A novel peptide–SH3 interaction

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SH3 domains constitute a family of protein–protein interaction modules that bind to peptides displaying an X-proline-X-X-proline (XPXXP) consensus. We report that the SH3 domain of Eps8, a substrate of receptor and non-receptor tyrosine kinases, displays a novel and unique binding preference. By a combination of approaches including (i) screening of phage-displayed random peptide libraries, (ii) mapping of the binding regions on three physiological interactors of Eps8, (iii) alanine scanning of binding peptides and (iv) *in vitro* cross-linking, we demonstrate that a proline-X-X-aspartate-tyrosine (PXXDY) consensus is indispensable for binding to the SH3 domain of Eps8. Screening of the Expressed Sequence Tags database allowed the identification of three Eps8-related genes, whose SH3s also display unusual binding preferences and constitute a phylogenetically distinct subfamily within the SH3 family. Thus, Eps8 identifies a novel family of SH3-containing proteins that do not bind to canonical XPXXP-containing peptides, and that establish distinct interactions in the signaling network.

Keywords: Eps8/SH3/signal transduction

Introduction

Eps8 is an SH3-containing substrate for several receptor and non-receptor tyrosine kinases (Fazioli *et al.*, 1993; Wong *et al.*, 1994; Gallo *et al.*, 1997). Several lines of evidence implicated Eps8 and its SH3 in mitogenic signaling: (i) overexpression of Eps8 increased mitogenic responsiveness to the epidermal growth factor (EGF) (Fazioli *et al.*, 1993) and EGF-dependent transformation (Matoskova *et al.*, 1995); (ii) constitutive tyrosine phosphorylation of Eps8, but not of several other tyrosine kinase substrates, was detected in many tumor cell lines, indicating that Eps8 might be part of a pathway preferentially selected in tumor growth (Matoskova *et al.*, 1995); (iii) fibroblasts from Eps8 knock-out mice were impaired

in their ability to transduce signals from Ras to Rac (Scita *et al.*, in press); and (iv) re-expression of wild-type Eps8, but not of a mutant bearing a five-amino-acid deletion in its SH3, restored Ras to Rac signaling in Eps8-negative fibroblasts (Scita *et al.*, in press).

The SH3 fold consists of two antiparallel β sheets, packed against each other at an approximate right angle (for reviews see Mayer and Eck, 1995; Lim, 1996 and references therein). Three variable loops are present, named the RT, n-src and distal loops; the first two participate in the creation of the ligand-binding surface. The ligand binding site on SH3 domains is frequently referred to as the 'proline helix binding groove', because SH3-binding peptides are characterized by the presence of a general X-proline-X-X-proline (XPXXP) consensus (Feng *et al.*, 1994; Goudreau *et al.*, 1994; Lim *et al.*, 1994; Musacchio *et al.*, 1994; Teresawa *et al.*, 1994; Wittekind *et al.*, 1994; Yu *et al.*, 1994; Wu *et al.*, 1995; Morton *et al.*, 1996), and they adopt a polyproline type II (PP-II) helix conformation when complexed with SH3 domains. Specificity of recognition of a PP-II-containing peptide by a given SH3 domain is partly due to the presence of additional residues (frequently positively charged amino acids) in the binding peptide. PP-II-containing peptides can bind to SH3s in two opposite orientations (Feng *et al.*, 1994; Lim *et al.*, 1994). Thus, for example, the src SH3 domain can bind either to RXXPPXP (the so-called Class I) or to PXXPXR (Class II) peptides (Feng *et al.*, 1994). The orientation of the peptide appears to be determined by specific interactions of the arginine residue with negatively charged residues in the RT loop.

The crystal structure of the Eps8–SH3 domain revealed, unexpectedly, an intertwined dimer, characterized by 'strand exchange', in which the two anti-parallel β sheets that are characteristic of the SH3 fold are contributed by different polypeptide chains. Residues that in the monomeric forms of the SH3 domain correspond to the n-src loop assume an extended conformation in Eps8–SH3, and function as a connecting segment to allow the exchange between monomers. Surprisingly, these critical rearrangements result in half-dimers that contain structurally conserved SH3 folds, whose central regions superimpose on those of the previously reported monomeric SH3 domains with minimal deviations (Kishan *et al.*, 1997). However, an important consequence of the dimeric configuration of Eps8–SH3 is that it determines a partial occlusion of the proline-helix binding groove, due to the presence in its proximity of the side chains of residues belonging to the extended n-src loop.

We have shown previously that Eps8 can exist as a dimer *in vivo*, and that dimerization requires an intact SH3 domain (Kishan *et al.*, 1997). While this set of observations suggests that dimerization may have important implications for the function of Eps8, it also leaves

mEps8-SH3										hEps8-SH3										Hck-SH3									
K	L	P	H	P	D	Y	I	Q		R	F	P	X	R	D	Y	H	E			K	R	G	P	L	P	P	L	A
N	F	P	L	P	D	Y	D	P		H	R	P	P	I	D	Y	T	V			F	R	P	L	P	N	A	P	S
N	P	L	P	P	P	D	Y	G	P	L	S	I	P	E	P	D	Y	E			R	A	R	P	M	P	P	L	P
A	L	P	A	K	D	Y	P	H		S	Y	P	R	R	D	Y	T	L			V	K	F	P	P	P	P	Q	W
T	Q	V	P	N	V	D	Y	E		V	R	Y	P	P	P	D	Y	F			R	E	R	R	L	P	G	V	P
			P	I	R	D	Y	P	A	S	R				P	I	P	D	Y	E	G	T	V			I	R	S	L
			R	P	L	P	D	Y	A	A	P				L	I	P	Q	I	D	Y	A	W						
S	A	P	R	R	D	Y	A	P		K	Y	P	K	V	D	Y	S	T											
Y	Y	P	P	A	D	Y	A	R																					
Y	P	H	A	D	Y	G	S	P																					
E	L	P	N	M	D	Y	G	P																					
A	L	P	T	P	D	Y	A	Q																					
L	L	P	P	R	D	Y	T	E																					
Y	P	H	P	D	Y	G	S	P																					
P	G	P	D	Y	G	E	S	L																					
Y	L	P	T	V	D	Y	N	W																					

Fig. 1. Identification of the PXXDY consensus as the preferred binding motif for Eps8-SH3. Predicted amino acid sequence of peptides selected by screening of a random phage-displayed peptide library with murine and human GST-Eps8-SH3 (mEps8-SH3 and hEps8-SH3) and with GST-Hck-SH3. The PXXDY consensus is indicated by white letters on black.

several questions unanswered. First, is the Eps8-SH3 dimer that was revealed by crystallographic analysis capable of binding to proline-rich substrates? If so, then does the specificity of binding of the dimeric domain differ substantially from that of other monomeric SH3 domains? Furthermore, it remains unclear whether a stable monomeric form of the Eps8-SH3 domain exists, and if it does, whether the binding specificity of the monomeric domain is different from that of the dimeric form. The present studies were undertaken to search for the binding preferences of the Eps8-SH3. Surprisingly, we found that the monomeric form of the domain is capable of binding to peptides that conform to an entirely novel consensus sequence, while the dimeric form displayed little if any binding activity.

Results

Identification of a consensus sequence for binding to the SH3 domain of Eps8

To analyze the binding specificity of the Eps8-SH3 domain, we employed glutathione *S*-transferase (GST) fusion proteins (GST-Eps8-SH3) to screen a phage-displayed random nonapeptide library (Felici *et al.*, 1991). The peptide sequences of inserts isolated after two cycles of selection are displayed in Figure 1. All selected peptides conform to a novel consensus, PXXDY, where X can be any amino acid. The SH3 domain of Hck, an Src-family member that was used as a control in this experiment, selected sequences conforming to the classical XPXXP sequence, as expected.

Mapping of the Eps8-SH3 binding site on *in vivo* interactors

We mapped the sites of interaction of Eps8-SH3 with two known *in vivo* ligands: e3b1/Abi-1 (Shi *et al.*, 1995; Biesova *et al.*, 1997) and RN-tre (Matoskova *et al.*, 1996a,b). To this end, we generated GST fusion proteins of contiguous and/or overlapping fragments of these proteins (Figures 2A and 3A, respectively), and tested their ability to bind to Eps8 or to its SH3 in a variety of assays, including *in vitro* binding, Far-Western (overlay assays) and ELISA assays (see Materials and methods).

We found that a 12-amino-acid peptide from e3b1 (e3b1/7), encompassing amino acid positions 386–397 of e3b1/Abi-1, was necessary and sufficient for binding (Figure 2B–D). Similarly, a fragment of RN-tre, encompassing positions 695–760 (RN-tre/5), possessed all the determinants necessary and sufficient to bind to Eps8 (Figure 3B and C). Within this, a 16-amino-acid fragment (RN-tre/7, positions 719–734) displayed full binding activity in Far-Western assays and retained ~10% binding in *in vitro* bindings (Figure 3B and C). An internal nine-amino-acid fragment (RN-tre/8, positions 724–732) still retained substantial binding activity in Far-Western assays, albeit displaying markedly reduced binding activity in *in vitro* binding assays (Figure 3B and C). The discrepancy between results with Far-Western and *in vitro* assays is likely to be due to steric hindrance to peptide binding by the GST moiety in *in vitro* assays, which is overcome by GST denaturation in Far-Western assays.

A similar set of analyses was also applied to the study of cl.74, a partial cDNA clone isolated in expression library screenings with the Eps8 protein and displaying binding activity towards the SH3 domain of Eps8. A complete description of the protein encoded by the full-length cDNA, of which cl.74 represents a fragment, is to be reported elsewhere (E.Tassi, Z.Biesova, P.P.Di Fiore, J.S.Gutkind and W.T.Wong, manuscript submitted). The sequence of the product of cl.74 identifies a protein fragment related to *Caenorhabditis elegans* SULU serine/threonine kinase (a member of the Ste20 family), having a high propensity for α -helix formation and predicted to form a coiled coil for most of its 438 amino acids (Figure 4A). Consistent with its high propensity for helix formation, there are no proline-rich regions in cl.74 and only four isolated prolines.

We confirmed the ability of cl.74 to interact with Eps8 by showing that an immobilized GST fusion protein encompassing the entire cl.74 (74-FL) was capable of binding to Eps8 in cell lysates (not shown). Binding was directed to the SH3 domain, as 74-FL could bind to the SH3 domain of Eps8 in Far-Western assay (Figure 4). Next, GST fusions encompassing contiguous and/or overlapping fragments of the cl.74 proteins were engineered (Figure 4B). A fragment containing the last 23 amino acids (from

Fig. 2. Mapping of the region of e3b1 that binds to the SH3 of Eps8. (A) Schematic representation of the GST-e3b1 fusion proteins and their ability to bind to Eps8 or to the Eps8-SH3. Proline-rich regions are indicated by solid bars. The presence of an SH3 domain is also indicated. For the smallest GST-fusions (e3b1/5 and e3b1/8), the predicted amino acid sequence of the e3b1 fragment is indicated. Strength of binding is indicated in a semi-quantitative fashion (+, strong binding; +/-, weak binding; -, no binding or very weak binding; ND, not done). (B) *In vitro* bindings. The lanes 'lysate' were loaded with 50 μ g of total cellular lysate, to serve as a reference. The position of Eps8 is indicated by an arrow. (C) Far-Western assays. Top panel, detection with purified biotinylated Eps8-SH3; bottom panel, detection with an anti-GST antibody. Control blots were also performed with biotinylated GST, which did not stain any of the GST fusions (not shown). (D) ELISA assays. GST fusion proteins immobilized in the assays are indicated underneath. In (A-D), results are typical and representative of at least three independent experiments.

Fig. 3. Mapping of the region of RN-tre that binds to the SH3 of Eps8. (A) Schematic representation of the GST-RN-tre fusion proteins and their ability to bind to Eps8 or Eps8-SH3. The presence of a Tre-Homology (TrH) domain is indicated. Proline-rich regions are indicated by solid bars. For the smallest GST fusions (RN-tre/7 and RN-tre/8), the predicted amino acid sequence of the RN-tre fragment is also indicated. Strength of binding is as in Figure 2. (B) *In vitro* bindings. The lanes 'lysate' were loaded with 50 μ g of total cellular lysate, to serve as a reference. The position of Eps8 is indicated by an arrow. (C) Far-Western assays. Top panel, detection with purified-biotinylated Eps8-SH3; bottom panel, detection with an anti-GST antibody. Control blots were also performed with biotinylated GST, which did not stain any of the GST fusions (not shown). In (A-C), results are typical and representative of at least three independent experiments.

positions 416 to 438, identified as cl.74/8) retained full binding activity to biotin-conjugated Eps8-SH3 in overlay assays. A partially overlapping fragment, extending from positions 405 to 426 (cl.74/11), did not show any binding (Figure 4B and C). The prediction that the last 12 residues of cl.74 contain a binding site to Eps8-SH3 was confirmed by engineering a GST fusion containing the last nine amino acids of cl.74. This construct, cl.74/12, showed full binding activity to Eps8-SH3 (Figure 4B; see also Figure 5E).

When the sequences of the Eps8-SH3 binding regions from e3b1, RN-tre and cl.74 were compared, we found that they all contained sequences fitting the PXXDY consensus. Thus, the combination of independent approaches, such as peptide library screening and deletion

mapping of minimal binding sites on three interactors of the Eps8-SH3 domain, unequivocally establishes that a sequence PXXDY is the preferred binding site for Eps8-SH3.

Identification of amino acid residues critical for binding to the SH3 domain of Eps8

To assess the individual contribution to binding of residues in the PXXDY motif and its proximity, we performed alanine scanning mutagenesis of the e3b1/7 and RN-tre/8 peptides, engineered in a GST fusion background. The results of Far-Western and ELISA assays on the e3b1/7 peptide are shown in Figure 5A and B. The two most critical positions were D and Y (of the PXXDY consensus) at positions 7 and 8, respectively, of the peptide. Muta-

Fig. 4. Mapping of the region of c1.74 that binds to the SH3 of Eps8. **(A)** Schematic representation of the c1.74 and SULU proteins and structural predictions on the c1.74 protein. The kinase domain of SULU is indicated by a solid box. The region of homology between SULU and c1.74 is indicated by a filled box. The propensity for the formation of coiled coils (coils) in c1.74 was established by the COIL Program, available through the ISREC server, with a window of 21 amino acids. The propensity for the formation of α -helices was determined by secondary structure prediction using the Chou-Fasman algorithm (Chou and Fasman, 1978). Amino acid positions are indicated at the bottom. **(B)** Schematic representation of the GST-c1.74 fusion proteins and their ability to bind to Eps8-SH3. The predicted amino acid sequence of the shortest c1.74 peptide (c1.74/12) is also shown. **(C)** Far-Western assays. Top panel, detection with purified-biotinylated Eps8-SH3; bottom panel, detection with an anti-GST antibody. Representative fusion proteins are shown, whereas results obtained with all fusion proteins are summarized in (B). Control blots were also performed with biotinylated GST, which did not stain any of the GST fusions (not shown). In (B) and (C), results are typical and representative of at least three independent experiments.

genesis of either of these amino acids to Ala completely abolished binding. The P of the PXXDY consensus (position 4 of the peptide) also influenced binding, and its mutation to Ala caused an ~50% reduction in binding activity. Interestingly, a proline at position 1 (corresponding to position -4 with respect to the PXXDY consensus) also influenced binding, indicating that the full binding site may encompass other residues besides those contained in the PXXDY consensus.

Alanine scanning of the RN-tre peptide yielded similar results (Figure 5C and D). Mutagenesis of either P, D or Y of the PXXDY consensus (positions 2, 5 and 6 of the peptide, respectively) resulted in dramatically reduced binding. The ability of residues outside of this consensus to influence binding was confirmed by the fact that an I→A mutation at position 1 (corresponding to position -1 with respect to the PXXDY consensus) substantially reduced binding.

The influence of neighboring residues was further confirmed by experiments in which we engineered GST fusion proteins encompassing three PXXDY-containing peptides obtained from the phage-display screening (Figure 5E). These fusion proteins were tested for their

ability to bind SH3-Eps8 in a Far-Western assay. A hierarchy of strength of interaction was shown within the PXXDY-containing sequences, again indicating contributions to binding from neighboring residues. Four peptides selected from the RN-tre sequence, and containing a PXXP consensus, were also tested in these experiments. As expected, they were unable to bind to the Eps8-SH3 domain (Figure 5E).

A family of Eps8-related proteins with unique binding preferences

By screening the Expressed Sequence Tags (EST) database, we identified three human and two mouse sequences showing homology to Eps8. The corresponding clones were obtained and sequenced in their entirety. They all represented partial cDNA clones predicting SH3-containing proteins with homology to Eps8 extending beyond the SH3 domain (data not shown). The three human sequences were named hEps8R1, hEps8R2 and hEps8R3 (standing for human Eps8-Related gene 1, 2 and 3, respectively). The two mouse sequences probably represent the murine orthologues of Eps8R2 and Eps8R3, respectively (not shown). In addition, we identified a

Fig. 5. Alanine scanning of binding peptides from e3b1 and RN-tre, and the hierarchy of interaction of various PXXDY-containing peptides. (A) and (B) Amino acid positions 1–12 of the e3b1/7 peptide were alanine scanned and the resulting mutant peptides (engineered as GST fusions) analyzed either in (A) Far-Western or in (B) ELISA for binding to the Eps8-SH3 domain. Each lane corresponds to a peptide in which the indicated amino acid was mutated to Ala. GST, negative control performed with GST alone; WT, positive control consisting of the wild-type GST-e3b1/7 fusion protein. (C) and (D) The amino acids in positions 1–9 of the RN-tre/8 peptide were alanine scanned and the resulting mutant peptides (engineered as GST fusions) analyzed in either (C) Far-Western or (D) ELISA for binding to the Eps8-SH3 domain. Each lane corresponds to a peptide in which the indicated amino acid was mutated to Ala. GST, negative control performed with GST alone; WT, positive control consisting of the wild-type GST-RN-tre/8 fusion protein. (E) A number of GST fusions containing the peptides indicated on the right and displaying either a PXXDY or a canonical PXXP consensus were tested in a Far-Western assay for binding to purified biotinylated Eps8-SH3 (bottom) or detected with anti-GST (top) as a control. Control blots were also performed with biotinylated GST, which did not stain any of the GST fusions (not shown). In (A–E), results are typical and representative of at least three independent experiments.

C. elegans sequence, Y57G11C.24c, which represents a nematode homolog of Eps8. The alignment of the SH3 domains of these Eps8-like sequences, and of several other SH3 domains, is reported in Figure 6A. An interesting feature revealed by this alignment is that an almost invariant aromatic residue (Tyr or sometimes Phe), located immediately before the beginning of the β E strand (position 51 in our alignment, corresponding to position 584 of human Eps8), is not present in the Eps8-related SH3s and is substituted by an isoleucine. All of the Eps8-related SH3s were grouped into a phylogenetically distinct branch of the SH3 dendrogram (Figure 6B), together with two SH3 domains from yeast SLA1p, and one from yeast Bem1p.

To evaluate whether the SH3 domains from the new members of the Eps8 family also displayed unusual binding preferences, we screened a phage-displayed library with the SH3 domains of hEps8R2 and mEps8R2. As shown in Figure 6C, a great majority of selected phages contained inserts encoding peptides with a PXXDY motif. Interestingly, a second, less represented, unusual consensus could be identified, represented by a PPPY/W motif (Figure 6C). It is not clear whether this latter consensus identifies a true binding specificity *in vivo* or a mimotope.

Characterization of the interaction between Eps8-SH3 and PXXDY-containing peptides

We have shown previously that Eps8-SH3 can form dimers, raising the question of whether the unusual binding specificity of Eps8-SH3 could be explained by its ability to dimerize. Moreover, we were interested in understanding the potential effects of the Y/F to I substitution in the Eps8-SH3 family. Purified SH3 domains from Eps8, and from a mutant Eps8 in which the I at position 51 was mutagenized to Y (Eps8-SH3Y), were tested by size-exclusion chromatography. The fast-performance liquid

chromatography (FPLC) profiles of both domains consisted of two peaks, which eluted with retention times compatible, when compared against calibration standards, with those of monomeric and dimeric forms of the domain (Figure 7A).

Peaks corresponding to the putative monomers and dimers were collected. When individually subjected to size-exclusion chromatography, they gave rise to both 'monomeric' and 'dimeric' peaks, consistent with the notion that the two forms exist in a dynamic equilibrium (not shown). We also performed *in vitro* cross-linking. As shown in Figure 7B, the protein from the 'monomeric' peak migrated as bona fide monomeric SH3 in SDS-PAGE, both before and after cross-linking. Conversely, the 'dimeric' peaks were efficiently cross-linked and migrated in SDS-PAGE as true dimers. Thus, the SH3 domain of Eps8 exists as a mixture of monomers and dimers in solution. The I to Y mutant also appeared to be in a monomer-dimer equilibrium, indicating that the presence of an I at position 51 is not directly required for dimerization of the Eps8-SH3 domain.

To define whether the binding properties of Eps8-SH3 were influenced by the oligomerization state of the domain, we incubated purified monomeric and dimeric fractions of Eps8-SH3 with the RN-tre/8 peptide, and performed *in vitro* cross-linking. The monomeric form of the SH3 was readily and quantitatively cross-linked to the peptide. A control, unrelated peptide could not be cross-linked to Eps8-SH3, indicating that binding, and subsequent cross-linking, were specific (Figure 7C, top). An Src-SH3 domain also displayed no cross-linking to RN-tre/8 (Figure 7C, top). Conversely, the dimeric form of Eps8-SH3 displayed marginal ability to be cross-linked to the RN-tre/8 peptide (Figure 7C, top). Similar results were obtained when cross-linking was performed with the e3b1/7 peptide

Fig. 6. A family of Eps8-related proteins whose SH3s bind to PXXDY-containing peptides. **(A)** The SH3 domains of various human, murine and nematode Eps8-like proteins were aligned among themselves (top) or to other SH3s (bottom). Identity and conservation were scored at a plurality of >50% and are indicated by reverse printing and shading, respectively. The arrow points to the Ile for Tyr/Phe substitution. The sequences of the SH3 of hAbl, hLck, hNCF-2-2, hp85, hSrc and hVav-2 were edited by removing small insertions to maximize alignment. **(B)** Phylogenetic tree. More than 100 SH3 domains, representative of various subfamilies of proteins, were retrieved from public databases and aligned with the CLUSTALW algorithm. Phylogenetic trees were visualized by TreeView. **(C)** Predicted amino acid sequence of peptides selected by screening of a random phage-displayed peptide library with human and murine GST-Eps8R2-SH3 (hEps8R2-SH3 and mEps8R2-SH3). The PXXDY consensus is indicated by reverse printing. Numbers in parentheses indicate phages independently isolated more than once in the original screening.

(Figure 7C, bottom). These results indicate that at least under the conditions of *in vitro* cross-linking, a PXXDY-containing peptide binds preferentially to Eps8-SH3 monomers.

After ruling out its involvement in domain dimerization, we attempted to establish whether the Y/F to I substitution in Eps8-SH3 could account for the peculiar specificity of this domain. Interestingly, we found that the monomeric form of the Eps-SH3Y mutant was readily cross-linked to the e3b1/7 peptide (Figure 8A). This prompted us to evaluate whether the specificity for PXXDY recognition was retained by this mutant. Screening of a phage-displayed peptide library with Eps8-SH3Y again yielded the PXXDY consensus (Figure 8B). Eps8-SH3Y also appeared to bind to native e3b1, in *in vitro* binding experiments, with better efficiency than the wild-type SH3 (Figure 8C). The same was true if various phages, selected by either the wild-type or mutant SH3, were challenged in a phage ELISA assay against the two SH3s (Figure 8D). This observation was quantified by measuring the dissociation constant (K_d) of the interaction of Eps8-SH3,

and of the I→Y mutant, with the e3b1/7 peptide using an ELISA assay. Both K_d values were in the micromolar range. Surprisingly, however, the mutant displayed a >10-fold higher affinity for the peptide than the wild-type SH3 (Figure 8E). Thus, while the Y/F to I substitution does not affect the oligomerization state of Eps8-SH3, or its ability to recognize unusual ligands, it appears that this substitution has a remarkable effect on the binding affinity of the SH3-peptide interactions.

Discussion

SH3 domains recognize linear sequences containing an XPPXP core motif, which is preceded or followed by a second region of specificity, often constituted by a positively charged residue (Feng *et al.*, 1994). The SH3 domain of Eps8 displays a different specificity. Combining approaches such as mapping of the minimal binding regions on three physiological Eps8-SH3 ligands, screening of phage-displayed peptide libraries, alanine scanning mutagenesis and *in vitro* cross-linking of the identified

Fig. 7. PXXDY-containing peptides bind preferentially to the monomeric form of Eps8-SH3. **(A)** Elution profiles in size-exclusion chromatography of Eps8-SH3 and of its mutant Eps8-SH3Y (bearing a Y for I substitution). Peaks migrating as monomers (M) and dimers (D) are indicated. **(B)** Cross-linking of monomeric and dimeric peaks. Individual peaks (M, monomeric; D, dimeric) obtained from size-exclusion chromatography of Eps8-SH3, Eps8-SH3Y or Abl-SH3 (Control SH3) were collected and subjected to *in vitro* cross-linking with disuccinimidyl substrate (DSS). The positions of monomers (M) and dimers (D) in SDS-PAGE-tricine are indicated. **(C)** Monomeric and dimeric peaks from Eps8-SH3 were collected and incubated with either the R8 (top panel) or the E7 (bottom panel) peptides (indicated as 'peptide') at the concentrations indicated, followed by cross-linking with DSS. The control-irrelevant peptide was the DS1 peptide described in Materials and methods. The control SH3 domain was src-SH3 in the top panel and Abl-SH3 in the bottom panel. The relative positions of monomer (M), monomer cross-linked to peptide (M/pep), dimer (D) and dimer cross-linked to peptide (D/pep) are indicated. In (A–C), results are typical and representative of at least three independent experiments. It is of note that in some experiments when a monomeric fraction was cross-linked with DSS, a band migrating as a dimer was visible [as is visible in (C), bottom]. This is most likely to be due to the refolding of some monomer in the dimeric cross-linkable configuration. Similarly, when a dimeric fraction was cross-linked in the presence of peptide (Figures 7C and 8A), a band migrating as cross-linked monomer–peptide was visible. Again, this is most likely due to the refolding of some dimer into the monomeric configuration that can bind to and be cross-linked with the peptide.

peptides, we demonstrated unequivocally that motifs containing the PXXDY sequence are the preferred binding sites for Eps8-SH3. The DY motif in this sequence was absolutely required for binding, and the N-terminal proline contributed substantially to specificity. Mutations of residues immediately preceding this proline caused a decrease in binding efficiency, but we could not detect special sequence requirements for these positions. The importance of the DY sequence was underscored by its presence in all peptides isolated from phage-displayed

Fig. 8. The presence of an Ile for Tyr substitution in the SH3 of Eps8 influences the affinity, but not the specificity, of binding. **(A)** Monomeric and dimeric peaks from Eps8-SH3Y were collected and processed as in Figure 7C. The control SH3 in this experiment was src-SH3. The relative positions of monomer (M), monomer cross-linked to peptide (M/pep), dimer (D) and dimer cross-linked to peptide (D/pep) are indicated. **(B)** Predicted amino acid sequence of peptides selected by screening of a random phage-displayed peptide library with GST-Eps8-SH3Y (Eps8-SH3Y). The PXXDY consensus is indicated by reverse printing. **(C)** *In vitro* binding. One milligram of total cellular lysate was incubated with the GST fusions indicated. After recovery of the bound material, detection was performed by immunoblotting with an anti-E3b1 serum. The lane lysate was loaded with 50 μg of total cellular lysate. **(D)** A total of 10¹⁰ transducing units of phage clones displaying different peptides (indicated underneath) were adsorbed to a plastic microtiter plate and challenged with 5 pmol of GST-Eps8-SH3 (solid bars) or GST-Eps8-SH3Y (empty bars). Bound domains were identified with an anti-GST antibody and a secondary antibody linked to alkaline phosphatase. **(E)** An ELISA assay was performed in which increasing concentrations of a biotinylated E7 peptide were challenged against a fixed amount (0.4 nM, 50 μl/well; see Materials and methods) of immobilized Eps8-SH3 or Eps8-SH3Y. Binding data were analyzed by the LIGAND software (Munson, 1983) to determine the *K_d* of the interactions. In (A–E), results are typical and representative of at least three independent experiments.

libraries, whereas the SH3 domain from Hck selected peptides containing the XPXXP sequence, as expected for an SH3 domain belonging to the tyrosine kinase family. Taken together, these data indicate that Eps8-SH3 binds to PXXDY motifs both specifically and selectively.

As the Eps8-SH3 used in our experiments is in monomer–dimer equilibrium, it was important to establish whether the species responsible for the selection of the

unusual PXXDY-containing peptides is monomeric or dimeric. The fact that all Eps8-SH3 ligands obeyed the same consensus suggests that only one prominent species (monomer or dimer) is active in the selection process. The alternative possibility that monomers and dimers have the same binding specificity is unlikely, given the fact that, as explained in the Introduction, the process of dimerization introduces significant changes in the SH3 ligand-binding site. Our cross-linking experiments indicated that the species that is active in ligand binding is the monomer. Thus, although it was tempting to speculate that the unusual binding specificity of Eps8-SH3 may result from the reorganization of the ligand-binding site revealed by the crystal structure of the dimer (Kishan *et al.*, 1997), our results ruled out this possibility.

The ligand specificity of Eps8-SH3 must thus depend on the fine details of its three-dimensional structure. A remarkable feature of Eps8-SH3 is the presence of an Ile residue at a position of the alignment at which Tyr (or sometimes Phe) is predominant in other SH3 domains (in the PXNY motif, immediately preceding the β E strand). This residue is an important determinant of the geometry of the SH3 ligand-binding site. Moreover, its phenolic hydroxyl is involved in the formation of a hydrogen bond with the main chain of the proline-rich peptide substrate (Musacchio *et al.*, 1994). The Ile for Tyr substitution in Eps8-SH3 appears to be subjected to evolutionary pressure. We have identified several Eps8-related genes in the EST database (including a *C.elegans* homolog), and they all contain Ile at this position. Thus, we attempted to define the role of the Y/F to I substitution with respect to the 'deviant' behavior of the Eps8-SH3, namely, its ability to form dimers and its unusual binding specificity.

First, we established that the presence of this substitution is not responsible for the partial dimerization of the Eps8-SH3 observed in size-exclusion chromatography, *in vitro* cross-linking experiments and crystallographic analysis. We then tested directly the ability of an Ile to Tyr mutant to bind peptides containing the unusual consensus PXXDY. The prediction that this substitution may have dramatic effects on the binding specificity observed for the wild-type Eps8-SH3 domain was contradicted by our results. In addition, when we screened a phage-displayed library with the mutated Eps8-SH3, we obtained peptides with the same PXXDY consensus observed with the wild-type domain. Interestingly, the only apparent effect of the I $\rightarrow$ Y mutation was a significant increase, of more than one order of magnitude, in the affinity of the interaction with PXXDY peptides.

The three-dimensional structure of the monomeric form of the Eps8-SH3 domain is unknown. However, substantial amino acid conservation at positions involved in the formation of the hydrophobic core suggests very good structural conservation between this domain and other members of the SH3 family whose monomeric three-dimensional structures are known. As discussed, the peculiar specificity of Eps8-SH3 cannot be explained by its dimerization and/or Tyr to Ile substitution at position 584. A visual inspection of an alignment revealed no other major differences at conserved sites between the Eps8-SH3 domain and other SH3 sequences. Thus, the observed differences in specificity are probably due to the effect of residues that are not conserved in the SH3 family. Residues

in the variable SH3 RT loop play a role in binding positively charged residues in many SH3 ligands, and may play a role in selecting the DY motif in the case of Eps8-SH3. If this were true, we would expect a binding mode for the PXXDY peptides similar to that observed for class II peptides. The high-resolution structural characterization of the complex between Eps8-SH3 and ligands conforming to the PXXDY consensus is bound to extend our understanding of the fine details of the mechanisms that determine substrate specificity in protein-protein interactions.

Our results also suggest a peculiar modality of control of Eps8 action *in vivo*, i.e. through regulation of its monomer-dimer equilibrium. We have shown previously that a fraction of Eps8 is present in the cell as a dimer (Kishan *et al.*, 1997). When total cellular proteins were subjected to size-exclusion chromatography, the bulk of Eps8 eluted with an apparent molecular mass of ~200 kDa (G.Scita and P.P.Di Fiore, unpublished results). This size is compatible with the possibility that most Eps8 is present in the cell as a dimer. The formation of Eps8 dimers *in vivo* requires the presence of intact SH3 domains, since it has been shown that a mutant Eps8 bearing a five-amino-acid internal deletion in the domain was not able to dimerize (Kishan *et al.*, 1997). Finally, dimerization of Eps8 was not due to interactions between its SH3 domain and an SH3 binding site in Eps8 itself (Kishan *et al.*, 1997). Thus, the possibility that the prevalent Eps8 species *in vivo* consists of dimers kept together through the intertwined structure of their SH3 domains appears reasonable. Our present results indicate that such a species would be biologically inactive, at least in terms of interacting with SH3-binding partners. However, the physical SH3-mediated interaction of e3b1 with Eps8 is indispensable for the transduction of signals from ras to rac (Scita *et al.*, in press), indicating that, at least in this case, monomeric Eps8 must be present in the cell to transduce signals actively.

The question of how the Eps8 dimer-monomer equilibrium is regulated *in vivo* remains open. Since the majority of Eps8 appears to be present in the cell as a dimer, it is conceivable that high local concentrations of binding peptides might shift the equilibrium of Eps8 from the dimeric to the monomeric form. Such high local concentrations of binding sites might be the result of re-localization events of eps8-binding proteins, following intracellular transduction of signals. We also note that upon activation of growth factor receptors, Eps8 undergoes post-translational modifications, including tyrosine phosphorylation and increased serine phosphorylation (Fazioli *et al.*, 1993). If these modifications were to control the monomer-dimer ratio, they would represent an interesting novel modality of regulation of a signal transducer: a possibility that warrants further investigation.

Materials and methods

Production of recombinant proteins

GST fusion proteins containing large fragments of the proteins of interest were obtained by recombinant PCR of the appropriate fragment from the murine or human Eps8 (Fazioli *et al.*, 1993; Wong *et al.*, 1994), human e3b1 (Biesova *et al.*, 1997), human RN-tre (Matoskova *et al.*, 1996a,b) and human cl.74 (this paper), followed by cloning in the pGEX-KT expression vector, in-frame with the GST moiety.

GST fusions containing short peptides from the proteins of interest were obtained by annealing *in vitro* complementary oligonucleotides followed by cloning in a pGEX-KT vector. Sequences of the oligos and primers used are available on request. Purification of the GST fusion proteins onto glutathione-Sepharose 4b (Pharmacia) was performed as described previously (Wong *et al.*, 1995; Matoskova *et al.*, 1996a,b).

Antibodies

Antibodies used were a previously described anti-Eps8 polyclonal serum directed against amino acids 583–821 (Fazioli *et al.* 1993), a previously described anti-e3b1 polyclonal serum directed against amino acids 2–480 (Biesova *et al.*, 1997) and an affinity-purified anti-GST polyclonal serum (Matoskova *et al.*, 1995a).

Binding assays

Three different kinds of binding assay were employed, designed to detect interactions between Eps8 (or its SH3) and various binding proteins, under different conditions of folding of the proteins involved in binding. In *in vitro* binding assays, native GST fusions of putative Eps8 ligands were tested for their ability to bind to native Eps8 present in cell lysates. In Far-Western assays, a native purified Eps8 SH3 domain was tested for its ability to bind to immobilized, denatured putative binders. In the ELISA assays, a native purified Eps8 SH3 domain was tested for its ability to bind to native, immobilized putative binders.

For *in vitro* binding assays, 10 µg of immobilized purified GST fusion proteins, or wild-type GST, were incubated with 1–2 mg of total cellular proteins for 60 min at 4°C. After several washes, proteins were eluted and analyzed by immunoblotting, as described previously (Fazioli *et al.*, 1993; Matoskova *et al.*, 1995). Immunoblots were decorated with the appropriate primary antibody and detection was performed with horseradish peroxidase (HRP) conjugated with specific secondary antisera, followed by enhanced chemiluminescence reaction (Amersham).

For Far-Western experiments with recombinant GST fusion proteins, 50 ng of each protein were fractionated by SDS-PAGE and transferred onto nitrocellulose. Blots were blocked in 5% non-fat powdered milk in TTBS (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20) for at least 2 h at room temperature and then in reduced glutathione (Sigma, 3 µM) in TTBS with 0.5% w/v bovine serum albumin (BSA) for 1 h at room temperature. This latter step substantially reduced the background (not shown). Blots were then incubated with the biotinylated purified Eps8-SH3 (3 nM) in TTBS in the presence of reduced glutathione (3 µM) and BSA (0.5% w/v) for 1 h at 4°C. After extensive washing in TTBS, blots were detected with 0.5 µg/ml HRP-conjugated streptavidin (Pierce) followed by enhanced chemiluminescence (Amersham).

For ELISA assays, bacterially expressed GST fusions were purified by glutathione-Sepharose and quantitated by BCA assay (Pierce). GST fusions (0.4 nM, 50 µl/well) were then absorbed onto 96-well Nunc polysorp ELISA plates, for at least 12 h at 37°C. After three washes with TTBS, plates were blocked with 100 µl of 4% BSA in TTBS, left overnight at 4°C and then washed three times with TTBS. Biotinylated GST or GST-Eps8-SH3 was then added at the indicated concentrations (0.05–1.0 µM) in a total volume of 50 µl of 1% BSA in TTBS, and incubated at room temperature for 2 h. Wells were rapidly washed six times with ice-cold TTBS and then incubated with 50 µl (1 µg/ml) of Immunopure alkaline phosphatase-conjugated streptavidin (Pierce) in 1% BSA in TTBS for 2 h at room temperature. After six rapid washes with ice-cold TTBS, reactions were developed by incubating plates with 50 µl of alkaline phosphatase substrate (Pierce).

For biotinylation of the GST fusion proteins, Immunopure Sulfo-NHS-Biotin (Pierce) was used; 0.1–1.0 mg of GST fusion proteins were incubated in the presence of Sulfo-NHS-Biotin (0.1 mg/ml) in a final volume of 0.5 ml for 1 h at room temperature in 50 mM NaHCO₃ pH 8.5. Biotinylated fusion proteins were separated from free biotin on either NAP-5 or PD-10 columns (Pharmacia).

Screening of phage-displayed libraries

The peptide library utilized and the panning conditions were essentially as described by Felici *et al.* (1991), the main difference being that the target SH3 domains (3 µg of a GST fusion) were immobilized by binding to 20 µl of GST-Sepharose matrix (Pharmacia). Two panning cycles were carried out for each domain before sequencing of the clones that were identified as positive in an ELISA assay.

Cloning of the cl.74 cDNA

A GST fusion protein containing the isolated SH3 domain of Eps8 (GST-Eps8-SH3; Matoskova *et al.*, 1995) was used to screen a pCEV-LAC-based prokaryotic expression library from M426 human fibroblasts

(a kind gift of T.Miki). Recombinant plaques (5×10^6) were screened after induction with isopropylthio-β-D-galactopyranoside using a modification of the Far-Western assay developed to identify proteins interacting with GST fusions (Matoskova *et al.*, 1996a,b). One of the positive phages, cl.74, was plaque purified and its ~1.7 kbp insert excised from the recombinant phage and subcloned into pBluescript. DNA sequencing was performed by the dideoxy-termination method on both strands of the cl.74 cDNA, using a commercial kit (Sequenase). The DDBJ/EMBL/GenBank databases were screened with the BLAST program (Bairoch and Boeckmann, 1994).

FPLC purification of SH3 domains and cross-linking experiments

SH3 domains were prepared by thrombin digestion of agarose-immobilized GST fusion proteins and purified by gel filtration using a Superdex 75 PC 3.2/30 column connected to a SMART system (Pharmacia Biotech). The column was equilibrated with phosphate-buffered saline and run at room temperature with a 0.1 ml/min flow rate.

Monomeric and dimeric forms of SH3 domains (operationally defined as such on the basis of their retention time in size-exclusion chromatography) were collected separately and cross-linked with a 10-fold molar excess of DSS (Pierce) in dimethylsulfoxide. The reaction was incubated at room temperature for 30 min, followed by quenching with 20 mM Tris-HCl. In some experiments, SH3 domains (50–100 mM), from either the monomeric or dimeric peaks, were incubated with the target peptides E7 (corresponding to the sequence of e3b1/7) or R8 (corresponding to RN-tre/8), or an unrelated peptide DS1 (TICIGCLAREDL) in excess molar ratio (from 4- to 8-fold, typically) and cross-linked with DSS (1–2.5 mM). Samples were analyzed by SDS-PAGE and Coomassie Blue staining.

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