Dynamic regulation of ubiquitylation and deubiquitylation at the central spindle during cytokinesis

Aiko Mukai¹, Emi Mizuno¹, Kaoru Kobayashi¹, Masaki Matsumoto², Keiichi I. Nakayama², Naomi Kitamura¹ and Masayuki Komada¹,*

¹Department of Biological Sciences, Tokyo Institute of Technology, 4259-B-16 Nagatsuta, Midori-ku, Yokohama 226-8501, Japan
²Department of Molecular and Cellular Biology, Medical Institute of Bioregulation, Kyushu University, Fukuoka 812-8582, Japan
*Author for correspondence (e-mail: makomada@bio.titech.ac.jp)

Accepted 6 February 2008
Journal of Cell Science 121, 1325-1333 Published by The Company of Biologists 2008
doi:10.1242/jcs.027417

Summary
During cytokinesis, the central spindle, a bundle of interdigitated anti-parallel microtubules between separating chromosomes, recruits various cytokinetic regulator proteins to the cleavage region. Here, we show that the level of protein ubiquitylation is strikingly and transiently elevated in Aurora B kinase-positive double-band regions of the central spindle during cytokinesis. Two deubiquitylating enzymes UBPY and AMSH, which act on endosomes in interphase, were also recruited to the cleavage region. Whereas UBPY was detected only in the final stage of cytokinesis at the midbody, AMSH localized to a ring structure surrounding the mitotic kinesin MKLP1-positive region of the central spindle and midbody throughout cytokinesis. Depletion of cellular UBPY or AMSH led to defects in cytokinesis. VAMP8, a v-SNARE required for vesicle fusion in cytokinesis, localized to the central spindle region positive for ubiquitylated proteins, and underwent ubiquitylation and deubiquitylation by both UBPY and AMSH. Our results thus implicate the ubiquitylation/deubiquitylation of proteins including VAMP8 in cytokinesis.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/121/8/1325/DC1

Key words: Central spindle, Cytokinesis, Deubiquitylation, Deubiquitylating enzyme, SNARE, Ubiquitylation

Introduction
Animal cell cytokinesis is initiated by ingression of the cleavage furrow and completed by scission of the midbody, a narrow intercellular bridge between two daughter cells (Glotzer, 2005; Eggert et al., 2006; Barr and Gruneberg, 2007). Membrane fusion and fission are implicated in several aspects of this cellular event.

Fusion of secretory vesicles and recycling endosomes with the plasma membrane at the cleavage furrow supplies membranes to increase the surface area of prospective daughter cells (Matheson et al., 2005; Baluska et al., 2006). Their homotypic fusion at the midbody in the final stage of cytokinesis completes abscission, a process that severs two daughter cells at the midbody, by sealing off the plasma membrane at the cytokinetic space (Matheson et al., 2005; Baluska et al., 2006). Many proteins in the membrane fusion machinery have been identified as essential regulators of cytokinesis (Albertson et al., 2005). For example, the SNARE proteins VAMP8/endobrevin and syntaxin 2 localize to the central spindle, a bundle of interdigitated anti-parallel microtubules between separating chromosomes, and play essential roles in cytokinesis (Low et al., 2003).

Endosomes are an organelle that sorts cell-surface membrane proteins that have undergone endocytosis (Gruenberg and Stenmark, 2004). In this process, ubiquitylation of endocytosed cargo proteins serves as a signal for lysosomal trafficking (Saksena et al., 2007). The endosomal sorting of ubiquitylated proteins is executed by sequential actions of endosomal sorting complex required for transport (ESCRT) 0, I, II and III, all of which are complexes of class E vacuolar protein-sorting (Vps) proteins (Saksena et al., 2007). The ESCRT complexes, together with other class E Vps proteins, play essential roles not only in the sorting of ubiquitylated cargo proteins but also in the invagination and scission of the lumenal vesicles of multivesicular endosomes (MVEs). Recently, components of ESCRT-I and ESCRT-III, as well as other class E Vps proteins, were shown to localize to the midbody and be required for cytokinesis, suggesting that the scission of daughter cells during cytokinesis use the same molecular machinery as that for the scission of MVE lumenal vesicles (Carlton and Martin-Serrano, 2007; Morita et al., 2007).

ESCRT-0, which is composed of two ubiquitin (Ub)-binding proteins, Hrs and STAM, interacts with two deubiquitylating enzymes (DUBs): UBPY and AMSH (Komada and Kitamura, 2005; Clague and Urbe, 2006). These DUBs deubiquitylate cargo proteins on endosomes and regulate their lysosomal traffic (McCullough et al., 2004; Mizuno et al., 2005). UBPY is also required for maintaining the morphology of endosomes by deubiquitylating various proteins on the organelle (Mizuno et al., 2006; Row et al., 2006). We have recently shown that 14-3-3 proteins bind to Ser⁶⁸⁰-phosphorylated UBPY and inhibit its catalytic activity (Mizuno et al., 2007). In M phase, UBPY is dephosphorylated at Ser⁶⁸⁰, dissociated from 14-3-3 proteins, and catalytically activated (Mizuno et al., 2007). These findings suggested that the importance of M phase-specific UBPY function and prompted us to study the role of UBPY during cell division. In this study, we demonstrate a dynamic regulation of protein ubiquitylation and deubiquitylation at the central spindle, which are implicated in cytokinesis.
Results

Subcellular localization of UBPY during cytokinesis

Immunofluorescence staining of HeLa cells with a previously characterized anti-UBPY antibody (Mizuno et al., 2005) showed that endogenous UBPY is broadly distributed in the cytoplasm in M phase until the onset of cytokinesis (A.M., E.M., K.K., M.M., K.I.N., N.K. and M.K., unpublished). However, it exhibited a specific subcellular localization during cytokinesis. In this study, we classified cytokinesis into four stages according to the morphology of the cell, central spindle and chromosome (Fig. 1A).

Stage 1 (S1) is the earliest stage, when the cleavage furrow emerges. At stage 2 (S2), the cleavage furrow ingresses, central spindles undergo compaction, and chromosomes start de-condensation. At stage 3 (S3), compaction of central spindles and de-condensation of chromosomes proceed, but prospective daughter cells still exhibit a round shape. Stage 4 (S4) is the final stage when daughter cells start flattening on the culture dish and central spindles become thin. UBPY staining was diffuse in the cytoplasm until stage 3 of cytokinesis (A.M., E.M., K.K., M.M., K.I.N., N.K. and M.K., unpublished). At stage 4, however, UBPY was concentrated in a double-band region flanking the Flemming body, a phase-dense central region of the midbody negative for anti-tubulin staining (Fig. 1B-B′) (Gromley et al., 2005). The same staining pattern was observed with a second anti-UBPY antibody raised in another rabbit but not with pre-immune serum (A.M., E.M., K.K., M.M., K.I.N., N.K. and M.K., unpublished). FLAG epitope-tagged UBPY, transfected into HeLa cells, also localized to the midbody, as assessed by anti-FLAG immunofluorescence, confirming the specificity of the anti-UBPY staining (Fig. 1C-C′). In addition, UBPY was detected at the midbody also in monkey COS-7 and mouse NIH-3T3 cells (Fig. S1A-A′,B-B′, supplementary material).

Subcellular localization of AMSH during cytokinesis

AMSH is the other DUB that interacts with ESCRT-0 (Tanaka et al., 1999; McCullough et al., 2004). We next examined the subcellular localization of endogenous AMSH during cytokinesis. Immunofluorescence staining of HeLa cells with a previously characterized anti-AMSH antibody (Tanaka et al., 1999) showed that AMSH also localizes to the cleavage region in dividing cells. However, the pattern of localization was spatially and temporally different from that of UBPY. AMSH was faintly detected at the cleavage plane already at stage 1 and persistently observed throughout cytokinesis (Fig. 1D-G′). In contrast to the double-band staining pattern of UBPY (Fig. 1B,C), AMSH staining was observed as single-band (Fig. 1D,E,G) or ring-shaped (Fig. 1F; Fig. S1C, supplementary material) structures, suggesting that AMSH localizes to the ‘midbody ring’ containing various cytokinetic proteins (Gromley et al., 2005; Zhao et al., 2006). In interphase cells, AMSH was detected in the midbody remnants, a residual structure of the midbody inherited by either of the daughter cells (Fig. 1E,E′,F,F′, asterisks) (Mishima et al., 2002; Gromley et al., 2005). The same localization pattern was observed in COS-7 and NIH-3T3 cells (Fig. S1C-C′,D-D′, supplementary material).

Fig. 1. UBPY and AMSH localize to the central spindle and midbody during cytokinesis. (A) Classification of the four stages (S1–S4) of cytokinesis used in this study. HeLa cells were double-stained for microtubules (red) and DNAs (blue) with anti-tubulin antibody and TO-PRO-3, respectively. See text for details. (B–B′) HeLa cells at stage 4 of cytokinesis were triple-stained with anti-UBPY antibody (B), anti-tubulin antibody (B′, red) and TO-PRO-3 (B′, blue). (C–C′) HeLa cells were transfected with FLAG-tagged UBPY, and triple-stained with anti-FLAG antibody (C), anti-tubulin antibody (C′, red) and TO-PRO-3 (C′, blue) at stage 4 of cytokinesis. (D–D′) HeLa cells at stages 1 (D–D′), 2 (E–E′,F,F′) and 4 (G–G′) of cytokinesis were triple-stained with anti-AMSH antibody (D–G), anti-tubulin antibody (D′–G′, red) and TO-PRO-3 (D′–G′, blue). B′–G′ are merged images. Asterisks in E,E′,F,F′ indicate the midbody remnants in neighboring interphase cells. Insets show high-magnification images of the central spindle/midbody regions. Scale bars: 10 μm.
Subcellular localization of ubiquitylated proteins during cytokinesis

The localization of DUBs to the central spindle and midbody suggested the presence of ubiquitylated proteins that are to be deubiquitylated by these DUBs during cytokinesis. We therefore stained HeLa cells with FK2, a monoclonal antibody that specifically recognizes Ub when it is conjugated to target proteins or another Ub (Fujimuro and Yokosawa, 2005). It does not recognize free Ub and is commonly used to detect poly- and mono-ubiquitylated proteins in the cell (Fujimuro and Yokosawa, 2005). Before the emergence of the cleavage furrow, FK2 exhibited diffuse cytoplasmic staining at very low levels (A.M., E.M., K.K., M.M., K.I.N., N.K. and M.K., unpublished). The antibody started to stain the bilateral regions of the cleavage plane at stage 1 of cytokinesis (Fig. 2A–A′). The double-band FK2 staining reached a maximal level at stage 2 (Fig. 2B–B′), then decreased to lower and hardly detectable levels at stages 3 (Fig. 2C–C′) and 4 (Fig. 2D–D′), respectively. The same staining pattern was observed in COS-7 and NIH-3T3 cells (Fig. S1E–E′,F–F′, supplementary material).

To confirm that FK2 indeed recognized ubiquitylated proteins, we examined whether exogenously added Ub chains compete with the staining by FK2. When FK2 was pre-incubated with a ~70-fold molecular excess of Lys48- or Lys63-linked Ub chains, FK2 staining was significantly abolished (Fig. S2, supplementary material). We next examined the localization of HA epitope-tagged Ub in transfected HeLa cells using anti-HA antibody. During cytokinesis, the majority of HA-Ub localized to the central spindle, where the staining completely merged with that by FK2 (Fig. 2E–E′).

To elucidate the spatial relationship between the ubiquitylated proteins and AMSH at the central spindle, we double-stained HA-Ub-transfected HeLa cells with anti-HA and anti-AMSH antibodies at stage 2 of cytokinesis. The AMSH-positive ring structure was located between and in contact with the two bands positive for HA-Ub, suggesting that AMSH deubiquitylates its substrate proteins at the contact site of the ubiquitylated protein- and AMSH-positive regions (Fig. 2F–F′).

Ubiquitylated proteins, UBPY, and AMSH localize to specific central spindle regions

The central spindle is the region where cytokinetic proteins are recruited to promote cell division. Centralspindlin, a complex of a mitotic kinesin MKLP1 and a Rho family GTPase-activating protein MgcRacGAP, localizes to the center of the central spindle (Mishima et al., 2002). The other mitotic kinesin MKLP2 and the Aurora B kinase complex, on the other hand, localize bilaterally to the centralspindlin-positive central region (Grunenberg et al., 2004). In immunofluorescence, the staining for ubiquitylated proteins perfectly overlapped with that of Aurora B (Fig. 3A–A′) and sandwiched the MKLP1-positive region (Fig. 3B–B′). UBPY also co-localized with Aurora B in the midbody at stage 4 (Fig. 3C–C′). By contrast, the AMSH-positive ring was positioned in the central region, where it surrounded the MKLP1-positive region (Fig. 3D–D′). These results indicate that the central spindle regions critical for cytokinesis are the site of dynamic protein ubiquitylation and deubiquitylation.

UBPY and AMSH interact with the ESCRT-0 complex on endosomes (Clague and Urbe, 2006). We therefore examined whether its component Hrs also localizes to the cleavage region. Immunostaining of HeLa cells with two independent anti-Hrs antibodies showed that besides endosomes, Hrs localized to the central, but not the flanking, region of the midbody at stage 4 of cytokinesis (Fig. 3E–E′). In the earlier stages, the level of Hrs staining was much lower at the central spindle (Fig. S3A–A′, supplementary material). This localization pattern was similar to that of a cytokinetic regulator Cep55 (Fabbro et al., 2005; Zhao et al., 2006), which was recently shown to exhibit yeast two-hybrid interaction with Hrs (Morita et al., 2007). However, the pattern was...
μ

Insets show high-magnification images of the central spindle/midbody regions. Double-stained with anti-Hrs (E) and anti-tubulin (E) and TO-PRO-3 (D), punctate staining in green indicates endosomes. A triple-stained with anti-AMSH antibody (D), anti-MKLP1 antibody (D) and TO-PRO-3 (C), triple-stained with anti-UBPY antibody (C), anti-Aurora B antibody (C) and TO-PRO-3 (B), triple-stained with anti-HA antibody (A), anti-Aurora B antibody (A) and TO-PRO-3 (A). Fig. 3. Marker analysis of the cleavage regions positive for UBPY, AMSH and ubiquitylated proteins. (A-A") HeLa cells were transfected with HA-Ub, and triple-stained with anti-HA antibody (A), anti-Aurora B antibody (A', red) and TO-PRO-3 (A", blue) at stage 2 of cytokinesis. (B-B") HeLa cells at stage 2 of cytokinesis were triple-stained with FK2 (B), anti-MKLP1 antibody (B', red) and TO-PRO-3 (B", blue). (C-C") HeLa cells at stage 4 of cytokinesis were triple-stained with anti-UBPY antibody (C), anti-Aurora B antibody (C', red) and TO-PRO-3 (C", blue). (D-D") HeLa cells at stage 2 of cytokinesis were triple-stained with anti-AMSH antibody (D), anti-MKLP1 antibody (D', red) and TO-PRO-3 (D", blue). (E-E") HeLa cells at stage 4 of cytokinesis were double-stained with anti-Hrs (E) and anti-tubulin (E') antibodies. Cytosplasmic punctate staining in green indicates endosomes. A"-E" are merged images. Insets show high-magnification images of the central spindle/midbody regions. Scale bars: 10 μm.

Spatially or temporally different from those of UBPY and AMSH. We also stained dividing HeLa cells for endosome markers; EEA1 for early endosomes, LAMP1 for late endosomes and lysobisphosphatidic acid (LBPA) for MVEs. None of these molecules was detected in the central spindle and midbody at any stage of cytokinesis (Fig. S3B-D, supplementary material; A.M., E.M., K.K., M.M., K.I.N., N.K. and M.K., unpublished).

UBPY and AMSH are required for efficient cytokinesis
To study the significance of protein deubiquitylation in cleavage regions, we examined the effect of depleting UBPY and AMSH in cytokinesis in HeLa cells using RNA interference (RNAi). Transfection of two independent small interfering RNAs (siRNAs) for each DUB led to a significant reduction in their protein levels (Fig. 4A). The consequence of a failure in cytokinesis is the generation of multi-nucleated cells. We therefore stained siRNA-transfected cells with a fluorescent nuclear marker SYTOX green (Fig. 4B), and quantified the population of multi-nucleated cells by counting them under the fluorescence microscope. As shown in Fig. 4C, depletion of UBPY or AMSH resulted in a marginal increase in the population of multi-nucleated cells (7±2% in mock-transfected, 12±1% and 12±2% in UBPY siRNA-1 and -2 transfected, and 18±2% and 17±3% in AMSH siRNA-1 and -2 transfected cells). Combined depletion of the DUBs had no additive effect, with a multi-nucleated cell population of 17~20% (A.M., E.M., K.K., M.M., K.I.N., N.K. and M.K., unpublished). These results suggest that the UBPY- and AMSH-mediated protein deubiquitylation is required for efficient cytokinesis.

We also stained siRNA-transfected cells with anti-tubulin antibody and quantified the population of cells in stages 1~3 and stage 4 of cytokinesis by counting them. We did not find statistically significant differences in the population of these cells between control, UBPY-depleted, and AMSH-depleted cells (A.M., E.M., K.K., M.M., K.I.N., N.K. and M.K., unpublished). When AMSH was depleted, however, dividing cells with an atypically long central spindle (>~20 μm; Fig. 4D, arrow) were more frequently observed than in mock-transfected cells in which the length of the central spindle was usually ~10 μm, even in the final stage of cytokinesis (Fig. 1A, stage 4). In AMSH-depleted cells with the atypical central spindle, ubiquitylated proteins were detected at the midbody ring by FK2 staining (Fig. 4D, arrowhead). The counting of cells with the long central spindle (>~20 μm) showed that their population increased approximately twofold after AMSH depletion (Fig. 4E). The depletion of UBPY, by contrast, had no effect (Fig. 4E).

VAMP8 undergoes ubiquitylation
Inhibition of UBPY results in an accumulation of ubiquitylated proteins, which are likely to be the substrates for UBPY, on endosomes (Mizuno et al., 2006; Row et al., 2006). As an independent experiment to identify comprehensively these ubiquitylated proteins, we performed a proteome analysis. HeLa cells were transfected with UBPY-C748A, a catalytically inactive UBPY mutant that acts dominant negatively (Mizuno et al., 2006). The membrane fraction of the cells was solubilized, and membrane-associated ubiquitylated proteins were purified by affinity chromatography using FK2-conjugated beads. Analysis of the recovered ubiquitylated proteins, after trypsin digestion, using a two-dimensional liquid chromatography/tandem mass spectrometry system identified a peptide fragment GENLEHLR (MASCOT score=42~47; A.M., E.M., K.K., M.M., K.I.N., N.K. and M.K., unpublished) corresponding to amino acid residues 38-45 in VAMP8, a v-SNARE for endosome fusion and regulated exocytosis (Antonin et al., 2000; Wang et al., 2004). This suggested that VAMP8 is either ubiquitylated or associated with other ubiquitylated proteins in UBPY-inhibited cells. We focused on this protein.
Ubiquitylation in cytokinesis

because VAMP8 localizes to the Aurora B-positive region of the central spindle and is required for cytokinesis (Low et al., 2003; Zhao et al., 2006). Indeed, immunofluorescence staining showed that both endogenous and FLAG-tagged VAMP8 localize to the FK2-positive region at the central spindle, suggesting that VAMP8 is ubiquitylated at the region (Fig. 5A–A′/H11033, B–B′/H11033).

To examine whether VAMP8 undergoes ubiquitylation, we expressed FLAG-tagged VAMP8 in HeLa cells. As a negative control, we also constructed VAMP8 mutants in which the Lys residues are replaced by Arg residues individually or in combination (Fig. 5C). The K24R, K47R and K59R mutants harbor the mutations at Lys24, Lys47 and Lys57, respectively. 4KR harbors the mutations at Lys64, Lys68, Lys72 and Lys75, whereas K0 lacks all the seven Lys residues. When wild-type VAMP8 was immunoprecipitated from transfected cells with anti-FLAG antibody and immunoblotted with the same antibody, a ladder of bands that possibly correspond to VAMP8 species conjugated with 1~3 Ub molecules was detected (Fig. 5D, left, bands 1~3). These bands became fainter when VAMP8-4KR was expressed, and disappeared when VAMP8-K0 was expressed (Fig. 5D, left). Reprobing of the immunoblot membrane with FK2 confirmed that the bands 2 and 3 indeed correspond to ubiquitylated VAMP8 (Fig. 5D, right). The band 1 was undetectable with FK2, probably owing to a low affinity of FK2 to mono-ubiquitylated VAMP8. Bands 1~3 were still detected when cell lysates were prepared with a hot-lysis method, excluding the possibility that they are other ubiquitylated proteins associated with VAMP8 (A.M., E.M., K.K., M.M., K.I.N., N.K. and M.K., unpublished).

UBPY and AMSH deubiquitylate VAMP8

To examine whether ubiquitylated VAMP8 is a substrate for UBPY and AMSH, FLAG-VAMP8 was transfected into HeLa cells together with HA-tagged UBPY, UBPYC748A, AMSH or the catalytically inactive dominant-negative mutant AMSHD348A (McCullough et al., 2004). FLAG-VAMP8 was immunoprecipitated from their lysates with anti-FLAG antibody and immunoblotted with FK2. The levels of the bands 2 and 3 decreased when wild-type UBPY or AMSH was overexpressed, and increased when UBPYC748A or AMSHD348A was expressed (Fig. 6A, top). FK2 also slightly detected the band 1 when these DUBs were inhibited (Fig. 6A, top). Reprobing of the immunoblot membrane with anti-HA antibody showed that UBPY and AMSH are associated with VAMP8 in the cell (Fig. 6A, second from top). Ubiquitylation of FLAG-VAMP8 similarly increased when HeLa cells were depleted of endogenous UBPY or AMSH using RNAi (Fig. 6B, top). Ubiquitylation of FLAG-VAMP8 was also elevated when UBPYC748A or AMSHD348A was expressed (Fig. S4A, top), suggesting that VAMP8 undergoes deubiquitylation by both UBPY and AMSH at the central spindle.

Discussion

In this paper, we demonstrated that the level of protein ubiquitylation rises strikingly and transiently in the Aurora B-positive region of the central spindle during cytokinesis, suggesting the regulation of this dynamic cellular event by ubiquitylation (Figs 2 and 3). To our knowledge, this is the first report that implicates ubiquitylation in cytokinesis. The same localization pattern of ubiquitylated proteins in different cell types of different species suggests that it is a common regulatory mechanism at least in mammalian cells (Fig. S1, supplementary material). In addition, we showed that the
endosomal DUBs, UBPY and AMSH, also localize to the cleavage regions during cytokinesis (Figs 1 and 3). However, they exhibited distinct localization patterns from that of the ubiquitylated proteins (Figs 1-3). The site of AMSH localization was spatially different from, but in contact with, the regions positive for ubiquitylated proteins. UBPY localized to the same Aurora B-positive region as the ubiquitylated proteins, but was found only in the final stage of cytokinesis. Such a spatial and temporal difference in the localization of ubiquitylated proteins and the DUBs may facilitate the strict regulation of the site and timing of protein deubiquitylation during the cellular process.

RNAi-mediated depletion of UBPY and AMSH resulted only in a moderate increase in the population of multi-nucleated cells (Fig. 4). These results suggest that the DUB activities are required for increasing the efficiency of cytokinesis but are not crucial for the event. Deubiquitylation may therefore have an accessory role in the regulation of the ubiquitylated proteins at the central spindle. The population of cells in the final cytokinesis stage (stage 4), as well as those in stages 1–3, was not significantly affected by depletion of UBPY or AMSH (A.M., E.M., K.K., M.M., K.I.N., N.K. and M.K., unpublished). Moreover, cells with an atypically long central spindle were more frequently observed when AMSH was depleted (Fig. 4). These results suggest that the cleavage furrow ingression proceeds normally in UBPY- and AMSH-depleted cells, and they rather have a defect in abscission. However, we cannot fully exclude the possibility that the knockdown efficiency of the DUBs was not sufficient to inhibit their functions completely in siRNA-transfected cells, because UBPY and AMSH were still faintly detectable in the lysates of these cells (Fig. 4).

Both UBPY and AMSH interact with ESCRT-0. However, the pattern of Hrs localization was different from those of UBPY and AMSH during cytokinesis (Fig. 3; Fig. S3, supplementary material), suggesting that ESCRT-0 does not mediate the localization of these DUBs at the cleavage regions. These DUBs also interact with ESCRT-III (Clague and Urbe, 2006; Row et al., 2007). The ESCRT-III components exhibit a double-band localization pattern in the midbody that is similar to that of UBPY in the final stage of cytokinesis (Morita et al., 2007). Therefore, UBPY is possibly recruited to the midbody by ESCRT-III. However, this mechanism cannot be applied to AMSH, because its localization pattern was different from that of ESCRT-III. In addition, none of the endosome markers tested localized to the cleavage regions throughout cytokinesis, suggesting that the DUBs and ubiquitylated proteins are not associated with ordinary early–late endosomes in the regions (Fig. S3, supplementary material).

Our results suggest that VAMP8 undergoes ubiquitylation and deubiquitylation by UBPY and AMSH at the central spindle and midbody (Figs 5 and 6). We failed to identify the ubiquitylated Lys proteins. VAMP8 undergoes ubiquitylation. (A-B') HeLa cells were transfected without (A-A') or with (B-B') FLAG-VAMP8, and stained with anti-VAMP8 (A) or anti-FLAG (B) antibody, together with FK2 (A',B', red) and TO-PRO-3 (A', blue), at stage 2 or 3 of cytokinesis. A' and B' are merged images. Insets show high magnification images of the central spindle region. Scale bar: 10 μm. (C) Schematic structure of mouse VAMP8 and its mutants used in this study. Positions of the SNARE motif, transmembrane domain and the seven Lys residues are indicated. (D) FLAG-tagged VAMP8 and its mutants were transfected into HeLa cells, and immunoprecipitated with anti-FLAG antibody. The immunoprecipitates were immunoblotted with anti-FLAG antibody (left) or FK2 (right). Black arrowheads indicate the positions of non-ubiquitylated FLAG-VAMP8 (band 0, ~14 kDa) and FLAG-VAMP8 proteins conjugated with 1 (band 1, ~22 kDa), 2 (band 2, ~30 kDa) and 3 (band 3, ~38 kDa) Ub molecules. Asterisks indicate the IgG light (L) chain used for immunoprecipitation. The identity of the bands indicated by a white arrowhead is unknown. Ubiquitylated VAMP8 was often detected as a doublet band. The reason for this is unknown.

Fig. 5. VAMP8 undergoes ubiquitylation. (A–B’) HeLa cells were transfected without (A–A’) or with (B–B’) FLAG-VAMP8, and stained with anti-VAMP8 (A) or anti-FLAG (B) antibody, together with FK2 (A’,B’, red) and TO-PRO-3 (A’, blue), at stage 2 or 3 of cytokinesis. A’ and B’ are merged images. Insets show high magnification images of the central spindle region. Scale bar: 10 μm. (C) Schematic structure of mouse VAMP8 and its mutants used in this study. Positions of the SNARE motif, transmembrane domain and the seven Lys residues are indicated. (D) FLAG-tagged VAMP8 and its mutants were transfected into HeLa cells, and immunoprecipitated with anti-FLAG antibody. The immunoprecipitates were immunoblotted with anti-FLAG antibody (left) or FK2 (right). Black arrowheads indicate the positions of non-ubiquitylated FLAG-VAMP8 (band 0, ~14 kDa) and FLAG-VAMP8 proteins conjugated with 1 (band 1, ~22 kDa), 2 (band 2, ~30 kDa) and 3 (band 3, ~38 kDa) Ub molecules. Asterisks indicate the IgG light (L) chain used for immunoprecipitation. The identity of the bands indicated by a white arrowhead is unknown. Ubiquitylated VAMP8 was often detected as a doublet band. The reason for this is unknown.
Ubiquitylation in cytokinesis

1331

residue(s) in VAMP8 because all the Lys-to-Arg mutants except for K0 exhibited ubiquitylation to various extent (Fig. 5), indicating that most, if not all, of the Lys residues are capable of undergoing ubiquitylation at least when other Lys residues are mutated. Anyway, these observations suggest that the VAMP8 function is regulated by ubiquitylation and subsequent deubiquitylation during cytokinesis. The significance of VAMP8 ubiquitylation in cytokinesis is unknown. To test this, we examined whether the VAMP8-K0 mutant acts in a dominant-negative manner in cytokinesis when overexpressed in HeLa cells. The population of multi-nucleated cells, however, was not significantly affected by VAMP8-K0 expression (A.M., E.M., K.K., M.M., K.I.N., N.K. and M.K., unpublished). Replacement of all the Lys residues might have caused a complete loss of the SNARE activity of VAMP8. It is also important to elucidate how ubiquitylation regulates the VAMP8 function. Co-immunoprecipitation experiments showed that ubiquitylated, as well as non-ubiquitylated, VAMP8 binds to syntaxin 2 and SNAP-23, the t-SNARE proteins shown to interact with VAMP8 (Low et al., 2003; Wang et al., 2004), suggesting that the ubiquitylation of VAMP8 does not inhibit its interaction with t-SNARE proteins (Fig. 5B, supplementary material). However, these experiments did not allow a quantitative comparison of the t-SNARE-binding affinity between ubiquitylated and non-ubiquitylated VAMP8. In addition, VAMP8 has been shown to form a homodimer (Mascia and Langosch, 2007), raising the possibility that ubiquitylated VAMP8 indirectly interacts with the t-SNARE proteins via non-ubiquitylated VAMP8. We also tested the possibility that ubiquitylation regulates the subcellular localization of VAMP8 by examining whether depletion of UBPY and AMSH affects the localization in immunofluorescence staining. The siRNA treatment, however, did not significantly affect the localization of endogenous VAMP8 at the central spindle/midbody regions (A.M., E.M., K.K., M.M., K.I.N., N.K. and M.K., unpublished). Further study is necessary to elucidate the role and significance of VAMP8 ubiquitylation in cytokinesis.

We do not speculate that VAMP8 is the only ubiquitylated protein at the Aurora B-positive central spindle region, because the level of FK2 staining was significantly higher in the region than in other regions of the cell such as the cytoplasm and nucleus (Fig. 2). Aurora B is a protein kinase that forms a complex with INCENP, survivin and borealin (Glotzer, 2005; Eggert et al., 2006), and is recruited to the central spindle by the mitotic kinesin MKLP2 (Gruneberg et al., 2004). These essential cytokinetic regulators are therefore other candidates for the ubiquitylated proteins detected by FK2 in this study. Previous studies have shown that Aurora B (Nguyen et al., 2005; Sumara et al., 2007) and survivin (Vong et al., 2005) undergo ubiquitylation during cell division. However, their ubiquitylation regulates earlier mitotic events at the chromosome and centromere before the onset of cytokinesis (Vong et al., 2005; Sumara et al., 2007). In addition, we failed to detect the ubiquitylation of endogenous Aurora B in HeLa cells even when UBPY and AMSH were inhibited by overexpressing UBPYC748A or AMSHD348A (A.M., E.M., K.K., M.M., K.I.N., N.K. and M.K., unpublished). These observations suggest that Aurora B and survivin are not ubiquitylated at the central spindle, although we can not fully exclude the possibility.
What is the role of protein ubiquitylation at the central spindle? It may regulate the fusion of vesicles required for cytokinesis, in which VAMP8 plays an essential role (Low et al., 2003). Recently, the ESCRT proteins were shown to be required for abscission (Carlton and Martin-Serrano, 2007; Morita et al., 2007), raising the possibility that the ubiquitylated proteins cooperate with the ESCRT machinery in this final cytokinetic event. In this case, the ubiquitylated proteins should function upstream of the ESCRT machinery, as the level of protein ubiquitylation was high in early cytokinesis stages before the ESCRT proteins appear at the midbody (Fig. 2) (Carlton and Martin-Serrano, 2007; Morita et al., 2007). A third possibility cannot be ruled out either: that the ubiquitylation serves as a proteasomal degradation signal for certain cytokinetic regulator proteins. To understand the role of protein ubiquitylation at the central spindle, it is necessary to identify the ubiquitylated proteins in a comprehensive manner. Elucidation of the ubiquitylated proteins, as well as the Ub ligase responsible for the ubiquitylation, will also make it possible to study the significance of the ubiquitylation in cytokinesis by examining the effects of inhibiting their functions.

Materials and Methods

Immunofluorescence

Cells were fixed with 100% methanol or 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 minutes on ice, permeabilized with 0.2% Triton X-100 or 0.05% saponin in PBS for 5 minutes (when fixed with paraformaldehyde), and stained with mouse monoclonal anti-multi-Ub antibody FK2 (1 μg/ml; MBL, Nagoya, Japan) (Fujimoto and Yokosawa, 2005), rabbit polyclonal anti-UBPY (1:500) (Mizuno et al., 2005), mouse monoclonal anti-AMSH (10 μg/ml; provided by N. Tanaka, Miyagi Cancer Center Research Institute, Natori, Japan) (Tanaka et al., 1999), mouse monoclonal anti-c-tubulin (40 μg/ml; Sigma-Aldrich, St Louis, MO), rabbit polyclonal anti-β-tubulin (1 μg/ml; Abcam, Cambridge, UK), rabbit polyclonal anti-FLAG (0.4 μg/ml; Sigma-Aldrich), rabbit polyclonal anti-HA (2 μg/ml; Sigma-Aldrich), mouse monoclonal anti-LAMP1 (200 μg/ml; BD Biosciences Transduction Laboratories, Lexington, KY), rabbit polyclonal anti-MKLP1 (10 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-Hrs (1:2,000; Komada et al., 1997), mouse monoclonal anti-EEA1 (1 μg/ml; BD Biosciences Transduction Laboratories, San Diego, CA), mouse monoclonal anti-LAMP1 (1:2,000; provided by Minora Fukuda, The Burnham Institute, La Jolla, CA) (Carlsson et al., 1988), mouse monoclonal anti-LBPA (1:10; provided by T. Kobayashi, RIKEN, Wako, Japan) (Kobayashi et al., 1998) and rabbit polyclonal anti-VAMP8 (1:30, Synaptic Systems, Gottingen, Germany) antibodies. Secondary antibodies were Alexa488- and Alexa594-conjugated anti-mouse IgG and anti-rabbit IgG antibodies (GE Healthcare, Piscataway, NJ). Blots were detected using the ECL reagent (GE Healthcare).

Large-scale preparation of ubiquitylated proteins

FLAG-UBPYC748A-transfected HeLa cells from eight 90 mm dishes were homogenized in 4 ml of 10 mM Tris-HCl (pH 7.4), containing 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A and 10 mM N-ethylmaleimide by passage through a 23-gauge needle 30 times. The post-nuclear supernatant, obtained after centrifugation at 1000 g for 5 minutes, was centrifuged at 100,000 g for 1 hour at 4°C. The pellet (membrane fraction) was solubilized in 0.3 ml of solubilizing buffer (50 mM Tris-HCl (pH 7.4), 300 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A and 10 mM N-ethylmaleimide), and centrifuged at 100,000 g for 1 hour. The supernatant was collected as the membrane protein fraction.

FK2 (2 μg, Nippon Bio-Test, Kobukunj, Japan) was conjugated to protein A-Sepharose (1 ml, GE Healthcare) using a chemical crosslinker, dimethyl pimelimidate (Pierce, Rockford, IL), as described previously (Matsumoto et al., 2005). The FK2-conjugated beads (0.5 ml) were incubated with the membrane protein fraction for 15 minutes at 4°C and washed with solubilizing buffer. Bound proteins were eluted with 100 mM glycine-HCl (pH 2.8) and neutralized with 1 M Tris-HCl (pH 8.0).

Liquid chromatography/tandem mass spectrometry

Ubiquitylated proteins prepared from UBPYC748A-transfected cells using the FK2 affinity beads were reduced with dithiothreitol, alkylated with iodoacetamide and digested with trypsin as described previously (Matsumoto et al., 2005). The resulting peptides were separated into 18 fractions using cation-exchange chromatography (Polybuffer 50 A, PolyLC, Columbia, MD), and each fraction was subjected to reverse-phase chromatography on a C18 column (L-column, Chemicals Evaluation and Research Institute, Saitama, Japan) with an HPLC system (Magic 2002, Michrom BioResources, Auburn, CA) that was coupled to an ion-trap mass spectrometer (Finnigan LCQ-Deca, Thermo Fisher Scientific, Waltham, MA) equipped with a nano-electrospray ionization source (AMR, Tokyo, Japan) as described previously (Matsumoto et al., 2005). Uninterpretable collision-induced dissociation (CID) spectra were compared with the Human International Protein Index ver. 3.16 (IPI, European Bioinformatics Institute) with the use of MASCOT algorithm.

We thank W. Hong, N. Tanaka, Mitsunori Fukuda, Minoru Fukuda, T. Kobayashi and T. Suzuki for cDNAs and antibodies. We thank H. Fujita (Kyushu University) for helpful advice regarding the mass spectrometric analysis. This work was supported by Grants-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan to M.K. (No. 18050011 and 19570178) and to N.K. (No. 18657040).

References

Albertson, R., Riggs, B. and Sullivan, W. (2005). Membrane traffic: a driving force in cytokinesis. Trends Cell Biol. 15, 92-101.

Antonin, W., Holroyd, C., Tikkkanen, R., Honing, S. and Jahn, R. (2000). The R-SNARE endoVAMP-8 mediates homotypic fusion of early endosomes and late endosomes. Mol. Biol. Cell 11, 3289-3298.

Baluska, F., Menzel, D. and Barlow, P. W. (2006). Cytokinesis in plant and animal cells: endosomes ‘shut the door’. Dev. Biol. 294, 1-10.

Barr, F. A. and Gruneberg, U. (2007). Cytokinesis: placing and making the final cut. Cell 131, 847-860.

Carlsson, S. R., Roth, J., Piller, F. and Fukuda, M. (1988). Isolation and characterization of human lysosomal membrane glycoproteins, h-lamp-1 and h-lamp-2. J. Biol. Chem. 263, 18911-18919.

Carlton, J. G. and Martin-Serrano, J. (2007). Parallels between cytokinesis and retroviral budding: a role for the ESCRT machinery. Science 316, 1908-1912.

Clague, M. J. and Urbe, S. (2006). Endocytosis: the DUB version. Trends Cell Biol. 16, 551-559.
Ubiquitylation in cytokinesis

1333

Eggert, U. S., Mitchison, T. J. and Field, C. M. (2006). Animal cytokinesis: from parts list to mechanisms. Annu. Rev. Biochem. 75, 543-566.

Fabbro, M., Zhou, B.-B., Takahashi, M., Sarcevic, B., Lal, P., Graham, M. E., Gabrielli, B. G., Robinson, P. J., Nigg, E. A., Ono, Y. et al. (2005). Cdk1/Erk2- and PI3K-dependent phosphorylation of a centrosome protein, Cep55, is required for its recruitment to midbody and cytokinesis. Dev. Cell 9, 477-488.

Fujimura, M. and Yokosawa, H. (2005). Production of antipolyubiquitin monoclonal antibodies and their use for characterization and isolation of polyubiquitinated proteins. Meth. Enzymol. 399, 73-86.

Glotzer, M. (2005). The molecular requirements for cytokinesis. Science 307, 1735-1739.

Gromley, A., Yeaman, C., Rosa, J., Redick, S., Chen, C.-T., Mirabelle, S., Guha, M., Gruenberg, J. and Stenmark, H. (2004). The biogenesis of multivesicular endosomes. Cytoskeleton 61, 753-759.

Gruneberg, U., Neef, R., Honda, H., Nigg, E. A. and Barr, F. A. (2004). Relocation of AMSH is an endosome-associated deubiquitinating enzyme. EMBO J. 23, 593-606.

McCullough, J., Clague, M. J. and Urbe, S. (2006). The ubiquitin isopeptidase UBPY regulates endosomal ubiquitin dynamics and is essential for receptor down-regulation. J. Biol. Chem. 281, 12618-12624.

Row, P. E., Prior, I. A., McCullough, J., Clague, M. J. and Urbe, S. (2006). The ubiquitin isopeptidase UBPY regulates endosomal ubiquitin dynamics and is essential for receptor down-regulation. J. Biol. Chem. 281, 12618-12624.

Mizuno, E., Iura, T., Mukai, A., Yoshimori, T., Kitamura, N. and Komada, M. (2005). Regulation of epidermal growth factor receptor down-regulation by UBPY-mediated deubiquitination at endosomes. Mol. Biol. Cell 16, 5163-5174.

Mizuno, E., Kobayashi, K., Yamamoto, A., Kitamura, N. and Komada, M. (2006). A deubiquitinating enzyme UBPY regulates the level of protein ubiquitination on endosomes. Traffic 7, 1017-1031.

Mizuno, E., Kitamura, N. and Komada, M. (2007). Evidence that late-endosomal SNARE complexes at the midbody is required for secretory-vesicle-mediated abscission. J. Cell Biol. 176, 457-466.

Wang, C.-C., Ng, C. P., Lu, L., Atlaslkhin, V., Zhang, W., Seet, L.-F. and Hong, W. (2004). A role of VAMP/endobrevin in regulated exocytosis of pancreatic acinar cells. Dev. Cell 7, 359-371.