Interaction of Insulin Receptor Substrate-2 (IRS-2) with the Insulin and Insulin-like Growth Factor I Receptors

EVIDENCE FOR TWO DISTINCT PHOSPHOTYROSINE-DEPENDENT INTERACTION DOMAINS WITHIN IRS-2*

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Insulin receptor substrate 2 (IRS-2) has recently been shown to be a substrate of the insulin receptor (IR). In this study we utilize the yeast two-hybrid system and assays of in vitro interaction to demonstrate that IRS-2 interacts directly with the IR and the insulin-like growth factor I receptor. We show that, like IRS-1, the region of IRS-2 that contains the putative phosphotyrosine binding and SAIN elements (188–591) is sufficient for receptor interaction and that this interaction is dependent upon the NP$^\gamma$ (where (p)Y is phosphotyrosine) motifs within the juxtamembrane domains of the receptors. In addition to this amino-terminal NPX(p)Y-binding domain, an additional domain of strong interaction was identified in the central region of IRS-2 and was localized between amino acids 591 and 733. This interaction was found to be dependent upon receptor phosphorylation but was NPX(p)Y-independent. This region does not appear to have either an SH2 or a phosphotyrosine binding domain. Both of the interactions could also be demonstrated in vitro using IRS-2 glutathione S-transferase fusion proteins. We conclude that IRS-2, unlike IRS-1, can interact with tyrosine-phosphorylated receptors such as the IR and insulin-like growth factor I receptor via multiple independent binding motifs. Our findings suggest the existence of a previously unidentified phosphotyrosine-dependent binding domain within the central region of IRS-2.

The insulin receptor (IR) and the related insulin-like growth factor I receptor (IGFIR) are believed to transduce signals at least in part by phosphorylation of cellular proteins such as IRS-1 and SHC, which, upon phosphorylation, serve as docking sites for downstream signaling proteins (3–5). Phosphorylation of IRS-1 upon multiple tyrosines leads to interaction with SH2 domain-containing proteins including Grb2, Syp, and the p85 subunit of phosphatidylinositol 3-kinase (PI 3-kinase) that lead to activation of various signaling cascades (6–8). Tyrosyl phosphorylation of SHC leads to its interaction with Grb2 and mediates activation of the guanine nucleotide exchange factor SOS and subsequent activation of the Ras signaling pathway (9–12). Both IRS-1 and SHC have been implicated in insulin-stimulated mitogenic signal transduction (13–15), and the relative role that each protein plays in mitogenic signaling by the IR and IGFIR is currently an area of active investigation (12, 16). IRS-1 has also been proposed to mediate some of the metabolic effects of the IR including regulation of glucose transport (17). The leading candidate for the regulation of glucose transport is PI 3-kinase, which interacts with and is activated by IRS-1 and to a lesser extent by the IR (18, 19).

Recently, mice have been generated in which the IRS-1 gene has been disrupted by homologous recombination. Surprisingly these mice were found to have only a slight insulin-resistant phenotype (20, 21). Muscle and adipose tissue from these mice were found to express an alternative ~190-kDa substrate which, like IRS-1, could interact with and activate PI 3-kinase in an insulin-dependent manner. Recently, a protein that possesses similar characteristics to IRS-1 was cloned (22). This molecule was first identified as a protein that was tyrosine-phosphorylated in response to interleukin 4 stimulation and was initially termed 4PS (23). The 4PS protein was shown to be tyrosine-phosphorylated in myeloid progenitor cells in response to interleukin 4 and insulin and to interact with and activate PI 3-kinase (24, 25). Using the p85 subunit of PI 3-kinase as an affinity matrix, the 4PS protein was purified from FDC-P2 cells and its cDNA was isolated (22). Sequence analysis revealed that 4PS possessed extensive homology with IRS-1, and it was therefore redesignated as IRS-2. Reconstitution experiments have confirmed that IRS-2 is a substrate of the IR and that it associates with PI 3-kinase and Grb2 (26).

We have recently characterized the interactions of the IR and IGFIR with IRS-1 and SHC using the yeast two-hybrid system and other assays of protein-protein interaction (27–30). The interactions of IRS-1 and SHC with the IR were found to be phosphotyrosine-dependent and also to require an intact NPX(p)Y motif within the juxtamembrane domains of the IR and IGFIR. We have localized the region within IRS-1 that is involved in interaction with the IR and IGFIR to the amino terminus of IRS-1 between amino acids 160 and 516 (30). Further deletions within this region suggested the presence of an essential region at the amino-terminal end of this domain, since deletion of amino acids 160–174 totally eliminated interaction (27). This essential region was subsequently shown to be the most highly

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§ The abbreviations used are: IR, insulin receptor; IGFIR, insulin-like growth factor I receptor; IRS, insulin receptor substrate; (p)Y, phosphotyrosine; RBD, receptor binding domain; PTB, phosphotyrosine binding; GST, glutathione S-transferase; CHO, Chinese hamster ovary; SH2, Src homology 2; PI 3-kinase, phosphatidylinositol 3-kinase; pH, Piekstrin homology.

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conserved region within the IRS-1 and IRS-2 proteins and has been suggested to contain a phosphotyrosine binding (PTB) motif (22). Although it is not yet clear whether this region contains a true PTB domain, these findings nevertheless support our previous identification of this region as an essential element of interaction (27, 30). We initially termed the region just downstream of the potential PTB domain the SAIN domain (for SHC and IRS-1 NPXY binding) on the basis of a limited homology to the SHC PTB domain (28). This region appears to be important for high level interaction yet is not essential for the interaction (27, 28, 30). Since our data clearly show that the SAIN elements in IRS-1 (and IRS-2, see below) are important for interaction but do not appear to contain the core binding element, the mechanism by which this domain enhances the interaction with the IR and IGFIR is currently unclear.

In this study, we characterize the interaction of IRS-2 with the IR and IGFIR. We demonstrate that IRS-2 interacts with the IR via two discrete domains, the first interacting in an NPXY(p)-dependent manner very similar to IRS-1 via an amino-terminal domain within IRS-2. We also identify a second receptor interaction domain located between 591 and 733 of IRS-2, which interacts in a phosphotyrosine-dependent but NPXY(p)-independent manner, unlike IRS-1.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—The yeast strain EGY40 (a, trp1, ura3-52, his3, leu2-3,112) and all yeast expression plasmids were provided by the laboratory of Roger Brent and have been previously described (28, 30–33). All routine growth and maintenance of yeast strains was as described (34). Plasmid transformation of yeast was either by the lithium acetate method (35) or by electroporation (36). The IR, IGFIR, and IRS-1 two-hybrid fusions have been previously reported (27, 28, 30). All site-directed mutants were generated using the method of Kunkel (37) using customized primers. The IRS-2 constructs were produced by standard methods, and detailed cloning strategies are available upon request.

β-Galactosidase Assays—The solution β-galactosidase assays were performed as described (38), and the units of β-galactosidase activity were calculated by the method of Miller (39). The values shown for the positive colonies represent the average of 3–10 assays (each assay representing an independent colony). Assays were performed on at least three different days to ensure the reliability of the data, and the values obtained varied ±10% from day to day. Statistical analysis was performed to determine the significance of the activity of each receptor mutant in Fig. 3B. Specifically the units of activity were compared by one-way analysis of variance, and significance levels were determined using the Newman-Keuls test.

In Vitro Interactions with the IR—GST fusion proteins were generated with various regions of IRS-1 and IRS-2 using the pGEX-5X-1 (Pharmacia Biotech Inc.) or pGSTag (40) vectors. All GST fusions were expressed in DH5α bacterial cells and purified on glutathione-agarose beads using standard techniques (41). The beads that contained immobilized fusion protein were then incubated with cell lysates derived from CHO.T cells (which overexpress the IR) prior to or after insulin stimulation (10 min, 100 nM). Lysates were prepared by lysis for 30 min on ice in 50 mM HEPES (pH 7.6), 1% Triton X-100, 1 mM EDTA, 10 mM NaF, 20 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 10 μM leupeptin followed by spinning at 10,000 × g for 10 min to remove insoluble material. The resulting supernatants were incubated with the immobilized GST proteins for 4 h. After extensive washing with 50 mM HEPES (pH 7.6), 150 mM NaCl, 0.1% Triton X-100, the proteins that coprecipitated with the IRS-1, IRS-2, or control GST proteins were analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting with either anti-IR (IR-CT1) (a gift from Ken Siddle) or anti-phosphotyrosine antibodies (PY20) (Transduction Labs).

RESULTS AND DISCUSSION

IRS-2 Interacts with the IR via Two Distinct Domains—To investigate whether IRS-2 interacts directly with the IR and IGFIR, we initially utilized the yeast two-hybrid system. As shown schematically in Fig. 1A, we utilized full-length and various subdomains of IRS-2 to generate activation domain hybrids. These hybrid proteins were assayed for interaction with the cytoplasmic domains of either the IR or IGFIR, which were expressed as DNA binding hybrids. As we have previously shown, these receptor hybrids are kinase active and autophosphorylate properly (27, 30). As summarized in Fig. 1B, expression of an essentially full-length (amino acids 3–1321) IRS-2 hybrid with either the IR or IGFIR resulted in very high activity in the two-hybrid assay as measured by either the colony color assay or the solution assay for β-galactosidase activity (300–500 units). To delineate the region(s) of IRS-2 that interacted with the IR and IGFIR hybrids, we produced 11 activation domain hybrid proteins containing various regions of IRS-2. When we expressed either the amino- (amino acids 3–591) or carboxyl- (amino acids 591–1321) terminal halves of IRS-2 as fusions, both gave significant activity (Fig. 1B). This suggested that IRS-2, unlike IRS-1, contained two domains capable of independently interacting with the IR and IGFIR.

We first focused on the amino-terminal receptor interaction domain that we term RBD1 for receptor-binding domain 1. As shown in Fig. 1B, expression of 10 IRS-2 hybrid proteins within this region provided evidence that the minimal domain possessing full activity was located between amino acids 188 and 591. Deletion of the carboxyl portion of this fragment to amino acid 429 or 326 reduced activity by at least 70%, and further deletion to amino acid 263 eliminated all activity. Amino-terminal deletion of amino acids 188–214 within the 188–591 fragment totally eliminated activity. We conclude that the amino-terminal subdomain within RBD1 forms an essential ele-
ment for interaction with the IR or IGFIR while the carboxyl portion of this domain is important but not essential for interaction. These data are entirely consistent with our previous delineation of the IR-binding domain of IRS-1 (27, 30). Not surprisingly, the amino-terminal subdomain of the RBD1 is the most highly conserved region between IRS-1 and IRS-2 (22). Furthermore, this region has been suggested (22) to contain a putative PTB domain (28, 42, 43) and has been shown to interact with the IR in vitro (44). Our data are in agreement that this region contains an essential element of the RBD1. Whether or not the IRS molecules contain regions that are structurally related to the classical PTB domain remains to be proven.

To better characterize the binding activity observed within the carboxyl end (591–1321) of IRS-2, we expressed nine regions of this protein and determined their activity in the two-hybrid assay. As shown in Fig. 2, the four carboxyl-terminal deletions showed that removal of amino acids 734–1321 had no significant effect upon activity. Further deletion to amino acid 697 eliminated all activity. Amino-terminal deletions showed that removal of amino acids 591–697 or 591–732 markedly reduced activity. These data suggest that IRS-2 contains a receptor binding domain located between amino acids 591 and 733, which mediates a strong interaction with both the IR and IGFIR.

The Amino-terminal RBD1 Domain Interacts in a Phosphotyrosine-dependent Manner — To better characterize the site(s) within the IR and IGFIR that were mediating interaction with IRS-2, we expressed four IRS-2 hybrids with a number of IR and IGFIR hybrids containing specific mutations or deletions. As shown in Fig. 3A, all four IRS-2 hybrids showed significant interaction with the wild-type IR and IGFIR hybrid proteins. Conversely, none of the IRS-2 hybrids showed any interaction with either the kinase-dead IR (K1018A) or IGFIR (K1003A) mutants in which the critical Lys within the ATP binding pockets had been mutated to Ala. This shows that interaction of IRS-2 with these receptors is kinase-dependent and that both RBD1 and RBD2 require receptor kinase activity for interaction. We next tested interaction of these IRS-2 hybrids with a number of IR and IGFIR hybrids in which specific Tyr residues had been altered. As shown in Fig. 3A, mutation of Tyr-960 within the IR or the corresponding Tyr-950 within the IGFIR eliminated interaction with the IRS-2 (3–591) hybrid protein. These tyrosines are located within the NPXY motif of the IR and IGFIR and have been previously shown to be essential for IRS-1 interaction with the receptors (28–30). These data strongly suggest that the RBD1 requires an intact NPX(p)Y motif for interaction. Conversely, the same Y960F IR or Y950F IGFIR substitutions had no effect upon interaction of the IRS-2 hybrids containing the RBD2 domain. In an attempt to identify the site(s) of interaction of RBD2, we examined interaction of RBD2-containing proteins with IR mutants in which specific tyrosine residues had been altered or deleted. Mutation of Tyr-953 within the juxtamembrane domain had no effect upon interaction of any of these proteins. Likewise, expression of an IR deletion mutant in which the 30 carboxyl amino acids were removed by insertion of a stop codon (Δ30CT) had no effect upon the interaction. Thus it is unlikely that the two carboxyl-terminal tyrosines (1316 and 1322) are essential for interaction. We also tested two IR mutants that contained mutations within the "triple tyrosine" region of the kinase domain, which has been implicated in IR kinase activation. The Y1146F (FYY) IR mutant showed undiminished interaction with RBD2-containing proteins. Conversely, a Y1150F/Y1151F

2 The numbering of amino acids of the IR corresponds to the sequence of the receptor of Ullrich et al. (1). These differ from that of Ebina et al. (2) by being 12 amino acids less.
(YFF) double mutant showed markedly reduced activities with RBD2 in the two-hybrid assay (data not shown). However, this YFF receptor mutant has been found to have markedly reduced activities for all interacting proteins (the p85 subunit of PI 3-kinase, SHC, and IRS-1) and does not appear to be efficiently autophosphorylated (not shown). This finding is consistent with a previous report that examined these receptor mutants in CHO cells, which overexpress the IR before and after insulin (INS) stimulation, and incubated with either a control GST or a GST-IRS-2 fusion protein as described under “Experimental Procedures.”

Reconstitution of IRS-2 Interactions in Vitro—We next investigated whether the interactions between the IR and the RBD1 and RBD2 domains of IRS-2 could be demonstrated in vitro. We produced seven GST fusions, which together encompass the entire IRS-2 coding sequence. These are shown schematically in Fig. 4A. Immobilized GST fusion proteins were incubated with lysates from CHO-IR cells that were either unstimulated or stimulated with insulin (100 nM for 10 min). After extensive washing, the coprecipitating proteins were separated by SDS-polyacrylamide gel electrophoresis and subsequently immunoblotted with either anti-phosphotyrosine or anti-IR antibodies. As shown in Fig. 4A, both domains previously identified in the two-hybrid assay showed clear phosphotyrosine-dependent interaction with the IR in vitro. Specifically, IRS-2 RBD1 hybrids containing amino acids 3–326 or 188–591 were able to interact with the IR in vitro, whereas the 327–591-amino acid hybrid was not. Two GST hybrids containing the RBD2 sequences (amino acids 591–785 or 591–894) were also found to interact efficiently with the IR in vitro. The lack of coprecipitation observed with the 3–189 amino acids and 895–1321 amino acid constructs suggests that the PH domain and extreme carboxyl end of IRS-2 do not interact significantly with the IR.

For comparison, we also produced four GST fusion proteins containing similar regions of the IRS-1 protein and assayed their ability to interact with the IR in vitro. As shown in Fig. 4B, the two GST fusions that contained the essential domain of interaction within RBD1 (amino acids 21–400 and 108–516) were efficiently coprecipitated with the IR, whereas the region of IRS-1 corresponding to RBD2 of IRS-2 showed no interaction. These data are consistent with our two-hybrid data and suggest that IRS-1 does not contain this second domain of IR interaction. This is not surprising since these regions show only minimal sequence similarity (22).

Models of IRS-2 Interaction with the IR—Our findings raise several questions regarding the role of RBD1 and RBD2 in...
IRS-2 mediated signaling. First, it is possible that the RBD2 domain represents a new phosphotyrosine binding domain analogous to the SH2 or PTB domains. This possibility is shown schematically in Fig. 5 (Model 1). RBD2 does not appear to contain either an SH2- or a PTB-like sequence, and searches of the available data bases do not reveal significant similarity to proteins other than IRS-1-related proteins. Therefore, RBD2 binding to phosphotyrosine-containing sequences appears to represent a novel type of interaction. However, our inability to identify a specific tyrosine within the IRS that mediates this interaction (except possibly tyrosines 1150 and 1151) does not allow us to make this conclusion definitively. It is also possible, for example, that IRS activation exposes a region of the receptor binding to phosphotyrosine-containing sequences appears to represent a novel type of interaction. However, our inability to identify a specific tyrosine within the IRS that mediates this interaction (except possibly tyrosines 1150 and 1151) does not allow us to make this conclusion definitively. It is also possible, for example, that IRS activation exposes a region of the receptor that is masked in the unstimulated receptor and becomes exposed after insulin binding. This region of the IR may thus interact with RBD2 in a manner that is dependent upon a phosphorylation-induced conformational change but independent of a specific phosphotyrosine residue. This possibility is shown schematically in Fig. 5 (Model 2). Finally, it is possible that RBD2 can interact with several phosphotyrosines. Thus, any single tyrosine mutation may not effectively eliminate the interaction. Further studies will be needed to address these possibilities.

The existence of two distinct phosphotyrosine binding elements within IRS-2 is intriguing in a number of other ways. It seems likely, for example, that such a binding element should allow IRS-2 to interact with additional signaling proteins and receptors that do not contain NPxxY/pY elements thus expanding the repertoire of potential signaling partners utilized by the IRS family of proteins. Whether a receptor has one or two sites of interaction with IRS-2 may also allow for differential signal transduction, which might theoretically be mediated by differences in the stability of the interaction or differential phosphorylation of IRS-2 tyrosine residues. It is also possible that each binding element within IRS-2 may interact in vivo with distinct tyrosyl-phosphorylated substrates. It will be interesting to determine whether the differences which we have observed between IRS-1 and IRS-2 allow these highly related proteins to play distinct functional roles in vivo.

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