Processing of N-linked glycans during endoplasmic-reticulum-associated degradation of a short-lived variant of ribophorin I

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Recently, the role of N-linked glycans in the process of ERAD (endoplasmic reticulum-associated degradation) of proteins has been widely recognized. In the present study, we attempted to delineate further the sequence of events leading from a fully glycosylated soluble protein to its deglycosylated form. Degradation intermediates of a truncated form of ribophorin I, namely RI_{332}, which contains a single N-linked oligosaccharide and is a substrate for the ERAD/ubiquitin-proteasome pathway, were characterized in HeLa cells under conditions blocking proteasomal degradation. The action of a deoxymannojirimycin- and kifunensine-sensitive α1,2-mannosidase was shown here to be required for both further glycan processing and progression of RI_{332} in the ERAD pathway. In a first step, the Man_8 isomer B, generated by ER mannosidase I, appears to be the major oligomannoside structure associated with RI_{332} intermediates. Some other trimmed N-glycan species, in particular $Glc_1Man_7GlcNAc_2$, were also found on the protein, indicating that several mannosidases might be implicated in the initial trimming of the oligomannoside. Secondly, another intermediate of degradation of RI_{332} accumulated after proteasome inhibition. We demonstrated that this completely deglycosylated form arose from the action of an N-glycanase closely linked to the ER membrane. Indeed, the deglycosylated form of the protein remained membrane-associated, while being accessible from the cytoplasm to ubiquitinating enzymes and to added protease. Our results indicate that deglycosylation of a soluble ERAD substrate glycoprotein occurs in at least two distinct steps and is coupled with the retro-translocation of the protein preceding its proteasomal degradation.

Key words: α1,2-mannosidase, endoplasmic reticulum-associated degradation (ERAD), N-glycan, oligomannoside structure, peptide N-glycosidase (PNGase), ubiquitin proteasome.

INTRODUCTION

In recent years, the ER (endoplasmic reticulum) has been recognized as a quality-control compartment, where newly synthesized polypeptides delivered by co- and/or post-translational translocation undergo a sequence of biochemical modifications monitored by a number of chaperones, enzymes and folding factors [1–3]. In essence, only proteins that have attained a properly folded state are competent to leave the ER for further progression through the secretory pathway. Proteins that have remained partially folded or assembled and those with misfolded conformations are recognized as aberrant and, in most cases, are targeted for ERAD (ER-associated degradation). In this process, the proteins are retro-translocated to the cytoplasm and subjected to degradation by the ubiquitin–proteasome system [4,5]. For glycoproteins, the N-linked oligosaccharides have been implicated in quality control and ERAD in various ways [2,6–8]. One important feature of the N-glycan is to confer to glycoproteins the capacity for temporary and regulated retention in the ER, through transient binding of the monoglucosylated form of the N-glycan to the ER lectins calnexin and/or calreticulin. A cycle of binding to and release from these chaperones is determined by the sequential action of UDP-glucose-glycoprotein glucosyltransferase and glucosidase II, which adds and removes a terminal glucose from the oligomannose [2,9,10]. This mechanism is believed to support the folding of newly synthesized glycoproteins. Additionally, in the case where the protein does not acquire its native structure, the ER α1,2-mannosidase I has been proposed to act as a timer for the exit of the substrate glycoprotein from the calnexin cycle by producing a Man_8 oligomannoside [11–13]. This N-linked glycan structure is considered to be implicated in the binding to a Man_8-specific ER lectin, an interaction that may play a role in the delivery of the glycoprotein to the degradative pathway, both in yeast and in mammals [14–18].

This well-recognized role of ER mannosidase I in generating a Man_8 structure that functions as a signal for ERAD often relies on the observation that different ERAD substrates are stabilized in cells treated with specific α1,2-mannosidase inhibitors. However, in most cases, the precise N-glycan structures adopted by the glycoprotein to be degraded were not determined. Remarkably, such a Man_8 structure also occurs on properly folded glycoproteins that leave the ER, and other mannosidases from ER or post-ER compartments could account for ERAD pathways [19–22]. For instance, the endomannosidase which cleaves the terminal mannose from the linear branch of the N-glycan only when it is glucosylated [19] could contribute to the release of incompletely folded glycoproteins from the calnexin cycle. Specific inhibitors of α1,2-mannosidases could inactivate enzymes with different fine specificities such as the ER mannosidases I and II and the Golgi mannosidase I. Accordingly, for the stabilization of the H2a and H2b subunits of the asialoglycoprotein receptor by α1,2-mannosidase inhibitors, it has been proposed that a $Man_6GlcNAc_2$

Abbreviations used: CHO, Chinese-hamster ovary; ConA, concanavalin A; CST, castanospermine; dMJ, deoxymannojirimycin; endo H, endoglycosidase H; ER, endoplasmic reticulum; ERAD, ER-associated degradation; HA, haemagglutinin; IEF, isoelectric focusing; Kif, kifunensine; NEM, *N*-ethylmaleimide; Ni-NTA, Ni^{2+}-nitrilotriacetate; PNGase F, peptide N-glycosidase F; RI, ribophorin I.

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structure could serve as a degradation signal [20]. ER mannosidase II has also been described to act in the ERAD of an α1-antitrypsin variant, but through a proteasome-independent pathway [21]. Finally, our previous work using a glycosylation-defective cell line that synthesizes a truncated N-glycan lacking the mannose linkage specifically recognized by the ER mannosidase I showed stabilization of an ERAD substrate in the presence of α1,2-mannosidase inhibitors. We then developed a procedure that allowed us to describe, for the first time, the composition of the N-glycan associated with a glycoprotein during its degradation. This analysis revealed the implication of mannosidases with specificities similar to those of the Golgi mannosidase in this glycosylation-defective cell line [22].

In view of these different observations, it was of importance to clarify the involvement of different enzymes implicated in N-glycan processing during ERAD of a glycoprotein in cells with a wild-type glycosylation phenotype. Therefore in the present study, we analysed in HeLa cells the N-glycan structures associated with degradation intermediates of a model substrate, RI_{332}, which contains one N-linked glycan. RI_{332} is a soluble truncated form of the rough ER-specific type I transmembrane glycoprotein RI (ribophorin I) which is rapidly degraded in CHO (Chinese-hamster ovary) and HeLa cells by the classical ERAD pathway dependent on the ubiquitin–proteasome system [23,24]. In addition, our previous results obtained with a temperature-sensitive CHO-cell mutant deficient in ubiquitination have suggested that processing intermediates produced during RI_{332} degradation could originate in the lumen of the ER [23]. We show here by electrophoretic analysis that, when proteasomal degradation is inhibited in HeLa cells, two distinct intermediates of RI_{332} accumulated in comparable amounts, one being glycosylated and the other unglycosylated. N-glycan analysis revealed that the glycosylated intermediate contains the Man_8 isomer B structure, expected to be formed by the action of the ER α1,2-mannosidase I. In addition, significant levels of other trimmed N-glycan species were also detected that might be generated by other mannosidases. We also demonstrate that the second intermediate in the degradation pathway that corresponds to a deglycosylated form of the protein was produced by the complete removal of the N-linked glycan, thus establishing the role of an N-glycanase activity in this process.

EXPERIMENTAL

Reagents

The pQE-30 His_6-tag expression vector, the *Escherichia coli* strain M15 [pREP4] for bacterial expression of His_6-tagged proteins and Ni-NTA (Ni^{2+}-nitrilotriacetate) Sepharose CL-6B beads were from Qiagen (Hilden, Germany). The T/A cloning vector pCR2.1 was purchased from Invitrogen (Groningen, The Netherlands). Oligonucleotides were from VBC Genomics (Vienna, Austria). Minimal essential medium, methionine-free RPMI 1640 and other cell culture reagents were obtained from Gibco BRL/Invitrogen Life Technologies (Paisley, Renfrewshire, Scotland, U.K.). Aprotinin, acetyl-L-leucyl-L-leucyl-L-argininal (leupeptin), L-leucyl-L-leucyl-L-leucine, and PMSF protease inhibitors, bovine pancreatic trypsin and NEM (*N*-ethylmaleimide) were from Sigma (Deisenhofen, Germany), and ZLLL and ZLL-Nva were from Peptides International (Louisville, KY, U.S.A.). Endo H (endoglycosidase H), PNGase F (peptide N-glycosidase F) and restriction enzymes were purchased from Roche Diagnostics (Mannheim, Germany). CST (castanospermine), dMJ (deoxymannojirimycin) and Kif (kifunensine) were from Toronto Research Chemicals (Toronto, Canada). Tran^{35}S-label metabolic labelling reagent containing [^{35}S]methionine and [^{35}S]cysteine (40.7 TBq/mmol) and aqueous [2-^{3}H]D-mannose (777 GBq/mmol) were obtained from ICN (Irvine, CA, U.S.A.). Protein A–Sepharose CL-4B beads and ConA (concanavalin A)–Sepharose 4B beads were from Amersham Biosciences (Uppsala, Sweden). [2-^{3}H]D-mannose (884.3 GBq/mmol) for the HPLC analysis of N-glycans and En^{3}Hance solution were purchased from NEN^{TM} Life Science Products (Boston, MA, U.S.A.). Ampholyte solutions were from Fluka (Buchs, Switzerland). X-Omat Blue XB-1 and BioMax MR X-ray films were purchased from Eastman Kodak (Rochester, NY, U.S.A.).

Antibodies

To prepare the rabbit anti-RI-lum antiserum, the cDNA corresponding to the luminal domain of mature rat RI was amplified by a standard PCR using the pWS-RI construct [24] cut with *Sac*I as template and the primers AAACGGATCCTCTTCGGAGGCTCCGCCGCTGG and AGGGGGTACCAGGCTCCTGCAGCATGAGCACC. At the same time, *Bam*HI and *Kpn*I restriction sites were introduced so as to facilitate the cloning of the DNA fragment obtained, after a T/A cloning step utilizing the pCR2.1 vector, into the pQE-30 vector, using the *Bam*HI and *Kpn*I sites. The resulting polypeptide that contains a His_6 tag at its C-terminus was produced in *E. coli* M15 [pREP4] cells and purified by affinity chromatography on Ni-NTA Sepharose CL-6B beads under denaturing conditions (8 M urea). The purified polypeptide was used to immunize a rabbit as described in [22].

The anti-RI-lum antibody works on SDS-denatured cell extracts under stringent conditions (0.6 % SDS and 1 % Triton X-100) [23]. The polyclonal rabbit anti-RI-LB antibody was described previously [22].

Cell lines

The H3-2 and H3*-8 clones were obtained by transfecting HeLa cells with the cDNAs encoding the glycosylated RI_{332} and the non-glycosylated RI_{332}-Thr soluble variants of RI respectively as already described [24]. HeLa transfectants were grown in 5 % CO_2 at 37 °C and in minimal essential medium supplemented with 7 % (v/v) fetal calf serum.

Treatment of cells with drugs, cell labelling, immunoprecipitations and sequential precipitations

The transfected HeLa-RI_{332} (H3-2) and HeLa-RI_{332}-Thr (H3*-8) cells were plated at a density of 7×10^5 in a 35 mm dish and grown overnight. Cells were preincubated at 37 °C with 1 mM CST, 2 μg/ml Kif, 2 mM dMJ or 50 μM ZLLL for 1 h in complete medium and subsequently for 30 min in methionine- and serum-free medium. Then the cells were pulse-labelled for 30 min with 500 μCi/ml of Tran^{35}S-label radioactive [^{35}S]Met–[^{35}S]Cys amino acid mixture and chased for the indicated periods of time in the continuous presence or absence of the drugs.

For immunoprecipitations with the anti-RI-lum antibody, the cells were lysed with 2 % (w/v) SDS, and the lysates were processed for immunoprecipitation under stringent conditions [0.6 % SDS and 1 % (v/v) Triton X-100] [23]. Alternatively, the cells were lysed with 1 % Triton X-100 in NET buffer (150 mM NaCl/50 mM Tris/HCl, pH 7.4/5 mM EDTA), supplemented with protease inhibitors (1.7 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml L-leucyl-L-leucyl-L-leucine and 5 mM PMSF), which was subsequently adjusted to 0.6 % SDS. Specific immunoprecipitations were performed overnight with 40 μl of Protein A–Sepharose and 5 μl of anti-RI-lum antiserum. After three washes with

SDS wash buffer (95 mM NaCl/25 mM Tris/HCl, pH 7.4/3 mM EDTA/1.25 % Triton X-100/0.2 % SDS/protease inhibitors as above) and two with PBS, the immunoprecipitates were eluted with reducing sample buffer containing 3 % (v/v) 2-mercaptoethanol. Where indicated, immunoprecipitates were treated with endo H as described previously [25]. For treatment with PNGase F, immunoprecipitates were washed with deionized water and incubated with 1 % (v/v) Nonidet P40, 50 mM sodium phosphate (pH 7.5) and 3 μl of PNGase F at 37 °C for 4 h. Samples were analysed by SDS/PAGE (10 % gels), followed by fluorography.

For sequential precipitations with ConA lectin and anti-RI antibodies, the cell lysates obtained from cells treated with 1 % Triton X-100 in NET buffer, were initially incubated with 40 μl of ConA–Sepharose beads overnight. Subsequently, adsorbed proteins were released by boiling the beads twice in 150 μl of SDS lysis buffer (95 mM NaCl/25 mM Tris/HCl, pH 7.4/3 mM EDTA/1 % SDS/protease inhibitors) and diluted with 1 ml SDS wash buffer. The following immunoprecipitations with anti-RI-lum were performed as above.

Cell fractionation, protease protection and ubiquitination analysis of ConA-separated material

Analyses of cell fractions, protease protection using trypsin and ubiquitination were performed as described previously [23].

Briefly, H3-2 cells labelled with Tran^{35}S-label radioactive [^{35}S]Met–[^{35}S]Cys amino acid mixture treated with or without ZLLL were lysed by Dounce homogenization in iso-osmotic buffer. Membrane and soluble fractions were separated by ultracentrifugation at 100 000 ***g*** and then RI_{332} was immunoprecipitated with anti-RI-lum antiserum and processed for SDS/PAGE analysis. Samples tested for protease protection were incubated with 10 μg/ml trypsin for 30 min at 30 °C; then protease inhibitors were added before immunoprecipitation. Ubiquitination of RI_{332} was evaluated on cell lysates prepared in the presence of 0.2 % SDS and 5 mM NEM. Ubiquitinated material was also analysed after separation of glycosylated and non-glycosylated RI_{332} by selective adsorption on ConA. Samples were then subjected to immunoprecipitations using anti-RI-lum antiserum, all in the presence of NEM, and analysed by SDS/PAGE and fluorography.

Isoelectric focusing (IEF)

For IEF analyses, immunoprecipitates were additionally washed twice with deionized water and taken up in 20 μl IEF sample buffer (9.5 M urea/2 % Triton X-100/0.8 % ampholytes, pH 3–10/5 % 2-mercaptoethanol). IEF gel electrophoresis under denaturing conditions was performed in slab gels [9.2 M urea/2 % Triton X-100/4 % (w/v) acrylamide/0.22 % bisacrylamide/4 % (v/v) ampholytes, pH 5–7/1 % ampholytes, pH 3–10/0.02 % ammonium persulphate/0.1 % *N*,*N*,*N*′,*N*′-tetramethylethylenediamine] with 20 mM H_3PO_4 as anode buffer. To all wells of the gel, 5 μl of overlay buffer (IEF sample buffer, diluted 1:3 and supplemented with 0.01 % Bromophenol Blue) was added and then 20 mM NaOH, which was also used as a cathode buffer. After the separation of the proteins at 10 mA for 14 h, the gels were fixed [30 % (v/v) methanol and 10 % (v/v) acetic acid] for 30 min, soaked in En^{3}Hance solution for 10 min, washed with water for 10 min, dried and used for fluorography.

HPLC analysis of oligosaccharide structures

Procedures to prepare oligomannosides bound to total glycoproteins [26] or to a soluble variant of RI during its degradation [22] have already been described. In the present study, approx. 10^7 H3-2 cells starved for 30 min in a medium with low glucose concentration (0.5 mM) were thereafter metabolically labelled with 100 μCi of [2-^{3}H]D-mannose for 45 min in glucose-free medium. After a 10 min chase in complete medium, total proteins were extracted with organic solvent as described by Kmiécik et al. [27]. N-glycans were released from protein by PNGase F, and then analysed by HPLC on an ASAHIPAK-NH2-P-50 column (Asahi, Kawasaki-ku, Japan) after elution in an acetonitrile gradient at a flow rate of 1 ml/min and monitored with a Flo-one β detector (Flotec, France).

To determine the composition of the N-glycans specifically associated with RI_{332}, mannose-labelled glycoproteins were purified on Protein A–Sepharose beads coupled with anti-RI-LB antiserum. N-glycans were released directly from such purified RI_{332} by treating the immunoprecipitated sample with PNGase F before HPLC analysis, as described in [22]. To study oligomannosides bound to RI_{332} shortly after synthesis, Triton X-100 extracts were prepared from H3-2 cells labelled in the same way as described for total glycoproteins, except that 300 μCi of [2-^{3}H]D-mannose was used. To study the N-glycan structures linked to RI_{332} during its degradation, H3-2 cells were pretreated for 1 h with 40 μM ZLL-Nva proteasome inhibitor and the drug was maintained in each subsequent step, namely 30 min starvation, 45 min mannose labelling and the 4 h chase. Under these conditions, intermediates of degradation of RI_{332} are known to accumulate. HPLC analysis of N-glycans released from RI_{332} was then performed as described above.

Oligomannoside derivatization and HPLC analysis to determine Man_8 isomer

The major N-glycan species associated with RI_{332} during its degradation were separated and purified by TLC. N-glycans were prepared from RI_{332} immunoprecipitates of extracts from radiolabelled H3-2 cells treated and chased for 4 h with ZLL-Nva as described above for HPLC analysis. The cell lysate was precleared with Protein A–Sepharose beads coupled with non-immune serum before specific immunoprecipitation with anti-RI-LB antiserum. The oligomannosides were released by endo H treatment of total proteins or of immunoprecipitated RI_{332}, and then analysed by TLC. Samples were loaded on to a silica-coated layer and separated in n-propanol/acetic acid/water (3:3:2) solvent for 24 h before visualization by fluorography as described previously [28].

The material corresponding to the $Man_8GlcNAc_1$ spot was recovered from the TLC plate by water extraction. These oligomannosides were derivatized with 2-aminopyridine as described by Hase et al. [29], and then desalted on a Biogel-P2 column. The derivatized oligosaccharides and standards (5×10^3–10^4 c.p.m.) were injected on to a Hypersil ODS2 column (Supelco, Bellefonte, PA, U.S.A.; 250 mm × 4.5 mm) for analysis by HPLC. The column was eluted isocratically with 5 mM ammonium acetate (pH 4.0) at a flow rate of 0.5 ml/min using a Waters solvent delivery 600 system coupled with a Packard 150 TR flow scintillation analyzer. The isomers were identified by comparison with the elution time of known standardized material.

RESULTS

Degradation of the truncated RI_{332} is mannosidase and proteasome dependent in HeLa cells

To study the relationships between N-glycosylation and glycoprotein degradation, we used as a model the truncated RI variant, RI_{332}, an ERAD substrate that requires the ubiquitin–proteasome

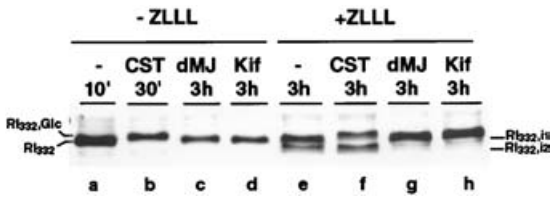

Figure 1 Dependence of the initial processing of RI_{332} and its degradation on the activity of an α1,2-mannosidase, but not on that of a glucosidase

[^{35}S]Methionine and [^{35}S]cysteine metabolically labelled H3-2 cell cultures were left untreated (lane a) or were treated with 1 mM of the glucosidase inhibitor CST (lanes b and f) or with either 2 mM dMJ (lanes c and g) or 2 μg/ml Kif (lanes d and h) to inhibit α1,2-mannosidases. These incubations were performed in the absence (lanes a–d) or presence (lanes e–h) of ZLLL (50 μM) as an inhibitor of proteasomal degradation. Samples treated with drugs stabilizing RI_{332} were chased for 3 h (lanes c–h). When RI_{332} is rapidly degraded, the cells were chased for a short time of 10 min (lane a) or 30 min (lane b). Cells were lysed with SDS-containing buffer and processed for immunoprecipitation with the anti-RI-lum antiserum. The immunoprecipitates were analysed by SDS/PAGE (10 % gel) followed by fluorography. RI_{332},Glc indicates a slower migrating form of RI_{332}, which is generated in the presence of CST and is expected to bear three glucose residues.

system for its degradation [22–24]. Intermediates in the degradation pathway were characterized in the [^{35}S]Met/Cys-labelled H3-2 clone, a HeLa cell transformant expressing RI_{332} that was treated with the proteasome inhibitor ZLLL and/or inhibitors of N-glycan processing. Anti-RI immunoprecipitates were then analysed by SDS/PAGE (Figure 1). RI_{332} is rapidly degraded in untreated cells with a half-life of approx. 1 h, as described previously [23], and is detected as a unique band with an apparent molecular mass of approx. 40 kDa after 10 min chase (Figure 1, lane a). In contrast, two intermediates of degradation, $RI_{332}i_1$ and $RI_{332}i_2$, accumulate when cells are chased for 3 h in the presence of ZLLL (Figure 1, cf. lane e with lane a). The upper intermediate, $RI_{332}i_1$, is essentially indistinguishable in its migration on SDS gels from the RI_{332} protein observed in untreated cells. The lower one, $RI_{332}i_2$, appears to be approx. 2 kDa smaller and results from the processing of the single N-linked oligosaccharide on RI_{332} [23]. The $RI_{332}i_2$ intermediate was also obtained when the cells were treated with the glucosidase inhibitor CST in addition to ZLLL (lane f). This indicates that glucosidase activity is not required for the formation of this intermediate. It is noticeable that in CST-treated cells, the presence of the three glucose residues on the N-linked glycan leads to a small electrophoretic shift of both the glycosylated form of RI_{332} without ZLLL and the glycosylated $RI_{332}i_1$ intermediate accumulating in the presence of the proteasome inhibitor (RI_{332},Glc; lanes b and f).

Next, to explore the requirement of an α1,2-mannosidase activity for ERAD of RI_{332}, H3-2 cells were treated with two specific α1,2-mannosidase inhibitors, dMJ and Kif, for 3 h. The inhibition of α1,2-mannosidase activity resulted in the stabilization of RI_{332} (Figure 1, lanes c and d), consistent with earlier observations [24]. Interestingly, the formation of the faster migrating $RI_{332}i_2$ intermediate, detected only in the presence of proteasome inhibitors, was essentially prevented when the cells were treated with either dMJ or Kif in addition to ZLLL (lanes g and h). These results demonstrate that an α1,2-mannosidase activity is a prerequisite for the processing on RI_{332}, so as to generate the intermediate $RI_{332}i_2$.

These findings together with the prevailing view that a Man_8 structure of the N-linked oligosaccharide is critical on glycoproteins destined for ERAD [11–13,30,31] prompted us to analyse the N-glycan structures present on RI_{332}.

The major N-glycans associated with the degradation of RI_{332} in H3-2 cells are isomer B of $Man_8GlcNAc_2$ and $Glc_1Man_7GlcNAc_2$

To understand better the processing of the N-glycan associated with the intermediates of degradation of RI_{332} accumulating in H3-2 cells in the presence of proteasome inhibitors (ZLL-Nva), the oligomannoside composition was determined by HPLC. After mannose labelling, oligomannosides were released by PNGase F treatment, either from newly synthesized total glycoproteins or from specifically immunoprecipitated RI_{332} at 10 min chase, and then compared with the N-glycans accumulating on RI_{332} when degradation is blocked for a 4 h chase in the presence of ZLL-Nva (Figure 2A). Whereas two RI_{332} degradation intermediates accumulated in equal amounts, as seen on SDS/PAGE (Figure 1, lane e) in H3-2 cells treated with ZLLL, only one prevalent N-glycan moiety (and not two species in equal amounts) eluting as a Man_8 derivative, was detected by HPLC (Figure 2A, black bars). In comparison, the major N-glycan species associated with the newly synthesized proteins at 10 min chase (either total glycoproteins or RI_{332}; Figure 2(A), white and grey bars respectively) were $Man_9GlcNAc_2$ moieties. These results show that the $RI_{332}i_1$ intermediate observed after ^{35}S-labelling, in the presence of proteasome inhibitors, contains mainly the Man_8 derivative, whereas the lower $RI_{332}i_2$ intermediate presumably does not possess N-glycans.

In addition to the accumulation of Man_8 derivatives accounting for 58 % of all the oligomannoside species linked to RI_{332} degradation intermediates, our HPLC analysis also revealed, even after 10 min chase, the presence of significant amounts of shorter N-glycans that are not detected on total glycoproteins (Figure 2A, cf. grey and white bars). The quantity of these trimmed N-glycan structures slightly increased on RI_{332} accumulating in H3-2 cells chased for 4 h in the presence of the proteasome inhibitor and reached a level of 12 % for Man_7, 12 % for Man_6 and 7 % for $Man_5GlcNAc_2$ (black bars).

The Man_8 structure found associated with proteins during their degradation is considered to be derived from the action of the ER α1,2-mannosidase I [11–13]. The present finding of trimmed oligomannoside structures (Man_7 to Man_5) on RI_{332} during its degradation (Figure 2A) and our previous observations in a glycosylation-defective cell line [22], point to the possible role of other α1,2-mannosidase activities in this process. According to the different specificities of α1,2-mannosidases described so far, three Man_8 isomers could indeed occur. The ER mannosidase I that cleaves the middle branched terminal mannose produces an isomer B of Man_8, whereas the ER mannosidase II specific for the terminal mannose of the α1,6-branched antennae generates an isomer C [32]. Also, the endomannosidase specific for the terminal α1,2-mannose linkage of the linear part of the high mannose structure, when it is glucosylated, could lead to the formation of Man_8 isomer A [19]. Golgi mannosidase I was also reported to have a high affinity for this terminal mannose [33,34]. Therefore we next determined the isomeric structure of the major oligomannoside moiety associated with RI_{332} degradation intermediates obtained after 4 h of chase in the presence of the proteasome inhibitor. For isomer determination, the Man_8-containing structures were extracted from TLC plates (see Figure 2B), derivatized with 2-aminopyridine, and then analysed by reversed-phase HPLC (see Figure 2C). TLC analysis of endo H-released glycans revealed that the oligosaccharide material corresponding to the migration profile of standard $Man_8GlcNAc_1$ did not appear homogeneous, suggesting the presence of different Man_8 isomers or glucosylated Man_7 species. Radioactive material migrating at the level of Man_8 (indicated by a bar in Figure 2B, lane 3) was eluted from the TLC plate and derivatized. As shown in

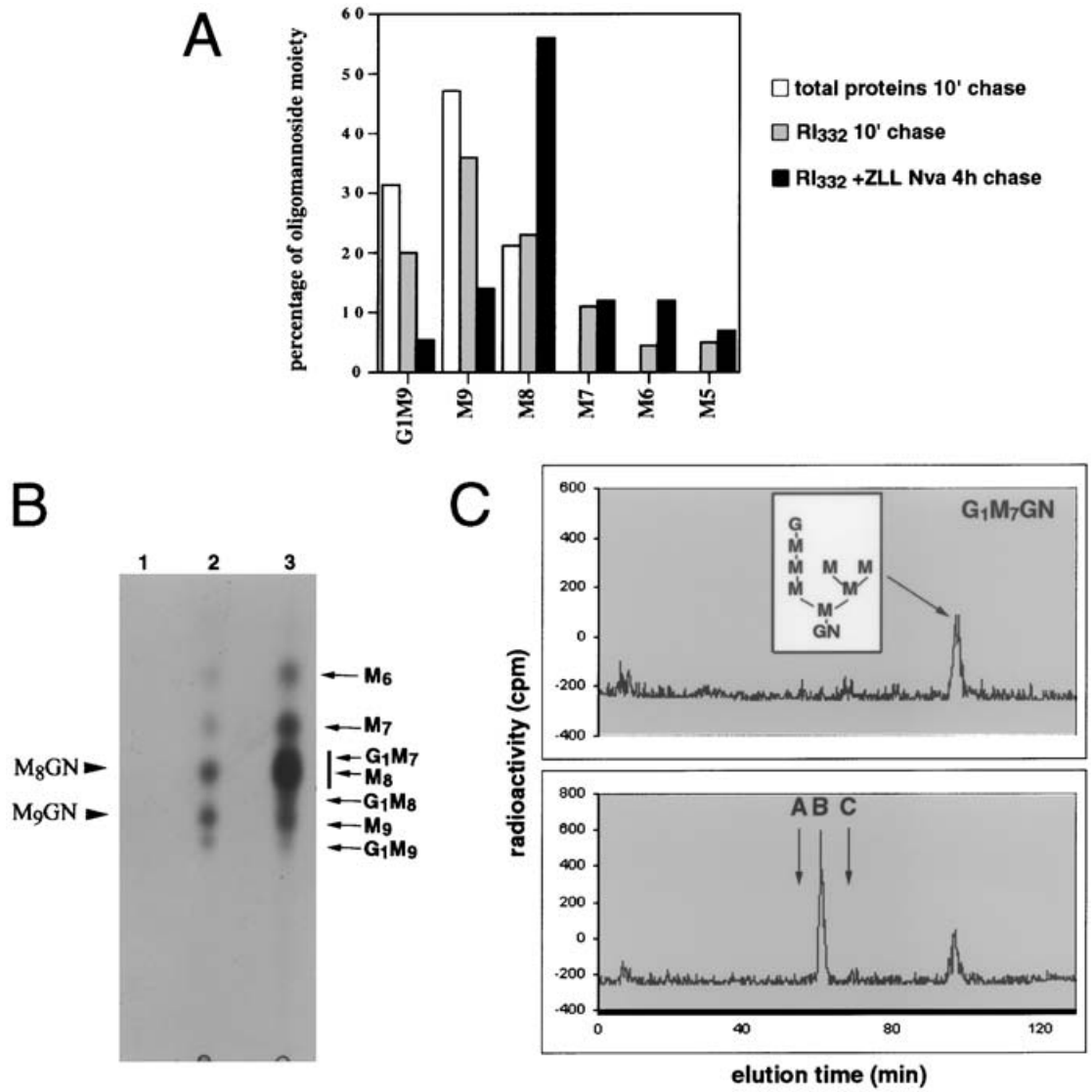

Figure 2 Presence of $Man_8GlcNAc_2$ isomer B and $Glc_1Man_7GlcNAc_2$ in the degradation intermediates of RI_{332} accumulating in H3-2 cells treated with a proteasome inhibitor

(**A**) HPLC profiles of the N-glycans associated with RI_{332} during its degradation in H3-2 cells are illustrated. The cells were pulse-labelled with [2-^{3}H]D-mannose for 45 min and then chased for 10 min. Oligomannosides were released by PNGase F treatment either from total proteins (white bars) or from RI_{332} obtained by immunoprecipitation with the anti-RI-LB antibody (grey bars). To accumulate RI_{332} degradation intermediates, H3-2 cells were also treated with the proteasome inhibitor ZLL-Nva for a 4 h chase period. Oligomannosides were then released from the immunoprecipitated RI_{332} intermediates (black bars). The histograms represent the percentages of the indicated oligomannoside species with a $GlcNAc_2$ at their reducing end: G1M9, Glc_1Man_9; M9, Man_9; M8, Man_8; M7, Man_7; M6, Man_6; M5, Man_5. The percentages were calculated from the amount of radioactivity in each peak monitored by HPLC corrected according to the number of mannose residues. (**B**) TLC analysis of oligomannosides released by endo H treatment from either total proteins after 10 min chase (lane 2) or from immunoprecipitated RI_{332} stabilized by a 4 h treatment with ZLL-Nva (lane 3), prepared as in (**A**). In control to lane 3, no material was released from a preclear of the same cell lysate with non-immune serum (lane 1). The mobility of M_9GN ($Man_9GlcNAc_1$) and M_8GN ($Man_8GlcNAc_1$), used as standards, is indicated by arrowheads on the left side. The extrapolated positions of the different oligomannoside species in the sample are indicated on the right side of the TLC and correspond to moieties with one GlcNAc at their reducing end. The bar indicates the region from which the oligosaccharides, obtained from RI_{332} when its degradation is inhibited, were eluted and taken for further analysis by reversed-phase HPLC after derivatization with 2-aminopyridine (shown in **C**, lower panel). The elution positions of standard aminopyridinylated Man_8 oligosaccharides (isomers A–C) are indicated. The G_1M_7GN ($Glc_1Man_7GlcNAc_1$) structure (upper panel) was generated by aminopyridinylation of the *A. Saito* mannosidase digestion product of $Glc_1Man_7GlcNAc_1$.

Figure 2(C), two compounds were observed by HPLC analysis (lower panel): the predominant species co-migrated with $Man_8GlcNAc_1$ isomer B and the less abundant compound, representing approx. 35% of the analysed material, was found to co-migrate with a $Glc_1Man_7GlcNAc_1$ standard (upper panel). It is to be noticed that monoglucosylated Man_9 and Man_8 derivatives are also detected in the TLC analysis (Figure 2B, lane 3).

Altogether, these observations add to the complexity of the N-glycan processing relating to glycoprotein degradation of a particular ERAD substrate, which may involve the participation of different mannosidases.

The lower intermediate of RI_{332} does not contain mannose and is the product of an N-glycanase activity

We were then interested in elucidating the nature of the faster migrating intermediate, $RI_{332}i_2$, only observed after proteasome

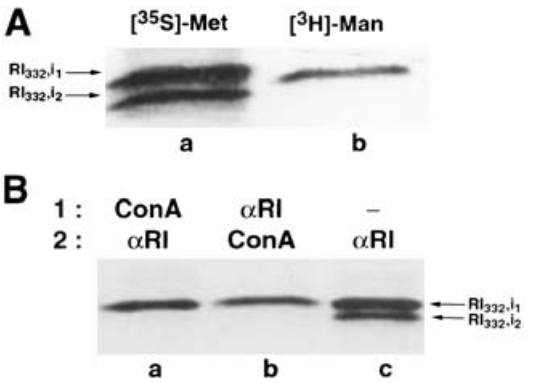

Figure 3 Absence of mannose in the processing intermediate $RI_{332}i_2$ observed in the presence of ZLLL

(**A**) H3-2 cells treated with ZLLL were metabolically labelled either with [^{35}S]Met/Cys (lane a) for 30 min or with [2-^{3}H]D-mannose (lane b) for 45 min, followed by chase incubations for 4 h. Cell lysates were then subjected to immunoprecipitation with anti-RI-lum antiserum under stringent conditions. Both samples were analysed by SDS/PAGE (10 % gel) and fluorography. (**B**) H3-2 cells were treated with ZLLL and metabolically labelled with [^{35}S]Met/Cys for 30 min, followed by a 3 h chase incubation. Cells were then lysed in a Triton X-100 containing buffer, and the lysates were subjected to sequential precipitations first with ConA–Sepharose beads followed by anti-RI-lum/Protein A–Sepharose beads or vice versa. The first precipitates were eluted from the ConA or anti-RI-lum-coupled beads with a buffer containing 2 % SDS and used for a second precipitation under stringent conditions with anti-RI-lum/Protein A–Sepharose (lane a) or ConA–Sepharose (lane b) respectively. An immunoprecipitation of the sample using only anti-RI-lum is shown as a control (lane c). All samples were analysed by SDS/PAGE (10 % gel) and fluorography.

inhibition by SDS/PAGE analysis. We first tested whether [2-^{3}H]D-mannose could be incorporated into this intermediate. As shown in Figure 3(A), [2-^{3}H]D-mannose is incorporated only into the upper band but not into the faster migrating intermediate ($RI_{332}i_2$), whereas both bands are visible to a similar extent after [^{35}S]methionine incorporation (cf. lane b with lane a). In another experimental approach, extracts from ZLLL-treated [^{35}S]methionine-labelled cells were subjected to sequential precipitations with either ConA, the mannose-reactive lectin, and then the anti-RI antibodies or vice versa. In either sequence, only the $RI_{332}i_1$ band is recovered but not the lower RI_{332} intermediate (Figure 3B, lanes a and b) that is readily observed by direct anti-RI immunoprecipitation (lane c). These results indicate that the $RI_{332}i_1$ intermediate constitutes a glycosylated form of the protein interacting with ConA, whereas the $RI_{332}i_2$ intermediate does not contain mannose.

Furthermore, to understand how the $RI_{332}i_2$ intermediate might be generated, anti-RI immunoprecipitates of lysates prepared from ZLLL-treated and -untreated cells were subjected to digestions with endo H and PNGase F before SDS-gel analysis. As shown in Figure 4, after endo H treatment, RI_{332} is converted into a deglycosylated form (cf. lane d with lane a) with a mobility slightly faster than the $RI_{332}i_2$ intermediate from ZLLL-treated cells (lane b). This is well illustrated when RI_{332} immunoprecipitated from ZLLL-treated cells is digested with endo H (cf. lane b with lane c). In this case, the $RI_{332}i_2$ band is maintained while the glycosylated $RI_{332}i_1$ intermediate shifts to a position slightly lower than the $RI_{332}i_2$. This is consistent with the observation that *N*-acetylglucosamine (GlcNAc) left on asparagine sites after endo H treatment was not detected on the $RI_{332}i_2$ intermediate, since the latter does not bind to wheatgerm agglutinin and is resistant to *N*-acetylglucosaminidase digestion (results not shown). Therefore $RI_{332}i_2$ is not the product of an endoglycanase.

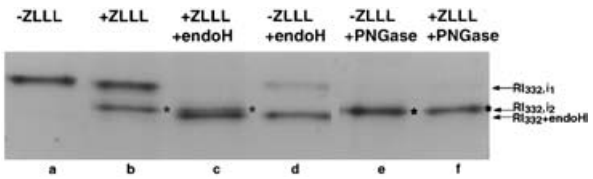

Figure 4 Identical electrophoretic mobility value for the processing intermediate $RI_{332}i_2$ observed in the presence of ZLLL and the PNGase F-digested material

H3-2 cells metabolically labelled for 30 min with [^{35}S]Met/Cys were either left untreated (− ZLLL: lanes a, d and e) or were treated with ZLLL (+ ZLLL: lanes b, c and f), and then chased for 10 min (untreated samples) or 3 h (ZLLL-treated samples). Cell lysates were subjected to immunoprecipitation with anti-RI-lum antiserum. The immunoprecipitates were mock-digested (lanes a and b), digested with endo H (lanes c and d) or with PNGase F (lanes e and f) before analysis by SDS/PAGE. The deglycosylated form of RI_{332} obtained after a PNGase F treatment is indicated by a ⋆ and also the $RI_{332}i_2$ intermediate, which migrates at the same position.

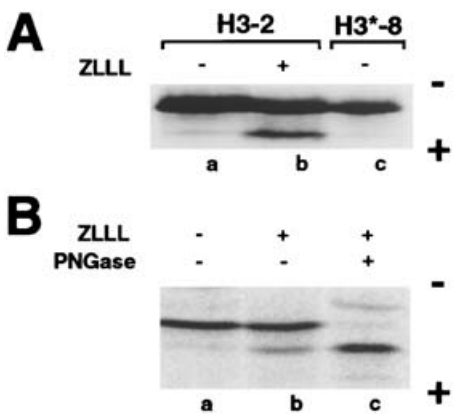

Figure 5 The more acidic nature of the deglycosylated intermediate $RI_{332}i_2$ compared with the fully glycosylated and the non-glycosylated forms of RI_{332}

Variation in the pIs of RI_{332} degradation intermediates labelled and immunoprecipitated in the same way as in the previous Figures was evaluated by IEF. The cathode and anode are indicated by − and + respectively, on the right. (**A**) RI_{332} from H3-2 cells left untreated (lane a) or treated with ZLLL (lane b), as well as the non-glycosylated RI_{332}-Thr variant of RI expressed in H3*-8 cells (lane c), are compared. (**B**) IEF analysis of RI_{332} from untreated H3-2 cells (lane a) or cells treated with ZLLL (lane b), as compared with the PNGase F digestion product of the degradation intermediates from ZLLL-treated cells (lane c).

By contrast, PNGase F treatment of RI_{332} samples from both ZLLL-treated and -untreated cells, led to a unique band harbouring the same electrophoretic mobility when compared with $RI_{332}i_2$. This observation led us to speculate that $RI_{332}i_2$ could be generated by the action of an N-glycanase-like activity. Such an enzyme is known to cleave the N-linked glycan from the asparagine residue to which it is attached and by doing so generate an aspartic acid. This modification changes the pI of the protein. For the mature RI_{332} protein, we obtained a pI of 6.07. For the corresponding form in which one asparagine residue was converted into aspartic acid, a pI of 5.94 is expected. IEF experiments were therefore performed using anti-RI immunoprecipitates prepared from different H3-2 cell lysates treated with or without with PNGase F (Figure 5). An additional band corresponding to a more acidic protein was observed only in cells treated with ZLLL (Figure 5A, lane b). This band migrates more towards the anode when compared with the other form also recovered from untreated cells (lanes a and b). The non-glycosylated variant of RI_{332}, where the asparagine residue of the N-glycosylation sequon had been replaced by a threonine (RI_{332}-Thr) and expressed in HeLa cells (H3*-8), was used as control. As

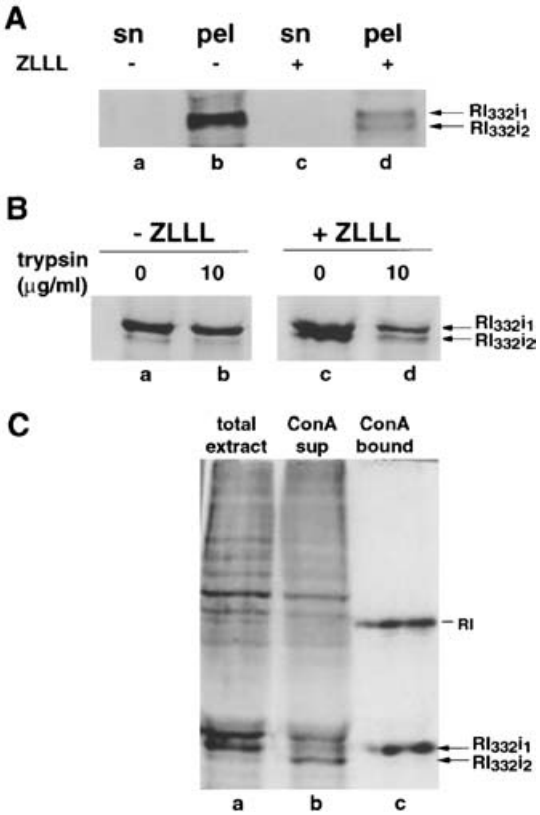

Figure 6 Membrane-associated intermediates of degradation of RI_{332}, including protease-sensitive and ubiquitinated deglycosylated forms

Anti-RI-lum immunoprecipitates from [^{35}S]Met/Cys metabolically labelled H3-2 cells were prepared from untreated (− ZLLL) and ZLLL-treated (+ ZLLL) cells and analysed by SDS/PAGE (10 % gel), as described in the previous Figures. (**A**) Untreated (lanes a and b) and ZLLL-treated cells (lanes c and d) were fractionated. The cells were lysed in iso-osmotic buffer by Dounce homogenization. Equal amounts of proteins from membrane (pel) and soluble (sn) fractions were separated by ultracentrifugation at 100 000 ***g*** before anti-RI-lum immunoprecipitation. (**B**) Untreated and ZLLL-treated H3-2 cells were lysed in an iso-osmotic buffer by Dounce homogenization, and total membrane fractions were either kept as untreated controls (lanes a and c) or incubated with 10 μg/ml trypsin (lanes b and d) for 30 min at 30 °C. (**C**) Microsomes from ZLLL-treated H3-2 cells were lysed in the presence of 0.2 % SDS and 5 mM NEM, and one sample was used directly for immunoprecipitation with anti-RI-lum antiserum (lane a). Another sample was precipitated with ConA–Sepharose, and the supernatant (lane b) and the material bound to and released from the ConA–Sepharose beads (lane c) were then subjected to immunoprecipitations using anti-RI-lum antiserum, all in the presence of NEM to preserve ubiquitinated forms. The additional prominent band in lane c corresponds to the endogenous RI.

expected, this protein showed the same mobility in the IEF gel as its glycosylated counterpart (lane c). Figure 5(B) shows that the more acidic form of RI_{332}, identified as the $RI_{332}i_2$ intermediate in ZLLL-treated cells, corresponds to the one obtained after PNGase F digestion of RI_{332}. Taken together, our results demonstrate that the $RI_{332}i_2$ intermediate constitutes a deglycosylated form of RI_{332} produced by the action of an N-glycanase.

The smaller degradation intermediate of RI_{332} is associated with the ER, en route to the cytosol

Finally, we investigated the intracellular localization of the N-glycanase activity detected during RI_{332} degradation. A cell fractionation assay revealed that both the glycosylated intermediates $RI_{332}i_1$ and the deglycosylated product $RI_{332}i_2$ in ZLLL-treated H3-2 cells (Figure 6A, lanes c and d), as well as the glycosylated form of RI_{332} in untreated cells (lanes a and b), are associated almost exclusively with microsomal membranes. This is in agreement with similar results previously obtained in the mutant ts20 CHO cell line [23]. Figure 6(B) indicates that, after digestion of the microsomes with trypsin, most of the deglycosylated intermediate, $RI_{332}i_2$, is sensitive to the protease, whereas the glycosylated form remains resistant (lanes a and b). Furthermore, a ConA separation of N-glycosylated proteins from those devoid of oligomannosides was performed. Total or ConA-separated proteins from the microsomal fraction were precipitated with anti-RI antibodies in the presence of NEM to prevent de-ubiquitination of proteins. Under these conditions, a number of high-molecular-mass bands were visible in the sample prepared from total microsomes, typical for poly-ubiquitinated proteins (Figure 6C, lane a). In this sample, the band corresponding to glycosylated RI_{332} is easily discernable. In contrast, the deglycosylated $RI_{332}i_2$ form is almost undetectable suggesting that this form may be ubiquitinated to a large extent and shifted to high-molecular-mass bands. This is confirmed by results from the ConA fractionation. Indeed, the high-molecular-mass forms are present only in the supernatant of the ConA-treated sample, enriched in deglycosylated $RI_{332}i_2$ (lane b), but not in the pellet from the ConA-retained material, where only the glycosylated form of the protein is found (lane c). This demonstrates that only the deglycosylated form of RI_{332} is ubiquitinated. Endogenous, glycosylated RI also appears to be enriched in the ConA-retained fraction.

In summary, these results provide evidence that deglycosylation of RI_{332} occurs when the protein is still associated with the microsomes. However, it is not clear whether the implicated N-glycanase exerts its activity on the luminal or cytoplasmic face of the ER.

DISCUSSION

The importance of N-linked glycan structures, in particular the isomer B of $Man_8GlcNAc_2$, as intermediates and regulatory devices in the pathway of ERAD has been widely recognized [2,3,9,35,36]. However, the precise N-glycan structure targeting proteins to ERAD and the mechanism leading to dislocation of glycoproteins from the ER to the cytosol for proteasomal degradation, remain open questions. In an attempt to understand better the requirement for N-glycans in the process of glycoprotein degradation, we analysed the processing of the single N-linked oligosaccharide on the ERAD substrate RI_{332}, a soluble truncated form of RI, during the course of its disposal. Normally, endogenous full-length RI, a long-lived ER-resident glycoprotein, possesses predominantly a fully mannosylated oligosaccharide chain. In fact, pulse–chase studies showed that $Man_9GlcNAc_2$ is the major structure on RI up until 7 h of chase [25]. In the present study, we show that an N-glycan corresponding to $Man_8GlcNAc_2$ isomer B, but interestingly also other shorter species, $Glc_1Man_7GlcNAc_2$ $Man_6GlcNAc_2$ and $Man_5GlcNAc_2$, are associated with RI_{332} during its degradation. Then, in a second step, deglycosylation of the RI_{332} ERAD substrate by an N-glycanase occurs in a mannosidase-dependent fashion and is tightly coupled with retro-translocation of the glycoprotein, preceding proteasomal degradation.

The first main conclusion drawn from our findings is the requirement of the action of a dMJ- and Kif-sensitive mannosidase for the degradation of RI_{332}. This is in agreement with previous observations by others and by us [12,13,24]. Interestingly, we found that both complete deglycosylation and degradation of RI_{332} do not occur if this mannosidase activity is compromised.

Based on genetic evidence in yeast [11,16,37] and on numerous studies with inhibitors in mammalian cells [6,12,13,24,38], it was concluded that ER mannosidase I is the central player in this process. However, most of the conclusions on the role of ER-mannosidase I were drawn from indirect observations such that different ERAD substrates accumulated in the presence of specific α1,2-mannosidase inhibitors. It is probable that degradative pathways involving other α1,2-mannosidases may also contribute to generating different N-glycan structures [21]. Supporting this view, our previous determination of the N-glycan structures associated with an HA (haemagglutinin)-epitope-tagged form of RI_{332} (RI_{332}-3HA) during its degradation in the glycosylation-defective CHO cell line, MadIA214, has demonstrated a requirement of an α1,2-mannosidase activity different from that of the ER-mannosidase I [22]. Indeed, in this cell line, truncated $Man_5GlcNAc_2$ are added to proteins, but this oligomannoside is not expected to be a substrate for the highly specific ER mannosidase I [33]. The simple N-glycan structure found in the glycosylation mutant cell line allowed us to speculate that the monoglucosylated N-linked $Man_5GlcNAc_2$ accumulating in the presence of mannosidase inhibitors could prolong retention of the improperly folded glycoprotein within the calnexin cycle. The trimmed $Man_4GlcNAc_2$ and $Man_3GlcNAc_2$ intermediates, detected when proteasomes are inhibited, could then arise by the action of a Golgi mannosidase after cycling of RI_{332}-3HA through the ER and *cis*-Golgi. Our hypothesis was that this activity would impair reglucosylation of the ERAD substrate and consequently its calnexin binding, thus favouring disposal of the glycoprotein [22].

This observation of a role for the mannosidase activity different from the ER-mannosidase I activity in ERAD could still be specific for the MadIA214 mutant cell line. Therefore in the present study, we questioned whether other mannosidases could also act on an ERAD substrate in cells synthesizing a complete high mannose core ($Glc_3Man_9GlcNAc_2$). The mannosidase activity revealed in mutant CHO cells could produce the A isomer or related structures of $Man_8GlcNAc_2$ if operating in wild-type cells. Various ER and Golgi mannosidases, for which a role has yet to be ascribed, may actually perform different functions in processing and degradation of glycoproteins [20,36,39]. This could also be achieved by the endomannosidase detected in the *cis*-Golgi of certain cell types, which produces an isomer A of $Man_8GlcNAc_2$ from monoglucosylated $Man_9GlcNAc_2$ [19]. In addition, results obtained *in vitro*, which remain to be confirmed *in vivo*, suggest that at very high concentrations the specificity of the ER mannosidase I could be broader than described previously [40]. Here we analysed the structures of the N-linked oligosaccharides recovered from the ERAD substrate RI_{332} expressed in HeLa cells after a proteasomal block, which comprise intermediates in the degradation pathway that accumulate under these conditions. The finding that most of the $Man_8GlcNAc_2$ corresponds to the isomer B is consistent with the earlier proposal that ER mannosidase I is the predominant mannosidase acting on glycoprotein ERAD substrates. Moreover, since in our analysis the N-linked $Glc_1Man_7GlcNAc_2$ was also observed on RI_{332}, it appears that this glycoprotein is also a substrate for other mannosidases, such as ER mannosidase II. Other N-glycans, including $Man_6GlcNAc_2$ and $Man_5GlcNAc_2$, were additionally found associated with purified RI_{332} in the course of its degradation. The occurrence of these latter structures can be explained by the action of α1,2-mannosidases similar to those observed in MadIA214 glycosylation mutant cells, possibly the Golgi mannosidase I or ER-Man_9 mannosidase or the endomannosidase which has been shown to be expressed in several human cell lines [19]. Thus it is remarkable that for one given ERAD substrate several mannosidases might be involved. From previous work, the degradation of RI_{332} in HeLa cells appears to be biphasic, with a first step depending on calnexin interaction; accordingly, the non-glycosylated variant of RI_{332} is more rapidly degraded [23]. The presence of N-linked $Glc_1Man_7GlcNAc_2$, and of monoglucosylated $Man_9GlcNAc_2$ and $Man_8GlcNAc_2$ derivatives, although in smaller amounts, strengthened the role of calnexin in the degradation pathway. This is also in agreement with the observation that inhibiting calnexin binding by CST treatment accelerates the degradation of RI_{332} [24]. In contrast, mannosidase inhibitors stabilized the glycoprotein, possibly favouring the reglucosylation of the N-glycan, thus causing its prolonged retention in the calnexin cycle.

Altogether, these results indicate that the RI_{332} ERAD substrate is trimmed in the ER by both the ER mannosidases I and II, leading to N-linked $Man_9GlcNAc_2$, $Man_8GlcNAc_2$ and $Man_7GlcNAc_2$. All of them can be reglucosylated and retained by calnexin. Therefore $Glc_1Man_7GlcNAc_2$ observed in the presence of proteasome inhibitors appears to be a critical structure, still allowing calnexin binding, but from which further trimming of an α1,2-mannose residue could be the signal for proteasomal degradation. Indeed, the smaller N-glycans, $Man_6GlcNAc_2$ and $Man_5GlcNAc_2$ generated by a mannosidase capable of cleaving the terminal mannose residue that serves as a substrate for reglucosylation are also observed. We propose that these structures are probably those required for targeting of glycoproteins to degradation by the proteasome as supported by our previous observations on RI_{332}-3HA intermediates of degradation in the MadIA214 glycosylation-defective cell line. Such a scenario is consistent with the results from the literature pointing to the role of a dMJ- and Kif-sensitive α1,2-mannosidase, however different from ER mannosidase I. Therefore, the postulated lectin EDEM, specific for the B isomer of $Man_8GlcNAc_2$ N-glycans and required for the disposal of the ERAD substrate, will be required to have a broader specificity to accommodate our present findings. After submission of this paper, a report by Lederkremer and co-workers [41] appeared, which supports our observation of highly trimmed N-glycans ($Man_{6-5}GlcNAc_2$). Another possibility to explain our results could be that two pathways depending on two different α,1,2-mannosidases participated in the disposal of RI_{332}: one would involve the B isomer of $Man_8GlcNAc_2$ and would depend on EDEM and the other would involve the $Man_6GlcNAc_2$ structure resulting in the exit of the substrate protein from the calnexin cycle and would be independent of the EDEM lectin. This study also indicates that the degradation process is more complex than initially anticipated and that the use of specific inhibitors and electrophoretic analyses are insufficient to identify N-glycan structures that are involved in ERAD.

Our results also show that the final step in the deglycosylation process of RI_{332} during its degradation pathway is effected by an N-glycanase. This activity cleaves the complete N-glycan from the glycoprotein and converts the asparagine residue, to which it was attached, into aspartic acid [42,43]. At present, it is not clear whether the N-glycanase action requires previous trimming of the N-linked oligosaccharide by an α1,2-mannosidase, or whether trimming needs to occur for progression in the pathway before the N-glycanase step, e.g. to allow recognition by a sensing and/or conveying molecule, such as a specific ER lectin [11,14–18]. In this context, our observation that CST treatment permits the fully deglycosylated intermediate to be produced may indicate that avoiding calnexin binding favours mannosidase processing for further progression in the ERAD pathway. These results and the observation that inhibitors of α1,2-mannosidases block not only degradation but also deglycosylation processes are compatible

with our hypothesis on the role of a mannose-trimmed structure that cannot be reglucosylated as an intermediate of degradation.

Nevertheless, the membrane topology and the mechanism of glycoprotein dislocation are not fully understood. The occurrence and properties of the deglycosylated form of RI_{332} raised the question as to where it is produced. Our previous studies with CHO cells [23] and the present study using HeLa cell transformants are compatible with a luminal cleavage of the N-glycan. An N-glycanase activity in the lumen of the ER has indeed been reported [35,44,45]. Alternatively, it is also conceivable that the N-linked oligosaccharide of RI_{332} is cleaved by a cytoplasmic PNGase, after the glycoprotein engages with the translocation channel. A cytoplasmic PNGase was described in yeast [46] and in mammals [43] based on cloning of the genes and characterization of the corresponding gene products. Interestingly, a role for N-glycanase in the cytosolic turnover of glycoproteins has very recently been proposed in yeast [47] and in mammals [48]. In such a scenario, the N-glycan of RI_{332} might be exposed at the cytoplasmic side of the ER membrane, becoming accessible to the cytoplasmic PNGase. This might also explain why the deglycosylated intermediate RI_{332} appears to be only loosely associated with the ER membrane, as it is largely sensitive to exogenously added protease and may readily become a substrate for ubiquitination.

Although several ERAD substrates have been demonstrated to proceed through a deglycosylated intermediate [49–52], this is not always the case. It is noteworthy that after inhibition of the proteasome, we were unable to observe the deglycosylated intermediate of the RI_{332}-3HA-tagged version of RI_{332} ([22] and our unpublished results). The N-linked glycosylation site is located only 57 amino acids from the C-terminus of the RI_{332} protein, whereas RI_{332}-3HA contains an additional 35-amino-acid-long peptide at its C-terminus corresponding to the three repeats of an HA-epitope. In this context, it is interesting to note that a PNGase from hen oviduct was described to deglycosylate specifically only the more C-terminally located N-glycan (Asn^{311}) out of two (Asn^{292}, Asn^{311}) on ovalbumin, a secreted protein of 385 amino acids [53]. These observations might indicate that the N-linked glycan has to be at a certain distance from the C-terminus to be excised by a cytoplasmic PNGase. However, at no instance were the orientation and the folding state of the glycoprotein during dislocation elucidated and models have been proposed [54,55]. Therefore it cannot be excluded that differences in the folding between the tagged and the untagged protein could affect the accessibility to the PNGase, which then could not be easily revealed in the presence of a proteasome inhibitor. Further experiments will be required to distinguish between these possibilities.

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REFERENCES

1 Leitzgen, K. and Haas, I. G. (1998) Protein maturation in the ER. Chemtracts **11**, 423–445
2 Ellgaard, L., Molinari, M. and Helenius, A. (1999) Setting the standards: quality control in the secretory pathway. Science **286**, 1882–1888
3 Roth, J. (2002) Protein N-glycosylation along the secretory pathway: relationship to organelle topography and function, protein quality control, and cell interactions. Chem. Rev. **102**, 285–303
4 Bonifacino, J. S. and Weissman, A. M. (1998) Ubiquitin and the control of protein fate in the secretory and endocytic pathways. Annu. Rev. Cell Dev. Biol. **14**, 19–57
5 Plemper, R. K. and Wolf, D. H. (1999) Retrograde protein translocation: eradication of secretory proteins in health and disease. Trends Biochem. Sci. **24**, 266–270
6 Parodi, A. J. (2000) Role of N-oligosaccharide endoplasmic reticulum processing reactions in glycoprotein folding and degradation. Biochem. J. **348**, 1–13
7 Cabral, C. M., Liu, Y. and Sifers, R. N. (2001) Dissecting glycoprotein quality control in the secretory pathway. Trends Biochem. Sci. **26**, 619–624
8 Cabral, C. M., Liu, Y., Moremen, K. W. and Sifers, R. N. (2002) Organizational diversity among distinct glycoprotein endoplasmic reticulum-associated degradation programs. Mol. Biol. Cell **13**, 2639–2650
9 Parodi, A. J. (2000) Protein glucosylation and its role in protein folding. Annu. Rev. Biochem. **69**, 69–93
10 Ellgaard, L. and Helenius, A. (2001) ER quality control: towards an understanding at the molecular level. Curr. Opin. Cell Biol. **13**, 431–437
11 Jakob, C. A., Burda, P., Roth, J. and Aebi, M. (1998) Degradation of misfolded endoplasmic reticulum glycoproteins in *Saccharomyces cerevisiae* is determined by a specific oligosaccharide structure. J. Cell Biol. **142**, 1223–1233
12 Yang, M., Omura, S., Bonifacino, J. S. and Weissman, A. M. (1998) Novel aspects of degradation of T cell receptor subunits from the endoplasmic reticulum (ER) in T cells: importance of oligosaccharide processing, ubiquitination, and proteasome-dependent removal from ER membranes. J. Exp. Med. **187**, 835–846
13 Liu, Y., Choudhury, P., Cabral, C. M. and Sifers, R. N. (1999) Oligosaccharide modification in the early secretory pathway directs the selection of a misfolded glycoprotein for degradation by the proteasome. J. Biol. Chem. **274**, 5861–5867
14 Hosokawa, N., Wada, I., Hasegawa, K., Yorihuzi, T., Tremblay, L. O., Herscovics, A. and Nagata, K. (2001) A novel ER α-mannosidase-like protein accelerates ER-associated degradation. EMBO Rep. **2**, 415–422
15 Jakob, C. A., Bodmer, D., Spirig, U., Battig, P., Marcil, A., Dignard, D., Bergeron, J. J., Thomas, D. Y. and Aebi, M. (2001) Htm1p, a mannosidase-like protein, is involved in glycoprotein degradation in yeast. EMBO Rep. **2**, 423–430
16 Nakatsukasa, K., Nishikawa, S., Hosokawa, N., Nagata, K. and Endo, T. (2001) Mnl1p, an α-mannosidase-like protein in yeast *Saccharomyces cerevisiae*, is required for endoplasmic reticulum-associated degradation of glycoproteins. J. Biol. Chem. **276**, 8635–8638
17 Molinari, M., Calanca, V., Galli, C., Lucca, P. and Paganetti, P. (2003) Role of EDEM in the release of misfolded glycoproteins from the calnexin cycle. Science **299**, 1397–1400
18 Oda, Y., Hosokawa, N., Wada, I. and Nagata, K. (2003) EDEM as an acceptor of terminally misfolded glycoproteins released from calnexin. Science **299**, 1394–1397
19 Karaivanova, V. K., Luan, P. and Spiro, R. G. (1998) Processing of viral envelope glycoprotein by the endomannosidase pathway: evaluation of host cell specificity. Glycobiology **8**, 725–730
20 Ayalon-Soffer, M., Shenkman, M. and Lederkremer, G. Z. (1999) Differential role of mannose and glucose trimming in the ER degradation of asialoglycoprotein receptor subunits. J. Cell Sci. **112**, 3309–3318
21 Cabral, C. M., Choudhury, P., Liu, Y. and Sifers, R. N. (2000) Processing by endoplasmic reticulum mannosidases partitions a secretion-impaired glycoprotein into distinct disposal pathways. J. Biol. Chem. **275**, 25015–25022
22 Ermonval, M., Kitzmuller, C., Mir, A. M., Cacan, R. and Ivessa, N. E. (2001) N-glycan structure of a short-lived variant of ribophorin I expressed in the MadIA214 glycosylation-defective cell line reveals the role of a mannosidase that is not ER mannosidase I in the process of glycoprotein degradation. Glycobiology **11**, 565–576
23 de Virgilio, M., Weninger, H. and Ivessa, N. E. (1998) Ubiquitination is required for the retro-translocation of a short-lived luminal endoplasmic reticulum glycoprotein to the cytosol for degradation by the proteasome. J. Biol. Chem. **273**, 9734–9743
24 de Virgilio, M., Kitzmuller, C., Schwaiger, E., Klein, M., Kreibich, G. and Ivessa, N. E. (1999) Degradation of a short-lived glycoprotein from the lumen of the endoplasmic reticulum: the role of N-linked glycans and the unfolded protein response. Mol. Biol. Cell **10**, 4059–4073
25 Rosenfeld, M. G., Marcantonio, E. E., Hakimi, J., Ort, V. M., Atkinson, P. H., Sabatini, D. and Kreibich, G. (1984) Biosynthesis and processing of ribophorins in the endoplasmic reticulum. J. Cell Biol. **99**, 1076–1082
26 Ermonval, M., Cacan, R., Gorgas, K., Haas, I. G., Verbert, A. and Buttin, G. (1997) Differential fate of glycoproteins carrying a monoglucosylated form of truncated N-glycan in a new CHO line, MadIA214, selected for a thermosensitive secretory defect. J. Cell Sci. **110**, 323–336
27 Kmiécik, D., Herman, V., Stroop, C. J. M., Michalski, J.-C., Mir, A.-M., Labiau, O., Verbert, A. and Cacan, R. (1995) Catabolism of glycan moieties of lipid intermediates leads to a single $Man_5GlcNAc$ oligosaccharide isomer: a study with permeabilized CHO cells. Glycobiology **5**, 483–494

28 Moore, S. E. and Spiro, R. G. (1994) Intracellular compartmentalization and degradation of free polymannose oligosaccharides released during glycoprotein biosynthesis. J. Biol. Chem. **269**, 12715–12721
29 Hase, S., Ibuki, T. and Ikenaka, T. (1984) Reexamination of the pyridylamination used for fluorescence labeling of oligosaccharides and its application to glycoproteins. J. Biochem. (Tokyo) **95**, 197–203
30 Tokunaga, F., Brostrom, C., Koide, T. and Arvan, P. (2000) Endoplasmic reticulum (ER)-associated degradation of misfolded N-linked glycoproteins is suppressed upon inhibition of ER mannosidase I. J. Biol. Chem. **275**, 40757–40764
31 Wang, J. and White, A. L. (2000) Role of calnexin, calreticulin, and endoplasmic reticulum mannosidase I in apolipoprotein(a) intracellular targeting. Biochemistry **39**, 8993–9000
32 Weng, S. and Spiro, R. G. (1996) Endoplasmic reticulum kifunensine-resistant α-mannosidase is enzymatically and immunologically related to the cytosolic α-mannosidase. Arch. Biochem. Biophys. **325**, 113–123
33 Tremblay, L. O. and Herscovics, A. (1999) Cloning and expression of a specific human α1,2-mannosidase that trims $Man_9GlcNAc_2$ to $Man_8GlcNAc_2$ isomer B during N-glycan biosynthesis. Glycobiology **9**, 1073–1078
34 Tremblay, L. O. and Herscovics, A. (2000) Characterization of a cDNA encoding a novel human Golgi α1,2-mannosidase (IC) involved in N-glycan biosynthesis. J. Biol. Chem. **275**, 31655–31660
35 Spiro, R. G. (2000) Glucose residues as key determinants in the biosynthesis and quality control of glycoproteins with N-linked oligosaccharides. J. Biol. Chem. **275**, 35657–35660
36 Herscovics, A. (2001) Structure and function of Class I α1,2-mannosidases involved in glycoprotein synthesis and endoplasmic reticulum quality control. Biochimie **83**, 757–762
37 Knop, M., Hauser, N. and Wolf, D. H. (1996) N-glycosylation affects endoplasmic reticulum degradation of a mutated derivative of carboxypeptidase yscY in yeast. Yeast **12**, 1229–1238
38 Liu, Y., Choudhury, P., Cabral, C. M. and Sifers, R. N. (1997) Intracellular disposal of incompletely folded human α1-antitrypsin involves release from calnexin and post-translational trimming of asparagine-linked oligosaccharides. J. Biol. Chem. **272**, 7946–7951
39 Daniel, P. F., Winchester, B. and Warren, C. D. (1994) Mammalian α-mannosidases – multiple forms but a common purpose? Glycobiology **4**, 551–566
40 Herscovics, A., Romero, P. A. and Tremblay, L. O. (2002) The specificity of the yeast and human class I ER α1,2-mannosidases involved in ER quality control is not as strict as previously reported. Glycobiology **12**, 14G–15G
41 Frenkel, Z., Gregory, W., Kornfeld, S. and Lederkremer, G. Z. (2003) Endoplasmic reticulum-associated degradation of mammalian glycoproteins involves sugar chain trimming to $Man_{6-5}GlcNAc_2$. J. Biol. Chem. **278**, 34119–34124
42 Tarentino, A. L. and Plummer, Jr, T. H. (1994) Substrate specificity of *Flavobacterium meningosepticum* endo F2 and endo F3: purity is the name of the game. Glycobiology **4**, 771–773
43 Suzuki, T., Park, H. and Lennarz, W. J. (2002) Cytoplasmic peptide:N-glycanase (PNGase) in eukaryotic cells: occurrence, primary structure, and potential functions. FASEB J. **16**, 635–641
44 Weng, S. and Spiro, R. G. (1997) Demonstration of a peptide:N-glycosidase in the endoplasmic reticulum of rat liver. Biochem. J. **322**, 655–661
45 Karaivanova, V. K. and Spiro, R. G. (2000) Effect of proteasome inhibitors on the release into the cytosol of free polymannose oligosaccharides from glycoproteins. Glycobiology **10**, 727–735
46 Suzuki, T., Park, H., Hollingsworth, N. M., Sternglanz, R. and Lennarz, W. J. (2000) PNG1, a yeast gene encoding a highly conserved peptide:N-glycanase. J. Cell Biol. **149**, 1039–1052
47 Chantret, I., Frenoy, J. P. and Moore, S. E. (2003) Free-oligosaccharide control in the yeast *Saccharomyces cerevisiae*: roles for peptide:N-glycanase (Png1p) and vacuolar mannosidase (Ams1p). Biochem. J. **373**, 901–908
48 Hirsch, C., Blom, D. and Ploegh, H. L. (2003) A role for N-glycanase in the cytosolic turnover of glycoproteins. EMBO J. **22**, 1036–1046
49 Wiertz, E. J., Jones, T. R., Sun, L., Bogyo, M., Geuze, H. J. and Ploegh, H. L. (1996) The human cytomegalovirus US11 gene product dislocates MHC class I heavy chains from the endoplasmic reticulum to the cytosol. Cell (Cambridge, Mass.) **84**, 769–779
50 Halaban, R., Cheng, E., Zhang, Y., Moellmann, G., Hanlon, D., Michalak, M., Setaluri, V. and Hebert, D. N. (1997) Aberrant retention of tyrosinase in the endoplasmic reticulum mediates accelerated degradation of the enzyme and contributes to the dedifferentiated phenotype of amelanotic melanoma cells. Proc. Natl. Acad. Sci. U.S.A. **94**, 6210–6215
51 Huppa, J. B. and Ploegh, H. L. (1997) The α chain of the T cell antigen receptor is degraded in the cytosol. Immunity **7**, 113–122
52 Johnston, J. A., Ward, C. L. and Kopito, R. R. (1998) Aggresomes: a cellular response to misfolded proteins. J. Cell Biol. **143**, 1883–1898
53 Suzuki, T., Kitajima, K., Emori, Y., Inoue, Y. and Inoue, S. (1997) Site-specific de-N-glycosylation of diglycosylated ovalbumin in hen oviduct by endogenous peptide: N-glycanase as a quality control system for newly synthesized proteins. Proc. Natl. Acad. Sci. U.S.A. **94**, 6244–6249
54 Shamu, C. E., Story, C. M., Rapoport, T. A. and Ploegh, H. L. (1999) The pathway of US11-dependent degradation of MHC class I heavy chains involves a ubiquitin-conjugated intermediate. J. Cell Biol. **147**, 45–58
55 Fiebiger, E., Story, C., Ploegh, H. L. and Tortorella, D. (2002) Visualization of the ER-to-cytosol dislocation reaction of a type I membrane protein. EMBO J. **21**, 1041–1053

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