Binding of Shc to the NPXY Motif Is Mediated by Its N-terminal Domain*

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Shc is an SH2-containing adapter protein that binds to and is phosphorylated by a large number of growth factor receptors. Phosphorylated Shc is able to interact with the Grb2-Sos complex which is responsible for mediating nucleotide exchange on Ras. We have shown previously that binding of Shc to the epidermal growth factor (EGF)-like receptor, c-ErbB-3, is through an NPXY motif (Prigent, S. A., and Gullick, W. J. (1994) EMBO J. 13, 2831-2841) shared by middle T antigen, TrkA, and EGF receptor. It has recently been reported that a region distinct from the SH2 domain is able to bind to tyrosine-phosphorylated proteins. In this paper we have used fusion proteins of various Shc domains to show that it is the N-terminal domain of Shc that is primarily responsible for binding EGF receptor and c-ErbB-3. Furthermore, by competition studies with synthetic phosphopeptides we have shown that this N-terminal domain binds to the previously identified NPXY motif.

The Shc gene product was isolated from cDNA libraries by screening with probes corresponding to SH2 domain sequences (1). It contains three distinct domains: a single SH2 domain in the C-terminal portion of the protein; a region with homology to collagen, which contains a tyrosine phosphorylation site at position 317 responsible for binding the adapter protein Grb2; and an N-terminal region of poorly defined structure and function. In contrast to Grb2, the Shc protein is transforming when expressed in NIH 3T3 fibroblasts. Association of Shc with Grb2 appears to be essential for this transforming ability since mutants lacking the Tyr-317 phosphorylation site lose their ability to induce neoplastic transformation (2). The Shc protein is ubiquitously expressed and is involved in the signaling pathways of many different classes of proteins. Tyrosine kinase receptors including those of the EGF receptor (3–5), insulin receptor (6–8), platelet-derived growth factor receptor (9), and nerve growth factor receptor families (10) utilize Shc as do non-receptor kinases such as v-Src, v-Fps, and p56LC (11–13). Other receptors that form complexes with Shc include the interleukin receptors (14–16), the B-cell antigen receptor (17, 18), the T-cell receptor (19), and the erythropoietin receptor (20). Recently it has been shown that Shc is phosphorylated in response to endothelin, which utilizes a heterotrimeric G-protein-coupled receptor (21). Shc has also been demonstrated to bind to middle T antigen and is involved in middle T antigen-induced transformation (22). Shc would therefore appear to be a highly versatile molecule involved in the function of diverse families of proteins.

We have previously identified the binding site for Shc on the type I growth factor receptor c-ErbB-3 as tyrosine 1309 (4). This tyrosine is contained in a motif Asn-Pro-X-Tyr (NPXY). Comparison of this site with the then reported Shc binding sites on TrkA and middle T antigen (4, 22, 23) led us to propose that this binding motif represents a consensus binding site for Shc (4). These studies were performed by generating NIH 3T3 cell lines expressing a chimeric EGF receptor/c-ErbB-3 protein, which contained the extracellular portion of EGF receptor linked to the intracellular domain of c-ErbB-3. Following activation by EGF, we were able to detect binding of Shc to the EGF receptor/c-ErbB-3 chimera, which could be inhibited by introducing a phosphopeptide corresponding to tyrosine 1309 of c-ErbB-3 into the cells by streptolysin O permeabilization.

The SH2 domain of Shc has been shown to bind directly to a number of receptors including the EGF receptor, platelet-derived growth factor receptor, and T-cell receptors but not to c-ErbB-3. The binding preference of the Shc SH2 domain has been mapped using a degenerate peptide library to the generalized motif phosphotyrosine-hydrophobic-X-hydrophobic (24). This consensus sequence is inconsistent with binding to the NPXY motif previously identified and as a result has generated confusion in the literature. In addition, for all previously identified SH2 interactions, the three residues C-terminal to the phosphotyrosine residue have been identified as the critical residues for binding. Very recently it has been reported that the N-terminal region of Shc contains a novel domain capable of binding phosphotyrosine residues (25, 26). It was therefore the objective of this study to determine whether the N-terminal domain of Shc is responsible for binding to the NPXY motif of c-ErbB-3 and EGF receptor.

MATERIALS AND METHODS

GST-Shc Fusion Proteins—GST-Shc constructs in the pGEX2T vector were as described previously (27) and are illustrated in Fig. 1. Fusion proteins were expressed in the protease-deficient Escherichia coli strain BL21 by induction with 0.1 mM isopropyl-1-thio-β-D-galacto-pyranoside overnight at 30°C. Bacteria were lysed using 1 mg/ml lysozyme in a buffer containing 50 mM Tris/HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 10 μg/ml each of the protease inhibitors leupeptin, aprotinin, and pepstatin, and fusion proteins were recovered by binding to glutathione-agarose. Following extensive wash-

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Lysis and Immunoblotting—Cell monolayers in 150-mm dishes were washed and incubated for 5 h at 37°C with 2% SDS, 100 mM NaF, 10 mM sodium pyrophosphate, 2 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 100 mM Na3VO4, 50 mM Tris/HCl, 150 mM NaCl, 0.1% Triton X-100, and 1 mM benzamidine. Lysates were then stripped for 1 h at 60°C with 2% SDS, 100 mM NaCl, 1% Triton X-100, and 50 mM Tris/HCl, pH 7.4, and analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting with antibodies specific for phosphotyrosine, EGFR, and c-ErbB-3.

RESULTS

Binding of EGF receptor and c-ErbB-3 to Shc N terminus—To determine which domain of Shc was primarily responsible for binding to the EGF receptor and c-ErbB-3, GST fusion proteins of Shc subdomains were used to precipitate receptors from EGF-stimulated NIH 3T3 cells expressing either EGF receptor or a c-ErbB-3 chimera. Binding of EGF receptor and c-ErbB-3 to Shc domains was detected using antibodies specific for phosphotyrosine, EGFR, and c-ErbB-3. Inhibition of binding to the NCH domain using antibodies specific for phosphotyrosine (αPY) and c-ErbB-3 (49.3) and c-ErbB-3 could only be detected bound to the NCH fusion protein using antibodies specific for phosphotyrosine (αPY) and c-ErbB-3 (49.3) and could not be detected in association with the SH2 domain or the collagen homology region. Phosphorylated EGFR receptor also bound predominantly to the fusion protein containing the N terminus of Shc. A small amount of binding to the SH2 domain could be detected with the anti-phosphotyrosine antibody and with the EGFR receptor antibody (12E). EGFR receptor binding to the SH2 domain was only seen in the EGF-stimulated lane indicating that it is a weak but phosphorylation-dependent association (Fig. 2). A very faint EGFR receptor band was visualized bound to the collagen homology domain following prolonged exposure to autoradiography film (PY3), which is probably a nonspecific interaction due to the large amount of EGFR receptor present in the cell lysate. This band was not seen with the EGFR receptor antibody due to the reduced sensitivity of this antibody relative to the anti-phosphotyrosine antibody.

Inhibition of Binding to N-terminal Domain Using Synthetic Phosphopeptides—Since the major Shc binding site on EGFr and c-ErbB-3 is contained within an NPXY motif, we used a synthetic phosphopeptide corresponding to this region (peptide 5) to determine whether receptor binding to the N terminus of Shc was mediated through this site. Precipitates from EGF-stimulated cells were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting with an anti-phosphotyrosine antibody. Complex formation between the NCH fusion protein and EGF receptor or the EGFr/c-ErbB-3 chimera was completely abolished by the presence of a synthetic peptide containing the NPXY sequence (Fig. 3A). In contrast, when pre-
concentration of 30 of the peptides described were completely reproducible. At a...circulation was performed in the presence of a tyrosine phosphopeptide containing an unrelated sequence encoding a p85 binding site (peptide 4), no reduction in binding was observed. Binding of endogenous EGF receptor in the parental NIH 3T3 cells was also eliminated by the presence of peptide 5.

To further assess the specificity of the interaction between the Shc N-terminal domain and the NPXY motif of EGF receptor and EGF receptor/c-ErbB-3, precipitations were performed in the presence of two concentrations of peptide 5 and in the presence of two additional peptides 11 and 12 in which the proline and asparagine residues of the NPXY motif are replaced by alanine. To control for any experimental variation in the amount of protein analyzed, immunoblots were probed with an antibody recognizing the glutathione S-transferase portion of the fusion proteins used. The results illustrated in Fig. 3B are representative of a number of experiments in which the inhibitory effects of the peptides described were completely reproducible. At a concentration of 30 µM, peptide 5 completely inhibited binding of the NCH protein to EGF receptor and EGF receptor/c-ErbB-3 and significantly reduced binding to both receptors at a concentration of 3 µM. Replacement of the proline residue by an alanine residue abolished the inhibitory effect of this peptide (peptide 11). Peptide 12, in which the asparagine residue is replaced by alanine, was able to partially inhibit binding of EGF receptor and EGF receptor/c-ErbB-3 to the NCH protein at a high concentration (30 µM) but had little or no effect at lower concentration. The inhibition obtained with peptide 12 at a 30 µM concentration was comparable with that seen with peptide 5 at a 10-fold lower concentration. None of these peptides had any effect on the EGF receptor/SH2 domain interaction. An apparent reduction in the EGF receptor binding to the SH2 domain in the presence of 3 µM peptide 12 reflects a decrease in protein loaded in this case.

DISCUSSION

There is an increasing body of evidence implicating Shc in the mechanisms responsible for Ras activation; however, its precise mechanism of action remains unclear. It appears that the complexes formed following growth factor stimulation depend on the growth factor in question (14, 27, 31). The demonstration recently that the N-terminal domain of Shc is able to interact with phosphorylated tyrosine residues in addition to its SH2 domain has provided some insight into the mechanisms by which Shc could recruit different subsets of proteins with potentially different consequences depending on the cellular environment (25).

In this paper we show that the N-terminal region of Shc binds to EGF receptor and to c-ErbB-3 and that it binds to the NPXY motif first defined by us (4). Various Shc GST-fusion proteins were examined for their ability to precipitate EGF receptor and EGF receptor/c-ErbB-3. In addition to the fusion proteins described, we attempted to express a shorter fragment of the N-terminal domain (amino acids 1–238) in bacteria but were unable to obtain material of sufficient quality to use in our experiments. The construct encompassing the N-terminal domain and the collagen homology region combined (NCH) was more readily expressed and produced a major product of the appropriate molecular weight on Coomassie-stained gels and GST immunoblots. We therefore chose to use the NCH protein for these studies and to perform precipitations in parallel with the CH domain, lacking the N terminus for comparison. Only the protein containing the N terminus (NCH) was able to precipitate EGF receptor/c-ErbB-3, and this interaction was completely abolished by the presence of an excess of phosphopeptide containing the c-ErbB-3 sequence spanning the NPXY motif. While the SH2 domain of Shc specifically precipitated a small amount of EGF receptor, the most striking interaction of EGF receptor was seen with the fusion protein containing the N terminus of Shc. As was the case for the EGF receptor/c-ErbB-3 chimera this interaction was completely abolished by competition with an NPXY-containing peptide. Thus we have clearly demonstrated that it is the N terminus of Shc that is primarily responsible for its interaction with EGF receptor and c-ErbB-3. The NPXY peptide had no effect on binding of EGF receptor to the Shc SH2 domain providing further evidence that this binding occurs through a different site. In addition we have shown that the most critical residue in the NPXY motif is the proline in the −2 position since alteration of this amino acid abolished the inhibitory effect of the peptide on receptor/Shc-NCH binding. A peptide in which the asparagine was changed to alanine still retained some inhibitory property at high concentration indicating that some variability in this residue may be permitted. Although these studies were performed in vitro, this result should help to clarify the current confusion in the literature regarding the EGF receptor binding sites for Shc and the role of the Shc SH2 domain.
While one study (3) using mutant EGF receptors reported the major binding site for Shc to be Tyr-1148 (NPXYQDQ), another study (32) using the Shc-SH2 domain reported tyrosines 1173 (NAGYLRVA) and 992 (ADEYLIPQ) to be the most important. Our data would support the notion that Tyr-1148, contained in (NAGYLRVA) and 992 (ADEYLIPQ) to be the most important. While other interactions between the SH2 domain of Shc and different sites on the same or a different receptor may have important functional consequences. Using the yeast two-hybrid system, Gustafson et al. (33) have demonstrated that Tyr-960 of the human insulin receptor, also contained in an NPXY sequence, is also able to bind to residues 1-238 of Shc in a phosphorylation-dependent manner. Similar results have been obtained for Tyr-950 of the IGF-1 receptor, which is also within an NPXY sequence (34). Interestingly, the same sites on the insulin receptor and IGF-1 receptor are also able to bind to IRS1 (35). While one study (3) using mutant EGF receptors reported the major binding site for Shc to be Tyr-1148 (NPDYQQD), another study (32) using the Shc-SH2 domain reported tyrosines 1173 (NAGYLRVA) and 992 (ADEYLIPQ) to be the most important. Our data would support the notion that Tyr-1148, contained in (NAGYLRVA) and 992 (ADEYLIPQ) to be the most important.