Sequence Requirements for Association of Protein-tyrosine Phosphatase PEP with the Src Homology 3 Domain of Inhibitory Tyrosine Protein Kinase p50\textsuperscript{csk}\textcopyright

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Previously, we reported that the inhibitory tyrosine protein kinase p50\textsuperscript{csh} is physically associated with the protein-tyrosine phosphatase PEP in hematopoietic cells. This interaction was shown to involve the Src homology 3 (SH3) region of Csk and a proline-rich sequence of PEP termed P1 (SRRTDDEIPPPLPERTPESFIVVEE). In this report, we have attempted to understand the structural basis for the highly specific association of these two molecules in vivo. Our studies revealed that the proline-rich core of the P1 region of PEP (PPPLPERT) was necessary but not sufficient for binding to p50\textsuperscript{csh}. Additional sequences located carboxyl to this motif were also needed for binding to the Csk SH3 domain in vitro and in vivo. Further analyses revealed that two aliphatic residues (isoleucine 625 and valine 626; PESFIVVEE) were especially important for this effect. In addition to clarifying the molecular basis for the selective ability of PEP to associate with Csk, these results constitute further evidence that sequences outside proline-rich cores dictate the specificity of SH3 domain-mediated interactions in vivo.

Src homology 3 (SH3)\textsuperscript{d} domains are modules of ~60 amino acids found in a variety of signaling molecules, as well as in certain constituents of the cytoskeleton (1) (reviewed in Refs. 2–4). There is accumulating data that these motifs mediate protein-protein interactions (reviewed in Refs. 2–4). Screening of synthetic peptide libraries and phage display libraries revealed that SH3 domains primarily recognize proline-rich stretches (5, 6). Crystallographic studies and nuclear magnetic resonance analyses further showed that these proline-rich sequences adopt a left-handed polyproline II (PPII) helical conformation, in which the core motif is XP\textsubscript{a}XP (where XP represents SH3 domain-binding dipeptides and p is a scaffolding residue, usually a proline) (7–9). The two core prolines (XP\textsubscript{a}XP) make direct contact with the SH3 domain, while the immediately surrounding residues control the conformation of the PPII helix, thereby affecting the stability of the interaction. A frequent basic residue located either amino-terminal (K/R\textsubscript{a}XP; class I ligands) or carboxyl-terminal (XP\textsubscript{a}XP\textsubscript{a}XKR; class II ligands) to the core prolines also forms a salt bridge with the SH3 domain and further enhances the affinity of the association (10). The position of this basic residue also determines the orientation of binding to the SH3 domain (8, 9).

The mechanisms determining the specificity of SH3 domain-mediated interactions in vivo are beginning to be understood. Initial studies led to the proposal that variable residues within the proline-rich core were responsible for the specificity of these interactions (2–4). However, as short proline-rich peptides typically bound numerous SH3 domains in vitro, it was subsequently postulated that sequences outside the PPII helix dictated the selectivity of these associations in vivo (11, 12). While definite proof of this notion is not yet available, it is supported by in vitro studies (13, 14). Most convincingly, crystallographic analyses demonstrated that amino acids positioned carboxyl to a PPII helix in the human immunodeficiency virus Nef protein provided additional contact with a mutant Fyn SH3 domain, thus dramatically augmenting the affinity of the association between these two molecules (13, 15). Similar observations were made by analyzing the interaction of the SH3 domains of Src and phosphatidylinositol 3’-kinase with synthetic peptides (13).

Csk is a 50-kDa cytoplasmic tyrosine protein kinase expressed in all cell types (16) (reviewed in Ref. 17). It contains amino-terminal SH3 and SH2 domains, in addition to a carboxyl-terminal catalytic region. Significant interest toward p50\textsuperscript{csh} stems from its ability to repress the activity of Src family tyrosine protein kinases (reviewed in Ref. 17). There is mounting evidence that the inhibitory effect of Csk requires the presence of an intact SH3 sequence (18, 19) (reviewed in Ref. 17). Even though the exact function of this domain remains to be elucidated, we previously found that it mediates the association of Csk with PEP, a nonreceptor protein-tyrosine phosphatase expressed in hematopoietic cells (20, 21). Approximately 25–50% of PEP molecules is bound to Csk in T cells, B cells, and macrophages (20). Structure-function analyses demonstrated that the SH3 domain of p50\textsuperscript{csh} binds a proline-rich sequence in the noncatalytic domain of PEP, termed P1 (see Fig. 1). This interaction is highly specific, as a closely related proline-rich motif in PEP, named P2 (Fig. 1), is unable to bind the Csk SH3 region. Furthermore, the Csk SH3 domain was found to associate with no other proline-rich proteins, with the exception of the recently uncovered PTP-PEST, a protein-tyrosine phosphatase structurally related to PEP (22, 23). Our preliminary data indicate that PEP cooperates with Csk to inhibit antigen re-

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\textsuperscript{a} The abbreviations used are: SH3, Src homology 3; PPII, polyproline II; PCR, polymerase chain reaction; GST, glutathione S-transferase; mAb, monoclonal antibody; HA, hemagglutinin.

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ceptor-mediated signal transduction in T cells.²

In this report, we have attempted to understand better the structural basis for the exquisite ability of PEP to associate with p50⁻csk in hematopoietic cells. The results of our studies revealed that the proline-rich core of the P1 region of PEP was necessary but not sufficient for binding to the Csk SH3 domain. We found that unique residues located carboxyl to the proline-rich region of PEP P1 were also required for the association between Csk and PEP. These findings provided further evidence that sequences outside proline-rich cores can govern the capacity of ligands to interact with SH3 domains in vivo.

MATERIALS AND METHODS

**Bacterial Fusion Proteins—**TrpE fusion proteins encompassing the proline-rich regions P1 (trpE-P1) and P2 (trpE-P2) of PEP were reported previously (20). The amino acid residues corresponding to these domains are depicted in Fig. 1. Mutant versions in which individual residues were replaced by alanines, or in which either amino-terminal or carboxyl-terminal truncations were created, were generated by PCR. A schematic representation of the truncations studied here is shown in Fig. 1. All constructs were verified by sequencing (data not shown). A glutathione-S-transferase (GST) fusion protein bearing the SH3 domain of Csk was described elsewhere (19, 20).

**In Vitro Binding Assays—**TrpE fusion proteins were produced in bacteria as outlined earlier (20). Bacterial lysates containing trpE-fusion proteins were eluted in sample buffer, boiled, and resolved in 8% SDS-polyacrylamide gels. TrpE fusion proteins were detected by immunoblotting with a mouse monoclonal antibody (mAb) directed against trpE (Oncogene Science) and 125I-goat anti-mouse IgG (ICN). Immunoreactive products were detected by autoradiography and quantitated with a PhosphorImager (BAS 2000; Fuji).

**Peptide Competition Assays—**Peptides corresponding to short (KDEIPPPLPERT) and long (KDEIPPPLPERTPESFIVVEE) versions of PEP P1 were commercially synthesized at the University of Toronto, Toronto, Ontario, Canada. For competition assays, immobilized GST-Csk SH3 domains (∼5 μg) were preincubated for 30 min at 4 °C with increasing concentrations (0.15–150 μM) of peptide, prior to addition of bacterial lysates containing trpE-3N P1 (5 μg). Binding assays were performed as outlined above. cDNA Mutants and Transient Transfections—A modified mouse pep cDNA coding for a PEP protein with a Myc epitope at the amino terminus (Myc-PEP) was described previously (20). A mutant in which the sequence PPLPERT of PEP P1 was deleted (ΔP1 PEP) was also reported (20). Additional mutants in which individual residues in PEP P1 were replaced by alanines were created by PCR. All constructs were verified by sequencing to ensure that no unwanted mutations had been introduced in the process (data not shown). The myc-pec cDNAs were cloned in the vector pXM139, which contains the SV40 origin of replication, as described under “Materials and Methods.”

**RESULTS**

**Elements in the Proline-rich Core of PEP P1 Required for Binding to the Csk SH3 Domain—**We previously reported that a trpE bacterial fusion protein encompassing amino acids 605–629 of mouse PEP (SRRTDEIPIPPPLPERTPESFIVVEE; Fig. 1), also termed PEP P1, was capable of associating with the Csk SH3 domain in vitro (20). To identify which residues in the proline-rich core of P1 were needed for this interaction, the individual amino acids in PPPLPERT were replaced by alanines through PCR. The resulting P1 mutants were expressed as trpE fusion proteins in bacteria and tested for their ability to associate with a GST fusion protein containing the Csk SH3 region, as described under “Materials and Methods.” The association of the trpE fusion proteins with GST-Csk SH3 domains was monitored by immunoblotting with anti-trpE antibodies (Fig. 2).

In agreement with earlier results (20), wild-type trpE-P1 (Fig. 2A, top panel, lane 1) was efficient at binding the Csk SH3 domain in these assays. Replacement of either the second (lane 3) or the fourth (lane 6) proline in PPPLPERT by alanine completely abolished binding to the Csk SH3 domain. In contrast, however, mutation of the first (lane 2) or third (lane 4) proline had no appreciable impact on this association. We also observed that substitution of the leucine (lane 5), the arginine (lane 8), or the threonine (lane 9) in PPPLPERT eliminated the association with the SH3 region of Csk, whereas mutation of the glutamate (lane 7) had no effect. We wished to ensure that our inability to observe any change in binding after mutating the first or third proline was not due to a lack of linearity in the assays. Hence, experiments were also performed using serial dilutions of trpE-expressing bacterial lysates (Fig. 2B). These analyses showed that mutation of the first (lanes 4–6) or third proline (lanes 7–9) of P1 had no influence on binding to the SH3 region of Csk. Importantly, we also established that the differences in binding of the various fusion proteins to GST-Csk SH3 domains were not caused by variations in their levels of expression, as demonstrated by a parallel immunoblot of total bacterial lysates with anti-trpE antibodies (Fig. 2A, bottom panel).

The basis for the slower electrophoretic mobility of some of the fusion proteins (lanes 5 and 8) is not known. However, it is likely to relate to differences in the conformation of these products in SDS-polyacrylamide gels.

**Sequences Carboxyl to the Proline-rich Core of PEP P1 Are Also Necessary for Binding to the Csk SH3 Domain—**While the proline-rich core of PEP P1 is clearly involved in the interaction with the Csk SH3 domain, our previous findings implied that this sequence may not be sufficient for a stable association between Csk and PEP (20). This was suggested by the observation that another polyproline motif with an identical proline-
We wished to quantify the impact of the carboxyl-terminal sequences in PEP P1 on its affinity for the Csk SH3 domain. To this end, peptide competition experiments were conducted. Peptides encompassing the proline-rich core of PEP P1 (KDEIPPPLPERT), in the absence or presence of the sequence PESFIVVEE at the carboxyl terminus, were synthesized. Various concentrations of the two peptides were then tested for their capacity to inhibit binding of trpE-ΔN P1 to GST-Csk SH3 domains (Fig. 4). This experiment revealed that the peptide with the carboxyl-terminal extension (Fig. 4A, lanes 9–15) could compete the interaction between trpE-ΔN P1 and GST-Csk SH3 domains. The association was reduced by ~50% with a concentration of peptide of 0.7 μM and was almost totally abolished when the peptide concentration was raised to 7 μM. By comparison, the shorter peptide (lanes 2–8) did not significantly affect the association of trpE-ΔN P1 with the Csk SH3 domain, even when a concentration of 150 μM was used (lane 8). On the basis of these results, we estimated that the presence of the carboxyl-terminal sequence enhanced the ability of PEP P1 to interact with the Csk SH3 domain by at least 200-fold (Fig. 4, A and B; data not shown). The data are presented graphically in Fig. 4B.

Two Aliphatic Residues in the Carboxyl-terminal Sequence of PEP P1 Largely Determine the Efficiency of Binding to the Csk SH3 Domain.—To better understand the function provided by the carboxyl-terminal sequence of PEP P1, smaller truncations were created to delineate the residues affecting Csk binding (see Fig. 1 for a description of the mutants). Deletion of the last three amino acids of PEP P1 (VEE) caused no more than a 5-fold reduction of the ability of trpE-P1 to associate with the Csk SH3 domain (Fig. 5A, lanes 8–10). However, truncation of three additional residues (FIV; lanes 11–13) abolished the capacity of trpE-P1 to bind the SH3 domain. We also verified whether additional residues located carboxyl to PEP P1 and which were not included in the original trpE-P1 construct could further influence binding to the Csk SH3 domain. It was observed that the presence of 20 supplementary residues at the carboxyl terminus of trpE-P1 (residues 630–649) had no measurable consequence on the extent of binding to the Csk SH3 domain (lanes 5–7).

For a more precise determination of the carboxyl-terminal residues involved in binding to the Csk SH3 domain, the amino acids in PESFIVVEE were individually replaced by alanines, and the impact of these mutations on the binding to the Csk SH3 domain was tested (Figs. 6, A and B). This experiment showed that most of the mutations in this region had little or no impact on binding to the Csk SH3 domain. However, replacement of the isoleucine (Fig. 6, A, lane 6, and B, lanes 19–21) or first valine (Fig. 6, A, lane 7, and B, lanes 22–24) was consistently found to diminish the interaction. Mutation of these residues reduced binding to the SH3 domain by 10- and 5-fold, respectively (data not shown). Substitution of the phenylalanine (Fig. 6, A, lane 5, and B, lanes 16–18) also diminished the association with the SH3 region of Csk, although the effect of this mutation was more variable when comparing Fig. 6, A and B. We also evaluated the impact of combining the mutations of the isoleucine and first valine (Fig. 6C). Whereas replacement of either residue caused a partial diminution in binding to the Csk SH3 domain (lanes 3 and 4), alteration of both amino acids (lane 5) completely abolished the association with the Csk SH3 region.

Sequence Requirements for the Csk-PEP Interaction in Vivo.—Finally, we wanted to verify that the requirements outlined above also applied to the association of PEP with p50CSK in vivo. To this end, alanine substitutions were introduced in the P1 region of the full-length PEP molecule by PCR. The PEP rich core sequence, i.e. PEP P2 (Fig. 1), was inefficient at associating with the Csk SH3 domain in vitro and in vivo. To obtain a better estimate of the relative affinity of these two related proline-rich sequences for the Csk SH3 domain, binding assays were conducted with serial 10-fold dilutions of either trpE-P1 or trpE-P2 lysates (Fig. 3A). With this approach, it was determined that the affinity of trpE-P1 for the Csk SH3 domain was 500–1000-fold greater than that of trpE-P2. Hence, the proline-rich core PPPLPERT is not sufficient for optimal binding to the SH3 domain. Presumably, other sequences in PEP P2 may inhibit the capacity of PPPLPERT to interact with the Csk SH3 domain.

To distinguish between these two possibilities, truncations of either the amino-terminal or the carboxyl-terminal sequences flanking the proline-rich core of P1 and P2 were engineered by PCR (see Fig. 1 for details). The resulting polypeptides were expressed as trpE fusion proteins and tested for their ability to bind the Csk SH3 domain as above. This experiment revealed that, unlike wild-type P1 (Fig. 3C, lanes 1–9), a P1 variant devoid of carboxyl-terminal flanking sequences (∆C P1; lanes 8–10) failed to associate detectably with the Csk SH3 domain. By contrast, a P1 mutant lacking the flanking amino-terminal residues (∆N P1; lanes 5–7) was still capable of interacting with the Csk SH3 sequence. Removal of either the amino-terminal (∆N P2; lanes 11–13) or the carboxyl-terminal (∆C P2; lanes 14–16) sequences in PEP P2 did not correct its inability to react with the Csk SH3 region (lane 4).
variants were also engineered to carry a Myc epitope at their amino terminus, to allow detection with anti-Myc antibodies. The myc-pep cDNAs were transiently transfected in Cos-1 cells, in the absence or presence of cDNAs coding for an HA-tagged version of Csk (Csk-HA). After cell lysis, the ability of the Myc-PEP variants to associate with Csk-HA was ascertained (Figs. 7, A and B). By contrast, mutation of the first (PEP
I625A
) proline in PPPLPERT, or of proline
P614A
, had no impact on the Csk-PEP association. The importance of the isoleucine (I625) and first valine (V626) located in the carboxyl-terminal region of PEP P1 was also demonstrated a lowered binding to p50
Csk
 (~25–40% of wild-type). By contrast, mutation of the first (PEP P613A; lane 5) or third (PEP P615A; lane 7) proline in PPPLPERT, or of proline 621 (PEP P621A; lane 12), had no impact of the Csk-PEP association.

The importance of the isoleucine (I625) and first valine (V626) located in the carboxyl-terminal region of PEP P1 was also ascertained (Figs. 7, A and B). Whereas replacement of isoleucine 625 by an alanine (PEP I625A; Fig. 7, A, lane 13, and B, lane 5) caused a ~50–70% reduction in binding to Csk, mutation of both isoleucine 625 and valine 626 (PEP IV—AA; Fig. 7B) caused a ~50–70% reduction in binding to Csk.
DISCUSSION

We reported elsewhere that, via its SH3 domain, Csk interacts with PEP, a protein tyrosine phosphatase accumulating in hematopoietic cells (20). This interaction was shown to be highly specific and to involve a single proline-rich sequence located in the noncatalytic domain of PEP, termed P1. In the present study, we wished to dissect in greater detail the molecular basis for this interaction. First, we examined which residues in the proline-rich core of PEP P1 (PPPLPERT) were needed for binding to the Csk SH3 domain. Through an alanine scan and in vitro binding studies, it was determined that the second and fourth prolines of the sequence PPPLPERT played crucial roles in this interaction. Moreover, the leucine, arginine and threonine were also important for binding to the Csk SH3 domain, whereas the first and third prolines, as well as the glutamic acid, appeared dispensable.

In combination, these findings were consistent with those made for other ligands of SH3 domains (7–9, 14). Assuming that the proline-rich core of PEP P1 adopts a PPII helical structure, the relative importance of the residues in PPPLPERT observed herein could be explained by their position in the PPII helix. Accordingly, the second and fourth proline would be essential for binding to the SH3 domain as they would correspond to the core prolines in the motif XpXp. The adjacent leucine, arginine and threonine would also be part of the surface of the PPII helix that contacts the SH3 domain, thus explaining their requirement for stable interaction with the Csk SH3 domain. By contrast however, the first and third prolines, as well as the glutamic acid, appeared dispensable.

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suffice for stable complex formation either in vitro or in vivo. This notion was initially suggested by our earlier finding that PEP P2, which possesses the same proline-rich core as PEP P1, was unable to negotiate binding to the Csk SH3 domain in the absence of P1 (20). Through the creation of truncated versions of PEP P1 and PEP P2, we uncovered that sequences located carboxyl to the polyproline motif of P1 were necessary for binding to the Csk SH3 domain. In peptide competition assays, these sequences increased by at least 200-fold the ability of PEP P1 to compete the interaction with the Csk SH3 domain. By performing an alanine scan, we further determined that two aliphatic residues, isoleucine 625 and valine 626, were especially important for stable binding of PEP P1 to the Csk SH3 domain. Mutation of either of these residues caused a noticeable (>80%) reduction in binding of PEP P1 to the Csk SH3 domain in vitro, while replacement of both amino acids completely abrogated this association. Importantly, mutation of these two residues also prevented binding of full-length PEP to p50cSk in transfected cells, indicating that these requirements also applied to the Csk-PEP interaction in vivo.

The exact roles of isoleucine 625 and valine 626 in the Csk-PEP association remain to be clarified by structural studies. Nonetheless, analyses of other SH3 domain-ligand interactions provide clues regarding their potential function (13–15). Most notable is the study of Lee et al. (13), in which the crystal structure of a mutant Fyn SH3 domain complexed with the human immunodeficiency virus Nef protein was solved. These authors observed that a sequence located 6–42 residues carboxyl to the proline-rich core of Nef made contact with the Fyn SH3 domain. Specifically, the carboxyl-terminal flanking sequence of Nef formed two α helices that bound the RT loop of the Fyn SH3 domain. This interaction augmented the affinity between the two molecules by approximately 300-fold (15), an effect quantitatively similar to what we have reported herein. Therefore, it is plausible that the carboxyl-terminal region of PEP P1 is also involved in an analogous interaction. Along these lines, computer-based predictions suggest that it may form a β strand, which would be disrupted by mutation of isoleucine 625 and/or valine 626 to alanine(s) (data not shown).

Throughout our studies, the results obtained in in vitro binding assays with bacterial fusion proteins generally correlated well with those observed in mammalian cells expressing full-length PEP and Csk proteins. However, it is noteworthy that the fourth proline and the leucine of the proline-rich core of PEP P1 were not absolutely required for the association in vivo, while they were necessary for the interaction in vitro. Therefore, it is plausible that additional features in the full-length PEP and/or Csk molecules, or in the cellular environment, can further stabilize their binding and partially compensate for the absence of these residues. Future studies will be needed to test this possibility.

Taken together, these results yield an understanding of the molecular basis of a highly specific SH3 domain-mediated interaction in vivo. While the proline-rich core of PEP P1 is essential for the association with Csk, stable binding of these two molecules in vivo and in vitro is also dependent on the presence of a short stretch of amino acids located outside the polyproline motif. Based on these observations and previously published findings (13–15), it is attractive to speculate that the specificity of other SH3 domain-mediated interactions in vivo may be defined by extended sequences positioned outside the proline-rich core of the ligand. This notion may explain the relative lack of binding specificity observed for proline-rich ligands identified from peptide libraries. Moreover, it raises the possibility that the true physiological ligands of several SH3 domains have yet to be uncovered.

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