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# Research Article

# Endomannosidase processes oligosaccharides of $\alpha$1-antitrypsin and its naturally occurring genetic variants in the Golgi apparatus

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**Abstract.** Endomannosidase provides an alternate glucose-trimming pathway in the Golgi apparatus. However, it is unknown if the action of endomannosidase is dependent on the conformation of the substrate. We have investigated the processing by endomannosidase of the $\alpha$1-antitrypsin oligosaccharides and its disease-causing misfolded Z and Hong Kong variants. Oligosaccharides of wild-type and misfolded $\alpha$1-antitrypsin expressed in castanospermine-treated hepatocytes or glucosidase II-deficient Phar 2.7 cells were selectively processed by endomannosidase and subsequently converted to complex type oligosaccharides as indicated by Endo H resistance and PNGase F sensitivity. Overexpression of endomannosidase in castanospermine-treated hepatocytes resulted in processing of all oligosaccharides of wild-type and variants of $\alpha$1-antitrypsin. Thus, endomannosidase does not discriminate the folding state of the substrate and provides a back-up mechanism for completion of *N*-glycosylation of endoplasmic reticulum-escaped glucosylated glycoproteins. For exported misfolded glycoproteins, this would provide a pathway for the formation of mature oligosaccharides important for their proper trafficking and correct functioning.

**Keywords.** Endomannosidase, glucosidase II, Golgi apparatus, *N*-glycosylation, protein folding.

## Introduction

Folding and glycosylation are closely related important processes for *de novo* synthesized proteins. They are assisted and monitored by chaperones, the endoplasmic reticulum (ER) lectins calnexin and calreticulin, glucosidase I and II, and UDP-glucose:glycoprotein glucosyltransferase, which are components of the quality control of protein folding [1, 2]. Immature or improperly folded glycoproteins will be recognized and re-glucosylated by glucosyltransferase, enter the calnexin/calreticulin cycle and exit it through glucose-trimming by glucosidase II. Thus, re-glucosylation and de-glucosylation are important aspects in the quality control of glycoprotein folding [3–5]. An alternate glucosidase-independent pathway of glucose trimming is provided by endo-$\alpha$-mannosidase [6–9]. Endomannosidase is not inhibited by glucosidase inhibitors and compensates for the glucosidase II-deficiency in Phar 2.7 cells, and therefore maintains the ability to synthesize complex type oligosaccharides [10]. Importantly, endomannosidase has been shown to carry out a substantial amount of de-glucosylation *in vivo* [11]. It acts not only on mono-glucosylated but also on di- and tri-glucosylated oligosaccharides, even with truncated mannose chains, which are poor substrates for glucosidase II [6, 9, 10]. The endomannosidase-generated $Man_{7-4}GlcNAc_2$-R oligosaccharide is not a substrate for the glucosyltransferase but for Golgi mannosidases. The

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processing activity of these mannosidases is essential for the maturation to hybrid and complex oligosaccharides.
Endomannosidase is located in *cis* and *medial* Golgi cisternae and to lesser amounts in pre-Golgi intermediates [12], which is different from the distribution of glucosidase II [12, 13] and glucosyltransferase [14]. This indicates that glucose trimming of *N*-linked oligosaccharides is not restricted to the ER but also occurs in the Golgi apparatus and pre-Golgi intermediates by endomannosidase. This would ensure that glucosylated glycoproteins exported from the ER based on ER-assisted folding [15] can be de-glucosylated and processed to mature oligosaccharides. Alternatively, endomannosidase could function in quality control of glycoprotein folding in compartments distal to the ER, *i.e.* pre-Golgi intermediates or Golgi apparatus. However, it is not known if endomannosidase discriminates misfolded from correctly folded glycoproteins similar to glucosyltransferase [1].
The function of endomannosidase as a processing enzyme was established by *in vitro* experiments analyzing the processing of the *N*-linked oligosaccharides of the vesicular stomatitis virus G protein of virus-infected cells [16]. We have established an expression system using the well-characterized human hepatic $\alpha$1-antitrypsin and its naturally occurring genetic variants, the Z and Hong Kong variants. Misfolded $\alpha$1-antitrypsin variants cause $\alpha$1-antitrypsin deficiency because, due to different degree of misfolding [17, 18], they are either completely retained and degraded or partially secreted by hepatocytes [19]. This *in vivo* situation resembles a cell culture system for transthyretin disease-associated variants in which global protein energetics were found to determine the extent of protein export [15]. Thus, $\alpha$1-antitrypsin represents a convenient model glycoprotein to investigate whether the endomannosidase action is dependent on the conformation of the substrate. We expressed the wild-type and mutant $\alpha$1-antitrypsin in rat hepatocytes and in endomannosidase-deficient CHO cells under glucosidase blockade as well as in glucosidase II-deficient Phar 2.7 cells. We show that endomannosidase acts on both native and misfolded variants of $\alpha$1-antitrypsin, establishing its function as a Golgi apparatus-located back-up mechanism for protein *N*-glycosylation.

## Materials and methods

**Reagents and antibodies.** Restriction enzymes, T4 DNA ligase, endoglycosidase H (Endo H), recombinant *N*-glycosidase *F* (PNGase F) and protease inhibitor tablets were purchased from Roche Diagnostics (Rotkreuz, Switzerland), reverse transcription system from Promega (Wallisellen, Switzerland), Taq DNA polymerase, 5′ RACE system, expression vectors pcDNA3.1 and pcDNA6/myc-His, competent *E. coli* DH5$\alpha$ cells, Lipofectamine 2000, cell culture media and fetal bovine serum from Invitrogen (Basel, Switzerland), expression vector pMAMneo from Clontech (Basel, Switzerland), QIAquick PCR Purification Kit and Plasmid Midi Kit from QIAGEN (Basel, Switzerland), and TRI reagent from Lucerna Chem (Lucerne, Switzerland). Oligonucleotides were synthesized by Microsynth (Balgach, Switzerland). Castanospermine, kifunensine and swainsonine were obtained from Toronto Research Chemicals (Toronto, Canada) and MG 132 from Calbiochem (Lucerne, Switzerland). $^{35}$S-labeled methionine was purchased from Anawa Trading SA (Wangen, Switzerland), protein A magnetic beads from Dynal (Hamburg, Germany), *N*-butyl deoxynojirimycin and all other chemicals of analytical grade from Sigma (Buchs, Switzerland).
Polyclonal rabbit anti-rat endomannosidase antibody was kindly provided by Dr. R. G. Spiro (Boston, MA), polyclonal rabbit anti-human $\alpha$1-antitrypsin antibody was from Dako (Zug, Switzerland), monoclonal mouse anti-myc antibody from Promega, affinity-purified Fab fragments of goat anti-rabbit IgG, goat anti-mouse IgG antibodies and rhodamine red-X-conjugated affinity-purified Fab fragments of goat anti-rabbit IgG from Jackson ImmunoResearch Laboratories (West Grove, PA), and Alexa 488-conjugated (Fab′)$_2$ fragments of goat anti-rabbit IgG from Molecular Probes (Eugene, OR).

**Cloning of $\alpha$1-antitrypsin and site-directed mutagenesis.** Total RNA from human HepG2 cells was isolated using the TRI reagent and reverse transcribed with AMV reverse transcriptase using random primers. For subsequent PCR amplification, Taq DNA polymerase was used under standard conditions with the following $\alpha$1-antitrypsin-specific oligonucleotides. Forward primer: 5′-GAATTCGAATTCACAATGCCGTCTTCTGTCTCG, reverse primer: 5′-GAATTCCTCGAGTTATTTTTGGGTGGGATTCAC. The $\alpha$1-antitrypsin Z variant was generated by a PCR-based site-directed mutagenesis strategy. The following oligonucleotide containing the specific mutation was designed. For $\alpha$1-antitrypsin Z variant (E342K): 5′-ACCATCGACAAGAAAGGGACT. The PCR products were subcloned into the *Eco*RI and *Xho*I sites of the expression vector pcDNA3.1. Competent *E. coli* DH5$\alpha$ cells were transformed with these constructs. Plasmid DNA was prepared using the QIAGEN plasmid DNA purification kit. The construct pcDNA3.1/$\alpha$1-antitrypsin Hong Kong was kindly provided by Dr. R. Sifers (Dallas, TX).

**Rat endomannosidase constructs.** The 5′ end of the cDNA encoding rat endomannosidase was isolated with a 5′-rapid amplification of cDNA ends (5′ RACE) approach. For the 5′ RACE, a cDNA from rat liver RNA was synthesized containing a ligation anchored oligonucleotide terminus. For PCR, an antisense gene-specific

primer binding to position 282–289 of the rat endomannosidase (accession no. AF023657) in combination with the 5′ anchor primer was used. By a RT-PCR approach, full-length rat endomannosidase was amplified, the stop codon TAA deleted and a *Hin*dIII and a *Xho*I site was introduced at the 5′ and at the 3′ end of the cDNA, respectively. Then, it was subcloned into the expression vector pcDNA6/myc-His.

**Cell lines and transfections.** The mouse lymphoma cell line Phar 2.7 was kindly provided by Dr. I. Trowbridge (San Diego, CA). HepG2, Clone 9 and BRL3A hepatocyted as well as CHO-K1 cells hepatocytes were obtained from American Type Culture Collection (Manassas, VA). BRL3A, HepG2 and Phar 2.7 cells were cultured in DMEM medium containing 10% fetal bovine serum (FBS) and clone 9 rat hepatocytes as well as CHO-K1 cells in Ham's F12 medium containing 10% FBS.

Transfections of clonal lines of BRL3A and clone 9 rat hepatocytes and of Phar 2.7 and CHO-K1 cells with the pcDNA3.1/$\alpha$1-antitrypsin constructs were performed using Lipofectamine 2000 according to the manufacturer's instructions. For selection, cells were grown in medium containing G418 (100 µg/ml) and clonal cell lines were established in 96-multiwell plates. Individual clones were tested for the expression of $\alpha$1-antitrypsin by immunofluorescence and by RT-PCR.

Phar 2.7 cells were also stably transfected with a vector containing an inducible promoter. For this, the cDNA coding for $\alpha$1-antitrypsin was subcloned into the *Sma*I and *Xho*I site of the expression vector pMAM/neo containing the inducible mouse mammary tumor virus promoter. Cells were selected in medium containing G418 (250 µg/ml).

Clone 9 rat hepatocytes, expressing wild-type or mutant $\alpha$1-antitrypsin were co-transfected with the pcDNA6/endomannosidase-myc constructs. Clonal cells were selected in medium containing blasticidin (8–10 µg/ml) and G418 (100 µg/ml) and individual clones were tested for the expression of recombinant myc-tagged endomannosidase by immunofluorescence.

**Metabolic labeling, inhibitor treatments, immunoprecipitation and SDS-PAGE.** Clonal lines of transfected BRL3A, clone 9, CHO or Phar 2.7 cells, grown in flasks (25 $cm^2$) to 70–80% confluence, were incubated in methionine-free DMEM containing dialyzed FBS for 30 min at 37 °C. For experiments with inhibitors, the cells were pre-incubated at 37 °C for 1 h with medium containing one of the following inhibitors: castanospermine (100 µg/ml), *N*-butyl deoxynojirimycin (100 µg/ml), swainsonine (10 µg/ml), kifunensine (300 µM) and tunicamycin (1 µM). For pulse labeling, the cells were incubated in fresh medium, with or without inhibitors, containing 100 µCi/ml [$^{35}$S]methionine for 20 min at 37 °C. For the chase, the radioactive medium was removed and the cells were cultured in medium containing cold methionine (1 mM), in the absence or presence of inhibitors. For proteasome inhibition, MG 132 (10 µM) was added to the medium during the chase period. Subsequently, cells were washed with ice-cold PBS, mechanically removed, sedimented by centrifugation and resuspended in PBS containing protease inhibitors. Proteins were extracted with Triton X-100 (1%, 1 h at 4 °C) and cell debris removed by centrifugation. The cell extract or the corresponding culture medium was added to protein A magnetic beads conjugated with rabbit anti-human $\alpha$1-antitrypsin antibody and incubated for 2 h at 4 °C. The immunoprecipitates were washed with PBS containing 0.1% Triton X-100 and with PBS. Immunoprecipitated proteins were released by boiling in Laemmli buffer and separated in 8% SDS-polyacrylamide gels. Radioactivity was visualized by autoradiography or using a phosphorimager (FUJI Film Corp., Japan). In addition, pulse-chase experiments in clone 9 cells expressing the wild-type and Z variant of $\alpha$1-antitrypsin were performed at 42 °C.

**Isolation of Golgi membranes and Western blotting.** Transfected or non-transfected clone 9 hepatocytes were homogenized in 250 mM sucrose, 10 mM Tris-HCl (pH 7.4) containing protease inhibitors. Cell debris and nuclei were removed by centrifugation at 1000 *g* for 5 min at 4 °C, and the supernatant centrifuged at 100 000 *g* for 45 min at 4 °C. The microsomal pellet was resuspended in PBS containing protease inhibitors, passed several times through a 27-G needle, and the supernatant used for Western blot analysis. Proteins (20 µg protein/lane) were separated in 10% SDS-polyacrylamide gels, transferred onto nitrocellulose membranes using a semidry blotting apparatus [20] and probed for endomannosidase. The membranes were blocked with PBS containing 1% BSA and 0.05% Tween 20 for 1 h at ambient temperature and incubated with 1 µg/ml rabbit anti-rat endomannosidase antibody or with 0.4 µg/ml mouse monoclonal anti-myc antibody overnight at 4 °C followed by the incubation with the corresponding alkaline phosphatase-conjugated secondary antibody. The color reaction was performed as described previously [21].

**Glycosidase digestions.** For Endo H digestion, immunoprecipitates were boiled in 30 µl 50 mM Tris-HCl (pH 6.8) containing 0.1% SDS and 0.1% 2-mercaptoethanol for 5 min. Then, 25 µl sodium acetate buffer (0.1 M, pH 5.6) containing protease inhibitors was added followed by the addition of 5 mU Endo H. For PNGase F digestion, immunoprecipitates were denatured as above and 3 µl HEPES buffer (0.5 M, pH 7.4), 2 µl 10% Igepal, 2 µl 1% AEBSF and 2 U PNGase F were added. All samples

were incubated at 37 °C for 12–16 h before SDS-PAGE analysis.

**Confocal immunofluorescence microscopy.** Cells grown as monolayer on glass cover slips were formaldehyde-fixed and saponin-permeabilized as described [22]. For double immunofluorescence, cells were incubated with rabbit anti-rat endomannosidase (0.4 μg/ml) for 2 h at ambient temperature followed by rhodamine red-X-conjugated Fab fragments of goat anti-rabbit IgG (1000-fold diluted) for 1 h. After rinses, free rabbit IgG was blocked with unlabeled goat anti-rabbit Fab (12 μg/ml) followed by rabbit anti-human α1-antitrypsin antibody (2 μg/ml) for 1 h and Alexa 488-conjugated $(Fab')_2$ fragments of goat anti-rabbit IgG (1000-fold diluted) for 45 min. Double immunofluorescence for endomannosidase and Golgi mannosidase II was performed as described [12]. In addition, the monoclonal anti-myc antibody (1.3 μg/ml) with the corresponding secondary antibody was used for clone 9 hepatocytes transfected with pcDNA6-constructs. After rinses in buffer and double-distilled water, coverslips were embedded in Moviol. Immunofluorescence was observed with a Leica confocal laser scanning microscope TCS SP2 using the 100 × objective (1.4). In the double-immunofluorescence overlays, effects of pixel shift were excluded. The z-axis resolution of the equipment was at maximum 300 nm per voxel and the x; y settings were between 50 and 250 nm per voxel.

**Immunoelectron microscopy.** Clone 9 rat hepatocytes were fixed in 3% paraformaldehyde in PBS for 10 min at 37 °C, followed by fixation for 20 min at ambient temperature. Mechanically detached cells were sedimented by centrifugation, enclosed in agarose (2%) and infiltrated with sucrose (2.0 M) containing 15% polyvinyl pyrrolidone (10 kDa) in the presence of 1% paraformaldehyde. Pieces were mounted on aluminum pins, frozen and stored in liquid nitrogen. Frozen ultrathin sections were prepared according to Tokuyasu [23, 24], picked up on formvar-coated nickel grids and stored overnight on 2 M sucrose containing 15% polyvinyl pyrrolidone at 4 °C. Immunogold labeling for α1-antitrypsin was performed on ultrathin frozen sections using the protein A-gold technique [25].

## Results

To study the possible function of endomannosidase in the quality control of protein folding or as a back-up mechanism of protein *N*-glycosylation, we stably expressed human α1-antitrypsin and two naturally occurring genetic variants of α1-antitrypsin in clone 9 rat hepatocytes and transiently in BRL3A hepatocytes. The oligosaccharide processing by the alternate endomannosidase trimming pathway was analyzed *in vivo* under conditions of castanospermine-induced glucosidase I and II blockade. Both clone 9 and BRL3A hepatocytes possess high endomannosidase activity [12] as compared with other cell lines [16], and lack endogenous α1-antitrypsin based on Western blot analysis and RT-PCR (data not shown). Furthermore, glucosidase II-deficient Phar 2.7 cells [10, 26, 27] and CHO cells lacking endomannosidase activity [28] were stably transfected.

**Wild-type human α1-antitrypsin is a substrate for endomannosidase.** Metabolic labeling of clonal lines of transfected clone 9 (Fig. 1a) or BRL3A hepatocytes (Fig. 1b) showed that the human α1-antitrypsin was synthesized as a 52-kDa glycoprotein and rapidly secreted as a 56-kDa form (Fig. 1a, b), as in liver hepatocytes. The 52-kDa form represents intracellular α1-antitrypsin with a high-mannose oligosaccharide structure due to processing of the oligosaccharide precursors by glucosidases as previously reported for α1-antitrypsin in HepG2 cells [29]. The higher molecular mass of secreted α1-antitrypsin is due to the presence of complex-type oligosaccharides (see also below). When synthesized in the presence of the glucosidase I and II inhibitor castanospermine, α1-antitrypsin had a molecular mass of 54 kDa because of the preserved $Glc_3Man_9GlcNAc_2$ structure of its three oligosaccharides and after secretion a slightly higher molecular mass (Fig. 1a, b). In the presence of castanospermine, the secretion of wild-type α1-antitrypsin was marginally reduced (Fig. 1a, b).

To study the processing of the *N*-linked oligosaccharides of α1-antitrypsin in clone 9 and BRL3A hepatocytes, immunoprecipitates were subjected to Endo H digestion [30]. Intracellular α1-antitrypsin in clone 9 hepatocytes (Fig. 1c) was Endo H sensitive, as indicated by a shift in molecular mass from the 52-kDa polypeptide, representing the high mannose-type form, to the 48-kDa form, as observed for endogenous α1-antitrypsin in human HepG2 hepatocytes (data not shown). The α1-antitrypsin secreted by clone 9 hepatocytes was Endo H resistant, indicating that its oligosaccharides were of complex type due to Golgi processing (Fig. 1c). In contrast, α1-antitrypsin secreted from castanospermine-treated clone 9 or BRL3A hepatocytes had three Endo H-resistant and one Endo H-sensitive species (Fig. 1c, d). This indicated that the tri-glucosylated oligosaccharides of α1-antitrypsin were processed by endomannosidase, which was followed by further glycosylation reactions in the Golgi apparatus as demonstrated by their PNGase sensitivity (Fig. 1c). The appearance of three Endo H-resistant species reflects selective processing of the oligosaccharides of α1-antitrypsin by endomannosidase, *i.e.* one out of three oligosaccharides of α1-antitrypsin, two out of three or all oligosaccharides were of complex type due to the action of endomannosidase. The Endo H-pattern did not

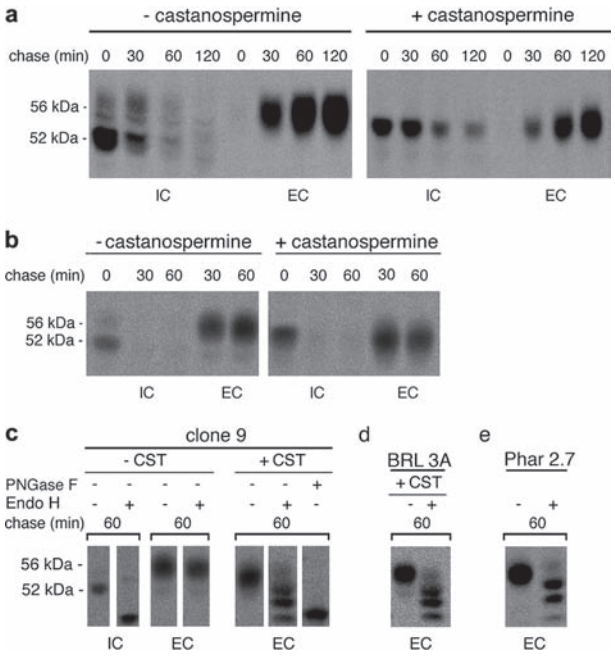


**Figure 1.** Human $\alpha$1-antitrypsin expressed in rat hepatocytes is processed by endomannosidase. Clone 9 rat hepatocytes (*a*) or BRL 3A hepatocytes (*b*) were pulsed for 20 min in the absence (–) or presence (+) of castanospermine and chased for the time period indicated. Cell lysates (IC) and culture medium (EC) were immunoprecipitated with anti-human $\alpha$1-antitrypsin antibody and analyzed by 8% SDS-PAGE/fluorography. (*c*) In the absence of castanospermine, the intracellular $\alpha$1-antitrypsin was sensitive to Endo H, whereas the secreted extracellular form was Endo H resistant. In the presence of castanospermine, Endo H digestion of the secreted $\alpha$1-antitrypsin yielded three Endo H-resistant forms and one sensitive form, which were PNGase F sensitive (*d*). Endo H treatment of secreted $\alpha$1-antitrypsin of BRL3A hepatocytes in the presence of castanospermine gave the same result. (*e*) Endo H treatment of secreted human $\alpha$1-antitrypsin expressed in the glucosidase II-deficient Phar 2.7 cells resulted in three Endo H-resistant and one Endo H-sensitive species. The higher band intensities as compared with (*c*, *d*) reflect the differences of *in vivo* substrate specificity of endomannosidase.

result from an incomplete digestion *in vitro* since both prolonged incubation and higher Endo H concentrations did not change the pattern (not shown). This is also fully supported by endomannosidase overexpression experiments (see below).

The $\alpha$1-antitrypsin expressed in glucosidase II-deficient Phar 2.7 cells was synthesized as a glycoprotein with a molecular mass of about 54 kDa since it possessed oligosaccharides with the $Glc_2Man_{9-8}GlcNAc_2$ structure, which are a substrate for endomannosidase [6, 9, 10]. While immunoprecipitated intracellular $\alpha$1-antitrypsin was fully Endo H-sensitive (data not shown), secreted $\alpha$1-antitrypsin showed three Endo H-resistant and one Endo H-sensitive species (Fig. 1e), similar to $\alpha$1-antitrypsin secreted from the castanospermine-treated clone 9 and BRL3A hepatocytes (Fig. 1c, d). Taken together, this demonstrated that during chemical glucosidase blockade or in glucosidase II-deficient Phar 2.7 cells, oligosaccharides of wild-type $\alpha$1-antitrypsin were processed by endomannosidase that generated an oligosaccharide substrate for further processing in the Golgi apparatus and resulted in the formation of Endo H-resistant, complex-type oligosaccharides.

**Misfolded mutants of $\alpha$1-antitrypsin are detectable in endomannosidase containing organelles.** Previously, we have shown that endomannosidase is present in *cis* and *medial* Golgi apparatus cisternae and in pre-Golgi intermediates [12]. Castanospermine treatment did not alter the inherent distribution of endomannosidase, or that of Golgi mannosidase II, as shown in Figure 2a–c. Next, we studied the mutual distributions of $\alpha$1-antitrypsin mutants and endomannosidase in castanospermine-treated cells. By double confocal immunofluorescence, the Z variant of $\alpha$1-antitrypsin (Fig. 2d–f) as well as the Hong Kong variant (data not shown) partially overlapped with staining for endomannosidase. This was confirmed by immunogold electron microscopy demonstrating labeling for the two variants of $\alpha$1-antitrypsin in the ER, pre-Golgi intermediates and the Golgi apparatus (Fig. 3a–c). The studied clonal cell lines expressed similar amounts of the $\alpha$1-antitrypsin variants based on pulse-labeling experiments (data not shown). Together, these results demonstrate that mutant $\alpha$1-antitrypsin is detectable in endomannosidase positive structures.

**Z variant of $\alpha$1-antitrypsin is processed by endomannosidase.** The Z variant expressed in clone 9 hepatocytes (Fig. 4a), in BRL3A hepatocytes (data not shown) and CHO cells (Fig. 4b) had a molecular mass of 52 kDa for

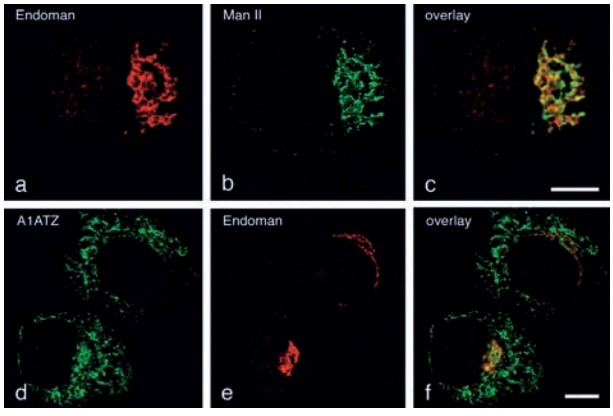


**Figure 2.** Confocal immunofluorescence of endomannosidase and human $\alpha$1-antitrypsin in clone 9 rat hepatocytes during glucosidase blockade. (*a–c*) A single confocal section reveals a perinuclear crescent-shaped staining for endomannosidase (*a*) and for Golgi-mannosidase II (*b*) which overlaps partially (*c*) in castanospermine-treated clone 9 hepatocytes. The additional punctate endomannosidase immunofluorescence is indicative of its presence in pre-Golgi intermediates. (*d–f*) In clone 9 hepatocytes, stably expressed Z variant of $\alpha$1-antitrypsin (*d*) and endomannosidase (*e*) exhibit partial co-distribution (*f*). Bars, 10 µm.

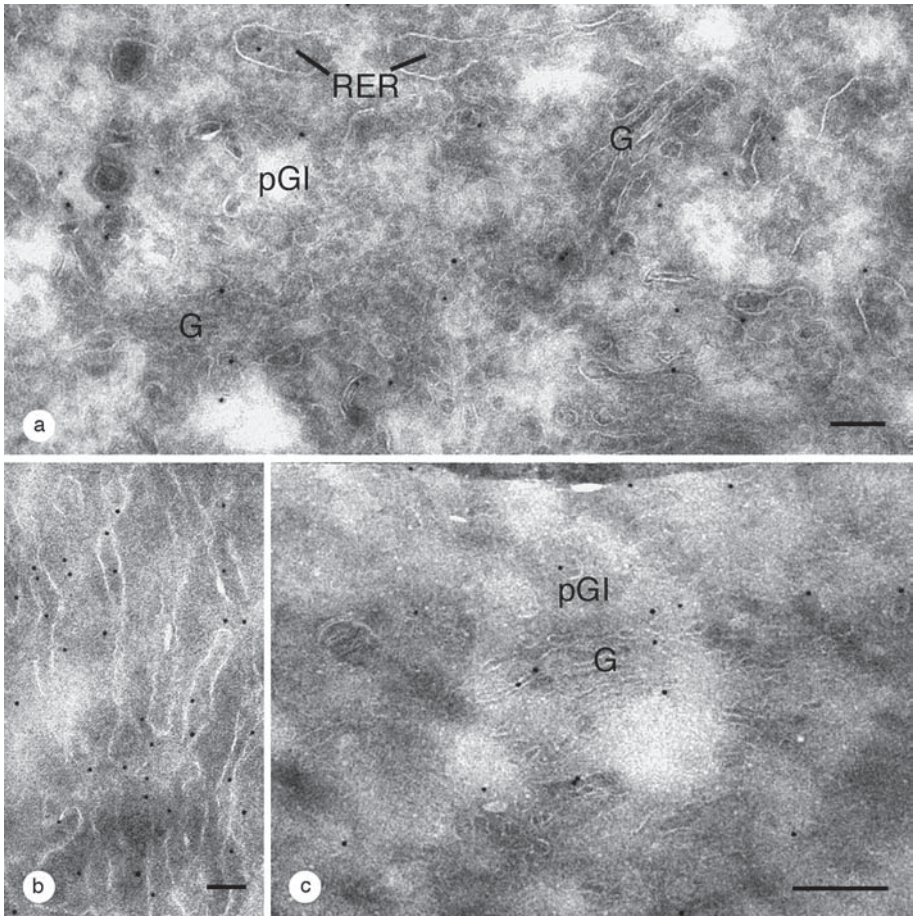


**Figure 3.** Immunogold localization of mutant $\alpha$1-antitrypsin in castanospermine-treated clone 9 hepatocytes. Immunogold labeling for the Z variant (*a*) and Hong Kong variant (*b*, *c*) of $\alpha$1-antitrypsin is detectable in the rough endoplasmic reticulum (RER), pre-Golgi intermediates (pGI) and the Golgi apparatus (*g*). Bars, 0.1 µm (*a*, *b*), 0.27 µm (*c*).

the intracellular form and 56 kDa for the secreted one (Fig. 4a, b) with the latter being Endo H resistant (data not shown), like wild-type $\alpha$1-antitrypsin (Fig. 1a, b). The Z variant in clone 9 and BRL3A hepatocytes started to become secreted after a lag period of 1 h with no detectable protein after a 30-min chase (Fig. 4a). However, in CHO cells only minor amounts were secreted after a lag period of 3 h (Fig. 4b). The small shift in molecular mass of the intracellular Z variant (Fig. 4a, b) was due to trimming by ER-mannosidase I since it could be inhibited by its specific inhibitor kifunensine [31] (Fig. 4c) but not by the Golgi mannosidase II inhibitor swainsonine [32] (data not shown).

In castanospermine-treated hepatocytes, the Z variant was synthesized as 54-kDa glycoprotein and secreted with the same kinetics as in untreated cells. Endo H treatment of the immunoprecipitated secreted Z variant, after 1 h of chase, gave three Endo H-resistant and one Endo H-sensitive species. After 3 h of chase, oligosaccharides of the Z variant were essentially Endo H-resistant (Fig. 4d). A similar result was found in cells pretreated with the glucosidase I inhibitor *N*-butyl deoxynojirimycin (Fig. 4d). It has been shown that the loop-sheet polymerization, *i.e.* misfolding, of the Z variant is accelerated at elevated temperatures such as 42 °C [18]. When synthesized at 42 °C in castanospermine-treated clone 9 cells, the secreted Z variant was a substrate for endomannosidase as indicated by its Endo H resistance (Fig. 4e).

In additional experiments, we tested the effect of overexpression of endomannosidase on the oligosaccharide processing of $\alpha$1-antitrypsin. The myc-tagged rat endomannosidase was at least threefold overexpressed in clone 9 cells based on densitometric evaluation of Western blots (data not shown) without effecting its inherent cellular distribution (Fig. 5a). The secretion of the Z variant of $\alpha$1-antitrypsin remained unchanged but all its three *N*-linked oligosaccharides were processed as indicated by the presence of a single Endo H-resistant, PNGase F-sensitive band (Fig. 5b). Similarly, the oligosaccharides of the wild-type $\alpha$1-antitrypsin were completely processed (data not shown). These data clearly indicated that endogenous endomannosidase activity levels resulted in a selective processing of the three *N*-linked oligosaccharides of the wild-type and Z variant of $\alpha$1-antitrypsin,

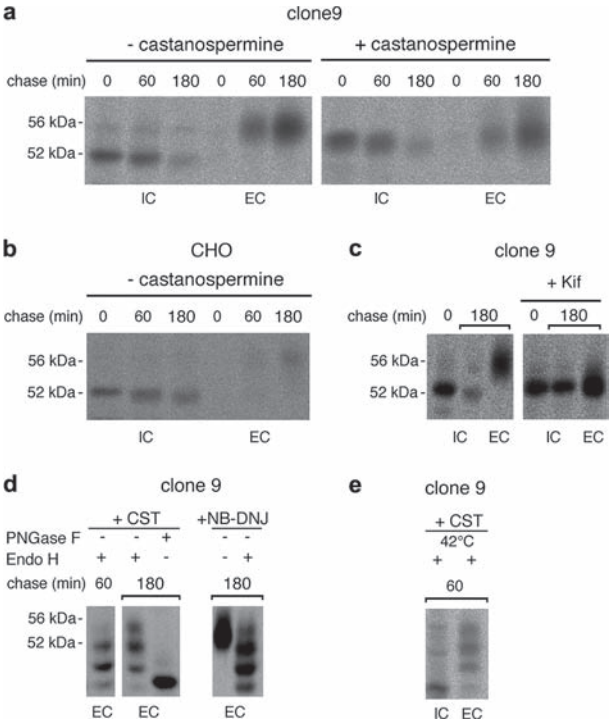


**Figure 4.** Processing of the Z variant of $\alpha$1-antitrypsin in clone 9 cells. (*a*) After a chase period of 3 h, the Z variant of $\alpha$1-antitrypsin was secreted in the medium as a 56-kDa protein in the absence of castanospermine and as a 54-kDa protein in the presence of castanospermine. (*b*) In contrast, most of the Z variant expressed in CHO cells was retained intracellularly. (*c*) ER-mannosidase I inhibition by kifunensine (Kif) abolishes the slight decrease in molecular mass of the intracellular Z variant during the chase. (*d*) Endo H digestion of secreted Z variant in castanospermine (CST) or *N*-butyl deoxynojirimycin (NB-DNJ)-treated clone 9 hepatocytes yielded three Endo H-resistant and one sensitive species. (*e*) Endo H treatment of secreted Z variant synthesized at 42 °C gave the same result as observed at 37 °C (see *d*).

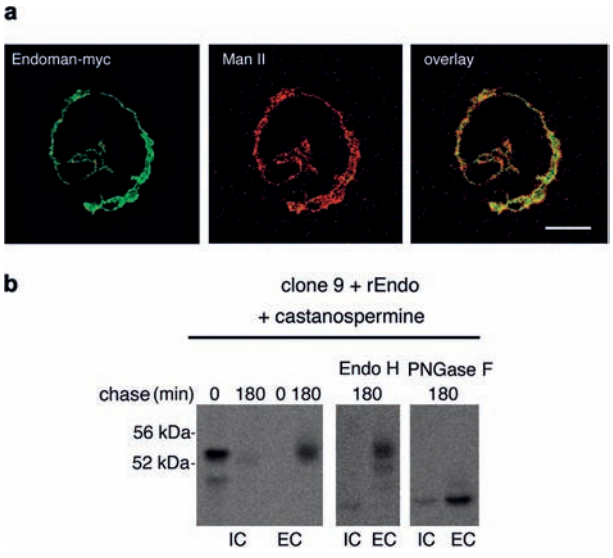


**Figure 5.** Overexpression of myc-tagged rat endomannosidase in clone 9 hepatocytes resulted in complete processing of the oligosaccharides of the Z variant of $\alpha$1-antitrypsin. (*a*) Double confocal immunofluorescence for endomannosidase-myc and Golgi-mannosidase II showed overlapping distributions. Bar, 10 µm. (*b*) The secreted Z variant of $\alpha$1-antitrypsin in endomannosidase-overexpressing, castanospermine-treated clone 9 hepatocytes was Endo H-resistant and PNGase F-sensitive.

which is no longer observed when endomannosidase is overexpressed.

**Terminally misfolded Hong Kong variant of $\alpha$1-antitrypsin is a substrate of endomannosidase.** The Hong Kong variant, a truncated $\alpha$1-antitrypsin [33] synthesized in clone 9 hepatocytes, was retained intracellularly as a 45-kDa protein (Fig. 6a) and partially subjected to proteasomal degradation (Fig. 6b). Probably, autophagy is involved, as previously shown for the Z variant of $\alpha$1-antitrypsin [34]. On treatment with castanospermine, the Hong Kong variant with a molecular mass of 47 kDa was gradually secreted (Fig. 6a). On treatment with Endo H, three Endo H-resistant and a minor Endo H-sensitive species were detectable (Fig. 6c), similar to that observed for the wild-type and Z variant of $\alpha$1-antitrypsin. These data indicated that the oligosaccharides of the Hong Kong variant were trimmed by endomannosidase and further processed by Golgi enzymes.

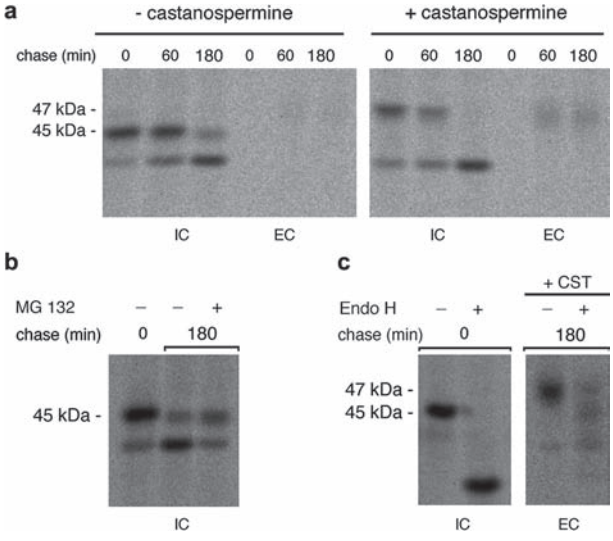


**Figure 6.** Castanospermine treatment resulted in secretion and processing of the Hong Kong variant of $\alpha$1-antitrypsin. (*a*) In the absence of castanospermine, the Hong Kong variant was synthesized as a 45-kDa protein and not secreted. The 43-kDa band represents the deglycosylated form of the Hong Kong variant of $\alpha$1-antitrypsin. In the presence of castanospermine, a 47-kDa protein was secreted after a lag period. (*b*) Addition of the proteasome inhibitor MG132 interfered the degradation of the Hong Kong variant. (*c*) In the absence of castanospermine, the Hong Kong variant was not secreted and Endo H sensitive, whereas in castanospermine-treated clone 9 hepatocytes it was secreted and yielded three Endo H-resistant and a minor Endo H-sensitive species.

## Discussion

We have previously established that endomannosidase, a glucose trimming endoglycosidase, resides in the Golgi apparatus and in pre-Golgi intermediates [12], leaving open the question of its possible function either as a sensor of the folding state of glycoproteins or as a back-up mechanism for completion of *N*-glycosylation of ER-escaped glucosylated glycoproteins. Here, we have demonstrated by a combination of biochemical and morphological approaches that Golgi localized endomannosidase acts on glucosylated oligosaccharides of both wild-type and misfolded variants of $\alpha$1-antitrypsin. In cells with a blocked glucosidase I and II trimming pathway, the alternate endomannosidase pathway produces an oligosaccharide substrate for trimming by Golgi mannosidases and elongation by Golgi glycosyltransferases. Thus, the present studies demonstrate that glucose trimming by endomannosidase serves as a back-up mechanism for protein *N*-glycosylation in the Golgi apparatus. The action of endomannosidase provides a mechanism by which glucosylated oligosaccharides of ER-escaped glycoproteins can be further processed to mature, functional ones and establishes the presence of a previously unknown function of the Golgi apparatus.

The present analysis of oligosaccharide processing by pulse-chase experiments with the use of Endo H and PNGase F in glucosidase I and II-inhibited rat hepatocyte cell lines and glucosidase II-deficient Phar 2.7 cells showed that wild-type $\alpha$1-antitrypsin is a substrate for endomannosidase. Furthermore, the genetic variants of $\alpha$1-antitrypsin studied were processed by endomannosidase and overexpression of the enzyme increased the effect. Although there was a difference in the time course of secretion between wild-type and Z variant of $\alpha$1-antitrypsin, both were processed by endomannosidase under conditions of castanospermine blockade of trimming glucosidases. This difference in secretion was reported earlier by others [35, 36] and can be best explained by the intracellular retardation of the Z variant by the ER quality control. The Hong Kong variant was fully secretion incompetent, but, as shown in the present study, was detectable not only in the ER but also in the pre-Golgi intermediates, which in contrast to the ER, containing endomannosidase [12]. This confirms and extends data demonstrating that misfolded proteins can escape the quality control in the ER proper and enter more distal parts of the secretory pathway from where they may be recycled, degraded or eventually secreted [37–39]. This relates to the existence of multiple checkpoints for quality control that extend beyond the ER [40–45]. As a case in point, in humans suffering from $\alpha$1-antitrypsin deficiency, the Z variant of $\alpha$1-antitrypsin not only becomes partially secreted but is also active as a serine protease inhibitor [36, 46, 47]. Under our experimental conditions, the Z variant of $\alpha$1-antitrypsin was secreted after a lag period. On the other hand, the Hong Kong and the S variant of $\alpha$1-antitrypsin [unpublished data] or misfolded T126M aquaporin2 [21] were recognized by the quality control of clone 9 hepatocytes, retained intracellularly and degraded via the ubiquitin proteasome system. Therefore, we conclude that the components necessary for quality control are present in clone 9 rat hepatocytes. In this context, it is of interest that in HEK293 cells about 40% of the Z variant is secreted [36], but in mouse hepatoma Hepa 1a cells [48] and CHO cells only about 15% is secreted, similar to that seen in patients suffering from $\alpha$1-antitrypsin deficiency [49]. These findings support the notion that cell-specific folding and quality control systems exist. Furthermore, the molecular mechanism of recognition appears to depend on the glycoprotein and type of mutation. For instance, misfolded T126M aquaporin 2 is retained in the ER, whereas misfolded E258K is misrouted to endo/lysosomes via the Golgi apparatus [21, 50]. Recently, it was reported that numerous pathological transthyretin variants were secreted with wild-type-like efficiency and only the most highly destabilized variants were subjected to ER-associated degradation in certain tissues [15]. It was concluded, that an ER-assisted folding pathway based on global energetics of the protein fold as well as protein chaperones, rather than a quality control based on wild-type stability, determined the extent of secretion. The proposed ER-assisted folding pathway might explain why in our experiments the Z variant was secreted, whereas the Hong Kong variant was subjected to ER-associated degradation. It would also provide an explanation for the differences in secretion among $\alpha$1-antitrypsin variants observed in patients.

We obtained data for the substrate specificity of endomannosidase by studying the physiologically relevant $\alpha$1-antitrypsin. The oligosaccharides on wild-type and variant $\alpha$1-antitrypsin located at position 46, 83 and 247 [49] were differentially processed by endomannosidase when present as tri- or di-glucosylated species. We observed that endomannosidase processed both wild-type and misfolded variants of $\alpha$1-antitrypsin, which indeed excludes a function as folding sensor. Classically, the ER is considered as the site of glucose trimming [51]. Endomannosidase can exist in complexes with calreticulin in the Golgi apparatus and pre-Golgi intermediates [12, 52]. Dissociation of calreticulin-glycoprotein complexes in this location can occur through endomannosidase [12–14]. Functionally, de-glucosylation by endomannosidase as compared with glucosidase II has greatly differing consequences. The resulting $Man_{\leq 8}$ isomer A oligosaccharide is no longer a substrate for re-glucosylation by glucosyltransferase [3]. Thus, the action of endomannosidase will prevent entry into another calnexin/calreticulin cycle. This difference determines the fate of the glycoprotein since its oligosaccharides can now be processed to mature

ones by Golgi mannosidases and glycosyltransferases. As shown here for $\alpha$1-antitrypsin, both the secreted variants and wild-type glycoproteins bear complex type oligosaccharides. Misfolded variants of $\alpha$1-antitrypsin, but also of other glycoproteins such as aquaporin 2, alpha-galactosidase A or the CFTR chloride channel possess biological activity and their function and proper trafficking may depend on proper glycosylation [53–56]. Thus, trimming by endomannosidase and the subsequent maturation of oligosaccharides in the Golgi apparatus will be potentially important in case of partially secreted variants of misfolded glycoproteins or when pharmacological chaperones are applied to rescue ER-retained misfolded glycoproteins [56–59].

In summary, we obtained evidence that endomannosidase acts on both correctly folded and misfolded $\alpha$1-antitrypsin, and propose that endomannosidase provides a back-up mechanism for de-glucosylation of ER-escaped glycoproteins so that their oligosaccharides can be processed to mature ones. For misfolded glycoproteins, this would provide a pathway for the formation of mature oligosaccharides probably important for their proper trafficking and correct functioning.

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