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AMSH is required to degrade ubiquitinated proteins in the central nervous system

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ABSTRACT

Deubiquitination is a biochemical process that mediates the removal of ubiquitin moieties from ubiquitin-conjugated substrates. AMSH (associated molecule with the SH3 domain of STAM) is a deubiquitination enzyme that participates in the endosomal sorting of several cell-surface molecules. AMSH impairment results in missorted ubiquitinated cargoes *in vitro* and severe neurodegeneration *in vivo*, but it is not known how AMSH deficiency causes neuronal damage in the brain. Here, we demonstrate that AMSH$^{-/-}$ mice developed ubiquitinated protein accumulations as early as embryonic day 10 (E10), and that severe deposits were present in the brain at postnatal day 8 (P8) and P18. Interestingly, TDP-43 was found to accumulate and colocalize with glial marker-positive cells in the brain. Glutamate receptor and p62 accumulations were also found; these molecules colocalized with ubiquitinated aggregates in the brain. These data suggest that AMSH plays an important role in degrading ubiquitinated proteins and glutamate receptors *in vivo*. AMSH$^{-/-}$ mice provide an animal model for neurodegenerative diseases, which are commonly characterized by the generation of proteinaceous aggregates.

1. Introduction

The highly dynamic endosomal sorting process determines a membrane-bound protein's fate by either recycling it back to the cell surface or delivering it into endosomal network pathways. Membrane proteins destined for lysosomes are tagged with ubiquitin. Endosomal sorting complexes required for transport (ESCRT) recognizes and dictates cargo selection. ESCRT produces intraluminal vesicles (ILVs) that originate by inward budding from the limiting membrane of the sorting endosome [1]. This process creates a multivesicular body (MVB), which leads to lysosome-dependent cargo degradation through the subsequent MVB-lysosome fusion event.

Balanced ubiquitination and deubiquitination of cargos is a prerequisite for protein homeostasis. Ubiquitin modifications are reversed through the isopeptidase activities of deubiquitinating enzymes (DUBs), with most of the DUBs studied deconjugating only a small number of targets [2]. In fact, deubiquitination, a term used here to refer to both ubiquitin and ubiquitin-like deconjugation, is emerging as a regulatory process in signaling pathways, chromatin structure, endocytosis, and apoptosis [2], and is important for physiological activities such as development, immunity, and neuronal function [3].

We identified AMSH (associated molecule with the SH3 domain of STAM) [4] while screening for an ESCRT-bound molecule. AMSH is an endosomal DUB in the JAMM metalloprotease family, and plays a role in MVB/late endosomes. Recombinant AMSH has been shown to deubiquitinate epidermal growth factor receptor (EGFR) and to cleave lysine 63 (K63)-linked, but not lysine 48 (K48)-linked, polyubiquitin chains into ubiquitin monomers [5]. In a previous study, we found that AMSH binds the ESCRT-III subunit CHMP3 and plays a role in MVB/late endosomes [6]. AMSH also binds the ESCRT-III subunits CHMP1A, CHMP1B, and CHMP2A [7]. This intimate relationship between AMSH and ESCRT prompted us to investigate AMSH's *in vivo* roles. We have reported that AMSH knockout mice (AMSH$^{-/-}$) exhibit postnatal growth retardation and die between postnatal day 19 (P19) and P23. AMSH$^{-/-}$ mice exhibit severe neuronal damage, specifically neuron loss and increasing numbers of apoptotic cells, that is almost en-

Abbreviations: AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; AMSH, associated molecule with the SH3 domain of STAM; AMPAR, α-amino-3-hydroxy-5-methyl-isoxazolepropionic acid receptor; CHMP, chromatin modifying protein; ESCRT, endosomal sorting complexes required for transport; FTD, frontotemporal dementia; MVB, multivesicular body; NMDAR, *N*-methyl-D-aspartate receptor; PD, Parkinson's disease; DUB, deubiquitinating enzyme.

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tirely confined to the CA1 subfield of the hippocampus [8]. Despite the severity of these AMSH-deficient neuronal phenotypes, the pathophysiology is not fully understood.

Most age-related neurodegenerative diseases are characterized by accumulations of aberrant protein aggregates in affected regions of the brain. In particular, ubiquitin-positive proteinaceous deposits are a hallmark of neurodegeneration; such deposits include Lewy bodies in Parkinson's disease (PD), neurofibrillary tangles in Alzheimer disease (AD), Bunina bodies in amyotrophic lateral sclerosis (ALS), and Pick bodies in frontotemporal dementia (FTD) with parkinsonism [9]. Since principal function of ubiquitination is to maintain protein homeostasis inside a cell, these neuronal pathologies may indicate a failure to clear unwanted proteins [9]. A recent report suggests that ESCRT-III dysfunction is associated with neurodegeneration resembling age-dependent neurodegenerative diseases such as FTD [10]. A certain percentage of FTD is known as chromosome 3-linked FTD (FTD3), which is attributed to a genetic disorder or mutation of the ESCRT-III molecule CHMP2B [11]. In a previous study using neuron-specific knockout mice, we found that the ESCRT-0 protein Hrs plays a pivotal role in neural cell survival by clearing ubiquitinated proteins in neurons [12]. A growing body of evidence suggests that insufficient ESCRT function leads to the accumulation of ubiquitinated proteins and to human neurodegenerative disease [13]. Nevertheless, little is known about how an ESCRT-associating DUB is involved in ubiquitinated protein degradation in the central nervous system. Here, we demonstrate that ubiquitinated protein accumulations are present in brain lesions found in AMSH$^{-/-}$ mice, and that AMSH is crucial for the proper degradation of both ubiquitinated proteins and glutamate receptors in the central nervous system.

2. Materials and methods

2.1. Cell fractionation

Cerebral tissues were washed with phosphate-buffered saline (PBS), suspended with homogenization buffer (10 mM HEPES, 3 mM imidazole, and 250 mM sucrose), and dissociated by passage through a 22-G needle. The cells were centrifuged at 3000*g* for 10 min at 4 °C, and the supernatants were ultracentrifuged at 100,000*g* for 30 min at 4 °C. The supernatants were regarded as the cytoplasmic fraction, and the pellets as the membrane fraction. The pellets were resuspended with IP buffer (10 mM Hepes, pH 7.2, 0.5% Triton-X, 150 mM NaCl) and centrifuged at 10,000*g* for 30 min at 4 °C. The supernatants were filtered through a polyvinylidene difluoride (PVDF) membrane (0.45 μm, PALL Life Sciences, NY) and regarded as a membrane fraction. These fractions were quantified using the Bio-Rad Protein assay (Bio-Rad, CA) according to the manufacturer's protocol.

2.2. Western blotting

Immunoblotting was conducted as previously described [4]. In brief, mouse brain lysates were fractionated as described above, then separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto PVDF membranes (Millipore, MA). After being blocked with 5% nonfat milk in Tris-buffered saline (TBS) containing 0.1% Tween 20, the membranes were probed with the primary antibodies indicated below, washed again, and probed with horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling, MA).

2.3. Immunofluorescence reactions and immunohistochemistry

For immunofluorescence studies, mice were perfused with 4% paraformaldehyde, and 50-μm sections were prepared using a microslicer (VT1000S, Leica, Nussloch, Germany). The antibodies and dilutions used were as follows: Anti-ubiquitin mouse monoclonal Ab (mAb) 1B3 (MBL, Nagoya, Japan), 1:100; anti-ubiquitin mouse mAb FK2 (BIOMOL, NY), 1:100; anti-TDP-43 mouse mAb (TARDBP) (Proteintech Group, IL), 1:100; anti-p62 (C-terminal specific) guinea pig polyclonal antibody (pAb) (American Research Products, MA), 1:100; anti-GFAP mouse mAb (Chemicon, CA), 1:200; anti-tyrosine hydroxylase rabbit pAb (AB152, Chemicon), 1:1000; anti-microtubule-associated protein 2 goat pAb (MAP2, [14]), 1 μg/mL; anti-calbindin rabbit pAb [15], 1 μg/mL. Appropriately, coupled secondary antibodies (Alexa Fluor, Molecular Probes, CA) were used for double-labeling. For immunohistochemistry, we used the Histofine mouse stain kit or Histofine simple stain mouse MAX-PO(R) (Nichirei, Japan), according to the manufacturer's protocols.

3. Results

Because AMSH is a deubiquitinating enzyme with endosome functions, we analyzed ubiquitinated protein accumulation in the soluble (cytoplasmic) and insoluble (membrane) fractions of the AMSH$^{-/-}$ brain. Western blot analysis using the anti-ubiquitin antibody P4D1 revealed no difference in ubiquitinated protein levels in the soluble fractions from control or AMSH$^{-/-}$ brains (Fig. 1, left panels). In the insoluble fraction, however, the ubiquitinated protein levels were higher in the AMSH$^{-/-}$ brain than in the control (Fig. 1, right panels). The ubiquitinated protein levels in the insoluble fraction increased slightly from embryonic day 10 (E10) to postnatal day 8 (P8) in the control brain, and the levels were higher in the AMSH$^{-/-}$ than the control brain during E10 to P18, near the end of the AMSH$^{-/-}$ mouse lifespan. By P8 the ubiquitinated protein levels in the AMSH$^{-/-}$ brain had increased markedly. These data suggest that AMSH deficiency leads to the progressive accumulation of ubiquitinated proteins in the membrane fraction of the brain.

Histopathological examination of the hippocampus, the brain region most affected in AMSH$^{-/-}$ mice, showed evident neurodegeneration in the CA1 subfield in P6 mice [8]. Confirming the pres-

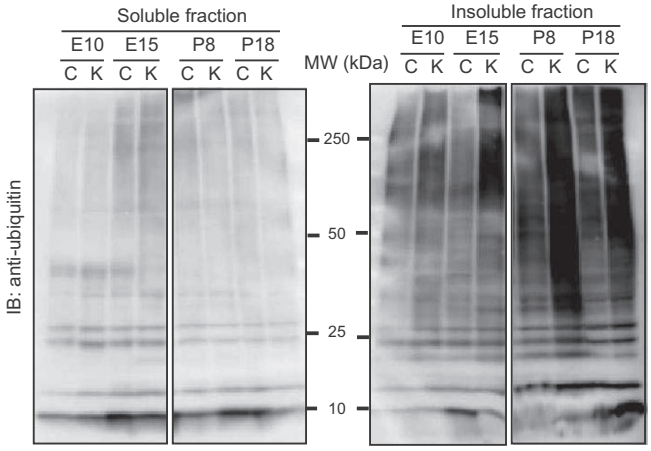

Fig. 1. Ubiquitinated proteins increase with aging in the AMSH knockout mouse brain. Western blots were performed on soluble and insoluble brain fractions of control (C) and AMSH knockout (K) mice at the ages indicated (see Section 2) using the anti-ubiquitin antibody P4D1. Gels were loaded with equal amounts of protein. IB, immunoblotting.