Phosphotyrosine-independent Binding of SHC to the NPLH Sequence of Murine Protein-tyrosine Phosphatase-PEST

EVIDENCE FOR EXTENDED PHOSPHOTYROSINE BINDING/PHOSPHOTYROSINE INTERACTION DOMAIN RECOGNITION SPECIFICITY

(Received for publication, October 26, 1995, and in revised form, January 16, 1996)

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The phosphotyrosine binding (PTB) or phosphotyrosine interaction (PI) domain of the proto-oncoprotein p52SHC binds to an NPXY consensus sequence found in several growth factor receptors (Kavanaugh, W. M., Turck, C. W., and Williams, L. T. (1994) Science 268, 1177-1179). The amino-terminal region of p52SHC, which includes the PTB/PI domain, has been previously shown to associate with protein-tyrosine phosphatase-PEST (PTP-PEST) in vivo (Habib, T., Herrera, R., and Decker, S. J. (1994) J. Biol. Chem. 269, 25243-25246). We report here the detailed mapping of this interaction in a murine context using glutathione S-transferase fusion protein binding studies and peptide competition assays. We show that the interaction between murine SHC and murine PTP-PEST is mediated through the PTB/PI domain of murine SHC and an NPLH sequence found in the carboxyl terminus of murine PTP-PEST. Since this interaction is not dependent on the presence of a tyrosine-phosphorylated residue in the target sequence, this reveals that the PTB/PI domain of SHC can recognize both tyrosine-phosphorylated sequences and non-tyrosine-based recognition motifs.

External stimuli are often transduced into intracellular events via specific cascades of protein tyrosine phosphorylation and dephosphorylation, which are modulated by the presence and availability of adaptor molecules, protein-tyrosine kinases, and PTPases. The cytoplasmic adaptor molecule SHC is among many mediators that act downstream of receptor tyrosine and cytoplasmic protein-tyrosine kinases. SHC can transform fibroblasts and differentiate PC12 cells in a Ras-dependent manner. SHC regulates these signaling events through its own tyrosine phosphorylation on residue Tyr137 and by mediating the assembly of tyrosine-phosphorylated signaling complexes via its SH2 and phosphotyrosine binding/ phosphotyrosine interaction (PTB/PI) domains (1-3). The p52SHC NH₂-terminal PTB/PI domain is a novel phosphotyrosine recognition motif that is structurally unrelated to SH2 domains and that was shown to bind with high affinity to the autophasphorylation sites of c-Erb B2 Tyr1227 (4, 5), TrkA Tyr499 (6-8), EGF receptor Tyr1148 (6, 7, 9, 10), c-Erb B3 Tyr1309 (9), insulin-like growth factor 1 receptor Tyr950 (11), insulin receptor Tyr960 (12), and the phosphorylated Tyr250 residue of polyoma middle T antigen (13). Peptide competition assays and screening of phosphotyrosine peptide libraries have demonstrated that the PTB/PI domain of p52SHC preferentially binds to the sequence NPXY (where X represents any amino acid and Y indicates a phosphotyrosine residue) with high affinity (5, 9, 10, 13, 14). The PTB/PI domain represents a novel mechanism whereby signaling proteins can interact with tyrosine-phosphorylated protein targets.

MPTP-PEST is a ubiquitously expressed, stable, cytosolic PTPase of 112 kDa that is characterized by the presence of four Pro, Glu, Ser, and Thr-rich PEST domains within the COOH terminus (15). MPTP-PEST is heavily phosphorylated on serine and threonine residues, and the enzymatic activity for the human homologue can be modulated by phosphorylation on specific serine residues (16). Another possible mode of regulation by which the activities of protein-tyrosine phosphatases might be controlled is via association with other proteins (for review see Ref. 17). Recently, it has been demonstrated that the human PTP-PEST protein interacts with the human p52SHC protein in vivo (18). In this report we demonstrate that the murine p52SHC protein binds to MPTP-PEST via its PTB/PI domain and that this binding is not dependent on the presence of a phosphotyrosine residue in the target sequence.

MATERIALS AND METHODS

MPTP-PEST and mSHC GST Fusion Proteins—GST fusion proteins were constructed using standard recombinant DNA technology. The constructs created by polymerase chain reaction were sequenced using Sequenase version 2.0 (Amersham Corp.).

Cell Culture, Immunoprecipitation, and Immunoblotting—NIH 3T3 and COS-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics. Transient transfection of COS-1 cells with hp52SHC cDNA and HA-MPTP-PEST DNAs was achieved by electroporation as described previously (15). Cell lysate preparations and immunoprecipitation experiments were performed according to previously published protocols (19). Immunoblotting on polyvinylidene difluoride membranes (Millipore) using antibodies described below and horseradish peroxidase-conjugated secondary antibodies was performed with chemiluminescence reagents (DuPont NEN or Amersham) according to the manufacturer's protocol.

§ This work was supported by operating grants from the Medical Research Council (MRC) of Canada (to C. J. M.) and from the National Cancer Institute of Canada (NCIC) and MRC of Canada (to M. L. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: PTPase, protein-tyrosine phosphatase; SH2, Src homology 2; EGF, epidermal growth factor; EGFR, EGF receptor; GST, glutathione S-transferase; PTB/PI domain, phosphotyrosine-binding/phosphotyrosine interaction domain; HA, hemagglutinin antigen; PTP-PEST, protein-tyrosine phosphatase-PEST; MPTP-PEST, murine PTP-PEST; mSHC, murine SHC.
Antibodies—The anti-SHC antibodies used in this study were a monoclonal antibody, S14620 (Transduction Laboratories), directed against residues 359–473 of human SHC, and a rabbit polyclonal antibody raised against a GST-SH2 domain fusion protein of human p52SHC (1). The anti-MPTP-PEST polyclonal antibody and the anti-HA tag monoclonal antibody 12CA5 have been described elsewhere (15). These antibodies were used for immunoprecipitation and immunoblotting procedures as described in the figure legends.

Binding Studies—The GST fusion proteins were expressed in bacteria and affinity-purified on glutathione-Sepharose beads (Pharmacia Biotech Inc.) according to published protocols (20). Protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad) according to the manufacturer's protocol. Peptide concentrations were determined spectrophotometrically at \(A_{205}\) (21). Human SHC or HA-MPTP-PEST-transfected COS-1 cell lysates were incubated with the appropriate GST fusion proteins bound to glutathione-Sepharose beads for 90 min at 4°C in HNMEM buffer (19) supplemented with protease and phosphatase inhibitors. The beads were then washed 4 times in HNTG buffer (19), separated by SDS-polyacrylamide gel electrophoresis, and immunoblotted with the appropriate antibody as described above. Peptide competition studies were performed as described above except that the peptides were preincubated with the GST fusion proteins for 30 min at 4°C prior to the addition of cell lysates.

RESULTS

Murine p52SHC Binds to MPTP-PEST in Vivo—A recent report demonstrated the in vivo interaction between the NH\(_2\) terminus of the human p52SHC protein with the COOH terminus of the human PTP-PEST protein using a yeast two-hybrid system and co-immunoprecipitation studies (18). We sought to determine if this interaction also exists in a murine context since the COOH termini of human and murine PTP-PEST enzymes display weak (74%) sequence identity relative to the highly conserved NH\(_2\)-terminal catalytic domains (98% identity).

Using an affinity-purified antibody that recognizes SHC proteins, we immunoprecipitated endogenous mSHC from NIH 3T3 cells under conditions where protein complexes have been shown to co-immunoprecipitate (19). The anti-mSHC immunoprecipitates were screened for the presence of endogenous MPTP-PEST by Western blotting using an anti-MPTP-PEST antibody (Fig. 1). An immunoactive band of 112 kDa (corresponding to the electrophoretic size of MPTP-PEST) is present in the anti-mSHC immunoprecipitates. In addition, screenings of a mouse embryonic cDNA library in a yeast two-hybrid system using p52SHC as a probe resulted in the isolation of cDNA clones coding for MPTP-PEST (data not shown). These results indicate that SHC proteins interact with MPTP-PEST in vivo.

SHC Binds to MPTP-PEST via Its NH\(_2\)-terminal PTB/PI Domain—In order to delineate the minimal binding require-
ments that are involved in the interaction between mSHC and MPTP-PEST, GST-mSHC fusion protein constructs were used in an in vitro binding assay. Using these GST fusion proteins representing different regions of mSHC (Fig. 2, A and B), we identified the portion of mSHC that binds to HA-MPTP-PEST (Fig. 2C). The segment of mSHC that lies between amino acids 47 and 209 represents the minimal region required for binding to HA-MPTP-PEST in vitro. Since the boundaries of this region in p52SHC coincide with those of the recently characterized PTB/PI domain (4, 6, 7), we used a mutated mSHC PTB/PI domain GST fusion protein that is defective in binding tyrosine-phosphorylated residues (6) in order to determine if the binding of p52SHC to MPTP-PEST is mediated through the PTB/PI domain. As shown in Fig. 2C, the mutated mSHC PTB/PI domain GST fusion protein does not bind to HA-MPTP-PEST (compare the lanes labeled GST-Shc-1–209 with GST-Shc-1–209D107–116). These results represent evidence that the PTB/PI domain of p52SHC interacts with MPTP-PEST.

The PTB/PI Domain of SHC Binds to MPTP-PEST via an NPLH Sequence—It has been previously shown that the binding of human SHC to the COOH terminus of human PTP-PEST occurs through a region situated between amino acids 416 and 775 of human PTP-PEST (18). We found that the mSHC/MPTP-PEST interaction is mediated by the PTB/PI domain of mSHC, a domain that was shown to be dependent on the presence of a phosphotyrosine residue on the target sequence. However, since no tyrosine residues are present in the equivalent sequence of MPTP-PEST, this interaction raises the possibility of a novel, tyrosine-independent target sequence binding.

Using a series of GST-MPTP-PEST fusion proteins representing different portions of the MPTP-PEST COOH terminus (Fig. 3, A and B), the region of MPTP-PEST required for binding to p52SHC was determined by an in vitro binding assay. The segment located between amino acids 576 and 613 of MPTP-PEST is sufficient for binding to p52SHC (Fig. 3C). Interestingly, this region contains a sequence (599NPLH602) that closely resembles the p52SHC PTB/PI domain binding consensus motif (NPXpY). In order to verify if the 599NPLH602 sequence of MPTP-PEST is involved in the interaction with mSHC, a triple point mutant (N599I, P600A, H602L) was created in the GST-MPTP-PEST-471–613 fusion protein background (Fig. 3, A–C, lanes labeled NPXHmut) and analyzed for binding to p52SHC in vitro. By mutating all three amino acids (N599I, P600A, and H602L), the binding of GST-MPTP-PEST-471–613 fusion protein to p52SHC was completely abolished (Fig. 3C). These results indicate that the sequence NPXH, found in the COOH terminus of MPTP-PEST, is required for binding to SHC in vitro.

The same mutations (N599I, P600A, and H602L) were recreated in a full-length HA-MPTP-PEST protein, and the effects of these substitutions were studied in vivo. Immunoprecipitation of SHC proteins in COS-1 cells transfected with either wild type or NPXH mutant HA-MPTP-PEST cDNAs were probed for the presence of associated HA-MPTP-PEST molecules by Western blot analysis (Fig. 4). An immunoreactive band of 120 kDa corresponding to HA-MPTP-PEST co-immunoprecipitated with SHC in cells transfected with the wild type HA-MPTP-PEST cDNA but not in the NPXH mutant cDNA transfectants despite an equal amount of both HA-MPTP-PEST proteins present in the lysates (Fig. 4B). This demonstrates that mutating all three amino acids (N599I, P600A, and H602L) in the context of a full-length HA-MPTP-PEST protein eliminates binding to SHC proteins in vivo.

Residues Asn599 and Pro600 of the 599NPLH602 Sequence of MPTP-PEST Are Essential for Binding in Vitro—In order to
determine which residues within the NPX sequence are necessary for binding, we studied the ability of synthetic peptides (corresponding to the amino acid sequence of MPTP-PEST located between residues 594 and 607) to compete for binding of the mSHC PTB/PI domain to full-length HA-MPTP-PEST in vitro. Tyrosine-phosphorylated and nonphosphorylated peptides corresponding to a previously characterized SHC PTB/PI domain recognition site on the EGF receptor (10) were used as controls in the same assay. The phosphorylated EGF receptor peptide (EGFR-P-1148) is capable of competing with full-length HA-MPTP-PEST for binding to the PTB/PI domain of mSHC (Fig. 5) at concentrations (between 50 and 500 nM) similar to those reported in other systems (10). Accordingly, its nonphosphorylated counterpart (EGFR-1148) is inefficient at competing with HA-MPTP-PEST for binding. A peptide representing the MPTP-PEST wild type sequence (PEST-WT) competes but at concentrations that are intermediate to those of the phosphorylated and nonphosphorylated EGF peptides (Fig. 5).

Similar peptide competition assays (10, 13) and phosphopeptide library screenings (14) have previously demonstrated that the 23Asn, 22Pro, and the phosphorylated tyrosine residue in the NPXpY motif are essential for SHC PTB/PI domain interaction with the autophosphorylated sites of growth factor receptors. To determine the contribution of these residues in the NPXH-mediated binding of SHC to MPTP-PEST, mutant peptides were used in the same in vitro competition binding assays. An MPTP-PEST mutant peptide bearing a N599D substitution is incapable of competition (Fig. 5). A peptide with a P600G mutation is also inefficient at competing for binding even at the highest concentration (Fig. 5). This suggests that both the Asn599 and Pro600 residues are involved in the binding of the wild type peptide. Finally, a peptide with a scrambled amino acid sequence is incapable of competing for binding. These results indirectly demonstrate that the binding of the PTB/PI domain of SHC to the NPLH602 sequence of MPTP-PEST in vitro is dependent on both the Asn599 and Pro600 amino acid residues.

Residue His602 Is Necessary for Binding in Vivo—The affinities with which the WT and H602A MPTP-PEST peptides bind to the PTB/PI domain of SHC in vitro are intermediate to those
displayed by the phosphorylated and nonphosphorylated EGF peptides (Fig. 5). This difference could represent inherent variations in the affinities of the SHC PTB/PI domain for phosphorylated EGF receptor and MPTP-PEST target sequences, in which case the His\(^{602}\) residue would not be an essential binding component in vitro. Conversely, this difference could reflect a situation where absent histidine post-translational modification is necessary for high affinity binding. In this case, the presence of a histidine residue at amino acid position 602 would be essential for binding.

In order to determine the importance of the His\(^{602}\) residue for binding to SHC proteins in vivo, we mutated the histidine to an alanine residue in the context of a full-length HA-MPTP-PEST. This H602A mutant was assayed for binding to SHC proteins in vivo under the same conditions described in the legend to Fig. 4. Anti-HA-MPTP-PEST immunoblot analysis of SHC immunoprecipitates reveals that the wild type but not the H602A mutant co-immunoprecipitated with SHC proteins (Fig. 6). Upon longer exposure of Fig. 6 (upper panel), less than 5% of the H602A HA-MPTP-PEST co-immunoprecipitated with SHC proteins (data not shown). This demonstrates that changing the histidine 602 to an alanine residue drastically reduces (>95%) the binding of SHC proteins to HA-MPTP-PEST in vivo, suggesting that the His\(^{602}\) residue is essential for binding to SHC proteins in vivo.

DISCUSSION

Our data show that the PTB/PI domain of SHC binds to the murine protein-tyrosine phosphatase-PEST via an NPXH sequence. Computer aided searches of sequence data bases have revealed that several proteins contain regions that have sequence similarity to the PTB/PI domain of p52\(^{SHC}\) (22). However, their ability to bind to tyrosine-phosphorylated residues in a sequence-specific manner and/or to non-phosphotyrosine-containing recognition sequences remains to be determined. The amino acids immediately surrounding the NPLH sequence in human and murine PTP-PEST proteins are identical (data not shown) and could underscore the importance of this region given the relatively low overall identity between the two COOH termini.

Previous peptide competition assays reveal that distinct amino acids located at different positions in the SHC PTB/PI domain binding sites of the EGF receptor, polyoma middle T antigen, and c-Erb B2 proteins are essential for binding (5, 10, 13). In all three examples, the Asn at position –3 and the phosphorylated tyrosine residue are absolutely essential for high affinity binding, but the location of other required amino acids seems to be protein-specific (data not shown). Although these experiments were performed by different groups and under slightly different conditions, the results nevertheless demonstrate that the modality of binding for the PTB/PI domain of SHC does not appear to be universal. This diversity and the recent NMR studies of both the PTB/PI domain of SHC (23) and of a PTB/PI domain recognition binding sequence (13) suggest that structural components of the SHC PTB/PI domain and its recognition binding site are critical. Therefore, a detailed mutagenic analysis of the surrounding amino acids of the \(^{599}\)NPLH\(^{602}\) sequence of MPTP-PEST could define the requirements for binding to the SHC PTB/PI domain of the nonphosphotyrosine target sequence of MPTP-PEST. Such analysis could unmask unique residues required for binding in a nonphosphotyrosine-dependent manner.

The necessity for the His\(^{602}\) residue in MPTP-PEST for binding to SHC proteins in vivo, in addition to the lower binding affinities displayed by the nonmodified wild type and H602A mutated MPTP-PEST peptides in vitro, suggest that the His\(^{602}\) could be post-translationally modified. Phosphohistidine is an important intermediate in prokaryotic signal transduction pathways involved in processes such as chemotaxis (24) and porin expression (25). Phosphohistidine has been found in many eukaryotic proteins and has recently been implicated in eukaryotic signal transduction in platelets (26). The cytoplasmic tail of P-selectin (a leukocyte adhesion molecule) undergoes rapid and transient histidine phosphorylation on the peptide sequence \(^{768}\)NPHSH\(^{772}\), where both histidine residues are po-
tential sites of phosphorylation following thrombin or collagen stimulation of platelets (26). It is therefore possible that the sequence NPLH of MPTP-PEST could be phosphorylated on the His residu...on the specific post-translational modification is required to achieve high affinity binding to SHC proteins much in the same way phosphorylation of tyrosine residues leads to an increase in binding affinity. Phosphorylation, be it on histidine or other phosphorylatable amino acids, may be an essential recognition parameter by which PTB/PI domain binding occurs in a tyrosine-independent manner. Our data demonstrating a phosphotyrosine-independent binding for this domains serves to underscore the need to re-evaluate the specificity of this interaction.

In conclusion, we demonstrate that the PTB/PI domain of SHC interacts with the non-phosphotyrosine-based NPLH sequence of MPTP-PEST. In vivo and in vitro studies established that the Asn and Pro residues at positions −3 and −2, respectively, are essential for binding and that the histidine residue at position 0 may require post-translational modification for high affinity binding to SHC proteins.

Acknowledgments—We are grateful to Dr. A. Veillette (McGill University) for critical review of the manuscript. We are also indebted to B. L. Margolis (University of Michigan Medical Center, Ann Arbor, MI) for the GST-SHC-1–209 210 construct, to Mei Y.-M. Duan, A. Veillette, and B. L. Margolis (University of Michigan Medical Center, Ann Arbor, MI) for critical review of the manuscript. We are also indebted to Dr. A. Veillette (McGill University) for helpful discussions.

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J. Biol. Chem. 1996, 271:8424-8429.
doi: 10.1074/jbc.271.14.8424

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