The Insulin Receptor Substrate (IRS)-1 Pleckstrin Homology Domain Functions in Downstream Signaling*

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The pleckstrin homology (PH) domain of the insulin receptor substrate-1 (IRS-1) plays a role in directing this molecule to the insulin receptor, thereby regulating its tyrosine phosphorylation. In this work, the role of the PH domain in subsequent signaling was studied by constructing constitutively active forms of IRS-1 in which the inter-SH2 domain of the p85 subunit of phosphatidylinositol 3-kinase was fused to portions of the IRS-1 molecule. Chimeric molecules containing the PH domain were found to activate the downstream response of stimulating the Ser/Thr kinase Akt. A chimera containing point mutations in the PH domain that abolished the ability of this domain to bind phosphatidylinositol 4,5-bisphosphate prevented these molecules from activating Akt. These mutations also decreased by about 70% the amount of the constructs present in a particulate fraction of the cells. These results indicate that the PH domain of IRS-1, in addition to directing this protein to the receptor for tyrosine phosphorylation, functions in the ability of this molecule to stimulate subsequent responses. Thus, compromising the function of the PH domain, e.g. in insulin-resistant states, could decrease both the ability of IRS-1 to be tyrosine phosphorylated by the insulin receptor and to link to subsequent downstream targets.

A variety of experimental approaches have also been utilized to test the role of the PH and PTB domains of the IRS proteins in their interactions with the IR and the subsequent tyrosine phosphorylations. Mutant IRS molecules in which either the PH or PTB domain have been deleted or replaced with homologous structures of other proteins have been expressed in mammalian cells (15–18). These studies have documented a primary role for the PH domain in the subsequent ability of IRS-1 to be tyrosine phosphorylated in intact cells after insulin stimulation. In either in vitro studies or yeast two-hybrid systems, a primary role of the IRS-1 PTB domain has been documented in receptor interactions (15, 19). In the case of IRS-2, a more central region of the molecule has also been identified as playing a role in receptor interactions (20, 21). Although numerous studies have documented the roles of the PH and PTB domains of the IRS proteins in the interactions with the IR and the subsequent tyrosine phosphorylation of the IRS proteins, no studies have tested the roles of these two domains on subsequent signals induced by the IRS-PI 3-kinase complex. Because mutations in the PH and PTB domains interfere with the interaction of the IRS with the IR and its subsequent tyrosine phosphorylation, it is impossible to determine the effect of these mutations on the downstream signals emanating from the IRS-PI 3-kinase complex. To overcome this obstacle, we now describe a constitutively active form of IRS-1. In this chimeric molecule, the inter-SH2 domain of the p85 regulatory subunit of PI 3-kinase is attached to various portions of the IRS-1 molecule. The inter-SH2 domain of p85 has
previously been documented to bind to the 110-kDa catalytic unit of the PI 3-kinase (22). Thus the resultant chimeric IRS-1 molecules now constitutively bind to the PI 3-kinase. By using these chimeric molecules, we have tested the role of different regions of the IRS-1 molecule in eliciting a subsequent biological response, the stimulation of the Ser/Thr kinase Akt. We demonstrate that the PH domain alone is sufficient to target the constitutively active PI 3-kinase to induce the activation of Akt and that this requires the lipid binding properties of the PH domain.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Cell culture medium DME-H21 and oligonucleotides were from Life Technologies, Inc. Glutathione-agarose beads and monoclonal anti-GST antibodies were from Sigma, protein A-Sepharose was from Repligen (Cambridge, MA). 12CA5 monoclonal anti-HA antibodies were from Roche Molecular Biochemicals (Mannheim, Germany), rabbit polyclonal anti-Akt antibodies directed against the PH domain of Akt1 were produced as described (23). 1,1-diphenyl-2 picrylhydrazyl, H2O2, 3-methyladenine, NADP, and D-glucose-6-phosphate dehydrogenase were from Sigma, and were used in a standard assay. DNA polymerase (Elongase 900), restriction endonucleases, and Taq DNA polymerase were from Life Technologies, Inc. or from New England BioLabs (Beverly, MA). The GSK-3 inhibitors SB, 25-gauge needle, incubated on ice for 10 min, and then spun at 4 °C for 10 min at 3000 rpm on a table top centrifuge. Supernatants were removed and subjected to ultracentrifugation at 100,000 × g (80,000 rpm) for 1 h at 4 °C using a TLA-100.3 rotor (Beckman Instruments, Inc.). Supernatants in the high-speed spin, designated cytosol (C), were immunoprecipitated with anti-HA antibodies. Pellets were resuspended in 1 ml of the homogenization buffer and spun at 100,000 × g for 1 h. Supernatants obtained in the second high-speed spin were discarded, and pellets were solubilized on ice in homogenization buffer containing 1% Triton X-100, 1 M NaCl. Cytosol and cytosolic fractions were used for immunoblotting with 12CA5 antibodies and ECL. Protein bands were quantified using an NIH image program. Ratios of the total particulate fraction over cytosol were calculated for each construct.

**Role of IRS-1 PH Domain in Downstream Signaling**

**Immunoprecipitation**—3T3-L1 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% calf serum at 37 °C, 5% CO2. The cells were grown to confluency and serum-starved 2–4 h and then rocking at 120 rpm at 37 °C, 5% CO2 to generate the viral supernatant. Viral infection of 3T3-L1 cells was performed using supernatants collected from the transfected Phoenix cells. 6-well plates with 50% confluent 3T3-L1 cells were infected with 1 ml of each viral supernatant. The plates were spun at 2,500 rpm for 90 min and then cultured until confluent. Cells were selected in complete medium containing 500 μg/ml hygromycin for a total of 8 days. The total pool of selected cells was used in all subsequent experiments. Western Blot Analyses—3T3-L1 fibroblasts overexpressing IRS-1 single constructs were grown in 10-cm plates to confluence and lysed in Act lysis buffer (see Akt kinase assay below). After removal of the insoluble matter by centrifugation, the cell lysates were immunoprecipitated using anti-HA epitope antibodies (12CA5) bound to protein A-Sepharose beads. Precipitated complexes were washed three times with cold buffer containing 50 mm Heps, pH 7.6, 150 mm NaCl, and 0.1% Triton X-100. Twenty microliters of gel loading buffer were added to each immunoprecipitate, and the samples were boiled for 5 min and loaded on 10% SDS-polyacrylamide gels. The proteins were transferred to nitrocellulose membranes and blotted with 12CA5 antibodies, and bound mouse antibodies were visualized using an anti-mouse antibody conjugated to horseradish peroxidase and ECL. 3T3-L1 cells stably transfected with the empty pWZL vector were used as a negative control.

**Subcellular Fractionation—** 3T3-L1 cells expressing the PH-domain constructs were grown to confluence and serum-deprived for 2 h. The cells were washed rapidly with ice-cold TES (20 mM Tris, pH 7.4, 5 mM EDTA, 250 mM sucrose), and then harvested in 1 ml of PI 3-kinase lysis buffer (137 mM NaCl, 20 mM HEPES, 10 mM NaF, 1 mM sodium fluoride, 1 mM EDTA, 1 mM dithiothreitol, 100 nM okadaic acid). The cell suspension was homogenized by passing 10 times through a 25-gauge needle, incubated on ice for 10 min, and then spun at 4 °C for 10 min at 3000 rpm on a table top centrifuge. Supernatants were removed and subjected to ultracentrifugation at 100,000 × g (80,000 rpm) for 1 h at 4 °C using a TLA-100.3 rotor (Beckman Instruments, Inc.). Supernatants in the high-speed spin, designated cytosol (C), were immunoprecipitated with anti-HA antibodies. Pellets were resuspended in 1 ml of the homogenization buffer and spun at 100,000 × g for 1 h. Supernatants obtained in the second high-speed spin were discarded, and pellets were solubilized on ice in homogenization buffer containing 1% Triton X-100, 1 M NaCl. Cytosol and cytosolic fractions were used for immunoblotting with 12CA5 antibodies and ECL. Protein bands were quantified using an NIH image program. Ratios of the total particulate fraction over cytosol were calculated for each construct.

**Akt and PI 3-Kinase Assays—** 3T3-L1 cells expressing IRS-1 single constructs were grown to confluence, serum-deprived for 2 h and stimulated with insulin as indicated. Cells were then washed once with cold HBS buffer (50 mM Heps, pH 7.6, 150 mM NaCl) and lysed in either PI 3-kinase lysis buffer (137 mM NaCl, 20 mM Tris-HCl, pH 8.0, 1 mM MgCl2, 1 mM CaCl2, 10% glycerol, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 400 μM vanadate, 1 mM diithiothreitol) or Akt lysis buffer (50 mM Heps, pH 7.6, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM vanadate, 2 mM sodium pyrophosphate, 30 mM sodium fluoride, 1 mM EDTA, 1 mM diithiothreitol, 100 mM okadaic acid). For the PI 3-kinase assays, cell lysates were immunoprecipitated with the anti-HA epitope antibodies (12CA5) captured on protein A-Sepharose beads. The precipitates were assayed for PI 3-kinase activity as described (27) using phosphatidylinositol as a substrate. The amounts of PI 3-kinase activity in the cells transfected with pWZL vector were subtracted from experimental values. Data shown are the means of three independent experiments. In the Akt kinase assays, endogenous Akt was immunoprecipitated from cell lysates using an antibody to the PH domain of Akt bound to protein A-Sepharose. Akt assays were performed as described in Ref. 28 using the GSK-3 peptide (GRPRTSFAEG) as a substrate. The phosphorylated peptide was separated on a...
RESULTS

Construction and Expression of Chimeric IRS-1 Molecules—To generate IRS-1 molecules that would constitutively bind and activate PI 3-kinase without being tyrosine phosphorylated, we constructed cDNAs that encode different portions of the IRS-1 molecule fused in frame with the inter-SH2 domain of the p85 regulatory subunit of the PI 3-kinase because this peptide (22) has previously been documented to be sufficient to bind to the 110-kDa catalytic unit of the PI 3-kinase (Fig. 1). In addition, each chimera contained an HA tag at the carboxyl terminus. Stable cell lines expressing each of these constructs were generated, and the amount of the expressed construct was examined by immunoprecipitation and Western blotting (Fig. 2). The construct only encoding the inter-SH2 domain was found to be poorly expressed and not studied further (Fig. 2). In contrast, constructs encoding either the PH domain alone or both the PH and PTB domains were found to be well expressed although the construct encoding the PH domain alone was expressed at a level of about one-half that of the PH-PTB construct (Fig. 2).

Measurement of the Amount of PI 3-Kinase Activity Associated with the Different Chimeric IRS-1 Molecules—To determine whether the chimeric IRS-1 molecules did contain an associated PI 3-kinase activity, lysates of cells infected with the empty virus (pWZL) are included as a control. The positions of the PTB, PH, and PH-PTB chimeric molecules as well as molecular weight markers are indicated. Also indicated are the Ig heavy chain (Hc) and light chain (Lc) of the anti-HA antibodies used in the precipitations.

Expression of the chimeric IRS-1 molecules in 3T3-L1 cells. Stable cell lines expressing the different constructs were obtained by retroviral infections. Cell lysates were immunoprecipitated with anti-HA epitope antibodies, and the precipitates were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting with the anti-HA antibodies. Precipitates from lysates of cells infected with the empty virus (pWZL) are included as a control. The positions of the PTB, PH, and PH-PTB chimeric molecules as well as molecular weight markers are indicated. Also indicated are the Ig heavy chain (Hc) and light chain (Lc) of the anti-HA antibodies used in the precipitations.
only the PH domain of the IRS-1 had ~50% of the PI 3-kinase activity of the PH-PTB molecule, whereas the PTB and iSH2 constructs had lower amounts of PI 3-kinase activity. The PH 3-kinase activities associated with these constructs (Fig. 3) were proportional to the amounts of each protein expressed (Fig. 2).

Measurement of the Amount of Active Akt Induced by the Chimeric IRS-1 Molecules—To test the role of the different domains of IRS-1 in the ability of the chimeric molecule to stimulate a downstream response, we examined the amount of active endogenous Akt in the cell lines expressing the different constructs. Confluent cells were serum-deprived for 2 h, and then cells expressing the pWZL vector were stimulated with insulin to determine the maximal levels of active endogenous Akt in these cells. Cells were lysed, and the Akt was immunoprecipitated and assayed for enzymatic activity (28). Cells expressing the chimeric IRS-1 molecules were lysed, and the endogenous Akt was precipitated and assayed for enzymatic activity. Results shown are mean ± S.E. from three independent experiments normalized to the amount of activity present in the precipitates from control cells treated for 5 min with 1 μM insulin.

Testing the Effect of the Mutations in the PH Domains on the Subcellular Distribution of the Chimeric IRS-1 Molecules—To test the effect of the point mutations in the PH domains on the subcellular localization of the chimeric IRS-1 molecules, we fractionated cells expressing either wild-type PH-PTB construct, PH-(R28C)-PTB or PH-(TM)-PTB. The fractions were tested for the presence of the constructs by Western blotting. We found that the wild-type PH-PTB IRS-1 chimera was present in the particulate fraction to a much greater degree then either the single mutant (PH-(R28C)-PTB) or the triple mutant (PH-(TM)-PTB); the particulate to cytosolic ratio of the wild-type PH-PTB chimera was 2.5-fold greater then the comparable ratio for either PH-(R28C)-PTB or PH-(TM)-PTB (Fig. 6).

DISCUSSION

The principal mechanism whereby the insulin receptor activates PI 3-kinase appears to be via its ability to stimulate the tyrosine phosphorylation of various endogenous proteins including the different IRS molecules, IRS-1 to 4 (1, 2). Activation of PI 3-kinase plays a critical role in subsequent biological responses, such as stimulation of glucose uptake and regulation of gene transcription (5, 6). A number of downstream targets of the PI 3-kinase and the lipids it generates have been identified. These include several Ser/Thr kinases such as the PDK-1, the family of Akt/PKB kinases, and several atypical protein kinase C including PKC ζ and λ. The exact role of these different kinases in eliciting subsequent biological responses has been debated (5, 6).
Extensive studies have investigated the mechanism whereby the IRS proteins are localized to the insulin receptor. All four IRS molecules contain both a PH domain and a PTB domain (1, 2). The PH domain binds various phospholipids, including PI 4,5-P2 as well as PI 3,4,5-P3 (9, 10). In addition some studies have suggested that the PH domain may also interact with various proteins (11). In contrast, the PTB domains bind phosphotyrosines, in particular, a phosphotyrosine in the juxtamembrane region of the insulin receptor (12, 13). Based on the crystal structure of the PH and PTB domains of the IRS-1 molecule, a model has been proposed of the two domains binding cooperatively to localize IRS-1 at the membrane in association with the insulin receptor (14).

In the present studies, we have tested the role of these two domains in the ability of the IRS-1 molecule to elicit a subsequent biological response, the activation of the Ser/Thr kinase Akt. To accomplish this, we produced novel chimeric molecules, which contain these two domains fused to the inter-SH2 domain of the p85 subunit of the PI 3-kinase. This peptide has previously been shown to be sufficient to bind and activate the PI 3-kinase (22). By adding these residues to the different domains of the IRS-1 molecule, we were able to produce chimeric molecules that were constitutively bound to active PI 3-kinase. This was necessary because prior studies have shown that the PH domain of the IRS-1 molecule was critical for its subsequent tyrosine phosphorylation and association with the PI 3-kinase. By adding the inter-SH2 domain to the different domains of the IRS-1 molecule, we were able to produce chimeric molecules that were active in the absence of insulin stimulation and receptor-mediated tyrosine phosphorylation.

In the present work we have shown that the PH domain is critical for the subsequent ability of these constitutively active chimeric molecules to activate Akt. The most convincing data for this conclusion comes from the studies of the point mutants of the PH-PTB domain chimeric molecule. The wild type and mutants were expressed to comparable levels and had almost identical associated PI 3-kinase activities. However, the wild type activated the endogenous Akt whereas both the single and triple PH domain mutants had a dramatically decreased ability to activate the enzymatic activities of the endogenous Akt (i.e. the introduction of either a single mutation or 3 mutations in the PH domain caused an approximate 75% decrease in the ability of the PH-PTB construct to activate Akt). Both mutations also had a similar decreased ability to bind to PI 4,5-P2 in comparison to the wild-type protein when they were expressed as PH-PTB-GST fusion proteins. The simplest interpretation of these data are that the PH domain function (i.e. binding to PI 4,5-P2 or a negatively charged protein) is critical to elicit the downstream functions. This conclusion is also supported by the

FIG. 5. Measurement of the lipid binding activities of the IRS-1 molecules. GST fusion proteins of the wild-type PH-PTB domain as well as the single (R28C) mutant and triple mutant (TM, R28C/K21L/K23L) were purified, and equivalent amounts of protein were incubated with phospholipid vesicles containing labeled PI 4,5-P2. The fusion proteins were captured on glutathione-agarose, washed, and eluted. The amount of bound PI 4,5-P2 was determined by liquid scintillation counting. A control of the GST protein alone was included. Results shown are mean ± S.E. from three independent experiments.

FIG. 6. Subcellular localization of the different chimeric IRS-1 molecules. Cells expressing the PH-PTB•iSH2 constructs were homogenized and subjected to differential fractionation to obtain cytosolic (C), soluble particulate (SP) and insoluble particulate (IP) fractions as described under "Experimental Procedures." The insoluble particulate and immunoprecipitates of cytosolic and solubilized particulate fractions were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting using anti-HA antibodies (A). The amounts of the different chimeric molecules in the subcellular fractions were quantified using NIH image program and are plotted in B as the ratio of the particulate (SP+IP) to cytosolic fraction (C). Data shown are representative of two independent experiments.
finding that a chimeric molecule with only the PH domain of IRS-1 was also capable of stimulating the activation of the endogenous Akt. The somewhat weaker activity of this chimera in comparison to the PH-PTB molecule is consistent with its lower expression and somewhat decreased amount of associated PI 3-kinase activity. Because the construct, which only contained the PTB domain was expressed at lower levels and therefore had lower associated PI 3-kinase activity, it is not possible to conclude from this construct alone whether the PTB domain alone would have been capable at higher levels to induce the activation of Akt. However, the finding that the PH-PTB domain chimeric molecules whose PH domain function was impaired by either single or triple point mutations were incapable of eliciting the activation of the Akt argue that the PTB domain alone would not be sufficient to elicit this response because the PTB domain function in these chimeric molecules is still intact.

A potential role of the PH domain of IRS-1 is to help localize the protein in the correct intracellular compartment. To test this hypothesis, we examined the subcellular localization of the wild-type and two mutant chimeric molecules by biochemical fractionation of the cells. The wild-type PH-PTB chimeric molecule was found to be in the particulate fraction to a much greater degree than the chimeric molecules containing the mutations in the PH domain. These results are consistent with the recent report that the IRS-1 molecule is at least partially present in a particulate fraction of the cell, possibly because of its association with the cytoskeletal complex (29) as well as with a recent report that the same mutant we have studied (R28C) blocks the insulin induced translocation of a IRS-GFP construct to the membrane (10). Thus, the PH domain may serve to localize the chimeric molecules in the subcellular localization required for the subsequent activation of Akt.

In conclusion, the present work presents the first evidence that a functional PH domain of the IRS-1 molecule is required for its ability to signal to downstream molecules such as activation of Akt. This requirement is presumably because of the ability of the PH domain of IRS-1 to direct the PI 3-kinase to the correct subcellular compartment. Compromising the function of the PH domain, for example in insulin-resistant states, could therefore both decrease the ability of IRS-1 to be tyrosine phosphorylated by the insulin receptor as well as to link to subsequent downstream targets (2).

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