Calnexin Phosphorylation Attenuates the Release of Partially Misfolded α1-Antitrypsin to the Secretory Pathway*§

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Calnexin is a type I integral membrane phosphoprotein resident of the endoplasmic reticulum. Its intraluminal domain has been deduced to function as a lectin chaperone coordinating the timing of folding of newly synthesized N-linked glycoproteins of the secretory pathway. Its C-terminal cytosolic oriented extension has an ERK1 phosphorylation site at Ser563 affecting calnexin association with the translocon. Here we find an additional function for calnexin phosphorylation at Ser563 in endoplasmic reticulum quality control. A low dose of the misfolding agent L-azetidine 2-carboxylic acid slows glycoprotein maturation and diminishes the extent and rate of secretion of newly synthesized secretory α1-antitrypsin. Under these conditions the phosphorylation of calnexin is enhanced at Ser563. Inhibition of this phosphorylation by the MEK1 inhibitor PD98059 enhanced the extent and rate of α1-antitrypsin secretion comparable with that achieved by inhibiting α-mannosidase activity with kifunensine. This is the first report in which the phosphorylation of calnexin is linked to the efficiency of secretion of a cargo glycoprotein.

It has generally been accepted that the quality control system of the endoplasmic reticulum (ER)3 prevents the exit of newly synthesized misfolded proteins to the secretory pathway (1). The calnexin cycle (1–3) has been extensively studied to deduce the mechanisms that regulate the selection and sorting of misfolded glycoproteins for their targeting to the proteasome for ER-associated degradation (ERAD). A prolonged time of association of constituents of the calnexin cycle with misfolded glycoproteins coincides with their targeting to ERAD (4). Similar to entry into the calnexin cycle for productive folding, entry to ERAD appears to be based on a sugar code for misfolded glycoproteins with the former being glucose-based and the latter being mannose-based (4–6).

The mannosidase EDEM/Htm1p (7, 8), whose expression is induced by the unfolded protein response (9–11), selectively processes ManN,GlcNAc, glycans on misfolded glycoproteins. This yields glycans containing a terminal α1,6-linked mannose residue. It is this sugar linkage that is recognized by the lectin Yos9p (12) for retrotranslocation of misfolded glycoproteins out of the ER and their degradation by the proteasome (see as well the work of Christianson et al. (13) for mammalian OS-9). An association between calnexin and EDEM facilitates the efficient presentation of misfolded glycoproteins to ERAD (9).

The dogma that ER quality control is highly efficient at preventing the access of newly synthesized misfolded proteins to the cargo exit machinery has been challenged by observations indicating that misfolded variants of transthyretin were efficiently secreted after expression in baby hamster kidney cells or murine hepatocytes (14). These authors further formalized a hypothesis for quality control in which competition of a misfolded protein for the folding machineries of the ER (ER-assisted folding) or the degradation machineries (ERAD) would define its degree of secretion. Transthyretin, however, is nonglycosylated and would not be expected to use the calnexin-dependent quality control machinery.

To manipulate the trafficking of misfolded secretory glycoproteins, we designed a cell-based assay to select conditions that allowed the partial secretion of misfolded glycoproteins. To this end, we chose the well studied human liver HepG2 cell line that secretes plasma proteins including the glycoprotein α1-antitrypsin (AAT), which is a well characterized cargo of the calnexin cycle and ER quality control (9, 15–17). Here we show that a low concentration of the proline analog L-azetidine 2-carboxylic acid (Azc) for a short time results in the misfolding of newly synthesized AAT (AATAzc), as deduced by its markedly enhanced time of association with calnexin and degradation via the proteasome. We also show that under these conditions inhibition of the mannosidase EDEM/Htm1p by kifunensine leads to the abrogation of AATAzc degradation and its enhanced secretion, revealing for the first time an in situ effect on quality control by affecting the mannose code for glycoprotein presentation to ERAD.

In addition, calnexin is phosphorylated on its cytosolic domain by casein kinase II at Ser534 and Ser544 and the mitogen-activated protein kinase ERK1 at Ser663 (18, 19). ERK1 is also activated by MEK1 under conditions of protein misfolding (20).

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3 The abbreviations used are: ER, endoplasmic reticulum; AAT, 1-antitrypsin to the secretory pathway*; GST, glutathione S-transferase; GST-CNXcyto, GST-calnexin cytosolic domain fusion protein; Htm1p, homologous to mannosidase-like protein; IRE1, inositol-requiring enzyme 1; KIF, kifunensine; CRT, calreticulin; EDEM, ER degradation enhancing α-mannosidase-like protein; EDEM; ER degradation enhancing α-mannosidase-like protein; Endo H, Endo-β-N-acetylglucosaminidase H; ERAD, ER-associated degradation; ERGIC, ER-Golgi intermediate compartment; ERK1, extracellular signal-regulated kinase 1; GST, glutathione S-transferase; GST-CNXcyto, GST-calnexin cytosolic domain fusion protein; Htm1p, homologous to mannosidase 1 protein; IRE1, inositol-requiring enzyme 1; KIF, kifunensine; MEK1, mitogen-activated protein kinase ERK1 kinase 1; NHK, null (Hong Kong) mutant of AAT; WT, wild type; XBp1, X-box binding protein 1; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid.
The cell-based assay was therefore also used to test for any role of calnexin phosphorylation in quality control. Using a monospecific antibody to Ser563 of phosphocalnexin, we show an enhanced phosphorylation at this site under the same protein misfolding conditions which led to prolonged AATAzc association with calnexin and its degradation by the proteasome. Inhibition of protein misfolding-induced phosphorylation of calnexin was revealed by the MEK1 inhibitor PD98059. Similar to the action of the mannosidase inhibitor kifunensine, PD98059 resulted in the attenuation of AATAzc degradation and enhanced secretion, revealing for the first time a role for calnexin phosphorylation at Ser563 in quality control.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—Human hepatoma (HepG2) cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum and antibiotics in a 37 °C incubator containing 5% CO2. Transient transfection of the plasmid encoding the human α1-antitrypsin null (Hong Kong) mutant (Dr. I. Wada, Fukushima Medical University School of Medicine, Fukushima, Japan) was performed with FuGENE HD (Roche Applied Science), and the cells were used ~44 h post-transfection. For the generation of stably transfected HepG2 cell lines, the cells were transfected using Lipofectamine (Invitrogen) and either the empty vector (pcDNA3) or the same vector containing the mutant IRE1 cDNA previously described by Nguyén et al. (20). Transfected cells were selected by resistance to geneticin (G418, Invitrogen), and polyclonal populations were selected and amplified.

**Phosphocalnexin Antibody**—The synthetic phosphopeptide KAEEDEILNRpS563PRNRKPRRE of human calnexin was coupled to activated keyhole limpet hemocyanin, and two chickens were each given a primary injection (day 0). At day 21 post-primary injection, the chickens were given a booster injection, and 21 days later the eggs were collected. The egg yolks were delipidated and tested for their specific immunoreactivity against the phosphopeptide (as compared with the nonphosphorylated form). The positive egg yolks were pooled and IgY-purified (Hyperomics Farma Inc.). For each assay, the anti-phosphocalnexin IgY was affinity-purified on nitrocellulose membranes spotted with the phosphopeptide and used immediately after purification.

**Metabolic Pulse-Chase Radiolabeling, Immunoprecipitation, and Endo H Digestion**—For metabolic radiolabeling, HepG2 cells were preincubated for 60 min in methionine/cysteine-free Dulbecco’s modified Eagle’s medium (Invitrogen), followed by a 10-min metabolic pulse radiolabeling with 100 μCi/ml Tran35S label (specific activity, 1048–1504 Ci/mmol; ICN) or EXPRESS35S label (specific activity, 1175 Ci/mmol; PerkinElmer Life Sciences) and then chased for various times in Dulbecco’s modified Eagle’s medium containing an excess of nonradiolabeled methionine/cysteine. Azc (Sigma-Aldrich) was included only in the 60-min preincubation and 10-min radiolabeling medium. KIF (2 μg/ml; Toronto Research Chemicals) and PD98059 (50 μM; Cell Signaling Technology) were included in the preincubation, radiolabeling, and chase medium. Lactacystin (25 μM; Calbiochem) and CHX (20 μg/ml; Sigma-Aldrich) were included only in the chase medium. At the end of the chase period the cells were placed on ice, and the medium was collected. The cells were lysed in 2% CHAPS (Roche Applied Science) as previously described (21). The supernatants were recovered following centrifugation at 435,000 × gmax for 30 min at 4 °C, and the protein content was determined by the Bradford method (22). Aliquots of supernatant were immunoprecipitated with rabbit polyclonal antibody to calnexin or calreticulin. Sequential immunoprecipitations using antibody to AAT and solubilized immune complexes from cell lysates that had first been immunoprecipitated using antibody to calnexin were performed as previously described (15). Alternatively, aliquots of supernatants from cells and media were immunoprecipitated with AAT antibody (Dako). For Endo H digestion, duplicate aliquots of cell and media supernatants were immunoprecipitated with antibody to AAT; one set was then incubated overnight at 37 °C in 150 mM sodium acetate (pH 5.5) and Endo H (Roche Applied Science) at a final concentration of 300 milliunits/ml and compared with mock digestions of immune complexes incubated overnight in the presence of buffer alone. The digestions were stopped by the addition of Laemmli sample preparation buffer followed by heating at 65 °C for 10 min.

**In Vitro Kinase Assay**—*In vitro* kinase assays were performed as previously described (19). Casein kinase II was purchased from Roche Applied Science. ERK1 was immunopurified from serum-stimulated HepG2 cells. For this, the cells were serum-starved overnight followed by the addition of 10% fetal bovine serum for 30 min. The cells were then lysed, and the clarified lysate supernatants were immunoprecipitated with antibody to ERK1 (Santa Cruz Biotechnology). *In vitro* kinase assays were then performed using either casein kinase II or the ERK1 immune complex as the kinase source and the cytosolic domain of calnexin (GST-CN1cyt0, 0.5 μg) (19) as substrate in the presence of ATP. The reactions were stopped by the addition of Laemmli sample preparation buffer and subjected to SDS-PAGE followed by Western blotting (see Fig. 6C). To determine the ERK1 kinase activity in HepG2 cells treated with Azc or Azc+CHX (see Fig. 6D), the cells were lysed, and the clarified lysate supernatants were immunoprecipitated with antibody to ERK1. *In vitro* kinase assays were then performed on the immune complexes using myelin basic protein (0.5 μg) as substrate and [γ-32P]ATP (specific activity 3000 Ci/mmoul). The reactions were stopped with Laemmli sample preparation buffer and subjected to SDS-PAGE followed by radiography. The bands on the radioautogram were quantified by scanning densitometry.

**SDS-PAGE and Quantification**—Metabolically radiolabeled proteins were resolved on 8% Laemmli SDS-polyacrylamide gels, processed for fluorography, dried, and exposed to Kodak X-Omat AR or Biomax MR-2 film. Myelin basic protein was resolved on 15% SDS-polyacrylamide gels, dried, and exposed to Kodak X-Omat AR film. Bands on the radioautograms were quantified by scanning densitometry. The densitometry values for intracellular AAT were expressed as percentages of the maximum intracellular AAT with the exception of Fig. 1C, where the values for intracellular AAT were expressed as percentages of the initial value. For Fig. 7B the densitometry values for intracellular AAT were normalized to the same cell lysate.
protein concentration and expressed as percentages of the maximum. The densitometry values for secreted AAT (Endo H-resistant + Endo H-sensitive) at any time was expressed as percentages of the maximum intracellular AAT. The values were then normalized to the maximum secreted control, which was set to 100% (Figs. 2D and 5A). Alternatively, the densitometry values for the secreted AAT were normalized to the same cell lysate protein concentration and expressed as percentages of the maximum secreted AAT (Fig. 7C). The percentage of AAT degraded (Fig. 4B) was defined as that amount of the maximum intracellular AAT, which was not recovered in the lysate + media at any time. For Fig. 7A the densitometry values for calnexin-associated AAT were normalized to the same cell lysate protein concentration and expressed as percentages of the maximum calnexin-associated AAT. For Fig. 4C all of the densitometry values were calculated to the same protein concentration and the same percentage of original cell lysate volume. The corrected values for calnexin-associated AAT and intracellular AAT were then expressed as percentages of maximum intracellular AAT. The amount of intracellular AAT that was non-calnexin-associated was obtained by subtracting the value for the calnexin-associated AAT from the value for the intracellular AAT.

**Alkaline Phosphatase Assay and Western Blotting**—Equal aliquots of HepG2 cell lysate supernatants were immunoprecipitated with antibody to calnexin. The immune complexes were then incubated in the presence of 10 units of shrimp alkaline phosphatase or buffer alone for 40 min at 37 °C. The reaction was quenched by the addition of Laemmli sample preparation buffer, and the immune complexes were separated by SDS-PAGE, followed by Western blot analysis using either the anti-phosphocalnexin antibody specific to the phospho-Ser563 site of calnexin or anti-calnexin antibody. The bands were visualized using either horseradish peroxidase-conjugated rabbit anti-chicken antibody and enhanced chemiluminescence (ECL) for the anti-phosphocalnexin blots or horseradish peroxidase-conjugated goat anti-rabbit antibody and ECL for the anti-calnexin blots. Alternatively, the alkaline phosphatase assay was performed using purified GST-CNXcyto (0.5 μg) followed by SDS-PAGE and Western blotting as described above.

**Reverse Transcription-PCR**—HepG2 cells were lysed in TRIzol (Invitrogen), and the total RNA was isolated according to the manufacturer’s recommendations. RNA was converted to cDNA using oligo(dT)20 and the Thermoscript reverse transcription-PCR system (Invitrogen). Aliquots of the cDNA synthesis reaction were used for PCR analysis of EDEM, XBP1, and glyceraldehyde-3-phosphate dehydrogenase mRNA expression by amplification with Taq DNA polymerase (MBI Fermentas). The oligonucleotides used for PCR were: 5’-GCTCAACCCCATCCTAGCC and 5’-CAGAGGGAACGTCG for EDEM; 5’-CCGCCATGCTGAGGCC and 5’-CTCCAGGCGTTG for XBP1; and 5’-ACCACCATGGAGGCTG and 5’-CTGATGCTGCGGATG for glyceraldehyde-3-phosphate dehydrogenase (Alpha DNA). PCR-amplified products were collected during the linear part of the amplification reaction and separated on agarose gels. The bands were visualized by UV transillumination.

**Proteinase K Sensitivity Assay**—Metabolically pulse-radiolabeled HepG2 cells, pretreated either with PD98059 alone or PD98059 and Azc, were chased for 180 min in the presence of PD98059 only. The chase medium was collected, and equal aliquots were incubated for 60 min on ice in the presence of increasing concentrations of proteinase K (Roche Applied Science). The digestion was stopped by the addition of 2 μg phenylmethylsulfonyl fluoride. The samples were then immunoprecipitated with antibody to AAT, followed by SDS-PAGE and fluorography.

**RESULTS**

**Cell-based Assay**—To promote AAT misfolding in a manner that still allowed its partial secretion, we used the proline analog 1-azetidine 2-carboxylic acid (Azc). Incubation of HepG2 cells for 70 min in the presence of increasing concentrations of Azc followed by a chase in the absence of Azc (Fig. 1) revealed an inverse relationship between Azc concentration and secretion. A 5 mM Azc concentration was chosen for further studies because this dose still allowed appreciable AAT secretion. By contrast the null (Hong Kong) mutant of AAT (NHK-AAT), a well-established model for terminally misfolded glycoproteins that are targeted to ERAD (9, 23), was not secreted from the cells (Fig. 2B). Only the endogenous wild type AAT (WT-AAT) was observed in the medium.
Incubation of HepG2 cells for 70 min with 5 mM Azc led to the intracellular accumulation of the immature (ER) form of AAT and a slower rate and amount of secretion of mature AAT (Fig. 2, C and D). Hence productively folded AAT, AATAzc, and terminally misfolded NHK-AAT are readily distinguished by the rate and amount of their secretion as well as their acquisition of Endo H resistance.

**Kifunensine Enhances the Secretion of AATAzc**—KIF prevented the degradation of AATAzc (Fig. 4, A/Azc and H11001 KIF) as expected from its effect as an inhibitor of the mannose-6-phosphate (M6P) receptor. Furthermore, splicing of XBP1 mRNA and EDEM expression were enhanced under these same conditions of Azc treatment (Fig. 4, D). Taken together, we conclude that AATAzc was treated as a misfolded glycoprotein by the calnexin cycle with a prolonged association with the pre-existing (nonradiolabeled) calnexin and delivered to the proteasome for degradation.

**FIGURE 2.** Comparison of the wild type, NHK-AAT mutant, and Azc-misfolded AAT in processing and secretion in HepG2 cells. Processing and secretion of newly synthesized AAT (A), transiently transfected NHK-AAT mutant (B), and Azc-misfolded AAT (C) was assessed in radioautograms of intracellular and secreted AAT immunoprecipitated from HepG2 cells incubated for 60 min in the absence (A and B) or presence (C) of 5 mM Azc, followed by a 10-min metabolic pulse radiolabeling in the absence (A and B) or presence (C) of 5 mM Azc. The cells were then chased for the indicated times in medium lacking Azc. The immature (Endo H-sensitive) and mature (Endo H-resistant) forms of AAT are indicated. D, secretion efficiency for endogenous AAT from control cells (A, right side) and Azc-treated cells (C, right side) was calculated as the proportion of secreted AAT (Endo H-resistant/Endo H-sensitive) at any time t over the maximum intracellular AAT. All of the values were then normalized to the maximum secreted control, which was set to 100% (see “Experimental Procedures”).

**FIGURE 3.** Transient association of calnexin but not calreticulin with newly synthesized AAT. A, radioautograms of calnexin (left panel) and calreticulin (right panel) immunoprecipitations from HepG2 cells metabolically pulse-radiolabeled for 10 min and chased for the times indicated. The arrowhead denotes the mobility of calnexin (CNX), and the arrow indicates the mobility of calreticulin (CRT). Molecular mass markers are indicated in kDa to the left of each radioautogram. B, radioautograms of sequential immunoprecipitations performed using antibody to AAT and the solubilized immune complexes from cell lysates that had first been immunoprecipitated with antibody to CNX or calreticulin. The majority of the newly synthesized radiolabeled AAT is calnexin-associated.
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**FIGURE 4. Azc-misfolded AAT is targeted to ERAD via EDEM.** A, radioautograms of immunoprecipitations from HepG2 cell lysates using antibody to calnexin (CNX-associated proteins), and sequential immunoprecipitations using antibody to AAT and solubilized immune complexes from cell lysates that had first been immunoprecipitated using antibody to calnexin (CNX-associated AAT). The cells were incubated for 60 min in the absence (control, ctrl) or presence of 5 mM Azc, followed by a 10-min metabolic pulse radiolabeling in the absence (control) or presence of 5 mM Azc and chase for various times in the absence of Azc, as described under “Experimental Procedures.” The arrowhead indicates the mobility of CNX. Of note is the diminished radiolabeled band corresponding to the mobility of calnexin in Azc treated cells. As described previously, newly synthesized glycoproteins associate with the mature nonradiolabeled calnexin under these conditions of pulse-chase (15). Western blot analysis confirmed that total calnexin levels remained similar even after treatment of the cells with Azc (data not shown). Molecular mass markers are indicated in kDa to the left of the panel. B, extent of AAT loss in HepG2 cells following treatment with 5 mM Azc (closed circles) or 5 mM Azc and 2 μg/ml kifunensine (Azc + KIF, closed triangles). Azc treatment, metabolic pulse radiolabeling, and chase were performed as for A. KIF was present in the cell medium throughout the experiment. Immunoprecipitation of intracellular and secreted AAT were performed as described under “Experimental Procedures.” Radioautograms were quantified by scanning densitometry. The percentage of AAT degraded was defined as the amount of the maximum intracellular AAT that was not recovered in the lysate + medium at any time t. C, quantification by densitometry scanning of the radioautograms for calnexin-associated and intracellular AAT from HepG2 cells treated with 5 mM Azc followed by a chase for 180 min in the presence or absence of the proteasome inhibitor lactacystin (Lct). The AAT observed at 180 min of chase is the misfolded AAT because no intracellular AAT is observed at this chase time in untreated (control) cells (see Fig. 2A). Azc treatment and metabolic pulse radiolabeling of the cells were performed as for A. Immunoprecipitation of intracellular AAT was performed as described under “Experimental Procedures.” Sequential immunoprecipitations for the calnexin-associated AAT were performed as for A. All of the densitometry values were calculated to the same protein concentration and the same percentage of original lysate volume. The corrected values for calnexin-associated and intracellular AAT and intracellular AAT were then expressed as a percentage of the maximum intracellular AAT. The amount of intracellular AAT that was non-calnexin-associated was obtained by subtracting the value for the calnexin-associated from the value for the intracellular AAT. D, reverse transcription-PCR analysis of EDEM, XBP1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression in HepG2 cells incubated for 60 min in the absence (control) or presence of 5 mM Azc, followed by a 180-min incubation without Azc (see “Experimental Procedures”). The bands were visualized by UV transillumination of the ethidium bromide-stained agarose gels. uXBPI, unspliced XBPI; sXBPI, spliced XBPI.

sidase activity of EDEM/Htm1p. Therefore degradation of AATAzc was considered a consequence of the mannosidase activity of EDEM/Htm1p. KIF treatment also enhanced the secretion of AATAzc (Fig. 5A), albeit at a slower rate than that of wild type AAT (Fig. 2D, control). However, this was not due to an enhanced dissociation of AATAzc from calnexin (Fig. 5B). Rather calnexin association with AATAzc was more prolonged (Fig. 5B, Azzc + KIF) coincident with the inhibition of AATAzc degradation by KIF (Fig. 4B). Indeed the majority of total intracellular AATAzc remained as an immature form upon KIF treatment (Fig. 5C, Azzc + KIF). Taken together, the simplest explanation is that inhibition of EDEM mannosidase activity prevented the presentation of AATAzc to the proteasome (see “Discussion”) and led to an increased intracellular pool that with time was able to access the secretory pathway.

**Enhanced Calnexin Phosphorylation at Ser563 by Azc—** incubation of HepG2 cells with Azc led to the enhanced phosphorylation of calnexin as shown by a pharos-specific antibody to Ser563 of calnexin (Fig. 6A, upper blot, lane 2). Azc-induced phosphorylation of calnexin was not blocked by the casein kinase II inhibitor 5,6-dichlorobenzimidazole riboside as deduced by P-32 incorporation into calnexin (data not shown). Even after washout of Azc, enhanced phosphorylation was maintained for 60 min and did not need ongoing protein synthesis (Fig. 6A, upper blot, lane 3). Quantification revealed that the greatest phosphorylation at Ser563 was observed when the cells were treated with Azc and then chased for 60 min with cycloheximide alone (Fig. 6A, histogram, Azc + CHX); enhanced phosphorylation was also observed with Azc treatment alone (Fig. 6A, histogram, Azc). Antibody recognition of phosphorylated calnexin was abrogated by prior treatment of anti-calnexin immunoprecipitates with alkaline phosphatase (Fig. 6B, upper
Furthermore, the antibody did not recognize the cytosolic domain of calnexin phosphorylated in vitro by casein kinase II (Fig. 6C, upper blot, lane 3). Only the calnexin cytosolic domain phosphorylated by ERK1 was recognized by the antibody (Fig. 6C, upper blot, lane 4). Therefore we conclude that the antibody is specific to phospho-Ser\(^{563}\) of calnexin.

Ser\(^{563}\) has been previously documented as an ERK1 kinase substrate (18). Azc alone, or after washout and a chase with CHX, led to enhanced ERK1 kinase activity that was inhibited by the MEK1 inhibitor PD98059 (Fig. 6D). Enhanced phosphorylation of calnexin at Ser\(^{563}\) upon incubation of cells with Azc was inhibited by PD98059 (Fig. 6E, lane 4). This phosphorylation event was attributed to IRE1 activation because dominant negative IRE1 expression prevented Ser\(^{563}\) phosphorylation of calnexin under conditions of protein misfolding by Azc (Fig. 6E, lane 6). These results show an ~2.5-fold increase in calnexin phosphorylation at Ser\(^{563}\) with Azc and a >3-fold increase with Azc + CHX (Fig. 6A) and are consistent with the magnitude of the increases in ERK1 kinase activity observed in independent experiments (Fig. 6D).
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Taken together we conclude that phosphorylation of calnexin at Ser^{563} is mediated by ERK1 activation during conditions of protein misfolding and inhibited by the MEK1 inhibitor PD98059. This would occur downstream of IRE1 activation upon accumulation of misfolded proteins in the ER. The CHX effect to enhance calnexin phosphorylation even after Aza washout followed by a chase with CHX was unexpected.

Inhibition of Ser^{563} Phosphorylation Leads to the Secretion of AATAzc—The effect of PD98059 on calnexin association of AATAzc was tested (Fig. 7). PD98059 did not appreciably affect the initial selection of newly synthesized glycoproteins, whether Aza-misfolded or not (Fig. 7A, 0 min of chase; quantification, right side). However, at 60 min of chase, a marked effect of PD98059 on calnexin association with AATAzc was apparent (Fig. 7A). This dissociation was not dependent on protein synthesis because dissociation was also seen in the presence of CHX during 60 min and even 180 min of chase after Aza washout. Dissociation from calnexin was not limited to AATAzc because all calnexin-associated Aza-misfolded glycoproteins revealed enhanced dissociation in the presence of PD98059 at these chase times (supplemental Fig. S1, left panel).

Dissociation of AATAzc from calnexin in the presence of PD98059 coincided with increased ER exit as measured by the maturation of AAT (Fig. 7B, quantification, right side). This also coincided with secretion of Endo H-resistant mature AAT (Fig. 7C, quantification, right side). No secretion of the NHK mutant of AAT was observed with PD98059 (Fig. 7C, left side), even in the presence of Aza (Fig. 8D, right panel), although small effects on the amount of NHK-AAT associated with calnexin (Fig. 8A, NHK-AAT) and in the intracellular pool (Fig. 8B, NHK-AAT) were observed at 0 min of chase. By contrast, AATAzc in the same cells showed enhanced secretion of the mature Endo H-resistant form upon PD98059 treatment (Fig. 8D, right panel, WT-AAT).

The AAT secreted from control and Aza-treated cells showed only a small difference in their sensitivity to increasing concentrations of proteinase K (Fig. 9). This may indicate that AAT-Aza has a conformation that is similar to the AAT in untreated (non-Aza) cells.
DISCUSSION

AAT is a well characterized cargo protein of the calnexin cycle whose association with calnexin has been shown to be N-glycan-dependent (15, 16). We concluded that AATAzc was misfolded after the 70-min incubation of HepG2 cells with Azc because a marked increase was observed in the time of association of newly synthesized radiolabeled AATAzc with calnexin (see as well Ref. 15), and inhibiting the activity of the proteasome by treatment of the cells with lactacystin led to an accumulation of intracellular AATAzc. Because degradation of AATAzc was inhibited by KIF, the pathway of AATAzc presentation to the proteasome via the mannosidase EDEM/Htm1p was concluded to be operational.Remarkably, KIF also led to the slow but ultimately nearly complete secretion of radiolabeled AATAzc. This is noteworthy because the terminally misfolded null (Hong Kong) mutant of AAT has been reported to be refractory to KIF rescue (24).

Nyfeler et al. (25) have used a protein fragment complementation assay to detect an association between the mannos lectin ERGIC-53 and wild type AAT, but in contrast the null (Hong Kong) AAT mutant showed little association. Because a knockdown of ERGIC-53 in HepG2 cells impaired the secretion of wild type AAT, Nyfeler et al. concluded that ERGIC-53 was a transport receptor for wild type but not mutant AAT. They also demonstrated that ERGIC-53 association with AAT was not affected by treatment of the cells with KIF. As the postulated transport receptor, it may be the competition between access to the secretory pathway via the mannos lectin ERGIC-53 and the degradation (ERAD) pathway via the mannosidase/mannose lectins EDEM (Htm1p)/Yos9p (OS-9), which defines the efficiency of ERGIC-53-mediated transport of AAT to the Golgi apparatus and then to the cell surface for extracellular discharge. This would be in agreement with the model of Sekijima et al. (14) as applied to secretory glycoproteins.

The cell-based assay using KIF and a titrated dose of Azc enabled testing of the sugar-based code for AATAzc presentation to the proteasome and any effect on its secretion. Even though this is the first report of KIF-mediated rescue of secretion for a misfolded glycoprotein (AAT), the results are predicted by current accumulated data on quality control.

The use of a phospho-Ser563-specific antibody to calnexin enabled a test of calnexin phosphorylation and ER quality control. The antibody revealed specificity for calnexin phosphorylated at Ser563 and complements previous work by us and others using mass spectrometry to deduce calnexin phosphorylation at this site (18, 26–28). With this antibody an effect of PD98059 on the rescue of AATAzc secretion coincided with an inhibition of phosphorylation at Ser563 of calnexin. Enhanced calnexin phosphorylation at Ser563 in HepG2 cells under conditions of glycoprotein misfolding coincided with ERK1 activation. This result is consistent with our previous finding that ERK1 is associated with calnexin and that ERK1 activation leads to the recruitment of calnexin to membrane-bound ribosomes (19). Indeed, an increased requirement for the control of protein folding at the ER may result from the detection of misfolded protein accumulation in the ER lumen by unfolded pro-
Inhibition of calnexin phosphorylation at Ser\(^{563}\) by PD98059 in Azc-treated cells inhibited presentation of AAT\(^{Azc}\) to the proteasome but enhanced the dissociation of AAT\(^{Azc}\) from calnexin. This coincided with enhanced secretion of AAT\(^{Azc}\). Remarkably, secreted AAT\(^{Azc}\) in PD98059-treated cells showed a similar sensitivity to proteinase K digestion as that of AAT secreted from non-Azc PD98059-treated cells, suggesting that AAT\(^{Azc}\) had attained a near wild type conformation. Further experiments will be needed to test this conjecture.

Our results predict a further level of regulation of quality control for the calnexin pathway, here involving calnexin phosphorylation. It may very well represent a fine tuning mechanism uncovered here through the differential incorporation of Azc into AAT during the 70-min pulse with Azc. Cycloheximide may augment the selection by Ser\(^{563}\) of phosphocalnexin for misfolded AAT intermediates better capable of rescue by prolonged repeated rounds of association with calnexin. An extension of these experiments in cells where Ser\(^{563}\) mutant calnexin has replaced the endogenous calnexin may enable further testing of this hypothesis, as would an extension to AAT mutants other than the terminally misfolded null (Hong Kong) to test for clinical relevance.

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