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Characterization of Ubiquitinated Lysosomal Membrane Proteins in *Acanthamoeba castellanii*

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Ubiquitinated proteins in lysosomes were characterized by using two monoclonal antibodies (mAbs): LYS8-1, a mAb to lysosomal proteins, and NYA124, a mAb to ubiquitin. LYS8-1 stained lysosome-like vesicles in immunofluorescence microscopy of *Amoeba proteus* and *Acanthamoeba castellanii*. In immunoblotting, LYS8-1's antigens (LYS proteins) were detected as 68-kDa and 77-kDa proteins in *A. proteus*, and as 30-kDa and 39-kDa proteins in *A. castellanii*. In immunoprecipitation of *A. castellanii*, at least four distinct LYS proteins, LYS35p, LYS39p, LYS42p, and LYS46p, were detected and accumulated upon inhibition of lysosome functions but not upon that of 26S proteasome functions. They were all found to be ubiquitinated, and were recovered in the lysosome fractions in subcellular fractionation experiments. In chemical fractionation analyses, LYS35p and LYS39p were demonstrated to be peripherally associated with lysosome membrane, while LYS42p and LYS46p tightly bound to the membrane. These results suggest that the LYS proteins become associated to lysosomal membrane upon ubiquitination.

Ubiquitin is an 8.6-kDa protein and is highly conserved in most eukaryotes. The covalent modification of target proteins with ubiquitin, i.e., ubiquitination, has important functions in diverse cellular processes such as cycle control, DNA repair, and muscle atrophy (Hershko and Ciechanover. 1998; Hochstrasser, 1996). While it is well-known that ubiquitination is responsible for the degradation of target proteins by 26S proteasomes in cytosol, increasing evidence shows that ubiquitination is involved in other cellular events in membranous cellular organelles as well (Bonifacino and Weissman, 1998; Hochstrasser, 1996). For example, recent studies showed that ubiquitination triggers endocytosis of several plasma membrane proteins both in *Saccharomyces cerevisiae* and in mammalian cells (Bonifacino and Weissman, 1998; Hicke, 1997). Stress-induced autophagic-lysosomal proteolysis is also dependent on an active ubiquitination system at least in mammalian cells (Gropper et al., 1991). In normal conditions, existence of ubiquitinated proteins in membranous organelles has been demonstrated in fibroblasts (Doherty et al., 1989), polymorphonuclear neutrophils (Laszlo et al., 1991), *S. cerevisiae* (Simeon et al., 1992), *Arabidopsis thaliana* (Beers et al., 1992), and insect Sf9 cells (Low et al., 1993), although the identity and the origin of these proteins are largely unknown. Ubiquitination is even involved in peroxisome biogenesis of *S. cerevisiae* (Wiebel et al., 1992) and *Pichia pastoris* (Crane et al., 1994). Finally, it is known that the modification with SUMO-1, a small ubiquitin-related protein, renders RanGAP1 localized at nuclear membrane in mammalian cells (Sternsdorf et al., 1997; Matunis et al., 1996).

In the present study, LYS8-1, a monoclonal antibody (mAb) raised against lysosomes, was characterized in *Amoeba proteus* and *Acanthamoeba castellanii*, both of which continuously exchange materials through their membranes. Our data provide evidence that the antigens of LYS8-1 are ubiquitinated lysosomal proteins in the cytoplasmic side of lysosome membrane, suggesting a novel function of ubiquitination in the membrane association of target proteins.

Materials and Methods

Cultivation of protozoans

Amoeba proteus (tD strain) was cultivated in a modified Chalkley's solution in Pyrex baking dishes (35 x 22×4 cm) at 24℃ (Jeon and Jeon, 1975) and fed daily with *Tetrahymena pyriformis* (GL strain) which was cultured axenically at 24℃ in the medium described by Goldstein and Ko (1976). *Acanthamoeba castellanii* (Castellanii strain, ATCC number 50374), provided by Dr. D. I. Chung (Kyungpook National University), was propagated axenically in the PYG medium (ATCC Culture Medium 712) at 27℃ as trophozoite.

Monoclonal antibodies

Isolation of lysosomes from *A. proteus* and production of monoclonal antibodies (mAbs) against them were performed as previously described (Yoo et al., 1996). Of these mAbs, LYS8-1 was used throughout this

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study, together with NYA124, an anti-ubiquitin mAb (Lee et al., 1998b), and an anti-β-galactosidase mAb (Lee et al., 1998a). Hybridoma cells were cultured in Dulbecco's modified Eagles medium (GIBCO BRL) supplemented with 20% bovine calf serum (HyClone Laboratories Inc.) at 37℃ under 5% CO_2. Cloned hybridoma cells were introduced into female BALB/c mice that had previously been injected with pristane (Sigma Chemical Co.) to induce ascites fluid (Harlow and Lane, 1988).

Immunofluorescence microscopy

Immunofluorescence microscopy was carried out according to the method of Kim et al. (1992). Briefly, *A. proteus* cells were flattened and fixed between a slide glass and a siliconized cover glass with a drop of 45% acetic acid for 10 min on dry ice. After the cover glass was flipped off, cells were permeabilized with absolute methanol for 10 min at -20℃ and rehydrated with phosphate buffered saline (PBS). They were then incubated with LYS8-1 (diluted 1:400 in PBS), and subsequently with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (diluted 1:200 in PBS) (Jackson ImmunoResearch Laboratories) for 1 h each in a moist chamber at room temperature. Cells were washed with PBS and mounted in Gelvatol. Specimens were observed using an ECLIPSE E600 fluorescence microscope (Nikon Co.). For immunofluorescence of *A. castellanii*, the cells were cultured on a sterilized slide glass for two days and washed with PBS. Fixation, permeabilization, and subsequent processes were the same as the above.

SDS-PAGE and immunoblotting

Proteins were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) with the buffer system of Laemmli (1970). After the electrophoresis, proteins in gels were stained with Brilliant Coomassie Blue (BCB, Sigma Chemical Co.) or transferred electrophoretically onto nitrocellulose (NC) membrane (PROTRAN BA85; Schleicher & Schuell) for immunoblotting (Towbin et al., 1979). Then the NC membrane was immunostained with LYS8-1 (diluted 1:1,000 in PBS) or NYA124 (diluted 1:500 in PBS), and subsequently with horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) (diluted 1:10,000 in PBS). Bound antibodies were visualized using a chemiluminescence reagent (ECL, Amersham International).

Treatment of proteolysis inhibitors

For the treatment of proteolysis inhibitors, *A. castellanii* cells were grown in 60 mm dishes for two days to near confluence. Before experiments, cells were treated for 3 h with 50 μM *N*-acetyl-leucyl-leucyl-norleucinal (ALLN) (Sigma Chemical Co.) or with 50 mM ammonium chloride (NH_4Cl) (Sigma Chemical Co.). It is known that ALLN, a peptide aldehyde, inhibits the chymotrypsin-like activity of the 26S proteasomes (Lee and Goldberg, 1998), and that NH_4Cl disrupts lysosomal functions by elevating pH of the lumen (Strous et al., 1996). In parallel, cells were incubated without these inhibitors for a control.

Subcellular fractionation

A. castellanii cells grown to confluence in five 60 mm dishes were harvested by centrifugation for 2 min at 170×g. For differential centrifugation, cells were resuspended in 2 ml of homogenizing medium (HM: 0.25 M sucrose, 10 mM triethanolamine, 10 mM acetic acid, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride [PMSF]) and homogenized on ice using a Dounce homogenizer (50 strokes) with a tight fitting pestle (pestle type A). The homogenate was spun for 10 min at 1,000×g, which yielded the 1,000×g pellet. The resulting post nucleus supernatant (PNS) was either used for chemical extraction of organellar peripheral membrane proteins or subjected to 10,000×g centrifugation for 30 min, which yielded the 10,000×g pellet. Finally, the 10,000×g supernatant was spun in a P90AT rotor of a Hitachi CP100α ultracentrifuge for 1 h at 100,000×g, which resulted in the 100,000×g pellet and the 100,000×g supernatant (cytosol). Alternatively, the PNS was spun directly for 1 h at 100,000×g to yield the cytosol fraction (supernatant) and total membrane fraction (pellet), the latter of which was used in Triton X-114 phase separation. For subcellular fractionation on discontinuous sucrose density gradients, cells were resuspended in 2.45 ml of HM and homogenized as the above. The homogenate was spun for 10 min at 1,000×g, and the supernatant (PNS) was layered on a discontinuous sucrose gradient consisting of 1 ml of 2 M sucrose, 3.4 ml of 1.3 M sucrose, 3.4 ml of 1.0 M sucrose, and 2.75 ml of 0.6 M sucrose that were prepared in 10 mM triethanolamine, 10 mM acetic acid, 1 mM EDTA, and 1 mM PMSF. After centrifugation in a P40ST rotor of Hitachi CP100α ultracentrifuge for 3 h at 280,000×g, 1 ml fractions were collected from the top to the bottom of the tube.

Immunoprecipitation

Immunoprecipitation was performed for the centrifuged subcellular fractions. Pellets of the differential centrifugation were lysed directly in radio immunoprecipitation assay buffer (RIPA: 150 mM NaCl, 1.0% Nonidet P-40 [NP- 40], 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl pH 8.0, and 1 mM PMSF), and the supernatants and sucrose density gradient fractions were adjusted to a concentration of 1.0% NP-40, 0.5% deoxycholate, and 0.1% SDS. After centrifugation for 30 min at 16,000×g, the resulting supernatants were incubated with 4 μl of LYS8-1 or anti-β-galactosidase mAb ascites fluid and subsequently with protein A-sepharose CL-4B

(Pharmacia Biotech.) slurry swollen in RIPA (20 μl bed volume) for 1 h each at 4℃. Immunoprecipitates were collected by spinning, washed 5 times with RIPA and 3 times with PBS, and subjected to SDS-PAGE and immunoblotting (Harlow and Lane, 1988).

Peptide sequencing

Immunoprecipitates were resolved by SDS-PAGE and electrophoretically transferred onto polyvinylidene difluoride membrane (WESTRAN, Schleicher & Schuell). The membrane was stained with BCB, and then submitted to Korea Basic Science Institute Seoul Branch for peptide sequencing. The determined amino acid sequences were compared with other sequences in databases using the FASTA3 program.

Acid phosphatase assay

Acid phosphatase (EC 3.1.3.2) is a marker enzyme for endosomes and lysosomes, and the activity in the fractions of the sucrose density gradient was measured using *p*-nitrophenol phosphate (Sigma Diagnostic) as a substrate (Yoo et al., 1996).

Triton X-114 phase separation of organellar proteins

Triton X-114 phase separation was carried out essentially as described by Bordier (1981) with minor modification. In brief, the total membrane fraction was obtained from the homogenate of NH_4Cl-treated *A. castellanii* as described above. Then membranes were resuspended in 1.0% Triton X-114 in HM and incubated for 15 min at 30℃. The solution was separated into the detergent and the aqueous phases by spinning for 15 min at 16,000×g *at room temperature.*

Chemical extraction of organellar peripheral membrane proteins

The NH_4Cl-treated *A. castellanii* cells were homogenized and PNS was obtained as described above. Aliquots of the PNS were incubated with 2.5 M urea, 0.1 M sodium carbonate (pH 11.0), or 1.0% Triton X-100 in HM for 30 min on ice, and subsequently centrifuged for 1 h at 100,000×g. Supernatants of urea or sodium carbonate treated sample were dialyzed against 50 mM Tris-HCl pH 8.0.

Miscellaneous

Total lysates of cells were prepared using NP-40 lysis buffer (1% NP-40, 50 mM Tris-HCl pH 8.0, 150 mM NaCl, and 1 mM EDTA) and the protein content was determined according to Lowry et al. (1951).

Results

Ubiquitinated LYS proteins in lysosomes

The LYS8-1 mAb is one of mAbs raised against lysosomes of *A. proteus.* In the indirect immunofluorescence, sporadic structures of *A. proteus* were stained by the mAb (Fig. 1a). When we examined the presence of LYS8-1's antigen in *A. castellanii*, LYS8-1 was found to be bound to sporadic structures in *A. castellanii* as well (Fig. 1b). In immunoblot analyses of total cellular proteins, LYS8-1 recognized 68 kDa and 77 kDa proteins in *A. proteus* and 30 kDa and 39 kDa proteins in *A. castellanii* (Fig. 2). These results showed that the

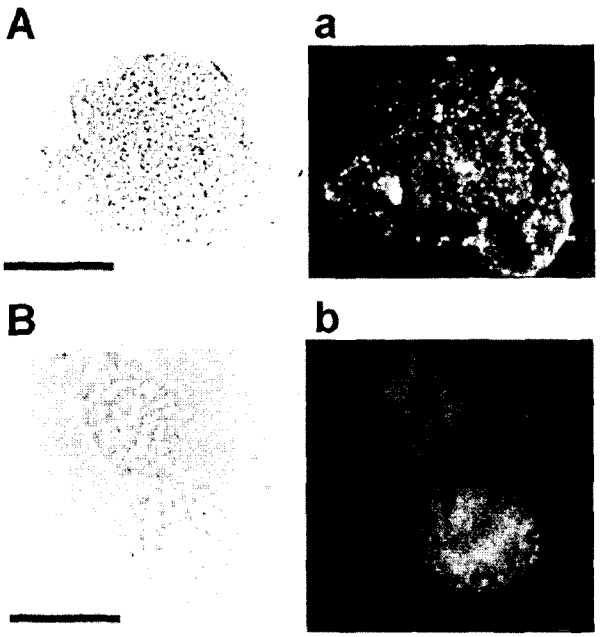

Fig. 1. Phase contrast (left) and indirect immunofluorescence (right) micrographs of *Amoeba proteus* (A) and *Acanthamoeba castellanii* (B) stained with the LYS8-1 mAb. Scale bars=10 μm (B) and 50 μm (A).

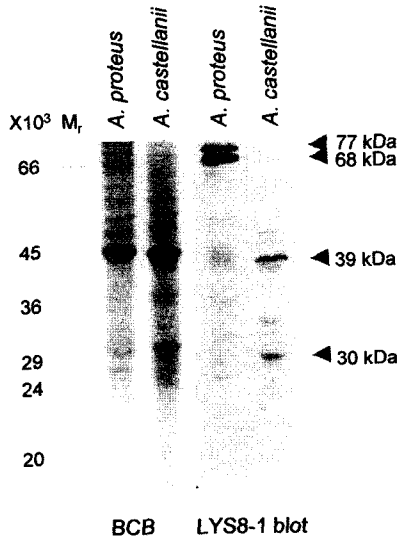

Fig. 2. Brilliant Coomassie Blue-stained SDS-polyacrylamide gel of proteins from *Amoeba proteus* and *Acanthamoeba castellanii* (BCB) and a corresponding immunoblot probed with the LYS8-1 mAb (LYS8-1 blot). Arrowheads indicate the proteins recognized by LYS8-1 whose molecular masses were calculated on the basis of Mr markers.

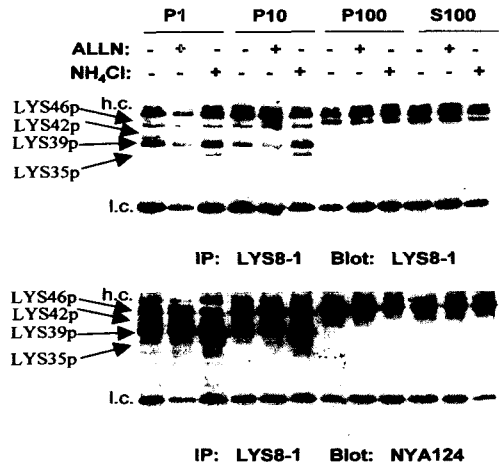

Fig. 3. Immunoblots of proteins by immunoprecipitated with LYS8-1 to the subcellular fractions *Acanthamoeba castellanii* treated with inhibitors. Immunoblots were probed either by LYS8-1 or by NYA124. P1, 1,000 *g* pellet. P10, 10,000 *g* pellet. P100, 100,000 *g* pellet. S100, 100,000 *g* supernatant. h.c., antibody heavy chain. l.c., antibody light chain.

mAb LYS8-1 recognizes its antigens in organelle-like structures of at least two related species, *A. proteus* and *A. castellanii*. From the ~9 kDa differences in molecular masses, we speculated that the antigens may be ubiquitinated proteins.

In order to test ubiquitination and to determine subcellular location of antigens, we chose *A. castellanii*, which is more appropriate for the following biochemical experiments. Firstly, *A. castellanii* cells were treated with proteolysis inhibitors, ALLN or NH_4Cl, for 3 h. Then, cells were homogenized and subcellular fractions were obtained by differential centrifugation. For each fraction, we carried out immunoprecipitation with LYS8-1 and subsequently immunoblotting with LYS8-1 or NYA124, an anti-ubiquitin mAb (Lee et al., 1998b). As a result, four different antigens of LYS8-1 (LYS proteins) appeared to have the ubiquitin epitope since they reacted to NYA124 (Fig. 3). They also seemed to reside in dense cellular organelles, considering their recovery in the 1,000×g pellets (nucleus and undistrupted cell fractions) and the 10,000×g pellets (dense organelle fractions) but not in the 100,000×g pellets (microsome fractions) and 100,000×g supernatants (cytosol fractions) (Fig. 3). In addition, they were somehow stabilized by inhibition of lysosome functions with NH_4Cl, but the effect of ALLN on 26S proteasomes was negligible (Fig. 3). We named them after their molecular masses as LYS35p, LYS39p, LYS42p, and LYS46p, respectively. Among them, the LYS39p was the major species, but we could not detect the 30-kDa antigen of *A. castellanii*. One possibility is that LYS8-1 can precipitate the four species of antigens including LYS39p but not the 30-kDa deubiquitinated one efficiently.

Since the two immunoblots were very similar (Fig. 3), we wondered if the epitope of LYS8-1 was identical to that of NYA124. Thus, *ApPUBI*, the polyubiquitin gene

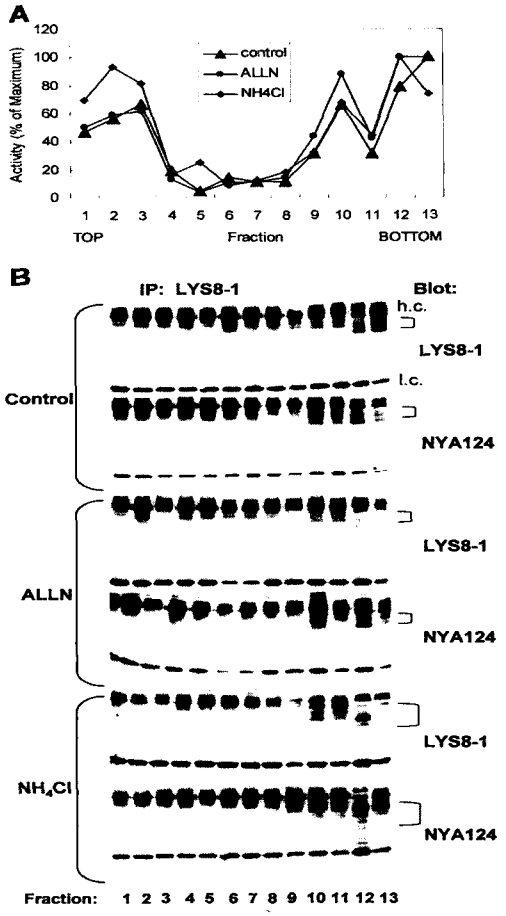

Fig. 4. Distributions of acid phosphatase (A) and antigens (B) in the sucrose density gradient fractions of *Acanthamoeba castellanii* homogenate treated with inhibitors. Acid phosphatase activities are presented as the percents of the maximum. Fractions were immunoprecipitated with LYS8-1 and immunoblots were probed either by LYS8-1 or by NYA124. Brackets in right denote the LYS proteins. h.c., antibody heavy chain. l.c., antibody light chain.

of *A. proteus*, was expressed in transformed *E. coli* DH5α with IPTG (Lee et al., 1998b), and the lysate was immunoblotted with the either mAb. From the result that LYS8-1 did not react with the induced polyubiquitin gene products while NYA124 did (data not shown), we concluded that the epitope of LYS8-1 was apparently different from that of NYA124.

The N-terminal amino acid sequence of LYS39p was determined by microsequencing. When compared with other proteins in databases using FASTA3 program, the N-terminal sequence, QVKFTIKTHYSVYN, matched with 60-70% similarities to a number of different kinds of proteins such as human uracil-DNA glycosylase, human synaptotagmin, ferredoxin of *Cyanobacterium anabaena*, and so forth. However, we could not determine the exact identity of LYS39p.

To further define intracellular location of LYS proteins, the cell homogenates were subjected to discontinuous sucrose density gradient centrifugation to separate membranous organelles. The activity of acid pho-

sphatase, an endosome/lysosome marker, was assayed for each subcellular density fraction. Endosomes and lysosomes were separated into one peak near the top and two peaks near the bottom, respectively, in the activity profile, indicating good separation of cellular organelles (Fig. 4A; Yoo et al., 1996). ALLN or NH_4Cl treatment had little effect on the activity profile, reflecting that organelles were preserved intact upon these treatments (Fig. 4A). For each subcellular fraction, immunoprecipitation with LYS8-1 was performed, followed by immunoblotting with LYS8-1 or NYA124. Distribution of the ubiquitinated LYS proteins was consistent with that of lysosomes, and the NH_4Cl treatment strengthened intensity of the bands on both immunoblots (Fig. 4B). This experiment confirmed that the ubiquitinated LYS proteins are located at lysosomes and stabilized upon failure of the lysosomal functions.

The membrane association of LYS from the cytoplasmic side

A. castellanii homogenates were chemically fractionated and analyzed with immunoprecipitation and immunoblotting to elucidate the biochemical nature of LYS proteins and to topographically distinguish one another.

Firstly, the total membrane fraction from NH_4Cl-treated homogenate was subjected to Triton X-114 phase separation. As a result, ubiquitinated LYS proteins were divided into low molecular weight and hydrophilic (aqueous phase) species, LYS35p and LYS39p, and high molecular weight and hydrophobic (detergent phase) species, LYS39p, LYS42p, and LYS46p (Fig. 5A). Since LYS39p was recovered in both phases, it may have approximately equal proportion of hydrophilic and hydrophobic regions. It is also likely that LYS42p and LYS46p are somehow modified to become sufficiently hydrophobic to associate with the membrane. As for LYS35p, it is possible that it is either a peripheral membrane protein or a lumen protein with a majority of hydrophilic region inside the lysosomes.

Secondly, the post nucleus supernatant of NH_4Cl-treated homogenate was treated with urea, sodium carbonate (pH 11.0), or the nonionic detergent Triton X-100. Urea and sodium carbonate would extract peripheral membrane proteins and Triton X-100 would solubilize integral membrane proteins. After the incubation, the solution was centrifuged at 100,000×g, and the extracted proteins were recovered in the supernatant. Upon the treatment with urea or sodium carbonate, LYS42p and LYS46p remained in the pellet, confirming that they are integral membrane proteins (Fig. 5B). Consistent with this, the Triton X-100 treatment released them from the membrane albeit partially (Fig. 5B). On the other hand, LYS39p and LYS35p were completly extracted with sodium carbonate and partially with urea, even though Triton X-100 did not solubilize all of them (Fig. 5B). Thus, LYS39p and LYS35p were identified as cytoplasmic peripheral membrane proteins.

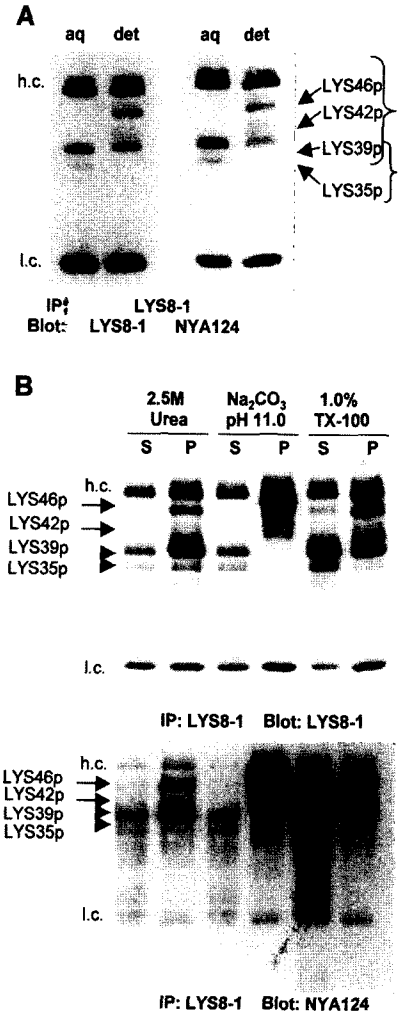

Fig. 5. Immunoblots of chemical fractions of *Acanthamoeba castellanii* immunoprecipitated with LYS8-1. Immunoblots were probed either by LYS8-1 or by NYA124. A, Triton X-114 phase separation of total cellular membrane from of *A. castellanii* treated with NH_4Cl. B, Chemical extraction of the membranes of *A. castellanii* treated with NH_4Cl. det, detergent phase. aq, aqueous phase. S, supernatant. P, pellet. h.c., antibody heavy chain. l.c., antibody light chain.

Discussion

Ubiquitinated lysosomal proteins have been found in many cell types, such as fibroblasts (Doherty et al., 1989), insect Sf9 cells (Low et al., 1995), and *S. cerevisiae* (Simeon et al., 1992). In the present study, we found another series of ubiquitinated lysosomal proteins, LYS, in *A. castellanii*. Although we failed to show the exact identities of LYS proteins as in many other reports on ubiquitinated lysosomal proteins, we found that LYS proteins somehow accumulate when the lysosome functions fail by NH_4Cl. It was also demonstrated that some of LYS proteins are peripheral membrane proteins on the cytoplasmic side and the others are integral membrane proteins, which suggests

a new role of ubiquitination.

In our experiments, NH_4Cl appeared to have affected the level of ubiquitinated LYS proteins not by direct inhibition of lysosomal enzyme activities but by other mechanisms. It is because LYS proteins are not located at the lumen of lysosomes where the lysosomal proteases are effective (Fig. 5). In fact, it is known that lysosomal inhibitors increase the expression of some heat shock genes (Ananthan et al., 1986) and that the polyubiquitin gene of chicken is under the control of a heat shock promoter (Bond and Schlesinger, 1985). Furthermore, it was reported that lysosomal inhibitors including NH_4Cl elicit transcription of a polyubiquitin gene in 3T3-L1 cells (Doherty et al., 1989). Therefore, it seems that NH_4Cl somehow increased the intracellular level of ubiquitin, resulting in further modification of LYS proteins with ubiquition.

Our results suggest a new role of ubiquitination, other than leading target proteins to breakdown either in 26S proteasomes or lysosomes (Bonifacino and Weissman, 1998; Hershko and Ciechanover, 1998). It is evident that the ubiquitination of LYS proteins is not required for degradation of proteins. In our data, ALLN, a 26S proteasome inhibitor, had little effect on the level of the proteins, suggesting that LYS proteins are not degraded at 26S proteasomes. In addition, since LYS proteins are not located at the lumenal side of lysosome membranes, lysosomal proteases would not be accessible to LYS proteins.

In fact, there is growing evidence for these nondegradative roles of ubiquitination (Hershko and Ciechanover, 1998). For example, when the FCRI receptor (IgE) binds with its ligand, this receptor is ubiquitinated at its cytosolic domain, which plays a role in activation, rather than down regulation (Paolini and Kinet, 1993). Although little is known about the mechanism for its stability at the plasma membrane, there is no evidence for the selective degradation of the stimulated receptors, and it becomes rapidly deubiquitinated upon removal of cross-linking agents (Hicke, 1997; Paolini and Kinet, 1993). Another well-known example is ubiquitination of histone H2A, which is stable all the time of cell cycle without degradation.

One possible role of the ubiquitination of LYS proteins is that the ubiquitin moieties make them tightly associated with membrane proteins. The membrane association of ubiquitinated LYS proteins appears to be very similar to that of RanGAP1 (Mahajan et al., 1997). Once SUMO-1, a small ubiquitin-like modifier, is attached, the RanGAP1 is translocated from cytosol to nuclear pore complex (NPC) in the nuclear membrane. Additionally, even though the mechanism is not clear, studies on viral lipid-anchored ubiquitins can account for the association of LYS proteins with the lysosomal membranes. It is reported that baculovirus virion contains phosphatidyl ubiquitin as well as free ubiquitin (Guarino et al., 1995). The virion of African swine fever virus (ASFV) also has membrane-anchored ubiquitin, which can be conjugated to the early expressed proteins by a virus-encoded ubiquitin conjugating enzyme (UBCv1) during infection (Hingamp et al., 1995). Therefore, we propose here that some ubiquitinated lysosomal proteins including the LYS proteins be anchored to membranes by the membrane-anchored ubiquitins or their equivalents.

In conclusion, it is possible that blocking of the lysosomal function leads to an increase in ubiquitin expression as well as in ubiquitination of LYS proteins in *A. castellanii*. In addition, the membrane association of LYS proteins allows us to hypothesize another nondegradative role of ubiquitin. We expect that this new function of ubiquitin may explain a lot about the protein targeting to organelle membranes.

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References

Ananthan J, Goldberg AL, and Voellmy R (1986) Abnormal proteins serve as eukaryotic stress signals and trigger the activation of heat shock genes. *Science* 232: 522-524.

Beers EP, Moreno TN, and Callis J (1992) Subcellular localization of ubiquitin and ubiquitinated proteins in *Arabidopsis thaliana*. *J Biol Chem* 267: 15432-15439.

Bond U and Schlesinger MJ (1985) Ubiquitin is a heat shock protein in chicken embryo fibroblasts. *Mol Cell Biol* 5: 949-956.

Bonifacino JS and Weissman AM (1998) Ubiquitin and the control of protein fate in the secretory and endocytic pathways. *Annu Rev Cell Dev Biol* 14: 19-57.

Bordier C (1981) Phase separation of integral membrane proteins in Triton X-114 solution. *J Biol Chem* 256: 1604-1607.

Crane DI, Kalish JE, and Gould SJ (1994) The *Pichia pastoris* PAS4 gene encodes a ubiquitin-conjugating enzyme required for peroxisome assembly. *J Biol Chem* 269: 21835-21844.

Doherty FJ, Osborn NU, Wassell JA, Heggie PE, Laszlo L, and Mayer RJ (1989) Ubiquitin-protein conjugates accumulate in the lysosomal system of fibroblasts treated with cystein proteinase inhibitors. *Biochem J* 263: 47-55.

Goldstein L and Ko C (1976) A method for the mass culturing of large free-living amoebas. *Meth Cell Biol* 13: 239-246.

Gropper R, Brandt RA, Elias S, Bearer CF, Mayer A, Schwartz AL, and Ciechanover A (1991) The ubiquitin-activating enzyme, E1, is required for stress-induced lysosomal degradation of cellular proteins. *J Biol Chem* 266: 3602-3610.

Guarino LA, Smith G, and Dong W (1995) Ubiquitin is attached to membranes of baculovirus particles by a novel type of phospholipid anchor. *Cell* 80: 301-309.

Harlow E and Lane D (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York.

Hershko A and Ciechanover A (1998) The ubiquitin system. *Annu Rev Biochem* 67: 425-479.

Hicke L (1997) Ubiquitin-dependent internalization and down-regulation of plasma membrane proteins. *FASEB J* 11: 1215-1226.

Hingamp PM, Leyland ML, Webb J, Twigger S, Mayer RJ, and Dixon LK (1995) Characterization of a ubiquitinated protein which is externally located in African swine fever virus. *J*

Virol 69: 1785-1793.
Hochstrasser M (1996) Protein degradation or regulation: Ub the judge. *Cell* 84, 813-815.
Jeon KW and Jeon MS (1975) Cytoplasmic filaments and cellular wound healing in *Amoeba proteus*. *J Cell Biol* 67: 243-249.
Kim KJ, Choi EY, and Jeon KW (1992) Use of antibodies as probes in the study of amoebae. In: Lee JJ and Soldo AT (ed), *Protocols in Protozoology*, The Society of Protozoologists, pp D-5.1-D.5.3.
Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head bacteriophage T4. *Nature* 227: 680-685.
Laszlo L, Doherty FJ, Watson A, Self T, Landon M, Lowe J, and Mayer RJ (1991) Immunogold localization of ubiquitin-protein conjugates in primary (azurophilic) granules of polymorphonuclear neutrophils. *FEBS Lett* 279: 175-178.
Lee DH and Goldberg AL (1998) Proteasome inhibitors: valuable new tools for cell biologists. *Trends Cell Biol* 8: 397-403.
Lee JE, Ahn EY, and Ahn TI (1998a) Development of a plasmid vector for overproduction of β-galactosidase in *Escherichia coli* by using genetic components of *groEx* from symbiotic bacteria in *Amoeba proteus*. *J Micobiol Biotech* 8: 509-516.
Lee SY, Kim HJ, Yoo SY, and Ahn TI (1998b) Characterization of a monoclonal antibody and a cDNA for polyubiquitin of *Amoeba proteus*. *J Euk Microbiol* 45: 431-438.
Low P, Doherty FJ, Sass M, Kovacs J, Mayer RJ, and Laszlo L (1993) Immunogold localization of ubiquitin protein conjugates in Sf9 insect cells: implications for the biogenesis of lysosome-related organelles. *FEBS Lett* 316: 152-156.
Lowry OL, Rosenberg NJ, Farr AL, and Randall RT (1951) Proteins measurement with phenol reagent. *J Biol Chem* 193: 265-275.
Mahajan R, Delphin C, Guan T, Gerace L, and Melchior F (1997) A small ubiquitin-related polypeptide involved in targeting RanGAP1 to nuclear pore complex protein RanBP2. *Cell* 88: 97-107.
Matunis MJ, Coutavas E, and Blobel G (1996) A novel ubiquitin-like modification modulates the partitioning of the Ran-GTPase-activating protein RanGAP1 between the cytosol and the nuclear pore complex. *J Cell Biol* 135: 1457-1470.
Paolini R and Kinet JP (1993) Cell surface control of the multiubiquitination and deubiquitination of high-affinity immunoglobulin E receptors. *EMBO J* 12: 779-786.
Simeon A, van der Klei IJ, Veenhuis M, and Wolf DH (1992) Ubiquitin, a central component of selective cytoplasmic proteolysis, is linked to proteins residing at the locus of non-selective proteolysis, the vacuole. *FEBS Lett* 301: 231-235.
Sternsdorf T, Jensen K, and Will H (1997) Evidence for covalent modification of the nuclear dot-associated proteins PML and Sp100 by PIC1/SUMO-1. *J Cell Biol* 139: 1621-1634.
Strous G, van Kerkhof P, Govers R, Ciechanover A, and Schwartz AL (1996) The ubiquitin conjugation system is required for ligand-induced endocytosis and degradation of the growth hormone receptor. *EMBO J* 15: 3806-3812.
Towbin H, Staehelin T, and Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets; procedures and some applications. *Proc Natl Acad Sci USA* 76: 4350-4354.
Wiebel FF and Kunau WH (1992) The Pas2 protein essential for peroxisome biogenesis is related to ubiquitin-conjugating enzymes. *Nature* 359: 73-76.
Yoo SY, Choi JY, Kim H, and Ahn TI (1996) Lysosomes are in subpopulations in *Amoeba proteus*. *Mol Cells* 6: 316-324.

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