A Molecular Basis for Insulin Resistance

ELEVATED SERINE/THREONINE PHOSPHORYLATION OF IRS-1 AND IRS-2 INHIBITS THEIR BINDING TO THE JUXTAMEMBRANE REGION OF THE INSULIN RECEPTOR AND IMPAIRS THEIR ABILITY TO UNDERGO INSULIN-INDUCED TYROSINE PHOSPHORYLATION*

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Tumor necrosis factor α (TNFα) or chronic hyperinsulinemia that induce insulin resistance trigger increased Ser/Thr phosphorylation of the insulin receptor (IR) and of its major insulin receptor substrates, IRS-1 and IRS-2. To unravel the molecular basis for this uncoupling in insulin signaling, we undertook to study the interaction of Ser/Thr-phosphorylated IRS-1 and IRS-2 with the insulin receptor. We could demonstrate that, similar to IRS-1, IRS-2 also interacts with the juxtamembrane (JM) domain (amino acids 943–984) but not with the carboxy-terminal region (amino acids 1245–1331) of IR expressed in bacteria as His6 fusion peptides. Moreover, incubation of rat hepatoma Fao cells with TNFα, bacterial sphingomyelinase, or other Ser(P)/Thr(P)-elevating agents reduced insulin-induced Tyr phosphorylation of IRS-1 and IRS-2, markedly elevated their Ser(P)/Thr(P) levels, and significantly reduced their ability to interact with the JM region of IR. Withdrawal of TNFα for periods as short as 30 min reversed its inhibitory effects on IR-IRS interactions. Similar inhibitory effects were obtained when Fao cells were subjected to prolonged (20–60 min) pretreatment with insulin. Incubation of the cell extracts with alkaline phosphatase reversed the inhibitory effects of insulin. These findings suggest that insulin resistance is associated with enhanced Ser/Thr phosphorylation of IRS-1 and IRS-2, which impairs their interaction 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 the insulin receptor signal. Moreover, the reversibility of the TNFα effects and the ability to mimic its action by exogenously added sphingomyelinase argue against the involvement of a proteolytic cascade in mediating the acute inhibitory effects of TNFα on insulin action.

The insulin receptor (IR) is an 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 a tyrosine-specific protein kinase (IRK) that undergoes autophosphorylation following insulin binding (reviewed in Ref. 1). Autophosphorylation activates the IRK (2) and enables it to phosphorylate endogenous protein substrates, including Shc (3) and the insulin receptor substrates IRS-1 (4) and IRS-2 (5), to further propagate the insulin signal. IR-1 and IRS-2, two related protein substrates of IRK, have a highly conserved amino terminus, which contains a pleckstrin homology domain and a phosphotyrosine-binding (PTB) domain, and a poorly conserved carboxyl terminus with several tyrosine phosphorylation motifs. IRS-1 and IRS-2 also contain over 30 Ser/Thr residues in consensus phosphorylation sites (4, 5). The relative roles of IRS-1 and IRS-2 in mediating insulin action is presently unknown, although IRS-2 functions as an alternative substrate of IR in IRS-1 null mice (6), which manifest a mild form of insulin resistance.

Insulin resistance is a state in which target cells fail to respond to ordinary levels of circulating insulin (7). At the molecular level, impaired insulin signaling results from mutations or post-translation modifications of the insulin receptor itself or any of its downstream effector molecules. A major negative regulatory role to insulin action is attributed to agents that enhance Ser/Thr phosphorylation of either the receptor itself or of its downstream effectors, which reduce IRK activity or its ability to phosphorylate substrate proteins (see Refs. 8 and 9 for reviews). For example, insulin’s counter-regulatory hormones such as epinephrine or glucagon increase cAMP levels, activate the cAMP-dependent protein kinase, and increase the Ser(P)/Thr(P) content of the insulin receptor, which results in an insulin-resistant state. Similarly, okadaic acid, an inhibitor of protein phosphatases, inhibits insulin-induced Tyr phosphorylation of IRS-1 while increasing the Ser/Thr phosphorylation level of this protein (10, 11). Tumor necrosis factor-α (TNFα), a mediator of insulin resistance in infection, tumor cachexia, and obesity, also acts in a similar manner. TNFα diminishes insulin-induced Tyr phosphorylation of IRS-1 while it induces Ser/Thr phosphorylation of IRS-1, which decreases

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† The abbreviations used are: IR, insulin receptor; IRK, insulin receptor kinase; CT, carboxy-terminal; JM, juxtamembrane; TNFα, tumor necrosis factor α; SMase, sphingomyelinase; TPA, 12-O-tetradecanoylphorbol-13-acetate; PTB, phosphotyrosine-binding; PAGE, polyacrylamide gel electrophoresis; TNFαR, TNFα receptor.
its electrophoretic mobility (12, 13). These effects of TNFα are not the result of increased protein-tyrosine phosphatase activity but are presumably mediated through inhibition of Ser phosphatases or activation of Ser kinase(s) (12). The effects of TNFα are mimicked by treating cells with sphingomyelinase or after addition of cell-permeable ceramide analogs (C2 and C3) (14, 15), suggesting that TNFα may utilize the sphingomyelin pathway (16, 17) to impair insulin action. Overall, these findings suggest the existence of converging desensitization mechanisms for IR and IRS-1 that involve Ser/Thr phosphorylation of these proteins.

A question still unresolved is how Ser/Thr phosphorylation of IR or its substrates affects their mutual interactions and what is the relative contribution of each of the elements in this signaling complex to the impairment in insulin signal transduction. This question is difficult to tackle in studies carried out in intact cells, because agents that enhance cellular Ser/Thr phosphorylation often affect both IR and its substrates. Hence, impaired signal transduction could result from enhanced Ser/Thr phosphorylation of IR itself, its downstream effectors (e.g., IRS-1 and IRS-2), or both. In the present study we undertook to address this issue by eliminating the effects of Ser/Thr phosphorylation of IR, focusing only on changes in IRS-1 or IRS-2. We took advantage of our previous findings that the isolated juxtamembrane (JM) domain of IR (amino acids 943–984) but not its carboxy-terminal (CT) region (amino acids 1245–1331), is sufficient to mediate interactions between IR and IRS-1 (18). Here we present evidence that similar to IRS-1, IRS-2 also interacts with the JM but not the CT region of IR. Moreover, insulin, TNFα, and other Ser/Thr phosphorylation-inducing agents significantly reduce the ability of IRS-1 or IRS-2 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 the insulin receptor signal, thus providing us with a possible molecular mechanism for the induction of an insulin-resistant state.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant human insulin was a gift from Novo-Nordisk (Copenhagen, Denmark). Recombinant murine TNFα was kindly provided by Dr. G. Adolf (Boehringer Ingelheim, Vienna). SMase (neutral, Staphylococcus aureus), okadaic acid, alkaline phosphatase (Sigma F3681), and calycin A were purchased from Sigma. ProBond Ni2+ beads were obtained from Invitrogen (San Diego, CA). Monoclonal Tyr(P) (PY-20) antibodies were obtained from Transduction Laboratories (Lexington, KY). Polyclonal IRS-1 antibodies were prepared as described (19). Polyclonal antibodies directed against a GST fusion protein containing amino acids 967–1094 of mouse IRS-2 were purchased from Upstate Biotechnology Inc. (Lake Placid, NY).

**Peptide Expression and Purification**—(His)6-tagged fusion peptides corresponding to 41 amino acids (amino acids 943–984) of the juxtamembrane region of IR (His)6-JM or 86 amino acids (amino acids 1245–1331) of the carboxy-terminal region of IR (Hist)6-CT were generated in bacteria and purified over ProBond Ni2+ beads (Invitrogen) as described (18). Synthetic peptides with the following sequence were used for binding-inhibition experiments: PIP10 pep (PLILLSNPEYLSSAS-NH2) and S1013 pep (SYEHHPPYTHMNG-NH2). Peptides were synthesized as described (20), purified by reverse-phase high performance liquid chromatography, and quantitated by amino acid analysis (21).

**Precipitation of Cytosolic Proteins Derived from Fao Cells by (His)6-tagged Fusion Peptides**—Cytosolic extracts were prepared from rat hepatoma Fao cells. Cells were grown in RPMI medium supplemented with 10% fetal calf serum. Confluent cells grown in 60-mm dishes were deprived of serum for 16 h prior to each experiment. The medium was removed and the cells were incubated with different agents (e.g., TNFα, SMase, and TPA) in serum-free medium for different time intervals at 37 °C. Cells were then incubated with or without 100 nM insulin for 1 min at 37 °C. Cells were washed three times with phosphate-buffered saline and harvested in 300 μl of buffer A (25 mM Tris-HCl, 2 mM sodium orthovanadate, 0.5 mM EGTA, 10 mM NaF, 10 mM sodium pyrophosphate, 80 mM β-glycerophosphate, 25 mM NaCl, 10 mM MgCl2, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 10 μg/ml trypsin inhibitor, and 1 mM phenylmethylsulfonyl fluoride, pH 7.4). After three freeze-thaw cycles, the cells were centrifuged at 12,000 × g for 30 min at 4 °C, and the supernatants were collected. 300 μg of ~300-μl aliquots from the supernatants were incubated for 1 h with 2 μg of purified JM peptide and 500 μl of ProBond Ni2+ beads prewashed in buffer A at 4 °C. The beads were washed four times with buffer A containing 200 mM imidazole and 0.1% Nonidet P-40, washed twice with buffer A, and boiled in 60 μl of Laemmli “sample buffer” (22). Samples were resolved by means of SDS-PAGE and subjected to Western blotting with the appropriate antibodies as described (18).

**Binding Inhibition Assays**—Cytosolic extracts were prepared from serum-deprived Fao cells. Cells were grown in 60-mm dishes, deprived of serum for 16 h prior to each experiment. The medium was removed, and cells 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 of buffer B (25 mM Tris-HCl, 2 mM sodium orthovanadate, 0.5 mM EGTA, 25 mM NaCl, 10 mM MgCl2, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 10 μg/ml trypsin inhibitor, and 1 mM phenylmethylsulfonyl fluoride, pH 7.4). After three freeze-thaw cycles, the cells were centrifuged at 12,000 × g for 15 min at 4 °C, and the supernatant was collected. Aliquots (300 μg, ~100 μl) were incubated with 5 μl (1000 units) of alkaline-phosphatase for 1 h at 37 °C. Following incubation, samples were subjected to precipitation using immobilized JM peptide (see above), boiled in 60 μl of Laemmli sample buffer (22), resolved by means of SDS-PAGE, and subjected to Western blotting with IRS-1 or IRS-2 antibodies (18).

**Immunoprecipitation**—Cells were solubilized at 4 °C with lysis buffer (50 mM Tris-HCl, 1% Nonidet P-40, 0.5% octylpolyoxy-cholate, 150 mM NaCl, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin). Cell lysates were centrifuged at 12,000 × g for 15 min at 4 °C. Aliquots of the supernatants (0.5–1.0 mg) were incubated for 3 h at 4 °C with IRS-1 and IRS-2 antibodies, and the immunocomplexes were captured by adding a 100-μl mixture of protein G-Sepharose and protein A-Sepharose beads (1:1) and incubating for 1 h at 4 °C. Immunocomplexes were washed three times with lysis buffer, suspended in Laemmli sample buffer (22), boiled for 5 min, resolved by 6.0–7.5% SDS-PAGE under reducing conditions (22), and transferred into nitrocellulose papers for Western blotting with the appropriate antibodies. Immunoblotting was performed as described previously (23), using an ECL kit purchased from Amersham Corp. and horseradish peroxidase-conjugated protein A according to the manufacturer’s instructions.

**RESULTS**

**IRS-2 Specifically Interacts with the JM Peptide**—We have previously shown that IRS-1 selectively interacts with an immobilized polypeptide corresponding to 41 amino acids of the juxtamembrane region of IR ([Hist]6-JM) (18), although it fails to interact with an 86-amino acid polypeptide, corresponding to the carboxy-terminal region of this receptor ([Hist]6-CT). To determine whether IRS-2 behaves in a similar manner, [Hist]6-JM and [Hist]6-CT peptides were incubated with extracts of rat hepatoma (Fao) cells that contain both IRS-1 and IRS-2 (5). As shown in Fig. 1A, IRS-2 specifically associated with the immobilized JM peptide, as detected by Western blotting with IRS-2 antibodies. About 25% of the total IRS-2 protein remained bound to the beads, and similar to IRS-1 (18), no difference was observed between IRS-2 derived from control or insulin-treated (10−7 M, 1 min) cells. In contrast, IRS-2, like IRS-1 (18), failed to associate with the CT peptide. As shown in Fig. 1B, both IRS-1 and IRS-2 bound to immobilized JM peptides with similar affinities. Half-maximal binding of either protein, present in 300 μg of cell extracts, required ~0.5 μg of immobilized peptide, whereas maximal binding (25–35% of the total) was obtained with 2 μg of peptides. These results suggest
that IRS-2 selectively interacts with the JM region with affinity similar to that of IRS-1.

To further assess the specificity of interactions of IRS proteins with the JM region, peptide competition assays were carried out. As shown in Fig. 1C, a 14-amino acid peptide (PLYASSNPEYLELSAS) denoted F$^{85}$pep, which forms part of the JM region, effectively competed with binding of IRS-1 and IRS-2 to (His)$_n$-JM. Half-maximal binding inhibition occurred in the presence of 0.3 $\mu$M F$^{85}$pep, whereas maximal binding inhibition took place in the presence of 1.0 $\mu$M peptide. In contrast, a 13-amino acid peptide (SYEHHIPYTMGST), corresponding to the carboxyl-terminal region of IR failed to inhibit IRS-1 binding even when present at a concentration of 100 $\mu$M. These observations further support the notion (18) that IRS-1 and IRS-2 specifically interact with the JM region and that these interactions might occur independent of the phosphorylation state of Tyr$^{206}$.

Okadaic Acid and Calyculin A. Inhibitors of Ser(P)/Thr(P) Phosphatases, Reduce the Association of IRS-1 and IRS-2 with the JM Peptide—We have previously shown (18) that the Tyr-phosphorylated form of IRS-1 interacts to a lower extent with the JM region. To determine whether elevated Ser/Thr phosphorylation also affects IRS-1 and IRS-2 interactions with the JM peptide, Fao cells were incubated either with okadaic acid or with calyculin A, which inhibit Ser(P)/Thr(P) phosphatase activities (24). Consistent with previous findings (11), okadaic acid and calyculin A inhibited insulin-induced Tyr phosphorylation of a protein having a molecular mass of 180 kDa (pp180). This band (Fig. 2, A and D) corresponds to IRS-1 and IRS-2, which are not well resolved by SDS-PAGE under these conditions (25). The decrease in Tyr phosphorylation was accompanied by decreased electrophoretic mobility of IRS-1 and IRS-2 (not shown), suggesting that both proteins indeed were subjected to enhanced Ser/Thr phosphorylation. The enhanced Ser/Thr phosphorylation of IRS-1 resulted in 60–80% reduction in its ability to interact with the JM peptide (Fig. 2, B and E). Moreover, the reduction in Tyr(P) content of p180 correlated with the reduced association of IRS-1 with the JM peptide. As shown in Fig. 3, half-maximal effects of both phenomena were achieved once the cells were incubated with 20–40 $\mu$M calyculin A. These results are compatible with the notion that the Ser/Thr-phosphorylated IRS-1 has a reduced affinity to the JM region of IR, which turns it into a poorer substrate for the IRK.

Similar results were obtained when we studied the capacity of immobilized JM peptide to interact with IRS-2 derived from calyculin A- or okadaic acid-treated Fao cells (Fig. 2, C and F). Hence, both forms of IRS exhibited a similar decrease in affinity toward the JM peptide (Fig. 3) once they were subjected to enhanced Ser/Thr phosphorylation.

TNFα Reduces the Association of IRS-1 and IRS-2 with the JM Peptide—Although the above findings already suggested that enhanced Ser/Thr phosphorylation of IRS-1 and IRS-2 diminishes their interaction with the JM domain of IR, we wished to determine whether physiological ligands that induce insulin resistance, such as TNFα, also affect critical Ser/Thr residues whose phosphorylation impedes interactions of IRS-1 (or IRS-2) with the JM region. For this purpose extracts of TNFα-treated Fao cells were prepared, and the extent of interaction of IRS-1 and IRS-2 present in these extracts with immobilized JM peptides was evaluated. In accordance with our previous findings (12), incubation of Fao cells with TNFα enhanced Ser/Thr phosphorylation of IRS-1 (12) and IRS-2 (Fig. 4B), as manifested by their decreased electrophoretic mobility. This was accompanied by a marked inhibition (~75%) of insulin-induced Tyr phosphorylation of pp180 (Fig. 4A) and a parallel reduction in the capacity of IRS-1 (Fig. 4D) and IRS-2 (Fig. 4F) to interact with the JM peptide, with both effects being obtained with 4–6 $\mu$g TNFα. Although TNFα is a known activator of a protease cascade (26), it did not affect the cellular content of either IRS-1 or IRS-2 within the time frame (30 min) of our experiments (Fig. 4, C and E).

SMase Mimics the Inhibitory Effects of TNFα on IR-IRS Interactions—The sphingomyelin pathway is a newly described signal transduction pathway mediating the action of several cytokines including TNFα. Indeed, we have previously shown (14) that incubation of Fao cells with bacterial SMase, which causes membrane hydrolysis of sphingomyelin, or with the cell-permeable ceramide analogs (C$_2$ and C$_6$) leads to a time- and dose-dependent decrease in insulin-induced tyrosine phosphorylation of IRS-1 and a parallel decrease in its electro-
Fig. 2. Effects of calyculin A and okadaic acid on insulin-stimulated Tyr phosphorylation of IRS-1 and IRS-2 and their ability to interact with the JM peptide. Fao cells were incubated for 30 min at 37 °C in the absence or in the presence of the indicated concentrations of calyculin A (A–C) or 100 nM okadaic acid (D–F). Cells were then incubated with 100 nM insulin for 1 min at 37 °C. A and D, cytosolic extracts were prepared as described under "Experimental Procedures." Samples (100 μg) were resolved by means of 6% SDS-PAGE and immunoblotted with Tyr(P) antibodies. Quantitation of the intensity of the bands, corresponding to Tyr-phosphorylated pp180, was presented as a bar graph. B and E, samples (300 μg) were incubated with 2 μg of purified JM peptide and 50 μl of Ni²⁺ beads for 60 min at 4 °C. After extensive washes, the beads were boiled in 60 μl of Laemmli sample buffer, and samples were resolved by means of 6% SDS-PAGE and immunoblotted with IRS-1 antibodies. Quantitation of the intensity of the bands corresponding to IRS-1 that remained bound to the immobilized JM peptide is presented as a bar graph. C and F, same as B and E except for the fact that Western blotting was carried out with IRS-2 antibodies. The results are of a representative experiment reproduced three times (mean ± S.D. ± 8%).

Correlation between the effects of calyculin A on Tyr phosphorylation of IRS-1 and IRS-2 and their interaction with the JM peptide. Fao cells were incubated with increasing concentrations of calyculin A for 30 min at 37 °C. Cells were then incubated with 100 nM insulin for 1 min at 37 °C. Quantitation of the intensity of the bands corresponding to tyrosine-phosphorylated pp180 (Fig. 2A) (▲), IRS-1 (Fig. 2B) (●), and IRS-2 (Fig. 2C) (○) complexes is presented.

The Inhibitory Effects of TNFα on IR-IRS Interactions Are Reversible—Because the inhibitory effects of TNFα involve enhanced Ser/Thr phosphorylation of IRS-1 and IRS-2, one would expect them to be reversible. To address this possibility we studied the effects of TNFα withdrawal on IR-IRS interactions. Fao cells were treated with TNFα for 30 min and then were extensively washed free of this agent. Cells were then maintained in TNFα-free medium for increasing periods of time prior to insulin stimulation. As shown in Fig. 6 removal of TNFα reversed its inhibitory action. Comparable levels of insulin-induced Tyr phosphorylation of pp180 were observed in control untreated or TNFα-treated cells that were washed and maintained for 30 min in TNFα-free medium (Fig. 6A). Similarly, comparable levels of either IRS-1 (Fig. 6B) or IRS-2 (Fig. 6C) were found associated with the JM peptide whether they were derived from control untreated or TNFα-treated and washed cells. These observations support the notion that TNFα effects on IR-IRS interactions involve a reversible signal transduction cascade rather than activation of an irreversible proteolytic process.

Protein Kinase C Inhibits Interactions of IRS-1 and IRS-2 with the JM Region—Although in Fao cells TNFα activates a Ser/Thr kinase different from Ca²⁺- and phosphatidylinerine-activated protein kinase C (12), we wished to determine whether other Ser/Thr kinases such as protein kinase C could also exert an inhibitory effect on IR-IRS interactions. For this
Interactions between the Insulin Receptor, IRS-1, and IRS-2

FIG. 4. Effects of TNFα on insulin-stimulated Tyr phosphorylation of IRS-1 and IRS-2 and their ability to interact with the JM peptide. Fao cells were incubated with the indicated concentrations of TNFα for 30 min at 37 °C. Cells were then incubated with 100 nM insulin for 1 min at 37 °C, A, cytosolic extracts were prepared. Samples (100 μg) were resolved by means of 6% SDS-PAGE and immunoblotted with Tyr(P) antibodies. Quantitation of the intensity of the bands, corresponding to Tyr-phosphorylated pp180 is presented as a bar graph. B, samples (500 μg) from control or TNFα-treated (6 nM) cells were subjected to immunoprecipitation (IP) with IRS-2 antibodies. Precipitated proteins were resolved by means of 6% SDS-PAGE and immunoblotted with Tyr(P) antibodies. C and E, same as in A save for the fact that IRS-1 (C) or IRS-2 (E) antibodies were used for Western blotting. Binding of IRS-1 (D) or IRS-2 (F) to the JM peptide was determined as described in the legend to Fig. 2. The results are of a representative experiment reproduced three times (mean ± S.D. ± 6%).

purpose the effects of TPA, a direct activator of protein kinase C, were studied. Incubation of Fao cells with TPA markedly inhibited insulin-induced Tyr phosphorylation of pp180, reduced the electrophoretic mobility of IRS-1 and IRS-2, and inhibited by 70% their ability to interact with the JM peptides (Fig. 7). Again, the authenticity of pp180 with IRS-1 and IRS-2 was verified when we could demonstrate similar inhibitory effects of TPA on immunoprecipitates of Tyr-phosphorylated IRS-1 and IRS-2 (Fig. 5C). These findings support the notion that IRS-1 and IRS-2 presumably serve as substrates for several different Ser/Thr kinases, all acting to impair their interaction with the JM region of IR.

Chronic Insulin Treatment Stimulates Ser/Thr Phosphorylation of IRS-1 and IRS-2, Which Inhibits Their Interaction with the JM Region—Incubation of cells with insulin induces a rapid Tyr phosphorylation of IRS-1, which is followed by a slower increase in its Ser(P)/Thr(P) content (27). Although the nature of the insulin-stimulated Ser/Thr kinase(s) is still unknown, this phenomenon is presumably one of the underlying causes of insulin resistance induced by hyperinsulinemia. To determine whether insulin-stimulated Ser/Thr kinases act as negative feedback regulators of IR-IRS interactions, we studied how they affect the capacity of IRS-1 and IRS-2 to maintain interactions with the JM region of IR. As shown in Fig. 8, incubation of Fao cells with 10−7 M insulin reduced in a time-dependent manner the extent of insulin-induced Tyr phosphorylation of pp180. This was accompanied by a decrease in the electrophoretic mobility of IRS-1 and IRS-2 (not shown) and an acute (>50%) reduction in their ability to interact with the JM peptide (Fig. 8B). The inhibitory effects of insulin could be reversed upon incubation of the cell extracts with alkaline phosphatase. As shown in Fig. 9, such treatment restored the ability of IRS proteins derived from cells treated with insulin for 60 min to interact with the JM peptide. These findings lend support to our hypothesis that reversible Ser/Thr phosphorylation of the IRS proteins reduces their ability to interact with the JM region of IR. Interestingly, the reduced binding of IRS-1 and IRS-2 to the JM peptide occurred subsequent to their insulin-induced Tyr phosphorylation (Fig. 8). These findings are consistent with previous studies (8), demonstrating that insulin-induced activation of Ser/Thr kinases occurs more slowly than the immediate activation of the IR and the Tyr phosphorylation of its substrate proteins. This slower activation of Ser/Thr kinases, which results in the dissociation of IRS proteins from the receptor, could serve as a reversible delayed negative feedback control mechanism to turn off insulin signaling.

DISCUSSION

In this study we present evidence that insulin resistance at the molecular level is induced at least in part by enhanced Ser/Thr phosphorylation of both IRS-1 and IRS-2, which impedes their interaction with the juxtamembrane region of the insulin receptor, turning them into poorer receptor substrates that fail to undergo appropriate Tyr phosphorylation. Impaired Tyr phosphorylation eliminates the ability of IRS-1 and IRS-2 to recruit downstream effector molecules such as phosphatidylinositol 3-kinase (12) and results in severe impairment of insulin signal transduction.

Several lines of evidence support such a model. First, we could demonstrate that IRS-2, like IRS-1, specifically interacts with the isolated JM region of IR but not with its isolated CT region. Moreover, 14-amino acid-long peptides, derived from the JM, but not the CT region, effectively compete with binding of the IRS proteins to the JM region. Second, in line with previous studies (9, 11, 12, 14, 28–30), we have shown that agents such as TNFα, SMase, TPA, okadaic acid, and calyculin A, all known to counteract insulin action, increase the Ser(P)/Thr(P) content of IRS-1 and IRS-2, as exemplified here by a decrease in their electrophoretic mobility. Third, we established an inverse correlation between the extent of Ser/Thr phosphorylation of IRS-1 and IRS-2 (manifested by their reduced mobility) and the ability of these proteins to interact with the isolated JM region of IR. Fourth, we were able to demonstrate the reversibility of these events, at least with regard to TNFα, again emphasizing the involvement of post-translation modifications in the form of Ser/Thr phosphorylation in modulating the affinity of IRS-1 and IRS-2 to the JM region.
Ser/Thr phosphorylation of IRS proteins may represent a junction where cross-talk between signals emitted by insulin and other hormones takes place. Of special interest is the attenuation in insulin signaling in response to TNFα, because the latter was recently suggested to be a cause and a link to obesity-induced insulin resistance (11, 14, 31, 32). TNFα binds with a high affinity to two receptors having different intracellular domains (17, 33). These receptors, p55 TNFαR (TNFαR1) and p75 TNFαR (TNFαR2), are transmembrane glycoproteins devoid of any enzymatic activity that associate with different intracellular effectors (34). Activation of TNFαR1 is sufficient to mediate most biological responses by TNFα, including insulin-resistant states (35). As of now, the TNFα receptors have been shown to utilize distinct mechanisms to couple to proximal cytoplasmic signaling molecules. Recruitment of the signal transducer FADD (also known as MORT1) to the TNFαR1 complex mediates apoptosis through activation of an intricate protease cascade (26, 36), whereas two other signal transducers, RIP and TRAF2, mediate both Jun amino-terminal kinase and NF-κB activation (36). Similarly, FAN, a novel protein containing a WD-repeat motif, couples TNFαR1 to neutral SMase (17) whose stimulation results in the production of ceramide (33), which in turn activates several kinases (16, 37, 38). Stimulation of these kinase(s) (14), which impairs IR-IRS interactions, occurs after only 15 min of TNFα treatment of Fao cells, suggesting that TNFα mediates insulin resistance in these cells via post-translational modification of an existing protein. Moreover, TNFα effects are mimicked by bacterial SMase and are reversible in nature, again suggesting involvement of signaling elements different from proteases.

TNFα, SMase, and ceramide did not affect Tyr phosphorylation of IRS-2 in 32D cells overexpressing both IR and IRS-2 (32D-IR/IRS-2 cells) (15). These observations have led to the
conclusion that IRS-1 but not IRS-2 is involved in the mechanism by which TNFα, SMase, and ceramides inhibit insulin signaling (15). In contrast to the above findings, our results clearly indicate that the insulin-induced Tyr phosphorylation of both IRS-1 and IRS-2, as well as their interactions with IR are subjected to inhibition either by TNFα or by SMase. The reason for these conflicting results is presently unknown, but they could be accounted for by the fact that our studies were carried out in insulin-responsive rat hepatoma cells rather than myeloid 32D cells. Hence, it might well be that part of the endogenous machinery responsible for negative feedback control of IRS-2 is missing from 32D cells, which were not designed physiologically to respond to insulin signals.

IRS-2 shares substantial structural homology as well as biological activity with IRS-1 (5). Both proteins interact with downstream effectors of the insulin signaling like Grb2, the p85α subunit of phosphatidylinositol 3-kinase, and SH-PTP2 (5, 6). Moreover, IRS-2 functions as an alternative substrate of the IR in IRS-1-deficient mice (5). Our results add another layer of similarity between the two proteins, showing that IRS-2, like IRS-1, is subjected to regulation by TNFα and other Ser(P)/Thr(P) elevating agents, at least in insulin-responsive hepatoma cells. It has recently been proposed that through the induction of Ser/Thr phosphorylation TNFα converts IRS-1 to an inhibitor of IRK (15). Our results offer an alternative explanation for the role of Ser/Thr-phosphorylated IRS-1 and IRS-2. According to our model TNFα-induced insulin resistance is associated with enhanced Ser/Thr phosphorylation of IRS-1 and IRS-2, which results in impaired interaction of these proteins with the insulin receptor. Such impaired interaction abolishes the ability of these proteins to undergo insulin-induced Tyr phosphorylation and further propagate the insulin signal.

The redundancy of kinases capable of inhibiting IR-IRS interactions is not surprising in view of the fact that different stimuli, having in common the ability to elevate intracellular Ser(P)/Thr(P) content, all induce insulin resistance. Some stimuli, like TNFα or TPA, are activators of specific kinases, whereas others such as calyculin A or okadaic acid elevate Ser(P)/Thr(P) content through inhibition of protein phosphatases. Prolonged exposure of cells to insulin itself increases the Ser(P)/Thr(P) content of IRS-1 and IRS-2 and impedes their interaction with the JM domain. Of note is the fact that the reduced binding of IRS-1 and IRS-2 to the JM peptide in insulin-treated cells occurred at a slower rate when compared with their rate of insulin-induced Tyr phosphorylation. These findings are compatible with the notion that insulin-induced activation of Ser/Thr kinases occurs more slowly when compared with the immediate activation of the IRK and the Tyr phosphorylation of its substrate proteins. Our results further suggest that initiation of negative feedback control mechanisms in the form of enhanced Ser/Thr phosphorylation and dissociation of receptor-substrates complexes is designed to commence with a delayed onset, thus allowing enough time for the insulin signal

**FIG. 8.** Effects of insulin on Tyr phosphorylation of IRS-1 and IRS-2 and their ability to interact with the JM peptide. Fao cells were incubated at 37 °C with or without 100 nM insulin for the indicated time periods. Tyr phosphorylation of pp180 (A) and the extent of binding of IRS-1 and IRS-2 to the JM peptide (B) were determined as described in the legend to Fig. 2. The results are of a representative experiment reproduced three times (mean ± S.D. ± 11%).

**FIG. 9.** Effects of dephosphorylation of IRS proteins on their ability to interact with the JM peptide. Fao cells were incubated at 37 °C with 100 nM insulin for 1 or 60 min. Cytosolic extracts were obtained and were treated with or without alkaline phosphatase as described under “Experimental Procedures.” The extent of binding of IRS-1 and IRS-2 to the JM peptide was then determined as described in the legend to Fig. 2. The results are of a representative experiment reproduced twice that yielded similar findings each time.

**SCHEME I.** Effects of phosphorylation of IRS proteins on their interaction with the JM region of IR. A model is presented whereby Ser/Thr phosphorylation of IRS proteins serves either as a physiological negative feedback control mechanism or as a mean to induce an insulin-resistant state under pathological conditions (see details in text).
to propagate before it is turned off. The reversible nature of this feedback control mechanism is emphasized by the fact that protein phosphatases are capable of neutralizing this process, at least in vitro.

The presence of multiple potential Ser/Thr phosphorylation sites on IRS-1 and IRS-2 makes the identification of specific residues involved in impaired IR-IRS interactions a formidable task. Nonetheless, recent insights into structure-function aspects of IRS proteins turns the PTB domain, present both within IRS-1 and IRS-2, into a likely candidate. This domain, which shares 75% sequence identity between IRS-1 and IRS-2, mediates their interactions with the JM region of IR (39). Alignment of the PTB domains (amino acids 162–316 of IRS-1 and amino acids 192–351 of IRS-2 (5)) reveals the presence of 16 conserved Ser/Thr residues whose phosphorylation might affect the interactions of the PTB domain with the JM region. It has recently been suggested that IRS-2 interacts with IR through an additional domain (amino acids 591–786) that presumably binds to the kinase region of IR (40). Although the contribution of this domain to the overall IR-IRS-2 interactions was not addressed in the present work, the correlation between diminished IRS-2 phosphorylation and diminished interactions of this protein with the JM region suggests that the PTB domain constitutes a significant contributor to the IR-IRS-2 interactions. In that respect, our results are somewhat at variance with those of Yenush et al. (41), who demonstrated that deletion of the PTB domain only partially inhibits (−30%) the ability of IRS-1 to undergo insulin-stimulated phosphorylation (at 10−7 M insulin) in 32D cells. The reason for the discrepancy could be attributed to the fact that the high IRS-1/IR ratio in 32D cells, which overexpress IRS-1 while having only 50% insulin receptors/cell, enables generation of IRS-1 complexes and phosphorylation of IRS-1 even when an important domain for interaction, such as the PTB region, is deleted. Indeed, at low concentrations of insulin (10−9 M) Tyr phosphorylation of PTB-deleted IRS-1 is severely impaired (41).

Deletion of the pleckstrin homology domain of IRS-1 impairs its ability to undergo insulin-stimulated phosphorylation in cultured cells (42) without impairing its ability to interact with the JM peptide in vitro (18). Hence, both the pleckstrin homology and PTB domains are required to maintain IRS-1-IR interactions in insulin-responsive cells. IRS-1 or IRS-2, phosphorylated either on Tyr or Ser/Thr residues tend to dissociate more readily from the JM peptide. This is compatible with the idea (Scheme I) that once IRS proteins are phosphorylated on Tyr residues they translocate away from the receptor to other cellular regions to further propagate the insulin signal. In contrast, Ser/Thr phosphorylation interferences with binding of IRS-1 or IRS-2 to the JM region and prevents their subsequent Tyr phosphorylation.

Ser/Thr phosphorylation appears to be a general mean to down-modulate insulin receptor signaling both under physiological and pathological states. Hence, Ser/Thr kinases stimulated by insulin act as negative feedback regulators that phosphorylate IRS proteins and induce a conformational change that diminishes the interactions between IRS-PTB domains and the JM region of IR. Other Ser/P/Thr/P'-elevating agents (e.g. TNFα) utilize the same principle to inhibit insulin signal transduction and establish an insulin-resistant state (Scheme I). Similarly, insulin-induced Ser/Thr phosphorylation of mSos results in the dissociation of Sos-Grb2 complexes and attenuation of the Shc/Grb2/Sos/Ras/MAP kinase cascade (43, 44). Hence, the two major insulin signaling pathways, those mediated by IRS proteins and those mediated by Shc, are subjected to negative feedback control in the form of Ser/Thr phosphorylation. This conclusion targets us toward potential pharmacological interventions in disease states where this mechanism can be the underlying cause of insulin resistance, such as the prevalent form of obesity-induced diabetes.

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