Analysis of the endoplasmic reticular Ca^{2+} requirement for α_1-antitrypsin processing and transport competence

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Depletion of Ca^{2+} sequestered within the endoplasmic reticulum (ER) of HepG2 hepatoma cells results in the luminal accumulation of immature α_1-antitrypsin possessing Man_{8-9} $GlcNAc_2$ oligosaccharide side chains. This study explores the basis for this arrest and describes consequent alterations in the size and rate of secretion of the complex endoglycosidase H-resistant form of the protein. Inhibition of glucosidase I and II with castanospermine or α-1,2-mannosidase with 1-deoxymannojirimycin produced altered ER processing intermediates that were rapidly secreted. Subsequent mobilization of ER Ca^{2+} stores resulted in the appearance and retention of slightly larger related forms of these intermediates. Retention of glycosylated intermediates was not ascribable to an association with α1,2-mannosidase or lectin-like chaperones, the intermediates were not degraded and all evidence of ER retention or size alterations produced by Ca^{2+} depletion was quickly reversed by Ca^{2+} restoration. Cells that were Ca^{2+} depleted for 2 h slowly secreted an abnormal slightly smaller complex oligosaccharide form of α_1-antitrypsin at approximately the same rate as the non-glycosylated protein generated by treatment with tunicamycin. The hypothesis that Ca^{2+} affects the folding and ER transport competence of glycosylated forms of α_1-antitrypsin is discussed.

INTRODUCTION

The endoplasmic reticulum (ER) functions prominently in the post-translational processing of proteins destined for secretion or insertion into cellular membranes. The organelle integrates various steps of early protein processing including ribosomal docking and co-translational translocation, post-translational modifications including disulphide bond formation and N-linked glycosylation, the folding and assembly of proteins into multimeric structures, and the targeting of improperly folded proteins for degradation [1–5]. Exit of proteins from the ER is currently thought to constitute a rate-limiting step in intracellular transport and maturation [4,6]. Proteins that are folded, assembled or glycosylated incorrectly are generally retained by the ER [4]. Extensive evidence has also developed that the ER possesses a signalling system to co-ordinate rates of mRNA translation with rates of protein processing. Key components of this system include the accumulation of underprocessed protein in the ER and an associated depression of eIF-2 cycling mediated by the double-stranded RNA-activated eIF-2α kinase (PKR) [7–12].

Ca^{2+} sequestered by the ER is essential for diverse processing events in the organelle. Calnexin and calreticulin are examples of ER-associated Ca^{2+}-binding proteins that assist folding of monoglucosylated glycoprotein intermediates and may function in retention of misfolded intermediates [13–16]. Depletion of ER-sequestered Ca^{2+} has been found to inhibit the oligomerization of viral proteins, the intracellular sorting of immunoglobulin M and the turnover of T-cell antigen receptor β and asialoglycoprotein receptor H2b subunits [17–20]. Depletion of the cation also suppresses translational initiation through activation of PKR [10–12] and signals induction of expression of the ER-resident protein chaperones glucose-regulated stress protein 78 or immunoglobulin-binding protein (GRP78/BiP) and GRP94 [21]. GRP78 functions in translocation and early folding events [4,6,21,22], and induction of its expression confers translational tolerance to depletion of Ca^{2+} stores [23–25].

In HepG2 hepatoma cells with depleted ER Ca^{2+} stores, processing and export of α_1-antitrypsin and several other glycoproteins are inhibited whereas the transport of albumin, a non-glycoprotein, is minimally affected [26,27]. Ca^{2+}-mobilizing drugs were found to block the oligosaccharide processing of α_1-antitrypsin, which possesses three asparagine-linked side chains, at conversion from the 49 kDa high-mannose form into the 54 kDa complex oligosaccharide form and to promote retention of the 49 kDa precursor within the Ca^{2+}-depleted ER. Retained forms of α_1-antitrypsin contained $Man_{8-9}GlcNAc_2$ oligosaccharide side chains predominantly [28]. Maturation of α_1-antitrypsin possessing $Man_{5-6}GlcNAc_2$ side chains was unaffected by ER Ca^{2+} depletion. Forms of α_1-antitrypsin identical with those retained with Ca^{2+}-mobilizing agent were observed on treatment with the α-1,2-mannosidase inhibitor 1-deoxymannojirimycin, whereas somewhat larger forms of the protein were produced during treatment with castanospermine, an inhibitor of ER glucosidases [28]. Neither 1-deoxymannojirimycin nor castanospermine impaired transport or secretion of α_1-antitrypsin, indicating that inhibition of oligosaccharide processing *per se* did not cause ER retention. Treatment with brefeldin A, which promotes redistribution of Golgi enzymes to the ER [29], permitted α_1-antitrypsin to mature to an endoglycosidase (Endo) H-resistant form, but prevented secretion [28]. However, maturation did not occur in cells exposed to brefeldin A in conjunction with either 1-deoxymannojirimycin or a Ca^{2+}-mobilizing agent.

Various mechanisms have been proposed [28] to explain retention of α_1-antitrypsin bearing high-mannose side chains in the Ca^{2+}-depleted ER. Inhibition of ER α-1,2-mannosidase activity after Ca^{2+} mobilization could derive from effects expressed on either the enzyme or the substrate. Potential enzyme-directed effects include a Ca^{2+} requirement for catalytic activity, substrate binding or substrate release. Putative substrate-directed effects involve either α_1-antitrypsin retention by an ER chaperone or folding of the protein to a conformation unsuitable for either mannosidase action or transport. The present study was under-

Abbreviations used: ER, endoplasmic reticulum; Endo H, endoglycosidase H; GRP78/BiP, glucose-regulated stress protein 78 or immunoglobulin-binding protein; PKR, double-stranded RNA-activated protein kinase.

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taken to discriminate among these possibilities. Ca^{2+} mobilization both delayed exit of α_1-antitrypsin from the ER and impaired mannose trimming. However, retention was found to occur independently of any association with ER α-1,2-mannosidases and did not require that glucose residues be removed. Cells subjected to continued ER Ca^{2+} depletion secreted an under-processed form of α_1-antitrypsin at approximately the same rate as tunicamycin-treated cells secreted the non-glycosylated protein.

EXPERIMENTAL

Materials

Ionomycin was purchased from Calbiochem and thapsigargin from LC Services Corp. L-[^{35}S]Methionine was obtained from ICN. Endo H, streptococcal neuraminidase and castanospermine were purchased from Genzyme, Boston, MA, U.S.A. Tunicamycin, (+)-1-deoxymannojirimycin and ionophore A23187 were obtained from Sigma. IgGSorb was from the Enzyme Center, Malden, MA, U.S.A. Goat antiserum to human α_1-antitrypsin was purchased from Cappel Research Reagents, Durham, NC, U.S.A., and rabbit antiserum to human albumin was obtained from US Biochemicals. Other reagents were from Sigma.

Culture and pulse-labelling of HepG2 proteins

Human HepG2 hepatoma cells were cultured in minimal essential medium supplemented with Earle's salts and 10% fetal bovine serum. Subconfluent monolayers were incubated briefly (< 1 min) with dilute trypsin solution (0.025% in saline) at room temperature. Detached cells were diluted in PBS, pelleted by low-speed centrifugation and suspended in serum-free Ham's F-10 medium modified to contain 100 μM $CaCl_2$, 3 μM methionine and 4.5 g/l glucose. By the criterion of Trypan Blue exclusion, viability of cells harvested in this manner was 85–90%. After a 15 min equilibration period at 37 °C, [^{35}S]methionine (25 μCi/ml) was added and pulse-labelling of proteins was conducted for 5–10 min. Unlabelled methionine (400 μM) was then added to arrest labelling, and cell suspensions were incubated with drugs as described in the text. When Ca^{2+} ionophores were employed, EGTA (1.1 mM) was added to hasten depletion of Ca^{2+} stores. To reverse effects of ionomycin, fatty acid-free BSA (2 mg/ml) was added, the incubation was continued for 5 min, and cells were then pelleted and resuspended in medium containing 5 mM $CaCl_2$.

Pretreatments with inhibitors of glycoprotein processing

Monolayer cultures in serum-free minimal essential medium were pretreated for 2 h at 37 °C with 12.5 μg/ml tunicamycin or 500 μg/ml castanospermine. Pretreatment media were then collected, and cells were harvested as described above for pulse-labelling. For pretreatment with 1-deoxymannojirimycin, cells were first harvested, washed and resuspended to 2×10^6/ml in low-methionine F-10 medium as described above. 1-Deoxymannojirimycin (4 mM) was added to the cell suspensions, and preparations were pretreated for 1 h at 37 °C before addition of [^{35}S]methionine.

Immunoprecipitation and electrophoresis

After specific treatments, aliquots (5 ml) of the cell suspension were chilled to 4 °C, and cells were separated from their respective media by centrifugation at low speed. Cells were washed once with ice-cold PBS and lysed in PBS, pH 7.2, containing 1% Triton X-100, 1 mM PMSF and 10 μg/ml each of leupeptin, aprotinin and antipain. After centrifugation for 5 min at 12400 ***g***, lysates were incubated for 5 min with IgGSorb and re-centrifuged to remove material that bound non-specifically to the adsorbent. Aliquots of precleared supernatants were then incubated with antibodies to α_1-antitrypsin or albumin at 1:100 dilution, and antigen–antibody complexes were precipitated by incubation with IgGSorb and collected by centrifugation. Samples of extracellular media were treated with antisera at 1:400 dilution, and antigen–antibody complexes were harvested as above. Complexes were washed twice by centrifugation and resuspension in PBS and then resuspended in Laemmli's sample buffer [30]. Immunoprecipitated proteins were extracted from antigen–antibody–Protein A complexes by boiling in sample buffer for 5 min, and samples were clarified by brief centrifugation. Proteins were resolved by one-dimensional SDS/PAGE (7.5% gel) of sample buffer supernatants from cell (20 μl) or medium (40 μl) samplings, and autoradiography was performed. Identification of the two immunoprecipitable species of α_1-antitrypsin displayed in Figure 1 as the high-mannose (48 kDa) and complex (54 kDa) forms of the glycoprotein has been described previously [27,28]; other molecular masses are estimated.

Digestions with neuraminidase and Endo H

For neuraminidase digestions, washed antigen–antibody complexes were suspended in 0.1 M sodium acetate buffer, pH 5.8, containing 0.2 or 1.0 m-unit/ml streptococcal neuraminidase. For Endo H digestions, the complexes were suspended in 50 mM sodium phosphate buffer, pH 5.5, containing 0.2% SDS, 1 M 2-mercaptoethanol and 1 mM PMSF. Samples were boiled for 5 min and this was followed by addition of 16 m-units of Endo H. Digestions with either enzyme were performed for 16 h at 37 °C and were terminated by the addition of sample buffer; this was followed by boiling for 5 min. Digests were clarified by centrifugation, and proteins of the clarified digests were separated by SDS/PAGE (7.5% or 9.0%) followed by autoradiography.

Quantification of α_1-antitrypsin and albumin retention

To determine the percentage of newly synthesized α_1-antitrypsin or albumin retained intracellularly as a function of specific treatments, autoradiography was performed for a standard time. For each treatment condition, the intracellular and extracellular forms of α_1-antitrypsin were quantified by densitometric scanning of autoradiograms using a Joyce–Loebl densitometer. Data obtained were corrected for local background and differences in sample loading volume. The percentage retained was calculated as labelled intracellular immunoprecipitate divided by total labelled immunoprecipitated protein.

RESULTS

Ca^{2+}-dependent transport of N-glycosylated forms of α_1-antitrypsin

HepG2 cells depleted of ER-sequestered Ca^{2+} retain an immature form of α_1-antitrypsin that is characterized by $Man_{8-9}GlcNAc_2$ oligosaccharide side chains, Endo H-sensitivity and a molecular mass (49 kDa) consistent with post-glucosidase but pre-mannosidase inhibition within the ER [26–28]. The identical form of α_1-antitrypsin is secreted from cells that are treated with 1-deoxymannojirimycin to inhibit α-1,2-mannosidase activity at the catalytic site [31,32]. It was of interest therefore to test the effect of this inhibitor on the retention of α_1-antitrypsin by Ca^{2+}-

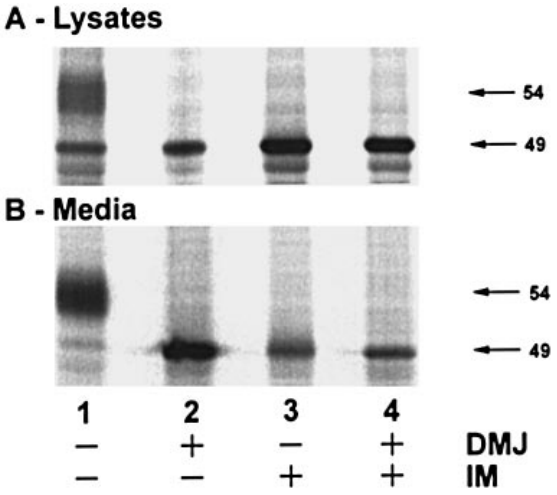

Figure 1 Intracellular retention of a 49 kDa form of α_1-antitrypsin after ionomycin treatment in the absence or presence of an α-1,2-mannosidase inhibitor

HepG2 cells were pretreated for 1 h in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 4 mM 1-deoxymannojirimycin (DMJ), followed by addition of [^{35}S]methionine for 5 min to pulse-label cellular proteins. Excess unlabelled methionine was then added and the chase incubation conducted for 60 min in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 1.5 μM ionomycin (IM). Cells were separated from their respective incubation media by centrifugation. α_1-Antitrypsin was immunoprecipitated from aliquots of cell lysate (**A**) and medium (**B**), and immunoprecipitates were subjected to SDS/PAGE (7.5% gel) followed by autoradiography.

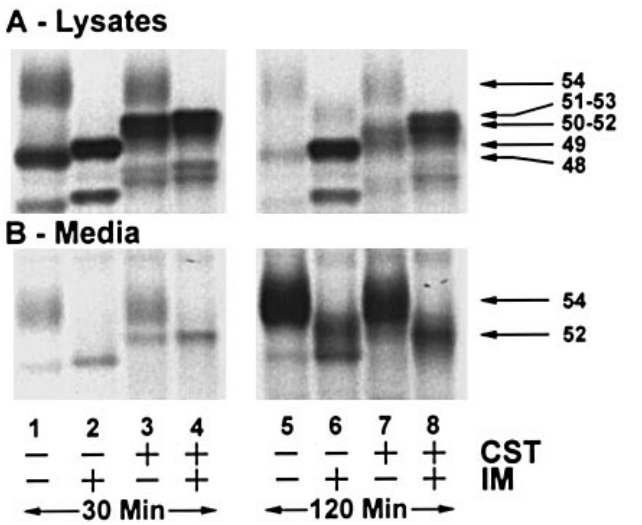

Figure 2 Forms of α_1-antitrypsin retained intracellularly after ionomycin treatment in the absence and presence of a glucosidase inhibitor

HepG2 cells were pretreated for 2 h in the absence (lanes 1, 2, 5 and 6) or presence (lanes 3, 4, 7 and 8) of 500 μg/ml castanospermine (CST), followed by pulse-labelling of cellular proteins with [^{35}S]methionine for 10 min. The chase incubation was then conducted for 30 min (lanes 1–4) or 2 h (lanes 5–8) in the absence (lanes 1, 3, 5 and 7) or presence (lanes 2, 4, 6 and 8) of 1 μM ionomycin (IM). Cells were separated from their incubation media by centrifugation. α_1-Antitrypsin was immunoprecipitated from aliquots of cell lysate (**A**) and medium (**B**), and immunoprecipitates were subjected to SDS/PAGE (7.5% gel) followed by autoradiography.

depleted HepG2 cells (Figure 1). The experimental strategy involved pretreatment of cells first with 1-deoxymannojirimycin, a relatively slow acting inhibitor, followed by Ca^{2+} depletion with ionomycin, a fast acting substance (approx. 6 min), added during the 1 h methionine chase period after pulse-labelling. α_1-Antitrypsin was immunoprecipitated separately from cell lysates and incubation media and analysed by autoradiography after SDS/PAGE. Untreated preparations efficiently processed the glycoprotein to the complex (54 kDa) form (lanes 1), with the majority being secreted into the medium (lane B1). Treatment with ionomycin arrested almost all of the protein in the high-mannose (49 kDa) form with little secretion into the medium (lanes 3). Treatment with 1-deoxymannojirimycin also produced the 49 kDa form, which was readily secreted into the medium without further modification (lanes 2). Treatment with both inhibitors (lanes 4) produced the 49 kDa protein with intracellular retention comparable with that found with ionomycin treatment alone. The retention of the 49 kDa α_1-antitrypsin intermediate by Ca^{2+}-depleted cells therefore did not appear to derive from binding to an ER α-1,2-mannosidase.

Retention of α_1-antitrypsin in the Ca^{2+}-depleted ER could potentially derive from an association with either calnexin or calreticulin, Ca^{2+}-binding chaperones that facilitate folding of glycoprotein intermediates containing $Glc_1Man_9GlcNAc_2$ structures [33,34]. Association with these chaperones is abrogated by the glucosidase inhibitor castanospermine, which inhibits hydrolysis of the two outermost glucose residues of the core oligosaccharide side chains [31,35]. The Golgi-situated endo-α-D-mannosidase present in HepG2 cells [36,37] provides a glucosidase-independent pathway for conversion into the mature complex oligosaccharide structure and subsequent secretion. The effect of Ca^{2+} depletion on the transport competence of the glucosylated α_1-antitrypsin intermediate was investigated (Figure 2). HepG2 cells were pretreated with castanospermine before pulse-labelling and chased for 30 min or 2 h in the absence or presence of ionomycin. Analysis of immunoprecipitates revealed that castanospermine treatment alone resulted in the formation of 50–52 kDa intracellular intermediates (lanes A3 and A7). These intermediates were sensitive to Endo H digestion (see Figure 4) and, like the 48 kDa intermediate produced by untreated controls (Figure 2, lanes A1 and A5), were subsequently processed to the 54 kDa mature form and exported to the medium (lanes B3 and B7 and B1 and B5). Ionomycin treatment by itself produced a 49 kDa intermediate that was retained intracellularly; by 2 h significant amounts of a diffuse approx. 52 kDa product was secreted (lanes 2 and 6). Combined treatment with castanospermine and ionomycin produced Endo H-sensitive α_1-antitrypsin intermediates that migrated at slightly higher molecular masses (51–53 kDa) than intermediates produced with castanospermine alone. The intermediates produced by the combined treatment were subjected to slow secretion (approx. 50% in 2 h) and were not processed further (lanes A4, A8, B4 and B8). Ca^{2+} depletion therefore resulted in the retention of $Glc_{1-3}Man_{7-9}GlcNac_2$ intermediates developed by castanospermine inhibition. Retention did not relate, however, to binding of these intermediates to the lectin-like chaperones specific for monoglucosylated oligosaccharides. It should be noted that the 51–53 kDa α_1-antitrypsin intermediates produced by joint treatment with castanospermine and ionomycin were never generated by ionomycin alone regardless of ionophore concentration tested or time of addition either before or after pulse-labelling (not shown).

The retention of α_1-antitrypsin by cells exposed to Ca^{2+}-mobilizing agents was investigated for an oligosaccharide side-chain requirement. Optimal folding, intracellular transport and secretion of various glycoproteins have been found to depend on

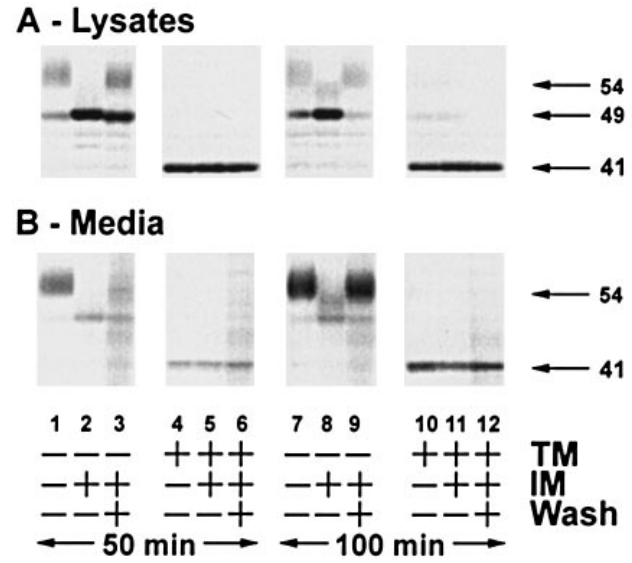

Figure 3 Differential effects of ionomycin on transport of glycosylated compared with non-glycosylated α_1-antitrypsin and reversibility of these effects

HepG2 cells were pretreated for 2 h in the absence (lanes 1–3 and 7–9) or presence (lanes 4–6 and 10–12) of 12.5 μg/ml tunicamycin (TM), followed by pulse-labelling of cellular proteins with [^{35}S]methionine for 5 min. The chase incubation was then conducted in the absence (lanes 1, 4, 7 and 10) or presence (lanes 2, 3, 5, 6, 8, 9, 11 and 12) of 1 μM ionomycin (IM). At 20 min of the chase, selected ionomycin-treated preparations (lanes 3, 6, 9 and 12) were adjusted with 2 mg/ml fatty acid-free BSA to bind ionophore, and the remainder of the chase was conducted in Ca^{2+}-enriched ionomycin-free medium (Wash). At 50 (lanes 1–6) or 100 (lanes 7–12) min of the chase, cells were separated from their incubation media by centrifugation. α_1-Antitrypsin was immunoprecipitated from aliquots of cell lysate (**A**) and medium (**B**), and immunoprecipitates were subjected to SDS/PAGE (7.5% gel) followed by autoradiography.

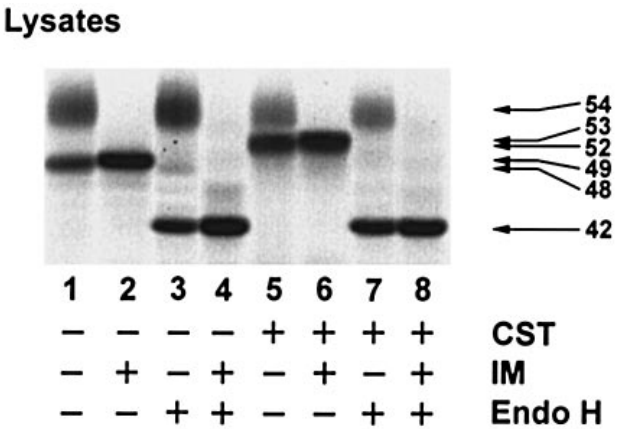

Figure 4 Endo H digestion products of α_1-antitrypsin synthesized after incubations in the absence or presence of castanospermine and with or without ionomycin

Cells were pretreated for 2 h in the absence (lanes 1–4) or presence (lanes 5–8) of 500 μg/ml castanospermine (CST), followed by pulse-labelling of cellular proteins with [^{35}S]methionine for 10 min. The chase incubation was conducted for 30 min in the absence (lanes 1, 3, 5 and 7) or presence (lanes 2, 4, 6 and 8) of 1 μM ionomycin (IM). α_1-Antitrypsin was immunoprecipitated from cell lysates, and immunoprecipitates were incubated in the absence (lanes 1, 2, 5 and 6) or presence (3, 4, 7 and 8) of 16 m-units of Endo H as described in the Experimental section. Aliquots of digests were subjected to SDS/PAGE (7.5% gel), followed by autoradiography.

an attached core oligosaccharide structure, whereas other glycoproteins are secreted regardless of glycosylation [33]. Non-glycosylated rat α_1-antitrypsin is subject to secretion, albeit at a greatly reduced rate compared with its glycosylated counterpart, indicating that glycosylation is not a prerequisite for secretion of this protein [38]. Tunicamycin, which inhibits the synthesis of the core oligosaccharide [31], was utilized to determine the disposition of non-glycosylated α_1-antitrypsin on depletion of ER Ca^{2+} stores. Cells were pretreated for 2 h with or without tunicamycin, pulse-labelled and chased for 50 or 100 min in the absence or presence of ionomycin followed by analysis of lysates and media for immunoprecipitable α_1-antitrypsin (Figure 3). In accord with a previous report [38], tunicamycin treatment generated a 41 kDa non-glycosylated form of the protein, which was slowly secreted into the medium (lanes 4, 10). Tunicamycin pretreatment followed by ionomycin chase resulted in a slight increase in intracellular retention (lane A11), and a comparably small decrease in secretion (lane B11), of the 41 kDa non-glycosylated α_1-antitrypsin. Conversely, a profound increase in intracellular retention was observed for the 49 kDa high-mannose intermediate when chased in the presence of ionomycin (lanes A2 and A8), as compared with its absence (lanes A1 and A7). Therefore Ca^{2+} mobilization affected the transport competence of the glycosylated form of α_1-antitrypsin more prominently than the non-glycosylated form of the protein.

The inhibition of α_1-antitrypsin maturation and export by ionomycin is fully reversible by a single fatty acid-free BSA wash provided that Ca^{2+} is available in the medium [27]. This reversal was utilized to compare the transport of the non-glycosylated and high-mannose forms of α_1-antitrypsin for Ca^{2+}-dependence (Figure 3). Tunicamycin-pretreated and non-treated HepG2 cells were exposed to ionomycin for 20 min during the methionine chase, washed with fatty acid-free BSA, and the chase completed in Ca^{2+}-enriched medium without ionophore. Ionomycin-treated cells exposed to this treatment regained the ability to process and export α_1-antitrypsin. Two α_1-antitrypsin conversions were noted in lysates. The initial change involved conversion of α_1-antitrypsin from the 49 kDa ionomycin-arrested form (lane A2) into a 48 kDa form after the BSA wash (lane A3). This 48 kDa form of α_1-antitrypsin corresponded to the intermediate detected in the untreated control (lane A1). Conversion from the 48 kDa high-mannose form to the 54 kDa complex secretable form was detectable by 50 min (lanes 3), and export of the fully processed mature form was nearly complete by 100 min (lanes 9). Cells from which ionomycin was not removed converted a minor fraction of the 49 kDa form into a secretable 52 kDa form (lanes 8). The modest effect of ionomycin on tunicamycin-pretreated cells was similarly reversed by fatty acid-free BSA wash and Ca^{2+} restoration (lanes 6 and 12). Since the effects of ionomycin on the retention of either the fully glycosylated or the non-glycosylated form of α_1-antitrypsin were readily reversible, it was apparent that neither retention arose from the formation of insoluble aggregates.

A potential association between transport-incompetent forms of α_1-antitrypsin and specific proteins in the Ca^{2+}-depleted ER was not detected. Co-immunoprecipitation with antibodies to α_1-antitrypsin conducted in the presence of various cross-linking agents, followed by SDS/PAGE and radioanalysis, revealed no preferential association of α_1-antitrypsin with ER chaperones or other cellular proteins after treatment with Ca^{2+}-mobilizing agent (not shown). Similar approaches employing an antibody to GRP78/BiP revealed no detectable association of this chaperone with α_1-antitrypsin in ionomycin-treated cells, although the same antibody efficiently co-immunoprecipitated unfolded forms of

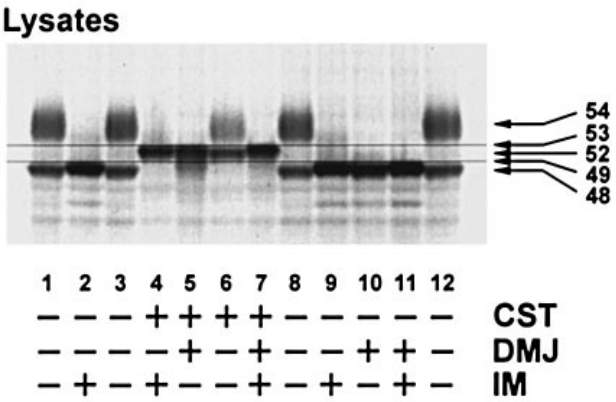

Figure 5 Similar effects of deoxymannojirimycin and ionomycin on the apparent molecular masses of glucosylated and de-glucosylated α_1-antitrypsin

HepG2 monolayers were pretreated for 2 h without inhibitor (lanes 1, 2, 3, 8, 9 and 12), with 500 μg/ml castanospermine (CST, lanes 4 and 6), with 4 mM deoxymannojirimycin (DMJ, lanes 10 and 11), or with both castanospermine and deoxymannojirimycin (lanes 5 and 7). Cells were harvested and suspended in pulse media containing pretreatment agents, and cellular proteins were pulse-labelled with [^{35}S]methionine for 5 min. The chase incubation was conducted for 30 min in the absence (lanes 1, 3, 5, 6, 8, 10 and 12) or presence (lanes 2, 4, 7, 9 and 11) of 1 μM ionomycin (IM). α_1-Antitrypsin was immunoprecipitated from cell lysates and resolved by SDS/PAGE (9% gel) followed by autoradiography. Parallel lines have been inserted to highlight differences in electrophoretic mobilities.

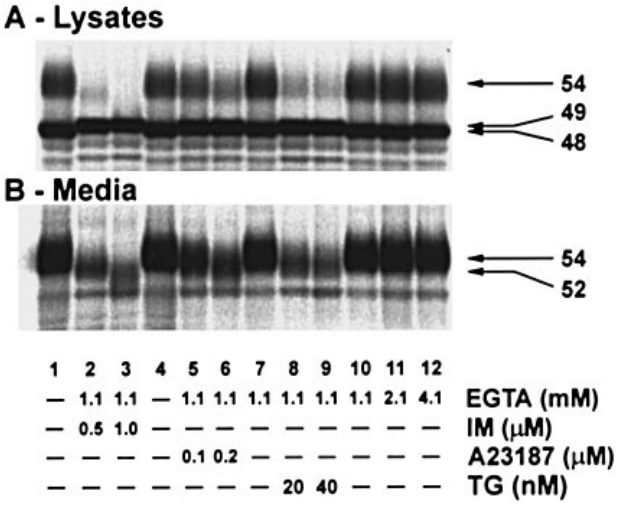

Figure 6 Secretion of an underprocessed form of α_1-antitrypsin by HepG2 cells with depleted ER Ca^{2+} stores

Pulse-labelling of cellular proteins with [^{35}S]methionine was conducted for 15 min. After 5 min of labelling, preparations were treated with 0.5 or 1 μM ionomycin (IM, lanes 2 and 3), 0.1 or 0.2 μM A23187 (lanes 5 and 6), 20 or 40 μM thapsigargin (TG, lanes 8 and 9), or 1.1, 1.1, 2.1 or 4.1 mM EGTA (lanes 7, 10, 11 and 12 respectively), or were left untreated (lanes 1 and 4). Excess unlabelled methionine was added 10 min after the addition of drugs, and chase incubations were conducted for 30 min or 2 h. α_1-Antitrypsin was immunoprecipitated from aliquots of cell lysate (**A**) at 30 min or from aliquots of medium (**B**) at 2 h. Immunoprecipitates were subjected to SDS/PAGE (7.5% gel) followed by autoradiography.

albumin from cells treated with a thiol-reducing agent (G. R. Cooper, C. O. Bostrom and M. A. Bostrom, unpublished work).

Effects of ionomycin on oligosaccharide processing

As noted earlier (Figures 2 and 3), mobilization of Ca^{2+} stores with ionomycin resulted in the formation of a high-mannose intermediate with a slightly higher apparent molecular mass (49 kDa) than the high-mannose intermediate (48 kDa) formed in the absence of ionomycin. Similarly, pretreatment with castanospermine generated a 52 kDa glucose-containing intermediate in the absence of Ca^{2+} mobilization and a larger 53 kDa intermediate in the presence of Ca^{2+} ionophore (Figure 2). Endo H digestion (Figure 4) of each of the intermediates of α_1-antitrypsin described above (48, 49, 52 and 53 kDa; lanes 1, 2, 5 and 6 respectively) yielded an identical product (approx. 42 kDa) as determined by SDS/PAGE and autoradiography (lanes 3, 4, 7 and 8). These data indicate that the observed differences in size resulted from altered processing of the oligosaccharide portion of α_1-antitrypsin in the presence of ionomycin and were not due to changes in other portions of the molecule.

The activity of the ER α-1,2-mannosidase has been reported to be stimulated by Ca^{2+} *in vitro* [32]. HPLC analysis revealed that α_1-antitrypsin intermediates of similar composition were generated by treatment with the mannosidase inhibitor 1-deoxymannojirimycin or by either of two Ca^{2+}-mobilizing agents [28]. The ability of 1-deoxymannojirimycin to cause the same increase in apparent molecular mass as seen with ionomycin (see Figure 2) was therefore examined (Figure 5). In addition, combined treatment with castanospermine and 1-deoxymannojirimycin was tested for the production of a 53 kDa intermediate comparable with that produced by exposure to castanospermine and ionomycin. Untreated controls chased in the absence of ionomycin produced the typical 48 kDa intermediate, much of which was converted into the mature 54 kDa species (lanes 1, 3, 8 and 12) within 30 min. The addition of ionomycin to the chase medium resulted in accumulation of the slightly larger 49 kDa intermediate (lanes 2 and 9). Pretreatment with 1-deoxymannojirimycin resulted in the production of an identical 49 kDa form whether chased with or without ionomycin (lanes 11 and 10 respectively). Castanospermine pretreatment alone resulted in formation of a 52 kDa species that was processed further to the mature 54 kDa product (lane 6). When castanospermine-pretreated cells were exposed to either 1-deoxymannojirimycin or ionomycin, or both, a larger 53 kDa intermediate was produced that was not further processed to the 54 kDa form (lanes 5, 4 and 7 respectively). The slight increase in molecular mass seen during the chase in the presence of ionomycin must therefore derive from impaired mannose trimming.

Secretion of underprocessed forms of α_1-antitrypsin by cells depleted of ER Ca^{2+}

Delayed secretion of a 52 kDa Endo H-resistant form of α_1-antitrypsin was observed when chase with ionomycin was extended beyond approx. 90 min (see Figure 2, lane B6). A similar Endo H-resistant underprocessed form of α_1-antitrypsin was reported by others to be secreted from HepG2 cells exposed to the Ca^{2+} ionophore A23187, but not with ionomycin [26]. That report concluded that A23187 selectively inhibited addition of terminal sialic acid residues in the Golgi in a manner that was unrelated to Ca^{2+} mobilization. It was therefore of interest to compare the effects of several agents that mobilize ER-sequestered Ca^{2+} on the production of the 52 kDa α_1-antitrypsin species using pulse–chase methodology (Figure 6). α_1-Antitrypsin was immunoprecipitated from cell lysates after 30 min and from

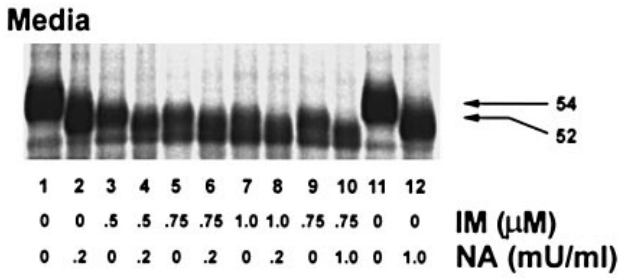

Figure 7 Neuraminidase sensitivity of the underprocessed form of α_1-antitrypsin secreted by HepG2 cells with depleted Ca^{2+} stores

Cellular proteins were pulse-labelled with [^{35}S]methionine for 5 min. The chase incubation was then conducted for 2 h in the absence (lanes 1, 2, 11 and 12) of ionomycin or with 0.5 (lanes 3 and 4), 0.75 (lanes 5, 6, 9 and 10) or 1 (lanes 7 and 8) μM ionophore. α_1-Antitrypsin was immunoprecipitated from aliquots of medium, and washed immunoprecipitates were incubated for 16 h without (lanes 1, 3, 5, 7, 9 and 11) or with 0.2 (lanes 2, 4, 6 and 8) or 1 (lanes 10 and 12) m-unit (mU) of streptococcal neuraminidase (NA) as described in the Experimental section. Aliquots of digests were subjected to SDS/PAGE (7.5% gel), followed by autoradiography.

extracellular media after 2 h of chase. As compared with controls chased for 30 min without the addition of Ca^{2+}-mobilizing drugs (lanes A1 and A4), synthesis of mature 54 kDa α_1-antitrypsin was inhibited by chase medium containing 0.5 or 1 μM ionomycin (lanes A2 and A3), 0.1 or 0.2 μM A23187 (lanes A5 and A6), or 20 or 40 μM thapsigargin, a selective inhibitor of ER Ca^{2+}-ATPases [39] (lanes A8 and A9). Conversion was not inhibited after a 30 min chase with 1.1, 2.1 or 4.1 mM EGTA (lanes A7, A10, A11 and A12), which acts extracellularly to lower cytosolic free Ca^{2+} rapidly (s) but mobilizes Ca^{2+} stores of HepG2 cells slowly [27]. At the later time of chase, processing and secretion of the 54 kDa complex form were largely complete for control preparations (lanes B1 and B4) or for preparations exposed to EGTA (lanes B7, B10, B11 and B12). Treatment with either of the ionophores or with thapsigargin resulted in delayed secretion of faster migrating forms of α_1-antitrypsin (lanes B2, B3, B5, B6, B8 and B9).

To explore the possibility that the faster migrating forms of α_1-antitrypsin possessed fewer sialic acid residues than the mature 54 kDa form, the sensitivity of the secreted forms to neuraminidase digestion was compared. Pulse-labelled preparations were chased for 2 h in the absence or presence of 0.5, 0.75 or 1 μM ionomycin, and secreted forms of α_1-antitrypsin were immunoprecipitated from the extracellular media. Immunoprecipitates were incubated in the absence or presence of 0.2 or 1 m-unit/ml streptococcal neuraminidase, followed by analysis by SDS/PAGE and autoradiography (Figure 7). The fully processed 54 kDa species secreted by control preparations (lanes 1 and 11) was digested to a more rapidly migrating form on incubation with either 0.2 or 1 m-unit/ml neuraminidase (lanes 2 and 12). Electrophoretic migration of secretable α_1-antitrypsin progressively increased with increasing ionomycin concentration during the chase (lanes 3, 5, 7 and 9). Digestion of these underprocessed forms with 0.2 or 1 m-unit/ml neuraminidase further increased migration (lanes 4, 6, 8 and 10), indicating the presence of sialic acid residues. The shift in apparent molecular mass after neuraminidase digestion of the fully processed 54 kDa form appeared to be somewhat greater than the shift observed after digestion of the underprocessed forms secreted by ionomycin-treated cells (compare the mid-points of the protein bands of lanes 11 and 12 with those of lanes 9 and 10).

DISCUSSION

The processing and secretion of such glycoproteins as α_1-antitrypsin, α-chymotrypsin, the C3 component of complement, the asialoglycoprotein receptor and various viral glycoproteins are clearly maintained by ER-sequestered Ca^{2+} [17,26,27,40], but the features of the Ca^{2+} requirement have not been clearly defined. The findings of the present report, in conjunction with previous results [28], support two distinct requirements for ER-sequestered Ca^{2+} in α_1-antitrypsin processing and export. These conclusions are based on alterations imposed by mobilization of the cation either with Ca^{2+} ionophores or thapsigargin. First, Ca^{2+} mobilization substantially slowed the exit of high-mannose forms of α_1-antitrypsin from the ER to rates approximating those observed for the non-glycosylated form produced during tunicamycin treatment. Second, Ca^{2+} mobilization inhibited the removal of mannose residues by ER mannosidases such that a slightly larger than normal form of α_1-antitrypsin (approx. 49 kDa) accumulated within the ER. Based on previous HPLC data, this form of α_1-antitrypsin was characterized by an increased content of $Man_9GlcNAc_2$ oligosaccharide side chains as compared with the normal processing intermediate (48 kDa), which contained predominantly $Man_8GlcNAc_2$ side chains. ER Ca^{2+} depletion clearly did not inhibit the hydrolysis of glucose residues from the oligosaccharide side chains by glucosidases I and II.

Potential physical retention of α_1-antitrypsin as a non-dissociable complex with a mannosidase at low ER Ca^{2+} was considered. Inhibition of mannosidases with 1-deoxymannojirimycin in conjunction with ionophore converted the rapid secretion of the 49 kDa α_1-antitrypsin intermediate to the slow rate seen with ionophore treatment alone. The inability of 1-deoxymannojirimycin to prevent the ionophore-mediated arrest of the 49 kDa intermediate established that retention of α_1-antitrypsin was not due to an association with a mannosidase. The possibility that retention of α_1-antitrypsin involved other components of the oligosaccharide-processing pathway was therefore examined.

Calnexin and the closely related protein, calreticulin, exemplify protein chaperones with lectin-like bonding properties that function in glycoprotein processing at steps thought to precede mannosidase action. The initial binding of various glycoproteins to calnexin and calreticulin appears to depend on trimming of the two outermost glucose residues to generate the monoglucosylated form [15,41]. Calnexin is known to interact in this manner with α_1-antitrypsin [14,42]. Another lectin-like component of the oligosaccharide-processing pathway, UDP-Glc–glycoprotein glucosyltransferase, specifically interacts with incompletely folded glucose-free high-mannose oligosaccharides [43]. Castanospermine, by inhibiting glucosidase catalysis [31], effectively prevents the initial association of glycoproteins with calnexin [15,35] and calreticulin [16]. Similarly, castanospermine should prevent the association of glycoproteins with UDP-Glc–glycoprotein glucosyltransferase by preventing glucose removal.

Treatment with castanospermine alone resulted in the formation of an Endo H-sensitive 50–52 kDa intermediate that was clearly distinguishable from either the 49 kDa high-mannose or 54 kDa complex oligosaccharide form of α_1-antitrypsin (Figure 2). This intermediate was readily transported to the Golgi, efficiently processed to the mature 54 kDa complex form and secreted. Glucosidase-independent conversion into the normal mature form presumably required catalysis by a Golgi endo-α-D-mannosidase activity that has been described for HepG2 cells [36,37]. Exposure of castanospermine-pretreated cells to Ca^{2+}-

mobilizing agents, however, resulted in the retention of α_1-antitrypsin as a slightly larger (51–53 kDa) Endo H-sensitive polyglucosylated species. Retention was therefore not explicable in terms of binding to lectin-like chaperones specific for monoglucosylated oligosaccharides. Longer-term treatment (2 h) of cells with castanospermine in conjunction with Ca^{2+}-mobilizing agents resulted in the slow secretion of a 52 kDa form of α_1-antitrypsin that resembled the product secreted during extended incubation with ionophore alone (Figure 2).

Retention of 51–53 kDa polyglucosylated α_1-antitrypsin intermediates by HepG2 cells treated with both castanospermine and ionomycin revealed that the forms of α_1-antitrypsin sensitive to Ca^{2+} mobilization were not restricted to the 49 kDa species retained with ionomycin alone. Polyglucosylated forms of α_1-antitrypsin were never generated by ionomycin in the absence of glucosidase blockade, even when ionomycin was added at different times before or after the [^{35}S]methionine pulse. This suggested either that glucose residues were removed before the effects of Ca^{2+} mobilization were imposed or that the affected protein remained accessible to glucosidases I and II. Pretreatment with castanospermine and 1-deoxymannojirimycin gave a 51–53 kDa α_1-antitrypsin intermediate that resembled the form generated by the combination of castanospermine and ionomycin, but was slightly larger than the 50–52 kDa form generated by castanospermine alone (Figure 5). This minor size differential indicated that the polyglucosylated protein was normally accessible to mannosidases, although, in the presence of Ca^{2+}-mobilizing agent or 1-deoxymannojirimycin, mannose trimming did not occur.

In the presence of tunicamycin, an inhibitor of N-linked glycosylation, many glycoproteins misfold and fail to exit the ER [33]. Pretreatment of HepG2 cells with tunicamycin completely eliminated glycosylation and substantially delayed secretion of the 41 kDa non-glycosylated α_1-antitrypsin such that export of the protein was observed only after 2 h of incubation. Based on densitometric scanning of autoradiograms (see the Experimental section), the extent of intracellular retention of non-glycosylated α_1-antitrypsin was routinely comparable with the retention of high-mannose forms of α_1-antitrypsin after Ca^{2+} mobilization (Figure 3 and results not shown). When tunicamycin pretreatment was combined with Ca^{2+} mobilization, only a modest increase in α_1-antitrypsin retention was observed. This slight differential (roughly 10%) was comparable with the effect of Ca^{2+} mobilization on secretion of albumin, a non-glycosylated protein ([24,25]; results not shown). Efficient exit of α_1-antitrypsin from the ER therefore required both the presence of oligosaccharide side chains and adequate sequestered Ca^{2+}. The simplest explanation for these observations is that both ER-sequestered Ca^{2+} and the attachment of oligosaccharide side chains facilitate the folding of α_1-antitrypsin to a transport-competent conformation. It is equally plausible, however, that the cation and the side chains satisfy the requirements of a putative ER-to-Golgi vesicular 'packaging' system that accelerates transport of the protein.

As discussed above, HPLC analyses of oligosaccharide side chains of α_1-antitrypsin synthesized by cells with depleted ER Ca^{2+} stores revealed impaired mannose trimming. The decreased electrophoretic mobilities observed for the high-mannose and polyglucosylated forms of the glycoprotein synthesized by Ca^{2+}-depleted preparations (Figure 2–6) were fully compatible with retention of mannose residues. Removal of the oligosaccharide side chains by Endo H digestion demonstrated that these electrophoretic alterations were manifested in the glycan portion of the molecules as opposed to the polypeptide backbone (Figure 4). It is notable that the activity *in vitro* of ER α-1,2-mannosidase, an enzyme possessing a Ca^{2+}-binding EF hand motif [44], is reported to be directly stimulated by Ca^{2+} [32]. An alternative that cannot be dismissed, however, is that ER Ca^{2+} depletion alters the conformation of α_1-antitrypsin to a form unsuitable for either mannosidase action or efficient transport.

Formation of the mature complex oligosaccharide structure ordinarily requires that mannose residues be removed from the high-mannose intermediate generated in the ER [45]. Although mannose trimming was clearly impaired in cells with depleted ER Ca^{2+} stores, a complex oligosaccharide form of α_1-antitrypsin that was slightly smaller (52 kDa) than the mature complex (54 kDa) species was nonetheless formed, albeit slowly (Figures 6 and 7). Extended treatments (2 h) with any of three different Ca^{2+}-mobilizing drugs resulted in the generation of this 52 kDa species. It was clear therefore that impaired mannose trimming of the glycoprotein in the ER was responsible for alteration of the final complex oligosaccharide product. The 52 kDa complex form of α_1-antitrypsin appeared to be readily secreted, as it did not accumulate intracellularly beyond the extent observed for the 54 kDa form. Neuraminidase digestion of the underprocessed form revealed the presence of fewer sialic acid residues than in the mature 54 kDa form (Figure 7). In the absence of definitive structural determinations, the unusual 52 kDa form was tentatively concluded to derive from Golgi processing of α_1-antitrypsin containing additional mannose residues.

The ability of Ca^{2+}-mobilizing agents to impede the exit of various secretory glycoproteins from the ER may explain some of the observed relationships between inhibition of glycoprotein processing, inhibition of translational initiation and induction of ER chaperones [7–12,27,28]. With the exception of Ca^{2+}-mobilizing agents and tunicamycin, traditional inhibitors of glycoprotein processing, including 1-deoxymannojirimycin, castanospermine, 1-*N*-methyldeoxynojirimycin and swainsonine, generally do not inhibit protein synthesis or cause induction of ER chaperones [8,28]. Brefeldin A exerts a slowly developing inhibition of protein synthesis over several hours [25,46], and, like ionomycin and tunicamycin, promotes the induction of GRP78/BiP [47]. These three inhibitors each appear to impair exit of glycoproteins from the ER, a property not shared by traditional inhibitors of oligosaccharide processing. The accumulation of underprocessed proteins within the ER appears to be the primary event signalling both inhibition of translational initiation and induction of ER chaperones.

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