Endoplasmic Reticulum Stress Response and Mutant Protein Degradation in CHO Cells Accumulating Antithrombin (C95R) in Russell Bodies

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The endoplasmic reticulum (ER) is the site of the synthesis and maturation of secretory proteins. Correct folding and assembly of newly synthesized secretory proteins are critically important for maintaining cell function and an efficient protein quality control system in the ER. When unfolded or aggregated proteins accumulate in the ER, certain signaling pathways such as the unfolded protein response (UPR) and ER-overload response (EOR) are functionally active in maintaining cell homeostasis. Recently we prepared Chinese hamster ovary (CHO) cells expressing mutant antithrombin (AT)(C95R) under control of the Tet-On system and showed that AT(C95R) accumulated in Russell bodies (RB), large distinctive structures derived from the ER. To characterize whether ER stress takes place in CHO cells, we examined characteristic UPR and EOR in ER stress responses. We found that the induction of ER chaperones such as Grp97, Grp78 and protein disulfide isomerase (PDI) was limited to a maximum of approximately two-fold. The processing of X-box-binding protein-1 (XBP1) mRNA and the phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) subunit were not induced. Furthermore, the activation of nuclear factor-kappa B (NF-xB) was not observed. In contrast, CHO cells displayed UPR and EOR when the cells were treated with thapsigargin and tumor necrosis factor (TNF)-α, respectively. In addition, a portion of the mutant AT(C95R) was degraded through proteasomes and autophagy. CHO cells do respond to ER stress but the folding state of mutant AT(C95R) does not appear to activate the ER stress signal pathway.

Key words endoplasmic reticulum (ER); ER stress; mutant antithrombin; unfolded protein response; ER-overload response; Russell body

MATERIALS AND METHODS

Materials The cell line (CHO-K1 Tet-On), hygromycin...
B and doxycycline were obtained from Clontech (Mountain View, CA, U.S.A.). Rabbit anti-human AT antibody, mouse anti-KDEL antibody, mouse anti-glyceraldehyde 3-phosphodehydrogenase (GAPDH) antibody, rabbit monoclonal anti-human phospho-eIF2α antibody and rabbit anti-human NF-κB p65 antibody were obtained from Athens Research and Technology (Athens, GA, U.S.A.), Calbiochem (San Diego, CA, U.S.A.), Ambion (Austin, TX, U.S.A.), Cell Signaling Technology (Danvers, MA, U.S.A.) and Santa Cruz (Dallas, TX, U.S.A.), respectively. Anti-rabbit and mouse immunoglobulin G (IgG) were obtained from Molecular Probes (Eugene, OR, U.S.A.). All other reagents were of biochemical or analytical grade.

**Cell Culture**
The preparation of CHO cells in which the expression of the wild type AT or mutant AT(C95R) is controlled with doxycycline has been described previously. The cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% (v/v) fetal calf serum, 100 µg/mL G418 and 100 µg/mL of hygromycin.

**Detection of XBP1 Splicing Variants**
Total RNA was isolated from CHO cells using the recommended protocol in the NucleoSpin® RNA kit (TaKaRa Bio, Shiga, Japan). The total RNA (1 µg) was used as a template for the first strand cDNA synthesis using the AffinityScript qPCR cDNA Synthesis kit (Agilent Technologies, Santa Clara, CA, U.S.A.) according to the manufacturer’s instructions. The cDNA was used to semi-quantify the relative level of the unspliced to spliced ratio XBP1 mRNA using the forward primer 5'-GCT TGT GAT TGA GAA ACC AGG-3' and reverse primer 5'-ACAGG GCC AACTGCTTAG-3' at 94°C for 30 s, 57°C for 20 s and 72°C for 30 s, and 30 cycles to amplify both splicing variants. As an internal standard, GAPDH mRNA was quantified using the forward primer, 5'-TTC ACC ACC AACTGCTTAG-3' and reverse primer 5'-GAT GCA GGG ATG ATG TCC-3', GAPDH was used as an internal standard.

**RESULTS**

**Expression of ER Chaperones during the Accumulation of Mutant AT(C95R)**
The synthesis of mutant AT(C95R) was initiated by the addition of doxycycline, and the time course of AT accumulation and ER chaperon induction were examined until 96 h. As shown in Fig. 1, the amount of mutant AT(C95R) in the CHO cells increased during the incubation with doxycycline. After 96 h, mutant AT(C95R) increased ca. 10-fold compared to 0 h. On the other hand, the induction of the three major ER chaperones was limited to a maximum of ca. 2-fold (2.2, 1.9 and 1.4-fold for Grp78, Grp97 and PDI, respectively), even after 96 h. These data suggest that the UPR is hardly activated in the CHO cells.

**The Accumulation of Mutant AT(C95R) Does Not Activate the UPR**
We next assessed the activation of the UPR by the splicing of XBP1 mRNA and phosphorylation of eIF2α. Upon an increase in the unfolded proteins in the ER, IRE1α, a proximal UPR sensor on the ER membrane, is activated and splices out the 26-nt intron in XBP1 mRNA, causing a frame shift of the coding region of XBP1. When control CHO cells as well as CHO cells expressing wild type or mutant AT(C95R) were incubated with doxycycline for 72 h, a small amount of spliced XBP1 mRNA was detected in the three cell lines, but the amount did not increase, even during the accumulation of AT(C95R) (Fig. 2A). Under the condition, the amount of wild type AT in the cells did not increase due to the secretion in the medium as seen in previous study (Fig. 2B). In contrast, the cells treated with 1 µg/mL thapsigargin for 4 h generated a more rapidly migrating species corresponding to the spliced XBP1 mRNA.

Another UPR signaling event occurs via the ER-localized kinase PERK. PERK is activated by autophosphorylation in
response to an increase of unfolded proteins in the ER and PERK phosphorylates eIF2α to arrest protein synthesis. As shown in Fig. 2C, when the CHO cells were incubated with doxycycline for 72 h, no increase in the phosphorylation of eIF2α was occurred. In contrast, treatment of the CHO cells with thapsigargin induced phosphorylation of eIF2α. These results suggest that the UPR is hardly activated in the cells, even though CHO cells possess the ability to respond to such signals.

**The Accumulation of AT(C95R) Does Not Activate the EOR** It has been reported that the accumulation of polymeric neuroserpin and the Z mutant of α₁-antitrypsin (E342K) in the ER activates NF-κB without any activation of the UPR. Therefore, we assessed whether the accumulation of mutant AT(C95R) activated this transcription factor. The amount of p65, a subunit of NF-κB, in the nuclear fraction, was examined in the cells expressing mutant AT(C95R). As shown in Fig. 3, when the cells were incubated with doxycycline for 72 h, p65 in the nuclear fraction did not increase. In contrast, p65 was increased by the treatment with thapsigargin and tumor necrosis factor (TNF)-α. These results suggest that the NF-κB signaling pathway was functional in the cells, but the accumulation of mutant AT(C95R) did not elicit the EOR response.

**Degradation of Mutant AT(C95R) in CHO Cells** The CHO cells did not show any sings of ER stress responses when the accumulation of mutant AT(C95R) was increased. We then examined how the degradation pathways function in response to mutant AT(C95R). The CHO cells were incubated with doxycycline for 12 h or 72 h. Doxycycline was removed and the cells were then incubated with or without MG132 (5 μM), rapamycin (100 nM) and 3-methyladenine (3MA) (5 mM) for 24 h. We chased these two conditions since the ER had normal reticulation structures and no RB was present in the CHO cells at of 12 h incubation, whereas large sized and a great many RB were present in the cells at 72 h of incubation. In the case of the 12 h incubation, the amount of mutant AT(C95R) was increased ca. 2-times by MG132, but not by rapamycin or 3MA (Fig. 4A), suggesting that proteasomes were selectively involved in the degradation of mutant AT(C95R) this stage. On the other hand, after 72 h of incubation, the amount of mutant AT(C95R) was increased by both MG132 and 3MA and decreased by rapamycin treatment (Fig. 4B), suggesting that both proteasomes and autophagy are involved in the degradation that takes place at this stage.

**DISCUSSION**

Recently, we expressed mutant AT(C95R) under the control of the Tet-On system in CHO cells and showed that mutant AT(C95R) accumulated in RB, unique membranous structures derived from the ER. In this study, we investigated the ER stress response by the accumulation and degradation of mutant AT(C95R) in CHO cells.

The following evidence suggests that both the UPR and EOR were essentially inactive in CHO cells. 1) During accumulation of mutant AT(C95R) up until 72 h, the splicing of XBP1 mRNA and phosphorylation of eIF2α were not activated (Fig. 2). 2) Under the same condition, the translocation of NF-κB in the nuclear fraction was not detected (Fig. 3). 3) Induction of the major ER chaperons such as Grp78, Grp98 and PDI was limited by ca. 2-times (Fig. 1). This is consistent with previous observations that calnexin was not evidently induced. The possibility that the CHO cells had lost the ability to respond to ER stress was excluded by the following findings. Both the UPR and EOR were activated in CHO cells by thapsigargin and TNF-α (Figs. 2, 3). In addition, the CHO cell ER-associated degradation (ERAD) of mutant AT(C95R) took place in the early stage of the accumulation, while ERAD and autophagy were functional in the late stage (Fig. 4). Recently, a CHO cell Tet-On system was also used for the analysis of the ER stress response to the accumulation of mutant α₁-antitrypsin. Therefore, the CHO cells expressing mutant AT(C95R) exhibit normal activity towards mutant proteins.

Unexpectedly, the ER stress response was not activated in the CHO cells expressing mutant AT(C95R). The reason for this is unknown at present, but one possibility is that the re-
cruit of Grp78 to mutant AT(C95R) from ER sensor proteins such as IRE1 and PERK might limit the capacity to activate the UPR and disturb Ca\(^{2+}\) homeostasis in the ER, since mutant AT(C95R) exists in a detergent soluble form and is folded as an oligomer.\(^9,10\) As mutant AT(C95R) lacks the cysteine residue required for disulfide bond formation, it did not fold correctly, but it may still be present as a folded structure. In fact, mutant AT(C95R) crosslinked dithiobis(succinimidyl propionate) (DSP) exhibited an approximate 200 kDa molecular mass upon sucrose gradient centrifugation.\(^10\)

Green fluorescent protein (GFP)-tagged mutant AT(C95R) is also present in a soluble form with a 28.7-nm hydrodynamic radius indicative of a large oligomer.\(^11\) Interestingly, fate of mutant \(\alpha_1\)-antitrypsin was divided into two groups.\(^7\) One is the polymeric Z mutant (E342K) and the polymeric King’s mutants (H334D). These mutants accumulated as detergent insoluble polymers in the ER and elicited the EOR, but not the UPR.\(^6\) In contrast, another is the null Hong Kong (NHK) mutation, which has the COOH-terminal 61 amino acids deleted by in a frame shift,\(^13\) did not fold properly, preventing the formation of an ordered polymer, and instead activated the UPR.\(^7\) In the case of mutant neuroserpin, mutant polymers have been shown to activate the NF-\(\kappa\)B pathway instead of the UPR.\(^8\) Taken together, it is suggested that the soluble oligometoric mutant AT(C95R) in CHO cells was nearly “silent” in terms of the UPR and EOR. In addition, it is shown for the first time that a mutant protein in the ER is degraded by proteasomes, instead of the protein in RB being degraded by autophagy. How the status of mutant proteins in the ER affects the activation of the UPR and EOR will be the subject of further research.

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Conflict of Interest The authors declare no conflict of interest.

REFERENCES


