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Competitive binding of UBPY and ubiquitin to the STAM2 SH3 domain revealed by NMR

Anja Lange, Mouhamad-Baligh Ismail, Gwladys Rivière, Maggy Hologne, Denis Lacabanne, Florence Guillière, Jean-Marc Lancelin, Isabelle Krimm, Olivier Walker *

Université de Lyon, Université Claude Bernard Lyon1, CNRS, UMR 5280 Institut des Sciences Analytiques, 43 Boulevard du 11 Novembre 1918, 69622 Villeurbanne, France

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ABSTRACT

To date, the signal transducing adaptor molecule 2 (STAM2) was shown to harbour two ubiquitin binding domains (UBDs) known as the VHS and UIM domains, while the SH3 domain of STAM2 was reported to interact with deubiquitinating enzymes (DUBs) like UBPY and AMSH. In the present study, NMR evidences the interaction of the STAM2 SH3 domain with ubiquitin, demonstrating that SH3 constitutes the third UBD of STAM2. Furthermore, we show that a UBPY-derived peptide can outcompete ubiquitin for SH3 binding and vice versa. These results suggest that the SH3 domain of STAM2 plays versatile roles in the context of ubiquitin mediated receptor sorting.

1. Introduction

The turnover of many membrane proteins is subtly regulated through the endocytic pathway, where receptor cargoes are directed to the lysosome for degradation or recycled. Modification of receptors by mono- or Lys63-linked polyubiquitination constitutes a trafficking signal that is deciphered and executed by the ESCRT machinery [1–3]. The ESCRT-0 complex, which is the most upstream component of the ESCRT system, is constituted of the STAM (signal transducing adaptor molecule) and Hrs (hepatocyte growth factor-regulated substrate) proteins. Each of them harbours multiple modular motifs known as ubiquitin binding domains (UBDs) [4], the function of which is to recognize the ubiquitin tag and usher cargoes along the endocytic pathway. To date, two UBDs known as VHS (Vps27/Hrs/STAM) [5] and UIM (ubiquitin interacting motif) [6] were identified in the STAM protein while Hrs possesses a VHS and a DUIM (double ubiquitin interacting motif) domain [7]. Removal of the ubiquitin (Ub) tag is achieved by deubiquitinating enzymes (DUBs) that are implicated in diverse cellular pathways [8]. Among the different DUBs, AMSH and UBPY are involved in lysosomal degradation and have been shown to interact directly with the ESCRT system and more precisely with the SH3 domain of STAM [9–11]. The interaction of DUBs with STAM is essential to drive activated receptors into the recycling pathway [9]. In addition, UBPY protects STAM and Hrs from proteasomal degradation [12], which is required for the proper functioning and integrity of the ESCRT machinery [13]. In the present article, NMR titration and spin relaxation experiments reveal, that both the UIM and SH3 domains of the STAM2 UIM-SH3 construct, can bind to a ubiquitin (Ub) molecule. This important finding demonstrates that the SH3 domain constitutes the third UBD of STAM2. By using a UIM^{I181E}-SH3 mutant, we also demonstrate that the SH3 domain can still bind Ub when Ub binding is abolished on the UIM domain. Furthermore, NMR competition assays show that a UBPY peptide can outcompete Ub for binding to the SH3 domain. Our results clearly demonstrate that the DUB binding function of the STAM2 SH3 domain is not exclusive and that SH3 could be involved in the concentration of ubiquitinated cargoes at the ESCRT-0 level as well.

Abbreviations: ESCRT, endosomal sorting complexes required for transport; Ub, monoubiquitin; UIM, ubiquitin interacting motif; SH3, Src homology 3 domain; UBD, ubiquitin binding domain; MVB, multivesicular body; Hrs, hepatocyte growth factor-regulated substrate; STAM, signal transducing adaptor molecule; VHS, Vps27/Hrs/STAM; DUB, deubiquitinating enzyme; UBPY/USP8, ubiquitin-specific protease Y/ubiquitin carboxyl-terminal hydrolase 8; AMSH, associated molecule with the SH3 domain of STAM

* Corresponding author. Fax: +33 4 72 43 13 95.
E-mail address: olivier.walker@univ-lyon1.fr (O. Walker).

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2. Materials and methods

2.1. Protein expression and purification

STAM2$^{162-265}$ (UIM-SH3) was purified as previously described for STAM2$^{1-149}$ (VHS) [14]. The TEV cleavage reaction left a N-terminal extensions GAMGM for STAM2$^{162-265}$. The Ub^{wt} was produced as described in refs [15–17]. The UIM^{I181E}-SH3 variant was engineered using site-directed mutagenesis. The UBPY peptide has been purchased from "Genosphere".

2.2. NMR experiments

The ensemble of NMR experiments was acquired at 288 K where the NMR samples were exchanged into a buffer containing 20 mM sodium phosphate at pH 6.8, 10% D_2O, 0.02% (w/v) NaN_3. Assignment and spin-relaxation experiments have been carried out on a Varian Inova Unity 600 equipped with a triple-resonance probe.

2.3. Protein resonance assignment

The backbone resonance assignment for UIM-SH3 was obtained using a combination of the following experiments: [^{15}N, ^{1}H]-HSQC, HNCA, HNCACB and 3D NOESY-[^{15}N, ^{1}H]-HSQC. Data were processed with NMRpipe [18] and analyzed with NMRview [19]. The UIM-SH3 sequence was renumbered according to the STAM2-VHS structure (PDB code 1X5B) by adding 7 to the STAM2 sequence (Uniprot accession number O75886).

2.4. Relaxation measurements and analysis

Relaxation measurements including ^{15}N longitudinal (R_1), transverse (R_2) relaxation and the ^{15}N-^{1}H cross-relaxation rates via steady-state ^{15}N{^{1}H}NOE were performed as previously described [20,21]. NMR spectra were recorded with spectral widths of 2000 Hz in the ^{15}N dimension and 9600 Hz in the ^{1}H dimension. For the R_1 measurements, we used twelve relaxation delays: 4, 20, 40, 80 (twice), 240, 480, 800 (twice), 1200, 1400, 1600, 1800 and 2000 ms with a recycling delay of 4 s. The R_2 CPMG measurements were performed with transverse relaxation period of 4, 12, 24, 32 (twice), 40, 48, 56, 80, 120 (twice), 160 and 200 ms and a relaxation delay of 4 s. For ^{15}N{^{1}H} NOE experiments, 2D spectra were recorded with and without presaturation of amide protons. The relaxation delay was set to six seconds in order to allow the bulk water magnetization to return as close as possible to its equilibrium value. All NMR data were processed with NMRpipe [18] and analyzed with SPARKY [22], and the relaxation rates were extracted using RelaxFit [21].

2.5. NMR titration studies

Interaction surfaces on ^{15}N-monoUb and ^{15}N-UIM-SH3 were characterized by means of chemical shift perturbations (CSPs) where a series of ^{1}H, ^{15}N-HSQC experiments were recorded upon addition of the (unlabeled) binding partner. To avoid possible aggregation of the proteins, we started from a ^{15}N-labeled protein at a concentration of 200 μM and added an increasing volume of a concentrated stock of unlabeled ligand protein until reaching saturation. To derive the corresponding binding constant, spectral perturbations were quantified as the combined amide CSPs: $\Delta\delta = [(\Delta\delta_H)^2 + (\Delta\delta_N/5)^2]^{1/2}$. The corresponding equations used to derive Kds from the UIM-SH3 or Ub side can be found in Supplementary data.

2.6. Homology modeling of UIM and UIM-SH3

The 3D structure of the UIM part in the UIM-SH3 construct was obtained by homology modeling following a methodology similar to the modeling of the VHS-UIM construct [23]. The amino acid sequences of STAM2-UIM and Vps27-UIM1 share 55% identity and 70% similarity. We used the UIM1 domain [24] of Vps27 (PDB code 1Q0V) to model the structure of the UIM part of the UIM-SH3 construct, while the STAM2 SH3 domain (PDB code 1X2Q) was used to model the SH3 part of the UIM-SH3 construct. Models were generated by using the Modeller program [25]. After alignment of the query and template sequences with Align2D, they were used as input in Modeller. A total of ten structures were generated for the UIM-SH3 construct.

3. Results

3.1. Structural and dynamical properties of the UIM-SH3 construct

The structure of the UIM-SH3 construct has been obtained by homology modelling (see Section 2). The UIM domain folds into an α-helix, as supported by chemical shift index (CSI) [26] and Talos+ [27]. This result is in agreement with our previous structural prediction of the UIM part of the VHS-UIM construct [23]. The complete UIM-SH3 backbone assignment is available from BioMagResBank under the accession number 18403. To characterize the dynamical properties of the UIM-SH3 construct, we used spin relaxation measurement as it reports on the overall rotational diffusion and structure of molecules. Measurements of the ^{15}N longitudinal (R_1) as well as the transverse relaxation rate (R_2) indicate that the SH3 and the UIM domains tumble essentially independently (see Fig. S1). The average R_2 values measured for the SH3 and the UIM domains in UIM-SH3 are 10.4 ± 0.3 and 6.5 ± 0.1 s^{-1}, respectively. Moreover, the steady-state ^{15}N{^{1}H}NOE shows that the 16 amino acid linker separating the SH3 and UIM domain is highly flexible. Model-free analysis of relaxation data indicates that the UIM domain is highly dynamic in the ps-ns time scale. The average squared order parameter (S^2) for the UIM part is 0.67 ± 0.06, which reflects a greater amplitude of the backbone motion compared to the SH3 part (S^2 = 0.88 ± 0.05).

3.2. The SH3 domain of STAM2 interacts with ubiquitin

In the ^{1}H, ^{15}N-HSQC spectrum of UIM-SH3, the UIM part overlaps with its spectrum as isolated unit (see Figs. S2 and S3). This fact provides clear evidence that the SH3 and UIM domains do not interact with each other in the UIM-SH3 construct. To map the interaction of the UIM-SH3 construct with Ub we monitored chemical shift perturbations (CSPs) in ^{1}H, ^{15}N-HSQC spectra of ^{15}N-labeled UIM-SH3 or Ub upon addition of the corresponding unlabeled binding partner. As illustrated on Fig. 1A, several residues of the UIM part in the UIM-SH3 construct present significant CSPs while some showed a strong decrease of their signal intensities, indicative of intermediate exchange. Specifically, hydrophobic residues Ile177, Ala178, Ile181 and Leu185, as well as negatively charged residues Asp174 and Glu182 exhibit strong signal attenuations. Furthermore, based on CSPs, our results suggest a highly specific interaction surface between the SH3 domain and Ub, mainly mediated by hydrophobic contacts (see Figs. 1A,C and S4). The strongest perturbations include the hydrophobic residues Phe220, Ala222, Val223, Phe230, Trp247 and Leu267 as well as an adjacent region formed by negatively charged residues Asp219, Glu221, Glu227 and Glu234. From the ^{15}N-Ub side, the UIM-SH3 construct mainly targets hydrophobic residues including Ile13, Ile44, Leu50 and Val70 while Ala46 and Gly47 experience