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STAM–AMSH interaction facilitates the deubiquitination activity in the C-terminal AMSH ☆

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Abstract

Signal transducing adaptor molecule (STAM) complexed with hepatocyte growth factor regulated tyrosine kinase substrate (Hrs) works on sorting of cargo proteins in multivesicular body (MVB) pathway. Associated molecule with SH3 domain of STAM (AMSH), a zinc-containing ubiquitin isopeptidase, is thought to play a role in regulation of ubiquitin-mediated degradation by binding to STAM. We have found that AMSH requires the conformation of Px(V/I)(D/N)RxxKP sequence to bind SH3 domain of STAM with ~7 μM affinity, and that the isolated C-terminal domain of AMSH contains the isopeptidase activity. Deubiquitination by AMSH was assisted when ubiquitins were bound to STAM which can bind to AMSH simultaneously. With the specificity toward K63-linked ubiquitins, this facilitated ubiquitin processing activity of AMSH may imply a distinct regulatory mechanism for sorting and degradation through STAM binding.

Keywords: AMSH; Deubiquitination; Isopeptidase; Lysine-63 link; Metalloprotease; Multivesicular body pathway; SH3 domain; Signal transduction; STAM; Ubiquitin

Membrane trafficking is a dynamic process that is responsible for the maintenance of cellular metabolism, stress response, down regulation of cell-surface molecules, and signal transduction. Cell-surface molecules, including growth factor receptors that bind to specific ligands or macromolecules, are internalized into membrane compartments called endosomes. Although some cargoes are pinched off in recycling endosomes to be sent back to the cell surface, other receptors and molecules are pinched off into endosomes that contain such luminal vesicles called the multivesicular body (MVB) [1,2]. Ubiquitination is a sorting signal for endocytosed and newly synthesized lysosomal proteins to be incorporated into the late endosome/MVB that bud inward from its limiting membrane [3,4]. The late endosome/MVB then fuses with the lysosome, delivering the sorted proteins within the inner vesicles into the lumen of the lysosome. This trafficking route is referred to as the MVB pathway and conserved from yeasts to higher eukaryotes [2].

Hrs (hepatocyte growth factor-regulated substrate) and STAM (signal-transducing adaptor molecule) were both identified as tyrosine phosphorylated proteins in cells stimulated with growth factors and cytokines [5,6]. Detailed

☆ *Abbreviations:* AMSH, associated molecule with SH3 domain of STAM; C-AMSH, C-terminal region (residues 249–424) of AMSH; DUB, deubiquitinating enzyme; F-AMSH, full-length AMSH; Hrs, hepatocyte growth factor regulated tyrosine kinase substrate; ITC, isothermal titration calorimetry; JAMM, Jab1/MPN domain-associated metalloisopeptidase; MVB, multivesicular body; N-AMSH, N-terminal region (residues 1–173) of AMSH; P1, ^{194}**G**P**L**V**P**DLEK^{202}; P2, ^{226}**R**P**A**K**PP**VVDR^{235}; P2C-AMSH, AMSH construct from 2nd PxxP motif to C-terminal end (residues 216–424); P3, ^{230}**PP**V**V**D**R**SL**K**P**G**A^{241}; RTK, receptor tyrosine kinase; STAM, signal transducing adaptor molecule; Ub, ubiquitin; UBPY, Ub isopeptidase Y; UIM, ubiquitin interacting motif.

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characterization of these molecules has derived that the Hrs/STAM complex and its yeast ortholog Vps27/Hse1 sort ubiquitinated cargo proteins including ligand-activated receptor tyrosine kinases (RTKs) by recognizing their ubiquitin (Ub) moieties on the early endosome [7]. In mammalian cells, overexpression of Hrs or STAM induces the accumulation of ubiquitinated proteins and ligand-activated epidermal growth factor receptor on the endosome [8]. Neither Hrs mutants lacking ubiquitin interacting motif (UIM) nor STAM mutants lacking the VHS (Vps27/Hrs/STAM) domain or UIM can cause these effects, suggesting essential roles of these domains in recognizing ubiquitinated cargoes [9,10].

AMSH (associated molecule with the SH3 domain of STAM) was named after its character of binding STAM [11]. The deduced amino acid sequence of AMSH indicated the presence of at least three characteristic regions, two Pro-Xaa-Xaa-Pro (PxxP) motifs (located at Pro195–Pro198 and Pro227–Pro231) known as possible binding sites for the SH3 domain, and a JAMM (Jab1/MPN domain-associated metalloisopeptidase) motif. Other SH3 domain-binding sites have been reported and one of them is a novel sequence (Px(V/I)(D/N)RxxKP) found in UBPY (Ub isopeptidase Y), another STAM-binding deubiquitinating enzyme (DUB) [12]. Recently, AMSH has also been demonstrated to possess a JAMM motif–Zn^{2+}-dependent ubiquitin isopeptidase activity *in vitro*, which displays distinct properties from UBPY [13]. UBPY binds to the ligand-activated EGFR when it is able to bind to Hrs/STAM complex, but not stably associated with the complex. It down-regulates EGFR degradation by deubiquitinating directly on endosomes [14]. Depletion of AMSH by RNAi leads to the accelerated degradation of ligand-activated EGFR [13], which may imply AMSH is also playing a role in the regulation of EGFR, but it is not clear whether AMSH works as UBPY by direct deubiquitination.

We have investigated the binding interactions among STAM, AMSH, and Ubs, and how they are affecting the isopeptidation by AMSH. It still remains to be defined how Hrs/STAM and AMSH work on sorting of ubiquitinated proteins, and this study will help to propose a mechanism that STAM and AMSH influence ubiquitinated molecules on the MVB pathway.

Materials and methods

Constructs and purification of recombinant proteins. The full-length STAM was inserted into pET22b expression vector (Novagen) and pGEX 4T-1 vector (Amersham Bioscience) to generate 6-histidines and glutathione *S*-transferase (GST) fusion proteins. F-AMSH (full-length, residues 1–424), N-AMSH (N-terminal region, residues 1–173), C-AMSH (C-terminal region, residues 249–424), and P2C-AMSH (residues 216–424, from the 2nd PxxP motif to the C-terminal end) of AMSH were cloned into the pET-GST vector to generate the GST fusion construct with a TEV cleavage site. F-D348A and P2C-D348A mutants contain a point mutation (D348A) of full-length AMSH and P2C-AMSH, which were introduced by the QuikChange Site-directed mutagenesis.

The constructs were transformed in *Escherichia coli* DH5α cells, and the expression was induced by adding IPTG at 18 °C. Recombinant proteins were overexpressed as soluble forms, and they were purified using glutathione affinity column or Ni^{2+}-chelated column (Amersham Bioscience). If necessary, the proteins were further purified using Q-Sepharose ion-exchange column and gel filtration on a HiLoad 26/60 Superdex-75 prep-grade column. All steps were carried out at 4 °C. The purified proteins were concentrated using Amicon concentrator and stored with 5% glycerol at −80 °C until use.

Isothermal titration calorimetry (ITC) and peptide synthesis. Experiments were performed using the VP-ITC system (MicroCal Inc.). The SH3 domain of STAM or P2C-AMSH was diluted to a concentration of 50 μM in the ITC buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl), degassed for 14 min by Thermovac (MicroCal Inc.), and placed in the sample cell. P2, P3 or SH3 domain was diluted to a final concentration of 1 mM in the ITC buffer and placed in the injection syringe. In each titration experiment, 36 aliquots of P2, P3 or SH3 domain (8 μl each) were injected under computer control into the 1.43 ml sample cell at 25 °C. The experimental data were corrected for dilution by subtracting the curve obtained by titration into buffer alone and then fit by least squares regression assuming a one-site binding model using the ORIGIN software provided with the instrument. Used peptides were chemically synthesized (Anygen Co. Ltd., Korea); P1 = ^{194}**G**P**L**V**P**DLEK^{202}, P2 = ^{226}R**P**AK**PP**VVDR^{235}, and P3 = ^{230}**PPVVDRSLKP**GA^{241}.

Ubiquitin binding and deubiquitination assay. The purified recombinant STAM or AMSHs were coupled to glutathione beads as GST fusion proteins. To examine the binding, Ubs (poly-, tetra-, or di-; K63- and K48-linked; Boston Biochem Inc.) were incubated with the beads in PBS buffer containing 1% Triton X-100 for 16 h at 4 °C. Bound Ubs and the pulled-down GST fusion proteins were detected by Western blotting. After washing the beads, SDS-loading buffer containing 100 mM DTT was added then boiled for 5 min. The immunoprecipitants were applied to SDS–polyacrylamide gel for electrophoretic separation. The proteins were transferred onto a PVDF membrane at a constant current. All procedures after blotting were carried out at room temperature. The membrane was blocked, incubated with the primary antibody, then with the species-specific secondary antibody conjugated to the horseradish peroxidase enzyme for 1 h at each step, and then treated with the enzyme enhanced chemiluminescence kit (Pierce) for the exposure to films.

K48-linked poly-Ub chains (500 ng; Boston Biochem) or K63-linked poly-Ub chains and K63-linked tetra-, di-Ub chains (500 ng; Boston Biochem) were incubated at 37 °C or 4 °C for 2 h in DUB buffer (50 mM Tris–HCl, pH 7.2, 25 mM KCl, 5 mM $MgCl_2$, and 0.1 mM DTT) with AMSH or mutant AMSHs for deubiquitination assay. For checking the isopeptidase activity on STAM-bound Ubs, GST-STAM and K63 poly-Ub chains were mixed with the beads first. After washing the beads, bound STAM-Ub chains were incubated at 4 °C for 2 h in DUB buffer with or without AMSHs. Processed Ubs were detected by Western blotting as described above.

Results and discussion

AMSH needs a Px(V/I)(D/N)RxxKP sequence conformation for binding to STAM-SH3 domain

PxxP sequence has been known as the SH3-binding motif [15,16], and other sequences rather than proline-rich peptides have also been seen in SH3 binding [17–19]. When ^{227}PAKP^{230}, ^{231}PVVDRSLKP^{239}, or ^{235}RSLKP^{239} was deleted from AMSH in mammalian cells in a previous study, the following pull-down assays with the SH3 domain of STAM showed that only ^{227}PAKP^{230} sequence deleted mutant bound to the SH3 domain [12]. Other deletions did not allow AMSHs to bind STAM-SH3 domain as another deletion mutant lacking ^{227}PAKPP^{231} [11]. The crystal structure of STAM2-UBPY peptide complex gives

an assumption that the preceding residue to the non-proline-rich sequence (P^{231}–P^{239}) might be important since TPMVNR (underlined sequence is a conserved residue in the non-proline-rich sequences) was involved in the interaction with SH3 domain [20].

To clarify the site for binding, we verified the interaction of PxxP motifs and the novel sequence in AMSH with the purified recombinant SH3-domain by ITC, since the second PxxP motif and a non-proline-rich sequence are continuously located in AMSH (Fig. 1). The synthesized peptides were used; P1 containing the first PxxP motif, P2 containing the second PxxP motif and N-terminal part of the novel sequence, and P3 containing the novel sequence. When the affinity was measured by isothermal calorimeter, peptides P1 and P2 did not bind to the purified SH3 domain of STAM, however, P3 showed the affinity similar to the affinity of P2C-AMSH containing C-terminal part of AMSH from P2 motif (Fig. 1B).

The K_d between STAM-SH3 domain and the novel sequence P3 was 7.23 μM (K_d with P2C-AMSH = ~6 μM), and is in similar range to the case of UBPY, another STAM-binding DUB, with $K_d = 27$ μM measured by fluorescence microscopy [20]. This result confirms that AMSH has the same novel SH3-binding sequence as in UBPY. In addition, C-terminal portion of this P3 sequence is needed for effective binding to SH3 as in the study by Kato et al. [12], since P2 containing the N-terminal five residues of the novel sequence could not bind to SH3 domain, even though the crystal structure of STAM2–UBPY complex shows the interaction between the first half of motif and SH3 domain [20]. Taking all deletion experiments *in vivo* and peptide binding *in vitro*, it seems that AMSH cannot bind to SH3 when a conformation was disrupted in the protein by any deletion in the binding sequence, but the peptide needs a proper sequence for making a right conformation in order to bind. Further structural studies of full-length protein complex will reveal how AMSH-STAM interaction affects the functions by the changes in their binding interface, possibly with a different manner from UBPY–STAM2.

The isopeptidase activity of AMSH resides in the C-terminal part

AMSH has been recently reported to have the isopeptidase activity against K63-linked poly-Ubs [13]. It has the Zn^{2+}-binding JAMM motif in its 270–375 residues, which made us speculate that AMSH might be a protein

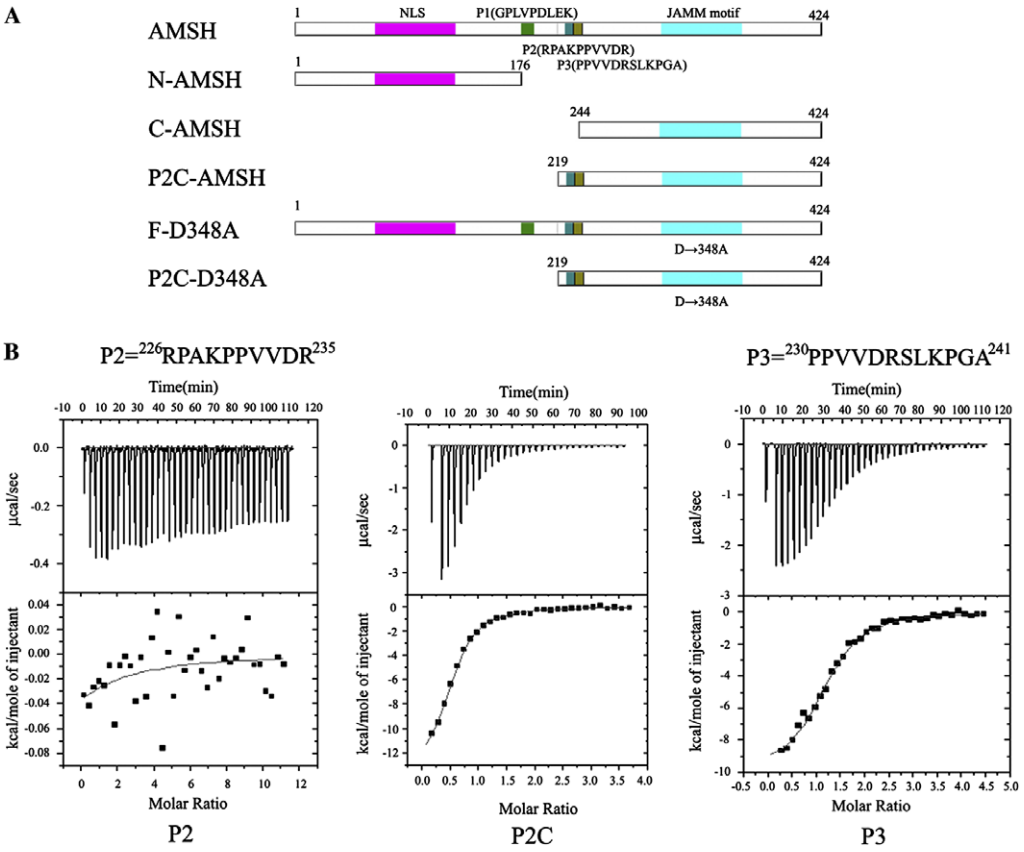

Fig. 1. AMSH–STAM binding detected by ITC. (A) Schematic representation of the recombinant proteins used for the binding assay; NLS (nuclear localization signal) in N-terminal region of AMSH is colored as magenta and JAMM motif in C-terminal domain as cyan. Three potential STAM-interacting segments, P1, P2, and P3 are colored as green, blue, and gold, respectively. (B) AMSH containing the non-proline-rich sequence (P2C-AMSH and P3) binds to STAM; isothermal calorimetric data with P2, P3 peptides and P2C-AMSH on SH3 domain of STAM.

of functionally discrete modules. Proteins working in the signal transduction are in many cases modular, to play roles to connect sending and receiving molecules in possibly different locations. The deubiquitination assay using recombinant AMSHs clearly showed that the isopeptidase activity is on the C-terminal part of AMSH, and N-AMSH did not have the activity at all (Fig. 2A). C- and P2C-AMSH were as active as F-AMSH. The processed mono-Ubs are not detected well enough, compared to the band intensity of input poly-Ubs. Deubiquitination by the C-terminal AMSH occurred only for K63-linked poly-Ubs and none of AMSHs had the ability to process K48-linked poly-Ubs (Fig. 2A; right panel) as it has been reported with full-length AMSH [13]. This isopeptidase activity was prevented when the metal chelating agent (phenanthroline) was added, confirming that the C-terminal part including JAMM motif is the functional domain for deubiquitination (Fig. 2B).

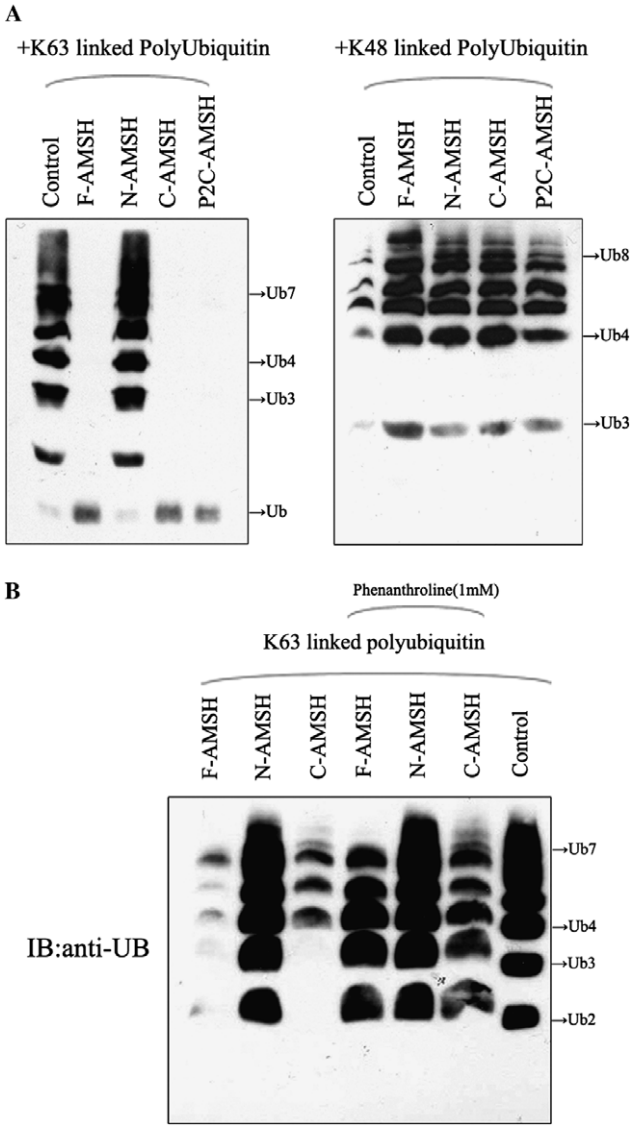

Fig. 2. C-terminal AMSH containing JAMM domain deubiquitinates K63-linked poly-Ubs. (A) Isopeptidase assay with K63-linked poly-Ubs (left panel) and K48-linked poly-Ubs (right panel). (B) Isopeptidation of K63-linked poly-Ubs by C-terminal AMSH is inhibited by metal-chelating reagent.

DUBs are specific to substrates and form several classes including JAMM/MPN proteases [21]. AMSH as a JAMM motif-containing isopeptidase shows different properties from UBPY, a cysteine protease [13]. AMSH is specific to K63-linked Ub processing compared to the fact that UBPY is working on both K48- and K63-linked Ubs [22]. It can be assumed that these two DUBs may function with STAM/Hrs for different purposes in cells. So, when STAM/Hrs complex moves ubiquitinated receptors to several directions, it may need to be checked by binding certain DUBs not to destroy ubiqutins and also to send out some cargo proteins.

AMSH–STAM binding enhances the deubiquitination of STAM-bound K63-linked ubiquitins

STAM binds to Ubs and also to AMSH, which makes a possibility that STAM can be another factor affecting the activity of AMSH in deubiquitination. We investigated the isopeptidase activity by adding different AMSH constructs after binding Ubs to STAM (Fig. 3). As described earlier, N- and C-AMSH without the STAM-interacting non-proline-rich sequence (P3), and P2C-AMSH containing from the P2 motif in front of C-AMSH were used to make sure the STAM binding (Fig. 1A). F- and P2C-AMSH proteins including P2 and P3 (continued sequence in ^{226}R–^{241}A) showed the binding to STAM (Fig. 3A). The null mutant in isopeptidase activity (D348A) had similar binding to STAM when it was the full length (F-D348A) or P2C-AMSH (P2C-D348A), implying that the STAM binding is not influenced by the deubiquitination activity in AMSH.

Toward free K63-linked Ubs, F-AMSH and C-AMSH showed almost same processing activities with less completeness at 4 °C than in the assay at room temperature (Fig. 3B; left side of left panel). P2C-AMSH also showed a similar result for free Ubs with a little attenuated activity (Fig. 3B), but N-AMSH showed no activity as seen in previous result at 37 °C (Fig. 2A). STAM-bound, K63-linked Ubs were digested better, compared to free Ubs cut by F-AMSH (Fig. 3B; right side of left panel). More interestingly, C-AMSH, which does not have any residues for STAM binding, could not deubiquitinate STAM-bound K63-linked Ubs almost as non-catalytic N-AMSH. Compared to this, P2C-AMSH which has the extended sequence to P2 motif binding to STAM showed the same activity as full-length AMSH. There was no activity in N-AMSH for STAM bound K63-linked poly-Ubs as for free K63-linked poly-Ubs. It was clear that STAM-bound Ubs are different from free Ubs upon deubiquitination by AMSH and that STAM makes deubiquitination easier or more efficient by AMSH. STAM-bound K48-linked poly-Ubs were not processed at all by any kind of AMSH constructs exhibiting same results for free K48-linked poly-Ubs

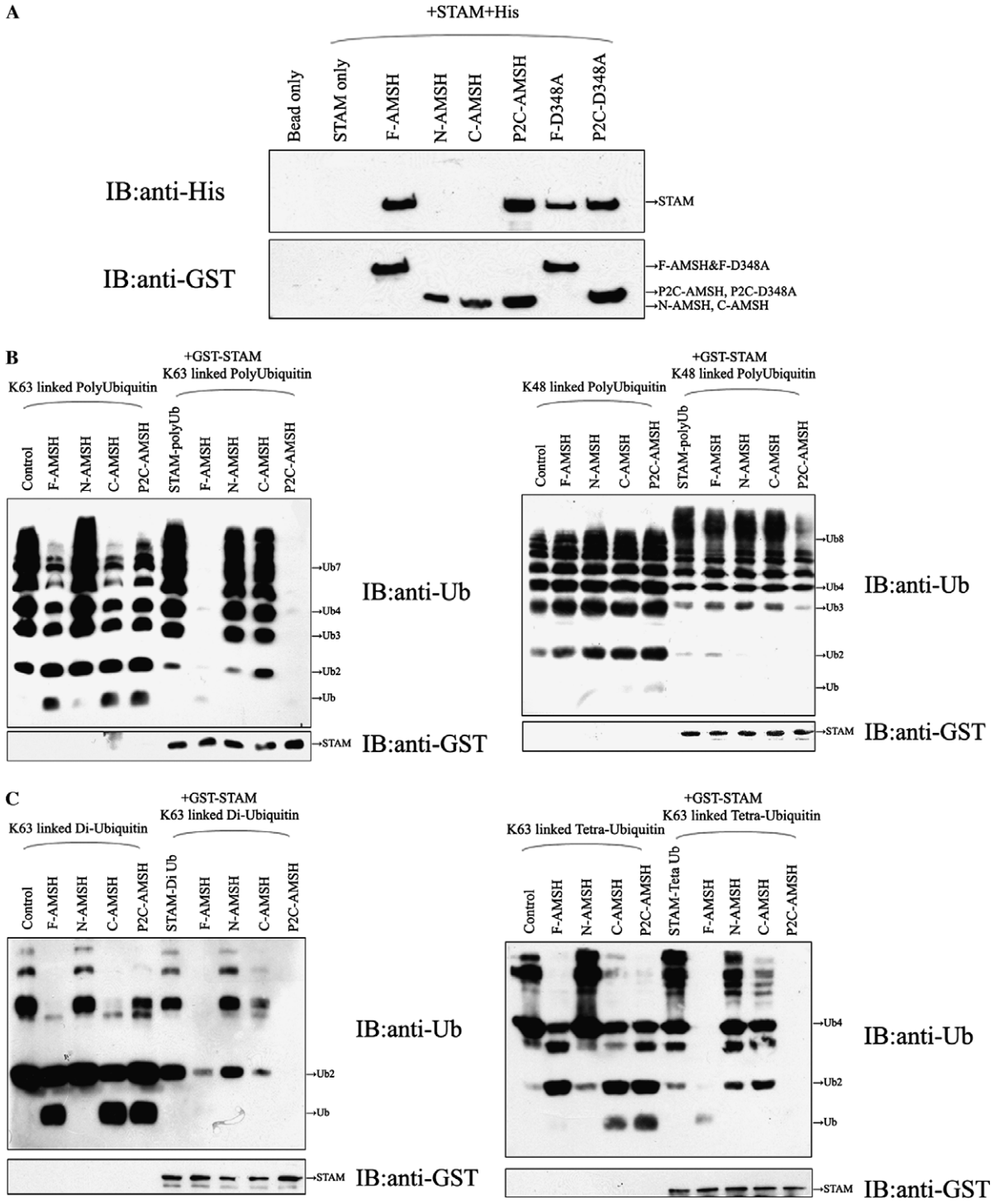

Fig. 3. Truncated AMSH with SH3-binding motif shows similar isopeptidase activity as the F-AMSH on STAM-bound Ubs. (A) Binding assay of truncated AMSHs to the full-length STAM. (B) Deubiquitination by AMSHs on free and STAM-bound K63-linked poly-Ubs (left panel), and K48-linked poly-Ubs (right panel). (C) Deubiquitination by AMSHs on free and STAM-bound K63-linked di-Ubs (left panel), and tetra-Ubs (right panel).

(Fig. 3B; right panel). The same assays with K63-linked di- and tetra-Ubs also showed similar results as with K63-linked poly-Ubs. The difference was that C-AMSH had the better effectiveness on processing the shorter Ubs bound to STAM (Fig. 3C). As seen in Fig. 2, the processed mono-Ubs were not detected well in the blots.

Conjugation with Ub serves as a sorting signal that determines the subcellular localization of various proteins such as the plasma membrane, *trans*-Golgi network (TGN), and the endosome [4,23]. Generally, the dynamic nature of Ub-protein conjugates provides a timing control for Ub-dependent events. Deubiquitination is required to

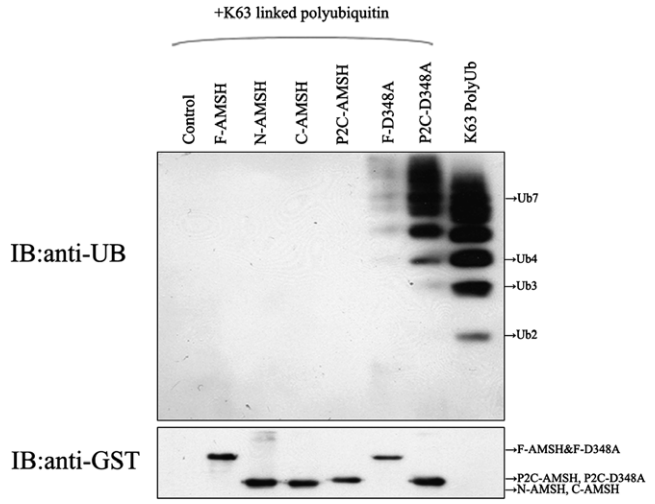

Fig. 4. Catalytically inactive AMSHs bind Ubs; AMSH bound poly-Ubs (upper panel) and the confirmation of expression level of mutant AMSHs (lower panel).

maintain the free Ub pool even though it is not mandatory for receptor sorting [24]. It was an interesting finding that the isopeptidase activity of C-terminal domain was increased when AMSH had a binding motif to STAM and Ubs were bound to STAM already (Fig. 3). The difference between P2C-AMSH and C-AMSH on processing of STAM-bound Ubs suggested that binding to STAM might be important for the function of AMSH inside the cells (Fig. 3B and C). It has been reported that pre-incubation with STAM stimulates AMSH's DUB activity on tetra-Ubs, which supports our data strongly [25].

There is an assumption that K63-linked poly-Ub chains may more closely resemble mono-Ub linked to substrate proteins with a more open configuration than K48-linked Ub [25]. Even though it was proposed that multiply mono-ubiquitinated proteins get selected for MVB pathway, our data show that mono-Ubs are not well captured after the processing by AMSH. It is worthy of mentioning that the other group had similar results of seeing that more K63-linked Ub dimers than monomers were accumulated after incubation with the AMSH fraction [14]. Maybe it has to be preceded to get more information about poly- and mono-ubiquitination on specific targets in protein degradation and sorting in order to know the criteria for distinguishing ubiqutinated proteins by certain DUBs.

STAM is holding ubiquitins for processing by AMSH

In order to confirm that the Ub binds to STAM only, the interaction between AMSH and Ubs was investigated (Fig. 4). We included D348A point mutants in JAMM domain (Fig. 1A), because this mutation has been reported to have the binding affinity to Ubs [13]. As we expected, AMSH did not show a binding activity to Ubs unless it has the mutation. It may imply that even though they can bind AMSH, binding time might be very short and AMSH's isopeptidase activity works so effectively that the bound Ubs are not detectable. When the activity was ablated by the mutation in JAMM domain which seems to be the key module for isopeptidation, Ubs were able to stay in bound state. It is interesting that the D348A mutant of F-AMSH shows less binding than that of P2C-AMSH. This could be related to a function of N-terminal domain of AMSH, which has the NLS sequence but does not show specific localization when AMSH was expressed in mammalian cells, as it appeared in the other study as well as in our data (not shown) [25].

STAM binds to ubiquitinated proteins which might have different conformation and then binding DUBs (AMSH or UBPY) may deubiquitinate specifically. When synthetic peptides P1 and P2 were added to STAM with K63-linked poly-Ubs, these 'trans' peptides did not help the deubiquitination by C-AMSH (data not shown). It seems that the weak interaction between STAM and AMSH does not change the conformation of STAM to help deubiquitination by AMSH, which only makes AMSH close enough to work on Ubs bound to STAM.

We have shown in this paper that JAMM domain containing C-terminal part of AMSH is the core for isopeptidase activity and that STAM helps this activity by holding Ubs simultaneously with AMSH. A mechanism can be suggested that AMSH functions on Ub recycling and the MVB pathway by working on the STAM bound, K63-linked Ubs or ubiquitinated proteins with a distinct manner from UBPY. The more information about the N-terminal part of AMSH will be helpful for defining the exact physiological function of AMSH.

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