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Retarded protein folding of the human Z-type α_1-antitrypsin variant is suppressed by Cpr2p

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ABSTRACT

The human Z-type α_1-antitrypsin variant has a strong tendency to accumulate folding intermediates due to extremely slow protein folding within the endoplasmic reticulum (ER) of hepatocytes. Human α_1-antitrypsin has 17 peptidyl-prolyl bonds per molecule; thus, the effect of peptidyl-prolyl isomerases on Z-type α_1-antitrypsin protein folding was analyzed in this study. The protein level of Cpr2p, a yeast ER peptidyl-prolyl isomerase, increased more than two-fold in Z-type α_1-antitrypsin-expressing yeast cells compared to that in wild-type α_1-antitrypsin-expressing cells. When *CPR2* was deleted from the yeast genome, the cytotoxicity of Z-type α_1-antitrypsin increased significantly. The interaction between Z-type α_1-antitrypsin and Cpr2p was confirmed by co-immunoprecipitation. *In vitro* folding assays showed that Cpr2p facilitated Z-type α_1-antitrypsin folding into the native state. Furthermore, Cpr2p overexpression significantly increased the extracellular secretion of Z-type α_1-antitrypsin. Our results indicate that ER peptidyl-prolyl isomerases may rescue Z-type α_1-antitrypsin molecules from retarded folding and eventually relieve clinical symptoms caused by this pathological α_1-antitrypsin.

1. Introduction

Human α_1-antitrypsin (α_1-AT) is synthesized in the liver and is secreted into the blood to protect tissues against indiscriminate proteolytic attacks from neutrophil elastases [1]. Among the more than 90 α_1-AT genetic variants reported, Z-type α_1-AT is most frequently found in patients with serious clinical problems such as liver cirrhosis and emphysema [2]. The structural feature of most deficient α_1-AT variants is conformational instability [3], leading to rapid clearance by endoplasmic reticulum (ER)-associated degradation [4]. However, some variants such as D256V, L41P, and Z-type (E342K) α_1-AT exhibit extremely retarded protein folding compared to that of the wild-type molecule [3]. Once folded, the stability and inhibitory activity of these variant proteins are comparable to those of wild-type α_1-AT. Retarded folding leads to the accumulation of folding intermediates, which are prone to forming intermolecular loop-sheet polymers in the ER of hepatocytes and which can cause liver cirrhosis. Indeed, only ~15% of newly synthesized Z-type AT molecules reach the extracellular compartment [5], and loop-sheet polymers of Z-type α_1-AT have been reported [6].

The folding of newly synthesized polypeptide chains is facilitated by folding-assistant proteins. For example, chaperonins (a major family of chaperones) facilitate the folding of limited numbers of client polypeptides [7]; protein disulfide isomerases catalyze the formation and exchange of disulfide bonds; and peptidyl-prolyl isomerases (PPIases) accelerate the rate-determining proline *cis–trans* isomerization step during protein folding [8]. As Z-type α_1-AT has no disulfide bonds and exhibits retarded folding, the possibility of folding assistance by an ER PPIase was evaluated in this study. PPIases are expressed in all organisms and are classified into three classes: cyclophilins, FK506-binding proteins, and parvulins. Cyclophilins are a major PPIase family that was originally known to form complexes with the immunosuppressant drug cyclosporin A and to block calcineurin-mediated immune responses involved in the rejection of transplanted organs [9]. Peptide bonds in native protein structures exist preferentially in the *trans* configuration, but approximately 7% of X-Pro peptide bonds are in the *cis* configuration [10,11]. The particular configuration of the protein must be acquired during folding; however, the required peptidyl-prolyl isomerization is a slow reaction because it involves rotation around a partial double bond [12]. Human

Abbreviations: PPIase, peptidyl-prolyl isomerase; α_1-AT, α_1-antitrypsin; YPD, 1% yeast extract, 2% peptone, and 2% glucose; YPGal, 1% yeast extract, 2% peptone, and 2% galactose.

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α_1-AT has 17 X-Pro peptide bonds of 393 peptidyl bonds, which may limit the folding rate of the protein.

Yeast is an ideal model system to investigate the contribution of cyclophilins due to the availability of high-throughput functional genomics methods [14]. Humans have seven major cyclophilins (hCypA, hCypB, hCypC, hCypD, hCypE, hCyp40, and hCypNK), whereas *Saccharomyces cerevisiae* possesses eight different cyclophilins (Cpr1–Cpr8) [13]. Yeast Cpr2p is localized to the ER and is induced by tunicamycin, an inhibitor of glycosylation, and heat shock [15,16], suggesting that this protein plays a role in the folding of secreted proteins [16]. Here, the contribution of Cpr2p to the folding of human Z-type α_1-AT was investigated in several biochemical and cellular analyses, including co-immunoprecipitation, *in vitro* folding assays, knockout studies, and secretion analyses.

2. Materials and methods

2.1. Materials

Rabbit anti-human α_1-AT antibody and goat anti-rabbit IgG antibody conjugated to peroxidase were purchased from Sigma (St. Louis, MO, USA). Protein A/G PLUS-Agarose was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Rat anti-Cpr2p antibody was from Aprogen Co. (Daejeon, Korea). Q-sepharose™ Fast Flow column, Hybond™ ECL™ nitrocellulose membrane, and PD-10 desalting column were purchased from Amersham Bioscience Co. (Piscataway, NJ, USA). Ni^{2+}-NTA (nitrilo-tri-acetic acid) agarose was from Peptron Co. (Daejeon, Korea). Curix CP-BU, a medical X-ray film, was purchased from Agfa Co. (Ridgefield Park, NJ, USA).

2.2. Yeast strains and transformation

Human wild-type and Z-type α_1-AT were overexpressed in *S. cerevisiae* BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) (Open Biosystems Inc., Huntsville, AL, USA). pYInu-AT, containing an inulinase (Inu) signal sequence under the control of the yeast *GAL10p* promoter, was used to express human α_1-AT in *S. cerevisiae* [17]. The cDNA coding for the Z-type α_1-AT variant replaced the wild-type α_1-AT gene on pYInu-AT; the resulting plasmid was named pYInu-ATZ. Wild-type BY4741 and the derived *cpr2Δ* yeast strain were transformed with pYInu-AT or pYInu-ATZ using the standard lithium acetate method [18]. Transformants were selected by growing cells in drop-out medium lacking uracil at 30 °C for 3 days.

2.3. Monitoring cell growth by a spotting assay

The *CPR2* knockout yeast strain transformed with pYInu-AT or pYInu-ATZ was cultured in YPD (1% yeast extract, 2% peptone, and 2% glucose) liquid medium at 30 °C overnight. Cultured cells were harvested and serially diluted to reach adequate cell densities. Ten microliter of each dilution was spotted on both YPD plates (which did not induce α_1-AT expression) and YPGal (1% yeast extract, 2% peptone, and 2% galactose) plates (which induced α_1-AT expression). The plates were further incubated at 30 °C for 2–3 days, and cell growth was observed.

2.4. Complementation analysis

The *CPR2* gene was amplified from *S. cerevisiae* Y2805 (*MATa pep4::HIS3 prb1-d can1 GAL2 his3 ura3-52*) genomic DNA by polymerase chain reaction (PCR) using *Pfu* polymerase (Promega Co., Madison, WI, USA). The forward primer was 5′-CTCCAAGCTTATGAAATTCAGTGGCTTGTGGTGTTGGTTG-3′ and the reverse primer was 5′-TCCAAAGCTTTCAAGAAGAGAGCTCAGGCGTCCACTCA-3′. The PCR products were digested using *Hind*III and cloned into the *Hind*III sites of a yeast expression vector, pACT2 AD (Clontech Laboratories Inc., Mountain View, CA, USA). The resulting plasmid was named pACT2-CPR2. The *cpr2Δ* yeast strain was co-transformed with pYInu-ATZ and pACT2-CPR2, and the co-transformants were selected in drop-out medium lacking leucine and uracil at 30 °C for 3–4 days. Cell growth was monitored by a spotting assay upon the expression of Z-type α_1-AT.

2.5. Co-immunoprecipitation of Z-type α_1-AT and Cpr2p from cell extracts

The *cpr2Δ* yeast strain harboring pYInu-ATZ and/or pACT2-CPR2 was cultured in YPGal liquid medium at 30 °C for 48 h. The cultured cells were harvested and resuspended in 1 ml of lysis buffer (50 mM HEPES, pH 7.0, 1% Triton X-100, 1 mM PMSF, and 1 μM aprotinin). Cells were lysed by vigorous vortexing with glass beads (425–600 μm in diameter), and the cell extracts were precleared with Protein A/G PLUS-Agarose beads at 4 °C for 2 h. Protein concentrations were determined using a Bio-Rad DC protein assay kit (Hercules, CA, USA). Crude lysates were then incubated with polyclonal rat anti-Cpr2p antibodies (1:100 dilution) at 4 °C overnight. Immune complexes were incubated with Protein A/G PLUS-Agarose beads for 2 h then collected by centrifugation. The immunoprecipitates were washed 4 times with IP wash buffer (50 mM HEPES, pH 7.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 1 mM PMSF), and the pellet was resuspended in sodium dodecyl sulfate (SDS) sample buffer. The proteins were resolved by 10% SDS–polyacrylamide gel electrophoresis (PAGE) then transferred to a nitrocellulose membrane. The blots were probed with rabbit anti-human α_1-AT antibodies, diluted 1:1000 in PBS containing 0.03% Tween 20 (PBST), and then with horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies diluted 1:10,000 in PBST. Bound antibodies were visualized by enhanced chemiluminescence on X-ray film using luminol as the substrate.

2.6. Purification of Cpr2p expressed in Escherichia coli

To express Cpr2p in *E. coli*, the *CPR2* gene without a signal sequence was amplified from *S. cerevisiae* Y2805 genomic DNA by PCR. The forward primer was 5′-CTCAGAATTCTCTGATGTG GGTGAGTTGATT GATCAGGAC-3′ and the reverse primer was 5′-TCCACTCGAGTCAAGAAGAGAGCTCAGGCGTCCA CTCA-3′. After digestion with *Eco*RI and *Xho*I, the *CPR2*-containing fragment was subcloned into the *Eco*RI and *Xho*I sites of the pET28a vector containing a polyhistidine-tag at the 5′ end of the coding region, generating pET28a-CPR2. *E. coli* BL21 (DE3) cells (Novagen Inc., Madison, WI, USA) were transformed with pET28a-CPR2 and grown at 37 °C in LB medium containing 50 μg/ml kanamycin to an optical density at 600 nm of approximately 0.6. Cpr2p expression was then induced by adding 0.1 mM isopropyl β-D-thiogalactoside followed by a 3-h incubation at 37 °C. The cells were harvested and disrupted in buffer (0.1 mM PMSF, 50 mM Tris–Cl, 250 mM NaCl, and 8 mM imidazole, pH 7.9) using a Bandelin sonicator at 43% power and 90% pulse for 2.5 min. The cell lysates were cleared by centrifugation at 14,000 rpm for 30 min in a Hanil Micro 17R+ centrifuge (Hanil Science Industrial Co., Seoul, Korea). The supernatants were loaded on a Ni^{2+}-NTA agarose column pre-equilibrated with binding buffer (50 mM Tris–Cl, 250 mM NaCl, and 8 mM imidazole, pH 7.9) and eluted by a linear gradient of 30–1000 mM imidazole in 50 ml of binding buffer. Purified Cpr2p protein was buffer-exchanged to IP wash buffer using a PD-10 desalting column. The concentrations of the purified proteins were measured with a Bio-Rad DC protein assay kit using bovine serum albumin as the standard.