Novel recognition mode between Vav and Grb2 SH3 domains

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Vav is a guanine nucleotide exchange factor for the Rho/Rac family that is expressed exclusively in hematopoietic cells. Growth factor receptor-bound protein 2 (Grb2) has been proposed to play important roles in the membrane localization and activation of Vav through dimerization of its C-terminal Src-homology 3 (SH3) domain (GrbS) and the N-terminal SH3 domain of Vav (VavS). The crystal structure of VavS complexed with GrbS has been solved. VavS is distinct from other SH3 domain proteins in that its binding site for proline-rich peptides is blocked by its own RT loop. One of the ends of the VavS β -barrel forms a concave hydrophobic surface. The GrbS components make a contiguous complementary interface with the VavS surface. The binding site of GrbS for VavS partially overlaps with the canonical binding site for proline-rich peptides, but is definitely different. Mutations at the interface caused a decrease in the binding affinity of VavS for GrbS by 4- to 40-fold. The structure reveals how GrbS discriminates VavS specifically from other signaling molecules without binding to the proline-rich motif.

Keywords: crystal structure/growth factor receptor-bound protein 2/protein–protein interaction/Src-homology 3 domain/Vav

Introduction

The Src-homology 3 (SH3) domain is a family of molecular modules conserved among diverse proteins (Birge *et al.*, 1996), which functions in protein–protein interactions for intracellular signal transduction. These interactions are mediated commonly among the SH3 domain-containing proteins through the recognition of a short proline-rich sequence embedded in proteins (Feng *et al.*, 1994; Lim *et al.*, 1994; Terasawa *et al.*, 1994). To accomplish the high specificity and proper affinity for the

SH3 domain, the peptide must adopt a polyproline-type II (PPII) helical conformation. The most famous example of this recognition is the interaction of growth factor receptor-bound protein 2 (Grb2) with son of sevenless (Sos), the guanine nucleotide exchange factor for Ras. Grb2 is an adaptor protein consisting of one SH2 domain flanked by two SH3 domains. It has been well established that Grb2 transmits the upstream signal through its association with proline-rich regions of Sos, via the SH3 domains (Egan *et al.*, 1993). As other targets of the Grb2 SH3 domains, proline-rich proteins such as Cbl, dynamin and WASP have been reported (reviewed in Buday, 1999). Vav is among the proposed targets of Grb2 (Machide *et al.*, 1995; Hanazono *et al.*, 1996; Miyakawa *et al.*, 1997).

Vav is a protein with a molecular mass of 95 kDa, which is expressed exclusively in hematopoietic cells (Katzav *et al.*, 1989). Vav contains a calponin-homology domain, a Dbl-homology domain, a pleckstrin-homology domain, an SH2 domain and two SH3 domains. Numerous biochemical studies have established that Vav plays pivotal roles in lymphocyte cell differentiation and proliferation, lymphokine production and cytoskeletal reorganization (reviewed in Bustelo, 2000). These cellular responses are induced by extracellular stimuli to various receptors, most of which trigger rapid tyrosine phosphorylation of Vav. The C-terminal region of Vav arranged in the order SH3–SH2–SH3 is required for its transforming activity (reviewed in Bustelo, 1996). The downstream molecules of Vav include members of the Rho/Rac family of small G proteins. The Dbl-homology domain of Vav is the guanine nucleotide exchange factor for Rac1 that activates the c-Jun N-terminal kinases (JNKs) (Crespo *et al.*, 1997). As suggested by its structure, an array of signaling proteins is associated with Vav. In manners that are dependent or independent of the extracellular stimuli, Vav is recruited to the multiple protein assemblies at the plasma membrane together with some of these proteins. Such signaling proteins include phosphatidylinositol 3-kinase (Lahesmaa *et al.*, 1995), Slp76 (Tuosto *et al.*, 1996) and Shc (Ramos-Morales *et al.*, 1994; Pedraza-Alva *et al.*, 1998) in T cells, Slp65 (Wienands *et al.*, 1998) in B cells, and Raf1 and mitogen-activated protein kinase (Song *et al.*, 1996) in mast cells. It is notable that Grb2 also participates in these multiple protein assemblies, supporting the importance of Grb2 as an assembler of Vav and other molecules in the signaling complexes. Recent studies have focused on the transition mechanisms of Vav from the cytoplasm to cholesterol-enriched membrane microdomains (GEMs) (Simons and Ikonen, 1997). Vav is proposed to be tethered to GEMs by Grb2 (Kim *et al.*, 1998; Salojin *et al.*, 2000).

The Grb2 recognition of Vav is notable as compared with the classical SH3 recognition of peptide ligands, because it is mediated through the dimerization of the SH3 domains in both molecules. Using a yeast two-hybrid

screening and a filter-binding assay, it was confirmed that Vav binds to the Grb2 C-terminal SH3 domain via its own SH3 domain located on the N-terminal side of the SH2 domain (Ye and Baltimore, 1994; Ramos-Morales *et al.*, 1995). To understand further the role of the Vav SH3 domain in signal transduction in the immune system, and to elucidate how Vav is recognized by Grb2 in the context of the entire domain, we have solved the crystal structure of the N-terminal SH3 domain of Vav complexed with the C-terminal SH3 domain of Grb2. On the basis of the crystal structure of the complex and the mutagenesis study, we present details of the protein–protein interaction by the SH3 domains independent of the recognition of the PPII helix.

Results

Structure determination

The crystal structure of the N-terminal SH3 domain of Vav (VavS: residues 595–660 of mouse Vav) (Adams *et al.*, 1992) complexed with the C-terminal SH3 domain of Grb2 (GrbS: residues 159–217 of human Grb2) was determined by multiple isomorphous replacement (MIR) (Table I). The complex was crystallized as one VavS and two GrbS molecules contained in an asymmetric unit. The final model at 1.68 Å resolution was refined to an *R*-factor of 20.7% (Table II). All of the non-glycine residues are located within the most favored or additionally allowed regions on the Ramachandran plot (Ramakrishnan and Ramachandran, 1965). The crystal structure of GrbS-free VavS was also solved by molecular replacement utilizing the structure of GrbS-bound VavS. The GrbS-free VavS crystal contains four VavS molecules in an asymmetric unit, and the model at 2.1 Å resolution was refined to an *R*-factor of 19.6%.

Overall structure of GrbS-bound VavS

The three-dimensional structure of GrbS-bound VavS and a stereo view of its α carbons are shown in Figure 1A and

B, respectively. VavS is folded into the canonical topology conserved among all SH3 domain-containing proteins (Noble *et al.*, 1993). Two antiparallel β -sheets, each consisting of three β -strands, are folded together so as to form a β -barrel as a whole. β A forms the smaller β -sheet together with β E and β B, while β C together with β B and β D forms the larger one. The two β -sheets are packed against each other at an approximate right angle. In accordance with previous reports, the loops consisting of residues 600–623, 630–635 and 642–646 are designated as the RT, n-Src and distal loops, respectively. There are two remarkable features of VavS as compared with known SH3 domain proteins.

First, the RT loop of VavS is particularly notable for its unusual extension (Figure 2). The loop is arranged against the β -barrel such that it caps one of the ends (Figure 1A), and contains a continuous tetraproline sequence (607–610). The tetraproline region, which is in the PPII helical conformation, forms a close hydrophobic contact on the surface of the β -barrel (Figure 3). The pyrrolidine ring of Pro609 packs tightly into a hydrophobic pocket that is formed on the β -barrel surface as framed by Tyr603, Phe613, Gly614, Phe616 and Trp636, and thus is a pivot of this contact. Pro607 is located proximately to the side chains of Tyr603, Trp636 and Arg654. Pro610 that immediately succeeds Pro609 stacks closely against the side chains of Phe613 and Trp636. A hydrogen bond between the carbonyl group of Pro607 and the side chain of Tyr603 appears to be important for the stability of the backbone conformation of the tetraproline region. In contrast, the side chain of Pro608 is projected to the direction opposite to the β -barrel surface and does not contribute to any intra-domain interaction.

Next, on another end of the β -barrel, the N- and C-terminal parts and the n-Src loop are assembled (Figure 1). This region includes the concave surface, which can be compared with a valley (Figure 5A and B). The valley runs on the base of the β -barrel and its dimensions are 15 Å in length, 8 Å in width and 5 Å in

Table I. Data collection and phasing statistics

Crystal	GrbS-bound VavS						GrbS-free VavS
Data collection statistics							
data set	native	KAu(CN) ₄ ·1	KAu(CN) ₄ ·2	(CH ₃ COO) ₂ Pb	(CH ₃ COO) ₂ Hg	cisplatin	
spacing range (Å)	39.5–1.68	45.1–1.99	20–3.3	100–2.7	20–3.0	100–3.0	100–2.1
concentration (mM)		15.9	15.9	saturation	1.0	saturation	
wavelength (Å)	1.0375	1.0375	1.5418	1.5418	1.5418	1.5418	1.5418
No. of reflections	293 792	106 665	14 062	21 608	19 559	12 907	84 381
No. of unique reflections	28 649	17 301	4093	6673	5417	4920	14 004
completeness	0.972	0.968	0.985	0.925	0.991	0.939	0.942
<i>R</i> _{merge} ^a	0.066	0.061	0.066	0.059	0.056	0.041	0.035
Phasing statistics							
spacing (Å)		2.5	3.3	2.7	3.0	3.0	
No. of sites		1	2	6	8	2	
<i>R</i> _{iso} ^b	0.156		0.258	0.218	0.262	0.243	
<i>R</i> _{Cullis} ^{c,d}		0.61/0.64	0.75/0.74	0.75/0.68	0.82/0.79	0.93/0.92	
mean figure of merit ^{d,e}				0.551/0.771			

^a*R*_{merge} = $(\sum |I_i - \langle I \rangle|) / \sum I_i$, where *I_i* is the intensity of the *i*th observation and $\langle I \rangle$ is the mean intensity.

^b*R*_{iso} = $\sum |F_{PH} - F_P| / \sum F_P$, where *F_P* and *F_{PH}* are the native and derivative structure amplitudes, respectively.

^c*R*_{Cullis} = $\sum |F_{PH} - |F_P \exp(i\phi_c)| + F_H| / \sum |F_{PH} - F_P|$, where *F_H* is the calculated heavy-atom structure factor and ϕ_c is the calculated phase.

^dValues on the left and right sides of the solidus are for the acentric and centric reflections, respectively.

^eFigure of merit = $\sum P(\alpha) \exp(i\alpha) / \sum P(\alpha)$, where α is the phase and *P*(α) is the phase probability distribution.

Table II. Refinement statistics

	GrbS-bound VavS	GrbS-free VavS
Spacing range (Å)	8.0–1.68	8.0–2.1
No. of reflections	27 162	13 692
Completeness ^a	0.931 (0.772)	0.934 (0.771)
R-factor ^b	0.207	0.196
Free R-factor ^c	0.235	0.253
No. of protein atoms	1481	2151
No. of water molecules	189	190
No. of MPD molecules	1	–
R.m.s. deviation		
bond lengths (Å)	0.009	0.011
angles (°)	1.395	1.562
Average temperature factor (Å ²)		
main chain atoms	15.4	10.1
side chain atoms	19.4	12.7
solvent molecules	32.1	18.4

^aValues in parentheses are for the data between 1.85 and 1.68 Å resolution (for GrbS-bound VavS) and between 2.2 and 2.1 Å resolution (for GrbS-free VavS), respectively.

^bR-factor = $\sum |F_{\text{obs}} - F_{\text{calc}}| / \sum F_{\text{obs}}$, where F_{obs} and F_{calc} are the observed and calculated structure amplitudes, respectively. The structure amplitudes over twice their standard deviations were included in the refinement.

^cFree R-factor was calculated for a randomly chosen 5% of the total reflections that was not included in the refinement.

depth. The valley is lined at its bottom with hydrophobic residues, Met597, Leu627, Ala630 and Trp637, and is bordered by two side walls, one of which is defined by the N- and C-terminal residues of Pro595 and Pro657, and the other by residues 631–634 in the n-Src loop. Regarding the four VavS molecules in the GrbS-free form, their structures are essentially identical to that of the GrbS-bound form. The most deviating regions from the GrbS-bound form are in the RT (611–613) and n-Src loops (631–634) (Figure 1B).

Implications for the recognition of proline-rich peptides

In principle, the PPII helix of the peptide ligand is recognized by the SH3 domain, with one face of the trigonal prism packing into the hydrophobic pockets named P₋₃, P₋₁, P₀, P₊₂ and P₊₃ (notated by Yu *et al.*, 1994). The PxxPxR motif (x = any amino acid) (minus orientation) is conserved as a minimum consensus in the proline-rich peptides, and two prolines and an arginine within the motif occupy the P₊₂, P₋₁ and P₋₃ sites, respectively (Feng *et al.*, 1994; Lim *et al.*, 1994; Terasawa *et al.*, 1994). To clarify the features of the corresponding region of VavS, a mouse Sos-derived peptide (PPPVPPR), which is bound to the C-terminal SH3 domain of Sem-5 in the minus orientation (Lim *et al.*, 1994), was placed on the expected binding site on the VavS surface (Figure 3B). Note that the numbering of the sites runs counter to the direction of the peptide ligand. Some structural features of VavS must make it impossible to bind to proline-rich peptides.

First, the binding of VavS to the external peptides would be occluded due to the steric hindrance with its own tetraproline region (Figure 3B). In order to recognize the proline-rich peptide, detachment of the tetraproline region from the molecular surface is necessary. However, the

intra-domain interactions around Pro609 are tight and seem to contribute significantly to the stability of the VavS core (Figure 3). Indeed, the mutation of Tyr603 to phenylalanine, which disrupts the hydrogen bond between the side chain of Tyr603 and the carbonyl group of Pro607, caused a large decrease in the protein's solubility (our unpublished result). The crystal structures of the GrbS-free VavS molecules also support this idea, because the structures and the intra-domain recognition modes of the tetraproline region of the four molecules are identical to those of the GrbS-bound form (Figure 1B), suggesting that this region remains rigid irrespective of the crystallization conditions. Next, even if the tetraproline region was detached from the intra-domain binding site, VavS could not bind to the peptide ligand because it lacks some prerequisites for PPII helix binding. The acidic residue, which is indispensable for the peptide binding through electrostatic interactions with an arginine of the peptide ligand, is absent at the expected P₋₃ site of VavS. The aromatic amino acids defining the P₊₂ and P₊₃ sites, which are phenylalanine or tyrosine in general, are also replaced by Arg654 and Gln601 in VavS. As a result, the hydrophobic interactions between the conserved aromatic side chains and the proline of the peptide ligand at the P₊₂ site, which are essential for the recognition of the peptide ligand, are lost (Nguyen *et al.*, 1998).

Biochemical studies are also consistent with this view derived from the present crystal structure. The C-terminal SH3 domain of Vav has been reported to bind to some proline-rich proteins (reviewed in Bustelo, 1996), whereas no targets for VavS other than Cbl-b have yet been proposed. For the binding of Cbl-b, both SH3 domains of Vav might function synergistically (Bustelo *et al.*, 1997). Therefore, we have demonstrated that the functional significance of VavS is not in the binding to proline-rich molecules.

Interfaces between the VavS and GrbS molecules in the complex crystal

In the complex crystal, VavS closely contacts two GrbS molecules, GrbS A and GrbS B, at two separate interfaces (Figure 4). The structures of GrbS A and GrbS B are essentially identical to those determined in a previous report (Maignan *et al.*, 1995). The areas of the solvent-accessible surface of VavS that decrease through contacts with GrbS A and GrbS B are 1044 and 877 Å², respectively (calculated using a 1.4 Å radius probe).

The VavS–GrbS A interface

At the VavS–GrbS A interface, the N-terminal part of the RT loop, a 3₁₀ helix between βD and βE, and βE of GrbS A are assembled together (Figure 4). This assembly forms a convex surface as a whole, which packs against the valley formed at the base of the VavS β-barrel (Figures 5B and 6A). The side chains of three GrbS residues, Leu164, Phe165 and Thr211, protrude from the convex surface into the valley. They are lined along the valley in the order Thr211, Leu164 and Phe165 from the entrance to the exit. In particular, Leu164 and Phe165 in the RT loop are in close contact with the hydrophobic cluster at the bottom of the VavS valley, thereby forming a core of the inter-domain interface. The side chain of Leu164 is positioned in close proximity to those of Leu627, Ala630 and Trp637

Fig. 1. Overall structures of VavS molecules. (A) The ribbon diagram for VavS in the complex crystal is shown in green. The β -barrel axis of VavS is horizontal and on the plane of the drawing. β -strands and loops are labeled with their identification codes, and the N- and C-termini are labeled with N and C, respectively. The figure was prepared using MOLSCRIPT (Kraulis, 1991) and RASTER3D (Bacon and Anderson, 1998). (B) Stereo view of VavS α carbons in the complex crystal superposed on those in the GrbS-free form. GrbS-bound VavS is drawn as a thick line in red, and the four GrbS-free VavS molecules are drawn as thin lines in yellow, green, blue and magenta, respectively.

of VavS. As for Phe165, its aromatic ring is tightly locked into the crevice formed by the side chains of Met597, Trp637 and Pro657. These three GrbS residues are not only in contact with the bottom of VavS, but are also grasped by both side walls of the valley. On one of the side walls, three successive GrbS residues, Arg207, Asn208 and Try209 in the 3_{10} helix, are placed. The guanido group of Arg207 loosely covers the face of the side chain of Pro595, while the aromatic ring of Tyr209 surrounds Pro657 together with Phe165 (Figures 5B and 6A). Of particular note is the space between the side chains of Phe165 and Tyr209 that is occupied by VavS Pro657, because it is utilized ordinarily as the P_{+2} and P_{+3} sites for PPII helix binding. On another side wall, three hydrogen bonds are formed between the polypeptide backbone of Ala632 and the side chains of Gln162 and Arg179. The side chain of Arg179 is juxtaposed with Phe165 and partially occludes the exit of the VavS valley.

The VavS–GrbS B interface

The VavS–GrbS B interface consists of two sites, which are discontinuous. The VavS component of each site is the RT loop and the n-Src loop, respectively (Figure 4). The RT loop of VavS accounts for three-quarters of the overall area of the VavS–GrbS B interface through interactions with the PPII helix-binding site of GrbS B (Figure 6B). The tetraproline region in the RT loop is bound to GrbS B in a manner similar to that of the proline-rich peptide bound to the SH3 domain in the minus orientation. Namely, Pro607, Pro608 and Pro610 occupy the pockets on GrbS that define the P_{+3} , P_{+2} and P_0 sites, respectively. The interactions at these sites exactly follow the canonical mode. It should be noted that the P_{-1} site of GrbS is occupied by Gly611 from VavS, which is surrounded by Asn192, Trp193, Pro206 and Asn208 of GrbS. In general, this site is occupied by a consensus proline residue of the peptide ligand. The polypeptide backbone of Gly611,

however, packs into the P_{-1} site and makes tight contact with the four surrounding residues. The electrostatic interactions expected at the P_{-3} site are deficient, since the VavS residues succeeding Gly611 are separated from the GrbS B surface and turn back to the VavS core. Note that Pro609 is recognized by the VavS core from the direction opposite to the GrbS B interface (Figure 3). In another site at the VavS–GrbS B interface, the n-Src loop of VavS interacts with the RT loop of GrbS (Figure 4). The interactions in this site are not as close as those in the former site, and are rather hydrophilic. The side chains of His634 and Asn635 hydrogen-bond to those of Glu171 and Asp168 of GrbS, respectively. The backbone amide group of Asn635 also hydrogen-bonds to the side chain of Gln170 (Figures 2 and 4).

Mutagenesis study of the VavS–GrbS A interface

To investigate which interface in the complex crystal has physiological significance, we analyzed the effect of point mutations at each interface on the binding affinity of VavS for GrbS by surface plasmon resonance (SPR) measurements. GST-fused GrbS was immobilized on a sensor chip, and VavS solutions ranging from 1 to 500 μ M were injected into the cells sequentially for the measurements. As the sensorgram of wild-type VavS shows, the association and dissociation rates of VavS for the immobilized GrbS were so rapid that the binding affinity was estimated based on the number of response units at equilibrium (Figure 7A). The Scatchard plot indicates that the binding of VavS to GrbS is monovalent (Figure 7B). As the targets for point mutations on VavS, we selected some residues based on the crystal structure. The elution profiles of size exclusion chromatography and CD spectra profiles ($\lambda = 200$ –260 nm) of all the mutants indicated that they maintain the folded architecture (data not shown). The results of the affinity measurements are summarized in Table III.

Fig. 2. Sequence alignment of VavS with other SH3 domain-containing proteins. The secondary structures of VavS (Vav_mouse), assigned using PROCHECK (Laskowski *et al.*, 1993), are shown above the sequences as arrows with their identification codes. 310 refers to the 310 helix occurring between β D and β E of most SH3 domain proteins. The numbering above the sequences is for VavS. The labels at both ends of each sequence are the residue numbers in the full-length protein. VavS and GrbS (Grb2C_human) residues involved in the VavS-GrbS A interface are colored in red and green, respectively. Some of the VavS residues at the interface that are conserved among N-terminal SH3s of other Vav members are also colored in their respective sequences. On the lower lines, C-terminal SH3s of Sem-5 (Sem5C) and Vav (VavC_human), N-terminal SH3 of Grb2 (Grb2N_human) and SH3s of tyrosine kinases are listed. If available, their three-dimensional structures were used for the sequence alignment.

First, since Trp637 is the major component at the bottom of the GrbS-binding valley, replacement of its side chain by a smaller one is supposed to abolish the inter-domain interactions. In addition, the importance of Trp637 for supporting one of the side walls is emphasized, because it forms a hydrogen bond with the polypeptide backbone of His634 via its side chain (Figure 6A). As expected, the substitution of Trp637 for tyrosine caused a large decrease (~40-fold) in the binding affinity. Secondly, two prolines defining one side wall, Pro595 and Pro657, were mutated to alanine. The large decrease (~9-fold) in the affinity caused by the Pro657Ala mutation agrees well with the tight interactions of its pyrrolidine ring with Phe165 and Tyr209 of GrbS. On the other hand, the side chain interaction of Pro595 with the GrbS residues is less tight than that of Pro657, and its mutation was relatively

tolerable. Finally, to investigate the role of the n-Src loop in GrbS binding, we mutated Ala632 to glycine. Ala632 is located at the tip of the n-Src loop, and its polypeptide backbone and methyl group both closely contact the GrbS residues at the side of the valley. The mutation resulted in an affinity decrease of ~10-fold. This large decrease might be caused not only by the loss of the side chain interactions, but also by the change of the backbone conformation around Ala632 to the forms unsuitable for GrbS binding as a result of the glycine replacement.

Mutagenesis study of the VavS-GrbS B interface

Among the VavS residues at the VavS-GrbS B interface, Pro608 and Gly611 are in the closest contact with GrbS B. The mutation of each residue to alanine or valine was expected to reduce the binding affinity, if the VavS-GrbS

Fig. 3. Tetraproline region and PPII helix-binding site of VavS. **(A)** The ribbon diagram for VavS in the complex crystal is shown with the tetraproline region close to the viewer. Residues 606–612 encompassing the tetraproline region, and the residues interacting with them or expected to form the PPII helix-binding site are drawn as rods in red and blue, respectively. **(B)** The molecular surface of VavS by GRASP (Nicholls *et al.*, 1991) is colored according to the local electrostatic potential, with colors ranging from blue (positive) to red (negative) through white (neutral). The tetraproline region is drawn as red rods, and the peptide ligand for the Sem-5 SH3 domain is superposed on the molecular surface (yellow rods). The expected binding sites of VavS for the proline-rich peptide are labeled with their identification codes.

B interface has physiological significance. The Pro608Ala mutation would remove the side chain interactions at the P_{+2} site of GrbS. On the other hand, the Gly611Val mutation could distort the backbone conformation of Gly611 to the forms unsuitable for GrbS binding by repulsion between the side chain and the carbonyl oxygen, or otherwise would introduce steric hindrance at the P_{-1} site (Figure 6B). However, both mutants retained binding affinities similar to that of wild-type VavS. Next, although the side chain of Pro609 does not face toward the GrbS residues, this residue is one of the key residues at the interface. The anchorage of its pyrrolidine ring in the hydrophobic pocket on VavS appears to support the backbone conformation around the residues interacting with GrbS, Pro607, Pro608, Pro610 and Gly611, from the back (Figure 3). However, the alanine substitution of Pro609 barely affected the binding affinity, as also found with the mutations of Pro608 and Gly611.

Discussion

Physiological binding site for GrbS

To provide the transient nature of the protein–protein interactions in signal transduction, recognition of the downstream proteins with the correct affinity is crucial (Nguyen *et al.*, 1998). The binding affinity of GrbS for VavS, estimated by SPR measurements, is of the same degree as those of the SH3 domains for proline-rich peptides (5.7–73 μ M; Feng *et al.*, 1994; Yu *et al.*, 1994). The VavS–GrbS B interface includes the inter-domain interactions that are reminiscent of classical PPII helix binding, albeit not in the optimal manner. Since there are previous reports on the intramolecular interactions of the

c-Src (Sicheri *et al.*, 1997) and Hck (Xu *et al.*, 1997) tyrosine kinases in which the PPII helix recognition does not follow the canonical mode precisely, we predicted at first that the VavS–GrbS B interface has physiological relevance. Feng (1994) indicated that an alanine substitution of each proline located at the P_{-1} and P_{+2} sites caused an ~10-fold decrease in the binding affinity of the Sos-derived peptide for the Sem-5 SH3 domain, thus emphasizing the requirement of the pyrrolidine ring at each position. Pro608 and Gly611 occupy the P_{+2} and P_{-1} sites of GrbS, respectively, with the former interacting with GrbS residues in the canonical manner, and the latter mimicking the proline–protein interactions by use of the polypeptide backbone. However, neither mutation caused a decrease in the affinity. These results indicate that the VavS–GrbS B interface is an artifact resulting from the high protein and precipitant concentrations under the crystallization conditions. Ramos-Morales *et al.* (1995) reported that the truncated version of Vav, which spanned the VavS region with the multiple mutations of the four prolines (607–610) to alanine, failed to bind to Grb2 in the yeast two-hybrid system. However, they reported that the double mutation of Pro608 and Pro610 did not disrupt the binding affinity in the same system. As the tetraproline region is part of the VavS core (Figure 3), it is probable that the multiple mutations of this region resulted in structural instability, and thus disrupted the binding affinity indirectly. In clear contrast to the VavS–GrbS B interface, all the four point mutations at the VavS–GrbS A interface caused a decrease in the binding affinity at a physiological concentration, which can be rationalized in terms of the crystal structure. Based on these results, we have demonstrated that the VavS–GrbS A interface actually reflects the interaction

Fig. 4. Ribbon diagrams for VavS and two GrbS molecules in the complex crystal. VavS, GrbS A and GrbS B are colored in green, red and blue, respectively. The view in (B) is rotated by 120° with respect to that in (A) around the vertical axis on the plane.

between VavS and GrbS. The significance of the involvement of the tetraproline region in the VavS core might be in strengthening the folded architecture so that it is rigid enough to expose its large hydrophobic surface to the solvent for GrbS binding. Among the GrbS-bound and four GrbS-free VavS molecules, there are few differences in the positions of the residues at the VavS–GrbS A interface except for the n-Src loop. In the n-Src loop, which approximately retains the backbone conformation, the positions of the Ala632 C α of the GrbS-free forms deviate from that of the GrbS-bound form by 1.6–4.2 Å toward the direction opposite to the VavS–GrbS A interface (Figure 1B). This indicates that some plasticity is tolerated around the region when VavS is free from GrbS.

Structural elements required for GrbS binding

Since members of the SH3 family share the same folded architecture, it is tempting to suppose that GrbS could recognize some SH3 domains other than those of VavS in a manner similar to that observed in the crystal structure. However, considering the stringent regulation of the immune system, mismatch in the recognition of downstream molecules must not be allowed. Using a set of SH3 domains as a probe (N- and C- terminal SH3s of Grb2 and Vav, SH3s of tyrosine kinases Abl, Btk, Fyn, Hck, Lck, Lyn and c-Src) (Figure 2), Ye and Baltimore (1994) tested the interactions between VavS and other SH3 domain proteins. In their report, only the interaction between GrbS and VavS was detected. What are the structural elements that confer on VavS the high specificity required for GrbS binding? In Figure 2, the N-terminal SH3 domains of four Vav members are listed. Vav2 and Vav3 are recently identified isoforms of Vav, and their biological functions are still not clear relative to Vav. However, most of the VavS residues crucial for the domain architecture are also

conserved or type-conserved among them, suggesting that they share similar structural features. Vav3 also forms a complex with Grb2 *in vivo* (Zeng *et al.*, 2000). Among the five residues of VavS that are in closest contact with GrbS A, Pro595, Met597, Ala632, Trp637 and Pro657, four residues are conserved in the Vav members. The exception is in Vav2, where Ala632 is replaced by proline. Although the binding of Vav2 has yet to be reported, the proline can be modeled into the VavS–GrbS A interface with only a minor movement of the Gln162 side chain of GrbS. This set of hydrophobic residues is not conserved among other SH3 domains. For a comparison, the molecular surfaces of the Abl and Hck SH3 domains that correspond to the GrbS A-binding site of VavS are shown in Figure 5C. In GrbS recognition, the importance of Trp637 and Pro657 of VavS is remarkable, as proved by the mutagenesis study. Since both residues are the key elements for forming the surface complementary to GrbS, the substitution of either for any other amino acid would prevent binding (Abl, Cys100 for Trp637 of VavS; Hck, Arg135 for Pro657 of VavS). Substitutions of Pro595 for longer side chains (Abl, Asn64; Hck, Ile81) should also perturb the entrance of the VavS valley, thereby hampering the docking of Leu164 and Thr211 of GrbS into the valley. In the same context, small residues at the Ala630 location should also be a prerequisite for GrbS binding. However, most of the SH3 domain proteins other than Vav have a bulkier residue at the corresponding location (Abl, Tyr93; Hck, Glu110). Moreover, the n-Src loop must be in the conformation to define one of the side walls of the valley. This loop is the most divergent region, in addition to the RT loop, among the members of the SH3 family. In the SH3 domain proteins other than Vav, the polypeptide backbone of the n-Src loop largely moves back from the binding surface, and is not in a conformation suitable for forming the complementary surface to GrbS.

Fig. 5. Schematic views of the VavS-GrbS A interface. (A) The molecular surface of VavS is shown as a transparent worm with the VavS-GrbS A interface close to the viewer. The VavS residues at the interface are drawn as green rods. For clarity, some residues that interact minimally with GrbS A are omitted (His634, Cys652, Val655 and His 656). The polypeptide backbone of the N-terminal tail derived from the expression vector is traced as a dotted line in white. (B) The side chains (rods) and polypeptide backbone (magenta tubes) of the GrbS residues at the interface are superposed on VavS. (C) The molecular surfaces of the Abl (left) (Musacchio *et al.*, 1994) and Hck (right) (Sicheri *et al.*, 1997) SH3 domains are shown in the same orientation as that of VavS in (A) and (B). Only the regions corresponding to residues 595–659 of VavS are shown.

Comparison of the recognition of VavS by GrbS with the classical recognition of the proline-rich peptide

As shown in Figure 8, the VavS-binding site of GrbS and its recognition in the VavS-GrbS A interface are definitely

different from those of the classical peptide binding of the SH3 domain. The binding of proline-rich peptides to SH3 domains is mediated through the recognition of some steric features of the PPII helix by a relatively narrow area of the domain surface ($\sim 350 \text{ \AA}^2$) (Figure 8B). In contrast,

Fig. 6. Stereo views of the two interfaces between VavS and GrbS. **(A)** The VavS-GrbS A interface is shown with the exit of the GrbS-binding valley close to and the entrance distant from the viewer. The VavS and GrbS residues are colored in green and red, respectively. The side chains involved in the inter-domain interactions and some main chains are drawn as rods. The α carbons are traced as tubes. For clarity, some VavS residues that minimally interact with GrbS are omitted. Hydrogen bonds are drawn as dotted lines in cyan. **(B)** The VavS-GrbS B interface is shown according to the same scheme as in (A). The viewing point is at the VavS core. Note that the orientation of the tetraproline region of VavS is rotated by $\sim 180^\circ$ around the vertical axis on the plane with respect to that in Figure 3. Water molecules are drawn as circles.

Table III. Dissociation constants of VavS mutants for GrbS

VavS mutant	K_d^a (μ M) (relative)	K_d^b	No. of interactions ^c
Wild type	16.8 ± 0.5	1.0	
VavS-GrbS A interface			
P595A	61.4 ± 1.7	3.7	9 (R207, N208, V210, T211)
A632G	165 ± 3	9.8	7 (Q162, A163, G180)
W637Y	630 ± 30	38	7 (L164, F165, R179)
P657A	155 ± 2	9.2	15 (F165, N208, Y209)
VavS-GrbS B interface			
P608A	20.6 ± 0.3	1.2	7 (F165, N208, Y209)
P609A	19.4 ± 0.7	1.2	—
G611V	16.1 ± 0.3	1.0	11 (P206, N192, W193, N208)

^a K_d is the mean value of three independent experiments with the error quoted as standard deviation.

^bThe dissociation constant of the mutants is represented by a value relative to that of the wild-type.

^cThe total number of the intradomain interactions (≤ 4.0 Å) of the VavS residue with the GrbS residues quoted in parentheses. Except for Gly611, the number is for the side chain of the VavS residue.

Fig. 7. Determination of the dissociation constant of wild-type VavS for GrbS. **(A)** Sensorgrams for SPR measurements, which were corrected after subtracting the background signals, are superposed. For clarity, only those for the loading of 1, 5, 10, 20, 60, 150 and 500 μM VavS are shown. The disorder at the end points of the injections stems from the subtraction procedure, since there was a time gap between the responses of the sample and blank cells with each injection. **(B)** Using the corrected response unit (cRU) and including all measurements, the dissociation constant was derived from the Scatchard plot. The SPR data are best fitted by a line defined by the equation, $y = -16.8x + 333$, and have a correlation coefficient of 0.998.

the global architecture of the VavS molecule is essential for assembling the key structural elements into the proper positions required for recognition by GrbS. The high specificity of GrbS for VavS is thus accomplished in the context of the entire domain architecture.

Among the GrbS residues that form the PPII helix-binding site, Phe165, Asn208 and Tyr209 participate in VavS binding (Figure 8A). Most of the VavS components contact these three residues at GrbS sites different from that for PPII helix binding. The exception is Pro657. The VavS-binding site of GrbS partially overlaps with its PPII helix-binding site at Pro657. The pyrrolidine ring of Pro657 packs into the pocket between the side chains of Phe165 and Tyr209, which is utilized as the P_{+2} and P_{+3} sites in the canonical recognition of the peptide ligand. A dipeptide moiety of the ligand, P_x or xP , is recognized at these sites by the aromatic side chains corresponding to Phe165 and Tyr209 of GrbS (Figure 8B). Although the disposition of VavS Pro657 toward them is not in agreement with either in the dipeptide moiety, it still interacts closely with the GrbS residues, and thus has a pivotal role in binding to GrbS, as shown by the mutagenesis study. As a consequence, it is explicit that

Fig. 8. Comparison of the VavS-GrbS A interface with the PPII helix-binding site of the Sem-5 SH3 domain. **(A)** Polypeptide backbones of GrbS and VavS are shown as tubes in magenta and green, respectively. Side chains of GrbS forming the PPII helix-binding site are shown as cyan rods. The four mutated residues of VavS at the VavS-GrbS A interface are shown in yellow. As a landmark, the tetraproline region of VavS is also shown in red. **(B)** The polypeptide backbone of the Sem-5 SH3 domain and the side chains forming the PPII helix-binding site are drawn according to the same scheme as in (A). The peptide ligand is drawn in yellow and the binding sites for the ligand are labeled with their identification codes.

GrbS could not simultaneously recognize Vav and other downstream molecules containing the proline-rich sequence such as Sos and WASP.

Conclusions

Responding to T-cell stimulations, Vav is translocated to GEMs, where it transmits the signals from upstream molecules to small G proteins. LAT, which is a mem-

brane-bound protein, is among the upstream targets of Vav, and its tyrosine phosphorylation by ZAP-70 is essential for the Grb2–Vav complex to be recruited into GEMs (Salojin *et al.*, 2000). Since the SH2 domain of Grb2, but not that of Vav, binds to phosphorylated LAT, the GrbS–VavS dimerization is postulated to serve as the molecular glue for assembling multiple proteins into the signaling complexes in GEMs. Likewise, Grb2 is proposed to transmit the signals from CD28 to Vav in GEMs through GrbS–VavS dimerization (Kim *et al.*, 1998). The mutation on CD28 that causes the loss of its binding to the Grb2 SH2 domain blocks the phosphorylation of Vav and the activation of JNKs.

Recent studies have extended the repertoire of SH3 recognition beyond the classical proline-rich peptide domain recognition. As precedents for the protein–protein interactions in which the SH3 domains are involved, the crystal structure of the complex of the Fyn SH3 domain and HIV-1 Nef (Lee *et al.*, 1996), and that of the 53BP2 protein and the core domain of the p53 tumor suppressor (Gorina and Palvetich, 1996) have been reported. In both complexes, other regions in the protein bear the major responsibility for accomplishing the high affinity binding in conjunction with the PPII helix-binding site. More recently, a peptide motif, RkxxYxxY, which deviates from the canonical category of peptide ligands, was also reported to bind to SH3 domains in a manner distinct from that of the canonical mode (Kang *et al.*, 2000). To recognize such a peptide motif or to function as part of a multiprotein assembly, the SH3 family utilizes its hydrophobic surface with considerable versatility.

Materials and methods

Protein preparations

The region encoding VavS was inserted into the pET-28a (+) vector (Novagen) using the *NdeI*–*EcoRI* restriction sites with the addition of a hexahistidine tag and a thrombin cleavage site at its N-terminus. The expression and the Ni²⁺-affinity column chromatography of the product were performed according to the protocol recommended by the manufacturer. After the hexahistidine tag was removed, VavS was purified by chromatography on Superdex 75 gel filtration and Mono Q anion-exchange columns (Pharmacia). The final yield of purified VavS was 5.0 mg from a 1 l culture. The sample was flash-frozen and stored at –80°C. The VavS mutants were prepared using the QuickChange site-directed mutagenesis kit (Stratagene), and the constructs used for SPR measurements were purified in the same way without thrombin cleavage. All the mutations were checked by protein sequence analysis or mass spectrometry. The preparation of GrbS was according to the previously reported procedure (Kohda *et al.*, 1994).

Crystallization and data collection of GrbS-bound VavS

Both VavS and GrbS solutions were exchanged with a crystallization buffer consisting of 10 mM Tris–HCl (pH 9.0), 150 mM NaCl, 10–20 mM dithiothreitol (DTT) and 1 mM EDTA. A plate-shaped crystal appeared within 2 weeks after the protein mixture containing 1.5 mM VavS and 3.0 mM GrbS was equilibrated against a reservoir consisting of 100 mM imidazole pH 6.5, 67% (v/v) 2-methyl-2,4-pentanediol (MPD), 75 mM MgCl₂ and 1 mM DTT at 20°C by the hanging drop vapor diffusion method. The crystal was grown to 0.5 × 0.3 × 0.1 mm by macroseeding. Mass spectrometry and N-terminal sequence analysis confirmed that the crystal contained VavS and GrbS molecules in a molar ratio of 1:2. This crystal is orthorhombic, belonging to the space group *C*222₁ with unit cell dimensions of *a* = 48.05 Å, *b* = 126.82 Å and *c* = 83.37 Å, and has a solvent content of 57.5%. Diffraction data up to 1.68 Å resolution were collected from a frozen crystal in a nitrogen cryostream at 100 K on a MarCCD165 detector (Mar research) at beamline 41XU of the Japan Synchrotron Radiation Research Institute (Nishi-Harima, Hyogo). A gold derivative was prepared by soaking a crystal in the harvesting buffer

containing a heavy atom reagent at 4°C. Both data sets of the native and gold derivative crystals were collected at 1.0375 Å, integrated using MOSFLM (Leslie, 1993), and scaled and merged by SCALA in the CCP4 program suite (CCP4, 1994). The other derivative crystals were subjected to CuK α radiation generated by a Rigaku rotating-anode X-ray generator in the home laboratory. All data sets were collected by an imaging plate detector, and were processed by DENZO and SCALEPACK (Otwinowski and Minor, 1997). The results of the data collections are summarized in Table I.

Structure determination of GrbS-bound VavS

The initial phasing was performed by MIR using five derivative crystals. All of the programs used for phasing were incorporated in the CCP4 program suite. After scaling was applied to the derivative data sets by FHSCAL, the heavy-atom parameters were refined and the MIR phases were calculated by MLPHARE using the data between 10 and 2.5 Å resolution. The two gold derivatives, collected by a synchrotron light source or in the home laboratory, were treated as different derivatives. The anomalous dispersion data of the gold derivative collected at a 1.0375 Å wavelength were also incorporated into the phasing. The mean figure of merit for 8915 reflections included in the phasing is 0.574 (Table I). After density modification by solvent flattening and histogram matching was applied to the MIR map using DM (Cowtan and Main, 1996), an initial model consisting of one VavS and two GrbS molecules was placed on the modified electron density map. Refinement of the model was performed by conjugate gradient minimization and simulated annealing with the slow-cooling protocol in X-PLOR version 3.1 (Brünger, 1992). For each cycle, the model was rebuilt manually by the molecular modeling program, Turbo-Frodo (Roussel and Cambillau, 1991). The final model consists of VavS with the vector-derived sequence, GSHM, at the N-terminus, GrbS A with the vector-derived serine at the N-terminus, GrbS B, one MPD molecule and 189 water molecules. Residue 660 of Vav, and residues 216–217 and 214–217 of Grb2 were omitted from VavS, GrbS A and GrbS B, respectively, because they were not defined on the electron density map.

Crystallization and data collection of GrbS-free VavS

A protein solution containing 5.0 mM of VavS was equilibrated against a reservoir consisting of 100 mM Tris–HCl pH 8.5, 30% (w/v) PEG 4000 and 9% (v/v) isopropanol at 4°C. A monoclinic crystal of the space group *P*2₁ (*a* = 32.21 Å, *b* = 101.12 Å, *c* = 39.71 Å and β = 91.34°) grew within 1–2 weeks. The crystal contains four VavS molecules in an asymmetric unit with a solvent content of 39.1%. After immersion in a cryoprotectant containing 15% (v/v) glycerol, diffraction data up to 2.1 Å resolution were collected by the home source from a flash-frozen crystal. The data set was processed by DENZO and SCALEPACK.

Structure determination of GrbS-free VavS

The VavS molecules in the monoclinic crystal were located by molecular replacement using VavS in the GrbS-bound form as a probe. Using the data between 10 and 2.5 Å resolution, four solutions with a correlation coefficient of 0.56 were obtained by rotation and translation function searches in AMoRe (Navaza, 1994). After rigid-body refinement, the model was refined in a manner similar to that used for GrbS-bound VavS. Throughout the refinement, non-crystallographic symmetry restraints (300 kcal/mol/Å²) were imposed on the main chain atoms of the four VavS molecules, except for the regions of residues 609–614, 631–633, 642–646 and 658–660. After the resolution was extended to 2.1 Å and 159 water molecules were incorporated into the model, the restraints were lifted. The final model consists of three VavS molecules with Pro595 being omitted, one VavS with the vector-derived methionine at the N-terminus, and 190 water molecules.

SPR measurements

GST-fused GrbS was immobilized on a CM5 sensor chip via an anti-GST antibody as recommended by the manufacturer (BIAcore). The fusion protein includes a linker of eight residues. The amount of the fusion protein on the chip was adjusted to ~1300 RU (resonance units) by monitoring the change in the refractive index. The flow rate and the temperature were kept at 5 μ l/min and 25°C. Prior to each injection of the VavS solution, the sensor chip was washed with loading buffer [10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% (v/v) polysorbate 20, and 2.5 mM 2-mercaptoethanol] for 20 min, which was sufficient for the recovery of the baseline of the sensorgram. The level of the SPR response was confirmed to be constant using a standard VavS solution before and after the 15 programmed runs. To measure the background response signal at equilibrium, a control experiment was

performed in parallel using a blank cell in which the same molar amount of GST as that of GST–GrbS was immobilized on the sensor chip. A set of the programmed runs was performed three times for calculating the mean dissociation constant. Prior to each set run, the cells were regenerated with 10 mM glycine pH 2.2, and GST and GST–GrbS were newly immobilized.

Accession numbers

The atomic coordinates and the structure factors of the refined models of GrbS-free VavS and GrbS-bound VavS have been deposited in the Protein Data Bank. The accession Nos are 1GCP and 1GCQ, respectively.

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