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Identification of a novel ubiquitin binding site of STAM1 VHS domain by NMR spectroscopy

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ABSTRACT

Interaction between the signal-transducing adapter molecule 1 (STAM1) Vps27/Hrs/Stam (VHS) domain and ubiquitin was investigated by nuclear magnetic resonance (NMR) spectroscopy. NMR evidence showed that the structure of STAM1 VHS domain resembles that of other VHS domains, especially the homologous domain of STAM2. We found that the VHS domain binds to ubiquitin via its hydrophobic patch consisting of N-terminus of helix 2 and C-terminus of helix 4 in which Trp26 on helix 2 plays a pivotal role in the binding. The binding between VHS and ubiquitin seems to be very similar to that between ubiquitin associated domain (UBA) and ubiquitin, however, the direction of α-helices involved in the ubiquitin binding is opposite. Here, we propose a novel ubiquitin binding site and the manner of ubiquitin recognition of the STAM1 VHS domain.

Structured summary
MINT-6804185: *STAM1* (uniprotkb:Q92783) *binds* (MI:0407) to *ubiquitin* (uniprotkb:P62988) by *nuclear magnetic resonance* (MI:0077)

1. Introduction

The covalent ubiquitination of proteins is a widespread regulatory post-translational modification. The carboxyl group of the C-terminal glycine of ubiquitin forms an isopeptide bond with ε-amino group of a lysine residue on a target protein. Ubiquitin may be attached to proteins as a monomer or as a polyubiquitin chain, where the consecutive ubiquitins are also linked via isopeptide bonds. Ubiquitination regulates a variety of cellular processes, including endocytosis, vesicular trafficking [1–3], cell-cycle control, stress response, DNA repair [4], signaling [5,6], transcription and gene silencing. So far at least sixteen domains were identified to interact with ubiquitin and many complex structures are determined (reviewed in Ref. [7]).

The signal-transducing adapter molecule (STAM) proteins form multifunctional complex with hepatocyte growth factor-regulated substrate (Hrs) protein, which sort the ubiquitinated cargo proteins from early endosomes to the endosomal sorting complex required for transport I (ESCRT-I) complex [8,9]. The STAM/Hrs complex has ubiquitin-binding domains such as Vps27/Hrs/Stam (VHS), ubiquitin interacting motif (UIM), GGAs and TOM (GAT) domains. The STAM proteins bind ubiquitin via the tandemly located the VHS and UIM domains, and play roles in the sorting of ubiquitinated proteins in the multivesicular body (MVB) pathway [10]. However, the mechanism of the interaction between VHS/UIM and ubiquitin including their binding sites remains unknown.

The STAM proteins consist of N-terminal VHS domain involved in vesicular trafficking [11], UIM involved in ubiquitination and ubiquitin metabolism [12], Src homology 3 (SH3) domain, a

Abbreviations: STAM, signal-transducing adapter molecule; VHS, Vps27/Hrs/Stam; NMR, nuclear magnetic resonance; UBA, ubiquitin associated domain; ESCRT, endosomal sorting complex required for transport; UIM, ubiquitin interacting motif; GAT, GGAs and TOM; MVB, multivesicular body; SH3, Src homology domain 3; GGA, golgi-localizing/γ-adaptin ear homology domain/ADP-ribosylation factor-binding protein; Hrs, hepatocyte growth factor-regulated substrate; Tom, target of Myb; Srcasm, Src-activating signaling molecule; TGN, transgolgi network; PDB, protein data bank; GST, glutathione S-transferase; IPTG, isopropyl β-D-1-thiogalactopyranoside; DTT, 1,4-dithiothreitol; PMSF, phenylmethanesulphonylfluoride; HSQC, heteronuclear single quantum correlation spectroscopy; DSS, 2,2-dimethyl-2-silapentane-5-sulfonic acid; CSI, chemical shift index; NOE, nuclear overhauser enhancement; K_d, equilibrium dissociation constant; CUE, coupling of ubiquitin conjugation to endoplasmic reticulum degradation; MIU, motif interacting with ubiquitin

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protein–protein interaction domain [13] and GAT domain, forming complex with GAT domain of Hrs protein [14]. The VHS domains consist of ~150 amino acids and are found in at least 60 proteins including golgi-localizing/γ-adaptin ear homology domain/ADP-ribosylation factor-binding (GGA1-3) proteins, Hrs, STAM (STAM1,2), target of myb 1 (Tom1), and Src-activating signaling molecule (Srcasm) [15]. Three-dimensional structures of several VHS domains except for STAM1 have been determined [16–19]. The domains form a super helix with eight α-helices which may serve as a scaffold for protein–protein interactions. Indeed, the interaction partners of the VHS domains are diverse. While the VHS domains from STAM1, STAM2, and Tom1 were reported to interact with ubiquitin respectively, the VHS domains of GGAs interact with some sorting receptors such as sortilin and mannose 6-phosphate receptors that traffic and transfer cargo between transgolgi network (TGN) and endosomal compartment [20,21]. However, the VHS domains in non-GGAs do not interact with the sorting receptors.

In this study, in order to understand the interaction between the STAM1 VHS domain and mono-ubiquitin, we employed nuclear magnetic resonance (NMR) spectroscopy. The structure of the STAM1 VHS domain seems to be very similar to that of the STAM2 VHS domain (protein data bank; PDB 1X5B). Two VHS domains from STAM1 and STAM2 share 74% sequence homology. NMR chemical shift perturbation experiments enabled the mapping of the binding interfaces between the STAM1 VHS domain and ubiquitin. The binding sites were the hydrophobic surface consisting of parts of helix 2 and helix 4 from the VHS domain where Trp26 plays an important role, and the hydrophobic patch around Ile44 of ubiquitin. Based on the homologous structure of STAM2 VHS domain, we propose that the STAM1 VHS domain shares the common ubiquitin recognition features with those of several helical ubiquitin-binding domains, such as ubiquitin associated domain (UBA), UIM, and coupling of ubiquitin conjugation to endoplasmic reticulum degradation (CUE). However, we found a distinct feature in the VHS–ubiquitin interaction. The distinct ubiquitin recognition feature of the STAM1 VHS domain is discussed.

2. Materials and methods

2.1. Preparation of proteins

The DNA sequences corresponding to the VHS domain (residue 2–139) of human STAM1 was cloned into the modified pET-glutathione S-transferase (GST) vector, which produced GST and thrombin cleavage site to the N-terminus of the target protein.

The plasmid was transformed into *Escherichia coli* strain BL21-CodonPlus (DE3)-RIL (Stratagene) for protein expression. The cells were grown at 37 °C in M9 minimal media and the protein expression was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at an optical density (600 nm) of 0.6, followed by additional growth at 37 °C for 4 h. Uniform labeling of the protein ([U-^{15}N] or [U-^{13}C, U-^{15}N]) with ^{13}C and ^{15}N was accomplished by including 99% $^{13}C_6$-D-glucose (1 g/l) and 98% $^{15}NH_4Cl$ (1 g/l) as the sole carbon and nitrogen sources, respectively. Samples, in which only Arg, Thr or Met labeled with ^{15}N, were prepared by the modified labeling protocol of LeMaster and Richards [22].

The GST-fused protein was purified using glutathione Sepharose 4B column (Amersham Biosciences). The GST was cleaved off by overnight incubation, at 20 °C, with 10 unit/mg of thrombin. Cleaved GST and uncleaved fusion protein were removed by another pass-over the glutathione Sepharose 4B column. The VHS protein was further purified by Superdex 75 size-exclusion column chromatography (Amersham Biosciences). The final protein contains additional SENLYFQGS sequences at its N-terminus. All sample solutions were exchanged into 20 mM sodium phosphate buffer, pH 6.8, with 1 mM 1,4-dithiothreitol (DTT), 0.1 M NaCl, 0.5 mM phenylmethanesulphonylfluoride (PMSF), 0.05 mM NaN_3 and 5% (v/v) D_2O. Sample concentrations were from 0.1 to 0.5 mM.

Unlabeled ubiquitin was purchased from Sigma–Aldrich and the expression of ^{15}N-labeled ubiquitin was done by the similar way of ^{15}N-labeled VHS production. Ubiquitin was cloned into a modified pET expression vector, which adds a polyhistidine tag and thrombin cleavage site to the N-terminus of the expressed protein, and expressed in BL21(DE3). A polyhistidine tagged protein was purified using Ni-NTA resin. Further purification was accomplished by heating up to 70 °C for 30 min. The polyhistidine tag was cleaved off and removed.

2.2. NMR spectroscopy

NMR experiments were conducted at 298 K using Bruker DRX 500, AVANCE 600 (with cryoprobe) and Varian VNMRS 900 spectrometers. The following experiments were recorded for the backbone assignment of VHS domain: 2D-[^{1}H—^{15}N]-heteronuclear single quantum correlation spectroscopy (HSQC), 3D-HNCA, HN(CO)CA, HNCACB, CBCA(CO)NH, and HNCO. The resonance assignment of ubiquitin was accomplished from 2D-[^{1}H—^{15}N]-HSQC, 3D-HNCA, HN(CO)CA, HNCACB, and HN(CO)CACB. A series of 2D-[^{1}H—^{15}N]-HSQC experiments were also recorded for ^{15}N-Arg, ^{15}N-Thr, and ^{15}N-Met labeled the VHS domains, respectively. ^{1}H chemical shifts were referenced to 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS) and ^{15}N and ^{13}C chemical shifts were referenced indirectly. Spectral data were processed using NMRPipe 2.3 [23] and analyzed using NMRViewJ [24].

2.3. VHS–ubiquitin affinity measurements and mapping of binding interfaces

The affinity of VHS–ubiquitin and the mapping of the binding interfaces were achieved by NMR titration experiments. 0.1 mM ^{15}N-labeled VHS domain and the same protein complexed with various concentration of ubiquitin (0.02, 0.04, 0.06, 0.1, 0.15, 0.2, 0.3, and 0.5 mM) were prepared. 2D-[^{1}H–^{15}N]-HSQC spectra of the free and ubiquitin-bound ^{15}N-labeled VHS domain were recorded. 2D-[^{1}H–^{15}N]-HSQC of the free and VHS-bound ^{15}N-labeled ubiquitin were also recorded. The concentration of ^{15}N-labeled ubiquitin was 0.05 mM and that of unlabeled VHS domain was 0.01, 0.03, 0.05, 0.1, 0.15, and 0.2 mM, respectively. Weighted average chemical shift differences, Δ_{ave}, were calculated using the formula, $\Delta_{ave} = \sqrt{\{[(\Delta_H)^2 + (\Delta_N/5)^2]/2\}}$ and plotted as a function of molar ratio. The equilibrium dissociation constants (K_d) were derived by performing non-linear regression assuming a single binding site using the fitting function in the program KaleidaGraph 3.5. The binding interfaces were judged from the chemical shift perturbation plot of the final complexes.

2.4. Significance of Trp26 in ubiquitin recognition

To address the significance of Trp26 in ubiquitin recognition of the STAM1 VHS domain, the VHS^{W26A} mutant where Trp26 was substituted by Ala was prepared by site-directed mutagenesis. The constructed plasmid was confirmed by DNA sequencing. After the purification using glutathione Sepharose 4B column, the GST–VHS^{W26A} fusion protein was used without the removal of GST.

25 μM GST–VHS, GST–VHS^{W26A} and GST were added to 50 μM ^{15}N-labeled ubiquitin, respectively. The measurements of 2D-[^{1}H,^{15}N]-HSQC were followed.

3. Results and discussion

3.1. Structural property of STAM1 VHS domain

The structural property of the STAM1 VHS domain was characterized and compared to that of other VHS domains. Based on the backbone resonance assignment, which has been deposited in the BioMagResBank (http://www.bmrb.wisc.edu) with the Accession No. 7321, we performed the prediction of the secondary structure of STAM1 VHS domain. Chemical shift index (CSI) [25] and Talos [26] analyses showed that the STAM1 VHS domain consists of 8 α-helices, which is consistent with the solution and crystal structure of other VHS domains [16–19]. Even though the three-dimensional structure of the STAM1 VHS domain was not determined yet, the structure seems to be very similar to that of the STAM2 VHS, judging from our preliminary structure calculation using long-range nuclear overhauser enhancement (NOE) restraints (data not shown).

3.2. Binding affinity of VHS–ubiquitin

Based on the assigned backbone ^{1}H and ^{15}N resonances of the STAM1 VHS (Fig. 1), we traced the chemical shift changes of the residues upon the addition of unlabeled ubiquitin. Fig. 2A shows the shift of Gly77 of VHS with the increasing amount of added ubiquitin. At the presence of 3 molar excess ubiquitin, the titration curves were saturated (Fig. 2B). The average K_d was 52 ± 10 μM.

Reciprocally, we also used ^{15}N-labeled ubiquitin as a bait to monitor the titration behavior upon the addition of the unlabeled VHS domain. The titration curves of ubiquitin residues were shown in Fig. 2C, from which the calculated K_d was 41 ± 6 μM. The discrepancy of K_d values seems to arise from the fact that the different version of ubiquitin was used in our experiments. For the unlabeled ubiquitin, we used six histidine-tagged protein, while the recombinant version of ubiquitin with additional N-terminal SENLYFQGS sequence from the plasmid was used for ^{15}N-labeled ubiquitin. Thus, we concluded that the affinity of VHS and ubiquitin is about 50 μM, which is relatively higher value among the ubiquitin related complexes. The affinities between ubiquitin and ubiqutin binding proteins are usually low with the range from 10 μM to 2 mM (summarized in Ref. [7]).

3.3. Mapping the interaction site between VHS domain and ubiquitin

From the final samples used in NMR titration experiments, e.g. ^{15}N-labeled VHS and unlabeled ubiquitin (1:5) complex and ^{15}N-labeled ubiquitin and unlabeled VHS (1:4) complex, the degree of

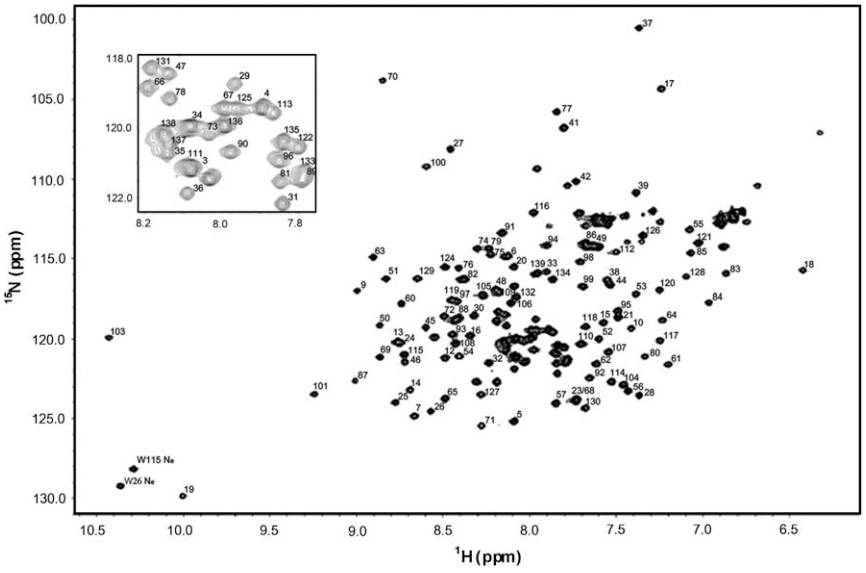

Fig. 1. 2D-[^{1}H–^{15}N]-HSQC spectrum of the STAM1 VHS domain. The assigned HN resonance of the STAM1 VHS domain was labeled on the right shoulder of each cross-peak with the residue number. The unlabeled cross-peaks are from the side-chain HN of Asn and Gln, or from the additional amino acids. Two εNH correlations from Trp26 and Trp115 were also indicated. The crowd region of the spectrum was expended on the left top for clear view.

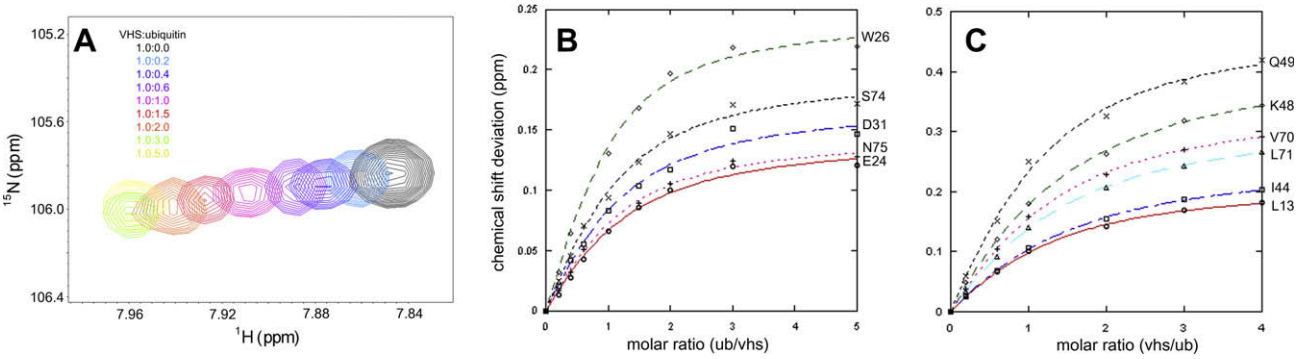

Fig. 2. NMR-based titration to identify the interaction of VHS–ubiquitin. (A) Overlays of an expanded region of 2D-[^{1}H–^{15}N]-HSQC spectra of the STAM1 VHS domain showing the amide chemical shift change of Gly77 as a function of added ubiquitin. The molar ratios of VHS:ubiquitin were indicated and color-coded. (B) Binding isotherm of selected residues from the STAM1 VHS domain with the molar ratio of [ubiquitin]/[VHS]. The concentrations of ^{15}N-labeled VHS were 0.1 mM, throughout the experiments. (C) Binding isotherm of selected ubiquitin residues. 0.05 mM ^{15}N-labeled ubiquitin were used.

chemical shift perturbation as a function of residue number were plotted (Fig. 3), and the binding interfaces on VHS and ubiquitin were identified.

Ubiquitin used its canonical hydrophobic patch around Ile44 for VHS binding. The significantly perturbed residues were not only Ile44 but also Leu13, Thr14, Arg42-Leu50, His68, Val70-Arg74, which are well known protein binding sites in ubiquitin [27–32].

The VHS domains of STAM1 and STAM2 were reported to interact with ubiquitin [10], however, the ubiquitin binding site was not identified yet. In this study, we identified the ubiquitin binding site of the STAM1 VHS domain using chemical shift perturbation experiments, which consists of Glu24-Trp26, Leu28, Ile29, Asp31, Ser74, Asp75, and Gly77. Those amino acids are conserved in the STAM2 VHS domain except for Ser74 (Ala74 in STAM2). Judging from the solution structure of STAM2 VHS (PDB 1X5B) and our preliminary structural data, those residues reside on N-terminus of helix 2 and C-terminus of helix 4 (Fig. 4A). To our knowledge, this result is the first residue-specific information about the VHS–ubiquitin interaction.

3.4. Role of Trp26 in ubiquitin recognition and implication of VHS–ubiquitin interaction

It is very reasonable to believe that the structure of the STAM1 VHS domain resembles that of the STAM2 VHS domain based on the sequence homology and the secondary structure information provided in this study. Our analysis of NOE cross-peaks also supports the structural resemblance between two VHS domains. The residues showing the significant chemical shift perturbation were presented on the structure of STAM2 VHS domain (Fig. 4A).

Between those residues, we focused on Trp26 since Trp26 showed the most intensive chemical shift perturbation not only for the backbone $^{1}H–^{15}N$ resonance but also the resonance of εNH side-chain, which was disappeared upon the addition of ubiquitin. Trp26 is partially solvent-exposed in the structure of STAM2 VHS domain and the most conserved residue with Asp25 (Fig. 4B). To confirm the role of Trp26 in ubiquitin recognition, we constructed the VHS^{W26A} mutant protein and compared its ubiquitin interaction to that of the wild-type VHS domain. Since the expression of the mutant protein in *E. coli* was low, we were not able to get sufficient amount of the STAM1 VHS^{W26A} mutant alone. Instead, we used GST–VHS^{W26A} fusion protein for the ubiquitin binding experiment. The purified GST–VHS, GST–VHS^{W26A}, and GST as a control, was added to ^{15}N-labeled ubiquitin, respectively. Fig. 4C shows the selected region from 2D-[$^{1}H—^{15}N$]-HSQC spectra of ^{15}N-labeled ubiquitin with or without the added proteins. While the addition of GST–VHS^{W26A} (green) and GST (blue) to ^{15}N-labeled ubiquitin did not show any difference in the backbone resonances of ^{15}N-labeled ubiquitin alone (black), GST–VHS clearly showed the interaction with ubiqiutin (red) with the similar manner of ubiquitin-STAM1 VHS chemical shift perturbation. Thus, Trp26 seems the most important residue in the ubiquitin recognition of the STAM1 VHS domain.

The structure of the suggested ubiquitin binding interface of the VHS domain from the STAM proteins was compared to that of the UBA and CUE domains. The UBA and CUE domains are three-helical bundles and bind to ubiquitin via conserved hydrophobic residues at the C-terminus of the α1 helix [27–32]. The interesting point is that the arrangement of helix 2 and 4 from STAM2 VHS domain is well overlapped to that of α1 and α3

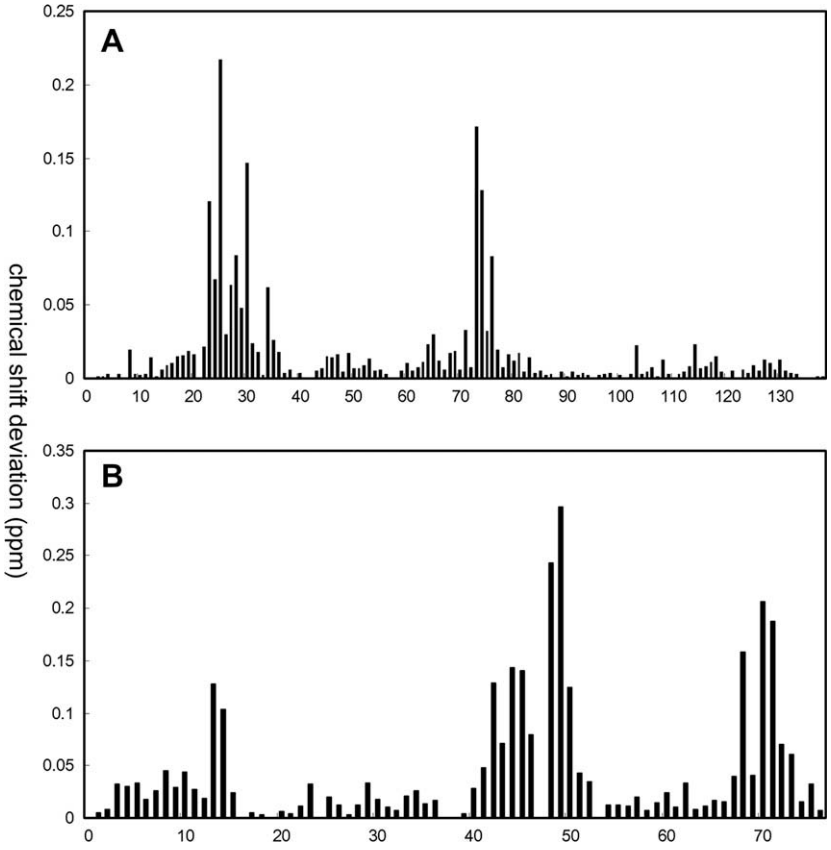

Fig. 3. Weighted average of chemical shift perturbation as a function of residues. (A) Chemical shift perturbation between the free and ubiquitin-bound VHS. (B) Chemical shift perturbation between the free and VHS-bound ubiquitin. Data were taken from the final complexes shown in Fig. 2 for both (A) and (B). The residues having the average value >0.05 were selected and mapped on Fig 4A.

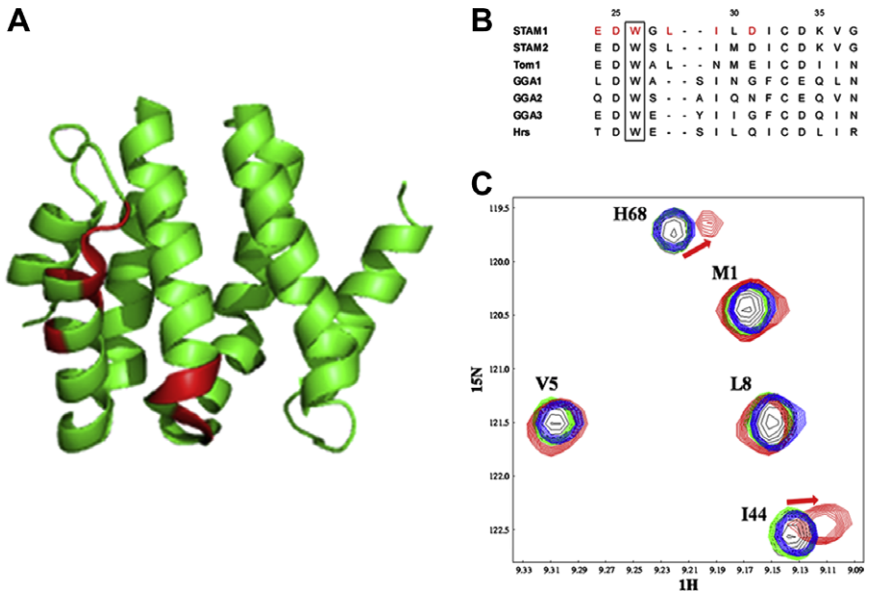

Fig. 4. Ubiquitin recognition of STAM1 VHS domain. (A) Ubiquitin binding sites of the STAM1 VHS domain were displayed in red. The structure of the STAM2 VHS domain was used (PDB 1X5B). (B) Sequence homology of helix 2 between the VHS domains. The residues with the significant chemical shift perturbation were colored in red on STAM1 and the conserved Trp residues are shown in the box. The residue numbers are from STAM1 sequence. (C). Selected region of 2D-[^{1}H–^{15}N]-HSQC spectra of ^{15}N-labeled ubiquitin alone (black) and ^{15}N-labeled ubiquitin with GST–VHS^{W26A} (green), GST (blue), and GST–VHS (red). The concentration of ubiquitin was 50 μM and those of the added proteins were 25 μM. The chemical shift change was only observed in the ubiquitin and GST–VHS mixture and the changes of Ile44 and and H68 of ubiquitin are shown (red arrow).

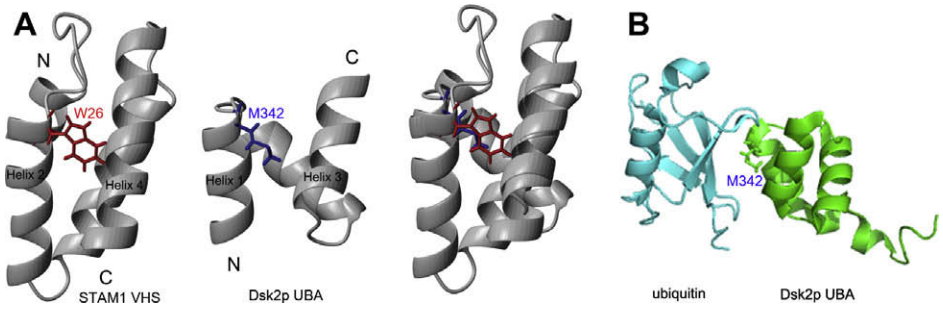

Fig. 5. Suggested binding mode of STAM1 VHS domain to ubiquitin. (A) Structure of ubiquitin binding region from VHS (left) and Dsk2p UBA (middle). The N- and C-terminus of each protein are indicated. The two domains are overlaid manually with reversed helical orientation (right). Side-chains of the critical residues for the ubiquitin recognition, Trp26 (STAM1 VHS) and Met342 (Dsk2p UBA), are shown. (C) Solution structure of Dsk2p UBA complexed with ubiquitin (PDB 1WR1). Ubiquitin is coded in cyan and Dsk2p in green. Met342 faces the hydrophobic patch of ubiquitin centered at Ile44.

helices of UBA domains of Dsk2p [29], even though the helical directions of VHS domain are opposite against those of the UBA domain (Fig. 5A). Indeed, the helices with the reversed orientation have been found to recognize the same site of ubiquitin previously. The single helical domains, UIM and motif interacting with ubiquitin (MIU), were reported as an example of the ubiquitin recognition motifs with the opposite helical orientations [33–36]. UIM and MIU recognized the hydrophobic patch around Ile44 of ubiquitin via the alanine residues in the center of respective helices.

The Met342 of Dsk2p UBA was suggested to play the most integral role in ubiquitin recognition by fitting its side-chain along β3 and β4 of ubiquitin and by packing its methyl group into a shallow hydrophobic pocket formed by the hydrophobic side chains of Ile44, Leu8, Gly47, His68 and Val70 of ubiquitin (Fig. 5). In the superimposed structures of ubiquitin binding region of the STAM2 VHS domain and the Dsk2p UBA domain, the position of Trp26 of VHS was completely matched to that of the Met342 of UBA. Thus, we propose that the manner of ubiquitin recognition of the VHS domain from STAM proteins is very similar to that of the Dsk2p UBA domain with the opposite helical orientation and Trp26 of the VHS domain is the key residue of ubiquitin recognition.

Moreover, the crystal structure of the VHS and FYVE domains of Hrs which forms multifunctional complex with the STAM proteins showed the intramolecular interaction between two domains, and the conserved Trp23 of the VHS domain, which corresponds to Trp26 of STAM1, plays a central role in the hydrophobic interaction with the loop between β3 and β4 strands of the FYVE domain [37]. In Hrs, the VHS domain did not show the ubiquitin interaction and the presence of the FYVE domain following the VHS domain was suggested to block the interaction of VHS–ubiquitin. It was also suggested that the VHS domains might interact with membrane and/or proteins of the endocytotic machinery [37]. Thus, Trp residue at the N-terminus of helix 2 of the VHS domain may mediate the multiple protein–protein interactions involved in the protein trafficking and signal transduction.

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