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A Novel Ubiquitin-like Protein Gene Expression During the Cyst Maturation in *Acanthamoeba castellanii*

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ABSTRACT

During periods of starvation or other stresses, trophozoites of *Acanthamoeba* undergo cellular differentiation into cysts. By using an anti-ubiquitin (Ub) monoclonal antibody (mAb) as a probe, we cloned a 2.68-kb cDNA encoding an open reading frame for a novel ubiquitin-like protein (Ublp94.4) composed of a ribosomal protein region (221-336 aa), an Ub domain (341 - 416 aa), a serine-rich region (426 - 440 aa), and a coiled-coil region (745 - 788 aa). The Ub domain showed 84 - 85% identity with other well-conserved Ub. In Northern blot analysis using the cDNA as a probe, we detected a 2.6 kb transcript in maturing cysts. In 72-h cysts, the level of the transcript was nearly 12 times that in trophozoites. In indirect immunofluorescence microscopy using the antiserum against Ublp94.4, labeled antigens were dispersed throughout the cytoplasm from 12-h encystment, but no antigen was detected in trophozoites or cells in 6-h encystment. The antiserum reacted with no protein in trophozoites, but with two proteins (35.0 kDa and 61.6 kDa) in cysts. The level of the two proteins increased with maturation of cysts. By immunoprecipitation analysis the 35 kDa protein was confirmed to bind the multi-Ub chain and to interact with 26S proteasomes. Thus, Ublp94.4 appeared to undergo post-translational modification to form a 61.6 kDa intermediate, and then to the active 35 kDa protein for proteasome-dependent proteolytic process in the cyst maturation.

Key words: cyst maturation, ubiquitin-like protein, posttranslational modification, cDNA, *Acanthamoeba castellanii.*

INTRODUCTION

The life cycle of *Acanthamoeba* is comprised of two distinct stages, the trophozoite and the cyst. When stressed, trophozoites of *Acanthamoeba* undergo encystment. In this organism encystment has been seen as a strategy for overcoming adverse conditions such as dehydration, extreme of temperature, lack of available food, and changes in salinity, nutrient or pH (Corliss and Esser, 1984). When *Acanthamoeba* are induced to encyst, a massive turnover of organelles and cellular membrane system takes place (Griffiths and Hughes, 1969). The initiation of encystment induces a reduction of metabolism so that the cell synthesizes a chemically and structurally complex cell wall. The wall synthesis is

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accompanied by a decrease in cytoplasmic mass and a gradual dehydration of the organism (Bowers and Korn, 1969). Studies on the chemical composition of cysts have shown that the formation of cyst wall involves production of cellulose and protein components of the wall structure that are not found in trophozoites (Neff and Neff, 1969). These changes let the cyst to more resistant to biocide than trophozoite. However, only two proteins, a cyst-specific protein (CSP21) (Hirukawa et al., 1998) and a subtilisin-like serine protease (Park et al., 2002) that display cyst-specific expression have been identified.

In eukaryotic cells, turnover of intracellular proteins is carried out by two distinct systems; the lysosomal system and the ubiquitin (Ub)-dependent proteolysis system. Ub-dependent proteolysis system accounts for the degradation of most short-lived, misfolded or damaged proteins (Ciechanover et al., 1984; Gropper et al., 1991). When cultured cells are subjected to a sudden increase in temperature, a burst of degradation of normally long-lived proteins is observed. The increase in proteolysis coincides with a reduction in free and histone-conjugated Ub and an increase in multi-Ub-protein conjugates (Parsell and Lindquist, 1993). The Ub-dependent proteolysis system also plays an important role in cyst formation of protozoan parasites (Gonzalez et al., 1999; Lopez et al., 2002). Treatment with the protease inhibitor, lactacystin, delayed encystment in *Entamoeba invadens* (Gonzalez et al., 1999). Glucosamine-6-phosphate isomerase, a key enzyme in encystment, appears to be controlled by ubiquitin attachment in *Giardia intestinalis* (Lopez et al., 2002). However, none of the cyst stage-specific Ubs has been identified in these organisms.

In a previous study to understand the cellular differentiation of *A. castellanii*, we followed changes in profiles of major proteins by 2-D PAGE. We found that 17 proteins among the 51 lost proteins and 2 proteins of the newly synthesized 16 proteins during encystment were reactive to anti-Ub mAb (Park et al., 2002). Identification of the lost and newly synthesized proteins with encystment would improve our understanding of encysting protozoan parasites. As an extension in this study, we cloned and identified a 2.68-kb cDNA encoding a novel Ub-like protein (Ublp). Here, we present our results and propose the role of Ublp94.4 in the proteolytic pathway to proteasome during encystment in *A. castellanii*.

MATERIALS AND METHODS

Cell culture

Trophozoites Acanthamoeba castellanii (Castellanii strain, ATCC No. 50374) was a gift from Dr. D. I. Chung (Kyungpook National University, Korea). Trophozoites were grown axenically in PYG medium [2% proteose peptone, 0.1% yeast extract, 1.8% glucose, 0.4 M $MgSO_4 \cdot 7H_2O$, 0.1 M sodium citrate, 5 mM $Fe(NH_4)_2(SO_4)_2$, 0.25 M Na_2HPO_4 and 0.25 M KH_2PO_4, pH 6.5]. Trophozoites from a stationary phase of culture (3.5×10^6 cells/ml) were inoculated into the PYG medium (7.4×10^3 cells/ml) and grown at 30°C in a shaking incubator agitated at 120 rpm.

Encystment Trophozoites in the stationary phase were collected aseptically by centrifugation for 2 min at 800 g, washed twice in encystment medium (EM) containing 95 mM NaCl, 5 mM KCl, 8 mM $MgSO_4$, 0.4 mM $CaCl_2$, 1 mM $NaHCO_3$ and 20 mM Tris-HCl, pH 9.0 (Lowry et al., 1951), inoculated into EM (3.5×10^6 cells /ml), and incubated at 30°C in a shaking incubator for varying time periods. The density of cells in suspension was determined by direct counts with a hemocytometer and averaged from four counts (Finley and Chau, 1991).

cDNA cloning

Using an anti-Ub mAb (Santa-Cruz Biotechnology, Inc) as a probe, the cDNA library of *Acanthamoeba* constructed in Lamda-uniZAP vector (Hong et al., 2000) was immunoscreened by plaque lift. Positive clones from the primary screening were processed for secondary and tertiary screenings until all plaques on the test plates were positive. Then, confirmed phage clones were autoexcised into pBluescript vectors using R408 helper phage and clones were maintained as plasmids in XL1-Blue strain of *E. coli*.

DNA sequencing and proteomics tools

The cDNA was extracted using a plasmid purification kit (Nucleogen) and submitted to the Macrogen (Seoul, Korea) for nucleotide sequencing. For similarity and homology analysis of amino acid sequences translated from the cDNA, NCBI protein databases were searched with BLAST. The protein sequence alignment was carried out using the CLUSTAL-X program. The pattern and profile of predicted amino acids, location of signal peptide cleavage sites in amino acid sequences, hydrophobicity and antigenicity were analyzed by ExPASy

proteomics tools.

Northern blot analysis

The total RNA prepared by guanidium isothiocyanate method from 0.5 ml packed cells (Chirgwin et al., 1979) was separated through 1.2% agarose gels containing 2.2 M formaldehyde and transferred onto NC membrane (Amersham International) by capillary transfer method and fixed by baking for 2 hrs. The cDNA probes were amplified by PCR using a forward primer 5′-ccaagca-cgtcatctac-3′ (nt. 1390-1406), a reverse primer 5′ -gtagtgagcagatctgtcgtgta-3′ (nt. 2010-2032) and 10 mCi/ml [α-^{32}P] dCTP and purified with Chroma spin columns (Clontech). After hybridization with ^{32}P-labeled cDNA probes in prehybridization solution (0.5 M sodium phosphate pH 7.2, 7% SDS, and 1 mM EDTA, pH 7.0) for 18-24 hrs at 42°C, the NC paper was washed in a solution containing 1 × SSC, 0.1% SDS for 10 min at room temperature and then in 0.5 × SSC, 0.1% SDS for 10 min at 68°C three times. The signals were visualized by autoradiography and quantified with the Bioimage analyzer BAS2000 (Fuji).

Production of polyclonal antiserum

E. coli cells transformed with the cDNA were collected by centrifugation, resuspended in NP-40 lysis buffer (1% NP-40, 50 mM Tris-HCl, 150 mM NaCl, and 1 mM EDTA, pH 8.0), and disrupted by French pressure cell at 1000 PSIG. Proteins were collected by centrifugation for 15 min at 14,000 g at 4°C and separated by SDS-PAGE in 2-mm-thick 10% slab gel. Polypeptide bands of 97.4 kDa were excised from the gel stained with Coomassie blue, electroeluted in a column for 4 hrs at 10 mA per column, precipitated with nine volumes of cold acetone (-20°C), and collected by centrifugation for 15 min at 9,000 g. The proteins were dissolved in PBS. Female BALB/c mice (4 weeks old) were immunized with electroeluted proteins as antigens. Proteins (50 μg in 100 μl) emulsified with an equal volume of complete Freund's adjuvant (Gibco BRL Life Technol.) were injected intraperitoneally. For boost injections, equal amounts of proteins mixed with incomplete Freund's adjuvant were injected at 2-week intervals. Two weeks after the final injection with proteins in PBS, blood was collected from lateral tail vein, allowed to clot for 60 min at 37°C, and then placed at 4°C overnight to allow the clot to contract. Serum was collected by centrifugation at 10,000 g for 10 min at 4°C, and stored at -20°C in aliquots of 100 μl.

Immunofluorescence microscopy

For immunofluorescence microscopy, cells were flattened and fixed between a slide glass and a siliconized cover glass with a drop of 45% acetic acid on dry ice for 10 min (Kim et al., 1992). Fixed cells were permeabilized with methanol for 10 min at -20°C and rehydrated with PBS. They were then incubated with antiserum against Ublp94.4 (diluted 1 : 2,500 in PBS), and subsequently with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (diluted 1 : 200 in PBS) (Jackson ImmunoResearch Laboratories) for 30 min 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.).

Protein sample preparation

Trophozoites and cysts were collected and washed twice with PBS by centrifugation at 800 g for 2 min. Trophozoites resuspended in 2 volume of lysis buffer (1% NP-40, 50 mM Tris-HCl pH 8.0, 150 mM NaCl, and 1 mM EDTA) were homogenized by sonication. Cysts resuspended in 2 volumes of lysis buffer were mixed with cold glass beads (Sigma, 0.425-0.60 mm diameter) and vigorously agitated with vortex mixer for 10 sec each time over a 20 min period at 4°C. The extent of homogenization was determined by phase-contrast microscopy. Subsequently, the homogenate was centrifuged for 15 min at 16,800 g and the supernatant was collected. The protein content was determined according to Lowry et al. (1951).

SDS-PAGE and immunoblotting

Proteins were separated by SDS-PAGE (Laemmli, 1970). After electrophoresis, proteins were stained with Brilliant Coomassie Blue (BCB) or transferred electrophoretically onto nitrocellulose (NC) membrane (Schleicher & Schuell) for immunoblot (Towbin et al., 1979). Then NC membrane was immunostained with anti-Ub mAb (diluted 1 : 1,000 in PBS) or antiserum against Ublp94.4 (diluted 1 : 2,500 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).

Immunoprecipitation

Immunoprecipitation was performed by using immunoprecipitation kit (Roche Co. Ltd.). After incubation for 72 hrs in encystment medium, cells were harvested, washed twice with PBS, suspended in lysis buffer (150 mM NaCl, 1.0% NP-40, 0.05% deoxycholate, 50 mM Tris-Cl pH 7.5, and the protease inhibitor cocktail), and lysed by vortexing with glass beads. To reduce background caused by non-specific adsorption of irrelevant cellular proteins to protein G-agarose beads, 50 μl of the homogeneous protein G-agarose suspension (25 μl bed volume) was added to the cell extracts (300 μl) and incubated for overnight at 4°C on a rocking platform. Supernatants were collected by centrifugation at 12,000 g for 20 sec in a microfuge, and incubated with 5 μl of antiserum against Ublp94.4 or anti-β -galactosidase mAb for control (Lee et al., 1998) and subsequently with 50 μl of protein G-agarose suspension (25 μl bed volume) for 3 hrs each at 4°C. Immunoprecipitates were collected by centrifugation, washed 2 times with lysis buffer (150 mM NaCl, 1.0% NP-40, 0.05% deoxycholate, 50 mM Tris-Cl pH 7.5, and the protease inhibitor cocktail), 2 times with wash buffer 2 (500 mM NaCl, 1.0% NP-40, 0.05% deoxycholate, and 50 mM Tris-Cl pH 7.5) and 1 time with wash buffer 3 (1.0% NP-40, 0.05% deoxycholate, and 10 mM Tris-Cl pH 7.5), and subjected to SDS-PAGE and immunoblot (Harlow and Lane, 1988). Alternatively, immunoprecipitation with antiserum against Ublp94.4 was carried out as described above except that cell extracts (300 μl) were incubated in ATP regeneration system (100 mM Tris-Cl pH 7.5, 1 mM dithiothreitol, 2 mM ATP, 2 mM $MgCl_2$, 20 mM creatine phosphate, and 3 unit of creatine kinase) at 37°C for 2 hrs (Haas and Rose, 1981), and subjected to SDS-PAGE and immunoblot with anti-19S proteasome subunit mAb (a gift from Dr. J. H. Chung, Seoul National University) to detect the interaction with 26S proteasome.

RESULTS

The cDNA encodes a novel ubiquitin-like protein

By screening the cDNA library of *Acanthamoeba* using an anti-Ub mAb as a probe, we obtained a 2.68-kb cDNA (GenBank Acc. AY279355) composed of an open reading frame (ORF; nt 21-2579) for a 94.4-kDa protein (Fig. 1A) in 2562 nucleotides and a poly-A tail. The protein had a ribosomal protein region (221-336 aa), followed by a Ub domain (341-416 aa), a serine-rich region (426-440 aa) and a coiled-coil region (745-788 aa) (Fig. 1B). The ribosomal protein region showed 25.4% identity with 60S ribosomal protein L4 of *Urechis caupo* (Fig. 2A). The Ub domain composed of 76 amino acids showed 84-85% identity with other well-conserved Ubs (Fig. 2B). However, the C-terminal boundary of the Ub domain was Gly-Cys^{416}. The serine-rich region contained 13 Ser in 15 residues. Thus, the protein appeared to correspond to Ublp of *A. castellanii* similar

(A)

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MALQL ANVTL YKNDL AFYEL VAPFP NNDTT PATSS APPTF CTKLD IPLKS KNLIV DTLSV (60)
RSPGL VLYNY DSELH ETIKA RDRHE ETFQF KSDSF ADFLK SCAGT PVEYT TQADS KEDHT (120)
VSGIV FTVED RVVPI ENGTL VEKQL CVLVN GNDLR RVLLK DLKSV RLLDT YLQGD LLQML (180)
SKLYE ARKPV QVATG TTSIH FSTTG PGYRA GGHFK YSYID RTEEW KCSYR LEIPS DESLE (240)
RSARA DELAK GKERA KEEPH QDKER RTGAE GNSSC GGPEL HLHMF GGVKN ASAQD VNSIR (300)
LRLVA NELEI SSNEV KKPAG AGGGG GSRSG AERTS ASGSS MQIFI KTLTG KSITL EVSSS (360)
DSIEA VKHKL QDKEG IPPDQ QRLIF AGKQL EESRT LAEYN IQKES TLHLV LRLRG GVAGE (420)
NDADT SSSSS SSSSS ASSRS GDEEF ESLDA AQMSG LAKHV IYDIS VPVTI RAKES CLVPI (480)
ASSAV EGDLV LVYDS RINEL NAYRA VHLKN STGQV LAPGL ISVLE DGRFV SQSQF TPMLP (540)
GDDQL IPYGY DSTVS IVKAM PSELQ EDNIK AIGIL YSTGS NRRPV GCRQT HLKVR RTRYT (600)
VKNNS TDRAI NKFYI DHNAD VNHGG FVIKT MESCI KSVTG FNRFQ FYLPP GGEIE FVVAE (660)
EATYT TDLLT TGSLV QFVKQ HAPDL LLAVA MEEKT LDIFK GIIIR EEAHA ALQSL ESGRF (720)
SDRQV SDWAS GSSVG GKLLD EKILT AAQKV LELRA ETAEM QRQID NHKRH IDKVF QNQNR (780)
LRENI RSLDK MTNSD LMKRY LNDLN VEEDD LISTR KEIDA IETAK LQRDK ELGDR QFALT (840)
KLARV AREAL TAS (853)
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(B)

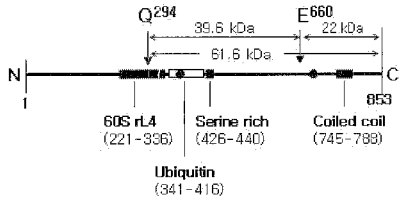

Figure 1. Deduced amino acid sequences (A) and schematic drawing (B) of Ublp94.4 protein encoded in a cDNA of *A. castellanii*. In A, amino acids for ubiquitin are underlined. Predicted proteolytic residues (^{294}Q and ^{660}E) by ExPASy proteomics tools are in underlined bold characters. The nucleotide sequence has been deposited with GenBank (AY279355). In B, schematic drawing was made on the basis of homology search. Numbers denote residue numbers of amino acids from N-terminus. The protein contains homologous domains for 60S-ribosomal protein L4 of *Urechis caupo* (GenBank Acc No. P49165), a ubiquitin, a serine rich region and a coiled coil region. Cleavage sites (Q and E) and two antigenicity domains (^{365}AVKHKLQ^{371} within Ub domain and ^{680}QHAPDLL^{686} within undefined region) predicted by ExPASy proteomics tools are indicated with arrows and solid circles, respectively.

(A)

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5'Region    RTEEVKCSYRLEIP- SDESLERSARADELAKGKERAKEEPHGDKERRTGAEQNSSCGQPELHLHMFGQVKNASAQ 294
60S rL4     RPSAEKSHYNLPMPKMTNTDLSRLLKSDEIQN----ALREPKKDKARRLQKKNPLKNYRVMMRLNPFAGAQKAAAK 339
            *.. *. *.* :*  :: .*.*  ::**: :    * .**:** **   :: .  :  ::*: *. .::*:*:

5'Region    DWNSIRLRLVANELEISSNEVKKPAGAGGGGGSRSGAERTSA   338
60S rL4     AVEQRRLKEKQAKLDQKRGIATPVEGAGKGRPRKTTAKPTKA   381
              :. **:   :*: . . ..  *** *  :: *: *.*
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(B)

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UB domain   MQIFIKTLTGKSITLEVSSSDSIEAVKHKLQDKEGIPPDQQRLIFAGKQLEESRTLAEYNIQKESTLHLVLRLRGC 416
ACACA       MQIFVKTLTGKTITLEVESSDTIENVKQKIQDKEGIPPDQQRLIFAGKQLEDGRTLADYNIQKESTLHLVLRLRGG 76
AGLNE       MQIFVKTLTGKTITLEVESSDTIENVKTKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGG 76
ACECL       MQIFVKTLTGKTITLEVESSDTVENVKSKIQDKEGIPPDQQRLIFAGKQLEDGRTLADYNIQKESTLHLVLRLRGG 76
CHLRE       MQIFVKTLTGKTITLEVESSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLADYNIQKESTLHLVLRLRGG 76
            ****:******:*****.***::* ** *:**********************:.***::******************
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Figure 2. Comparisons of the deduced amino acid sequences of the ribosomal protein region with 60S-ribosomal protein L4 of *Urechis caupo* (P49165) (A), and those of ubiquitin domain with other ubiquitins (B). ACACA: *Acanthamoeba castellanii* Ub-CEP (AC P4963-4), AGLNE: *Aglaothamnion neglectum* poly-Ub (AC P42740), ACECL: *Acetabularia cliftonii* mono-Ub (AC P42739), and CHLRE: *Chlamydomonas reinhardtii* mono-Ub (AC P14624).

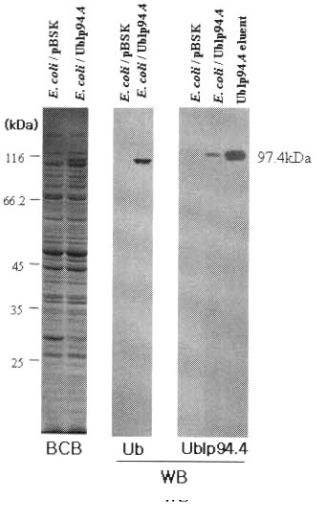

Figure 3. SDS-PAGE of proteins from *E. coli* transformed with the cloned cDNA stained with BCB and corresponding immunoblot (WB) stained with anti-Ub mAb (Ub) and antiserum against Ublp94.4. *E. coli* DH5α transformed with the cloned cDNA was induced with 1 mM IPTG. *E. coli* transformed with a pBSK vector was used for control.

to the polyprotein of *Bovine viral diarrhea virus-1* in that the ribosomal protein region fused upstream of the Ub domain. Thus, we named the protein Ublp94.4. ExPASy proteomics tools predicted Ublp94.4 to be a highly hydrophilic protein.

E. coli DH5α transformed with the cDNA produced an immunopositive 97.4-kDa protein (94.4 kDa from Ublp cDNA and 3.0 kDa from expression vector) reacting with the anti-Ub mAb. *E. coli* transformed with a pBSK vector alone for comparison did not produce any immunopositive protein (Fig. 3). We purified the protein from *E. coli* transformed with the cDNA and produced specific antibodies in BALB/c mice (Fig. 3). We fixed encysting *A. castellanii* cells at time intervals, stained them with the antiserum against Ublp94.4 as a primary antibody and FITC-conjugated goat anti-mouse IgG as a second antibody, and observed on a fluorescence microscope. We used preimmune serum as a control for primary antibody and found no fluorescence in cells.

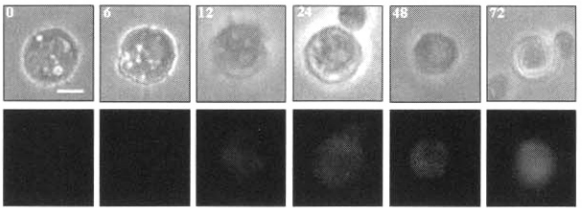

Figure 4. Phase contrast and corresponding indirect immunofluorescence micrographs of *A. castellanii* stained with antiserum against Ublp94.4. Numbers represent encysting time (h). The bar in 0 h (trophozoite) represents 10 μm for all pictures.

None of the trophozoites or cells kept in the encystment medium for 6 hrs showed any immunofluorescence. Immunofluorescence began to appear from 12 hrs of encystment and intensified to 72 hrs encystment. In mature cysts, fluorescence was found throughout the cytoplasm (Fig. 4).

Expression and processing of Ublp94.4 in encystment

The expression of Ublp94.4 gene during encystment was followed by Northern-blot and Western-blot analyses. For Northern blotting we made probes by PCR amplification of 0.6 kb domain (nt 1390-2032) to exclude unexpected detection of Ub gene expression. In the blot we detected a 2.6 kb RNA band close to the length of the predicted transcript from the cloned cDNA. The transcript was hardly detectable in early cyst stages (0-6 h cysts). During 6-12 h encystment, the level of the transcript increased gradually. Between 24-72 h encystment, the level of the transcript increased abruptly and was about 12 times that in trophozoites (Fig. 5).

In immunoblotting, the antibody against Ublp94.4 did not stain any protein in 0-h cyst (trophozoite). As

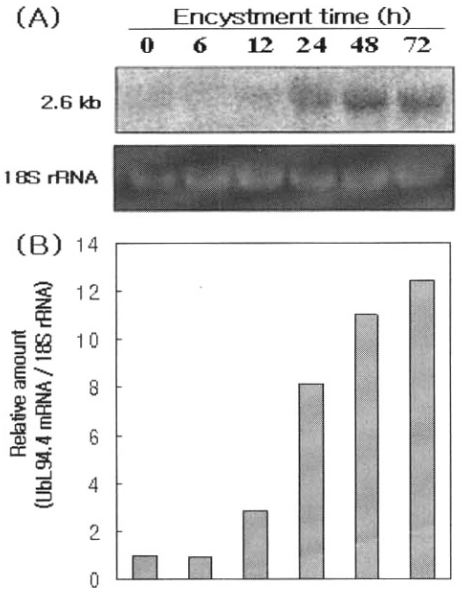

Figure 5. Northern blot (A) and densitometiric quantitation of the blot (B) for the detection of changes in the expression level of Ublp94.4 gene during encystment of *A. castellanii*. Total cellular RNAs prepared from 2×10^7 cells were analyzed in a 1.2% agarose gel containing 37% formaldehyde. Each lane was loaded with 10 μg RNA. The relative amount is a comparative value to 0-h cyst calculated by dividing the band density of Northern blot for 2.6-kb mRNA with that of 18S rRNA.

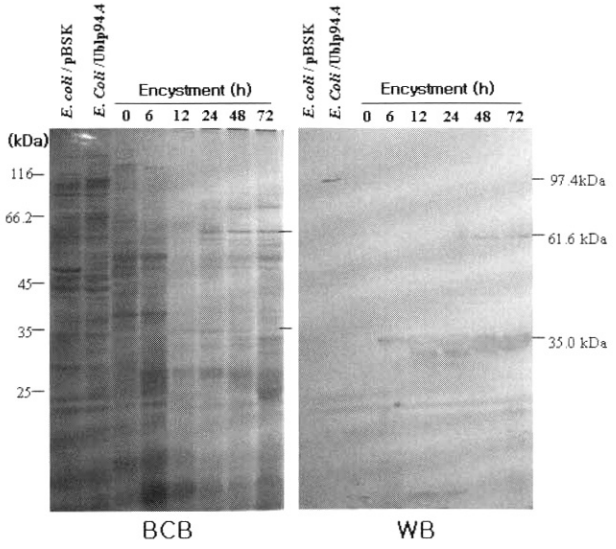

Figure 6. SDS-PAGE gel stained with BCB and corresponding immunoblot (WB) with antiserum against Ublp94.4 for the detection of changes in the level of Ublp94.4 proteins during encystment of *A. castellanii*. Ublp94.4 produced in *E. coli* was 97.4 kDa. In encysting amoebae the antiserum against Ublp94.4 stained a 35.0-kDa protein in early encysting cells and 61.6-kDa and 35.0-kDa proteins in matured cysts.

encystment proceeded, two immunopositive proteins with different molecular masses, 35.0 kDa and 61.6 kDa, respectively, were detected. The 35.0 kDa protein was first detected in 6 h-cyst and its level abruptly increased in 12 h-cysts. The 61.6 kDa protein was first detected in 24 h-cyst and its level increased with cyst maturation (Fig. 6). These results suggested that the expression of Ublp94.4 was negligible in trophozoites but increased with cyst maturation.

In the deduced amino acid sequence, the ExPASy proteomics tools, SignalP V 1.1, predicted signal peptide cleavage sites at Q^{294} and E^{660} with the cleavage value of 0.683 and 0.562, respectively. ProtScale predicted ^{365}AVKHKLQ^{371} within Ub domain and ^{680}QHAPD-LL^{686} within an undefined region for high antigenicity. These predictions and the results of immunoblot suggested that the precursor form of Ublp94.4 was cleaved at the first signal peptide cleavage site (Q^{294}) to make the first two fragments, one 32.8 kDa fragment (1-294 aa) and the other 61.6 kDa containing both predicted antigenicity domains and the Ub domain (295-853 aa). When Ublp61.6 was cleaved at the second signal peptide cleavage site (E^{660}), the product would be 39.6 kDa and 22.0 kDa in calculated molecular mass. In

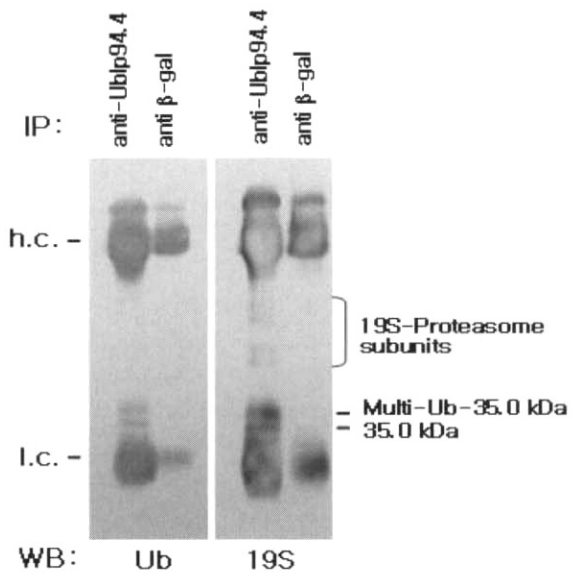

Figure 7. Immunoprecipitation analysis for the detection of multi-ubiquitinated 35.0 kDa protein and its targeting to proteasomes in vivo. The proteins of 72 h-cyst were immunoprecipitated with antiserum against Ublp94.4 or anti-β-gal mAbs for control and blotted with anti-ubiquitin (Ub) mAb or with anti-19S proteasome subunit (19S) mAb. The h.c and l.c are heavy chain and light chain of IgG, respectively.

the Western blot we detected a 35.0 kDa as the second immunopositive band. Thus, 39.6-kDa fragment appeared to contain antigenicity including the Ub domain, which would have been further processed to 35.0 kDa.

The 35.0 kDa protein is multi-ubiquitinated and targeted to proteasomes

Since the antibody against Ublp94.4 had a high specificity to Ublp94.4 and its processed products, we carried out an immunoprecipitation study to detect the multi-Ub form of 35.0 kDa proteins in vivo and their possible interactions with 26S proteasomes. The lysate of 72 h-cyst containing the most Ublp35 and Ublp61.6 was immunoprecipitated with the antibody against Ublp94.4. In the immunoblot, we detected a ladder of positive bands including the 35.0 kDa protein and multi-ubiquitinated form of 35.0 kDa protein using an anti-Ub mAb. The immunoprecipitate obtained with an anti-β-gal mAb for comparison did not have any protein to react with the anti-Ub mAb (Fig. 7). Therefore, the 35 kDa protein containing the Ub domain appeared to be the active form of Ublp94.4, which could be multiubiquitinated.

In another test, the lysate of 72 h-cyst was subjected to immunoprecipitation in the ATP regeneration system for the detection of interaction between the 35.0 kDa protein and proteasomes. In the Western blot of the precipitates with the anti-19S proteasome subunit mAb (Fig. 7) several 19S proteasome subunits were detected, whereas the immunoprecipitate with the anti-β-gal mAb for comparison did not react with anti-19S proteasome subunit mAb. These results suggested that the 35.0 kDa protein had activity for multiubiquitination and affinity toward the 19S regulatory subunit of the 26S proteasome in the presence of ATP.

DISCUSSION

In this study, we demonstrated that a novel Ublp94.4 in *Acanthamoeba* is a Ublp and its gene expression is enhanced with the maturation of cysts. A distinctive feature of Ublps is that it is always synthesized in the precursor form and is processed by post-translational modification (Hochstrasser, 1998). The Ublp94.4 protein produced in *E. coli* as a 97.4-kDa fusion protein did not undergo any change. In encysting amoebae we detected an mRNA having a corresponding length for the cloned cDNA. However, we could not detect Ublp94.4, and detected 61.6 kDa and 35 kDa in maturing cysts. Thus, Ublp94.4 appears to undergo post-translational modification to 35 kDa via 61.6 kDa. Since the 35.0 kDa fragment can be multiubiquitinated and associates with the 19S regulatory subunit of the 26S proteasome, we suggest that it has a role in targeting proteolytic substrates to the proteasomes during the encystment. However, further characterization of the fragmented polypeptides is necessary to confirm posttranslational modification of the Ublp94.4 proteins.

Proteins are usually targeted to proteasome after poly-ubiquitination by a sequential reaction of E1 (ubiquitin activating enzyme), E2 (ubiquitin conjugating enzyme) and E3 enzyme (ubiquitin-protein ligase) before degradation by multi-subunit ATP-dependent protease complex (26S proteasome) (Schauber et al., 1998). The Ub-dependent proteolysis system is a major control point for regulating cell-cycle progression (Pagano, 1997) and stage-specific gene transcription (Pahl and Baeuerle, 1996), and it is also essential for maintaining the differentiation of protozoan parasites (Gonzalez et al.,

1999; Lopez et al., 2002).

Recently, a number of Ublps have been identified. Ublps resemble ubiquitin in their amino acid sequences and often have similar means of attachment to their target proteins (Hochstrasser, 1998). In starving yeast Apg12, one Ublp plays a role in the autophagy pathway, which results in the engulfment and digestion of cytoplasm and organelles (Mizushima et al., 1998a; Mizushima et al., 1998b). Ublps are classified into type-I and type-II families (Hershko and Ciechanover, 1998; Hochstrasser, 1998). Type-I Ublps including SUMO (small-Ub-related modifier) and Rub1 (related-to-Ub 1) have conserved Gly-Gly sequences at the C-terminus of the Ub domains and compete with Ub in binding to substrate proteins. Binding of SUMO protects the substrate protein from Ub-mediated degradation (Desterro et al., 1998). SUMO can form a thiolester with a specific E2-like protein, Ubc9 (Desterro et al., 1997), and degrade proteins as with the Ub-dependent proteolysis system. Rub1 also can form a thiolester with E2-like protein, Ubc12, which is necessary for Rub1-protein conjugation. Rub1 plays an important role in the growth of plants, mammals, and other organisms (Hochstrasser, 1998).

Type-II Ublps including Rad23, Dsk2p, and Dbi1 do not have the conserved Gly-Gly sequences and contain Ub-like domain, and they interact with the 19S regulatory subunit of the 26S proteasome (Schauber et al., 1998; Russell et al., 1999). Rad23 binds Ub (Bertolaet et al., 2001; Chen et al., 2001), multi-Ub chains (Schauber et al., 1998) and ubiquitinated proteins (Ortolan et al., 2000) and interacts with the 19S regulatory subunit of the 26S proteasome (Schauber et al., 1998; Russell et al., 1999). Dsk2 and Dbi1 also contain a Ub-like domain. Dsk2 and Ddi1 could interact with Ub and multi-Ub chains (Funakoshi et al., 2002). Dsk2 and Ddi1 also could regulate the translocation of proteolytic substrates to proteasome.

Our results show that the C-terminal end of Ub domain in Ublp94.4 contains Gly-Cys instead of Gly-Gly and that the active 35 kDa form of Ublp94.4 is multiubiquitinated for interacting with proteasomes. A massive turnover of organelles and cellular membrane system takes place during the encystment of *Acanthamoeba* (Griffiths and Hughes, 1969). However, none of proteolytic systems or other relevant molecules has been reported in cysting protozoans, yet. The Ublp94.4 is the first ubiquitin like protein detected and confirmed to be expressed in cysting *Acanthamoeba*. Thus, we suggest that Ublp94.4 belongs to the type-II Ublp family and that it may play an important role in cyst maturation by regulating the proteolytic pathway.

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