Structural Insight into Modest Binding of a Non-PXXP Ligand to the Signal Transducing Adaptor Molecule-2 Src Homology 3 Domain*

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Although some exceptional motifs have been identified, it is well known that the PXXP motif is the motif of ligand proteins generally recognized by the Src homology 3 (SH3) domain. SH3-ligand interactions are usually weak, with ordinary $K_D \sim 10 \mu M$. The structural basis for a tight and specific association ($K_D = 0.24 \mu M$) between Gads SH3 and a novel motif, PX(V/I)(D/N)RXXKP, was revealed in a previous structural analysis of the complex formed between them. In this paper, we report the crystal structure of the signal transducing adaptor molecule-2 (STAM2) SH3 domain in complex with a peptide with a novel motif derived from a ligand protein, UBPY. The derived $K_D$ value for this complex is 27 $\mu M$. The notable difference in affinity for these parallel complexes may be explained because the STAM2 SH3 structure does not provide a specificity pocket for binding, whereas the Gads SH3 structure does. Instead, the structure of STAM2 SH3 is analogous to that of Grb2 SH3 which, in addition to normal PXXP ligands, has also been shown to moderately recognize the novel motif discussed herein. Thus, the extremely tight interaction observed between Gads SH3 and the novel motif is caused not by an innate ability of the novel motif but rather by an evolutionary change in the Gads SH3 domain. Instead, SH3 domains of STAM2 and Grb2 retain the moderate characteristics of recognizing their ligand proteins like other SH3 domains for appropriate transient interactions between signaling molecules.

In a wide variety of intracellular proteins in living cells, the SH3 domain is known as one of the ubiquitous protein-interaction modules that contribute to constructing complicated signaling networks (1). The SH3 recognizes a proline-rich sequence of its ligand protein (2) with a $\phi$PXX$\phi$P (PXXP) motif, which is usually further classified into $+\phi$PXX$\phi$P (class I) and $\phi$PXX$\phi$P+ (class II) (where $\phi$ and + are usually a hydrophobic residue and an arginine residue, respectively). Each motif adopts a left-handed type II polyproline (PPII) helix, known as a collagen chain conformation, and fits the ligand-binding site on the SH3 surface. Ligand recognition by an SH3 domain is not exclusive but rather promiscuous. An SH3 domain may interact with several ligand proteins in vivo and vice versa, which are due not only to the low ligand recognition specificity of the domain but also to the weak interactions, with $K_p$ (dissociation constant) values of $1 \times 10^{-5} \mu M$ in most cases (1). This results in the formation of a nonlinear, complicated network consisting of many SH3-containing proteins and their ligand proteins (1, 3).

The Grb2 C-terminal SH3 domain (SH3(C)) interacts with Sos, c-Cbl, Gab1, Gab2, SLP-76, and Vav in vivo and also interacts with UBPY and ASMH in vitro (4–10). The Sem5 (Grb2 in Drosophila) SH3(C) in complex with a Sos-derived class II peptide, PPVVPPRRR (where consensus residues are underlined), is a typical example of an SH3-ligand complex. In the structure, the ligand peptide adopts a PPII helix conformation, and the side chain of the underlined arginine residue electrostatically interacts with the conserved negatively charged pocket on the SH3 surface formed by its acidic RT loop (4).

It has, however, become apparent that some SH3 domains recognize motifs that do not contain the $\phi$PXX$\phi$P motif. PX(V/I)(D/N)RXXKP is a classic example of such a motif, first identified in a deubiquitinating enzyme UBPY (9, 11, 12), a binding partner of the SH3 domain of mouse STAM2 (also known as Hrs binding protein) (13–15). STAM2, in cooperation with Hrs, functions in the endocytic degradation pathway of growth factor-receptor complexes, which requires the SH3 domain of STAM2 (15). Moreover, a proteomics screening suggested that the SH3 domain of yeast Hse1p, a homolog of STAM (16), is most likely to associate with a deubiquitinating enzyme Ubp7, although its biological implications have yet to be elucidated (3).

In addition to the SH3 domains of STAM proteins, Gads (a Grb2-related protein) SH3(C) interacts with the novel motif, PX(V/I)(D/N)RXXKP, of SLP-76 very strongly, with a $K_p$ of 0.24 $\mu M$. This indicates an $10^5$ times higher affinity compared with ordinary interactions between SH3 domains and their PXXP ligands (17). The solved structure of Gads SH3(C) in complex with the novel motif revealed that, in addition to hydrophobic interactions with the (Val/Ile) residue of the motif, electrostatic interactions with the RXXK region enables this strong binding. Moreover, Grb2 SH3(C) requires the novel motif as a sequence in some of its ligands such as c-Cbl, Gab1, Gab2, and SLP-76.
However, the interaction between Grb2 SH3(C) and the novel motif is much weaker than its Gads SH3(C)-related counterpart, with an ordinary $K_D = 6-20 \mu M$ (5, 18). From the crystal structure of the STAM2 SH3 domain in complex with a UBPY-derived peptide reported here, we discuss the structural importance of a novel, but modest, interaction for ligand recognition.

**EXPERIMENTAL PROCEDURES**

**Preparation of the SH3 Domain—**The DNA sequence of the SH3 domain (residues 204–261) of mouse STAM2 was amplified by PCR and was cloned into the pTYB12 expression vector of the IMPACT-CN system (New England BioLabs) containing the N-terminal intein and chitin-binding domain unit. Proteins were expressed in *Escherichia coli* ER2566 cells at 18 °C for half a day after induction with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside. After being harvested, cells were lysed in standard buffer (20 mM HEPES, pH 8.2, 0.5 M NaCl, 1 mM EDTA) by sonication on ice, and the lysate was centrifuged at 4 °C. The supernatant was loaded onto the chitin bead column, and the column was washed with standard buffer. By incubating for 2 days at 20 °C for the on-column self-cleavage reaction with standard buffer supplemented with 50 mM dithiothreitol, the SH3 domain was cleaved from the fused tag and eluted. The eluted SH3 domain was further purified on a gel filtration column and concentrated in 20 mM Tris-HCl and 0.2 M NaCl, pH 8.0.

**Peptide Preparation—**Initially, we synthesized a UBPY-derived 9-mer peptide (PVMNRENKPP (700–708)) by the standard Fmoc method. Because crystallization of the SH3 domain in complex with this peptide had failed because of low affinity, a longer (11-mer) peptide (TPMVNRENKP (709)) was prepared (synthesized by Sigma). This peptide was dissolved in 2 mM Tris-HCl and used for the subsequent spectroscopic and co-crystallization experiments. A Ubp7-derived peptide and poly-L-proline (molecular weight range of 100 Da to 1 kDa), used in fluorescence spectroscopy, were purchased from Sigma.

**Fluorescence Spectroscopy—**Interaction between the STAM2 SH3 domain and peptides in solution was observed by fluorescence intensity change at 345 nm. Samples were excited at 295 nm to minimize the excitation of residues other than tryptophan at the STAM2 SH3 domain ligand-binding site (Trp-239 and Trp-240) (19). The dissociation constant was calculated according to a function described by Nguyen et al. (20). Non-linear least square fitting of the data to the function was carried out with GNUPLOT.

**Crystallography of the STAM2 SH3 Domain—**The SH3 domain was crystallized at 25 °C using the vapor diffusion method, with the drops consisting of a 1:1 mixture of 1 mM (~7 mg/ml) protein solution and the reservoir solution (0.1 M Tris-HCl and 1.9 M ammonium sulfate, pH 9.0). Prior to data collection, the crystal was flash-frozen in liquid nitrogen with 20% glycerol in the crystallization solution. A set of data was collected on beamline BL-6A at the Photon Factory and processed and merged with DPS (21). The initial phase was determined by the molecular replacement method using EPMR (22). The minimized mean structure of the Grb2 SH3(C) domain determined by NMR (PDB code 1GFC) was used as a search model (23). The structure was refined by using XcalYView (24) and CNS (25). Crystallographic statistics are as follows: space group $P2_1_2_1_2_1$; unit cell parameters $a = 64.1$, $b = 44.38$, $c = 52.74$, $\alpha = \gamma = 90^\circ$, $\beta = 116.0^\circ$; resolution = 2.5 Å; completeness = 99.9%; and $R_{merge} = 6.7%$.

**Crystallography of the SH3-peptide Complex—**The SH3 domain (4.6 mM) was mixed at a 1:2 molar ratio with the 11-mer UBPY peptide and subsequently crystallized at 25 °C using the vapor diffusion method. The drops consisted of a 1:1 mixture of the complex solution and the reservoir solution (0.08 M NaH2PO4 and 1.92 M K2HPO4). Prior to data collection, the crystal was flash-frozen in liquid nitrogen with 20% glycerol in the crystallization solution. A set of data was collected on L261B1 at SPring-8 and processed with MOSFLM (in CCP4; Ref. 26). The initial phase was determined by the molecular replacement method using EPMR. The unresolved structure of the STAM2 SH3 domain was used as a search model. The structure was refined using XcalYView, CNS, and Refmac5 (27). Molecular graphics in the figures were drawn with PyMOL (http://pymol.org), with the exception of Fig. 2A (drawn with GRASP; Ref. 28) and Fig. 2C (drawn with molscript (29) and Raster3D (30)). The Protein Data Bank codes of the SH3 domain models used for structural comparison with the STAM2 SH3 domain are 1H3H (Gads SH3(C); Ref. 17), 1GCQ (Grb2 SH3(C); Ref. 8), 1SEM (SEM-5 SH3(C); Ref. 4), and 1CKA (c-Crk SH3(C); Ref. 31). Crystallographic statistics are as follows: space group $P2_1_2_1_2_1$; unit cell parameters $a = b = 48.45$, $c = 58.58$, $\alpha = \gamma = 90^\circ$, $\beta = 120^\circ$; resolution = 1.7 Å; completeness = 97.9%; and $R_{merge} = 6.4%$.

**RESULTS**

**Binding Affinity of the Novel Motif to the STAM2 SH3 Domain—**Two sites containing the novel motif, PXV/I/K/D/N/RXXKP, have been found in mouse UBPY that interact with the STAM2 SH3 domain (9). PMYRNKENP (700–708) is more highly conserved among the species than PQYDRTKPK (405–413) (conserved residues are underlined). Thus, we prepared the ligand peptide containing the former sequence, 1TPMVRENKP11 (699–709). To begin with, we measured the dissociation constant ($K_D$) of the complex formed between STAM2 SH3 and the UBPY-derived peptide. The $K_D$ value, derived from tryptophan fluorescence experiments, was 27 μM (Fig. 1A). The affinity is significantly (100×) lower than the reported one between Gads SH3(C) and the novel motif (PMYRNKENP; 0.24 μM; Ref. 7). Instead, the $K_D$ of the STAM2 SH3-peptide complex is comparable to those of complexes between Grb2 SH3(C) and its novel motif-containing ligands ($K_D$ values ranging from 6 to 20 μM; Refs. 5 and 18), as well as those of ordinary interactions between SH3 domains and φPXφPφ ligands (1–10 μM; Ref. 1). These findings imply that the novel motif does not necessarily function to enhance binding to SH3 domains; rather, the interaction between Gads SH3(C) and the novel motif is considered extraordinary. In terms of the degree of binding affinity, the

![Fig. 1. Fluorescence change of STAM2 SH3 domain by peptide binding.](image)
interaction between STAM2 SH3 and its ligand is considered ordinary, despite its unusual ligand sequence.

**UBPY Peptide Binding Site and Peptide Conformation**—The differences in the SH3-ligand affinity are thought to have originated from their structural differences. To confirm this idea, we solved the crystal structure for the STAM2 SH3 domain in complex with the UBPY-derived peptide ligand at 1.7 Å resolution (Table I). The two C-terminal residues of the peptide are not included in the final model because they do not show definite peaks on an electron density map, probably because of disorder. The ligand-binding site of the STAM2 SH3 domain consists of a rather hydrophobic surface suitable for polyproline type II helix binding and an adjacent acidic region formed by the RT and n-Src loops, common among other SH3 domains (Fig. 2, A and B). Gads SH3(C) (MODEL1 in the NMR ensemble structure; Ref. 17), Grb2 SH3(C); Ref. 8), SEM-5 SH3(C); Ref. 4) and c-Crk SH3(C); Ref. 31) are superposable upon the structure of the STAM2 SH3 domain, with root-mean-square difference values for all C atoms of 1.3, 0.5, 0.7, and 0.9 Å, respectively. The root-mean-square difference value for Grb2 SH3(C) superposed with SEM-5 SH3(C) is 0.6 Å. These values imply that STAM2 SH3, Grb2 SH3(C), and SEM-5 SH3(C) share similar overall structures (Fig. 2C). The relatively large difference between the STAM2 and Gads SH3 domains may be attributed to drastic changes of the Gads SH3(C) around the hydrophobic pocket (see below) as well as the fluctuations observed in the NMR structure, which tends to cause larger differences than the crystal structures (32, 33).

Based upon the studies of the interactions of the SLP-76 peptide with the Grb2 and Gads SH3(C) domains, the UBPY peptide binds in the vicinity of the general STAM2 SH3 ligand-binding site, as predicted. The second half of the peptide adopts a right-handed 3_10 helix conformation, which has never been observed in pXpY motifs (Fig. 2B). Superposition of the SLP-76 peptide (1APSIDRSTKPA31) onto the UBPY peptide reveals a similarity between the two peptide structures (Fig. 2D). Notable differences are seen around Val-4 and the conformation of the Asn-5, Arg-6, and Lys-9 side chains involved in the mediation of essential hydrogen bonds (see below). Of the noted inconsistencies, the δ oxygen of the Asn-5 side chain provides two intramolecular hydrogen bonds connecting the oxygen with neighboring Arg-6 and Glu-7 nitrogen atoms of the main chain (Fig. 3A). Although the asparagine (Asn-5) can be replaced by an aspartic acid (Asp-5) in the novel motif (p[X/V/I]/D/N)pXpY; Ref. 24), these hydrogen bonds are probably retained and function to sustain the motif’s conformation.

**Interactions in which the First Half of the UBPY Peptide Is Involved**—Superposition of a quintessential complex, SEM-5-Sos, on the STAM2-UBPY complex shows that the overall position of the first half of the UBPY peptide (1TPMVNR8) is similar to that of the Sos peptide (1PPPVPP9; class II). 1TPMV residues and the tip of the Arg-6 side chain overlap with Sos residues (Fig. 2, C and E). The first two residues (Thr-1/Pro-2) and Val-4 of the UBPY peptide function in the same way as the first pXpY unit and second p of the pXpY motifs. Two hydrogen bonds connecting Arg-6 with Glu-220 in the RT loop contribute to binding by electrostatic interactions (Fig. 3A). However, because the novel motif lacks a residue corresponding to the second proline in the pXpY motif, the UBPY peptide binds more loosely around Asn-5 than the Sos peptide (Fig. 2E). Thus, it is expected that the binding of TPMVNR is not as tight as that of pXpYpX (class II). In fact, this observation is consistent with a study conducted on the interaction between Grb2 SH3(C) and Gab1-derived peptides containing the novel motif in which the first half of the motif is required but not sufficient for binding to Grb2 SH3(C) (10).

**Comparison with the “Second Pocket” of the Gads SH3(C) Domain**—Recognition of I4 in the SLP-76 peptide (1APSIDRSTKPA11) by Gads SH3(C) is achieved in a remarkable way. The Ile-4 fits into the hydrophobic pocket (referred to as the second pocket; Ref. 17) formed by Glu-275, Ser-299, Trp-300, Pro-313, and Asn-315 (Tyr-316 is not involved in this pocket, different from the original description; Ref. 17) (Fig. 4C). Although this area is usually a binding site for the second p di-peptide of the pXpY motif, one notable feature of Gads SH3(C) is that the pocket is enclosed on one side by the Glu-275 side chain, resulting in a smaller pocket. Liu et al. (17) explained that this pocket is too small to accommodate a pXpY unit and is, therefore, not a favorable binding site for pXpYpX ligands. In fact, the Sos-derived peptide (class II) has a 47-times lower affinity for Gads SH3(C) than does the SLP-76 peptide. However, the Glu-214 side chain of the STAM2 SH3 domain, corresponding to the Glu-275 of Gads, extrudes to solvent and does not interfere with the second p di-peptide binding region (Fig. 4A). The direction of phenylalanine side chains are also opposite. The phenylalanine corresponding to Phe-213 of STAM2 or Phe-274 of Gads is one of the most conserved residues in the hydrophobic core of the SH3 domain (32). Here, Phe-213 of STAM2 SH3 contributes to hydrophobic core formation, whereas, surprisingly, the corresponding Phe-274 of Gads SH3(C) is exposed to solvent. In short, the positions of the Phe-Glu pair are exchanged in the STAM2 and Gads SH3 domains. Fig. 4A shows that, if a UBPY peptide binds to Gads SH3(C), the position of UBPY Val-4 would be too close to Glu-275 of Gads, and UBPY would be repelled by steric repulsion. This finding demonstrates the highly specific ligand recognition ability of the Gads SH3 domain, whereas the STAM2 SH3 domain maintains lower recognition ability, much like that of ordinary SH3 domains.

**Association with a pXpYpX Peptide**—The modesty of STAM2 SH3 domain should not be treated as a disadvantageous property, as weak, transient interactions play a significant role in signal transduction. This property may allow other ligand proteins to bind to the STAM SH3 domain. To verify the proposal that STAM2 SH3 recognizes the PPII helix conformation, a binding experiment with synthetic poly-L-proline peptides (19) was conducted (Fig. 1B). Next, we prepared a peptide, RKRP-PPPPP; Ref. 24), originally found in yeast Ubp7 (see beginning of text). Although there has been no evidence that this sequence is related to SH3 domains, we selected it because it seems to be the most viable candidate in the Ubp7 sequence as a pXpYpX
Fig. 2. Ligand peptides binding to SH3 domains. A, surface potential of STAM2 SH3. Positive and negative potentials are shown in blue and red, respectively. Side chains of Arg-6 and Lys-9 of the peptide face toward the acidic RT-loop. B, ribbon representation of the complex. The peptide is shown in yellow. C, superposition of three SH3-ligand complexes. STAM2-UBPY, SEM-5-Sos, and c-Crk-C3G complexes are shown in orange-yellow, violet-gray, and green-cyan pairs, respectively. D, superposition of the SLP-76 peptide on the UBPY peptide. UBPY and SLP-76 are shown in yellow and green, respectively. SLP-76 structure from the most representative conformer is used for comparison. E, positions between Val-4 (UBPY) and Ile-4 (SLP-76) show the largest difference. Side chain directions of Arg-6 and Lys-9 are also opposite. F, position definition and sequences of the three ligand peptides. Residues corresponding to the P$_{-1}$ position are enclosed in blue. The wavy line denotes the 3$_{10}$ helix.
sequence. The STAM2 SH3 domain weakly associates with this class I (+X_{i}PX_{i}P) peptide ($K_D = 74 \mu M$) (Fig. 1A). This result indicates that STAM2 may interact with normal $\phi PX_{i}\phi P$ ligands in vivo.

Structural Basis for Understanding the Differences in Binding Affinity—As described above, neither STAM2 SH3 nor Grb2 SH3(C) are as tightly bound to the novel motif as Gads SH3(C). The exchange of the Phe-Glu positions may play a significant role in the difference of affinity. The Gads SH3(C) domain provides a deep pocket to catch Ile-4 of SLP-76 with adjacent Glu-275 side chains, resulting in a significantly stronger binding (Fig. 4C). Contrastingly, the corresponding region of STAM2 SH3 where Val-4 of the UBPY peptide binds is rather flat and there is no pocket (Fig. 4B). This is also true with the surface region of SEM-5 SH3, where the ligand valine (the second $\phi$ in the $\phi PX_{i}\phi P$) is positioned (Fig. 4D). As a result of the exchange of the Phe and Glu positions in the Gads SH3(C) domain, the interacting surface area between the side chain of Ile-4 and Gads extends to 235 $Å^2$, whereas it is only 150 $Å^2$ in the Val-4-STAM2 interaction. This suggests that, unlike the Gads SH3(C) domain, no evolutionary change favoring a higher affinity has occurred at the Val-4/Ile-4 binding site in either the STAM2 SH3 or Grb2 SH3(C) domains.

The Lysine-specific Interactions—The most notable structural feature of the novel motif is the $\beta_{10}$ helix of the RXXK (*REPENK*) of UBPY region. This helix enables Arg-6 and Lys-9 side chains to extend in parallel toward the RT loop establishing widespread electrostatic interactions (Fig. 2A) (17). In the present crystal structure, static hydrogen bonds can be identified. In addition to an extensive hydrophobic interaction with Trp-239, the Lys-9 side chain specifically binds to the STAM2 SH3 surface by hydrogen bonds connecting Lys-9 with a triad of oxygen atoms from the side chains of Glu-217, Gln-219, and Glu-220 (Fig. 3B). This interaction resembles that of the c-Crk SH3(C)-ligand (C3G peptide: PPAPLPKPKG) complex, in which the corresponding basic (+) residue in $\phi PX_{i}\phi P$ (class II) is not an arginine but a lysine (underlined in the above C3G sequence) (Fig. 3C) (31). Superposition of the C3G peptide on the STAM2-UBPY complex shows that the positions of the lysine amino groups correspond to one another (Fig. 2C). Thus, each of the basic residues in the RXXK region is thought to function in an equivalent manner as the residues present at the $P_{-3}$ position of usual motifs (Fig. 2F).

Despite the electrostatic interactions achieved by the RXXK region, which are thought to contribute to the increase in affinity between the novel motif and the SH3 domains, the interaction between STAM2 SH3 and the UBPY peptide is weak ($K_D = 27 \mu M$). This result may be explained by the fact that the novel motif lacks the residue corresponding to the second proline in $\phi PX_{i}\phi P$, which causes the UBPY peptide to bind loosely around Asn-5 than a $\phi PX_{i}\phi P$ peptide (Fig. 2E) and offsets the advantageous two-sword interaction accomplished by the Arg-6 and Lys-9 side chains.

**DISCUSSION**

The dynamic association as well as dissociation of intracellular signaling components is indispensable in accurately transmitting signals. High affinity interaction ensures tight association of signaling molecules, whereas low affinity interaction causes transient association of molecules with broader specificity. Interaction mediated through SH3 domains is generally modest in nature, resulting in construction of an entangled network of signaling molecules (1, 3).

The physiological significance of such a moderate interaction has recently been discussed (1). Binding partner proteins for an SH3 domain is not necessarily fixed beforehand but can be selected based on cellular contents, especially on local concentrations of SH3 domains and their ligands (34). The STAM2 SH3 domain has been reported to favor ligands with a $\phi PX_{i}\phi P$ sequence (9), which we were able to confirm structurally in the present study. Nevertheless, we also found that the STAM2 SH3 domain does not destroy the surface structure of the ligand-binding site suited for binding $\phi PX_{i}\phi P$ ligands. This was confirmed by fluorescent spectroscopy data. These results further suggested that STAM2 SH3 may interact with $\phi PX_{i}\phi P$ ligands, although no ligands other than UBPY have been identified in vivo thus far. Endosomal localization of STAM2 (35) may restrict the ligands that can interact with STAM2.

One may suppose that unique motifs exist to offer specific interactions, which are exhibited in the interaction with the Gads SH3(C) domain. However, the interactions with the STAM2 SH3 domain as well as with the Grb2 SH3(C) domain are not extraordinary in terms of their moderate association affinity. Non-PXXP ligand sequences other than PX(V/I)(D/E)...
NRXXKP, such as PXDDY or RKXXXXY, have also been reported (36, 37). Again, in both cases, interactions with SH3 domains are moderate, as indicated by their $K_d$ values (20–60 μM). As a consequence, unique motifs do not necessarily contribute directly to the increase in affinity of SH3 domains.

Structural analysis of the STAM2 SH3 domain confirmed the specialty intrinsic to the preceding structure of the Gads SH3(C) domain. As complete exposure of the conserved phenylalanine (Phe-274 of Gads) to solvent is exceptional and appears to be energetically unfavorable, there should have been strong evolutionary pressure for increasing the affinity between Gads SH3(C) and its ligand protein, SLP-76. On the contrary, the STAM2 SH3 domain is designed for moderate and transient association with UBPY, and also implies some potential to do something with unknown ligand proteins.

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