EDEM1 accelerates the trimming of α1,2-linked mannose on the C branch of N-glycans

Nobuko Hosokawa1,2, Linda O Tremblay3, Barry Sleno3, Yukiko Kamiya4,5, Ikuo Wada6, Kazuhiro Nagata7, Koichi Kato4,5,7, and Annette Herscovics3

1Department of Molecular and Cellular Biology, Institute for Frontier Medical Sciences, Kyoto University, Kyoto 606-8397, Japan; 2McGill Cancer Centre, Montreal, Quebec H3G 1Y6, Canada; 3Okazaki Institute for Integrative Bioscience, National Institutes of Natural Sciences, Okazaki 444-8787, Japan; 4Graduate School of Pharmaceutical Sciences, Nagoya City University, Nagoya 467-8063, Japan; 5Department of Cell Sciences, Institute of Biomedical Sciences, Fukushima Medical University School of Medicine, Fukushima 960-1295, Japan; and 7The Glycoscience Institute, Ochanomizu University, Tokyo 112-8610, Japan

Received on November 2, 2009; revised on January 4, 2010; accepted on January 5, 2010

Glycoprotein folding and degradation in the endoplasmic reticulum (ER) is mediated by the ER quality control system. Mannose trimming plays an important role by forming specific N-glycans that permit the recognition and sorting of terminally misfolded conformers for ERAD (ER-associated degradation). The EDEM (ER degradation enhancing α-mannosidase-like protein) subgroup of proteins belonging to the Class I α1,2-mannosidase family (glycosylhydrodase family 47) has been shown to enhance ERAD. We recently reported that overexpression of EDEM1 enhances glycoprotein ERAD with a concomitant increase in α1,2-mannosidase activity. This can be attributed to both wild-type and mutant EDEM1 inhibiting aberrant NHK dimer formation. We further analyzed the N-glycan profile of total cellular glycoproteins from HepG2 cells stably overexpressing EDEM1 and found that the relative amount of Man5GlcNAc2 isomer A, which lacks the terminal B and C branch mannoses, was increased compared to parental HepG2 cells. Based on this observation, we conclude that EDEM1 activity trims mannose from the C branch of N-glycans in vivo.

Keywords: EDEM (ER degradation enhancing α-mannosidase-like protein)/ERAD (ER-associated degradation)/glycoprotein/mannose trimming/N-linked glycan

Introduction
Quality control of proteins synthesized in the endoplasmic reticulum (ER) is coupled to processing of the N-linked oligosaccharides attached to nascent polypeptides (Anelli and Sitiia 2008; Caramelo and Parodi 2007; Hebert et al. 2005; Helenius and Aebi 2004). As nascent proteins emerge from the Sec61 translocation channel in the ER, the N-glycan Glc3Man9GlcNAc2 is attached to Asn residues within the consensus sequence Asn-X-Ser/Thr. Following trimming by glucosidases I and II, monoglycosylated N-glycans can associate with the lectin-chaperones calnexin and calreticulin which assist protein folding (Caramelo and Parodi 2007; Helenius and Aebi 2004). The entry of proteins into the monoglucosyl cycle is regulated by removal and readdition of the final glucose residue by glucosidase II and UDP-glucose:glycoprotein glucosyl transferase, respectively.

Proteins failing to attain their correct conformation are recognized as terminally misfolded and are retrotranslocated from the ER to the cytosol through the dislocation channel by a process known as ER-associated degradation (ERAD) (Meusser et al. 2005; Nakatsukasa and Brodsky 2008). Once in the cytosol, the N-glycans are removed by N-glycanase (Suzuki 2007), and the proteasome proceeds to degrade the misfolded polypeptides (Raasi and Wolf 2007). Although the recognition process of terminally misfolded proteins is not yet fully understood, the importance of mannose trimming in targeting glycoproteins for ERAD has been well established in both budding yeast and mammalian cells (Helenius and Aebi 2004; Jakob et al. 1998). In particular, ER mannosidase I (ER ManI) trimming of the mannose residue from the N-glycan B branch (Gonzalez et al. 1999; Tremblay and Herscovics 1999) triggers ERAD of misfolded glycoproteins in mammalian cells (Cabral et al. 2001; Hosokawa et al. 2003; Wu et al. 2003). Recent studies have also concluded that additional mannose trimming leading to the formation of Man7,GlcNAc2 is also required to elicit glycoprotein degradation (Lederkremer and Glickman 2005). ER ManI has been postulated to perform this additional mannose trimming in vivo (Avezov et al. 2008; Hosokawa et al. 2003), but ER degradation enhancing α-mannosidase-like protein 3 (EDEM3) and the Golgi1,2-mannosidases may also be involved due to recycling of ERAD substrates (Hosokawa et al. 2007).
Mammalian cells contain three distinct EDEM proteins in the ER: EDEM1 (Hosokawa et al. 2001), EDEM2 (Mast et al. 2005; Olivari et al. 2005) and EDEM3 (Hirao et al. 2006). All three mammalian EDEMs, as well as the budding yeast (Saccharomyces cerevisiae) ortholog Htm1p/Mnl1p (Jakob et al. 2001; Nakatsukasa et al. 2001), share substantial homology to the ER and Golgi α1,2-mannosidases (glycosylhydrolase group 47), and they have also been reported to accelerate glycoprotein ERAD (Kanehara et al. 2007; Olivari and Molinari 2007).

EDEM1 was hypothesized to enhance ERAD through its extraction of misfolded glycoproteins from the calnexin cycle (Molinari et al. 2003; Oda et al. 2003), but the precise mechanism by which this occurs has yet to be determined.

To date, studies seeking to characterize the α1,2-mannosidase activities of EDEMs and Htm1p/Mnl1p have yielded different results. In yeast, Htm1p/Mnl1p was reported to lack enzymatic activity in vivo since deletion of this gene did not affect oligosaccharide structures (Jakob et al. 2001). Furthermore, we were unable to detect EDEM1 α-mannosidase activity in vitro (Hosokawa et al. 2001). However, it was recently reported that EDEM3 displays processing α-mannosidase activity in vivo (Hirao et al. 2006). In cells overexpressing EDEM3, mannose trimming was observed on both misfolded and total cellular glycoproteins. On the contrary, EDEM2 has been reported to lack enzyme activity both in vivo and in vitro (Mast et al. 2005). More recently, overexpression of EDEM1 in vivo was reported to induce de-mannosylation activity (Olivari et al. 2006). Hence further studies are required to clarify the unique enzymatic activities of the individual EDEM proteins and elucidate their role in ERAD.

In the present study, we examined the mannose-trimming activity of EDEM1 in vivo on the ERAD substrate α1-antitrypsin null (Hong Kong) (NHK) (Liu et al. 1999). In cells transfected with EDEM1, we identified the formation of Glc1Man9GlcNAc isomer C by the release and fractionation of [3H]mannose-labeled N-glycans on NHK. However, in cells overexpressing EDEM3, mannose trimming was observed on both misfolded and total cellular glycoproteins. On the contrary, EDEM2 has been reported to lack enzyme activity both in vivo and in vitro (Mast et al. 2005). More recently, overexpression of EDEM1 in vivo was reported to induce de-mannosylation activity (Olivari et al. 2006). Hence further studies are required to clarify the unique enzymatic activities of the individual EDEM proteins and elucidate their role in ERAD.

In the present study, we examined the mannose-trimming activity of EDEM1 in vivo on the ERAD substrate α1-antitrypsin null (NHK) (Liu et al. 1999). In cells transfected with EDEM1, we identified the formation of Glc1Man9GlcNAc isomer C by the release and fractionation of [3H]mannose-labeled N-glycans on NHK. However, in cells overexpressing EDEM3, mannose trimming was observed on both misfolded and total cellular glycoproteins. On the contrary, EDEM2 has been reported to lack enzyme activity both in vivo and in vitro (Mast et al. 2005). More recently, overexpression of EDEM1 in vivo was reported to induce de-mannosylation activity (Olivari et al. 2006). Hence further studies are required to clarify the unique enzymatic activities of the individual EDEM proteins and elucidate their role in ERAD.

In the present study, the examination of the mannose-trimming activity of EDEM1 in vivo on the ERAD substrate α1-antitrypsin null (NHK) (Liu et al. 1999). In cells transfected with EDEM1, we identified the formation of Glc1Man9GlcNAc isomer C by the release and fractionation of [3H]mannose-labeled N-glycans on NHK. However, in cells overexpressing EDEM3, mannose trimming was observed on both misfolded and total cellular glycoproteins. On the contrary, EDEM2 has been reported to lack enzyme activity both in vivo and in vitro (Mast et al. 2005). More recently, overexpression of EDEM1 in vivo was reported to induce de-mannosylation activity (Olivari et al. 2006). Hence further studies are required to clarify the unique enzymatic activities of the individual EDEM proteins and elucidate their role in ERAD.
Results

Identification of Glc$_1$Man$_8$GlcNAc$_2$ isomer C formation on NHK in cells overexpressing EDEM1

To characterize the α-mannosidase activity of EDEM1, 293 cells coexpressing misfolded NHK and EDEM1 were labeled with $[^3]$H]mannose. Thereafter, the N-glycans on NHK were isolated and resolved by high-performance liquid chromatography (HPLC) (Hosokawa et al. 2003). Glc$_1$Man$_9$GlcNAc$_2$ and Man$_9$GlcNAc$_2$ were predominantly recovered along with relatively smaller amounts of Man$_8$-6GlcNAc$_2$. Throughout the chase period, progressive mannose trimming was observed. Importantly, a small unidentified peak eluting slightly earlier than Man$_9$GlcNAc$_2$ was also observed (Figure 1A, thick arrow). This unidentified peak had previously appeared in HPLC profiles of NHK N-glycans from EDEM1 overexpressing cells and most notably in the published profiles including the $[^{14}]$C Glc$_1$Man$_8$GlcNAc isomer B standard. The latter revealed that the unidentified peak elutes slightly later than the $[^{14}]$C Glc$_1$Man$_8$GlcNAc isomer B standard (Hosokawa et al. 2003). Thus to identify this unknown peak, $[^{14}]$C Glc$_1$Man$_8$GlcNAc isomer B and $[^3]$H]Glc$_1$Man$_8$GlcNAc isomer C standards were prepared, and a mixture of both standards was then confirmed to be separable by HPLC (Figure 1C, see Materials and methods). Thereafter, the $[^3]$H]Glc$_1$Man$_8$GlcNAc isomer C standard was added to an aliquot of the initial NHK N-glycan sample described in Figure 1A and resolved under the same HPLC conditions. This standard eluted within the same fraction as the unidentified peak (Figure 1B, thick arrow), thereby indicating overexpression of EDEM1 yields Glc$_1$Man$_8$GlcNAc$_2$ isomer C on NHK in vivo.

Characterization of the N-glycans on NHK in cells transfected with the EDEM1 E220Q mutant

To further investigate whether EDEM1 processing α-mannosidase activity specifically produces Glc$_1$Man$_8$GlcNAc$_2$ isomer C, we generated EDEM1 E220Q containing a conserved acidic amino acid mutation that was previously demonstrated to abolish α1,2-mannosidase enzyme activity (Lipari and Herscovics 1999; Hirao et al. 2006). Following cotransfection of cells with NHK and EDEM1 E220Q, the oligosaccharides on NHK were released by Endo H digestion, fractionated by HPLC and compared to those recovered from both mock and wild-type EDEM1 transfected cells (Figure 2). The peak of Glc$_1$Man$_8$GlcNAc isomer C detected in cells overexpressing wild-type EDEM1 was observed to increase during the chase period and accounted for approximately 10% of the total labeled N-glycan on NHK at

Fig. 2. Absence of Glc$_1$Man$_8$GlcNAc isomer C formation on NHK in cells overexpressing EDEM1 E220Q. 293 cells were cotransfected with NHK and pcDNA3.1 (mock), HA-tagged EDEM1 or HA-tagged EDEM1 E220Q. NHK N-glycans were extracted from $[^3]$H]mannose-labeled cells and fractionated by HPLC. Annotations are the same as in Figure 1.
3-h chase (middle panel, thick arrow). However, this peak was negligible in samples isolated from cells transfected with EDEM1 E220Q (lower panel), thereby indicating EDEM1 promotes α1,2-mannose trimming of N-glycans yielding Glc1Man8GlcNAc2 isomer C. These results suggest that EDEM1 possesses intrinsic α1,2-mannosidase activity.

Effect of the EDEM1 E220Q mutation on NHK ERAD

We then analyzed whether the α1,2-mannosidase activity of EDEM1 is required to enhance misfolded NHK ERAD. Cells co-expressing NHK and either wild-type or E220Q EDEM1 were pulse-labeled with [35S]methionine/cysteine, and NHK degradation was examined. We observed that EDEM1 enhanced NHK degradation, as reported previously (Hosokawa et al. 2001).

Moreover, EDEM1 E220Q also enhanced NHK degradation to nearly the same extent as wild-type EDEM1 (Figure 3A, quantified in B), indicating that the intrinsic α-mannosidase activity of EDEM1 is dispensable for the enhancement of NHK ERAD. Reciprocal coimmunoprecipitation of NHK with wild-type or E220Q EDEM1 was detected (Figure 3A) suggesting that the E220Q mutant retains its ability to interact with misfolded NHK. Since we previously reported NHK ERAD acceleration by EDEM1 is dependent on EDEM1 maintaining the retrotranslocation competence of NHK via inhibition of covalent dimer formation (Hosokawa et al. 2006), we proceeded to investigate the impact of the EDEM1 E220Q mutation on NHK dimer formation. Immunoprecipitation revealed the mutant inhibits NHK dimer formation to a similar extent as the wild-type EDEM1 (Figure 3C). Hence its ability to maintain misfolded NHK in a retrotranslocation competent state allows EDEM1 E220Q to promote ERAD acceleration. Western blotting analysis determined that similar levels of EDEM1 E220Q and wild-type EDEM1 were expressed in the transfected cells (Figure 4A). The level of overexpression was estimated to be approximately 25- to 50-fold higher than the endogenous protein (Figure 4B).
Analysis of N‐glycans from total cellular glycoproteins in HepG2 cells stably overexpressing EDEM1

We next examined the N‐glycosylation profiles of total cellular glycoproteins extracted from HepG2 cells stably overexpressing hemagglutinin (HA)‐tagged EDEM1 and compared their profiles to parental HepG2 cells. Pyridylaminated N‐glycans derived from the glycoproteins were fractionated using an amide‐silica column (Figure 5A), and the isomer composition of each fraction was further characterized. Man9GlcNAc2 isomer A (lacking terminal α,1,2‐mannose on both the B and C branches) and isomer C (lacking terminal α,1,2‐mannose on both the A and B branches) were separated from the Man9GlcNAc2 peak by octadecyl silica (ODS) column (Figure 5B). Isomer composition of N‐glycans of total cellular glycoproteins from HepG2 and EDEM1‐HepG2 cells was calculated and compared (Figure 5C). Importantly, overexpression of EDEM1 increased the relative amount of Man9GlcNAc2 isomer A, suggesting that EDEM1 preferentially trims the α,1,2‐linked mannose from the C branch. The increase of Man5GlcNAc2 and complementary decrease of Man9GlcNAc2 in EDEM1‐HepG2 cells indicate that mannose trimming was stimulated by the overexpression of EDEM1 (Figure 5C). Finally, the level of EDEM1 expressed in the stably transfected EDEM1‐HepG2 cells was compared to the level in transiently transfected HEK 293 cells (Figure 5D). Western blotting revealed that a similar amount of EDEM1‐HA was expressed in the detergent soluble fraction in both cell populations.

Discussion

This study demonstrates that Glc1Man9GlcNAc2 isomer C is formed on NHK in cells overexpressing EDEM1 as summarized in (Figure 6). Additionally, the formation of Glc1Man9GlcNAc2 isomer B on NHK in cells overexpressing ER ManI has been previously reported (Hosokawa et al. 2003) (Figure 6B). The formation of Glc1Man9GlcNAc2 isomer C is nearly abolished by the mutation of a conserved catalytic residue essential for α,1,2‐mannosidase activity of EDEM1, thereby indicating that the observed mannose trimming is probably due to EDEM1 processing α‐mannosidase activity. Notably, these results differ from those recently reported by Olivari et al. (2006), which suggest that EDEM1 de‐mannosylates the A branch. Importantly, the specificity reported by Olivari et al. was determined using a mutant cell line that produces truncated Man9GlcNAc2 N‐glycan structures containing a single terminal α,1,2‐linked mannose residue exclusively on the A branch (B3F7 Chinese hamster ovary mutant cells (Cacan et al. 1992)). The ability of EDEM1 to preferentially process α,1,2‐linked mannose from the C branch reported herein is further supported by the increase of Man9GlcNAc2 isomer A on total cellular glycoproteins from EDEM1‐HepG2 cells. Taken together, we propose that the mannose trimming on N‐glycans is catalyzed by a coordinated action of ER ManI and EDEM1, when EDEM1 is overexpressed (Figure 6C). It may be that EDEM1 also can process mannose from both A and C branches, since we observed an increase of Man5GlcNAc2 N‐glycan in total cellular glycoproteins from EDEM1‐HepG2 cells. A successful in vitro assay remains to be devised to further establish the preferred order of mannose trimming by EDEM1.

Furthermore, the mannose‐trimming activity of EDEM1 was found to be dispensable for the enhancement of NHK ERAD, since the rate of NHK degradation in cells expressing EDEM1 E220Q was similar to that in cells expressing wild‐type EDEM1. However, EDEM1 acceleration of NHK ERAD seems to be dependent on its ability to maintain NHK in a retrotranslocation competent form (Hosokawa et al. 2006), and this is not abolished by the E220Q mutation. Similarly, enhancement of misfolded BACE457Δ ERAD was not impacted by the EDEM1 E220Q mutation due to its dependence on the function of EDEM1 to reduce disulfide‐bonded aggregate formation that was not affected by the mutation (Olivari et al. 2006). These observations differ significantly from EDEM3 that requires intrinsic mannosidase activity to stimulate glycoprotein ERAD (Hirao et al. 2006). In particular, the relative amount of Glc1Man9GlcNAc2 isomer C formed in cells overexpressing EDEM1 is only 10% after a 3‐h chase period, whereas in cells overexpressing EDEM3 in excess of 50% of the N‐glycans on NHK are trimmed to form Man5‐7GlcNAc2 after 2 h.
Taking into consideration that transfected EDEM1 is expressed at a level that is at least 25-fold higher than the endogenous level, its processing α-mannosidase activity in vivo seems to be relatively weak. This resembles the Schizosaccharomyces pombe ER α-mannosidase which was reported to enhance glycoprotein ERAD, but has extremely feeble enzymatic activity in vivo and is undetectable in vitro (Movsicho et al. 2005). Additionally, EDEM2 which also enhances glycoprotein ERAD lacks enzymatic activity both in vivo and in vitro (Mast et al. 2005).

Alternatively, EDEM1 may require a cofactor for its optimal mannose processing activity. Htm1p/Mnl1p, the EDEM homologue in S. cerevisiae, was recently reported to associate with protein-disulfide isomerase, but whether this interaction is required for mannosidase activity is unclear at present (Clerc et al. 2009; Sakoh-Nakatogawa et al. 2009). Conversely, EDEM1 may serve as a cofactor for another α1,2-mannosidase as suggested for ER Man1 (Termine et al. 2009).

While this manuscript was in revision, Clerc et al. (2009) reported that Htm1p/Mnl1p acts as a processing α1,2-mannosidase capable of trimming the terminal mannose from the C branch of N-glycans downstream of ER mannosidase in S. cerevisiae which is consistent with our present study in mammalian cells. Furthermore, the sugar-binding specificities of the mannose 6-phosphate homology domains were recently characterized for yeast Yos9p and mammalian OS-9, both of which are involved in ERAD (Hosokawa et al. 2009; Quan et al. 2008). Specifically, removal of the terminal mannose from the C branch was determined to be an important degradation signal recognized by these lectins. Thus characterization of the mannose-trimming activity of the three mammalian EDEMs is fundamental to further elucidating the formation...
EDEM1 accelerates mannose trimming on C branch of N-glycans

and recognition of the N-glycan signals involved in the glycoprotein quality control machinery.

Materials and methods

Cell culture and transfection

Human embryonic kidney 293 cells were cultured in DMEM (Dulbecco’s modified Eagle’s medium) containing 1 g/L glucose supplemented with 10% fetal bovine serum and antibiotics (100 U/mL penicillin G and 0.1 mg/mL streptomycin). Plasmids were transfected using FuGENE6 transfection reagent (Roche Applied Sciences, Mannheim, Germany) according to the manufacturer’s protocol. Human hepatocellular carcinoma HepG2 cells were cultured as HEK 293 cells but were grown in DMEM containing 4.5 g/L glucose and additionally supplemented with nonessential amino acids (Invitrogen, Invitrogen Life Technologies, Carlsbad, CA) and were plated on poly-L-lysine-coated dishes. HepG2 cells stably overexpressing EDEM1-HA were established as described previously (Hosokawa et al. 2006) and maintained in medium supplemented with 30 μg/mL blasticidin S (Funakoshi, Tokyo, Japan).

Plasmid construction

The EDEM1 E220Q mutant was constructed by substituting Glu220 (GAA) with Gln (CAA) using Quick Change™ site-directed mutagenesis (Stratagene, La Jolla, CA). HA-tagged EDEM1 and the α1-antitrypsin NHK variant were prepared as described previously (Hosokawa et al. 2001).

Metabolic labeling and immunoprecipitation

Metabolic labeling and pulse-chase experiments were performed as described previously (Hosokawa et al. 2003). Briefly, for [3H]mannose labeling of NHK, cells were labeled with 7.4 MBq/mL D-[2-3H]mannose (PerkinElmer Life Sciences, Boston, MA) in DMEM containing 1 mM glucose and chased in DMEM containing 5 mM mannose and 25 mM glucose. For [35S]methionine/cysteine labeling, cells were incubated in DMEM lacking methionine/cysteine and containing 8.2 MBq/mL [35S]-Express Protein Labeling mixture (PerkinElmer Life Sciences) for 15 min after preincubation in medium lacking methionine/cysteine for 20 min. Chase incubations were performed in normal growth medium.

Cells were extracted with buffer containing 1% NP-40, and NHK or HA-tagged EDEM1 was immunoprecipitated using antibodies to α1-antitrypsin (DAKO, Glostrup, Denmark) or the HA-tag (Santa Cruz Biotechnology, Santa Cruz, CA) as described previously (Hosokawa et al. 2003). Metabolically labeled proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), exposed to a phosphor-imager, and the observed signals were quantified using ImageQuant (STORM, GE Healthcare Biosciences, Uppsala, Sweden). To detect NHK disulphide-bonded dimer, cells were treated with proteasome inhibitor lactacystin (20 μM) and maintained in medium supplemented with 30 μg/mL blasticidin S (Funakoshi, Tokyo, Japan). Oligosaccharide structures on NHK were analyzed by HPLC analysis of oligosaccharide on NHK.

Analysis of oligosaccharide on NHK

Oligosaccharide structures on NHK were analyzed by HPLC as described previously (Hosokawa et al. 2003; Romero et al. 1985). Briefly, [3H]mannose-labeled NHK was blotted onto polyvinylidene difluoride (PVDF) membrane subsequent to immunoprecipitation and separation by SDS-PAGE. The N-glycans on NHK were then released by Endo N-glycanase H (Endo H) digestion and resolved by HPLC.

Preparation of Glc3Man9GlcNAc standards

[3H]mannose-labeled Glc3Man9GlcNAc was obtained from rat liver glycoproteins as described previously (Jelinek-Kelly et al. 2005) and purified by MicroSpin G-25 columns (GE Healthcare, Uppsala, Sweden) before use. The purified standards were used as reference for HPLC analysis.
HPLC on an aminospherisorb column. [14C]glucose-labeled mL bovine serum albumin (BSA), 10 mM CaCl2 and 1 mM NaN3 in 50 mM 2-(108 cells were harvested and lyophilized. 

**Analysis of N-glycans of total cellular glycoproteins**

Pyridylamination and structural identification of N-glycans from total cellular glycoproteins were performed as described previously (Sriwilaijaroen et al. 2009). Briefly, approximately 1 × 106 cells were harvested and lyophilized. N-glycans were released by hydrazinolysis from delipidated samples and subsequently re-N-acetylated and then fluorescence-labeled with 2-aminopyridine (Wako Pure Chemical Industries, Osaka, Japan). The pyridylamidated glycans (PA-glycans) were fractionated using a TSK-gel Amide-80 column (Tosoh, Osaka, Japan). The elution times of the individual peaks on the amide-silica and ODS columns were normalised with respect to the degree of polymerization of PA-isomalto-oligosaccharide and are represented in units of glucose. N-glycan structures were identified based on their elution positions on the two HPLC columns (above) compared to the elution positions of PA-glycans described in the literature (Tomiya et al. 1991).

**Western blotting**

Cells were extracted using a buffer containing 1% NP-40. Following separation by 10% SDS-PAGE, the proteins were transferred onto a nitrocellulose or a PVDF membrane as described previously (Hosokawa et al. 2008). Membranes were blocked with Blocking-One solution (Nacalai Tesque, Kyoto, Japan). HA-tag (Santa Cruz Biotechnology), EDEM1 (Sigma-Aldrich, Saint Louis, MO) and actin (CHEMICON, Temecula, CA) antibodies were diluted in Can Get Signal solution (TOYOBO, Osaka, Japan) or phosphate-buffered saline supplemented with 0.1% Tween-20 and 5% Blocking-One solution. Peroxidase-conjugated secondary antibodies were then applied, and the proteins were detected by enhanced chemiluminescence (GE Healthcare Biosciences).

**Funding**

This work was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (N.H., Y.K., I.W., K.K. and K.N.), Hayashi Memorial Foundation for Female Natural Scientists (N.H.) and by an operating grant from the Canadian Institutes of Health Research (A.H.).

**Acknowledgements**

This paper is dedicated to the memory of Professor Annette Herscovics. We thank K. Kanamori (Kyoto University) for technical assistance.

**Conflict of interest statement**

None declared.

**Abbreviations**

BSA, bovine serum albumin; DMEM, Dulbecco’s Modified Eagle’s medium; EDEM, ER degradation enhancing α-mannosidase-like protein; Endo H, Endo N-glycanase H; ER, endoplasmic reticulum; ERAD, ER-associated degradation; ER ManI, ER mannosidase I; HA, hemagglutinin; HPLC, high-performance liquid chromatography; NHK, α1-antitrypsin null (Hong Kong); ODS, octadecyl silica; PA, pyridylamidated; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

**References**


EDEM1 accelerates mannosamine trimming on C branch of N-glycans


