Identification of Residues within the SHC Phosphotyrosine Binding/Phosphotyrosine Interaction Domain Crucial for Phosphopeptide Interaction*  

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Shc is an Src homology 2 (SH2) domain protein thought to be an important component of the signaling pathway leading from cell surface receptors to Ras. A new phosphotyrosine interaction (PI) domain (also known as the phosphotyrosine binding (PTB) domain) has been described in the amino terminus of Shc. The Shc PI domain binding specificity is dependent on residues lying amino-terminal to the phosphotyrosine rather than carboxy-terminal as is seen with SH2 domains. We randomly mutagenized the Shc PTB/PI domain in an effort to identify residues in the domain crucial for interaction with phosphotyrosine-containing peptides. We then screened the mutants for binding to the tyrosine-phosphorylated carboxy-terminal tail of the epidermal growth factor (EGF) receptor. Most striking were mutations that altered a phenylalanine residue in block 4 of the domain severely impairing PI domain function. This phenylalanine residue is conserved in all but one subfamily of PI domains that have been identified to date. Reconstitution of this phenylalanine mutation into full-length Shc created a protein unable to interact with the EGF receptor in living cells.

**EXPERIMENTAL PROCEDURES**

Random Mutagenesis of Shc Amino Acids 76–209—Using the oligonucleotides (forward 5′CCG GAA TTC ATG AAC AAG CTG AGT GGA3′ and reverse 5′CCG CTC GAG TCA CAG GTC CTC CTC GCT CTT GAT CAG CTT CTG CTC CTG CAG ATT CCT GAG ATC ATA CTG TTT GAAA3′) and plasmid Shc 1–209/pGStag as a template (4), standard PCR was used to create a Shc 1–209 construct containing an EcoRI site on the 5′-end and a PsiI site ifollowed by an in-frame Myc epitope tag, stop codon, and an XhoI site on the 3′-end (Fig. 1). This fragment was then cloned into the pTOPE vector (Novatope System, Novagen) using the EcoRI and XhoI restriction sites to create Shc 1–209/Myc pTOPE. The Shc 1–209 insert in pTOPE was then mutagenized by PCR (17) using pTOPE forward and reverse sequencing primers. The product of the PCR reaction (containing the mutagenized PI domain) was digested with BseEI and PsiI restriction enzymes and ligated into Shc 1–209/Myc pTOPE (Fig. 1A). The ligation was transformed into competent Novablue (DE3, Novagen) Escherichia coli strain and selected on carbenicillin/tetracycline agar plates.

**Screening of Mutants**—Clones containing the mutagenized PI domain were plated onto 10-cm plates, and approximately 1500 independent recombinants were screened. Colonies were lifted onto nitrocellulose and lysed in situ according to the manufacturer’s instruction for the Novatope system. Probing nitrocellulose filters with radiolabeled EGF receptor was performed as described previously (10). After probing with EGF receptor and detection of binding with autoradiography, the filters were then screened with an anti-Myc monoclonal antibody, 9E10 (18), using a biotin-avidin-linked alkaline phosphatase system (Vectastain, Vector Laboratories) as the readout. Clones that did not bind to the EGF receptor yet were positive for Myc antibody binding were selected for sequencing (Sequenase version 2, U. S. Biochemical Corp.).

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†The abbreviations used are: SH2, Src homology 2; EGF, epidermal growth factor; GST, glutathione S-transferase; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PI, phosphotyrosine interaction; PTB, phosphotyrosine binding.
clones with single mutations were selected for further analysis.

Dot Blot Analysis of Mutants—The selected Shc 1–209 mutants were cloned into the GST fusion protein expression vector, pGEX 4T-1, using EcoRI and XhoI. GST fusion proteins were expressed and purified as described previously (19). Eluted fusion proteins were quantitated using both the Bio-Rad protein detection kit and SDS-PAGE alongside BSA standards. Serial dilutions of 200, 100, and 50 ng of fusion protein were spotted onto duplicate nitrocellulose filters. After blocking the filters, they were probed with the 32P-labeled EGF receptor (EGFR), washed, and exposed to film. After autoradiography, the filters were reprobed with an anti-Myc antibody using colorimetric detection. The autoradiograms were then aligned with the Myc antibody-stained filters. The panel on the right shows screening for Myc tag expression using monoclonal antibody 9E10 against the Myc epitope. The panel on the left shows binding of labeled EGF receptor to the same filter. The arrow depicts a colony selected for further analysis with normal binding of the Myc antibody but defective EGF receptor binding.

RESULTS AND DISCUSSION

We subcloned the cDNA encoding the amino terminus of murine p52 Shc (Fig. 1A) into the pTOPE vector allowing expression in bacteria expressing T7 polymerase. We added a

**Fig. 2. Identification and characterization of PI domain mutants.** A, binding of 32P-labeled EGF receptor to PI domain mutants. PI domain mutants identified using the method in Fig. 1 were subcloned into pGEX-4T-1 vector. GST fusion proteins were prepared as described under “Experimental Procedures” with 200, 100, and 50 ng spotted on nitrocellulose. The wild type (WT) and mutant PI domains were then probed with 32P-labeled EGF receptor. For quantitation, squares were cut from the filters and Cerenkov counts determined. Duplicate filters spotted with GST fusion protein and probed with anti-Myc were utilized to ensure comparable amounts of protein were spotted for each protein (results not shown). B, sites of PI domain mutations. The PI domain mutations isolated by random mutagenesis are listed above the wild type Shc sequence. The numbers in parentheses represent the binding of GST fusion proteins containing these mutations to the EGF receptor as a percent of wild type control. The numbers represent an average of the binding to 100 and 200 ng of GST fusion protein. C, effects of PI and SH2 domain mutagenesis on binding to EGF receptor (EGFR). GST fusion proteins (3 μg of Shc PI domain; 10 μg of Shc SH2 domain) were immobilized on glutathione beads, and EGF-stimulated HER14 cell lysate was added (1.5 mg of protein). After incubation for 90 min at 4 °C, the beads were washed with radiimmune precipitation buffer and the bound proteins separated by SDS-gel. After transferring to nitrocellulose, the bound EGFR receptor was detected by immunoblotting with anti-phosphotyrosine antibodies. As a control, lysate (100 μg) was run directly on the gel (far right lanes). It should be noted that although the binding of the wild type SH2 and PI domain (PID) appear equivalent, three times as much SH2 domain was used.

0.1% Triton X-100) and boiled in 1 × sample buffer. The bound proteins were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-Myc and anti-phosphotyrosine (19). Blots were visualized using chemiluminescence (Renaissance, DuPont NEN).

**Panel 1**

**Panel 2**

**Panel 3**
Myc epitope in-frame at amino acid 209 that contained a unique XhoI site at its carboxy terminus and introduced a PstI site between the Myc epitope and the PI domain. We then created a library of PI domains with mutations running from amino acid 76 to 209 of p52 Shc but excluding the Myc epitope as described under “Experimental Procedures.”

Colonies expressing the library of PI domain mutants were lifted onto nitrocellulose and screened for EGF receptor binding and Myc epitope expression. Colonies selected for further study were subjected to DNA sequencing. Several of the mutants that had more than one mutation were not further analyzed. The binding of some of the PI domains with single point mutations and binding less than 50% of control are displayed in Fig. 2A while the sites of the mutations are shown in Fig. 2B. The binding percentage compared with wild type, displayed in Fig. 2B, represents an average of the binding to 100 and 200 ng of GST fusion protein.

Also displayed in Fig. 2A is the binding of two mutations that were created based on the previously published PI domain alignment (16). In this alignment two absolutely conserved residues were identified, a glycine at residue 58 and a serine at residue 151 of p52 Shc. The S151A mutation had impaired binding and mapped to a region of Shc where several of the random mutants were located. This mutation resulted in a binding approximately 1.0% of wild type. No binding was detected in the G58A mutation. However, a large fraction of this protein became insoluble when expressed in bacteria, suggesting that this mutation adversely affected the conformation of the protein.

The effects of two of the phenylalanine mutations on PI domain binding are shown using a different assay in Fig. 2C (left panel). In this assay, the GST fusion proteins were immobilized on glutathione-agarose, and cell lysates containing tyrosine-phosphorylated EGF receptor were added. Both of the MHC epitope -in-frame at amino acid 209 that contained a unique XhoI site at its carboxy terminus and introduced a PstI site between the MHC epitope and the PI domain. We then created a library of PI domains with mutations running from amino acid 76 to 209 of p52 Shc but excluding the MHC epitope as described under “Experimental Procedures.”

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The effects of two of the phenylalanine mutations on PI domain binding are shown using a different assay in Fig. 2C (left panel). In this assay, the GST fusion proteins were immobilized on glutathione-agarose, and cell lysates containing tyrosine-phosphorylated EGF receptor were added. Both of the phenylalanine mutations, F198V and F152L, markedly reduced the PI domain binding in agreement with the results shown in Fig. 2A. Shown for comparison in the right panel of Fig. 2C is the extent of mutating arginine 397 of the Shc SH2 domain to lysine. This is an arginine within the SH2 domain that is crucial for phosphotyrosine interaction. As expected this mutation completely abolished the ability of the Shc SH2 domain to bind to the EGF receptor.

The alignment presented in Fig. 3 maps the mutations we have generated to the conserved blocks found in PI domains (16). The blocks were confirmed by another round of sequence data base searches in which we were able to retrieve 10 more members of the PI domain family. Among them are the partial sequences of two new proteins, Est2 and FE6L, that have been identified in this work. FE6L (for FE65-like protein) belongs to the FE65 subfamily while Est2 appears to be yet another divergent member of the PI domain family. The other 8 sequences are probably orthologues of existing PI domain containing proteins which, nevertheless, indicate conserved regions in the alignment. The relationship of the PTB domains of IRS-1 and IRS-2 to our PI domain alignment is not clear (6, 22). We have not been able to include them without severely impairing the quality of the PI domain alignment.

Some of the mutations we identified mapped to relatively conserved residues while others did not. The mutations affecting phenylalanine at residue 198 were the most striking because they had the greatest effect on the binding of the Shc PI domain. This phenylalanine is conserved in all PI domains except those of the FE65 subfamily where it is replaced by a cysteine. The fact that mutagenesis of the conserved residues, Phe-198 and Ser-151, severely impairs phosphopeptide binding lends strong support to the validity of PI domain sequence alignment. The mutation of residues analogous to the Phe-198 and Ser-151 in other PI domains could lead to proteins with impaired PI domain function. The finding that one subfamily of PI domains, the FE65-like proteins, has cysteine instead of phenylalanine at the position equivalent to Phe-198 of Shc points to a possible diversification of PI domain binding specificity. Thus it is conceivable that the PI domain represents more of a general protein interaction domain, one subtype of which is involved in phosphotyrosine binding.
The PI domain, not the SH2 domain, is known to be crucial for the interaction of Shc with the nerve growth factor receptor, TrkA (11, 12). However, the relative roles of these domains in Shc interactions with EGF receptor are less clear because both the Shc SH2 and PI domains can bind the EGF receptor in living cells. We proceeded to reconstitute the PI domain and SH2 domain point mutations we had created into full-length Shc to analyze the role of these domains in the interaction of Shc with tyrosine-phosphorylated EGF receptor in living cells. We performed these studies by transiently transflecting four forms of Myc-tagged Shc into HeLa cells. These forms were wild type Shc, R397K Shc, F198V Shc, and a double mutant, F198V/R397K. The expressions of all these forms of Shc were equivalent as assessed by blotting cell lysates with anti-Myc antibody (Fig. 4, upper left panel). By immunoprecipitating with anti-Myc antibody we could analyze the associations of the wild type and mutant forms of Shc with endogenous EGF receptor present in HeLa cells. We found that while mutations in the SH2 domain had only minor effects on binding, mutations in the PI domain severely impaired binding (lower panel). Despite the abilty of PI domain mutations to interfere with Shc binding, we saw no significant effect of these mutations on EGF-induced Shc tyrosine phosphorylation (upper right panel). This is not surprising as there have been several previous studies demonstrating that Shc does not need to bind to EGF receptor to become tyrosine-phosphorylated (23–26). It may be that a low affinity interaction between Shc and the EGF receptor is all that is required for Shc tyrosine phosphorylation or that a separate EGF-responsive kinase can also phosphorylate Shc. Regardless, these results confirmed that the F198V mutation abrogates PI domain function in living cells and points to the important role PI domains play in Shc interactions with growth factor receptors. The isolation of these PI domain mutation will be an important tool to understand the role of this domain in Shc signaling as well as the role of the PI domain in other proteins.

Note Added in Proof—While this manuscript was in press, the structure of the PI/PTB domain bound to a phosphopeptide was published (Zhou, M.-M., Ravichandran, K. S., Olejniczak, E. T., Petros, A. M., Meadows, T. A., Pant, M. S., Satller, M., Harlan, J. E., Wade, W. S., Burakoff, S. J., and Fesik, S. W. (1995) Nature 378, 584–592). This structure indicates that Phe-198 is a crucial residue making direct contact with the phosphopeptide at residues 3 and 5 to the phosphotyrosine.

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Fig. 4. Mutagenesis of the PI domain blocks the interaction of Shc with EGF receptor in living cells. The mutations F198V and R397K either separately or together were reconstituted into full-length murine Shc containing a Myc tag epitope at its carboxyl terminus. Wild type and mutant Shc constructs were then transiently transfected into HeLa cells. Forty-eight hours after transfaction the HeLa cells were stimulated with EGF (250 ng/ml) for 5 min. Cells were then lysed in 500 μl of a 1% Triton-based lysis buffer containing protease and phosphatase inhibitors. The majority of the lysate (400 μl) was subjected to immunoprecipitation with anti-Myc antibody. After washing with HNTG, the immunoprecipitates (IP) were run on an SDS gel and transferred to nitrocellulose. The high molecular weight portion of the nitrocellulose was then blotted with anti-phosphotyrosine to detect EGF receptor while the lower molecular weights were probed with anti-Myc (lower panel). Lysate (20 μl) was also run directly on an SDS gel, transferred to nitrocellulose, and blotted with anti-Myc or anti-phosphotyrosine (Anti-PTyr, upper panels).
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