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Segregation and rapid turnover of EDEM1 by an autophagy-like mechanism modulates standard ERAD and folding activities

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ABSTRACT

EDEM1 is a crucial regulator of endoplasmic reticulum (ER)-associated degradation (ERAD) that extracts non-native glycopolypeptides from the calnexin chaperone system. Under normal growth conditions, the intralumenal level of EDEM1 must be low to prevent premature interruption of ongoing folding programs. We report that in unstressed cells, EDEM1 is segregated from the bulk ER into LC3-I-coated vesicles and is rapidly degraded. The rapid turnover of EDEM1 is regulated by a novel mechanism that shows similarities but is clearly distinct from macroautophagy. Cells with defective EDEM1 turnover contain unphysiologically high levels of EDEM1, show enhanced ERAD activity and are characterized by impaired capacity to efficiently complete maturation of model glycopolypeptides. We define as *ERAD tuning* the mechanisms operating in the mammalian ER at steady state to offer kinetic advantage to folding over disposal of unstructured nascent chains by selective and rapid degradation of ERAD regulators.

Most of the proteins synthesized in the ER are covalently modified at asparagines in Asn-Xxx-Ser/Thr motifs by the addition of pre-assembled glucose$_3$-mannose$_9$-*N*-acetylglucosamine$_2$-core oligosaccharides. Rapid removal of the two outermost glucose residues elicits association of the mono-glucosylated oligosaccharide displayed by nascent polypeptides with the lectin chaperones calnexin and calreticulin and the associated oxidoreductase ERp57. This facilitates the formation of native intra- and intermolecular disulfide bonds [1]. Folding-defective glycoproteins are not normally released from the ER into the secretory pathway. After a *lag* phase during which they have been subjected to *futile* folding-attempts, terminal α1,2-bonded mannose residues are removed from their *N*-glycans. De-mannosylation of non-native polypeptides is operated by several ER-resident members of the glycosyl hydrolase 47 (GH47) superfamily of mannosidases [2]. It is a signal of long retention in the ER lumen, it causes irreversible extraction from the calnexin chaperone system and translocation into the cytosol for degradation [3–5]. EDEM1 is an ER stress-inducible member of the GH47 family of α1,2-mannosidases and a crucial regulator of glycoprotein disposal from the ER. An increase in EDEM1 concentration in the ER lumen upon induction of the Ire1/Xbp1 ER-stress response pathway or upon ectopic expression facilitates glycoprotein's disposal by three distinct mechanisms: (i) by accelerating substrate de-mannosylation and extraction from the calnexin chaperone system; (ii) by inhibiting aggregation of misfolded polypeptides released from calnexin; (iii) by chaperoning ERAD candidates to the putative site of translocation across the ER membrane (reviewed in [2,6]).

Here, we show that EDEM1 has shorter half-life than ER-resident molecular chaperones and enzymes assisting protein folding. EDEM1 is segregated from the bulk ER into small, LC3-I-coated vesicles, the EDEMosomes, and is rapidly degraded by lysosomal enzymes. Cells with defective EDEM1 turnover contain increased amount of EDEM1 and are characterized by such an unphysiologically enhanced ERAD activity that maturation of cargo proteins may be prematurely interrupted. We postulate that rapid turnover of EDEM1 and of other ERAD regulators similarly characterized by short half-life is required to maintain appropriate function of the ER folding compartment in unstressed cells.

Materials and methods

Antibodies, expression plasmids, and inhibitors. Antibodies to BACE, calnexin, BiP, ERp72, and ERp57 and plasmids for expression of BACE457, BACE457NOG, and EDEM1-HA are described in [7,8]. The anti-EDEM used for immunoprecipitation was from Santa Cruz Biotech Inc (C-19, sc-27391). We acknowledge the generosity of several friends that shared antibodies to EDEM1 (K. Nagata and

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N. Hosokawa), ATG5 (M. Mizushima), Sec61alpha (R. Zimmerman), RibophorinI (S. High), PDI (I. Braakman), ERp29 (S. Mkrtchian) and the cDNA encoding for ATG5 and ATG5K130R (N. Mizushima). PS341 was a kind gift of R. Sitia.

Cell Lines, transient transfections, metabolic labeling, analysis of data. The *wt* and *atg5*$^{-/-}$ MEF (N. Mizushima) were grown in DMEM supplemented with 10% FBS. Transfections were performed with Lipofectamine 2000 (Invitrogen) according to the instructions of the manufacturer. Experiments were normally performed 17 h after transfections. Metabolic labeling is described in [9].

Separation of transitional and peripheral ER with self-generated Optiprep™ density gradients. Cells were carefully washed with ice-cold isotonic phosphate buffer, pH 7.4 (PBS), resuspended in 0.8 ml HB-EDTA (10 mM triethanolamine, 10 mM acetic acid, 250 mM sucrose, 1 mM EDTA and a protease inhibitors cocktail, pH 7.4), and scraped with a rubber policeman. Cells were broken to release intracellular compartments, organelles and proteins by 10 passages through a 25G1 needle. After centrifugation (2 times 1500*g*, 5 min) the post-nuclear supernatant (PNS) was collected, and loaded onto a linear 10–30% Optiprep™ (Nycomed) gradient prepared by mixing a 10% and a 30% Optiprep solution with a Gradient Master Biocomp™ (angle 80°, speed 20 rev/s, time 1 min e 30 s). Separation of intracellular organelles was performed by ultracentrifugation in an MLS-50 rotor (17 h, 29,000 rpm/94,000*g*). Fractions were collected from the top, separated in reducing 10% SDS–polyacrylamide gels, transferred on PVDF membranes and decorated with the appropriate antibody.

Immunofluorescence microscopy. Indirect immunofluorescence microscopy is described in [10]. All antibodies (rabbit polyclonal anti-EDEM1 (kind gift of K. Nagata and N. Hosokawa), monoclonal anti-LC3 (Nano Tools), monoclonal anti-Hsp47 (Stressgen), Alexa 488-labeled goat anti-rabbit, Alexa 594-labeled goat anti-mouse) were diluted (1:100).

Results and discussion

EDEM1 is a short-living ERAD regulator

Although specific studies have not been published, there is a general agreement that ER-resident molecular chaperones and folding enzymes are long-living proteins. To confirm this, the proteins expressed in mouse embryonic fibroblasts (MEF) were metabolically labeled for 30 min with ^{35}S-methionine and cysteine. After various chase times with unlabeled amino acids, labeled ER-proteins were immunoisolated from detergent extracts with specific antibodies. Variations in their content were determined by band densitometry in reducing SDS–PAGE. The amount of radioactive calnexin, BiP, ERp72, and ERp57 (shown as paradigmatic examples in Fig. 1A, B) did not decrease during the 120 min of chase, thus confirming their long half-life. For the ERAD regulator EDEM1, however, analysis of the intracellular stability revealed a much faster turnover (Fig. 1A–C, $T_{1/2}$ of circa 55 min).

EDEM1 turnover requires functional lysosomes and is inhibited by ATG5-deletion

EDEM1 turnover was only poorly inhibited by cell exposure to inhibitors of cytosolic proteasomes (PS341, Fig. 1C, lane 7) but it was substantially delayed upon cell exposure to lysosomotropic agents (chloroquine (CQ), Fig. 1C, lane 6). Thus, EDEM1 disposal requires the activity of lysosomal proteases.

EDEM1 is not transported through the secretory pathway en route to lysosomes [2,6]. We therefore determined whether macroautophagy, an inducible intracellular process in which membrane-bound compartments engulf organelles and macromolecules and deliver them to lysosomes for destruction [11–14] was required for the rapid, physiologic turnover of EDEM1. To this end, we determined whether inactivation of macroautophagy upon ATG5-deletion [15] abolished EDEM1 disposal.

Comparison of EDEM1 disposal in *wt* MEF and in MEF lacking ATG5 revealed that in *wt* cells roughly 55% of endogenous EDEM1 was degraded per hour (Fig. 1A–C and Fig. 1D, lanes 1 and 2). This percentage of degradation dropped to about 20% per hour in cells lacking ATG5 (Fig. 1D, lanes 3 and 4). Thus, inactivation of autophagy delayed, but it was not sufficient to prevent EDEM1 disposal. If macroautophagy would be involved in EDEM1 turnover, then its induction upon nutrient deprivation should accelerate EDEM1 disposal. Instead, induction of autophagy obtained by growing *wt* MEF in culture media lacking FBS and depleted of aminoacids abolished EDEM1 disposal (Fig. 1E, compare degradation of EDEM1 in untreated cells (lanes 1–3) vs. degradation of EDEM1 upon activation of macroautophagy (lanes 4–6)). We postulate that induction of autophagy leads to consumption/depletion of a cellular component (e.g., membrane-associted LC3-I, see below), which is required for EDEM1 turnover. Thus, ATG5-deletion slows EDEM1 turnover, but conventional macroautophagy is not involved in the process.

Analysis by immunoblot revealed that cells lacking ATG5 contain a substantially elevated concentration of EDEM1 (Fig. 1F). This could result from the defective EDEM1 disposal (as shown in Fig. 1D) and/or from enhanced EDEM1 synthesis. We excluded the second possibility by showing that ATG5-deletion did not activate the unfolded protein response. Consistently, the transcript (SFig. 1B) and protein levels (Fig. 1F) of several ER-stress markers were the same in cells with and without ATG5. The level of EDEM1 transcripts (SFig. 1B) and the new synthesis of EDEM1 (Fig. 1D, compare the amount of labeled EDEM1 expressed in *wt* vs *atg5*$^{-/-}$ MEF, lanes 1 and 3, respectively) were also unaffected upon ATG5-deletion. Thus, the slower turnover of endogenous EDEM1 in *atg5*$^{-/-}$ MEF results in unphysiologically elevation of the intralumenal content of EDEM1 (Fig. 1F and SFig. 1A).

EDEM1 co-localizes with ER and autophagosome markers

Electron microscopy and confocal immunofluorescence analysis recently revealed the presence of EDEM1 in small ER-derived vesicles that lack conventional ER markers and a recognizable cytosolic coat such as COPII. The destination or function of these vesicles has not been established, but the occasional presence of ERAD substrates led to propose a possible role of this vesicular transport out of the ER in removal of misfolded proteins from the ER lumen [16]. We now challenge this hypothesis and we propose that a vesicular transport out of the ER regulates rapid EDEM1 turnover to reduce the content of this crucial ERAD regulator in the protein folding compartment.

To verify our hypothesis, we first looked for markers that co-stain EDEM1-containing compartments in immunofluorescence. In *wt* MEF, part of the endogenous EDEM1 was in the bulk ER as shown by the co-localization with ER markers such as calnexin, BiP or Hsp47 (Fig. 2A, panel 1). A significant fraction of endogenous EDEM1 was scattered throughout the cytoplasm in small vesicles that, consistent with published data, were not stained with antibodies to ER markers (panel 1) nor with antibodies to COPII (data not shown and [16]). A screen for possible surface markers of intracellular organelles revealed that the ER-derived, EDEM1-containing vesicles were coated with LC3 (Fig. 2A, panel 2 and Fig. 2B), a marker for autophagosome membranes [17]. Cell incubation with chloroquine or deletion of ATG5, two conditions that inhibit EDEM1 turnover (Fig. 1) resulted in EDEM1 accumulation in the LC3-decorated vesicles (Fig. 2A, panel 3) and in EDEM1 enrichment in the ER lumen, respectively (SFig. 2A, panel 1). Some of the EDEM1-LC3 vesicles became substantially larger (1–1.5 μm, arrowheads) upon chloroquine-treatment as an indirect indication of their acidic luminal pH that