Phosphorylation of Insulin Receptor Substrate-1 on Multiple Serine Residues, 612, 632, 662, and 731, Modulates Insulin Action*

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Okadaic acid has been described previously as being a negative regulator of insulin signaling, as it inhibits insulin stimulation of glucose transport. In addition, this drug induces on insulin receptor substrate-1 (IRS-1) a decrease in tyrosine phosphorylation, concomitantly with an increase in serine/threonine phosphorylation. The present work was aimed at the identification of the serine/threonine residues that, upon phosphorylation, might be involved in modulating insulin signaling. To this end, we studied double-point mutants of IRS-1, in which serines 612/632 and 662/731 were replaced with alanine. These are four plausible sites of phosphorylation by mitogen-activated protein kinases and are in the immediate proximity of tyrosine residues, which are potential sites of interaction with the phosphatidylinositol 3-kinase Src homology 2 domains. Using transient expression in 293 E BNA cells, we demonstrate that serines 612, 632, 662, and 731 and mitogen-activated protein kinases are not involved in the okadaic acid effect on IRS-1. Rather, these serines appear to play a role in modulating basal and insulin-stimulated IRS-1 tyrosine phosphorylation, association of IRS-1 with p85, and phosphatidylinositol 3-kinase activity in the IRS-1-p85 immune complex, since mutation of these sites enhances these events. Our findings suggest the existence of an IRS-1 desensitization mechanism resulting from serine/threonine phosphorylation, occurring at least on serines 612, 632, 662, and 731.

Insulin exerts its biological effects by binding to its specific tyrosine kinase receptor on the cell surface. Thereafter, the activated receptor tyrosine kinase leads to its autophosphorylation and phosphorylation of cellular proteins (1, 2). A major substrate of the insulin receptor kinase is the insulin receptor substrate-1 (IRS-1). This cytoplasmic protein was first identified as a 185-kDa phosphoprotein in anti-phosphotyrosine immunoprecipitates from insulin-stimulated Fao hepatoma cells (3), and subsequently cloned from rat liver (4). IRS-1 is expressed in most cells and appears to play an important role in insulin signal transmission (5–7). After insulin stimulation, IRS-1 is phosphorylated on several tyrosine residues, most of them being located in YXXM or YXXM motifs (4, 8). This tyrosine phosphorylation step is crucial, since it allows Src homology 2 domain-containing proteins to interact with IRS-1, leading to the activation of several intracellular processes and resulting in the final effects of insulin. A growing list of proteins interacting with IRS-1 is appearing. Among these are growth factor receptor-bound protein 2, Grb2 (9), the tyrosine phosphatase SH-PTP2 (10, 11), Nck (12), p85α and p85β, the two isoforms of the PI 3-kinase regulatory subunit (13–15), and p55IKK (16).

In addition to tyrosine phosphorylation, IRS-1 also undergoes serine/threonine phosphorylation. Indeed, in SDS-PAGE, IRS-1 usually migrates with an electrophoretic mobility corresponding to 165–185 kDa, while its expected molecular mass is only 131 kDa. It is believed that this discrepancy is due essentially to serine/threonine phosphorylation of the protein in the basal state. Insulin leads to increased IRS-1 serine phosphorylation, and, to a lesser extent, threonine phosphorylation (5). These phosphorylation events occur on multiple sites on the protein and are probably due to several serine/threonine kinases activated during insulin action. Recently, our laboratory has demonstrated that okadaic acid, an inhibitor of serine/threonine phosphatases, is also able to increase IRS-1 serine/threonine phosphorylation (17). Moreover, after okadaic acid treatment, IRS-1 immunoprecipitated from 3T3-L1 adipocytes was less able to be tyrosine phosphorylated by the activated insulin receptor in a cell-free system. In vitro reconstitution experiments also showed a reduced PI 3-kinase activity associated with IRS-1 from okadaic acid-treated cells, compared to PI 3-kinase activity associated with IRS-1 from nontreated cells. These results provide an explanation for the observation that okadaic acid induces an insulin-resistant state. Indeed, a previous study from our laboratory showed an inhibitory effect of okadaic acid on insulin-induced glucose transport in isolated soleus muscle and 3T3-L1 adipocytes (18). In addition, in the presence of the phosphatase inhibitor, decreased insulin-stimulated IRS-1 tyrosine phosphorylation and decreased PI 3-kinase activity immunoprecipitated with antibodies to phosphotyrosine were observed. These effects of okadaic acid could be due to the increased IRS-1 serine/threonine phosphorylation seen with the drug. Very recently, tumor necrosis factor-α was found to cause a similar decrease in insulin-induced tyrosine phosphorylation of IRS-1 (19). Like okadaic acid, tumor necrosis factor-α induces insulin resistance and leads to the phosphorylation of IRS-1 on serine/threonine residues (19–21). Taken together, these data point to an important role of IRS-1 serine/threonine phosphorylation in the modulation of insulin signaling.

In the present study, we attempt to determine the serine and/or threonine residues implicated in the regulation of IRS-1 tyrosine phosphorylation and associated PI 3-kinase activity.
We focused on the residues that might be phosphorylated upon okadaic acid treatment of cells. The primary sequence of IRS-1 contains at least 35 potential sites for serine/threonine and tyrosine), (ii) their ability to associate the intact cells with respect to: (i) their phosphorylation state (serine/threonine and tyrosine), and MAP kinases (4, 22). The approach we used was to construct IRS-1 proteins mutated on serine residues. We chose to mutate four potential phosphorylation sites for MAP kinases, serine 612, 632, 662, and 731, for the following reasons. (i) MAP kinases are activated by okadaic acid (23). (ii) These four serine residues, located in the YMXMP sequence, are adjacent to potential tyrosine phosphorylation sites for the activated insulin receptor. (iii) Finally, these four tyrosine residues reside in potential binding sites for the Src homology 2 domains of the p85 subunit of PI 3-kinase, and three of them (608, 628, and 658) have been shown to participate in such an interaction (11, 24). In brief, we have replaced the four serine residues with alanine to obtain two double mutants, IRS-1 S612A/S632A and S662A/S731A. Both mutants, as well as wild-type IRS-1, were expressed in 293 EBNA cells, and we compared the effects of insulin and okadaic acid on the different IRS-1 proteins in intact cells with respect to: (i) their phosphorylation state (serine/threonine and tyrosine), (ii) their ability to associate the p85 of PI 3-kinase, and (iii) their associated PI 3-kinase activity.

EXPERIMENTAL PROCEDURES

Materials

Reagents for SDS-PAGE were purchased from Bio-Rad. Culture media and Genetin were from Life Technologies, Inc. Tran35S-label (1190 Ci/mmol) and 125I were from ICN Pharmaceuticals, Inc.; 125I-protein A was labeled using the chloramine-T method as described previously (25). Triton X-100, Nonidet P-40, leupeptin, benzamidine, pepstatin, and L-phenylmethylsulfonyl fluoride were from Serva (Heidelberg, Germany). Protein A-Sepharose was from Pharmacia Biotech Inc. Enzymes for molecular biology procedures were purchased from New England Biolabs. Antibodies to phosphotyrosine and to the p85 subunit of PI 3-kinase were from Santa Cruz Biotechnology, Inc. Insulin was a kind gift from Novo Nordisk (Copenhagen, Denmark). Enzymes for immunoprecipitation and immunoblotting was prepared in our laboratory by immunizing rabbits with a synthetic peptide derived from the C-terminus of IRS-1.

Methods

Production of IRS-1 Mutants—The rat IRS-1 cDNA subcloned into pBluescript II KS was obtained from M. F. White and C. R. Kahn (Joslin Diabetes Center, Boston, MA). For the obtention of IRS-1 with point mutagenesis, site-directed mutagenesis was performed using the Clontech (Palo Alto, CA) double-strand DNA strategy, based on the Deng and Nickoloff method (26). The mutations were verified by dideoxynucleotide sequence analysis. The mutant cDNAs obtained and the cDNA of wild-type IRS-1 were excised from pBluescript with SalI and SpeI restriction enzymes and subcloned into the eucaryotic expression vector pCEP-4 at the compatible XbaI and NheI sites, respectively.

Cell Culture and Transfections—Wild-type IRS-1 and IRS-1 mutants were transiently expressed in 293 EBNA cells, which are human embryonic kidney cells that constitutively express the EBNA-1 protein from the Epstein-Barr virus (Invitrogen, San Diego, CA). These cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 5% (v/v) fetal calf serum (FCS) at the appropriate XbaI and NheI sites, respectively.

Phosphatidylinositol 3-Kinase Activity—To 293 EBNA cells cultured in 9.5-cm2 dishes were transfected and stimulated or not with okadaic acid (2 μM, 40 min), and stimulation was performed in PBS supplemented with 0.2% (w/v) BSA. After 5 min before the end of the stimulation, the antibody to the p85 subunit of PI 3-kinase, the PI 3-kinase activity was measured as described previously (15). The radioactivity incorporated into phosphatidylinositol-3-phosphate was quantified using 125I-protein A followed by autoradiography.
Cells were pretreated or not with okadaic acid before being activate the lipid kinase activity of PI 3-kinase. Transfected IRS-1S612A/S632A, and IRS-1S662A/S731A—We compared wild-type IRS-1 and the two IRS-1 mutants for their ability to activate the lipid kinase activity of PI 3-kinase. Transfected cells were pretreated or not with okadaic acid before being stimulated with insulin for 5 min. Then, IRS-1 was immunoprecipitated and PI 3-kinase activity was measured as described under “Experimental Procedures” (Fig. 3A, upper panel). The PI 3-kinase activity associated with wild-type IRS-1 was increased 4.5-fold in the presence of insulin. Addition of okadaic acid led to a complete inhibition in the insulin-stimulated PI 3-kinase activity. Concerning the two IRS-1 mutants, okadaic acid induced the same effect as the one observed for wild-type IRS-1, i.e. a complete inhibition of the insulin-stimulated PI 3-kinase activity. It is noteworthy that okadaic acid alone had no effect on the basal PI 3-kinase activity associated with either wild-type or mutant IRS-1 proteins (not shown). Interestingly, we observed that, compared to wild-type IRS-1, the basal PI 3-kinase activity was increased in cells expressing IRS-1S662A/S731A and, to a lesser extent, IRS-1S612A/S632A and IRS-1S662A/S731A. Moreover, insulin-stimulated PI 3-kinase activity was enhanced in cells expressing the mutant proteins as compared to the wild-type IRS-1. It should be stressed that the amount of expressed IRS-1 proteins was comparable in all the conditions, as determined by immunoblotting, and that the action of okadaic acid was confirmed by the lower electrophoretic mobility of IRS-1 in cells treated with the drug (Fig. 3A, lower panel).

A statistical comparison of the basal and insulin-stimulated PI 3-kinase activities associated with both IRS-1 mutants and with wild-type IRS-1 was performed (Fig. 3B). For IRS-1S612A/S632A, we found that the basal associated PI 3-kinase activity was increased approximately 30% over the basal activity obtained with wild-type IRS-1, while the insulin-stimulated activity was not significantly different from that of wild-type IRS-1. Moreover, for this mutant the increment between insulin-stimulated and basal conditions was slightly, but significantly, decreased from 360% over basal for wild-type IRS-1 to 310% for IRS-1S612A/S632A (p < 0.005).

With IRS-1S662A/S731A, we observed an increase in the basal associated PI 3-kinase activity, as well as a significant increase in the insulin-stimulated one, which reached 80 and 130% over basal and insulin-stimulated activities obtained with wild-type IRS-1, respectively. As opposed to what we found for IRS-1S612A/S632A, the fold stimulation was significantly increased, from 360% for wild-type IRS-1 to 460% for IRS-1S662A/S731A (p < 0.005). This indicates that the increase in the PI 3-kinase activity we observed was not only due to an increase in the basal activity, but also to an increase in insulin’s effect. In conclusion, serine residues 612, 632, 662, and 731 are able to modulate the PI 3-kinase activity associated with IRS-1 in the absence as well as in the presence of insulin.

In Vivo Tyrosine Phosphorylation and Association of Wild-type IRS-1, IRS-1S612A/S632A, and IRS-1S662A/S731A.
with PI 3-Kinase—To determine whether PI 3-kinase activity and the association of IRS-1 with the p85 subunit of PI 3-kinase were correlated, we immunoprecipitated IRS-1 proteins from transfected cells incubated or not with okadaic acid, in the presence or absence of insulin. After transfer of the separated proteins, immunoblotting with an antibody to p85 or phosphotyrosine was performed (Fig. 4). By stripping the antiphosphotyrosine antibodies from the membrane (panel A) and blotting it again with antibodies to IRS-1, the amount of IRS-1 immunoprecipitated in each condition was visualized (panel C).

To perform quantitative analysis of this experiment, we scanned the autoradiogram and counted the radioactivity incorporated into the proteins. The values obtained were adjusted to take into account the differences in the expression level of the IRS-1 proteins. For the three IRS-1 proteins, insulin increased their association with p85 (Fig. 4, panel B, lanes 2, 5, and 8) for wild-type IRS-1, IRS-1 S612A/S632A, and IRS-1 S662A/S731A, respectively). Okadaic acid prevented this insulin-stimulated effect, which is similar to what we observed for the PI 3-kinase activity (see Fig. 3A). However, for IRS-1 S662A/S731A the association with p85 after insulin treatment was 1.7-fold higher than that found with wild-type IRS-1 (p < 0.05). For IRS-1 S612A/S632A, a slight increase was observed, but it was not significant. This demonstrates that, in these conditions, the association with p85 and the PI 3-kinase activity associated with IRS-1 were correlated.

We also compared in the same experiment the insulin-induced tyrosine phosphorylation of wild-type IRS-1 versus that of the two mutants, and the effect of okadaic acid on this tyrosine phosphorylation (Fig. 4, panel A). Similar to what we observed for the insulin-stimulated association of IRS-1 with p85, okadaic acid strongly inhibited insulin-induced tyrosine phosphorylation of wild-type IRS-1 and IRS-1 S612A/S632A (lanes 3 and 6). Compared to wild-type IRS-1, in insulin-stimulated conditions, a slight increment in phosphotyrosine was seen with IRS-1 S612A/S632A, but it was not statistically significant (compare lane 5 and lane 2). In contrast, the tyrosine phosphorylation of IRS-1 S662A/S731A was significantly increased (p < 0.025; compare lane 8 and lane 2), which is compatible with the increase in its association with p85 (Fig. 4, panel B). We also observed for this mutant an increased residual phosphotyrosine content in okadaic acid-treated cells, compared to that seen for wild-type IRS-1 and IRS-1 S612A/S632A (compare lane 9 to lanes 3 and 6). Nevertheless, the decrease in insulin-induced tyrosine phosphorylation of IRS-1 S662A/S731A was approximately the same as that obtained with wild-type IRS-1 and IRS-1 S612A/S632A (70% versus 80%), indicating that okadaic acid exerts the same effect on the three IRS-1 proteins.

In the experiment shown, the basal tyrosine phosphorylation, as well as the association with p85, was too weak to allow the detection of possible differences between the three IRS-1 proteins (panels A and B, lanes 1, 4, and 7). When the complete series of experiments was analyzed, the enhanced basal asso-
The fraction of p42 MAP kinase (about 50%) has been activated by lanes 1 corresponding to p42 MAP kinase instead of a single band seen with okadaic acid (while it totally inhibited the activation of p42 MAP kinase seen (reflected by a slower electrophoretic mobility on SDS-PAGE treatment (proteins were separated by SDS-PAGE, transferred to an Immobilon membrane. IRS-1 (panel A) and p42 MAP kinase (panel B) were revealed by antibodies to IRS-1 or to p42 MAP kinase, respectively, and [125I]-protein A. The experiment shown is representative of three independent experiments.

Effect of PD 098059 on Okadaic Acid-induced Stimulation of p42 MAP Kinase and IRS-1 Serine/Threonine Phosphorylation—Since we suspected that the MAP kinases could be involved in the effect of okadaic acid on IRS-1, we studied the effect of a specific inhibitor of MEK, PD 098059 (28), on the electrophoretic mobility of IRS-1 in the presence or in the absence of okadaic acid. To monitor the action of the inhibitor, we looked at its effect on the activation of p42 MAP kinase in the same conditions, i.e. with or without okadaic acid. Thus, after stimulation, 293 cells transfected with wild-type IRS-1, IRS-1 S612A/S632A, or IRS-1 S662A/S731A were lysed and the proteins were separated by SDS-PAGE, transferred to a membrane, and subjected to immunoblotting with antibodies either to IRS-1 or to p42 MAP kinase (Fig. 5). After okadaic acid treatment (panel B, lanes 2, 6, and 10), we observed two bands corresponding to p42 MAP kinase instead of a single band seen in basal conditions (lanes 1, 5, and 9). This indicates that a fraction of p42 MAP kinase (about 50%) has been activated by okadaic acid. PD 098059 alone had no effect (lanes 4, 8, and 12), while it totally inhibited the activation of p42 MAP kinase seen with okadaic acid (lanes 3, 7, and 11). However, the IRS-1 serine/threonine phosphorylation induced by okadaic acid, reflected by a slower electrophoretic mobility on SDS-PAGE (panel A, lanes 2, 6, and 10 for wild-type IRS-1, IRS-1 S612A/S632A, and IRS-1 S662A/S731A, respectively), was unchanged by PD 098059 for wild-type IRS-1 (lane 3) as well as for IRS-1 S612A/S632A (lane 7) or IRS-1 S662A/S731A (lane 11). This result indicates that the serine/threonine kinases, which are activated after okadaic acid treatment and which are responsible for the increase in IRS-1 serine/threonine phosphorylation, are distinct from the MAP kinases.

FIG. 5. Effect of the MEK inhibitor, PD 098059, on the electrophoretic mobility of wild-type IRS-1, IRS-1 S612A/S632A, and IRS-1 S662A/S731A induced by okadaic acid. 293 EBNA cells expressing different IRS-1 proteins were treated or not with okadaic acid (2 μM) for 40 min after an 18-h incubation in the presence or absence of PD 098059 (50 μM). The cells were lysed, 100 μg of proteins were separated on SDS-PAGE and transferred to an Immobilon membrane. IRS-1 (panel A) and p42 MAP kinase (panel B) were revealed by antibodies to IRS-1 or to p42 MAP kinase, respectively, and [125I]-protein A. The experiment shown is representative of three independent experiments.

DISCUSSION
Okadaic acid, a serine/threonine phosphatase inhibitor, is able to promote an insulin-resistant state in intact cells (18, 29). In addition, okadaic acid leads to an increase in serine/threonine phosphorylation of IRS-1 (17). These results suggest that both phenomena observed in the presence of okadaic acid, i.e. IRS-1 serine/threonine phosphorylation and altered insulin action could be causally linked. In the present study, we wished to identify the serinethreonine residues of IRS-1 implicated in this process. Therefore, we decided to mutate the four serine residues located in YMXXMP motifs and we constructed the IRS-1 mutants, IRS-1 S612A/S632A and IRS-1 S662A/S731A, in which the serine residues had been replaced with alanine residues two at a time. These mutants were expressed in 293 EBNA cells, and we compared their phosphorylation state and some of their biological properties to those seen with wild-type IRS-1.

It was described previously that okadaic acid induces a slower electrophoretic migration of IRS-1 on SDS-PAGE (17). This is thought to be due to the increased level of IRS-1 serine/threonine phosphorylation induced by okadaic acid, compared to the one seen in basal conditions. We found that upon okadaic acid treatment our IRS-1 mutants displayed the same decrease in electrophoretic mobility as wild-type IRS-1. This result suggests that IRS-1 mutants are still phosphorylated and, consequently, that the mutated serine residues are not involved, at least in a major fashion, in the okadaic acid-induced phosphorylation of IRS-1. However, we cannot exclude the possibility that the mutated serine residues are actually phosphorylated in wild-type IRS-1. Indeed, it could be possible that the decreased electrophoretic mobility seen with IRS-1 S612A/S632A and IRS-1 S662A/S731A was still maintained, because it might be due to other IRS-1 residues phosphorylated after okadaic acid treatment.

Analysis of the insulin-stimulated PI 3-kinase activity, tyrosine phosphorylation, and association with p85 of IRS-1 mutants led us to conclude that the potential serine phosphorylation sites removed in our IRS-1 mutants, i.e. serines 612, 632, 662, and 731, are not implicated in the insulin-resistant state induced by okadaic acid. Thus, for both mutants we found a total inhibition of these insulin-stimulated effects, comparable to that obtained with wild-type IRS-1. Finally, the specific inhibitor of MEK, PD 098059, which completely inhibited the activation of p42 MAP kinase induced by okadaic acid in 293 EBNA cells, was totally unable to reverse the effect of okadaic acid on the electrophoretic mobility of wild-type IRS-1 as well as of IRS-1 S612A/S632A and IRS-1 S662A/S731A. This demonstrates that okadaic acid-induced serine/threonine phosphorylation of IRS-1 does not involve the MAP kinase pathway. Taking our results together, we conclude that one or several serine/threonine kinases (distinct from the MAP kinases and MEK), are activated by okadaic acid and are responsible for the phosphorylation of IRS-1 under these conditions.

Interestingly, regarding the basal and insulin-induced PI 3-kinase activity associated with IRS-1 mutants, independently of okadaic acid action, we found that, compared to wild-type IRS-1, IRS-1 S612A/S632A had an increased basal PI 3-kinase activity and IRS-1 S662A/S731A showed a markedly increased basal and insulin-stimulated PI 3-kinase activity. For IRS-1 S662A/S731A, we found in addition an increase in its insulin-stimulated association with p85. This observation was correlated with an enhanced tyrosine phosphorylation of IRS-1 S662A/S731A in response to insulin. At least the following two non-mutually exclusive mechanisms could explain this regulatory effect of serine phosphorylation: (i) inhibition of IRS-1 tyrosine phosphorylation and/or (ii) a blockade of the subsequent binding to the p85 Src homology 2 domains. Our results seen with IRS-1 S662A/S731A in insulin-stimulated cells are compatible with the first hypothesis, since the tyrosine phosphorylation content of this mutant appeared to be increased. This increase occurs probably only on tyrosine 658 and/or 727, which are close to serines 662 and 731. Thus, our result indicates that both tyrosine residues are major phosphorylation sites in intact cells, since we were able to detect an increase in
the total amount of IRS-1 S662A/S731A tyrosine phosphorylation. However, it is possible that by itself the increase in IRS-1 tyrosine phosphorylation does not account for the enhanced association with p85 and PI 3-kinase activity. Indeed, these effects could also be due, at least in part, to the fact that p85 could interact more efficiently at least with tyrosines 658 and 727 of the IRS-1 mutant, since the phosphorylated serines 662 and 731 are absent.

We did not detect striking modulations of tyrosine phosphorylation and association with p85 for both IRS-1 mutants, in the basal state. However, we would expect that the same mechanisms as those proposed for insulin-stimulated conditions are responsible for the increase seen in basal PI 3-kinase activity associated with both IRS-1 mutants, compared to wild-type IRS-1. In such a scenario, we have to assume that part of the serine residues are phosphorylated by serine/threonine kinase(s) in the absence of insulin. This is compatible with the continuous serine/threonine phosphorylation of IRS-1 in the basal state (5). This phosphorylation would occur at least on serine 662 and 731, since the effect on PI 3-kinase activity seen with IRS-1 S662A/S731A is more pronounced than the one seen with IRS-1 S612A/S632A. The differences observed between both IRS-1 mutants after insulin stimulation could be due to a preferential phosphorylation of serine 662/serine 731 by the potential MAP kinase phosphorylation sites are likely to be with IRS-1S662A/S731A more pronounced than the one seen on IRS-1 mutant, compared to wild-type IRS-1.

In summary, in the present work, we were interested in studying the mechanisms participating in the modulation of insulin signaling. We hypothesized that MAP kinase phosphorylation sites, located next to tyrosine phosphorylation sites in YMXM motifs, serines 612, 632, 662, and 731, were obvious candidates for such a role. The two key contributions of the manuscript and G. Visciano for illustration work.

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