# *N*-glycan processing in ER quality control

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## Summary

**Glycosylation of asparagine residues in Asn-x-Ser/Thr motifs is a common covalent modification of proteins in the lumen of the endoplasmic reticulum (ER). By substantially contributing to the overall hydrophilicity of the polypeptide, pre-assembled core glycans inhibit possible aggregation caused by the inevitable exposure of hydrophobic patches on the as yet unstructured chains. Thereafter, *N*-glycans are modified by ER-resident enzymes glucosidase I (GI), glucosidase II (GII), UDP-glucose:glycoprotein glucosyltransferase (UGT) and mannosidase(s) and become functional appendices that determine the fate of the associated polypeptide. Recent work has improved our understanding of how the removal of terminal glucose residues from *N*-glycans allows newly synthesized proteins to access the calnexin chaperone system; how substrate retention in this specialized chaperone system is regulated by de-/re-glucosylation cycles catalyzed by GII and UGT1; and how acceleration of *N*-glycan dismantling upon induction of EDEM variants promotes ER-associated degradation (ERAD) under conditions of ER stress. In particular, characterization of cells lacking certain ER chaperones has revealed important new information on the mechanisms regulating protein folding and quality control. Tight regulation of *N*-glycan modifications is crucial to maintain protein quality control, to ensure the synthesis of functional polypeptides and to avoid constipation of the ER with folding-defective polypeptides.**

Key words: ER, *N*-glycans, Protein folding, Quality control, ER-associated degradation, calnexin, EDEM

## Introduction

The ER is the site of folding for proteins destined for compartments of the secretory and endocytic pathways (the ER, Golgi and endo-lysosomes), the plasma membrane and secretion. As nascent protein chains enter the ER lumen through the Sec61 translocon complex, their sequence is scanned by the luminal oligosaccharyltransferase (OST) for asparagines in consensus Asn-x-Ser/Thr motifs. These are modified covalently by the addition of a pre-assembled, tri-antennary core glycan composed of two *N*-acetylglucosamine, nine mannose and three glucose residues (Fig. 1A). *N*-glycans initially serve to increase the hydrophilicity of the as-yet-unstructured nascent polypeptides. Subsequently, but still co-translationally, *N*-glycosylated nascent chains enter a glycoprotein-dedicated chaperone system comprising calnexin, a type I ER membrane lectin, and calreticulin, its soluble relative (Ellgaard et al., 1999). Access to the calnexin/calreticulin system requires removal of the two outermost glucose residues displayed on branch A of the *N*-glycan by the sequential action of glycanases GI and GII. Whether there are further contacts, beyond the glycan, between glycoprotein substrates and calnexin/calreticulin is a matter of ongoing investigation (reviewed by Williams, 2006). GII-catalyzed removal of the third glucose residue follows the dissociation of folding substrates from calnexin and is required for release of native polypeptides from the ER and transport to their final destination. The folding sensor UGT1 adds back a terminal glucose to promote re-association of non-native polypeptides released from calnexin, thus prolonging their retention in the ER folding environment. Cycles of de-/re-glucosylation might be protracted until the polypeptide released from calnexin fulfils quality control requirements.

Folding-defective polypeptides are targeted by a system that progressively dismantles the *N*-glycan by sequentially removing terminal α1,2-linked mannose residues. Mannose removal initially reduces the activity of de- and re-glucosylating enzymes on *N*-glycans exposed by non-native proteins (Grinna and Robbins, 1980; Sousa et al., 1992). Cleavage of the terminal mannose on branch A, the only acceptor for the UGT1-catalyzed reglucosylation, results in the irreversible exclusion of the folding-defective polypeptide from the calnexin chaperone system. Hence this event interrupts futile folding attempts and prepares the terminally misfolded polypeptide for retrotranslocation into the cytosol and proteasome-operated disposal, a process known as ER-associated degradation (ERAD).

## Glycoprotein folding and quality control

### Entering the calnexin system

Sequential cleavage of the outermost α1,2-linked glucose residue by GI and of the first α1,3-linked glucose by GII generates the $Glc_1$-$Man_9$-$GlcNAc_2$ trimming intermediate that allows the polypeptide to associate with calnexin (and calreticulin) (Fig. 2, Step 1). The first de-glucosylation step catalyzed by GI (removal of glucose 1, Fig. 1A) occurs as soon as the core glycan has been transferred to the nascent chain by OST. Removal of glucose 2 by GII is slower and regulated (Daniels et al., 2003; Deprez et al., 2005; Wang et al., 2005). GII is an unusual glycosidase that comprises one catalytic subunit (α) and one mannose 6-phosphate receptor-like subunit

(β). According to a model recently proposed by Helenius and co-workers, the correct positioning of the first α1,3 glucose (glucose 2) in the active site of GII requires association of the GII β-subunit with the tetramannosyl branch of a second *N*-glycan on the polypeptide chain (trans-activation of GII trimming of the middle glucose) (Deprez et al., 2005). Therefore, the generation of the monoglucosylated calnexin ligand must await, at least in some cases, the correct positioning of a second *N*-glycan on the nascent chain or the engagement of a second *N*-glycosylated protein. Trimming of the second glucose is nevertheless accomplished before synthesis of the polypeptide chain is completed – i.e. in a matter of a few minutes. Hence, co-translational association of substrates with calnexin and calreticulin has been observed in microsomes and in living cells (Chen et al., 1995; Hebert et al., 1997; Molinari and Helenius, 2000).

## A folding cage

Calnexin and calreticulin each consist of a globular domain, which contains the sugar-binding site, and an extended arm (the P-domain), the tip of which weakly associates with ERp57, a member of the protein disulfide isomerase (PDI) superfamily (Frickel et al., 2002; Leach et al., 2002). Polypeptides emerging from the translocon complex enter a protected environment formed by the translocon, the OST subunits, the glucosidases, calnexin, ERp57 and other luminal factors that prevent the unstructured polypeptide interacting with other unstructured nascent chains. This substantially decreases the probability of forming aberrant homo- and hetero-oligomeric aggregates. Many proteins that enter the secretory pathway contain structure-stabilizing disulfide bonds. Formation of these is often the rate-limiting reaction in protein folding in the ER lumen and is catalyzed by members of the PDI family. The human ER lumen contains about 20 distinct PDI-family members (Ellgaard and Ruddock, 2005). We know little about their regulation, tissue-specific distribution, in vivo roles in protein biogenesis and/or turnover and any functional overlap with each other or ER-resident chaperones. However, the association of ERp57 with calnexin and calreticulin makes it the primary contender for the PDI that assists glycopolypeptide maturation (Molinari and Helenius, 1999; Oliver et al., 1997; Zapun et al., 1998). Importantly, ERp57 is the first oxidoreductase for which knockout mammalian cell lines have been reported (Garbi et al., 2006; Soldà et al., 2006) (see below).

## Insight from chaperone knockouts

Knocking out individual components of the calnexin chaperone system is paving the way for a deeper understanding of the mechanisms of protein folding and quality control in the mammalian ER. In recent years, murine fibroblasts lacking calreticulin (Mesaeli et al., 1999), calnexin (Denzel et al., 2002), UGT1 (Molinari et al., 2005) or ERp57 (Garbi et al., 2006; Soldà et al., 2006) have been reported. All of these knockouts result in embryonic lethality in mice, except for the calnexin knockout, which causes a severe and progressive pathology characterized by impaired growth and motor disorders that eventually lead to premature death (Denzel et al., 2002). By contrast, at the cellular level, all of these knockouts are at first sight well tolerated. Viability, proliferation, morphology and stress levels are indistinguishable between

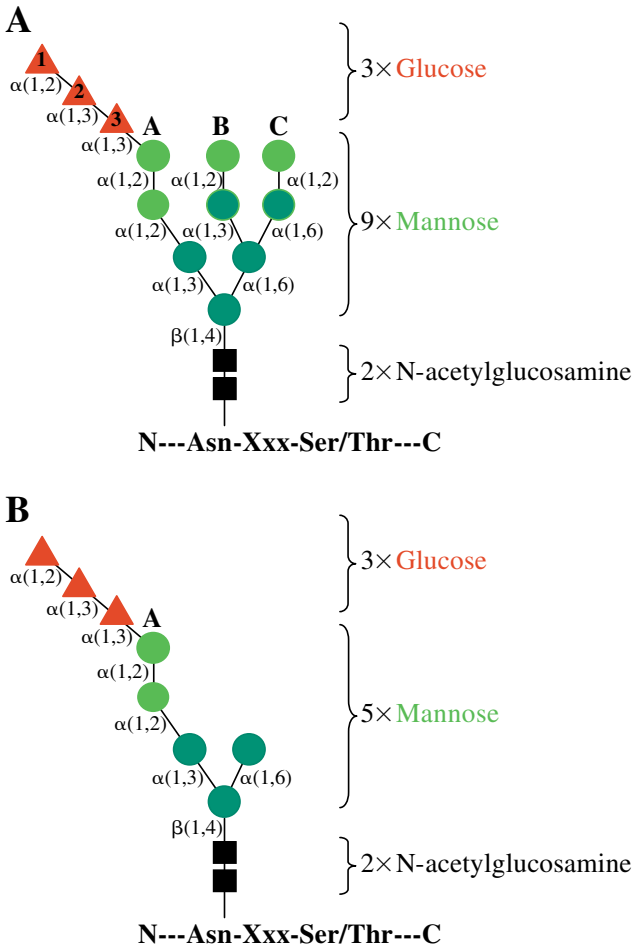


**Fig. 1.** Core glycans added on protein nascent chains. (A) The complete oligosaccharide is composed of two *N*-acetylglucosamine residues (black squares), nine mannose residues (green circles), and three glucose residues (red triangles). Linkages are shown and the three N-glycan branches are labeled A,B and C. (B) The absence of Dol-P-Man in the B3F7 cell line results in the transfer of a precursor structure missing four of the nine mannose residues but retaining the α1,2-mannose A.

wild-type and chaperone-deleted cells, which indicates that deletion of one member of the system does not cause large-scale protein misfolding. Although most polypeptides mature normally in cells lacking calnexin or calreticulin, there are at least two relevant exceptions: influenza virus hemagglutinin (HA) exhibits substantial folding defects when expressed in cells lacking calnexin (Molinari et al., 2004; Pieren et al., 2005); and MHC class I molecules show loading with sub-optimal peptides and premature release at the surface of calreticulin-deficient cells (Gao et al., 2002). Similarly, in ERp57-deleted cells, influenza HA (Soldà et al., 2006) and class I MHC molecules (Garbi et al., 2006) are the only model glycoproteins, among those analyzed so far, whose folding/export is significantly affected. Analyses of ERp57-deleted cells have led to the identification of at least one PDI family member, ERp72, which can act as a surrogate chaperone that catalyses intra- and inter-molecular disulfide bond formation when ERp57 is absent (Soldà et al., 2006). ERp72 shares the residues that in ERp57 are involved in association

with the calnexin/calreticulin P-domains (Ellgaard and Ruddock, 2005); however, in contrast to ERp57, ERp72 still associates with substrates following inhibition of their binding to calnexin (Soldà et al., 2006).

### Release from the calnexin chaperone system

Release from calnexin/calreticulin is followed by GII-catalysed cleavage of the innermost glucose residue from the *N*-glycan. The mammalian ER contains a folding sensor, UGT1, which checks the structure of the released polypeptides and adds back a glucose residue on the terminal α1,2-linked mannose residue on branch A of those polypeptides that have not yet attained their native conformation. There are conflicting data regarding a requirement for the proximity of the non-native region and the re-glucosylated *N*-glycan (Ritter et al., 2005; Taylor et al., 2004). In any case, re-glucosylation promotes re-association of the immature polypeptide with the ER lectins (Parodi, 2000). A re-glucosylation activity is absent in *Saccharomyces cerevisiae*. It is present, but dispensable for viability, in *Schizosaccharomyces pombe*, in which most polypeptides reach the native conformation in a single round of binding to calnexin (Fanchiotti et al., 1998). Less information is available about the requirement for mammalian glycoproteins to cycle in the calnexin chaperone system to acquire native conformation. At least two recombinant proteins, the T cell receptor α-subunit (Van Leeuwen and Kearse, 1997) and transferrin (Wada et al., 1997), are subjected to repeated, almost frenetic (the $t_{1/2}$ of the terminal glucose is <10 minutes)

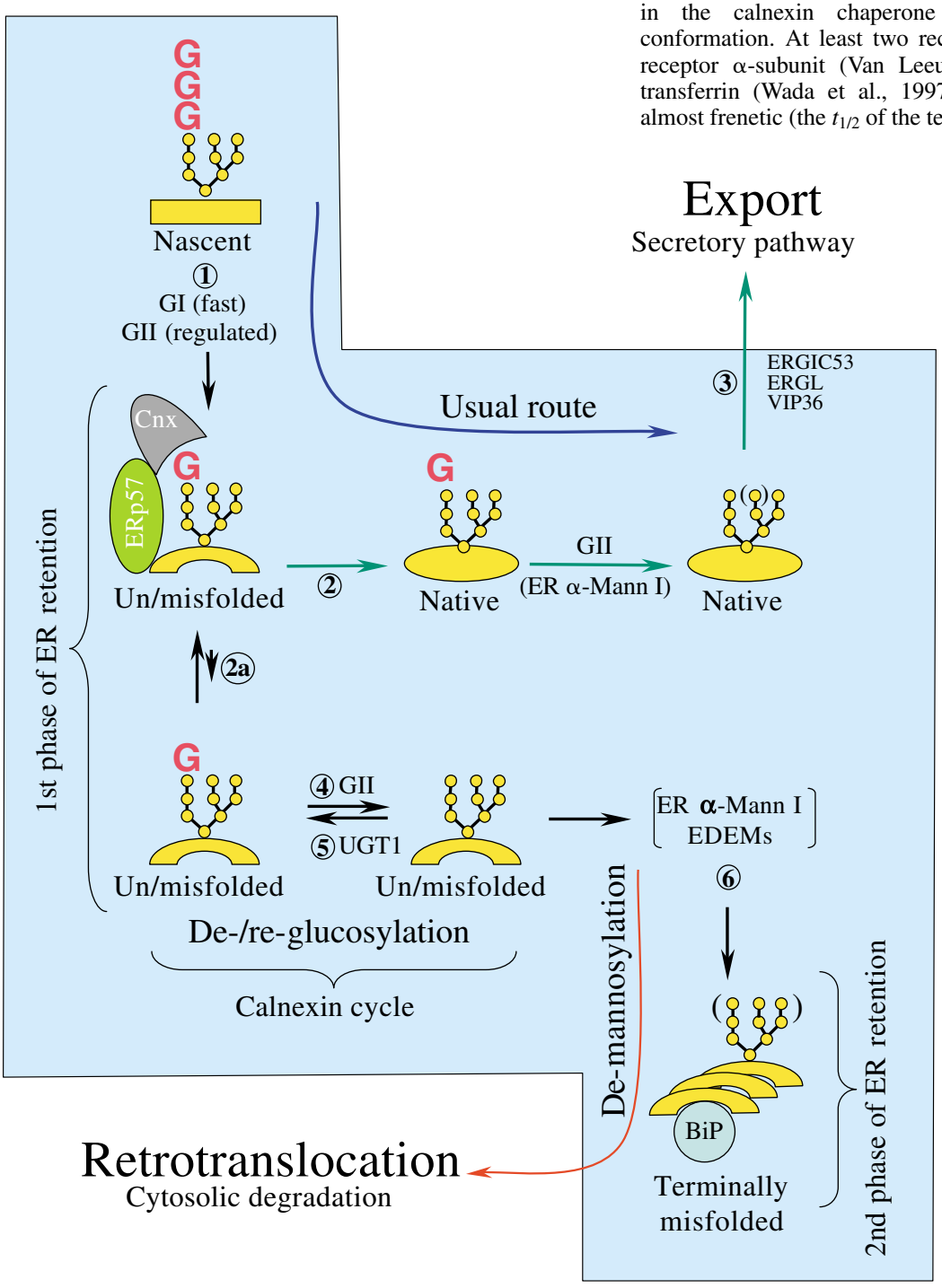


**Fig. 2.** Glycan processing determines the fate of the associated polypeptide chain. After addition of the pre-assembled oligosaccharide the two outermost glucoses are removed (step 1) and the nascent polypeptide associates with calnexin/calreticulin (Cnx) and the oxidoreductase ERp57. Most glycopolypeptides are probably released as native proteins (step 2) and exit the ER (step 3). Folding-defective polypeptides enter cycles of dissociation/re-association (steps 2a, 4 and 5) with Cnx. Folding attempts are eventually interrupted upon extensive substrate de-mannosylation and terminally misfolded polypeptides are retrotranslocated into the cytosol and degraded (step 6).

GII/UGT1-catalyzed, de-/re-glucosylation cycles during maturation. However, as in *S. pombe*, the majority of newly synthesized mammalian glycoproteins seems to acquire a transport-competent structure in a single round of association with the ER lectins. Hence, UGT1 deletion does not substantially accelerate the overall kinetics of polypeptide release from calnexin and the ER (Molinari et al., 2005) (T. Soldà, R. J. Kaufman and M.M., unpublished results).

Deletion of UGT1 does not result in a reduction in ER quality control, thus revealing that this enzyme is not the gatekeeper that prevents non-native polypeptides exiting the ER. Instead UGT1 delays the hand off of folding-defective polypeptides from the calnexin system to the systems that regulate the retrotranslocation of misfolded polypeptides into the cytosol for proteasome-mediated disposal (Molinari et al., 2005) (Fig. 2, step 6). Surprisingly, differences in the release of terminally misfolded substrates from calnexin in wild-type and UGT1-deficient cells emerge only upon persistent protein misfolding, 60 minutes after association of the substrate with calnexin. Thus, release from calnexin (steps 2 and 2a in Fig. 2) might initially be very slow and polypeptides are likely to explore conformations while trapped in the folding cage formed by calnexin and ERp57 (Molinari et al., 2005).

## ERAD: disposal of folding-defective glycopolypeptides

### Signalling folding incompetence

Newly synthesized polypeptides are eventually released from the ER either as native molecules to be transported to their site of action along the secretory pathway or as terminally misfolded polypeptides retrotranslocated into the cytosol for disposal. A crucial part of the quality control processes in the ER is therefore the recognition of terminally misfolded chains, their extraction from cycles of futile folding attempts, their unfolding and transport into the cytosol for proteasome-mediated degradation (Ellgaard et al., 1999; Meusser et al., 2005).

The finding that inhibition of mannose removal from *N*-glycans protects folding-defective polypeptides from ERAD (Su et al., 1993) led to the concept of the 'mannose-timer' (Helenius, 1994). According to this model, removal of mannose residues from *N*-glycans terminates the maturation phase and initiates a series of events, still incompletely characterized, that eventually lead to retrotranslocation of the terminally misfolded polypeptide into the cytosol. By contrast, protection from de-mannosylation prolongs the lag phase that precedes the onset of degradation of the misfolded glycoprotein by retarding release of the substrate from the calnexin chaperone system (de Virgilio et al., 1999; Molinari et al., 2002; Wu et al., 2003).

Numerous studies with inhibitors in mammalian cells revealed the active involvement of kifunensine-sensitive α1,2-mannosidase(s) in ERAD regulation (Cabral et al., 2001; Hebert et al., 2005; Helenius and Aebi, 2004; Herscovics, 2001; Lederkremer and Glickman, 2005). Other work has shown that *N*-glycans on folding-defective polypeptides are extensively processed to $Man_{5\text{-}6}$ configurations by the removal of α1,2-linked mannoses (light-green circles in Fig. 1A) (Ermonval et al., 2001; Foulquier et al., 2004; Foulquier et al., 2002; Frenkel et al., 2003; Hosokawa et al., 2003; Kitzmuller et al., 2003). Moreover, removal of an α1,2-linked mannose is required for degradation of misfolded proteins expressed in mutant cell lines [e.g. B3F7 and MadIA214 (Ermonval et al., 2001)] in which the only cleavable residue is the terminal mannose on chain A (Fig. 1B). Removal of this mannose residue, the only acceptor for UGT-catalyzed protein re-glucosylation, thus seems to be a potent signal for glycoprotein disposal in mammalian cells because it results in the irreversible interruption of futile folding attempts in the calnexin cycle. Which protein(s) perform the extensive mannose trimming observed during preparation of the folding-defective polypeptides for proteasomal disposal is still a matter of debate and extensive research. At least three possibilities exist: (1) co-sequestration of ERAD candidates and ER α-mannosidase I in specialized sub-regions of the ER (Frenkel et al., 2003) in which the mannosidase concentration reaches levels similar to those shown in vitro to cause extensive mannose removal (Herscovics et al., 2002; Wu et al., 2003); (2) intervention of Golgi endo-mannosidase(s); (3) intervention of a new class of recently characterized mannosidase-like proteins, EDEM1, EDEM2 and EDEM3 (Hirao et al., 2006; Mast et al., 2005; Molinari et al., 2003; Oda et al., 2003; Olivari et al., 2005; Olivari et al., 2006).

### The EDEM family

EDEM1 (for ER degradation enhancing α-mannosidase-like protein) was first described as a mannosidase relative containing a 450-residue domain that shares 35% sequence identity with the catalytic domain of ER α-mannosidase (Fig. 3A). EDEM1 overexpression accelerates disposal of model glycoproteins expressed in mammalian cells (Hosokawa et al., 2001) by accelerating the exit of polypeptides undergoing futile folding attempts from the calnexin chaperone system (Molinari et al., 2003; Oda et al., 2003). EDEM1 was initially described as a type 2 membrane protein that is embedded in the ER membrane by its uncleaved signal sequence, which also serves to form a complex with calnexin (Oda et al., 2003). This model has been challenged by data showing that, in most cell lines, the signal sequence is efficiently cleaved, releasing the mature EDEM1 protein into the ER lumen (Olivari et al., 2005). Recently, two relatives, EDEM2 and EDEM3, have been cloned and characterized (Fig. 3A) (Hirao et al., 2006; Mast et al., 2005; Olivari et al., 2005).

EDEM1, EDEM2 and EDEM3 are major targets of the ER-stress-induced Ire1/Xbp1 pathway (Olivari et al., 2005). Mammalian cells use this pathway to increase their capacity for ERAD, which is easily saturated (Travers et al., 2000), in response to an increase in cargo load and/or accumulation of misfolded polypeptides (Olivari et al., 2005; Yoshida et al., 2003; Yoshida et al., 2006). RNA interference directed against EDEM proteins (Gong et al., 2005; Molinari et al., 2003) and inactivation of the Ire1/Xbp1 pathway that regulates their intralumenal level both result in the sub-optimal disposal of misfolded glycoproteins (Yoshida et al., 2003) and eventually prevent protein folding and reduce secretory capacity (Eriksson et al., 2004), thus revealing important cross-talk between the folding and ERAD machineries. However, how upregulation of EDEM proteins actually facilitates the degradation of folding-defective glycopolypeptides remains unclear.

### Are EDEM proteins active α1,2 mannosidases?

Although EDEM1 is a mannosidase-like protein, it was

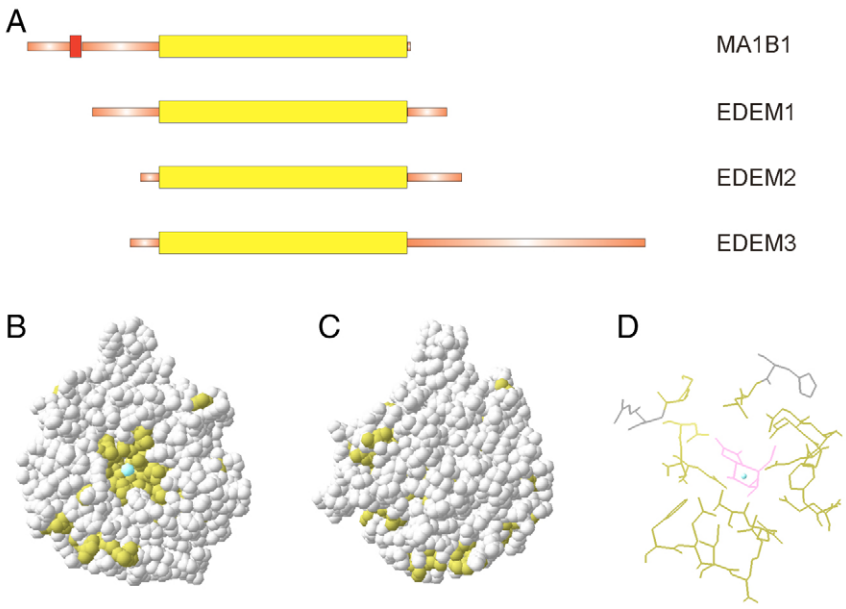


**Fig. 3.** Similarities between EDEMs and ER α-mannosidase I. (A) EDEM1, EDEM2, EDEM 3 and ER α-mannosidase I (MA1B1) share sequence similarity within a region containing the $(\alpha\alpha)_7$ barrel catalytic domain of class I mannosidases (family 47GH), shown in yellow. The N- and C-regions of the EDEM proteins beyond this domain share no similarity with MA1B1 or significant sequence similarity with each other. MA1B1 contains a transmembrane region, shown in red, which the EDEM proteins lack. The N-terminus of MA1B1 is cytosolic. (B) Sequence conservation between EDEM proteins and MA1B1. Residues highlighted in yellow on the structure of MA1B1 [based on Vallee et al. (Vallee et al., 2000)] are conserved; note the clustering of conserved residues around the active site. The calcium atom seen in the active site is light blue. (C) Back view of the protein in B. (D) Sequence conservation around the active site of the EDEM proteins and MA1B1. Residues within 6Å of kifunensine (pink) in the structure of MA1B1 with kifunensine bound [based on Vallee et al. (Vallee et al., 2000)] are shown. Residues highlighted in yellow are conserved; two residues whose side chains do not point towards kifunensine are not conserved (grey). The calcium ion is in light blue.

originally described as a putative lectin rather than an active mannosidase because it lacks a specific disulfide bond conserved in mannosidases (Hosokawa et al., 2001). Accumulating evidence now shows that EDEM proteins could actually be active mannosidases. First, the disulfide absent in the EDEM proteins is not conserved among all mannosidases and is dispensable for glycanase activity. Second, sequence similarity between EDEM proteins and ER α-mannosidase I is, significantly, restricted to the region containing the $(\alpha\alpha)_7$ barrel catalytic domain of class I mannosidases. Despite the low level of sequence identity (35%), all catalytic residues are conserved and structure modelling indicates no difference in their location (Hosokawa et al., 2001; Karaveg and Moremen, 2005; Karaveg et al., 2005; Tempel et al., 2004) (Fig. 3B). Residues involved in the binding of kifunensine, a specific inhibitor of α1,2 mannosidases, are also conserved (Fig. 3D). Third, elevation of the intralumenal level of EDEM1 (Olivari et al., 2006) and EDEM3 (Hirao et al., 2006) substantially accelerates de-mannosylation of folding-defective polypeptides. Acceleration of mannose removal upon upregulation of EDEM1 also occurs in B3F7 cells, which are characterized by addition of aberrant oligosaccharides lacking branch B and C to nascent polypeptide chains (Fig. 1B). Thus, EDEM1 (and possibly EDEM2 and EDEM3) can remove the terminal branch A mannose, the only cleavable terminal mannose present in glycoproteins in this cell line and the only saccharide that can be reglucosylated by UGT to prolong retention of folding-defective polypeptides in the calnexin cycle (Olivari et al., 2006).

## EDEM proteins as molecular chaperones

Recent evidence suggests that EDEM1 also accelerates ERAD by preventing the formation of disulfide-bonded dimers (Hosokawa et al., 2006) or covalent aggregates (Olivari et al., 2006) containing terminally misfolded glycoproteins released from calnexin. This chaperone-like activity is not linked to the capacity of EDEM to accelerate substrate de-mannosylation, because enzymatically inactive mutants of EDEM1 can still perform this function (Olivari et al., 2006).

## From the ER lumen into the cytosol

Despite extensive studies, the molecular machinery and the translocation channel(s) used to retrotranslocate terminally misfolded polypeptides into the cytosol remain incompletely characterized. However, several pathways for the degradation of terminally misfolded ER proteins have been identified in screens of yeast strains lacking steps of the ERAD process (Wolf and Schafer, 2005). In yeast, specific machineries are involved in the detection of structural protein defects. The ERAD-L machinery detects luminal defects, the ERAD-C detects cytosolic defects and the ERAD-M detects structural defects in the transmembrane regions of newly synthesized polypeptides (Ahner and Brodsky, 2004; Denic et al., 2006; Ismail and Ng, 2006; Vashist and Ng, 2004). Some of the components of these pathways (e.g. Kar2p and PDI) have orthologues in higher eukaryotes. Others – for example, the ER lectin Yos9p, which belongs to a multimeric complex involved in the recognition of proteins with luminal defects – do not yet have functional equivalents (reviewed by Ismail and Ng, 2006).

The mechanisms of ERAD in metazoans are less well characterized but certainly more complex than those of unicellular eukaryotes. In mammalian cells, a ‘hand off’ of folding-defective polypeptides from the calnexin/calreticulin system to the BiP chaperone system has been shown to exist (Cabral et al., 2002; Molinari et al., 2002; Molinari et al., 2005). The rate of the transfer of ERAD candidates from one chaperone system to another is regulated by sugar-processing events that are absent in yeast, such as substrate re-glucosylation that prolongs retention in the protective

calnexin/ERp57 folding cage and extensive de-mannosylation. In addition, the association of PDI with the misfolded polypeptide before retrotranslocation into the cytosol has been shown (Molinari et al., 2002). This has been interpreted as an involvement of this ER oxidoreductase in the reduction of disulfide bonds, which is required for retrotranslocation of ERAD candidates (Fagioli and Sitia, 2001; Tortorella et al., 1998) and of subunits of bacterial toxin, which co-opt the same route (Gillece et al., 1999; Tsai et al., 2001). The precise composition of the ER channel(s) used to retrotranslocate terminally misfolded polypeptides in the cytosol is still unknown. Retrotranslocation has long been known to involve Sec61, a proteinaceous channel also utilized by proteins entering co-translationally into the ER (Pilon et al., 1997; Plemper et al., 1997; Zhou and Schekman, 1999), even though exceptions are known (Huyer et al., 2004). Additional proteins termed derlins have recently been implicated (Lilley and Ploegh, 2004; Ye et al., 2004) and these associate with the EDEM proteins (Oda et al., 2006). The mechanisms connecting retrotranslocation and proteasome-mediated degradation are unclear, but many substrates accumulate in the ER lumen when proteasome activity is blocked, which indicates that retrotranslocation and degradation are coupled reactions (Mancini et al., 2000; Mayer et al., 1998; Molinari et al., 2002).

## Conclusions and Perspectives

The cellular machinery regulating protein folding and quality control in the ER is complex; it needs to be. The machinery must welcome a wide variety of growing nascent chains emerging in the ER lumen, assist in their folding and eventually extract terminally misfolded proteins for degradation without prematurely interrupting folding attempts of proteins that have not completed the folding program.

Over the past few years a number of significant advances have been made in our understanding of this field. Amongst the most relevant are certainly those that shed light on the important regulatory role of the polypeptide-associated oligosaccharides. Several recent reviews cover the roles of *N*-glycans and ER or intermediate compartment lectins in export of native proteins from the ER (Hauri et al., 2002; Hebert et al., 2005; Schrag et al., 2003) and that of cytosolic lectins in directing misfolded proteins to the cytosolic proteasome (Hebert et al., 2005; Yoshida, 2003). Here we have focused on the important role of *N*-glycans, ER-resident lectins and *N*-glycan-processing enzymes in protein quality control in the mammalian ER.

The glycoprotein-dedicated chaperone system is used by many, if not all, newly synthesized cellular glycoproteins. It is also exploited for maturation of glycosylated viral gene products expressed in virus-infected cells. The machinery is highly redundant, and surrogate chaperones and enzymes can be used by nascent chains. Indeed, deletion of calnexin (Denzel et al., 2002), calreticulin (Mesaeli et al., 1999), ERp57 (Garbi et al., 2006; Soldà et al., 2006) or UGT1 (Molinari et al., 2005) produces very little phenotypic effect in cell lines and only very few model substrates that dramatically suffer from individual deletions of members of this folding machinery have been identified. However, the devastating consequences of lack of these proteins in the corresponding murine models and the lethal outcome of an inherited glucosidase I deficiency show the importance of the glycoprotein-dedicated machinery for embryogenesis and/or homeostasis of specific tissues and organs (De Praeter et al., 2000; Pieren et al., 2005).

Despite the highly sophisticated chaperone machineries that assist protein biogenesis in the mammalian ER, a certain percentage of newly synthesized proteins [up to 30% in some studies (Schubert et al., 2000)] never acquires a native, transport-competent structure. In addition, mutations or changes in biophysical conditions can dramatically lower the protein folding efficiency; hence rapid disposal of aberrant products is crucial to maintain ER homeostasis. The consequences of the accumulation and deposition of misfolded proteins – for example, in human ER-storage diseases – are cell death, tissue damage and organ failure (Aridor and Hannan, 2000).

A range of tools are available to dissect the mechanisms of ER homeostasis – for example, viral products that elicit rapid degradation of class I MHC molecules, bacterial toxins that co-opt the retrotranslocation machinery to enter the host cell cytosol, model ERAD substrates to be expressed at high levels in the cell line of interest, organisms such as *S. cerevisiae* that are easy to manipulate genetically and that share a simplified version of the machineries and mechanisms operating in metazoans, and inhibitors of specific enzymatic pathways. Every experimental approach offers advantages and drawbacks, and caution must be used in any attempt to generalize the findings. For example, the disposal of class I MHC molecules triggered by cytomegalovirus gene products is an exceptionally fast process. It by-passes the requirement for mannose removal from the *N*-glycans on the ERAD substrate and causes accumulation of the protein to be degraded in the cytosol upon inhibition of the proteasome, whereas for most ERAD substrates retrotranslocation from the ER into the cytosol is coupled to degradation (Mancini et al., 2000; Mayer et al., 1998; Molinari and Sitia, 2005). In addition, one must be cautious about generalizing from data obtained from a single species or a single model substrate. Many components of the ER quality control machinery appear to be conserved, but several functions (e.g. the cycling in the calnexin chaperone system or the extensive de-mannosylation that ERAD substrates are subjected to) are only present in higher eukaryotes, and other more subtle differences may exist. Furthermore, similar caution must be exercised with the use of inhibitors (most of which have not been comprehensively tested for specificity), temperature shifts (which will have effects on many different processes) and conclusions drawn about normal physiological pathways on the basis of knockout and knock-down studies given the potential functional redundancy in the system. However, there is no doubt that the comparative quantitative study of all these model systems can be combined to give a deep understanding of the pathways that work over the range of conditions under which each cell must operate.

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