AMSH is an endosome-associated ubiquitin isopeptidase

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The JAMM (JAB1/MPN/Mov34 metalloenzyme) motif has been proposed to provide the active site for isopeptidase activity associated with the Rpn11/POH1 subunit of the 19S-proteasome and the Csn5-subunit of the signalosome. We have looked for similar activity in the molecule with the SH3 domain of STAM (AMSH), a JAMM domain–containing protein that associates with the SH3-domain of STAM, a protein, which regulates receptor sorting at the endosome. We demonstrate isopeptidase activity against K48-linked tetraubiquitin and K63-linked polyubiquitin chains to generate di-ubiquitin and free ubiquitin, respectively. An inactivating mutation (D348A) in AMSH leads to accumulation of ubiquitin on endosomes and the concomitant stabilization of a ubiquitinated form of STAM, which requires an intact ubiquitin interaction motif (UIM) within STAM. Short interfering RNA knockdown of AMSH enhances the degradation rate of EGF receptor (EGFR) following acute stimulation and ubiquitinated EGFR provides a substrate for AMSH in vitro. We propose that AMSH is a deubiquitinating enzyme with functions at the endosome, which oppose the ubiquitin-dependent sorting of receptors to lysosomes.

Introduction

The signal for endosomal sorting of many receptors is the covalent addition of ubiquitin mediated by a cognate E3 ligase. This signal is recognized by the endosomal protein Hrs (Hepatocyte growth factor regulated tyrosine kinase substrate, Vps27 in yeast), which can also recruit the endosomal-associated complex required for transport (ESCRT) I to endosomes, through direct interaction with TSG101 (Vps23 in yeast; for review see Clague and Urbé, 2003). Hrs is recruited to the endosome in a complex with signal transducing adaptor molecule (STAM)/Hrs binding protein, Hse1 in yeast. At the endosome it is concentrated in regions covered with a “bilayered” clathrin coat through direct interaction with clathrin (Raiborg et al., 2001; Sachse et al., 2002), where it may serve to concentrate ubiquitinated receptors before inward vesiculation (Urbé et al., 2003).

The Hrs binding partner STAM was initially identified as a mediator of interleukin stimulated myc induction and cell proliferation (Asao et al., 1997). Two further STAM binding partners have been identified, the deubiquitinating enzyme (DUB) UBPY (Naviglio et al., 1998; Kato et al., 2000) and associated molecule with the SH3 domain of STAM (AMSH; Tanaka et al., 1999). Both of these proteins associate with the SH3 domain of STAM through a shared STAM-binding PX(V/I)(D/N)RXXKP motif and must therefore be mutually exclusive (Kato et al., 2000).

Deubiquitination itself is not mandatory for receptor sorting to the yeast vacuole, as ubiquitin can confer sorting after being fused in frame with a cargo protein (Reggiori and Pelham, 2001), but it is required for maintenance of the free ubiquitin pool, upon which receptor trafficking depends. Depletion of the yeast DUB, Doa4, leads to an arrest of receptor sorting through depletion of free ubiquitin levels (Swaminathan et al., 1999; Amerik et al., 2000; Dupre and Haguenauer-Tsapis, 2001). Just as coordinated kinase action requires the opposing effect of phosphatases, ubiquitin ligation is opposed by multiple DUB activities (Wilkinson, 2000; Wing, 2003). Two classes of DUB enzymes have been characterized as cysteine proteases; the ubiquitin COOH-terminal hydrolases family and the ubiquitin specific proteases (UBP) family of which Doa4 and UBPY are members.

Recently, a new family of isopeptidase DUB enzymes has been predicted on the basis of a shared JAB1/MPN/Mov34 metalloenzyme (JAMM) motif (Hochstrasser, 2002; Maytal-Kivity et al., 2002). The DUB activity of the proteasome recycles ubiquitin and is coupled to protein degradation. It has been linked to the JAMM-containing Rpn11/POH1 subunit.

Abbreviations used in this paper: AMSH, associated molecule with the SH3 domain of STAM; DUB, deubiquitinating enzyme; EGF, EGF receptor; Hrs, Hepatocyte growth factor regulated tyrosine kinase substrate; JAMM, JAB1/MPN/Mov34 metalloenzyme; NEM, N-ethylmaleimide; siRNA, short interfering RNA; STAM, signal transducing adaptor molecule; UBP, ubiquitin specific proteases; UIM, ubiquitin interaction motif.
of the 19S proteasome lid, through mutational analysis and sensitivity to metal chelating agents (Verma et al., 2002; Yao and Cohen, 2002). Isopeptidase activity amongst the JAMM family is not confined to ubiquitin; the Jab1/Csn5 subunit of the COP9 signalosome has been implicated in cleavage of Nedd8 from Cul1 (Cope et al., 2002; Cope and Deshaies, 2003) and the motif is also present in eubacteria and archaeabacteria which do not possess ubiquitin (Maytal-Kivity et al., 2002). Definitive biochemical evidence for direct association of the JAMM domain–containing proteins with enzymatic activity has been lacking as no activity has so far been found with purified proteins outside of their respective multicomponent complexes. The structure of a JAMM motif–containing protein AfJAMM, from Archaeoglobus fulgidus, has recently been independently determined by two groups (Tran et al., 2003; Ambroggio et al., 2004), revealing a fold that resembles cytidine deaminase and an active site architecture that is shared with thermolysin, placing the JAMM domain in a superfamily of metal-dependent proteases.

AMSH contains a JAMM motif. In this paper, we have characterized a Zn$^{2+}$-dependent ubiquitin isopeptidase activity of purified AMSH in vitro, which displays distinct properties from UBPY. Our data also indicate a functionally significant association of AMSH with endosomes that influences the rate of EGF receptor (EGFR) degradation.

**Results and discussion**

**AMSH shows deubiquitinating activity in vitro**

We provide the first direct demonstration of isopeptidase activity attributable to an isolated JAMM-containing protein. GST-AMSH and GST-UBPY, purified from *E. coli*, were incubated with K48-linked tetra-ubiquitin (Fig. 1 a). DUB activity is reported by the generation of lower denomination forms of ubiquitin (mono-, di-, and tri-ubiquitin) resulting from cleavage of the amide bond between K48 and the COOH-terminal glycine of a linked ubiquitin. The cysteine protease UBPY showed modest DUB activity toward tetra-ubiquitin at physiological pH of 7.2 (unpublished data) but efficiently generated mono-ubiquitin at pH 8.3 as characterized previously (Naviglio et al., 1998; Hartmann-Petersen et al., 2003). AMSH possessed similar DUB activity at both pH values; we therefore routinely used pH 7.2 with this enzyme. UBPY provides for complete breakdown of the tetra-ubiquitin chain into component monomers of ubiquitin. Remarkably, AMSH produces barely detectable levels of mono-ubiquitin, but preferentially generates di-ubiquitin by a single cleavage of the tetra-ubiquitin chain (Fig. 1 a).

Other DUB enzyme families are cysteine proteases and consequently sensitive to the alkylating agent NEM. We found AMSH activity was inhibited by N-ethylmaleimide (NEM) treatment (Fig. S1 c, available at http://www.jcb.org/cgi/content/full/jcb.200401141/DC1), and also inhibited by zinc chelating agents (Fig. 1 b), as expected for a metalloprotease. Although we do not expect the requirement of a catalytic cysteine for AMSH activity, we note the presence of cysteine residues within the JAMM domain of AMSH, through which NEM may exert an inhibitory effect.

We next used K63-linked and K48-linked chains of heterogeneous length as substrate to probe for linkage-dependent specificity. Although K48-linked polyubiquitin specifies proteasomal degradation, K63 linkages have been linked to DNA repair mechanisms and to the endocytic pathway (Dupre and Haguenerau-Tsapis, 2001; Schnell and Hicke, 2003). AMSH completely processed the K63 chains to mono-ubiquitin, but was unable to process the K48 chains (Fig. 1 c). This surprising failure to process the K48-linked chains may reflect the presence of di-ubiquitin in the starting mixture, which may then exert product inhibition properties consistent with the data of Fig. 1 a. Remarkably, UBPY demonstrated a completely opposite specificity; no activity against K63 linkages and full processing of the K48 chains (Fig. 1 c). A point mutation (D348A) of a conserved aspartate residue within the JAMM domain of AMSH, ablated
enzymatic activity toward both K63-linked polyubiquitin and K48-linked tetra-ubiquitin (Fig. 1 c and Fig. 2). Expression of AMSH (D348A) leads to accumulation of endosomal ubiquitin. GFP-AMSH showed marked nuclear, cytosolic, and some plasma membrane labeling as well as association with a few dispersed punctae (Fig. 2). The strong nuclear localization of AMSH is consistent with its possession of a nuclear localization sequence. A cytosolic localization of epitope-tagged AMSH has also been described previously (Itoh et al., 2001). The association with punctae was much more pronounced following expression of the enzymatically inactive mutant GFP-AMSH (D348A; Fig. 2). Punctae correspond to endosomes as judged by colocalization with EEA1 (Fig. 2 a, C and D), internalized biotin-EGF (Fig. 2 b, C and D), and to a lesser degree with recycling endosomes (Fig. 2 a, E and F; transferrin receptor at steady state). Expression of catalytically inactive AMSH (D348A) led to an accumulation of ubiquitin on endosomes that clearly exceeds the levels seen in nontransfected cells and cells overexpressing wild-type AMSH (Fig. 2 b, E and F). Furthermore, the endosomal protein Hrs, which contains a ubiquitin interaction motif (UIM), was recruited to these punctae and correspondingly depleted from the cytosol (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200401141/DC1).

AMSH–STAM interaction

We have confirmed a previously reported interaction between AMSH and STAM (Kato et al., 2000), a protein
which is in dynamic equilibrium between cytosol and endosomal membranes. Co-immunoprecipitation was observed following coexpression of GFP-AMSH and HA-STAM in HeLa cells (Fig. 3 a). Expression of an inactive mutant GFP-AMSH (D348A) led to a marked increase in this association (Fig. 3 b). We also noticed that this mutant stabilized a minor form of total STAM that runs at a higher molecular mass (Fig. 3 c), is enriched in the bound fraction and can be detected with an anti-ubiquitin antibody (Fig. 3 d). STAM contains a UIM domain, which has been shown to both bind ubiquitin and specify mono-ubiquitination of endocytic proteins which contain this motif (Polo et al., 2002, 2003). A double point mutation in the UIM domain of STAM (L176A/S177A) reverses the enhanced interaction with AMSH (D348A) and the accumulation of the ubiquitinated higher molecular mass form of STAM (Fig. 3, c and d).

This leads us to propose that on top of the well-characterized interaction of the SH3-domain of STAM with AMSH, there are further interactions that are contingent on an intact STAM-UIM domain, which are only apparent with enzymatically inactive AMSH. We suggest this may be due to the UIM-dependent addition of an AMSH substrate (e.g., ubiquitin or ubiquitin-like molecule) onto STAM, which would normally be cleaved, either by active AMSH or UBPY, but will simply provide an additional binding site for enzymatically inactive AMSH. The D348A mutant of AMSH may therefore act as a "substrate-trap" mutant, analogous to catalytically inactive mutants of tyrosine phosphatase enzymes (Flint et al., 1997), by displacing both endogenous AMSH and UBPY, which share a binding site on STAM.

Short interfering RNA (siRNA) knockdown of AMSH accelerates EGFR down-regulation

An endosome-associated DUB may be expected to influence trafficking of ubiquitinated receptors. We used siRNA to specifically knockdown AMSH in HeLa cells (Fig. 4, a–c). If
AMSH influences the dynamics of EGFR trafficking through deubiquitination, then it should alter the rate of receptor degradation following acute stimulation with EGF. We consistently observed an increased rate of receptor degradation in AMSH knockdown cells. The relative amount of receptor remaining after 30 min of stimulation compared with control cells is 0.51 (± 0.04, n = 4). A second siRNA duplex designed to knockdown AMSH likewise enhanced the rate of EGF degradation (unpublished data). The E3-ligase Cbl has been shown to promote EGFR degradation through ubiquitination of the receptor, which promotes lysosomal sorting at the expense of recycling (Levkowitz et al., 1998; Thien and Langdon, 2001). Endosomal DUBs, such as AMSH, could be expected to reverse this modification and hence oppose lysosomal sorting. In support of this notion, we are able to show that EGFR, immunoprecipitated from EGF-stimulated Her14 cells, can be deubiquitinated by AMSH in vitro. Note the smearable appearance of EGFR is due to multiple mono-ubiquitination, rather than polyubiquitination (Haglund et al., 2003; Mosesson et al., 2003), allowing us to infer that mono-ubiquitin can provide a substrate for AMSH isopeptidase activity. Thus, knockdown of AMSH may enhance receptor degradation by removing an activity, which opposes that of the relevant E3-ligase as illustrated in Fig. 5. Although we cannot fully exclude effects on components of the sorting machinery, we do not observe any appearance of additional high molecular mass bands in AMSH knockdown cells for Hrs nor for STAM, suggesting that in the absence of AMSH, another DUB, possibly UBPY, controls the ubiquitination status of STAM (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200401141/DC1). In our proposed model (Fig. 5), the action of AMSH precedes absolute commitment of the receptor to the lysosomal pathway, allowing for the possibility that an alternative DUB such as UBPY may act to recycle ubiquitin from receptors after sorting but before sequestration in lumenal vesicles, as has been proposed for Doa4 in yeast (Amerik et al., 2000).

**Concluding remarks**

Our data is the first isopeptidase activity associated with a purified JAMM domain protein. Clearly not all JAMM domain proteins are deubiquitinating enzymes as they appear in evolution before development of the ubiquitin system. Others require embedding in macromolecular complexes for activity to be observed. AMSH DUB activity can be directed against K63-linked ubiquitin and it is noteworthy that another DUB, UBPY, is unable to process these chains. The results presented here which demonstrate AMSH ubiquitin isopeptidase activity toward ubiquitin chains and EGFR, an influence upon endosomal ubiquitin levels and the rate of acute down-regulation of the EGFR all point to a physiological role for this activity.

**Materials and methods**

**Plasmids**

Human AMSH cDNA was purchased from Origene Technologies Inc., sequenced and subcloned into pGEX4T2 and pEGFP-C. The D348A mutation was introduced by QuickChange Site-directed Mutagenesis (Stratagene). GST-UBPY and HA-STAM (pMIV-HA-Hbp) constructs were gifts from G. Draetta (European Institute of Oncology, Milan, Italy) and N. Kita-mura (Tokyo Institute of Technology, Yokohama, Japan), respectively. The UIM point mutant L176/S177A was generated by PCR-based mutagenesis using the following primers: 5’-gctaaagctattgaggcagcgttgcaagagcag-3’ and 5’-ctgctctgcaaacgctgcctcaatagctttagc-3’.

**Antibodies and other reagents**

Antibodies were purchased from Covance (HA, P4G7), Affinity Bio-Reagents, Inc. (FK2), Roche (transferrin receptor), Santa Cruz Biotechnology, Inc. (EGER; RI and 1005), and Sigma-Aldrich (ubiquitin, tubulin). Anti-GFP was a gift of F. Barr (Max Planck Institute of Biochemistry, Martinsried, Germany). Biotin-EGF and secondary antibodies were purchased from Molecular Probes and Sigma-Aldrich. Rabbit polyclonal AMSH antibody was generated against the peptide MSDHGDVSLPPEDRV (CovalAb). Rabbit polyclonal EEA1 and Hrs antibodies have been described previously (Mills et al., 1998; Sachse et al., 2002). Her14 cells (stably transduced with human EGFR) were provided by E. Klapisz (Institute of Biomembranes, University of Utrecht, Utrecht, Netherlands).

**Immunofluorescence**

Transfected cells were processed 24 h after transfection for immunofluorescence as described previously (Urbé et al., 2003). Dual stained confocal images were taken with a Laser-Sharp confocal microscope (Bio-Rad Laboratories; 63x Planapol 1.4 oil objective; Carl Zeiss Microimaging, Inc.). Triple-labeled samples were stained with AF594 and 633 coupled secondary antibodies and analyzed with a confocal SP2 AOB5 (Leica; HCX PL APO CS 63.0 × 1.40 oil objective).

**Bacterial expression and purification of recombinant proteins**

GST-AMSH, -D348A, and -UBPY were expressed in Rosetta (DE3) pLyS cells (Novagen) and batch purified with glutathione-Sepharose (Amersham Biosciences) according to the manufacturer’s instructions.
Deubiquitination assay
K48-linked tetra-ubiquitin (250 ng) or K48-linked polyubiquitin chains (500 ng), purchased from Affinity BioReagents, Inc., or K63-linked polyubiquitin chains (500 ng; Boston Biochem) were incubated at 37°C for 4 or 18 h in DUB buffer (50 mM Tris-HCl, pH 7.2 or pH 8.3, 25 mM KCl, 5 mM MgCl₂, 1 mM DTT) with or without GST-AMSH, GST-D348A, or GST-UBPY in 20 μl. The reaction was stopped by holding at 4°C for 10 min, followed by addition of SDS sample buffer. Ubiquitinated EGF was immunoprecipitated from lysates (25 mM Tris, pH 7.2, 100 mM NaCl, 50 mM NaF, 0.5% NP-40, 10 mM NEM, phosphatase inhibitor cocktail II; Sigma–Aldrich) prepared from starved or EGF-stimulated HeLa cells, washed, and incubated for 18 h in DUB-buffer, pH 7.2, with AMSH added as indicated.

Detection of ubiquitin
Proteins were resolved by 15% SDS-PAGE for 3 h at 90 V with a Tris-glycine-cathode anode buffer and a Tris-glycine-based anode buffer, blocked in 0.5% fish gelatin, 0.1% Tween 20 in PBS and pH 10, 20% methanol. The membrane was boiled for 30 min in de-ionized water, soaked in 2.3% SDS, 5% -mercaptoethanol, 63 mM Tris-HCl, pH 6.8, for 30 min. Resolved proteins were transferred to nitrocellulose (0.2 μm) for 45 min in 25 mM 3-(cyclohexylamino)-1-propane-sulfonic acid, pH 10, 20% methanol. The membrane was boiled for 30 min in de-ionized water, blocked in 0.5% fish gelatin, 0.1% Tween 20 in PBS and probed with a rabbit antibody to ubiquitin (Sigma–Aldrich) followed by ECL-based detection.

Cotransfection and immunoprecipitation
HeLa cells were transfected with Genejuice (Novagen) and lysed 24 h after transfection. Immunoprecipitations were performed as described previously (Urba et al., 2003).

Depletion of cellular AMSH by siRNA
HeLa cells were treated 24 h after seeding with either control siRNA duplex (nonspecific control VII) or AMSH-specific siRNA duplex (sense 5‘-UUAACAAAUCUGCUCAAUUC-3‘; antisense 5‘-PAUAAGACGCAGA-UUUAGAUU-3‘; Dharmacon) at 44.3 nM concentration using Oligo-取证in the absence of serum (Invitrogen). 4 h after transfection, FBS was added to a final concentration of 10%.

Online supplemental material
Fig. S1 is a comparison of GST-AMSH and GST-D348A activity on tetra-ubiquitin chains (500 ng; Boston Biochem) were incubated at 37°C for 18 h in DUB-buffer, pH 7.2, with AMSH added as indicated.

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