Distinct Modes of Interaction of SHC and Insulin Receptor Substrate-1 with the Insulin Receptor NPEY Region via Non-SH2 Domains*

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Insulin receptor substrate 1 (IRS-1) and src homology and collagen protein (SHC) are signaling proteins which are rapidly phosphorylated on tyrosines after insulin receptor (IR) activation. We have recently shown that both SHC and IRS-1 interact with the tyrosine-phosphorylated NPEY motif of the IR and insulin-like growth factor I receptor via non-SH2 domains (Gustafson, T. A., He, W., Craparo, A., Schaub, C. D., and O’Neill, T. J. (1995) Mol. Cell. Biol. 15, 2500–2508; O’Neill, T. J., Craparo, A., and Gustafson, T. A. (1994) Mol. Cell. Biol. 14, 6433–6442; Craparo, A., O’Neill, T. J., and Gustafson, T. A. (1995) J. Biol. Chem. 270, 15639–15643). In this study we characterize these interactions by examining the effects of 18 amino acid substitutions within and around the IR NPEY motif upon interaction with SHC and IRS-1. We confirm that Tyr-960 within the NPEY motif of the IR is essential for both IRS-1 and SHC interaction and that Asn-957 and Pro-958 are essential for IRS-1 interaction and important but not critical for SHC interaction. Additional mutations surrounding the NPEY motif revealed completely distinct patterns of interaction for SHC and IRS-1. Specifically, mutation of Leu-952 or Tyr-953 (at positions –7 and –8 from Tyr-960) markedly reduced IRS-1 interaction but had no effect upon SHC interaction. Likewise, mutation of Ala-963 (+3) reduced IRS-1 but not SHC interaction. Conversely, substitution of Leu-962 (+1) with either Ala or Arg reduced SHC interaction by 70 and 90% respectively, yet had no effect upon interaction with IRS-1. Our data show that the sequences within and surrounding the NPEY motif contribute differentially to either SHC or IRS-1 recognition. Our findings suggest mechanisms by which the different interaction of known receptors with IRS-1 and SHC may be mediated.

After insulin receptor (IR) activation, multiple signaling pathways are activated leading to diverse effects upon cellular metabolism and mitogenesis (4). One major substrate of the IR is IRS-1, which is phosphorylated on numerous tyrosines (5–7) leading to subsequent interaction with SH2 domain-containing effector molecules including the p85 subunit of phosphatidylinositol 3-kinase, GRB2, and SH-PTP2 (syp) (8–10). Another substrate of the IR is SHC, so named because of its src homology 2 (SH2) and collagen homology domains (11). Multiple SHC proteins exist, two of which (p52 and p46) result from the use of alternative translation start sites, while the origin of the p66 isoform is less clear. Phosphorylation of SHC upon Tyr-317 leads to interaction with the SH2 domain of GRB2, an interaction that results in activation of p21ras via an intermediate guanine nucleotide release protein (12–15).

We have recently shown that both IRS-1 and SHC require phosphorylated Tyr-960 located within the NPEY motif of the IR juxtamembrane domain for interaction (1, 2). We have made similar observations regarding the interaction of IRS-1 and SHC with the related insulin-like growth factor I receptor (3). We have identified a related motif within SHC and IRS-1, which appears to comprise a part of the IR- and insulin-like growth factor I receptor-binding domains. We have termed this motif the SAIN domain (for SHC and IRS-1 NPXY binding) (1). Others have also identified this domain within SHC (also termed PID or PTB) as being important for the phosphoryltyrosine-dependent interaction of SHC with the EGF, TrkA, and HER2/neu receptors as well as with a putative signaling protein pp145 (16, 17). A search of the protein data base for proteins homologous to the SHC SAIN domain has identified a loosely conserved consensus for this domain, and related sequences have been identified in a number of proteins (18). IRS-1 was not identified in this search although many of the apparently important residues are conserved within the IRS-1 and SHC SAIN domains. In the absence of any structural information, it remains unclear whether the IR-binding domain within IRS-1 represents another SHC-like family member or is more distantly related.

Both SHC and IRS-1 require the NPXY motif for interaction with the IR, yet it is clear that not all phosphorylated NPXY motifs interact with both SHC and IRS-1. For example, the EGF, c-ErbB-3, and TrkA receptors interact well with SHC via NPXY motifs but have not been reported to interact with IRS-1-like molecules (19–24). Conversely, the IL-4 receptor phosphorylates IRS-1-like molecules apparently through an NPXY-dependent mechanism (25–27) but does not appear to phosphorylate SHC proteins efficiently (28). Since SHC and IRS-1 appear to be ubiquitously expressed, these findings suggest that structural or sequence differences in and around the NPXY motifs of these receptors may determine the specificity of SHC versus IRS-1 interaction. To address this possibility we have generated a panel of 18 amino acid substitution mutants within and around the NPXY motif of the IR and analyzed their ability to interact with either IRS-1 or SHC in the yeast two-hybrid system. Our data show that amino acid sequences both upstream and downstream of the NPXY motif within the IR are important for determination of the specificity of IRS-1 and/or
As shown schematically in Fig. 1, we generated multiple amino acid substitution mutants within and around the NPEY motif of the IR using site-directed mutagenesis (35). In most cases we chose alanine as the replacement amino acid except: Tyr-960 (which we substituted with either Phe or Glu), Leu-961 (Ala or Arg), and Ala-963 (Asp). All mutant receptors were introduced into yeast vectors and expressed as LexA fusions as described previously (1, 2). As a control we assayed the interaction of each receptor mutant with a p85 activation domain hybrid, which contains both SH2 domains and has been previously shown to interact with the IR (1). As shown in Fig. 1, the p85 hybrid showed no significant differences in levels of β-galactosidase activity when coexpressed with all receptor mutants as compared with the wild-type receptor. This is most likely since p85 is thought to interact with C-terminal IR sequences (38) and thus should not be affected by mutations within the juxtamembrane domain of the IR as long as these mutations do not affect autophosphorylation of the remainder of the IR. The demonstration that p85 interacts with all receptor mutants together with our previous finding that p85 required IR autophosphorylation for interaction (1) clearly showed that overall IR structure and autophosphorylation remain intact in all mutant receptors.

As shown in Fig. 1, we next analyzed the interaction of two IRS-1 hybrids with the panel of IR mutants. We utilized a full-length IRS-1 hybrid (1–1242) and a hybrid containing amino acids 45–516, a region that contains the SAIN domain and retains high level interaction with the IR (1, 2). In confirmation of our previous data, mutation of Tyr-960 to Phe eliminated interaction with full-length IRS-1, and here we show that the smaller SAIN domain-containing hybrid also shows no interaction (1, 2). Identical results were obtained after substitution of Tyr-960 with Glu, thereby showing that introduction of a negatively charged amino acid at this site is not sufficient to reconstitute the interaction. This suggests that, like SH2 domains, the SAIN domains also interact specifically with phosphorylated tyrosines. Substitution of either Asn-957 or Pro-958 with Ala within the NPEY motif eliminated interaction of both IRS-1 hybrids with the IR. As would be predicted, a double substitution in which both Asn-957 and Pro-958 were changed to alanine showed no interaction with either IRS-1 hybrid protein. In addition to the NPEY motif itself, only 3 of the other 12 mutations had significant effects upon IRS-1 interaction. Specifically, substitution of either Leu-952 or Tyr-953 with Ala resulted in a 70% reduction in activity for both IRS-1 hybrids with the IR. As would be predicted, a double substitution in which both Asn-957 and Pro-958 were changed to alanine showed no interaction with either IRS-1 hybrid protein. In addition to the NPEY motif itself, only 3 of the other 12 mutations had significant effects upon IRS-1 interaction. Specifically, substitution of either Leu-952 or Tyr-953 with Ala resulted in a 70% reduction in activity for both IRS-1 hybrids with the IR. As would be predicted, a double substitution in which both Asn-957 and Pro-958 were changed to alanine showed no interaction with either IRS-1 hybrid protein.

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activation domain hybrid

A

B

Fig. 3. Model for SHC and IRS-1 interaction with the insulin receptor. A, schematic model of SHC and IRS-1 interaction with the IR. The NPEY motif is boxed. The lines represent amino acids, which are important for each interaction. The thickness of the lines is meant to represent the relative importance of the amino acid to the interaction. B, summary of sequences known or proposed to interact with either SHC or IRS-1-like molecules. The NP and Y residues are boxed as are the common amino acids proposed to be involved in IRS-1 interaction. The h refers to the tendency for bulky hydrophobic residues to be found at this position.

NPXY box and important Leu and Tyr residues at −7 and −8 (from Tyr-960). Conversely, SHC interaction is totally unaffected by these upstream Leu and Tyr substitutions. In addition, the Asn and Pro within the NPXY motif, while important, are not essential for SHC interaction. Unlike IRS-1, SHC interaction is markedly influenced by the identity of the amino acid immediately C-terminal to the phosphotyrosine, since substitution of Leu-961 with either Arg or Ala markedly reduced SHC interaction without affecting IRS-1 interaction. The apparent functional similarities of SAIN and SH2 domains make it tempting to speculate that the SAIN domain contains a phosphotyrosine binding pocket, which is surrounded by sequence-specific recognition pockets that recognize and interact with the Asn and Pro residues within the NPXY motif as well as with the other surrounding amino acids noted herein. In addition to the possibility that the individual amino acids that we have identified mediate a direct interaction with either SHC or IRS-1 we must also consider the possibility that these substitutions may cause other alterations in the local structure of the NPXY region and/or may result in decreased phosphorylation of Tyr-960. Although we cannot adequately address these issues experimentally, we do not favor the latter possibility, particularly in the case of the substitutions which affect IRS-1 or SHC interaction differentially, since interaction with the other protein is generally unaffected. In other words, given the absolute requirement for Tyr-960 phosphorylation it seems likely that this would be reflected by reduced interaction with both SHC and IRS-1. It is notable that the NPXY motif is thought to adopt a reverse turn structure, which is influenced by the Asn, Pro, and Tyr residues (39). Although it is unclear whether or not this structure is valid within an intact protein such as the IR, it remains possible that such a conformation unaltered with the phosphorylation of the tyrosine allows recognition of SHC and IRS-1 while the identity

Fig. 2. Effect of amino acid substitutions within and around the IR NPEY motif upon SHC interaction. The amino acid substitutions that were made are shown to the left. The interaction domain hybrids are shown above. The SHC hybrids contained the full-length (1–473) or the interaction domain only (1–238). β-Galactosidase activity was measured and is reported in Miller Units ± S.E. The data were analyzed as discussed in Fig. 1.

Table 3. Sequences known or proposed to interact with either SHC or IRS-1-like molecules. The NP and Y residues are boxed as are the common amino acids proposed to be involved in IRS-1 interaction. The h refers to the tendency for bulky hydrophobic residues to be found at this position.

A schematic model summarizing these data is presented in Fig. 3A. In this model the lines represent the amino acids whose substitution resulted in decreased interaction with either SHC or IRS-1, and the thickness of the lines represents their relative importance of each to the interaction. Our data suggest that the NPXY motif serves as a core binding site for both SHC and IRS-1 and that phosphorylation of Tyr-960 is essential for both interactions. IRS-1 (unlike SHC) appears to depend more heavily upon amino acids to the N-terminal side of Tyr-960 including essential Asn and Pro residues within the...
of the surrounding amino acids determines optimal specificity. Our finding that the majority of substitutions outside of the NPXY motif showed no changes in interaction with SHC or IRS-1 suggests that the overall structure of this region is not particularly sensitive to individual substitutions.

Our identification of amino acids which are important for SHC and/or IRS-1 interaction with the IR allows us to propose consensus sequences for IRS-1 and SHC interaction and furthermore to speculate upon the role that these sequences may play in determining whether SHC and/or IRS-1 interact with known receptors and other signaling proteins. These sequences are shown in Fig. 3B. In support of our identification of Leu-952 and Tyr-953 as being important for IRS-1 binding, the IR family of receptors and the IL-4 receptor all contain a Leu and either a Tyr or Val at these positions. Thus all of the receptors known to interact with IRS-1-like molecules have relatively bulky hydrophobic side chains at these positions. Conversely, neither the TrkA, EGFR receptor, c-ErbB-3, c-ErbB-2, or middle T antigen proteins contain similar amino acids at these positions, suggesting one potential reason why they do not appear to interact efficiently with IRS-1-like molecules. In addition, the Ala corresponding to IR 963 is conserved in the IR family of receptors and (although it is replaced by Phe in the IL-4 receptor) this position may be important for the specificity of IRS-1 interaction. Common sequences surrounding the NPXY motifs of the IR and IL-4 receptor were first noted by Keegan et al. (27) and were postulated to play a role in IRS-1/4PS (IRS-2) interaction with these proteins. Our data support the functional importance of some of the common amino acids noted by these authors, in particular the Leu and Tyr/Val at positions −8 and −9 from the Tyr and the NPXY motif itself. Other conserved amino acids such as the Pro at −10, Ala/Ser at −5, Ser at 2 and 4, and the Asp at 5 do not appear to be absolutely required for efficient IRS-1 interaction with the IR in the two-hybrid assay. Alternatively, alanine may be able to functionally substitute for these amino acids.

SHC interaction with the IR appears to be largely determined by interaction with the NPXY motif and a single amino acid at position 1 from the Tyr. Although the identity of this amino acid does not appear critical, most proteins that interact efficiently with SHC appear to contain bulky hydrophobic residues at this position. One of the few receptors that has been reported to interact inefficiently with SHC in vivo is the IL-4 receptor (28). This receptor contains an Arg at position +1, an amino acid which our data show clearly interferes with SHC interaction with the IR but has no effect upon IRS-1 interaction. It is also interesting to note that while SHC can be coprecipitated with the EGF (11, 13), TrkA (22, 23), c-ErbB-2 (16), and c-ErbB-3 (19) receptors and with polypeptide middle T antigen (20, 21), similar attempts by a number of groups to communoprecipitate SHC with the IR have proven unsuccessful (12–14, 40). The reason for this difference is unclear although it seems likely that SHC may interact more tightly with these other proteins because they contain amino acids surrounding the NPXY motif which result in a more stable interaction. In this regard, recent work has suggested that a hydrophobic residue (such as Ile or Leu) at position −5 from the phosphotyrosine within the NPXY motif is important for interaction with the EGF and nerve growth factor receptors (41, 42). It is possible that such a hydrophobic residue allows SHC coprecipitation, whereas the IR (which contains a Ser at this position) is unable to coprecipitate SHC.

Our conclusion that the SHC SAIN domain of SHC interacts with phosphorylated NPXY motifs (1) has recently been confirmed in vitro (43, 44). The first study, using SHC coprecipitation analysis combined with phosphopeptide competition, showed that the SHC SAIN domain (also termed PTB) interacts in vitro with three phosphorylated tyrosines within the c-ErbB-2/neu receptor (45). Two of these have the NPXY motif while the other has an NLYY sequence. Furthermore, these authors utilized phosphopeptide competition studies to show that the Asn at −3 and the Trp at +1 were very important for SHC interaction with the c-ErbB-2 receptor in vitro. Another group, analyzing peptide interaction with the SHC SAIN domain in vitro, has demonstrated the importance of the Asn and Pro residues at −3 and −2 as well as the importance of a hydrophobic residue at +1 (44). These findings, involving entirely different methodologies, are consistent with our data regarding the IR-SHC interaction that we have characterized using the two-hybrid assay.

The conclusions presented here, while consistent with the known interactions of SHC and IRS-1 with their upstream regulators, are preliminary and will need to be further tested in cells of higher eukaryotic origin to assure the physiological relevance of these findings. We will need to assess the effects of various IR receptor mutations upon the ability of the receptors to phosphorylate IRS-1 and SHC and to mediate downstream signaling events. Our findings should theoretically be useful for the production of IR mutants, which are relatively specific for either SHC or IRS-1 signaling. These receptor mutants should prove to be important reagents which will allow a critical examination of the relative roles of these two proteins in mediating the many effects of the IR, including regulation of cellular metabolism, mitogenesis and differentiation.

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