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Phosphotyrosine-Dependent Interaction of SHC and Insulin Receptor Substrate 1 with the NPEY Motif of the Insulin Receptor via a Novel Non-SH2 Domain

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The SHC proteins have been implicated in insulin receptor (IR) signaling. In this study, we used the sensitive two-hybrid assay of protein-protein interaction to demonstrate that SHC interacts directly with the IR. The interaction is mediated by SHC amino acids 1 to 238 and is therefore independent of the Src homology 2 domain. The interaction is dependent upon IR autophosphorylation, since the interaction is eliminated by mutation of the IR ATP-binding site. In addition, mutational analysis of the Asn-Pro-Glu-Tyr (NPEY) motif within the juxtamembrane domain of the IR showed the importance of the Asn, Pro, and Tyr residues to both SHC and IR substrate 1 (IRS-1) binding. We conclude that SHC interacts directly with the IR and that phosphorylation of Tyr-960 within the IR juxtamembrane domain is necessary for efficient interaction. This interaction is highly reminiscent of that of IRS-1 with the IR, and we show that the SHC IR-binding domain can substitute for that of IRS-1 in yeast and COS cells. We identify a homologous region within the IR-binding domains of SHC and IRS-1, which we term the SAIN (SHC and IRS-1 NPXY-binding) domain, which may explain the basis of these interactions. The SAIN domain appears to represent a novel motif which is able to interact with autophosphorylated receptors such as the IR.

The recent identification and cloning of proteins which interact with receptor tyrosine kinases (RTKs) has allowed much insight into the molecular basis for RTK signal transduction (9, 10, 18, 19, 24, 27, 54). These effector proteins contain Src homology 2 (SH2) domains of approximately 100 amino acids which interact directly with phosphotyrosine-containing regions of each RTK (21, 35). Upon receptor autophosphorylation, these SH2 domain-containing proteins interact with the RTK. Unlike most RTKs, the insulin receptor (IR) and the related insulin-like growth factor 1 receptor (IGFIR) appear to interact with and phosphorylate an intermediate signaling protein termed IRS-1, for insulin receptor substrate 1 (17). After tyrosine phosphorylation by the IR, IRS-1 is thought to interact with a variety of SH2 domain-containing proteins, including the p85 subunit of phosphatidylinositol 3-kinase, GRB-2, and SH-PTP2 (Syp) (45, 52). These proteins presumably mediate some of the effects of the IR.

Another substrate of the IR is the SH2 domain-containing protein SHC, so named because of its SH2 domain as well as its homology to collagen (36). 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. The SHC proteins have been implicated in mitogenic signaling by a variety of tyrosine kinases. These include the receptors for nerve growth factor (32), epidermal growth factor (EGF) (36), platelet-derived growth factor (PDGF) (59), and insulin (39). SHC has also been implicated in signaling by other classes of receptors, including the interleukin-2 receptor (40, 62) and the T-cell receptor (41). Other hormones which have been shown to cause phosphorylation of SHC include interleukin-3, granulocyte-macrophage colony-stimulating factor, and steel factor (28, 58).

SHC becomes rapidly phosphorylated upon tyrosines after IR stimulation of cells, resulting in rapid association of SHC with the GRB-2 adapter protein, which then allows $p21^{ras}$ activation via the guanine nucleotide release protein SOS (38, 39, 42, 60). It is generally thought that Ras activation is the mechanism by which SHC stimulates mitogenesis. GRB-2 has been shown to interact with IRS-1 and SHC after tyrosine phosphorylation of these proteins by the IR and other RTKs (45), although the interaction of GRB-2 with IRS-1 does not appear to be sufficient to explain mitogenic signaling via the IRS-1 molecule (30). Unlike the EGF and PDGF receptors, coprecipitation studies have not shown interactions either between SHC and the IR or between SHC and IRS-1 (22, 38, 39, 60). Since many protein-protein interactions may not be easily amenable to coprecipitation assays because of low affinities, high off rates, or susceptibility to the salts and detergents used in these assays, we explored the use of the two-hybrid assay to determine whether SHC could form a specific interaction with the IR. We have previously used this assay to begin to study the interaction between the IR and IRS-1 (34). Here we show that SHC and the IR form a specific complex which results in high-level activity in the two-hybrid assay. This interaction is dependent upon receptor tyrosine autophosphorylation and also upon an intact NPEY motif. We propose that SHC and IRS-1 interact directly with the tyrosine-phosphorylated NPEY motif and that the interaction is mediated at least in part by a homologous domain which we term the SAIN (SHC and IRS-1 NPXY-binding) domain.

MATERIALS AND METHODS

Yeast strains and plasmids. *Saccharomyces cerevisiae* EGY40 (α *trp1 ura3-52 his3 leu2*) and all yeast expression plasmids were provided by the laboratory of Roger Brent and have been previously described (13, 16, 34, 61). All procedures

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for routine growth and maintenance of yeast strains were as described previously (15). Plasmid transformation of yeast cells was either by the lithium acetate method (43) or by electroporation (5).

Plasmid construction. The cDNA fusions were produced by a variety of standard methods (1). These included the use of PCR, oligonucleotide linkers, prior shuttling into one or more intermediate vectors, and filling in or removing overhangs to allow the in-frame insertion into the yeast expression vectors. Detailed cloning strategies for each clone are available upon request and will not be presented here. All junctions were sequenced by using customized primers and Sequenase 2.0 (United States Biochemical Corporation, Cleveland, Ohio) sequencing protocols, as were all PCR-derived DNAs. The plasmids encoding the wild-type, Lys-1018→Ala (K1018A), and Tyr-960→Phe (Y960F) IR hybrids have been previously reported (34). All site-directed mutants were generated by the method of Kunkel (23), using customized primers.

Human SHC was cloned by PCR amplification from a human fetal cardiac cDNA library (Stratagene). The PCR primers were designed by using published sequences (36) to amplify the region of the SHC cDNA from the (first) start codon to the stop codon. This PCR product was then subcloned into the Bluescript plasmid vector for sequencing. We noted two differences from the published sequences which would lead to differences in coding potential. In particular, amino acid 16 was encoded by GAG (Glu), rather than the previously reported GGG (Gly), and amino acid 147 was encoded by ATA (Ile) rather than ATG (Met).

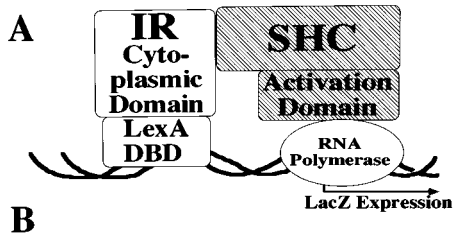
β-Galactosidase assays. The colony color β-galactosidase assay was performed as described previously (6, 34). Colony color assays were performed on a minimum of 5 to 10 independent colonies from each hybrid. The solution β-galactosidase assays were performed as described previously (7), and the units of β-galactosidase activity were calculated by the method of Miller (29). The values shown for the positive colonies represent averages of 3 to 10 assays ± standard error (SE) (each assay representing an independent colony). For the negative colonies, the values represent the averages of at least three assays. Assays were performed on at least three different days to ensure the reliability of the data.

Immunoblot analysis of hybrid protein expression. Expression of the activation domain hybrids was analyzed by using an antihemagglutinin antibody as previously described (34). The approximate predicted molecular masses of the hybrids are as follows: 1-473, 75 kDa; 1-369, 68 kDa; 1-238, 53 kDa; 1-210, 47 kDa; 1-117, 43 kDa; 57-210, 39 kDa; 46-238, 43 kDa; 57-238, 46 kDa; 84-238, 43 kDa; 117-238, 40 kDa; 157-238, 36 kDa; 240-473, 48 kDa; 240-368, 42 kDa; and 378-473, 33 kDa. The sizes of the SHC hybrids were as predicted, although it should be pointed out that the sizes of a few of the hybrids do not correlate to the size of the SHC insert. For example, the 46-238 hybrid has a larger SHC insert than the 84-238 insert, yet the hybrid protein has a lower molecular weight. These discrepancies are due to differences in the number of amino acids encoded by the linker sequences between the activation domain and the SHC sequences and to different numbers of amino acids at the carboxyl terminus derived from the vector. Our current and previous studies (34) have demonstrated that these extraneous peptides play no role in the interaction with the IR.

COS cell expression studies. For COS cell expression studies, the IR-binding domain of SHC (amino acids 2 to 238) was inserted into an IRS-1 expression construct from which the IRS-1 IR-binding domain (amino acids 45 to 516) had been deleted. The IRS-1 Δ45-515 deletion construct has been described elsewhere (34). These expression plasmids were used to transfect COS-7 cells by the DEAE-dextran procedure (1). The SHC and SHC/IRS-1 chimera plasmids were cotransfected with an IR expression plasmid (12). On the day after transfection, the cells were split into two plates and allowed to grow for 48 h. At this point, the cells were treated for 10 min with phosphate-buffered saline supplemented with 0.1% bovine serum albumin with or without 100 nM insulin. After 10 min at 37°C, the medium was removed and the cells were scraped into 300 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer. The cell lysates were boiled and briefly sonicated. Duplicate 7.5% gels were loaded, and following electrophoresis, proteins were blotted to nitrocellulose and analyzed with the ECL detection system (Amersham, Arlington Heights, Ill.), using either an antiphosphotyrosine antibody (PY20; Transduction Laboratories, Lexington, Ky.) or an antibody against the carboxyl terminus of IRS-1 (Upstate Biotechnology Inc., Lake Placid, N.Y.).

RESULTS

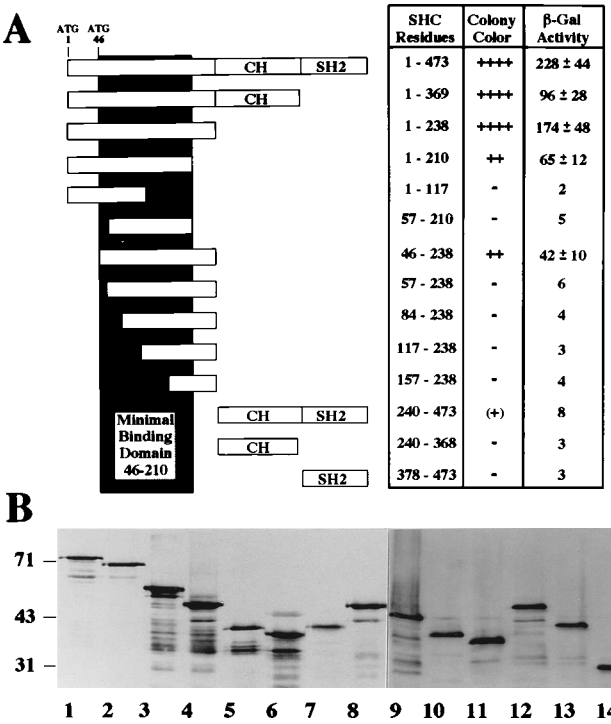
SHC interacts specifically with the IR in the two-hybrid assay. SHC has been shown to become phosphorylated upon tyrosines within seconds of IR activation and therefore appears to be a direct substrate of the IR in vivo (39, 60). SHC has also been shown to be a substrate of the EGF and PDGF receptors; furthermore, it has been shown that the SH2 domain of SHC is able to interact with these receptors in coprecipitation assays using glutathione *S*-transferase (GST)-SH2 domain fusion proteins (36, 59). In contrast, no coprecipitation of SHC was observed either with the IR or with IRS-1 after insulin stimulation of cells (22, 38, 39, 60). To investigate further these somewhat paradoxical findings, we tested whether a direct interaction between SHC and the IR could be detected in the yeast two-hybrid assay. We had previously shown that this assay can be used to study the interaction between the IR and IRS-1 (34), another interaction which is difficult to demonstrate by coimmunoprecipitation (2, 53). The IR bait hybrid protein, in which the entire cytoplasmic domain of the IR is fused to the DNA-binding region of LexA, has been described elsewhere (34). To produce the SHC hybrid, we fused the full-length SHC cDNA (amino acids 1 to 473) to the acidic activation domain (16, 61). A schematic of these hybrid proteins is shown in Fig. 1A. Plasmids expressing these hybrid proteins were introduced into yeast strain EGY40 along with the *lacZ* reporter plasmid, and transformants were identified by growth on the proper selective media. These colonies were first analyzed by the colony filter assay for β-galactosidase activity (6) after replica plating onto selective plates containing either glucose or galactose (in order to induce the galactose-specific expression of the SHC fusions) (16). Thus, β-galactosidase activity should be observed only when the yeast cells are grown in the presence of galactose. As summarized in Fig. 1B, no β-galactosidase activity was seen when the IR hybrid was expressed independently of the SHC hybrid, regardless of the carbon source. Conversely, when the SHC hybrid was coexpressed with the IR bait, dark blue colonies were observed, but only when grown on galactose-containing plates. To ensure that the SHC hybrid was not interacting with the LexA portion of the bait, it was coexpressed with a second bait hybrid protein which contains the identical LexA domain fused to a portion of the *Drosophila* bicoid protein (61). No β-galactosidase activity was observed with this combination, demonstrating that the SHC hybrid does not interact with the LexA portion of the bait hybrid. In addition, this result showed that the SHC hybrid could not induce β-galactosidase activity independently. To better quantitate the β-galactosidase activity, solution assays were performed with lysates from cells grown in either glucose



B

Plasmids		Colony Color		β–Gal Activity	
LexA Hybrid	Activation Hybrid	GLU	GAL	GLU	GAL
IR	-	-	-	2	2
IR	SHC 1-473	-	++++	3	196±42
Bicoid	SHC 1-473	-	-	2	2

FIG. 1. SHC interacts specifically with the IR in the two-hybrid assay. (A) Schematic representation of the hybrid proteins. The entire cytoplasmic domain of the human IR was fused to the LexA DNA-binding domain (DBD). The cDNA encoding full-length SHC (amino acids 1 to 473) was fused to the activation domain. Interaction of these hybrid proteins would be expected to drive the expression of the *lacZ* reporter gene. (B) The IR hybrid and the *lexAop-lacZ* reporter were introduced into yeast cells in the absence or presence of the SHC hybrid, and transformants were isolated on selective plates. Transformants were assayed for β-galactosidase activity by either the colony color or the solution assay as described in Materials and Methods. As a negative control, the SHC hybrid was coexpressed with the LexA-bicoid hybrid. The colony color assay showed either white (−) or dark blue (++++) colonies. β-Galactosidase (β-Gal) activity is reported by Miller units as mean ± SE.



SHC Residues	Colony Color	β-Gal Activity
1 - 473	++++	228 ± 44
1 - 369	++++	96 ± 28
1 - 238	++++	174 ± 48
1 - 210	++	65 ± 12
1 - 117	-	2
57 - 210	-	5
46 - 238	++	42 ± 10
57 - 238	-	6
84 - 238	-	4
117 - 238	-	3
157 - 238	-	4
240 - 473	(+)	8
240 - 368	-	3
378 - 473	-	3

FIG. 2. The amino-terminal region of SHC interacts with the IR. (A) Schematic of 14 SHC activation domain hybrids assayed in the two-hybrid system. The colony color assay showed either white (−), very light blue [(+)], medium blue (++), or dark blue (++++) colonies when grown on galactose-containing plates. When grown on glucose-containing plates, all colonies were white (data not shown). β-Galactosidase (β-Gal) activity (in Miller units) was quantitated by using the solution assay as described in Materials and Methods and is reported as mean ± SE. The locations of the alternative start sites are shown above. CH and SH2 refer to the locations of the collagen homology and SH2 domains. (B) Immunoblot analysis of expression of the SHC hybrid proteins. Expression of the SHC hybrids was analyzed by immunoblotting with the hemagglutinin epitope tag antibody as described in Materials and Methods. Lanes 1 to 14 are in the same order as the schematic (from top to bottom) and refer to hybrids 1–473 (lane 1) through 378–473 (lane 14). Identical amounts of cell lysate were analyzed for all samples.

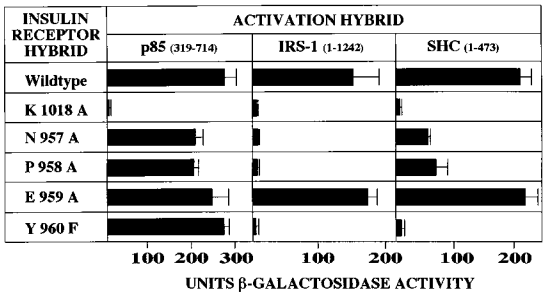


FIG. 3. Mutagenesis of the IR hybrid reveals the importance of receptor kinase activity and the NPEY motif to SHC and IRS-1 interaction. The alanine substitution IR mutants were coexpressed with activation hybrids containing either the region of p85 which contains both SH2 domains, full-length IRS-1, or full-length SHC. β-Galactosidase activity generated by the interaction between these proteins was determined as described in Materials and Methods and represents the average activity (in Miller units) from three to five independent colonies ± SE.

or galactose. As shown in Fig. 1B, these results confirmed those obtained from the colony color filter assay. Coexpression of the SHC and IR hybrids resulted in approximately 200 U of β-galactosidase activity. We conclude that the two-hybrid assay is able to detect a specific interaction between full-length SHC and the cytoplasmic domain of the IR, resulting in high-level β-galactosidase activity.

SHC amino acids 1 to 238 are sufficient for IR interaction. To delineate which region of SHC was responsible for the interaction, we generated a series of SHC deletion constructs and coexpressed them with the IR hybrid. As shown in Fig. 2A, carboxyl-terminal deletions clearly show that the region which mediates the interaction with the IR is located within the first 238 amino acids of SHC. Further deletion to amino acid 210 showed somewhat reduced activity, while further deletion to amino acid 117 eliminated all activity. Amino-terminal deletions within the 1–238 domain suggested that amino acids 1 to 46 were important for full activity but were not essential, since the 46-238 construct showed significant though reduced activity compared with the 1-238 hybrid. It has been noted in vivo that the p52 form of SHC is phosphorylated more efficiently and quickly than the p46 form (38, 39, 60). These findings are consistent with ours and suggest that amino acids 1 to 46 may be important for efficient IR interaction and subsequent phosphorylation. Further amino-terminal deletions of amino acids 46 to 56 eliminated activity, suggesting that an essential component of the interaction lies within this region. A very slight activity was observed with the 240-473 hybrid, while the SH2-domain hybrid itself showed no activity. To ensure that the low activities observed with the negative constructs were not due to lack of expression of the proteins, we examined their expression by immunoblotting with the hemagglutinin antibody, which recognizes all SHC hybrids. As shown in Fig. 2B, all hybrid proteins were of the expected sizes and were expressed at comparable levels. We conclude that SHC interacts with the IR in an SH2 domain-independent manner and that the minimal binding domain of SHC is located between amino acids 46 and 210.

IR tyrosine kinase activity is essential for SHC and IRS-1 interaction with the IR. We next investigated whether IR kinase activity was necessary for the interaction with SHC. We coexpressed the 1-473 SHC hybrid with a kinase-inactive IR hybrid in which the critical Lys (1018) within the ATP-binding site had been mutated to Ala. We have previously demonstrated that this hybrid receptor construct shows no tyrosine autophosphorylation when expressed in yeast cells (34). As shown in Fig. 3, the SHC 1-473 hybrid showed no interaction with the IR K1018A mutant. For comparison, we also examined whether a full-length IRS-1 hybrid could interact with the K1018A mutant and found that this hybrid also showed no activity. This observation is consistent with our previous demonstration that a similar IRS-1 hybrid containing amino acids 21 to 1242 showed no interaction with the K1018A receptor (34). Lastly, we coexpressed an activation domain hybrid fused to the region of the p85 subunit of phosphatidylinositol 3-kinase that contains both SH2 domains (amino acids 319 to 714), which we have shown interacts specifically with the IR in the two-hybrid assay (data not shown). We found that this hybrid was also unable to interact with the K1018A mutant. We conclude that the interaction of the IR with SHC, IRS-1, and p85 is absolutely dependent upon IR tyrosine kinase activity, suggesting strongly that IR autophosphorylation is required in order to form binding sites for these proteins.

IR Tyr-960 is essential for SHC and IRS-1 interaction with the IR but is not necessary for p85 interaction. It has been shown that expression of a mutant IR containing a Tyr-960-to-Ala substitution within the juxtamembrane domain showed severely impaired insulin-dependent tyrosine phosphorylation of SHC (60). We therefore tested whether a similar mutation in the IR hybrid (Y960F) would affect SHC interaction in the

two-hybrid assay. We have previously shown that this receptor hybrid retains the ability to autophosphorylate but is unable to interact with IRS-1 in the yeast assay system (34). As shown in Fig. 3, the IR Y960F mutant showed an almost complete loss of interaction with the SHC 1-473 hybrid protein. Identical results were obtained with the SHC 1-238 construct, and the interaction is therefore independent of the SH2 domain (data not shown). As predicted from our previous work (34), the full-length IRS-1 hybrid showed no interaction with the Y960F mutant in either assay. Lastly, we examined the ability of the p85 hybrid to interact with this Y960F mutant. We found that interaction with p85 was not affected by this mutation. This is due to the fact that p85 is thought to interact with the YXXM motif (YTHM at Tyr-1322) within the C terminus of the IR (reference 55 and our unpublished observations) and therefore should not be affected by the Y960F mutation. These results with p85 suggest that the Y960F mutation does not cause a global change in the structure of the IR hybrid. This idea is supported by our previous demonstration that the Y960F IR shows clear tyrosine autophosphorylation when expressed in yeast cells (34). We conclude that the interaction of SHC and IRS-1 with the IR is dependent on Tyr-960; this observation, together with the K1018A mutation data, suggests strongly that autophosphorylation of Tyr-960 is involved.

Mutational analysis of the NPEY motif demonstrates the importance of Asn and Pro (but not Glu) to IRS-1 and SHC binding. Because Tyr-960 is found within an NPXY motif which has been shown to be important for IRS-1 phosphorylation and similar motifs have been shown to be involved in SHC interaction, we addressed the importance of each of the amino acids within the NPEY motif of the IR to IRS-1 and SHC interaction in the two-hybrid assay. Alanine substitution mutants were generated by site-directed mutagenesis within the NPEY motif and analyzed for SHC and IRS-1 interaction. As shown in Fig. 3, both the N957A and P958A mutations resulted in the elimination of IRS-1 interaction with the IR. These mutations significantly reduced (by ~75%) the interaction of the IR with SHC, but surprisingly, unlike IRS-1, SHC retained some ability to interact with the IR despite these mutations. Thus, despite the overall apparent similarities in the interaction of SHC and IRS-1 with the IR, subtle differences appear to exist. In contrast to the Asn-957 and Pro-958 mutations, alteration of IR Glu-959 to Ala had no effect upon the interaction of either SHC or IRS-1. This finding is in clear support of the definition of the NPXY motif, in which the identity of the residue immediately to the amino-terminal side of the Tyr is not critical. Importantly, the interaction of the p85 hybrid was not affected significantly by any mutations within the NPEY motif. These findings suggest strongly that the receptor mutations within the NPXY motif do not affect receptor structure or the ability of the IR hybrids to autophosphorylate. We conclude that the efficient interaction of SHC and IRS-1 with the IR is dependent upon the Asn, Pro, and phospho-Tyr residues within the NPEY motif and that the various proteins interact in a similar yet distinguishable manner. Experiments are under way to analyze the effects of mutation of amino acids surrounding the NPEY motif.

The SHC IR-binding domain can substitute for the IRS-1 IR-binding domain in yeast and COS cells. We previously showed that an IRS-1 mutant in which amino acids 45 to 515 had been deleted was unable to undergo IR-mediated tyrosine phosphorylation when coexpressed with the IR in COS cells (34). Considering that both SHC and IRS-1 appear to interact directly with NPEY motif of the IR, we reasoned that replacement of IRS-1 amino acids 45 to 515 with SHC amino acids 2 to 238 should allow interaction of this chimeric protein with

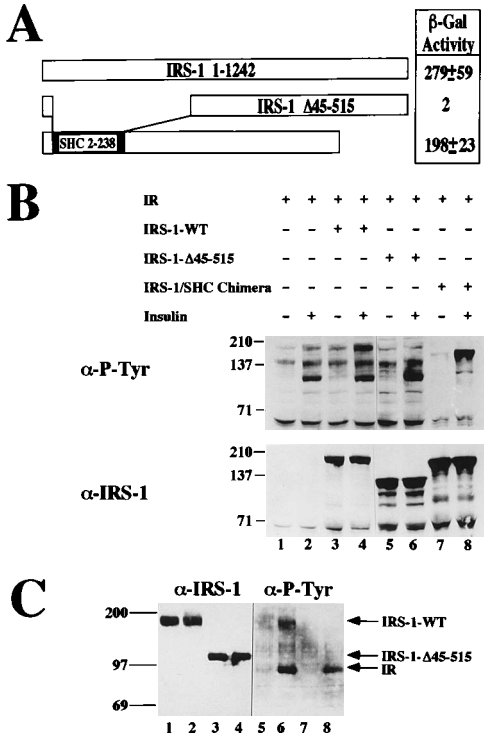


FIG. 4. SHC amino acids 2 to 238 can substitute for the IRS-1 IR-binding domain in yeast and COS cells. (A) Schematic representation of full-length IRS-1, the IRS-1 Δ45-515 deletion mutant, and SHC/IRS-1 chimeric cDNAs used for expression in yeast and COS cells. The yeast fusions were analyzed by using the solution β-galactosidase (β-Gal) assay as described in Materials and Methods. Activity (in Miller units) is reported as mean ± SE. (B) Analogous cDNAs were transferred to the expression vector pECE for expression in COS cells. These expression vectors were cotransfected with expression plasmids for the IR as shown above the immunoblots. Duplicate plates were treated with 0 or 100 nM insulin for 10 min as shown, and lysates were analyzed by immunoblotting with either an antiphosphotyrosine (α-P-Tyr) or anti-IRS-1 (α-IRS-1) antibody as described in Materials and Methods. (C) To better separate the IR phosphorylated band from that of the IRS-1 Δ45-515 hybrid (lanes 3, 4, 7, and 8) were left untreated (odd-numbered lanes) or were treated with insulin for 10 min prior to electrophoresis of cellular lysates and immunoblotting with either an anti-IRS-1 or antiphosphotyrosine antibodies.

the IR in COS cells. We predicted that the chimera would regain the ability to interact with the IR, resulting in insulin-dependent phosphorylation, since the majority of Tyr phosphorylation sites on IRS-1 are located between amino acids 516 and 1242 (52). The structures of the deletion mutant and the SHC/IRS-1 chimera are shown schematically in Fig. 4A. We first tested these constructs in the two-hybrid assay. As shown in Fig. 4A, the SHC IR-binding domain (amino acids 2 to 238) was able to functionally replace the 45–515 domain of IRS-1, restoring high-level activity in the two-hybrid assay.

We next produced analogous constructs for expression in COS cells. As shown in Fig. 4B, when the SHC/IRS-1 chimera was coexpressed with the IR in COS cells, it showed high-level insulin-dependent phosphorylation on tyrosines (Fig. 4B, lanes 7 and 8). In fact, the relative phosphorylation of the chimera was consistently greater than that of the full-length IRS-1 (lane 4), and it was necessary to load about one-fourth of the sample on the antiphosphotyrosine immunoblot (but not the IRS-1 blot) compared with the other lanes to avoid overexposure and smearing of this lane. For this reason, the phosphorylated IR band is not readily apparent but is clear on longer exposures (not shown). Full-length IRS-1 also showed significant insulin-

dependent phosphorylation compared with control cells (compare lane 4 with lane 2). Conversely, as we have previously shown, the IRS-1 deletion construct (which runs more slowly than the IR) showed little or no phosphorylation (lanes 5 and 6). The expression of the IRS-1 and IRS-1/SHC chimeric proteins was confirmed by immunoblotting of duplicate blots with an anti-IRS-1 antibody that was made to the C terminus of IRS-1 and would therefore recognize all IRS-1 constructs. To better separate the IR phosphorylated band from that of the IRS-1 Δ45-515 hybrid, we extended the electrophoresis time prior to immunoblotting as shown in Fig. 4C. Lysates from COS cells expressing the IR and either full-length IRS-1 (lanes 1, 2, 5, and 6) or the Δ45-515 hybrid (lanes 3, 4, 7, and 8) were analyzed by electrophoresis on the same gel prior to immunoblotting. It is clear from these data that the IRS-1 deletion construct does not comigrate with the IR and is not phosphorylated despite clear IR autophosphorylation. In support of these data, antiphosphotyrosine antibodies were unable to immunoprecipitate the SHC/IRS-1 chimeric protein after insulin stimulation (data not shown). Although we observed an apparent increase in phosphorylation of the SHC/IRS-1 chimera compared with the intact IRS-1 in two independent experiments, we cannot conclude from this finding that the SHC SAIN domain has a better IR-binding activity than the IRS-1 SAIN domain. This is due to the artificial nature of the COS expression system in which differences in transfection, protein expression, targeting, or processing of each expressed protein cannot be adequately controlled. We therefore consider this assay to be useful in determining whether a protein is phosphorylated in an insulin-dependent manner and not for quantitating phosphorylation. We conclude that the IR-binding domain of SHC (amino acids 2 to 238) is able to functionally substitute for the IR-binding domains of IRS-1 in the yeast two-hybrid assay and in COS cells. In COS cells, this interaction resulted in the restoration of tyrosine phosphorylation of the chimeric protein, presumably on the numerous tyrosines located between IRS-1 amino acids 516 and 1242, which are known to be the favored sites of IR phosphorylation (17, 44, 52, 53). Although our data show that SHC and IRS-1 compete for the same site for interaction with the IR, we cannot comment on the relative affinities of these two interactions. This is because the two-hybrid assay is not useful for affinity measurements and the COS cell system is also inherently difficult to control. It is likely that in vitro assays of interaction using purified components will need to be developed to adequately address this question.

Identification of a homologous region within the IR-binding domains of SHC and IRS-1. Because of the similar nature of the interactions of SHC and IRS-1 with the IR in the two-hybrid assay and the ability of the SHC IR-binding domain to substitute for the IRS-1 domain, we reasoned that these proteins must contain a common sequence motif which could explain these functional similarities. As shown in Fig. 5A, we have identified regions between SHC amino acids 41 to 200 and IRS-1 amino acids 313 to 462 which show a high degree of similarity. These regions show approximately 25% identity and 40% similarity when conservative substitutions are taken into account. As shown schematically in Fig. 5B, this region of SHC corresponds to essentially the entire minimal domain of interaction of SHC which we had delineated in the two-hybrid assay. Consistent with the potential importance of this domain in mediating this interaction with the IR, any significant deletion of either end of this domain of SHC showed no activity (Fig. 5B). Likewise, the homologous region of IRS-1 corresponds well to the carboxy-terminal IR-binding domain of IRS-1, whose deletion we have previously shown results in

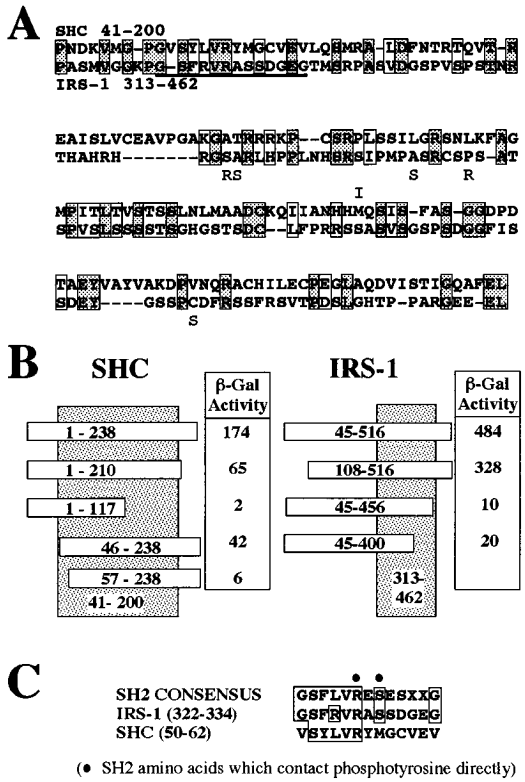


FIG. 5. Identification of a region of homology within the IR-binding regions of SHC and IRS-1. (A) SHC amino acids 41 to 200 are aligned with IRS-1 amino acids 313 to 462. Shaded boxes represent conserved residues; open boxes represent conservative substitutions (D, E) (R, K, H) (F, Y) (I, V, L, M) (S, T). Gaps have been inserted by eye to maximize the identity. The putative FLVRES-like sequence is underlined. The Ile (I) shown above the SHC sequence is the substitution which we identified in our SHC cDNA clone. The amino acids shown below the IRS-1 sequence represent differences which are found in rat IRS-1 (53) or within the IRS-1-like molecule cloned from a human hepatocellular carcinoma (31). (B) Schematic showing the regions of homology (shaded areas) in relation to the activities of SHC or IRS-1 hybrids in the two-hybrid assay. The β-galactosidase (β-Gal) activities (in Miller units) are summarized from this report and from reference 34. (C) Alignment of an SH2-FLVRES domain consensus and the region at the amino terminus of the IR-binding domains of SHC and IRS-1. This region is underlined in panel A.

severe reduction in interaction with the IR in the two-hybrid assay (34). Interestingly, although this domain of SHC appears sufficient and necessary for IR interaction, the corresponding domain of IRS-1 is important but not sufficient for interaction. Rather, IRS-1 appears to require additional amino-terminal sequences for full interaction. Nevertheless, this region of IRS-1 is extremely important for IRS-1 interaction, since its deletion reduces the interaction with the IR by ~95%. We term this domain the SAIN domain.

Although there appear to be no obvious recognizable sequence motifs within the SAIN regions of SHC and IRS-1, we did note one element which is reminiscent of the conserved FLVRES motif of SH2 domains which is critical for interaction with phosphotyrosine-containing peptides. These sequences are located at the amino-terminal end of the conserved regions and are shown in Fig. 5C. Other than this small motif, no other sequences characteristic of SH2 domains are evident within these regions of SHC or IRS-1. In support of this clearly speculative idea, this FLVRES-like element is located within the 46–56 region, whose deletion entirely eliminates activity of the SHC hybrids in the two-hybrid assay. As shown in Fig. 5C, both of these sequences contain significant differences from

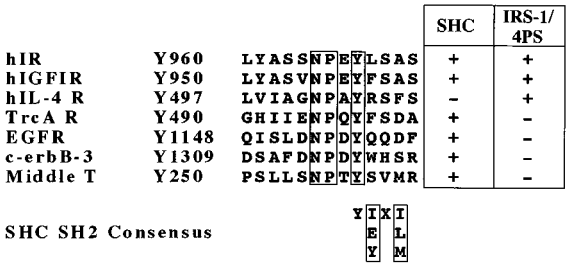


FIG. 6. Alignment of NPXY-containing regions of signaling proteins known or thought to interact with SHC and/or IRS-1/4PS. The N, P, and Y residues within the NPXY motif are boxed. The evidence for these interactions is discussed in the text. The SHC SH2 domain consensus as identified in vitro is also shown, and the alternative residues at positions +1 and +3 are boxed. The prefix "h" indicates human. EGFR, EGF receptor.

known FLVRES motifs, including the Arg-for-Leu substitution at position +1 (relative to the Phe) in IRS-1, the Met-for-Ser substitution at position +5 in SHC, and the lack of an acidic residue at position +4 in either protein. Nevertheless, the central Arg which has been shown to form two hydrogen bonds with the phosphotyrosine in SH2 domains is conserved. It is possible that this element plays an analogous role in SAIN domain interaction with phosphotyrosine-containing proteins. Additional experiments are under way to examine this idea.

DISCUSSION

Interaction of SHC with other signaling proteins with and without NPXY motifs. SHC has also been shown to interact with a variety of receptors and other signaling proteins. We will summarize these experiments and try to put these results in context regarding our findings with the IR. Our data suggest that SHC has the capability to interact with phosphotyrosine-containing peptides in at least two ways, either via its SH2 domain or via its SAIN domain. Previous experiments have sometimes assumed that these interactions were mediated by the SH2 domain of SHC and have largely ignored the remainder of the molecule. In regard to the SHC SH2 domain, a consensus for interaction has been proposed to be phospho-Y-(I, E, or Y)-X-(I, L, or M), using the in vitro methodology developed by Songyang et al. (47, 48). This motif has been suggested to be the basis for the interaction of SHC with the T-cell and PDGF receptors (40, 59), since an SHC SH2 domain-GST fusion protein could coprecipitate these receptors. The tyrosines thought to be involved in these interactions are consistent with the predicted SH2 domain-binding motif with hydrophobic residues at positions 1 and 3 and thus probably represent interactions with the SHC SH2 domain.

SHC has been shown to interact with NPXY motifs in at least three other signaling proteins, the TrkA (nerve growth factor) receptor (32, 49), the c-*erbB-3* proto-oncogene product (37), and the polyomavirus middle T antigen (MT) (8, 11). In addition we have demonstrated interaction of SHC with the NPXY motif of the IGFI receptor (10a). These NPXY motifs and the sequences surrounding them are shown in Fig. 6. In the TrkA receptor, the site of SHC interaction has been clearly demonstrated to be Tyr-490 (within an NPXY motif), using phosphopeptide competition and receptor mutagenesis approaches (32, 49). This sequence does not conform to the SHC SH2 domain consensus, and to our knowledge, the SHC SH2 domain has not been shown to be involved in this interaction. We predict that this interaction is mediated by the SAIN motif of SHC.

Similarly, the SHC-binding site within the c-ErbB-3 receptor has been mapped to Tyr-1309, which has the sequence NPDY (37). This study used synthetic phosphopeptides in permeabilized cells to compete for endogenous SHC interaction with the endogenous receptor. As discussed below, in our view, this approach appears to be more physiologically relevant than the more commonly used in vitro assays in which purified SH2 domain fusion proteins are added to cell lysates. Importantly, this group also reported that mutation of the Asn or the Pro residue to Ala within the within the NPXY motif of the phosphopeptide resulted in the total loss of inhibition of SHC binding. This finding is consistent with our view that the NPXY motif is involved, and we predict that it is the SAIN region of SHC which interacts with the c-ErbB-3 receptor.

It has recently been shown that SHC interacts with Tyr-250 of polyomavirus MT (8, 11). As shown in Fig. 6, this tyrosine is located within an NPXY motif. In these studies, it was shown that mutation of Tyr-250 eliminates SHC phosphorylation and SHC interaction with MT. Mutation of the Pro-248 residue within the NPXY motif to His also greatly reduced but did not eliminate SHC phosphorylation and MT coprecipitation (8). This finding is consistent with our demonstration that the Pro at position −2 in relation to the phosphotyrosine is important for efficient SHC interaction. These studies with MT suggested that the interaction was mediated by the SHC SH2 domain, since GST-SH2 domain fusions could precipitate a small amount of MT protein. Interestingly, the MT sequence might be predicted to interact with both the SH2 and SAIN domains of SHC, since it contains a Met at position +3. Whether the SAIN domain of SHC interacts with MT was not examined.

Studies of SHC interaction with the EGF receptor have not led to a clear consensus regarding the primary sites of interaction. As discussed below, we believe that this is due to differences in the methodologies used. One group had identified Tyr-1148 as the major SHC-binding site and Tyr-1173 as a less important site (33). Another group has suggested that Tyr-1173 and Tyr-992 are the main sites of interaction (4). These tyrosines have the following surrounding sequences: Tyr-1148 NPDYQQD, Tyr-1173 NAGYLRVA, and Tyr-992 ADEY LIPQ. Our data suggest that the reason for these disparate findings is that the former study analyzed the interaction with full-length SHC (33) whereas the latter study focused exclusively upon the SHC SH2 domain (4). Thus, the latter study did not observe any interaction with Tyr-1148 within the NPXY motif. This is consistent with our data, since Tyr-1148 would be predicted to bind to the SAIN motif of SHC but not the SH2 domain. In further support of this idea, the first study (33) showed that phosphopeptide competition of the Tyr-1148-mediated interaction required an intact NPXY motif, since a phosphotyrosine-containing peptide containing only the C-terminal four residues (from the Tyr) did not compete for EGF receptor interaction whereas a peptide extended to include the NPXY motif did compete. Neither Tyr-1173 nor Tyr-992 fulfills either the SH2 or SAIN domain-binding criteria, and their mode of interaction is unclear, although our results showing that mutation of the Pro in the IR NPXY motif reduces but does not eliminate SHC interaction, suggests that Tyr-1173 (NAGY) may also serve as a less efficient SAIN domain-binding site. Two other studies have suggested that mutation or deletion of all phosphorylated tyrosines in the EGF receptor showed phosphorylation of SHC and unimpaired mitogenic signaling (14, 46). The explanation for these different results is unclear. It appears that SHC can interact with the EGF receptor at a number of sites, via the SH2 or SAIN domain and perhaps via additional mechanisms.

As discussed above, many studies of SHC interaction with

receptors and other signaling molecules have focused on the SH2 domain and have not analyzed possible interaction with other SHC domains. This is especially true of in vitro studies in which purified SH2 domain-GST fusions are mixed with lysates from receptor-overexpressing cells. It is possible in these experiments that the SH2 domain interacts with a variety of phosphotyrosine-containing peptides of lower affinity, resulting in coprecipitation of nonphysiologically relevant proteins, especially since the SH2 domains are present in high concentrations. Second, many experiments rely on coimmunoprecipitation to assay interaction with SHC. Although SHC can be coprecipitated with the MT and the EGF and Trk receptors, the inability of a number of groups to demonstrate coprecipitation of SHC with the IR or with IRS-1 suggests that the interaction mediated by the SAIN domain may not always be amenable to the conditions required for coprecipitation (22, 38, 39, 60). Thus, these approaches may be consistently useful only for the study of SH2 domain interactions. Lastly, it should be noted that coprecipitation studies using cell lysates may lead to the identification of interactions between complexes of multiple proteins, and therefore coprecipitation is not necessarily an indication of a direct protein-protein interaction. In summary, the clear demonstration of the importance of the NPXY motifs within the EGF receptor, NGF receptor, c-*erbB-3* proto-oncogene product, and MT in regard to SHC signaling supports the physiological relevance of the SAIN domain in signal transduction via a wide variety of signaling molecules.

Another protein which may contain a SAIN domain is an IRS-1-like molecule termed 4PS, which is critical for mitogenic signaling by the interleukin-4 receptor (IL4R) (56, 57). This protein appears to be a distinct gene product which is structurally and functionally related to IRS-1. 4PS has been shown to interact with a region of the IL4R which contains an NPXY motif (Fig. 6), although the importance of tyrosine phosphorylation in this interaction is unclear (20). Since 4PS is phosphorylated by the IR and therefore presumably interacts with the IR in a manner similar to that of IRS-1, it will be interesting to compare the sequences of 4PS, IRS-1, and SHC in the regions which we propose to be important for interaction with the NPXY motif of the IR.

SHC, IRS-1, and the NPXY motif. We have presented evidence that SHC amino acids 1 to 238 are sufficient to mediate interaction of SHC with the IR in yeast cells and COS cells. This interaction therefore appears to have physiological relevance. The interaction has many characteristics of an SH2 domain-type interaction. For example, it does not occur if the IR has been rendered kinase inactive via mutation of the ATP-binding site, nor does it occur if Tyr-960 within the IR juxtamembrane domain has been mutated. Our finding that Tyr-960 is critical for SHC interaction in yeast cells is consistent with the results of Yonezawa et al. (60), who reported severely reduced tyrosine phosphorylation of SHC by a mutant IR containing a Tyr-960-to-Ala substitution. The simplest interpretation of these results is that autophosphorylation of Tyr-960 results in the formation of a direct binding site for SHC and IRS-1. This idea is supported by the demonstration that Asn-957 and Pro-958 are critical for IRS-1 and important (though not critical) for SHC interaction. Another explanation for our findings may be that these alanine substitutions result in loss of phosphorylation of Tyr-960 and thus show less interaction. We do not favor this possibility because SHC shows significant interaction with both the Asn and Pro mutant receptors, suggesting that Tyr-960 is phosphorylated. Second, as discussed more fully below, others have shown that Tyr-phosphorylated NPXY-containing peptides corresponding to the c-ErbB-3 protein in which Asn or Pro has been changed to alanine are unable to compete SHC interaction in vivo (37). This finding clearly shows that the Asn and Pro residues are important for efficient interaction of SHC with this receptor even in the context of a fully phosphorylated NPXY motif. Also, the interaction of SHC with MT antigen is severely reduced by mutation of the Pro to His without affecting Tyr phosphorylation within the NPXY motif (8). Thus, we favor the idea that the phosphorylated NPXY sequence forms part of a direct binding site for the SAIN domain. A schematic model of this interaction in comparison with the SH2 domain interaction is shown in Fig. 7A. Preliminary experiments (not shown) suggest that the SAIN domains of SHC and IRS-1 are much less soluble than SH2 domains when expressed as bacterial GST fusion proteins, and we have thus far been unable to examine in vitro interactions between these domains and the IR.

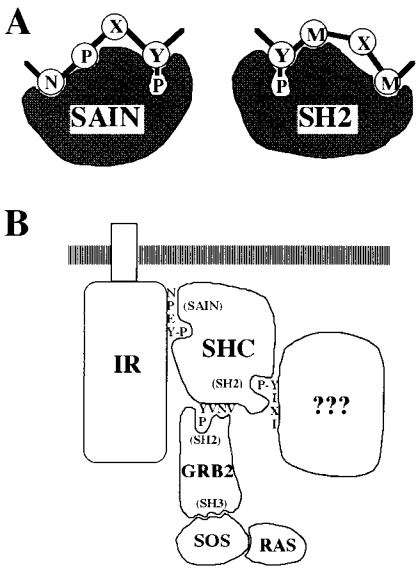


FIG. 7. Models of SAIN domain interaction with NPXY motifs and of IR signaling via SHC. (A) A theoretical schematic model of the SAIN domain as it might interact with a phosphotyrosine-containing peptide compared with an SH2 domain. The SH2 domain shown represents the p85 subunit of phosphatidylinositol 3-kinase, which prefers the YMXM motif. (B) A model of IR signaling via the SHC protein. In this model, insulin stimulates receptor autophosphorylation, which phosphorylates Tyr-960 within the juxtamembrane domain. Phosphorylation of this tyrosine results in the direct interaction with the SAIN domain of SHC encompassed by amino acids 1 to 238. This interaction may allow the proper presentation of SHC to the receptor kinase domain, resulting in phosphorylation of SHC on Tyr-317. This phosphotyrosine has been shown to interact with GRB-2 and to subsequently activate Ras via the guanine nucleotide release protein SOS. This model leaves the role of the SHC SH2 domain in IR signaling open for speculation.

Examination of the sequences surrounding the NPXY motif in proteins known to interact with SHC and/or IRS-1 reveals little conservation (Fig. 6). Yet, with the exception of the IR and IGFIR, the majority of the other signaling proteins have been reported to interact only with SHC or IRS-1/4PS. Conversely, the IL4R appears to interact with 4PS and IRS-1 but has not been reported to be a potent activator of SHC (58), suggesting that this sequence is not sufficient to interact efficiently with SHC. Conversely, a number of proteins which interact with SHC, including the Trk receptor, c-ErbB-3, and polyomavirus MT, have not been reported to interact with IRS-1-like molecules. This suggests that subtle differences between NPXY-containing signaling proteins which allow discrimination between IRS-1 and SHC must exist. It seems likely

that amino acids surrounding the NPXY motif will prove critical in the regulation of interactions between proteins which possess SAIN motifs.

A model for IR signaling via the SHC molecule. As shown schematically in Fig. 7B, the IR autophosphorylates Tyr-960 in a *trans* manner after insulin activation. This phosphorylation results in the formation of a direct binding site which is recognized by the SAIN domain of SHC. The resultant interaction of SHC with the IR presumably leads to the phosphorylation of Tyr-317 of SHC within the YXNX motif, which has been proposed to be the site of interaction with GRB-2. This interaction is thought to activate Ras and mediate at least some of the mitogenic effects attributed to SHC. This model leaves the role of the SHC SH2 domain open for speculation. It is possible that SHC is able to interact with other signaling proteins via the SH2 domain. In this regard, it has been shown that SHC is able to coprecipitate at least two other proteins. One is a 145- to 150-kD protein of unknown function (25, 26), and the second is a 100-kDa protein which appears to possess tyrosine kinase activity (39). Our model would predict that SHC has the potential to act as an adapter molecule which can serve to link a minimum of three distinct proteins together. Intrinsic to this sort of interaction lies a tremendous regulatory potential, both positive and negative.

Screening a cDNA library with the IR as bait also identifies SHC. We have recently begun to use the IR as bait to screen cDNA libraries to identify novel IR effector proteins. In our first screen of approximately 250,000 cDNAs, we were successful in the identification of 7 distinct cDNAs whose protein products interact specifically with the IR. Interestingly, one of these clones encoded a truncated SHC cDNA (data not shown). This SHC cDNA-containing plasmid appears to have undergone a recombination event such that only amino acids 26 to 245 were present in the hybrid protein. This observation is in clear support of our findings. Furthermore, the fact that SHC is one of very few cDNAs which were identified in this screen among a large population of cDNAs suggests that this interaction is not a result of a nonspecific interaction and is therefore likely to be of significance.

The SAIN domain as a potential phosphotyrosine-dependent binding domain. In addition to the well-characterized interaction of SH2 domains with phosphotyrosine-containing peptides, it is also clear that the protein tyrosine phosphatases (PTPases) interact directly with such peptides. The structures of a number of these PTPases have recently been presented (3, 50, 51). These structures are very different from that of SH2 domains. The sequences of these PTPases are quite distinct from one another, yet all are able to form similar binding pockets. The active sites of the PTPases have little in common with the phosphotyrosine-binding pocket of the SH2 domain. The only obvious common feature between these two motifs is that they both are relatively flat surfaces with an active site cleft which is able to fit the relatively large phosphotyrosine moiety. The SAIN domain is clearly not an SH2 domain and also is not homologous to the PTPases. We propose that the SAIN domain may represent a third motif which is able to specifically recognize phosphotyrosine-containing peptides. Considering the exquisite control of cellular functions which can be orchestrated by phosphorylation and dephosphorylation of tyrosine residues, it is perhaps not surprising that multiple distinct protein structures which can interact with phosphotyrosine-containing proteins have evolved.

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ADDENDUM

While this report was under review, Kavanaugh and Williams (18a) presented data showing that the SAIN domain of SHC interacts with a putative signaling molecule, pp145, in a phosphotyrosine-dependent manner. In addition, Blaikie et al. (5a) showed that the SAIN domain of SHC mediates the interaction with the EGF receptor as well as the TrkA and HER2/neu receptors. Both groups identified a region of SHC which corresponds almost exactly to the domain that we report here. As discussed herein, we predict that these interactions are most likely mediated by the NPXY motifs present in these receptors and that upon its cloning, pp145 will be also be found to contain such a motif.

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