Characterization of Mammalian Ecm29, a 26 S Proteasome-associated Protein That Localizes to the Nucleus and Membrane Vesicles*

Carlos Gorbea‡, Geoffrey M. Goellner‡§, Ken Teter¶, Randall K. Holmes§, and Martin Rechsteiner¶**

From the ‡Department of Biochemistry, University of Utah School of Medicine, Salt Lake City, Utah 84132 and the §Department of Microbiology, University of Colorado Health Sciences Center, Denver, Colorado 80262

In addition to its thirty or so core subunits, a number of accessory proteins associate with the 26 S proteasome presumably to assist in substrate degradation or to localize the enzyme within cells. Among these proteins is ecm29p, a 200-kDa yeast protein that contains numerous HEAT repeats as well as a putative VHS domain. Higher eukaryotes possess a well conserved homolog of yeast ecm29p, and we produced antibodies to three peptides in the human Ecm29 sequence. The antibodies show that Ecm29 is present exclusively on 26 S proteasomes in HeLa cells and that Ecm29 levels vary markedly among mouse organs. Confocal immunofluorescence microscopy localizes Ecm29 to the centrosome and a subset of secretory compartments including endosomes, the ER and the ERGIC. Ecm29 is up-regulated 2–3-fold in toxin-resistant mutant CHO cells exhibiting increased rates of ER-associated degradation. Based on these results we propose that Ecm29 serves to couple the 26 S proteasome to secretory compartments engaged in quality control and to other sites of enhanced proteolysis.

The covalent attachment of ubiquitin (Ub)1 to eukaryotic proteins is a post-translational modification of remarkable scope, complexity, and importance (1). The ubiquitin system regulates a vast array of physiological processes including transcription (2–4), cell cycle traverse (5), apoptosis (6), circadian rhythms (7), and memory (8), just to name a few. Although attachment of Ub can serve non-destructive purposes (9–11), the selective degradation of proteins is the principal way in which Ub controls cellular processes (12). The exquisite selectivity exhibited by Ub-mediated proteolysis is provided by several hundred Ub ligases, the E3 recognition components of the system (13–15). The Ub ligases collaborate with a smaller number of Ub carrier proteins, the E2s, to add poly(Ub) chains onto ϵ-amino groups of lysines in the protein substrates. The poly(Ub) chains in turn target the marked proteins to the 26 S proteasome, a large ATP-dependent protease, for degradation. The 26 S proteasome is formed by the binding of 19 S regulatory complexes (RCs) to one or both ends of the cylindrical 20 S proteasome, the proteolytic component of the 26 S enzyme. The 19 S RC provides subunits that recognize, unfold, and transfer substrate poly peptides to the central chamber of the 20 S proteasome where they are degraded (16).

The foregoing account would suggest that there are only two forms of the 26 S proteasome, one with a single 19 S RC cap and one doubly capped. However, a number of proteins associate either with the 19 S RC or with the 20 S proteasome. In eukaryotes, PA200 and PA28 (or REG) are two proteasome activators that, like the RC, bind the ends of the 20 S proteasome (17, 18). Since there are two ends to the cylindrical 20 S proteasome, the enzyme can simultaneously bind a PA200 or a PA28 and a 19 S RC, thereby forming hybrid proteasomes (19). The 19 S RC associates with a larger set of proteins, many of which are components of the Ub system. For example, Ub isopeptidases form reasonably stable associations with the 26 S proteasome (20), and several Ub ligases interact with 26 S proteasomes (21–24). An even larger number of proteins have been identified as interacting partners of individual RC subunits, especially the ATPases (25). Whereas some of these proteins may be substrates, others may function as adaptors that recruit substrates to the 26 S proteasome or localize the enzyme within cells (26). It now appears that 26 S proteasomes have a dynamic composition in which a core of about 30 different subunits associates with perhaps a greater number of accessory proteins that assist in substrate degradation.

Recently, several proteomic screens identified the yeast protein ecm29p as a 26 S proteasome-associated component (27, 28). The association of ecm29p and the 26 S proteasome was examined by Leggett et al. (29), who found the protein associated with either the 19 S RC or the 20 S proteasome depending upon conditions. ECM29 deletion produced yeast 26 S proteasomes that dissociated in the absence of ATP, leading Leggett et al. (29) to propose that ecm29p tethers the RC to the 20 S proteasome. Higher eukaryotic genomes contain a well conserved homolog of yeast ecm29p, and this allowed us to generate anti-peptide antibodies to human Ecm29. We have used these antibodies for the initial characterization of mammalian Ecm29.

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¶ Present address: Biomolecular Science Center, Dept. of Molecular Biology and Microbiology, University of Central Florida, Biomolecular Research Annex, 12722 Research Parkway, Orlando, FL 32826.

** To whom correspondence should be addressed: Dept. of Biochemistry, University of Utah School of Medicine, Salt Lake City, UT 84132.

† The abbreviations used are: Ub, ubiquitin; poly(Ub), polyubiquitin; ER, endoplasmic reticulum; ERGIC, endoplasmic reticulum-Golgi intermediate compartment; TGN, trans-Golgi network; ERAD, endoplasmic reticulum-associated degradation; TR, transferrin receptor; CHO, Chinese hamster ovary cells; GFP, green fluorescent protein; PBS, phosphate-buffered saline; MCA, 7-amino-6-methylcoumarin; RC, 19 S regulatory complex; NPAGE, native polyacrylamide gel electrophoresis; ESTs, expressed sequence tags; DTT, dithiothreitol; HANT, homology to adaptins N terminus; HEK, human embryonic kidney cells.

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EXPERIMENTAL PROCEDURES

Materials—Maleimide-activated ovalbumin and SuperSignal® Femto chemiluminescence reagent were from Pierce Biotechnology. Dulbecco’s-modified Eagles’s (DME) medium was purchased from In-vitrogen Life Technologies, Inc. F12 medium with L-glutamine was from Sigma. The HeLa derivative, D98/AH2, and Chinese Hamster ovary cells (CHO) were grown in DME and F12 media, respectively containing 10% fetal calf serum and penicillin-streptomycin. Complete® protease inhibitor mixture was purchased from Roche Applied Science. Suc-LLVY-MCA, anti-FLAG M2TM and anti-/H9253-tubulin monoclonal antibodies were from Sigma. Dextran (10,000 MW, anionic) conjugated to Alexa® 488, monoclonal anti-golgin-97, Alexa® 488, 568, and 647 goat anti-rabbit and anti-mouse IgG polyclonal antibodies and Vectashield® mounting medium with DAPI were purchased from Molecular Probes. Monoclonal antibodies to the KDEL retrieval sequence of ER-resident proteins were obtained from Calbiochem. Anti-mannose-6-phosphate receptor monoclonal antibodies were from abcam. A monoclonal antibody to the human transferrin receptor (TR) was purchased from Zymed Laboratories. Monoclonal antibodies MCP21, MCP102, and MCP168 to 20 S proteasome subunits /H9251, /H9252, and /H9252, respectively were purchased from Affiniti. Monoclonal antibodies to ERGIC-53 were a kind gift from Hans-Peter Hauri (Basel, Switzerland). Anti-6 (MCP20) monoclonal antibodies were a gift from Klavs Hendil (Copenhagen, Denmark). Antibodies to S10b and S14 were prepared as described (30).

Sequence of Ecm29—The human sequence was assembled using the sequence of the KIAA0368 protein (gi:30151586) and cDNA sequences (gi:34534077 and gi:34527903) contained in the GenBank™ data base. Sequences used to produce anti-peptide antibodies are underlined in red. A potential nuclear localization signal sequence is highlighted in green. One of three alternative N-terminal sequences in hEcm29 encoded by human, mouse, and rat ESTs derived from brain, retina, testis, and ovary is highlighted in black. Sequences homologous to conserved regions of β-adaptins are highlighted in orange. A region that bears resemblance to VHS domains is highlighted in pink. B, architecture of human Ecm29. We have proposed that Ecm29 is composed almost entirely of HEAT-like repeats (diamonds) (35). A region with homology to VHS domains (pink rectangle) and two helical regions homologous to conserved sequences in adaptins (orange ellipses) were identified by RPS-BLAST analysis of the oasis_sap.v1.58 data base. The VHS-like domain of hEcm29 was aligned with a structure-based alignment of the VHS domain of D. melanogaster Hrs (HrsD) (74, 75) and the structurally related ENTH domain of rat epsin-1 (76); both are shown below the schematic representation of hEcm29.
predicted KIAA0368 sequence (gi:37540467). A number of human, mouse, and rat expressed sequence tags (ESTs) (gi:3398354; gi:35193073; gi:5636575; gi:588268; gi:12755765; gi:15401157; gi:16454877; gi:28567689) encoded alternative N-terminal sequences with homology to the predicted N termini of Drosophila melanogaster (gi:17861783) or Oryza sativa (constructed from gi:32975471 and gi:32990505) Ecm29 sequences. The human Ecm29 sequence was used to perform an RPS-BLAST analysis of the oasi_sap.v1.58 database and PSI-BLAST reiterated searches.

**Ecm29 Antibodies**—Three peptides, Ecm29–1 (C-RNRKESTSE\[Q\]PSFPE); Ecm29–2 (C-RGRPLDDIIDKLPE); and Ecm29–3 (C-RPELEKKAALKTLKLE), corresponding to distinct regions of the human Ecm29 protein (see Fig. 1) were used for antibody production. Peptides were synthesized and coupled to maleimide-activated ovalbumin according to the manufacturer’s instructions through an added N-terminal cysteine residue. Peptide-coupled ovalbumin was sent to Harlan Bioproducts for Science, Inc (Indianapolis, IN) for antibody production.

**Distribution of Ecm29 in HeLa Cells and Mouse Organs**—HeLa cells or freshly harvested mouse organs were extracted in 1 ml of 5 mM Hepes, pH 7.2, 0.25 M sucrose, 0.2 mM EDTA containing protease inhibitors by 300 strokes using a tight-fitting Dounce homogenizer. A 200-μl supernatant was then sedimented on a 10–30% glycerol gradient for 4 h at 48,000 rpm using a Beckman SW50.1 rotor. 180-μl fractions were collected from the bottom and assayed for peptidase activity using suc-LLVY-MCA for 1 h at 37 °C to visualize the 26 S proteasome:Ecm29 ratio of 1.7 (v/v) in the presence of 1 mM ATP and 10% glycerol and incubated at 37 °C for 30 min. Samples were finally resedimented on 10–30% glycerol gradients, assayed for peptidase activity using suc-LLVY-MCA as substrate and analyzed by SDS-PAGE and immunoblotting with anti-Ecm29-3 antibodies.

**Partial Purification of Ecm29 from Bovine Brain**—Bovine brain was homogenized in 10 mM Tris-HCl, pH 7.0, 25 mM KCl, 10 mM NaCl, 1.1 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT containing 0.25% Triton X-100 plus protease inhibitors, and centrifuged at 13,000 × g. Glycerol was added to 10% and the sample (8 g) was batch-adsorbed to 500 ml DEAE and proteins were eluted with 5-column volumes of a 35 to 300 mM KCl linear gradient. Fractions containing Ecm29 were identified by SDS-PAGE and immunoblotting, pooled and loaded onto a 100-ml DEAE column. Proteins were eluted with 500 ml of a linear gradient from 35 to 300 mM KCl. Pooled fractions containing Ecm29 were concentrated and applied to a HiLoad 26/60 prep grade Superdex 200 column equilibrated in 25 mM Tris-HCl, pH 7.5, 75 mM KCl, 1 mM DTT, and 10% glycerol. Last, fractions containing Ecm29 were loaded on a Mono-Q HR 5/5 column equilibrated in homogenization buffer, pH 8.5 with 10% glycerol, and proteins were eluted with a linear gradient from 35 to 750 mM KCl. A 200-μl sample of the pooled Mono-Q fractions was layered atop a 5-ml 5–20% sucrose gradient and centrifuged at 39,000 rpm for 18 h at 4 °C using a Beckman SW50.1 rotor. Fractions were analyzed by SDS-PAGE and immunoblotting with anti-Ecm29-3 antibodies.

**Expression of Ataxin-7 Containing an 86-Residue Polyglutamine Tract**—Human HeLa and L4 prostate cells were grown on 18-mm circular coverslips and enucleated after treatment with cytochalasin B by the method of Prescott et al. (33) as described previously (34). Karyoplast and cytoplast fractions were extracted in 50 μl of 0.25% Triton X-100 in 10 mM Tris-HCl, pH 7.5, 1 mM ATP, 1 mM DTT plus protease inhibitors and subjected to SDS-PAGE and immunoblotting with anti-Ecm29-3 or antibodies to 26 S proteasome subunits.

**Expression of Tagged Versions of Mouse Ecm29 in Cultured Cells**—A cDNA encoding mouse Ecm29 (96% identity to human Ecm29) cloned into the pXY vector was purchased from Open Biosystems and used as template for the polymerase chain reaction. The resulting DNA was
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subcloned at the BamHI/SalI sites of pFLAG-CMV (Sigma) or pGFP-N1 (Clontech). Transfections of HeLa, HEK293, and COS7 cells were performed with Lipofectamine 2000™ according to the manufacturer’s instructions. For immunofluorescence, HeLa and COS7 cells grown on coverslips were transfected with 5 μg of total plasmid DNA, fixed in 100% methanol at −20 °C 2 days after transfection and processed as described below. HEK293 cells grown on T-25 flasks (Corning) were transfected with 10 μg of pFLAG-CMV-Ecm29 and harvested 19 h after transfection. Cells were homogenized in buffer containing 0.25% sucrose and sedimented through a 5-ml 10–30% sucrose gradient as described under “Experimental Procedures.” Fractions were concentrated, mixed at a 26 S proteasome:Ecm29 ratio of 1.7 (v/v) in the presence of 1 mM ATP and 10% glycerol, and incubated at 37 °C for 30 min. Samples were then resedimented on a 5-ml 10–30% glycerol gradient, assayed for peptidase activity in the presence (●) or absence (○) of ATP and analyzed by denaturing or non-denaturing gel electrophoresis followed by Western blotting using anti-FLAG M2™ monoclonal antibodies (1:400).

Immunofluorescence—HeLa cells grown on coverslips were washed briefly in phosphate-buffered saline (PBS) and fixed in 100% methanol at −20 °C for 10 min or in 2 or 4% paraformaldehyde in PBS for 15 min at 4 °C. When cells were fixed in methanol, Ecm29 antibodies were used at dilution of 1:100 for anti-Ecm29-1, 1:50 for anti-Ecm29-2 or 1:200 for anti-Ecm29-3. Conversely, when cells were fixed with paraformaldehyde to subcellular compartments were used as followed: anti-ERGIC-53, 1:500; anti-golgin-97, 1:25; anti-KDEL, 1:100; anti-manose-6-phosphate receptor, 1:50; anti-transferrin receptor, 1:25; and anti-γ-tubulin, 1:10,000. Endocytic vesicles were stained by continuous labeling in the presence of 1 mg/ml Alexa® 488-dextran conjugate for 30 or 60 min at 37 °C in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Polyclonal antibodies to S7 were used at 1:10. When cells were fixed in methanol, Ecm29 antibodies were used at dilution of 1:100 for anti-Ecm29-1, 1:50 for anti-Ecm29-2 or 1:200 for anti-Ecm29-3. Conversely, when cells were fixed with paraformaldehyde to subcellular compartments were used as followed: anti-ERGIC-53, 1:500; anti-golgin-97, 1:25; anti-KDEL, 1:100; anti-manose-6-phosphate receptor, 1:50; anti-transferrin receptor, 1:25; and anti-γ-tubulin, 1:10,000. Endocytic vesicles were stained by continuous labeling in the presence of 1 mg/ml Alexa® 488-dextran conjugate for 30 or 60 min at 37 °C in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Polyclonal antibodies to S7 were used at 1:10 whereas anti-β3 was used at 1:25. After paraformaldehyde fixation, cells were washed with PBS and permeabilized in 0.2% Triton X-100 in

Fig. 3 Association of human Ecm29 with the 26 S proteasome is reversible. A, HeLa cells were homogenized in buffer containing 0.25 M sucrose or B, were extracted in buffer containing 0.25% Triton X-100. Aliquots of the post-mitochondrial supernatants (~1.8 mg of total protein) were centrifuged on 5-ml 10–30% glycerol gradients as described under “Experimental Procedures.” Fractions were collected and assayed for peptidase activity in the presence (●) or absence (○) of ATP using succ-LYV-MCA as substrate (top panels). Samples were separated by non-denaturing electrophoresis, overlaid with 20 μl succ-LYV-MCA (second panels), transferred to nitrocellulose and immunoblotted with anti-Ecm29-3 antiserum (third panels). In A, samples were also subjected to SDS-PAGE and immunoblotted with anti-Ecm29-3 (bottom panel). B clearly shows that after extraction with Triton X-100, Ecm29 is no longer associated with the 26 S proteasome. C, bovine brain was homogenized in buffer containing 0.25% Triton X-100 plus protease inhibitors and centrifuged at 13,000 × g to remove debris. The supernatant fraction was then subjected to a series of chromatographic steps as described under “Experimental Procedures.” Samples were analyzed by Western blotting of non-denaturing (NATIVE) and SDS-PAGE gels using anti-Ecm29-3 antibodies. D, 200-μl sample of the pooled Mono-Q fractions was centrifuged on a 5-ml 5–20% sucrose gradient at 39,000 rpm for 18 h. Fractions were analyzed by SDS-PAGE and immunoblotting with anti-Ecm29-3. Sedimentation markers included catalase (232 kDa), aldolase (158 kDa), and bovine serum albumin (67 kDa). In A, samples were subjected to SDS-PAGE and immunoblotting with anti-Ecm29-3 to verify dissociation of Ecm29 from the proteasome (not shown). Fractions containing stripped 26 S proteasomes or “free” Ecm29 were pooled, bovine serum albumin was added to 0.1 mg/ml, and each sample was dialyzed to remove the detergent. Dialed samples were concentrated, mixed at a 26 S proteasome:Ecm29 ratio of 1.7 (v/v) in the presence of 1 mM ATP and 10% glycerol, and incubated at 37 °C for 30 min. Samples were then resedimented on a 10–30% glycerol gradient, assayed for peptidase activity in the presence (●) or absence (○) of ATP and analyzed by immunoblotting with anti-Ecm29-3 (bottom panel). After dialysis and concentration, both 26 S proteasome and Ecm29 sedimented as shown in the top and middle panels. Note that Ecm29 is absent from the stripped 26 S proteasome (middle panel). However, when the enzyme was mixed with stripped Ecm29 (26 S + Ecm29), a substantial amount of Ecm29 was found re-associated with the 26 S proteasome.
PBS for 10 min at room temperature. Cells were blocked with 10% nonfat milk in Tris-buffered saline, pH 8.0 with 0.05% Tween 20 (TBST) and incubated with primary or secondary antibodies in blocking buffer for 1 h at 37 °C. Fluorescent secondary antibodies were used at a dilution of 1:500. After incubation with antibodies, cells were washed six times in TBST for 5 min each at room temperature. Fluorescence confocal laser scanning microscopy was performed on an Olympus FXV confocal microscope equipped with argon 488 and krypton 568/647 lasers using a 60X PlanApo 1.4 NA oil objective and Fluoview 2.13.9 software. The relative amounts of Ecm29 and cellular markers at specific subcellular locations were estimated using Volocity v2.0, a volume rendering and three-dimensional quantification software (Improvision).

RESULTS

Amino Acid Sequence of Human Ecm29 (hEcm29)—From the human genome data base and EST libraries we assembled a putative full-length sequence for human Ecm29 (Fig. 1A) that is identical to the KIAA0388 protein reported in the latest GenBank™ release. Human Ecm29 is 1839 amino acids long, and Prosite analysis reveals the presence of a possible bipartite nuclear targeting signal (highlighted green in Fig. 1A). The N-terminal sequence of human Ecm29 (highlighted black in Fig. 1A) is one of three alternative N-terminal sequences encoded by human, mouse, and rat ESTs derived from brain, retina, testis, and ovary: MYHIDCRDqler (most highly represented); MAAAAASQDELNqler; and METGSDSDqler. All known Ecm29 sequences exhibit an apparent modular structure consisting of HEAT repeats (at least 25 in hEcm29; diamonds in Fig. 1D) (35), a region with sequence homology to VHS domains (36) (highlighted pink in Fig. 1A), and two potential helical regions with homology to conserved sequences in β-adaptins (highlighted orange in Fig. 1A) that we call HANT domains (homology to adaptins N terminus). PSI-BLAST reiterative analysis of the GenBank™ data base using the entire human Ecm29 sequence revealed that HEAT repeats within the C-terminal half of Ecm29 show highest similarity to HEAT motifs of the translational activator GCN1 and importin β2 (E values < e^-60 after 5 iterations) (35, 47). A similar computational analysis indicated highest similarity between the VHS-like region of human Ecm29 (residues 893–1038; pink rectangle in Fig. 1B) and the VHS domain of hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) (E values e^-27.e^-10 after 5 iterations). However, unlike the VHS domain of Hrs and other proteins, the VHS-like region of Ecm29 is not present at the N terminus of the molecule (Fig. 1).

Antibodies to Human Ecm29—Antibodies were generated to synthetic peptides corresponding to the three sequences underlined in red in Fig. 1. Western blots of HeLa cell extracts using the resulting antisera identified a 205-kilodalton polypeptide, the size expected for hEcm29 (Fig. 2A). A survey of mouse organs using the three anti-peptide antibodies revealed that the levels of Ecm29 vary considerably, being particularly high in testis and brain, low in liver, and almost undetectable in heart or kidney (Fig. 2B). By contrast, the levels of S10b, a 19 S RC ATPase, were highest in testis, lowest in pancreas, and relatively uniform in the rest of the organs (Fig. 2B). Two of the three antisera recognized a slower migrating form of Ecm29 in brain (arrows in Fig. 2B) and some mouse and human brain ESTs contain open reading frames extending beyond the initial Met residue of Ecm29 (not shown). Presumably, alternative splicing gives rise to a longer form as well as smaller Ecm29 species present in brain. The patterns generated by the three antibodies were similar in testis, lung, and spleen and in other organs (pancreas, liver, and kidney) differed at most in minor aspects leaving little doubt that Ecm29 is the protein that was detected by the three anti-peptide antibodies.

Association of hEcm29 with the 26 S Proteasome Is Detergent-sensitive and Reversible—To determine if human Ecm29 associates with the 26 S proteasome, HeLa cells were homogenized in 0.25 M sucrose, and the distribution of proteasome activity was determined after sedimentation of the post-mitochondrial supernatant fraction on a 10–30% glycerol gradient. Samples from the various fractions were subjected to native or SDS-PAGE electrophoresis followed by Western blotting. As shown in Fig. 3A, virtually all HeLa Ecm29 is bound to the 26 S proteasome after homogenization in sucrose. This is particularly clear by comparing the location of 26 S proteasomes evident from fluorogenic peptide overlay (second panel) with the Western blot of the same gel (third panel). Except for a minor immunoreactive band (possibly the 19 S RC), directly below the two enzymatically active species of 26 S proteasome, HeLa Ecm29 migrated with the large, ATP-dependent protease. A Western blot of SDS-PAGE transfers (bottom panel) confirmed that the immunoreactivity on the native gel transfer is present as the 205-kilodalton polypeptide expected for Ecm29 (Fig. 3A, bottom panel).

A much different distribution of Ecm29 was observed after extraction of HeLa cells in buffer containing Triton X-100. Ecm29 was no longer associated with the 26 S proteasome but sedimented with apparent molecular weights ranging from 200 to 600 kDa (Fig. 3B). Western blots of native gel transfers showed several forms of hEcm29 that migrated as a smear on native gels; faster sedimenting species present in fractions 15–20 barely entered the gel whereas slower sedimenting species found in fractions 21–25 formed a distinct band. During partial purification of Ecm29 from detergent extracts of bovine...
brain, the protein exhibited similar properties. Ecm29 from more purified preparations (i.e., after Mono-Q chromatography) sedimented as a monomer on a sucrose gradient and migrated fastest on a native gel (Fig. 3, C and D). Presumably, slower migrating and faster sedimenting detergent-released Ecm29 molecules either form multimers or are associated with proteasome subcomplexes or possibly with other cellular components. Fractions containing proteasome-free hEcm29 were pooled, dialyzed, concentrated, and mixed with detergent-stripped HeLa 26 S proteasomes. Following re-centrifugation substantial amounts of Ecm29 once again sedimented with the 26 S proteasome, indicating that detergent extraction does not prevent reassociation of Ecm29 with the 26 S proteasome (Fig. 3E).

Subcellular Localization of Mammalian Ecm29—Staining of HeLa cells with anti-Ecm29-1 or anti-Ecm29-3 resulted in predominantly cytoplasmic fluorescence that was virtually absent in cells stained with preimmune serum and was blocked by preincubation with the cognate peptide (Fig. 4). At low PMT/gain exposures there was intense juxtanuclear staining in most HeLa cells. Confocal images of cells stained with both anti-Ecm29 and anti-γ-tubulin identified this region as the centrosome (Fig. 5, A–C). In addition to the centrosomal location,
**FIG. 6.** *Ecm29 is present on endosomes.* HeLa cells grown on coverslips were continuously labeled with 1 mg/ml fluorescent dextran for 30 (B) or 60 (E, H, K) min and incubated with anti-Ecm29-1 (A) or anti-Ecm29-2 (D, J) antibodies. In panels G and J, HeLa cells were incubated with preimmune serum or with anti-Ecm29-2 antiseraum preincubated with 30 mg/ml immunizing peptide, respectively. Detection and fluorescence confocal laser scanning microscopy were performed as described under “Experimental Procedures.” The images correspond to single 0.2 μm optical sections. Centrosomes are indicated by white arrowheads (see Fig. 5, A–C). Scale bars, 10 μm.

**FIG. 7.** *Ecm29 is present on early and late endosomes.* HeLa cells grown on coverslips were fixed with −20 °C methanol and labeled with anti-Ecm29-3 antibodies (A, D, G) and monoclonal antibodies to the transferrin receptor (TR, B), the mannose-6-phosphate receptor (E) or both (H). Binding was detected with Alexa® 568 goat anti-rabbit IgG and Alexa® 488 goat anti-mouse IgG polyclonal antibodies, and visualized by confocal microscopy as described under “Experimental Procedures.” The images correspond to single 0.2 μm optical sections. Scale bars, 10 μm.
higher PMT/gain exposures showed that HeLa Ecm29 was present in a cytoplasmic vesicular pattern. To determine whether Ecm29 was localized to a particular set of membrane compartments we collected confocal images from HeLa cells stained with anti-Ecm29 and various compartment-specific antibodies. The images in Fig. 5, D–L show that Ecm29 partially overlapped with anti-KDEL, a monoclonal antibody against the retention signal of ER-resident proteins, and with ERGIC-53, a marker of the ER-Golgi intermediate compartment (ERGIC) (37). The extent of overlap between Ecm29 and the ER/ERGIC compartments increased substantially when both anti-KDEL and anti-ERGIC-53 were incubated simultaneously with anti-Ecm29 (Fig. 5, J–L) indicating that Ecm29 is present on both compartments. By contrast, there was little overlap between Ecm29 and golgin-97 (Fig. 5, M–P), a marker for the trans-Golgi network (TGN) (38).

Although these results suggest that Ecm29 is present on a subset of ER/ERGIC compartments in the secretory pathway, the dense packing of vesicles within the cytoplasm makes this conclusion tentative. Endosomes provide a set of vesicles that can readily be distinguished following the uptake of fluorescent markers. This feature coupled with the significant Ecm29 staining peripheral to the ER/ERGIC compartments prompted us to ask whether Ecm29 also associates with endosomes. HeLa cells were incubated in medium containing Alexa® 488-dextran (39, 40), fixed in 2% paraformaldehyde and stained with anti-Ecm29-1 or Ecm29-2 (the epitope for anti-Ecm29-3 is destroyed by paraformaldehyde). The confocal images shown in Fig. 6, A–F demonstrate that Ecm29 associates with endosomes. As expected, vesicle-specific Ecm29 staining was absent in cells incubated with preimmune serum, and abolished when antibodies were preincubated with cognate peptide (Fig. 6, G–L). Confocal imaging of HeLa cells stained with monoclonal antibodies to the transferrin and mannose-6-phosphate receptors confirmed that Ecm29 associates with both early and late endocytic vesicles (Fig. 7). Furthermore, in HeLa cells pulse-labeled with fluorescent dextran and chased for 2 h in serum-free medium, Ecm29 was associated with late juxtanuclear endosomal vesicles that had fused (data not shown). Since glycerol gradient fractionation indicated that all Ecm29 mole-
cules are present on 26 S proteasomes (Fig. 3), we expected that 26 S proteasomes would also be found on endosomes. This proved to be the case as shown by staining with antibodies to \(/H9252\), a 20 S proteasome subunit, and antibodies against S7 of the 19 S RC (Fig. 8, A and B). It should be noted that classical fractionation procedures involving homogenization in sucrose reveal all Ecm29 to be associated with \(/H9251\) soluble 26 S proteasomes (Fig. 3A), thereby precluding attempts to confirm the immunofluorescence data by traditional methods of subcellular fractionation. We did, however, make two additional measurements on the intracellular distribution of mammalian Ecm29. HEK293 cells were transfected with pFLAG-CMV expressing FLAG-tagged mouse Ecm29. Glycerol gradient sedimentation of sucrose extracts revealed that FLAG-Ecm29 associated exclusively with the 26 S proteasome (Fig. 9A), and immunofluorescence staining with anti-FLAG monoclonal antibodies produced a vesicular distribution like that observed with the three anti-peptide antibodies to Ecm29 (Fig. 9B). Furthermore, similar results were obtained upon transfection with a plasmid expressing an Ecm29-green fluorescent protein (GFP) fusion protein (see bottom panels in Fig. 12, below). Thus, we are confident that Ecm29 is a component of 26 S proteasomes that are localized on intracellular vesicles and, as shown below, present in the nucleus.

Ecm29 Is Up-regulated in Toxin-resistant CHO Cells Exhibiting Enhanced ER-associated Degradation (ERAD)—The presence of a putative VHS domain in Ecm29 and its localization on secretory compartments suggests that the protein may recruit 26 S proteasomes to membranes for the purpose of ERAD. In previous studies, mutagenized CHO cells were selected for simultaneous resistance to \(/H9262\) Pseudomonas aeruginosa exotoxin A and ricin (41). Since the two toxins are endocytosed by different pathways and modify different cellular targets, multitoxin resistance was expected to arise from changes in a common toxin processing mechanism. This was confirmed by finding that six of the isolated cell lines also exhibited non-selected resistance to cholera toxin (CT), which is thought to exploit ERAD for entry into the cytosol. In three of these toxin-resistant clones (5, 16, and 46) the established ERAD substrate \(/H9263\) α1-antitrypsin \(Z\) accumulated in the ER and was thus
degraded less efficiently than in the wild-type parental cells, while the other three clones (23, 24, and 38) degraded α-l-antitrypsin Z more rapidly than wild-type CHO cells. Clones 23 and 24 also degraded the catalytic CT polypeptide more rapidly than wild-type CHO cells. Clones 23 and 24 express 2–3-fold more Ecm29 protein than either wild-type CHO or the ERAD-deficient lines. Moreover, the increased coupling efficiency between toxin degradation in the cytosol (42). Since overall proteasome activity was not altered in clones 23 and 24, it was concluded that toxin resistance was caused by increased coupling efficiency between toxin translocation from the ER lumen into the cytosol and toxin degradation in the cytosol (42).

Given our suspicion that Ecm29 recruits the 26 S proteasome to secretory quality control compartments, we compared Ecm29 levels in wild-type CHO and in each of the 6 toxin-resistant clones. It is clear from the blots in Fig. 10A that clones 23 and 24 express 2–3-fold more Ecm29 protein than either wild-type CHO or the ERAD-deficient lines. Moreover, the increased ERAD in clones 23 and 24 is not accompanied by a general up-regulation of proteasome subunits (see Fig. 10B) or proteasome activity (42). Although the 205-kDa Ecm29 species is not increased in CHO clone 38, the other high ERAD mutant, a larger isoform of Ecm29 is uniquely present in this mutant, possibly being the same isoform as the one seen in mouse brain (see Fig. 2B). Even though the up-regulation of Ecm29 in toxin-resistant CHO mutants exhibiting enhanced ERAD lends support to the idea that Ecm29 couples the 26 S proteasome to quality control compartments in the secretory/endothetic pathways, Ecm29 was not induced when HeLa cells were treated with brefeldin A, monensin, tunicamycin, or inhibitors of ER glycosidases, compounds that induce the unfolded protein reponse (UPR) pathway (43) (data not shown).

Mammalian Ecm29 Is Present in Karyoplasts and on Intracellular Polyglutamine Inclusions—In most cases, nuclei stain for Ecm29 less intensely than the cytoplasm (Figs. 5, 6, and 8). However, significant nuclear staining was observed in human prostate L4 cells (see below), and the intensity of nuclear staining varied considerably in HeLa cells depending upon fixation methods as well as culture conditions (see Figs. 5–8). Because the nuclear envelope and densely packed chromatin can make immunodetection of intranuclear components difficult, we employed cytochalasin B-mediated enucleation of HeLa cells to measure the nuclear/cytoplasmic distribution of Ecm29. As shown in Fig. 11, HeLa Ecm29 was distributed evenly between cytoplasm and karyoplast fractions. The presence of Ecm29 in karyoplasts may reflect a substantial pool of intranuclear Ecm29 or Ecm29 may simply be enriched in perinuclear structures, e.g. centrosome or perinuclear ER. There is, however, no doubt that Ecm29 can enter the nucleus as shown by expression of polyglutamine-expanded ataxin-7.

Expression of proteins containing an expanded polyglutamine tract (polyQ) causes a number of neurodegenerative diseases that include Huntington’s and Kennedy’s disease and six spinocerebellar ataxias (SCAs). One of the hallmarks of these diseases is the formation of intranuclear polyQ inclusions that accumulate components of the ubiquitin-proteasome system (44–46). To determine whether Ecm29 accumulates in polyQ inclusions, we transfected human HeLa and L4 prostate cells with a plasmid encoding a His-tagged version of ataxin-7 containing 86 glutamines, or expressed in COS7 cells both the ataxin-7-Q86 protein and mouse Ecm29 fused to GFP. Fixed cells were stained with anti-His to localize polyQ aggregates and either stained with anti-Ecm29-3, or visualized for the fluorescence of the Ecm29-GFP fusion protein. As seen with other components of the Ub-proteasome system, Ecm29 is indeed present on polyQ intranuclear inclusions (Fig. 12).

The Intracellular Distribution of Ecm29: A Summary—Velocity v2.0, a volume-rendering and three-dimensional quantification software, was used to estimate the relative amount of Ecm29 in karyoplasts may reflect a substantial pool of intranuclear Ecm29 or Ecm29 may simply be enriched in perinuclear structures, e.g. centrosome or perinuclear ER. There is, however, no doubt that Ecm29 can enter the nucleus as shown by expression of polyglutamine-expanded ataxin-7.

Degradation of Ecm29 between nucleus and cytoplasm. HeLa cells grown on 18-mm circular coverslips were enucleated after treatment with cytochalasin B as described under “Experimental Procedures.” Karyoplast (K) and cytoplast (C) fractions were extracted in 50 μl of 0.25% Triton X-100 in 10 mM Tris-HCl, pH 7.5, 1 mM ATP, 1 mM DTT plus protease inhibitors, and subjected to SDS-PAGE (panel A) and immunoblotting with the indicated antibodies (panel B). W, whole cell extract; p80, 80-kDa cytoplasmic polypeptide recognized by anti-PA200 polyclonal antibodies (18); Nup62, nucleoporin 62. S10b, S14, S10h, and S14 are 19 S RC subunits; α6 is a 20 S proteasome subunit. The amount of Ecm29 in the karyoplast fractions was 47% (Exp. 1) and 48% (Exp. 2) as determined by densitometry.

**DISCUSSION**

Human Ecm29 Is a Non-stoichiometric Component of the 26 S Proteasome—Like its yeast homolog (29), mammalian (m) Ecm29 is a proteasome-associated protein. This is shown in Fig. 3 by the combined use of glycerol gradient sedimentation, native gel electrophoresis, and Western blotting. However, the properties of mEcm29 differ in two respects from those reported for yeast ecm29p. First, we have not observed HeLa or bovine brain Ecm29 bound to the 20 S proteasome either during purification or after attempts at reconstitution (Fig. 3 and

**Fig. 11.** Distribution of Ecm29 between nucleus and cytoplasm. HeLa cells grown on 18-mm circular coverslips were enucleated after treatment with cytochalasin B as described under “Experimental Procedures.” Karyoplast (K) and cytoplast (C) fractions were extracted in 50 μl of 0.25% Triton X-100 in 10 mM Tris-HCl, pH 7.5, 1 mM ATP, 1 mM DTT plus protease inhibitors, and subjected to SDS-PAGE (panel A) and immunoblotting with the indicated antibodies (panel B). W, whole cell extract; p80, 80-kDa cytoplasmic polypeptide recognized by anti-PA200 polyclonal antibodies (18); Nup62, nucleoporin 62. S10b, S14, S10h and S14 are 19 S RC subunits; α6 is a 20 S proteasome subunit. The amount of Ecm29 in the karyoplast fractions was 47% (Exp. 1) and 48% (Exp. 2) as determined by densitometry.
data not shown). Second, markedly variable expression levels in mouse organs (Fig. 2) rule out mEcm29 being a stoichiometric component of the 26 S proteasome. If mEcm29 clamps the 19 S RC to 20 S proteasomes as proposed for the yeast protein (29), it does so only in certain mammalian organs. Rather than functioning to stabilize the 26 S proteasome, we propose that mEcm29 is an adaptor that recruits the 26 S proteasome to specific intracellular locations requiring enhanced rates of protein degradation such as the centrosome, the cytoplasmic face of the ER and possibly polyQ aggregates.

Evidence That Mammalian Ecm29 Is an Adaptor—Several lines of evidence suggest that Ecm29 functions as an adaptor. Computer analyses of the Ecm29 sequence predict the protein to be composed almost exclusively of HEAT repeats (35). These protein modules form flexible, curved solenoids that enable HEAT-repeat-containing proteins to bind multiple protein ligands, and HEAT repeats are found in a number of adaptor proteins (47, 48). In addition, Ecm29 contains a region homologous to VHS domains found in Hrs, STAM, and GGAs, proteins that serve as adaptors in endosomal sorting and vesicular trafficking (36, 49). Finally, two regions in Ecm29 share homology to the \( \gamma \)H9252\( \gamma \)H9262 subunit of AP2 adaptor complexes, and potential clathrin-interacting motifs are present throughout the Ecm29 sequence (50). Thus, the sequence of Ecm29 suggests an adaptor function.

The intracellular distribution of Ecm29 also supports the idea that Ecm29 is an adaptor. Ecm29 and the 26 S proteasome are found on endosomes (Figs. 6–8), and the presence of a VHS-like domain in Ecm29 suggests that it recruits the 26 S enzyme to endosomal membranes. Recently, Dong et al. (51) observed direct interaction between the 20 S proteasome subunit XAPC7 and Rab7, a GTPase that controls late endocytic events and is found on late endosomes. Similarly, Ecm29 may function as a molecule that recruits proteasomes to endosomes.

Table I

| Marker | Location | % Ecm29 | % Marker that overlaps with Ecm29 |
|--------|----------|---------|---------------------------------|
| 6-Tubulin | Centrosome | 9 ± 1 (n = 30 cells) | ND |
| KDEL | ER | 31 ± 2 (n = 12 cells) | 33 ± 2 (n = 12 cells) |
| ERGIC-53 | ERGIC | 17 ± 1 (n = 17 cells) | 53 ± 2 (n = 17 cells) |
| golgin-97 | ERGIC | 5 ± 4 (n = 11 cells) | ND |
| KDEL + ERGIC-53 | ER/ERGIC | 44 ± 2 (n = 23 cells) | 48 ± 3 (n = 23 cells) |
| Dextran | Endosomes* | ND | ND |
| Nucleus* | 38 ± 2 (n = 28 cells) | ND |

* ND, not determined.
* The fraction of Ecm29 on endosomes could not be determined because of variable fixation of the fluorescent dextran and severe photobleaching during scanning.
* Cell nuclei were identified morphologically and digitally excised from the images to estimate the amount of nuclear Ecm29.
Although protease activity is known to regulate the trafficking of several plasma membrane receptors (52–58), the role of the protease in endocytosis remains a mystery. It has been proposed that 19 S RC isopeptidase activity is needed for proper trafficking of the EGF receptor (52). Alternatively, 26 S proteasomes may degrade components involved in the endocytic process, such as c-Cbl (52), or membrane scaffolding proteins, such as PSD95 (59).

Besides being found on endosomes, mEcm29 is located at two intracellular sites exhibiting high levels of Ub-dependent proteolysis: the centrosome and the ER/ERGIC compartments (Fig. 5 and Table I). It has long been established that the centrosome is enriched in proteases especially when cells synthesize large amounts of abnormal proteins or when proteasomes are inhibited (60–63). Proteasomes have likewise been shown to degrade secretory proteins in a process called ERAD (64–67). Our finding that the intracellular distribution of mEcm29 overlaps the ER and ERGIC compartments raises the possibility that Ecm29 recruits proteasomes to membranes within these organelles. In fact, a role for Ecm29 in the ERAD pathway is indicated by overexpression of the protein in mutant CHO cells that exhibit increased ERAD activity (Fig. 10A). Localization of Ecm29 within the nucleus and on intranuclear polyQ inclusions (Fig. 12) does not argue against its playing a major role in proteasomal degradation of secretory or endocytic substrates, because the nuclear function of Ecm29 and 26 S proteasomes may be independent of their function in the cytoplasm. Moreover, a number of proteins involved in endocytosis are known to shuttle between nucleus and cytoplasm (68–70). Among mouse organs, we have also shown that Ecm29-proteasomes are inhibited (60–63). Proteasomes have likewise been shown to degrade secretory or endocytic process, such as c-Cbl (52), or membrane scaffolding proteins, such as PSD95 (59).

In summary, we have shown that mEcm29 is a 26 S protease-associated protein with varying levels of expression among mouse organs. We have also shown that Ecm29-proteasome complexes are present at centrosomes, on endosomes, and on intranuclear polyQ inclusions. Up-regulation of Ecm29 in mutant CHO cells exhibiting increased ERAD and its apparent location on ERGIC compartments lead us to propose that Ecm29 couples proteasomes to the ERAD quality control pathway and to functions occurring on endosomes and other membrane vesicles (71–73).

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Characterization of Mammalian Ecm29, a 26 S Proteasome-associated Protein That Localizes to the Nucleus and Membrane Vesicles

Carlos Gorbea, Geoffrey M. Goellner, Ken Teter, Randall K. Holmes and Martin Rechsteiner

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