosphorylation of IRS-1 and IRS-14A. CHO-T cells were transiently transfected or not transfected with either pcDNA3-IRS-1 or pcDNA3-IRS-14A, as described under “Experimental Procedures.” Cells were incubated with 100 nM insulin for 1 or 60 min at 37 °C. Cellular extracts were prepared, and samples (100 μg) were resolved by means of 7.5% SDS-PAGE and immunoblotted with Tyr(P) antibodies. Results of two independent experiments are presented.

Phosphorylation of IRS Proteins by PKB

Effect of Vanadate on Tyr Phosphorylation of IRS-14A—To confirm that IRS-14A undergoes accelerated Tyr dephosphorylation, the effects of vanadate, a potent inhibitor of PTPs (39) were studied. As shown in Fig. 7, pretreatment of CHO-T cells overexpressing IRS-14A with vanadate, prior to a 60-min incubation with insulin, elevated the Tyr(P) content of IRS-14A to levels even greater than those observed following 2-min insulin treatment. These findings support the notion that IRS-14A is subjected to accelerated Tyr dephosphorylation following prolonged incubation with insulin. Hence, PKB-mediated phosphorylation of Ser residues within the PTB domain of IRS-1 presumably turns IRS-1 into a poorer substrate for PTPs and helps to maintain it in its Tyr-phosphorylated active conformation.

Fig. 7. Effects of vanadate on insulin-stimulated Tyr phosphorylation of IRS-14A. CHO-T cells were transiently transfected with pcDNA3-IRS-14A, as described under “Experimental Procedures.” Cells were incubated with or without 1 mM vanadate for 1 h at 37 °C. Cells were further incubated with 100 nM insulin for 1 or 60 min at 37 °C. Cellular extracts were prepared, and samples (100 μg) were resolved by means of 7.5% SDS-PAGE and immunoblotted with Tyr(P) antibodies. The intensity of the bands corresponding to the phosphorylated IRS-1 was quantitated using scanning densitometer. Results are mean ± S.D. of two independent experiments.

DISCUSSION

Our studies indicate that IRS-1 is a potential physiological substrate for PKB, which acts as a positive regulator of IRS-1 function. Analysis of the sequences of IRS proteins reveals the presence of four Ser residues within RXRXXS motifs that serve as potential PKB phosphorylation sites (37). All four motifs are localized in conserved regions within, or in close proximity to, the PTB domains of IRS-1 and IRS-2, implicating their potential importance for IRS signaling. Both the full-length IRS-1 and its isolated PTB domain, expressed as a glutathione S-transferase fusion protein, serve as in vitro substrates of PKB.

Furthermore, mutations of the Ser residues within the RXRXXS motifs reduce the ability of PKB to phosphorylate IRS-1 and its isolated PTB domain in vitro. The reduced ability of PKB to phosphorylate the mutated IRS proteins could not be attributed to gross structural alterations of IRS-14A, as a result of the mutation, since the wild-type and the mutated IRS interacted with the IR to a similar extent in vitro, and when overexpressed in CHO-T cells, they underwent Tyr phosphorylation to a similar extent following acute treatment with insulin. Two lines of evidence support the notion that IRS-1 might also serve as an in vivo substrate of PKB. First, overexpression of a constitutively active form of PKB induces a mobility shift of IRS-1, even in cells that were not treated with insulin. Second, PKB forms stable complexes with IRS-1 in vivo, and it is readily co-precipitated with IRS-1 specific antibodies. The association of PKB with IRS-1 is accelerated following insulin stimulation and is presumably the consequence of the Tyr phosphorylation of the IRS protein that leads to its interaction with the p85α regulatory subunit of PI3K and other downstream effectors (1, 13).

PKB-mediated phosphorylation of IRS-1 seems to act as a positive feedback mechanism of insulin signals. Overexpression of PKB attenuates the rate of Tyr dephosphorylation of IRS-1, which occurs following prolonged insulin treatment (21). Conversely, mutation to Ala of Ser residues within the PTB region of IRS-1, which serve as potential PKB phosphorylation sites, accelerates the rate of Tyr dephosphorylation of the IRS-1 protein. The enhanced dephosphorylation of IRS-14A occurred with no significant effect on IR-IRS-1 complex formation and could not be attributed to enhanced degradation of IRS-14A protein. Hence, phosphorylation of these Ser sites seems to protect the IRS-1 protein from the rapid action of protein Tyr phosphatases and maintains IRS-1 in its Tyr-phosphorylated form.

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active conformation. Indeed, when cells that overexpress the mutated form of IRS-1 are incubated with insulin in the presence of vanadate, a potent inhibitor of PTPs (39), the accelerated rate of Tyr dephosphorylation of the mutated IRS proteins is abolished. It is presently unclear why phosphorylation of Ser residues within the PTB domain of IRS-1 prevents its Tyr dephosphorylation. Most likely, Ser phosphorylation induces a conformational change, turning IRS-1 into a poorer substrate for PTPs. Alternatively, phosphorylation of Ser residues within the PTB domain of IRS-1 could induce translocation of IRS-1 (40) away from the relevant PTPs.

We have previously shown (21) that enhanced Ser/Thr phosphorylation of IRS proteins impedes their interaction with the JM region of the IR and turns them into poorer substrates for the insulin receptor kinase. Impaired Tyr phosphorylation eliminates the ability of IRS proteins to recruit downstream effector molecules, results in severe impairment of insulin signal transduction, and could provide a molecular basis for the induction of insulin resistance. Similarly, insulin-induced Ser/Thr phosphorylation of mSos results in the dissociation of Sos-Grb2 complexes and attenuation of the Shc/Grb-2/Sos/Ras/mitogen-activated protein kinase cascade (41, 42). Hence, Ser/Thr kinases, stimulated by insulin, act as negative feedback regulators to turn off insulin signals under physiological conditions.

The PTB domain, which shares 75% sequence identity between IRS-1 and IRS-2, mediates the interactions of IRS proteins with the JM region of the insulin receptor (12). Alignment of the PTB domains of IRS-1 and IRS-2 (4) reveals the presence of 16 conserved Ser/Thr residues, whose phosphorylation might affect the interactions of the PTB domain with the juxtamembrane region. In the present study, we could demonstrate that four Ser residues within the RXXRXS motif presumably act as positive effectors of IRS-1 functions. Still, this leaves 12 other Ser residues within the PTB domain alone as potential targets for Ser/Thr kinases that might act as negative regulators of IRS-1 function.

In the present study, we provide evidence that at least one of these negative regulators is also a wortmannin-sensitive Ser/Thr kinase, different from PKBα. Several lines of evidence support this conclusion. We could demonstrate that out of several inhibitors tested, only wortmannin, a PI3K inhibitor, effectively inhibited the dissociation of IRS proteins from the IR and the subsequent reduction in Tyr phosphorylation of IRS proteins, observed following a 60-min insulin treatment. Other inhibitors that selectively block the activities of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase, p38 mitogen-activated protein kinase, or several of the PKC isoforms (α, β, γ, δ, ε, and ζ) were ineffective in preventing the negative feedback control mechanism induced by insulin. Hence, a wortmannin-sensitive Ser/Thr kinase, different from PKBα, presumably acts as the feedback control regulator that turns off insulin signals. Activation of this kinase is expected to take place subsequent to activation of PKBα, which acts as a positive regulator of IRS-1 function.

Several Ser/Thr kinases, located downstream of PI3K are likely candidates to fulfill this role. These include the mammalian target of rapamycin (43) and p70s6 kinase (44), which are activated by phosphatidylinositol 3-kinase-dependent kinase 1 (45) and PKB (38). Indeed, mammalian target of rapamycin-mediated phosphorylation of IRS-1 on serines 632, 662, and 731 of IRS-1 was shown to inhibit insulin-stimulated Tyr phosphorylation of IRS-1 and its ability to bind PI3K (46, 47). Accordingly, membrane-targeted PI3K was found to stimulate Ser/Thr phosphorylation of IRS-1 and to inhibit IRS-1-associated PI3K activity (48). Other potential candidates could be members of the PKC family. Atypical PKCs, exemplified by PKCζ, were implicated as downstream effectors of PI3K (49); however, the possibility that PKC isoforms are effectors of an insulin-stimulated signaling cascade is somewhat controversial (50, 51). Although we have shown that two selective inhibitors of PKC, GF-109203X (Calbiochem; inhibits PKCa-β1, -β2, -γ1, -γ2, and -ε) and Go-6976 (Calbiochem; inhibits PKCa-β2, and -μ1), are ineffective in preventing the reduction in phosphorylation of IRS proteins following a 60-min insulin treatment, we cannot rule out the possibility that PKC isoforms insensitive to these inhibitors such PKCζ, -η, or -θ could mediate insulin’s effects. In fact, we have previously shown that 12-O-tetradecanoylphorbol-13-acetate, a potent activator of various PKC isoforms, effectively inhibits both IRS-1 interactions with the JM region of the IR and insulin’s ability to phosphorylate IRS proteins (21). Similarly, mutation of Ser612 of IRS-1 eliminates the ability of 12-O-tetradecanoylphorbol-13-acetate to induce IR-IRS dissociation, thus implicating PKCs as effective regulators of IR-IRS interactions (46, 52).

Other downstream effectors of PI3K are less likely to act as insulin-induced negative regulators of IRS-1 function. Glycogen synthase kinase 3 is capable of phosphorylating IRS-1, and this modification converts IRS-1 into an inhibitor of IR Tyr kinase activity in vitro (53); however, it is unlikely that glycogen synthase kinase 3 could act as an insulin-stimulated kinase of IRS-1, since glycogen synthase kinase 3 activity is inhibited by insulin (54). Other kinases in this category are the family of the phosphatidylinositol 3-kinase-dependent kinases (25–27). PKDs are downstream effectors of PI3K (25–27) and are stimulated in response to insulin (55). However, being upstream activators of PKB (25–27) turns them into less likely candidates for being negative regulators of IRS-1 function. Also, it still remains to be determined whether the substrate specificity of phosphatidylinositol 3-kinase-dependent kinases enables them to phosphorylate key Ser/Thr residues within the IRS-1 molecule. Finally, the possibility still exists that other PKB isoforms, not studied here (i.e. PKBβ and PKBγ (56)) might act as a negative feedback control regulator of IRS-1 in vivo. This possibility, however, seems less probable in view of the fact that the three PKB isoforms possess identical substrate specificity toward a range of peptides (56).

Collectively, our findings indicate that Ser/Thr phosphorylation of IRS protein following insulin stimulation has a dual role, either to enhance or to terminate insulin signal. Insulin activates a wortmannin-sensitive kinase, downstream of or independent from PKB, that phosphorylates as yet unidentified Ser/Thr residues within the IRS protein. Phosphorylation of these sites is part of the negative feedback control mechanism, induced by insulin, that leads to the dissociation of the IR-IRS complexes and results in the termination of insulin signal. Agents that induce insulin resistance, such as tumor necrosis factor, take advantage of this mechanism by stimulating the phosphorylation of IRS proteins on the same or similar Ser/Thr sites, whose phosphorylation results in the dissociation of IR-IRS complexes (21). In contrast, we have shown that Ser residues in the PTB domain of IRS-1, located within consensus PKB phosphorylation sites, presumably function as positive effectors of insulin signaling. Once phosphorylated by PKBα, they serve to protect IRS proteins from the rapid action of PTPs. In such a way, PKBα acts to propagate and accelerate insulin signaling by phosphorylating downstream effectors and by phosphorylating IRS proteins, thus generating a positive feedback loop for insulin action. Both Ser/Thr kinases that phosphorylate IRS-1, the positive regulator PKB and the wortmannin-sensitive negative regulator, are downstream effectors of PI3K. This suggests that their action should be orchestrated in a way that will enable sustained activation of IRS-1, as a
result of phosphorylation by PKB, prior to the activation of the negative regulator, whose action is expected to terminate insulin signal transduction. Further studies are required to unravel the mechanisms that control this intricate regulatory process.

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