Human Heat Shock Protein 105/110 kDa (Hsp105/110) Regulates Biogenesis and Quality Control of Misfolded Cystic Fibrosis Transmembrane Conductance Regulator at Multiple Levels*

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Heat shock protein 105/110-kDa (Hsp105/110), a member of the Hsp70 super family of molecular chaperones, serves as a nucleotide exchange factor for Hsc70, independently prevents the aggregation of misfolded proteins, and functionally relates to Hsp90. We investigated the roles of human Hsp105α, the constitutively expressed isoform, in the biogenesis and quality control of the cystic fibrosis transmembrane conductance regulator (CFTR). In the endoplasmic reticulum (ER), Hsp105 facilitates CFTR quality control at an early stage in its biosynthesis but promotes CFTR post-translational folding. Deletion of Phe-508 (ΔF508), the most prevalent mutation causing cystic fibrosis, interferes with de novo folding of CFTR, impairs its export from the ER and accelerates its clearance in the ER and post-Golgi compartments. We show that Hsp105 preferentially associates with and stabilizes ΔF508 CFTR at both levels. Introduction of the Hsp105 substrate binding domain potently increases the steady state level of ΔF508 CFTR by reducing its early-stage degradation. This in turn dramatically enhances ΔF508 CFTR cell surface functional expression in cystic fibrosis airway epithelial cells. Although other Hsc70 nucleotide exchange factors such as HspBP1 and BAG-2 inhibit CFTR post-translational degradation in the ER through cochaperone CHIP, Hsp105 has a primary role promoting CFTR quality control at an earlier stage. The Hsp105-mediated multilevel regulation of ΔF508 CFTR folding and quality control provides new opportunities to understand how chaperone machinery regulates the homeostasis and functional expression of misfolded proteins in the cell. Future studies in this direction will inform therapeutics development for cystic fibrosis and other protein misfolding diseases.

Hsp105/110, like Hsp70, consists of an amino-terminal ATPase domain and a carboxyl terminal substrate binding domain (SBD) (1). Hsp105 efficiently refolds denatured proteins in vitro in the absence of Hsc70 (2, 3). In the cytosol, Hsp105 forms high molecular weight complexes with Hsc70 (4) and functionally relates to Hsc70 (5, 6) as well as Hsp90 (7). Hsp105 facilitates the nucleotide exchange of Hsc70 (8, 9). In Yeast, Hsp105 homologue Sse1 collaborates with Hsp70 homologue Ssb or Ssa in regulating the co-translational or post-translational folding of cellular proteins, respectively (10). Sse1 is specifically required for Ssa1-mediated post-translational translocation of the yeast mating pheromone α-factor into the endoplasmic reticulum (ER) (6). Furthermore, Hsp105 stabilizes apolipoprotein B in the ER and promotes its secretion (11). There are two mammalian Hsp105 isoforms: Hsp105α and Hsp105β (12). Hsp105α is constitutively expressed and is further inducible by heat shock or stress. Hsp105β is an alternatively spliced form of Hsp105α and is strictly heat-induced. This study focuses on Hsp105α, which is referred to as Hsp105 below.

The cystic fibrosis transmembrane conductance regulator (CFTR) is an ATP binding cassette transporter whose deficiency leads to cystic fibrosis (CF) (13). CFTR is highly susceptible to misfolding due to mutations, which leads to the reten-
tion of the nascent channel protein in the ER (14–21) and its subsequent ER-associated degradation (ERAD) (22, 23). Most strikingly, the deletion of the phenylalanine at residue 508 (ΔF508) accounts for 70% of all CF-causing alleles and is seen in >90% of CF patients (14). Hsp70 facilitates the maturation (24, 25) and quality control of nascent CFTR (26–28). Inhibiting Hsp90 activity prevents CFTR maturation and promotes its degradation (29). A global proteomic profiling of CFTR-associated proteins revealed an extensive cytoplasmic chaperone network containing Hsp70, Hsp90, and multiple chaperonins including Hsp105 (30). Given the importance of Hsp70 and Hsp90 in CFTR biogenesis and the demonstrated roles for Hsp105 as an independent folding component as well as a chaperone for both Hsc70 and Hsp90, we hypothesize that Hsp105 plays an important role in regulating CFTR maturation and quality control.

We conducted a systematic functional analysis of Hsp105 in CFTR biogenesis. We found that Hsp105 regulates CFTR folding and quality control at multiple levels. HspBP1 and BAG-2, two other Hsc70 nucleotide exchange factors (NEFs), are reported to inhibit CFTR ERAD through co-chaperone CHIP (31, 32). In contrast, we found that Hsp105 facilitates CFTR quality control at an earlier stage during its biosynthesis. Moreover, Hsp105 promotes CFTR post-translational folding and preferentially associates with the misfolded ΔF508 CFTR in the ER and at the cell periphery. Overexpressing Hsp105 stabilizes ΔF508 CFTR in both the ER and cell periphery, leading to enhanced rescue at both reduced and physiological temperatures. Introduction of Hsp105 SBD potently inhibits the early stage of ΔF508 CFTR quality control in the ER, leading to improved cell surface functional expression in CF airway epithelial cells. Our findings reveal a pivotal role for Hsp105 in the cellular handling of misfolded CFTR by the cytoplasmic chaperone machinery.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Chemicals—**CFTR monoclonal antibodies (mAbs) used in this study include MM13-4, M3A7, 13-1, and 24-1. Other antibodies used include anti-Hsp105 mAb (Novoceastra, Newcastle upon Tyne, UK), anti-Hsc70, anti-Hsp90α, and anti-Hsp9αβ mAbs (Stressgen, Ann Arbor, MI), anti-Hsp70 polyclonal antibodies (Stressgen), anti-HspBP1 mAb (Abcam, Cambridge, MA), anti-HspBP1 polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), anti-BAG-2 antibody (Imgenex, San Diego, CA), and anti-actin mAb (Millipore, Temecula, CA). Restriction enzymes, mung bean nuclease, and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). Pfu DNA polymerase was purchased from Stratagene (La Jolla, CA). Protein G beads were purchased from GE Healthcare or East Coast Bio (North Berwick, ME), Glutathione-Sepharose 4B was from GE Healthcare, and other chemicals were from Sigma.

**Plasmids—**Hsp105 shRNA plasmid was constructed using IMG-800–1 vector (Imgenex) according to the manufacturer’s protocol. The Hsp105 target sequence used was 5′-CAG CCA TGT TGT TGA CTA AGC-3′. The control plasmid contains a non-targeting sequence 5′-TCA GTC ACG TTA ATG GTC GTT-3′.

The pCMV-SPORT6 harboring human full-length Hsp105α coding sequence (pCMV-SPORT6-Hsp105α) was obtained from ATCC (Manassas, VA) and was the basis for constructing Hsp105α mutants. Amino acid sequence alignment was performed using the Sequence Manipulation Suite software (33).

The pCMV-SPORT6-Hsp105α was digested with XhoI and NotI. Blunt ends were generated by treatment with mung bean nuclease, and the plasmid was re-circularized by ligation to produce pCMV-SPORT6-Hsp105α-MB. This removes the extra Xbal site in the multiple cloning sites region so that the Xbal site within the Hsp105α coding sequence becomes unique. The amino-terminal KpnI-XbaI fragment of the Hsp105α coding sequence was used as a cassette to introduce the G232D substitution by overlapping polymerase chain reactions (PCR) from pCMV-SPORT6-Hsp105α-MB. To construct the SBD (Met384–858) mutant, the carboxyl terminal Sall-PstI fragment of the Hsp105α coding sequence was used as a cassette. A methionine was added before residue 384 to serve as a start codon to generate pCMV-SPORT6-Hsp105α-SBD.

The shRNA-refractory Hsp105α expression plasmid was constructed by introducing three silent mutations in the shRNA target sequence of pCMV-SPORT6-Hsp105α-MB to yield 5′-CAG CCA TGC TGC TCA CTA AGC-3′. The mutations were introduced by overlapping PCR, and the DNA fragment was ligated back into the Sall-XbaI cassette of the same plasmid.

The pcDNA3-EGFP was obtained from Addgene (Cambridge, MA), and the EGFP coding sequence was subcloned into pcDNA3.1(+) through XhoI and XbaI sites to generate pcDNA3.1(+)–EGFP. The CFTR expression plasmids pcDNA3.1(+)-CFTR-WT, pcDNA3.1(+)–CFTR-ΔF508 and pcDNA3.1(+)–CFTR-DAA (34), and the pcDNA3.1(−)-GST–EGFP plasmid (35) have been described previously. The pcDNA3.1(−)-Myc–Rab3A plasmid was provided by Dr. William Balch (The Scripps Research Institute, La Jolla, CA) and was used as the control plasmid for Hsp105 overexpression experiments. We also repeated key experiments using pcDNA3.1(+)–EGFP as control, and similar results were obtained (data not shown). All constructs containing PCR-derived DNA fragments were confirmed by DNA sequencing.

**Cell Culture and Transfection—**Human embryonic kidney 293 (HEK) cells were cultured in Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum, and 100 units/ml each of penicillin and streptomycin. The HEK cells stably expressing ΔF508 (HEK-ΔF) or wild-type (HEK-WT) CFTR (34, 36) were maintained in the above medium supplemented with 150 μg/ml hygromycin B (EMD Chemicals, Gibbstown, NJ) as described previously (34). CF airway epithelial IB3-1 cells (37) were maintained in LHC-8 medium without gentamicin (Invitrogen), 5% fetal bovine serum, and 100 units/ml each of penicillin and streptomycin. Cell transfection was performed using Lipofectamine 2000 (Invitrogen) or Effectene (Qiagen, Valencia, CA). HEK cells were transfected with Hsp105 shRNA or control shRNA construct. Stable cell lines were generated by selection with 400 μg/ml G418. Once generated, the stable cell lines were maintained in appropriate media supplemented with 200 μg/ml G418.
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Cell Lysis and Quantitative Immunoblotting—Cells were lysed on ice in buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100 (v/v), and the Complete™ protease inhibitor mixture (Roche Diagnostics) for 30 min. Insoluble material was cleared by centrifugation at 16,000 × g for 20 min. Protein concentration was measured by Bradford assay. Proteins in cell lysates were separated on SDS-PAGE gel and transferred onto nitrocellulose membrane. Proteins of interest were detected with relevant antibodies and visualized by ECL. Protein loading was optimized based on its intracellular abundance so that the intensity of bands is within dynamic range. The protein bands were quantified by densitometry using ImageJ software (National Institutes of Health).

Metabolic Labeling and Pulse-Chase Analysis—Pulse labeling and pulse-chase analysis were performed in 60-mm culture dishes largely as described previously (35). Equal numbers of cells were seeded in each dish and cultured for at least 16 h to allow cell attachment. The cells were then transfected with CFTR alone or in combination with other expression plasmids. After 16–24 h, the CFTR-expressing cells were cysteine- and methionine-starved for 30 min, pulse-labeled with EasyTag Express 35S Protein Labeling Mix (PerkinElmer Life Sciences) for 30 min, and chased for the indicated time if necessary. CFTR from cell lysates were recovered by immunoprecipitation and resolved on SDS-PAGE gels. The radiolabeled CFTR were quantified by phosphorimaging using a Typhoon FLA7000 Imager (GE Healthcare).

Quantitative Co-immunoprecipitation—HEK cells transiently transfected with plasmids encoding different forms of CFTR were lysed as described above. CFTR was immunoprecipitated with Protein G-Sepharose beads coated with a CFTR mAb as described previously (38). For all co-immunoprecipitation experiments, HEK cells not expressing CFTR were included to control for nonspecific binding of proteins to the Protein G-Sepharose beads.

Fluorescence-based Iodide Efflux Assay—The CFTR-mediated, CAMP-activated iodide efflux was measured at 37 °C using the fluorescent dye 6-methoxy-N-(3-sulffopropyl)-quinolinium (SPQ) (TEF Labs, Austin, TX) as described by Munkonge et al. (39) with minor modifications (35). IB3-1 cells were grown on glass coverslips for at least 16 h before they were transfected with CFTR alone or together with other expression plasmids. Over 24 h post-transfection, the cells were loaded with SPQ by hypotonic shock and perfused with NaI solution until maximal quench of the SPQ fluorescence was achieved. Then the cells were stimulated by perfusion with 10 μM forskolin plus 100 μM isobutylmethylxanthine in NaNO₃ solution. The SPQ fluorescence was measured at 30-s intervals, and the fluorescence intensity at each time point (F) was normalized to the value at the maximal quenching (F₀) to facilitate comparison. Results were typically derived from a field of 15–27 cells, and multiple fields were recorded from the same or different coverslips. The initial rates of fluorescence dequenching were estimated by linear fits to the first 300 s of the cAMP-induced iodide efflux.

RESULTS

Hsp105 Facilitates CFTR Quality Control in ER at Early Stage—We tested the role of Hsp105 in CFTR biogenesis by reducing its expression using shRNA (Fig. 1). Based on pulse-chase analysis, Hsp105 knockdown increases the synthesis of wild-type CFTR by 24% during the 30-min pulse (Fig. 1A). In contrast, neither the rate of disappearance of the immature, ER-localized, core-glycosylated CFTR (band B) nor the rate of its conversion to the mature, Golgi-processed, complex glycoforms (band C) during the chase period was changed. Under the same conditions, the synthesis of ΔF508 CFTR was increased by 188%, and no major change in the rate of disappearance of band B was observed (Fig. 1B). As the initial levels of radiolabeled CFTR band B were different between the two sets (Cntrl and 105i) (Fig. 1, A and B, upper panels), we normalized the levels of radiolabeled CFTR bands B and C at all time points to the level of band B at 0-h chase within each set to facilitate comparison (Fig. 1, A and B, lower charts).

The Hsp105 knockdown caused an 80% reduction in Hsp105 level (Fig. 1C), which was not accompanied by a significant change in the levels of major cytoplasmic chaperones such as Hsp70, Hsc70, Hsp90α, and Hsp90β, although a slight but statistically insignificant decrease in Hsc70 was observed. The steady state levels of two other Hsc70 NEFs known to impact CFTR biogenesis, HspBP1 (31) and BAG-2 (32), were not affected by Hsp105 knockdown, nor did the level of the J-domain-containing Hdj-2 (24, 40) (Fig. 1C).

The much greater enhancement in the synthesis of ΔF508 CFTR than wild-type CFTR as a result of Hsp105 knockdown suggests that Hsp105 has a greater impact on the biogenesis of the misfolded ΔF508 CFTR. We found that the increase in ΔF508 CFTR synthesis by Hsp105 knockdown resulted in an increase in the steady state level of ΔF508 CFTR (Fig. 2A), and re-introducing the shRNA-refractory version of Hsp105 (r105) into the Hsp105 deficient cells (105i) led to a dose-dependent decrease in ΔF508 CFTR level. By pulse-chase analysis, we clearly observe a reversal of the increase in ΔF508 CFTR synthesis resulting from Hsp105 rescue (Fig. 2B).

To test if Hsp105 knockdown enhances general protein synthesis, we expressed a GST-EGFP fusion protein under the control of the same promoter as the CFTR expression plasmids in the control and 105i cells and subjected the cells to short pulse-labeling with increasing durations. The fusion protein was recovered using glutathione-Sepharose beads. The radioisotope incorporation into the GST-EGFP fusion protein was quantified by phosphorimaging. As shown in Fig. 2C, the rate of radioisotope incorporation into this cytoplasmic localized GST-EGFP fusion protein in the 105i cells is very similar to that in the control cells. Therefore, Hsp105 appears to selectively decrease the synthesis of CFTR, especially ΔF508 CFTR.

To explore the mechanism underlying the dramatic inhibition of ΔF508 CFTR synthesis by Hsp105, we blocked the ERAD of ΔF508 CFTR by incubating the ΔF508-expressing cells in proteasome inhibitor ALLN and compared the ΔF508 CFTR syntheses in the 105i cells with and without Hsp105 rescue. As shown in Fig. 2D, although Hsp105 rescue results in a significant decrease in ΔF508 CFTR synthesis in the absence of ALLN, in the presence of ALLN, the levels of ΔF508 CFTR synthesis between the control and Hsp105 rescue were not significantly different. This suggests that Hsp105 reduces the synthesis of ΔF508 CFTR through enhancing the ERAD of ΔF508 CFTR at
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Hsp105 promotes post-translational folding of CFTR—To further test the role of Hsp105 in CFTR biogenesis, we overexpressed the protein in HEK cells co-transfected with wild-type CFTR and found that it slightly increased the average steady state level of band C (Fig. 3A, p = 0.08, n = 3) but significantly reduced the level of band B. Under the same conditions, the level of ΔF508 CFTR band C was increased, but its band B level was unchanged (Fig. 3B). To explore the mechanism, we performed pulse-chase analyses and found that Hsp105 overexpression reduced the synthesis of both wild-type and ΔF508 CFTR (Fig. 3, C and D). With wild-type CFTR, Hsp105 overexpression did not impact the post-translational turnover of its band B but dramatically enhanced its conversion to band C (Fig. 3C). As the initial levels of the radiolabeled immature CFTR between the control and the Hsp105 overexpression (105) were quite different, the levels of both band B and band C were normalized to the level of band B at 0-h chase in each set to facilitate comparison of the two. In the case of ΔF508 CFTR, its reduced synthesis was offset by its enhanced stability in the ER (Fig. 3D), leading to an unchanged steady state level of band B (Fig. 3B). This was consistent with a role for Hsp105 in promoting the post-translational folding of both wild-type and ΔF508 CFTR. In the latter case, ΔF508 CFTR exits the ER inefficiently, and hence a larger foldable pool of the immature protein is present in the ER. Consistent with enhanced forward folding of ΔF508 CFTR, the steady state level of its band C was significantly increased (Fig. 3B). In HEK cells the residual processing of ΔF508 CFTR was greater than in other cell lines. After long exposure, we were able to detect its band C even at physiological temperature. As ΔF508 CFTR matures with greater efficiency at reduced temperature (41) and the chaperone machinery plays a critical part in this process (38), we tested if Hsp105 overexpression enhances the low temperature rescue of ΔF508 CFTR and found that it indeed did. This was reflected in a significant increase in band C but no change in the level of band B (Fig. 4). Taken together, Hsp105 overexpression enhanced the post-translational folding of CFTR in the ER.

Hsp105 preferentially associates with ΔF508 CFTR in ER—As Hsp105 appears to preferentially stabilize ΔF508 CFTR in the ER (Fig. 3), we assessed the extent of Hsp105 association with the ER-localized wild-type and ΔF508 CFTR by quantitative co-immunoprecipitation (Fig. 5). We treated the CFTR-expressing HEK cells with brefeldin A to block the ER-to-Golgi trafficking so that the vast majority of the CFTR molecules were retained in the ER. Although ΔF508 mutation is known to cause global misfolding in CFTR (34, 42–44), an ER exit code mutation (DAA) (45) was recently shown to impair CFTR export (34). As Hsp105 appears to preferentially stabilize ΔF508 CFTR, we thus focused on wild-type CFTR and found that it indeed did. This was reflected in a significant increase in band C but no change in the level of band B (Fig. 3B). Taken together, Hsp105 overexpression enhanced the post-translational folding of CFTR in the ER.

An early stage during its synthesis. Consistent with such a conclusion, Hsp105 has a much greater effect on ΔF508 CFTR than on wild-type CFTR simply because the former is subjected to ER quality control to a much greater extent than the latter.

FIGURE 1. Hsp105 knockdown increases the synthesis of wild-type and ΔF508 CFTR. A and B, HEK cells stably expressing a non-targeting shRNA (Cntrl) or an Hsp105 shRNA (105i) were transfected with wild-type (A) or ΔF508 (B) CFTR. Twenty-four hours post-transfection the cells were pulsed for 30 min and then chased for the indicated time periods. For all pulse-chase analyses, the levels of radiolabeled CFTR in band B or C were quantified by phosphorimaging and normalized to the level of CFTR band B at the 0-h chase in each set. The relative level of radiolabeled band B at the 0-h chase in each set was indicated below the autoradiograph. Shown are the means and S.E. of two independent experiments. C, the Cntrl and 105i cells were transfected with wild-type CFTR. Equal amounts of cell lysates were immunoblotted for the indicated chaperones and actin. For all figures, the steady state levels of CFTR or chaperones were quantified by densitometry, normalized to the levels of the actin loading control, and further normalized to the value of the Cntrl to facilitate comparison. The means and S.E. are shown. Unpaired, two-tailed t test was performed. Where indicated, * and ** denote p < 0.05 and 0.01, respectively. n = 3.
from equivalent amounts of cell lysates including the mock, and equal fractions of the immunoprecipitated proteins were loaded on SDS-PAGE gel for immunoblotting analysis. To quantitatively compare the relative levels of Hsp105 association among the three forms of CFTR, we subtracted the levels of associated Hsp105 by that of the mock and then normalized the values to the corresponding level of CFTR. We found that H9004F508 CFTR had a much greater association with Hsp105 than wild-type or DAA CFTR (Fig. 5). This is consistent with the findings that H9004F508 CFTR associates more extensively with Hsc70 and Hsp70 (34, 46) and Hdj-2 (24) than wild-type CFTR. The preferential association of Hsp105 with the misfolded H9004F508 CFTR in the ER is consistent with the preferential stabilizing effect of Hsp105 overexpression on H9004F508 CFTR (Fig. 3).
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processing at the steady state (34). We tested if Hsp105 is able to associate with ΔF508 CFTR in post-Golgi compartments (Fig. 6A). At 37 °C, the vast majority of ΔF508 CFTR exists in band B. To obtain the band C of ΔF508 CFTR, we incubated HEK-ΔF cells at reduced temperature to allow processing to occur and then treated the cells with cycloheximide to remove the band B. We assessed the association of Hsp105 with either the band B or the band C of ΔF508 CFTR by quantitative co-immunoprecipitation using a CFTR mAb. HEK cells expressing wild-type CFTR (HEK-WT) (34, 36) were treated in the same manner to control for the potential impact of low temperature on Hsp105-CFTR association. Because both bands B and C are present in wild-type CFTR at 37 °C, in the case of wild-type CFTR we quantitatively compared the overall Hsp105 association with bands B and C at 37 °C and the Hsp105-association with band C alone after incubation at reduced temperature followed by cycloheximide treatment.

As shown in Fig. 6A, the immunoprecipitated proteins from equivalent amounts of cell lysates were separated on SDS-PAGE and immunoblotted for CFTR, Hsp105, and other chaperones. Given the different steady state levels of CFTR after various treatments, after subtracting the mock value, we normalized the levels of co-immunoprecipitated chaperones to the level of CFTR to quantitatively assess the extent of chaperone association (Fig. 6, B and C). Strikingly, we observed a much greater association of Hsp105 with the post-Golgi form of ΔF508 CFTR than with its ER form (Fig. 6, A and B). This is not caused by the lowering of the temperature and/or the treatment with cycloheximide as, under the same condition, less association of Hsp105 was observed with band C of the wild-type CFTR at 30 °C than with the combination of bands B and C at 37 °C (Fig. 6, A and C), which suggests that the band B of wild-type CFTR has greater association with Hsp105 than its band C. Overall, we have observed much lower Hsp105 association with wild-type CFTR than with ΔF508 CFTR, and this is true for both band B and band C (Fig. 6, B and C). These findings suggest that Hsp105 preferentially associates with ΔF508 CFTR in post-Golgi compartments.

As a recent study demonstrated that cytoplasmic Hsc70, Hsp90α, and a number of cochaperones facilitate the clearance of ΔF508 CFTR from the cell periphery (48), we extended our analysis to include Hsc70 and Hsp90α. We found that both have a dramatically greater association with the band C of ΔF508 CFTR than with its band B (Fig. 6B). Again, the association of the same chaperones with wild-type CFTR is generally much lower than with ΔF508 CFTR for both bands B and C (Fig. 6, A–C). However, the functional relationship between Hsp105 and these chaperone proteins remains to be determined.

To test the effect of Hsp105 overexpression on the peripheral stability of ΔF508 CFTR, we measured the post-Golgi stability of ΔF508 CFTR in the absence and presence of Hsp105 overexpression by cycloheximide chase at 37 °C. As shown in Fig. 6D, Hsp105 overexpression dramatically improved the post-Golgi stability of ΔF508 CFTR as reflected in a much slower turnover of its band C when protein synthesis is blocked by cycloheximide.

Hsp105 SBD Rescues ΔF508 CFTR by Interfering with Its Early-stage ERAD—The ATPase domain of Hsp105 is essential for its NEF activity. The G233D substitution in yeast Hsp105 homologue Sse1 blocks its ATP binding, impairs its Hsp70 interaction, dramatically diminishes its NEF activity, abolishes Sse1 biological activity, and fails to rescue the ydj1–J151 temperature-sensitive growth defect (6, 8, 49, 50). Based on sequence alignment, we constructed the corresponding mutant of human Hsp105, G232D, or G-D (Fig. 7A). Expression of the G-D mutant at a similar level as wild-type Hsp105 abolishes its enhancement of ΔF508 processing (Fig. 7B), supporting that the enhanced ΔF508 processing is mediated through Hsp105 function.
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Due to the absence of the ATPase domain, Hsp105 SBD lacks NEF activity (8). The SBDs of both the mammalian Hsp105 and the yeast Sse1 were reported to possess full holdase activity (the activity of a chaperone to maintain substrate solubility and prevent aggregation) in vitro (3, 50). We found that introduction of Hsp105 SBD into HEK cells increased the steady state level of ΔF508 CFTR band C by 248% and band B by 57% (Fig. 7B). To test the mechanism, we performed pulse-chase analyses and found that Hsp105 SBD enhanced ΔF508 CFTR synthesis by 2-fold without affecting its post-translational turnover in the ER (Fig. 7C). To further test if Hsp105 SBD, like Hsp105 knockdown, increases ΔF508 CFTR expression through inhibiting its early-stage ERAD, we performed pulse analysis in the presence and absence of proteasome inhibitor ALLN (Fig. 7D). As Hsp105 SBD fails to increase the synthesis of ΔF508 CFTR in the presence of ALLN (Fig. 7D), Hsp105 SBD exerts its effect through reducing ΔF508 CFTR ERAD at an early stage during its synthesis in the ER.

Hsp105 SBD Enhanced Cell Surface Functional Expression of ΔF508 CFTR in IB3-1 CF Airway Epithelial Cells—To verify the impact of Hsp105 on ΔF508 CFTR rescue in CF airway epithelial cells, we transiently co-expressed ΔF508 CFTR with Hsp105 or its SBD in CF airway epithelial IB3-1 cells expressing one copy of the ΔF508 CFTR gene (37) (Fig. 8). In IB3-1 cells, Hsp105 overexpression increased the steady state level of ΔF508 CFTR band B by 71% and band C by 73% at 37 °C (Fig. 8A, middle panel), suggesting a prominent effect on the stabilization of the mutant CFTR in the ER. At reduced temperature, Hsp105 overexpression increased the level of ΔF508 band B to an even greater level (by 270%) (Fig. 8A, left panel), further underscoring its effect on stabilization of the mutant CFTR in the ER. The introduction of Hsp105 SBD produced an increase in ΔF508 CFTR band B by 463% and an increase in band C by 266% (Fig. 8A, right panel). To assess the impact of Hsp105 SBD on the cell surface functional expression of ΔF508 CFTR in IB3-1 cells, the cAMP-activated iodide efflux was measured using an SPQ fluorescence-based method. We observed a clear increase in cAMP-stimulated iodide efflux as a result of introduction of Hsp105 SBD into the cell (Fig. 8B). The estimated initial rate of iodide efflux after cAMP stimulation in Hsp105 SBD-expressing IB3-1 cells was significantly higher than the same cells expressing GFP (Fig. 8C). Remarkably, the estimated initial rate achievable by Hsp105 SBD expression in IB3-1 cells was ∼66% that of wild-type CFTR in the same cells in our experiments (Fig. 8C). These data support a potent enhancement of ΔF508 CFTR cell surface functional expression by Hsp105 SBD in CF airway epithelial cells.

DISCUSSION

The cytoplasmic chaperone machinery plays a critical role in the biogenesis of polytopic membrane proteins in the secretory pathway. Major cytoplasmic chaperones such as Hsp70 and Hsp90 have multiple roles in distinct stages of membrane protein biogenesis. Understanding their specific roles in each stage is an important but challenging task. A number of cochaperones functionally regulate the folding activities of Hsp70 and Hsp90. Hsp105 is a tri-functional chaperone protein in the cytosol, where it can act as a holdase, an Hsc70 NEF, and an Hsp90. Hsp105 is a tri-functional chaperone protein in the cytosol, where it can act as a holdase, an Hsc70 NEF, and an Hsp90 cochaperone. In this study, we examined the roles of Hsp105 in CFTR biogenesis. We found that Hsp105 regulates CFTR folding and quality control at three distinct levels: early-stage quality control during CFTR synthesis, post-translational folding, and the peripheral stabilization of the misfolded ΔF508 CFTR (Fig. 9).

Hsp105 reduces the synthetic yield of CFTR by enhancing its early-stage quality control. Consistent with this, both Hsp70 and Hsp105 have been implicated in co-translational folding of membrane proteins in the ER (10, 51). CFTR quality control in the ER can occur coincident with translation through RMA1 (52–54). Recently, DNAJ1B12, a J-domain-containing activator of Hsc70 ATPase, was found to cooperate with Hsc70 and RMA1 in early-stage ERAD of ΔF508 CFTR (55). As Hsp105 facilitates the nucleotide exchange of Hsc70 and promotes the early-stage ERAD of CFTR, it is possible that Hsp105 pairs with...
DNAJB12 in regulating Hsc70-mediated quality control of CFTR (Fig. 9). Interestingly, introduction of Hsp105 SBD into the cell potently increases the production of ΔF508 CFTR by decreasing its early-stage ERAD (Fig. 7). The mechanism by which Hsp105 SBD inhibits the early-stage ERAD of CFTR remains to be elucidated.

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Hsc70 is known to facilitate post-translational ERAD of CFTR through co-chaperone CHIP (26, 28). HspBP1 and BAG-2, two other Hsc70 NEFs, potently inhibit CHIP-mediated post-translational ERAD of CFTR in HEK cells (31, 32). In contrast, we have not observed any effect of Hsp105 on the post-translational ERAD of wild-type CFTR (Figs. 1 and 3). Hsp105 knockdown (Fig. 1B) or rescue (Fig. 2B) has minimal impact on ΔF508 CFTR post-translational ERAD. Nevertheless, when Hsp105 is overexpressed by >5 fold, a modest post-translational stabilization was observed for ΔF508 CFTR in the ER (Fig. 3D). It remains to be determined whether the latter is mediated through the inhibition of CHIP-dependent post-translational ERAD or through Hsp90-mediated enhancement in post-translational folding. It is unlikely that Hsp105 holdase activity plays a role in ΔF508 CFTR post-translational stabilization, as the expression of Hsp105 SBD, which retains its holdase activity but lacks the NEF activity, had no effect on ΔF508 CFTR post-translational turnover in the ER (Fig. 7C). Taken together, the above results argue that unlike the other two Hsc70 NEFs, Hsp105 does not have a major role in CHIP-mediated post-translational ERAD of CFTR but plays a critical role in CFTR quality control at an earlier stage during its synthesis (Fig. 9).

We have also identified a pro-maturation role for Hsp105 in wild-type CFTR biogenesis after Hsp105 overexpression (Fig. 3, A and C). The mechanism by which Hsp105 regulates CFTR maturation remains unclear. Yeast Hsp105 homologue Sse1 is an Hsp90 cochaperone (7) and plays an important role in the regulation of client protein maturation and degradation in the cytosol in an NEF-dependent manner (56). It is likely that an analogous mechanism exists on the cytoplasmic face of the ER membrane, where the Hsp70-Hsp90 chaperone system actively regulates the conformational maturation of CFTR (29, 30) (Fig. 9). Alternatively, enhanced stringency of early-stage quality control might increase the post-translational foldability of CFTR in the ER.

Unlike wild-type CFTR, the forward folding of the ΔF508 mutant is arrested in an immature conformational intermediate in the ER (42, 43, 57, 58). Upon rescue by low temperature and/or a second site mutation, the conformational maturation of ΔF508 CFTR is able to proceed, leading to enhanced export (34). Consistent with an important role for cytoplasmic chaperones in the temperature-rescue of ΔF508 CFTR (38), Hsp105 overexpression promotes the temperature rescue of ΔF508 CFTR in HEK cells (Fig. 4). Most strikingly, Hsp105 overexpression enhances the processing of ΔF508 CFTR even at physiological temperature in HEK cells (Figs. 3B).

In IB3-1 cells, Hsp105 overexpression appears to have a greater impact on ΔF508 stabilization than on its maturation (Figs. 3, B and C, and 8A). Although the precise mechanism remains unclear, the fold of Hsp105 overexpression achievable in IB3-1 cells is much lower than in HEK cells (Figs. 3B and 8A, middle panel). This leads to relatively lower level of ΔF508 band C but higher level of ΔF508 band B. However, the great net increase in the level of band C still results in increased cell surface functional expression of ΔF508 CFTR in IB3-1 cells (data not shown). Introduction of Hsp105 SBD into IB3-1 cells leads to an almost 6-fold increase in ΔF508...
band C, which translates into an increase in the initial rate of iodide efflux to 66% of the rate achievable by wild-type CFTR (Fig. 8, B and C).

Hsp105 preferentially associates with ΔF508 CFTR in both the ER (Fig. 5) and post-Golgi compartments (Fig. 6, A–C). Unlike wild-type CFTR, ΔF508 CFTR is trapped in a chaperone-protected folding intermediate (57). Increasing Hsp105 level will enhance its pro-folding effect, leading to ΔF508 CFTR stabilization in both the ER (Fig. 3D) and cell periphery (Fig. 6D). Such an effect in the ER is at least partially offset by the Hsp105-dependent enhancement of early-stage quality control of CFTR (Fig. 3, B and D).

The ΔF508 CFTR is unstable in post-Golgi compartments (47), resulting from accelerated endocytosis (59). Both Hsp70 and Hsc70 associate with mature CFTR at the cell surface (60). We found that Hsp105 associates extensively with rescued ΔF508 CFTR (Fig. 6, A and B). Both Hsc70 and Hsp105 have been implicated in clathrin basket dissociation (61–63). However, the general impact of Hsp105 on clathrin-mediated endocytosis cannot account for the dramatic difference in Hsp105 association between wild-type and ΔF508 CFTR in post-Golgi compartments (Fig. 6, A–C).

A recent study revealed that Hsc70, Hsp90α, and several cochaperones including Hop collaborate with the ubiquitination system in regulating ΔF508 CFTR internalization from the cell surface (48). Although knocking down Hsp105 does not appear to affect ΔF508 CFTR cell surface turnover (48), we found that overexpressing Hsp105 stabilizes ΔF508 CFTR in post-Golgi compartments in HEK cells (Fig. 6D). Consistent with published reports, our data indicate that, aside from Hsp105, both Hsc70 and Hsp90α associate extensively with mature ΔF508 CFTR (Fig. 6, A–C). Although Hsc70, Hsp90α, and multiple cochaperones promote the peripheral turnover of ΔF508 CFTR (48), Hsp105 stabilizes it (Fig. 6D). It is possible that Hsp105 regulates ΔF508 CFTR peripheral turnover by modulating Hsc70 and Hsp90 activities. Alternatively, Hsp105 can directly stabilize the rescued ΔF508 CFTR in cell periphery through its holdase activity (Fig. 9).
In eukaryotic cells two distinct sets of chaperones mediate the de novo folding of nascent polypeptides and the rescue of stress-denatured proteins (64). The former is stress-repressed, whereas the latter is stress-induced. Hsp105 family proteins belong to a subgroup of chaperones that is repressed by certain types of stresses but inducible by others including heat shock (64). We found that Hsp105 regulates both de novo folding of CFTR in the ER and the rescue of misfolded ΔF508 CFTR in both the ER and cell periphery. Balancing the two roles of Hsp105 is crucial to the effective rescue of ΔF508 CFTR. Many chaperone proteins promote ΔF508 CFTR rescue by regulating its ERAD. Reducing CHIP stabilizes CFTR in the ER (28). Knocking down Aha1 (30) or p97/VCP (65) has a similar effect. Histone deacetylase inhibitors such as 4-phenylbutyrate and suberoylanilide hydroxamic acid and endogenous bronchodilator S-nitrosoglutathione can modulate cellular chaperone levels, leading to increased ΔF508 CFTR cell surface expression (66–69). S-Nitrosoglutathione was recently found to rescue ΔF508 CFTR by down-regulating the level of co-chaperone Hop (70). Hop was shown to accelerate ΔF508 CFTR turnover at the cell periphery (48). Moreover, overexpression of specific fragments of CFTR either displaces chaperone binding to ΔF508 CFTR (71) or substitutes ΔF508 CFTR for degradation (72), leading to rescue. We uncovered a multilevel regulation of CFTR biogenesis and quality control by Hsp105 in collaboration with other folding components in the cell (Fig. 9). Understanding the mechanism of such regulation will lead to the identification of novel molecular targets for the rescue of CFTR misfolding in CF. Small molecule compounds that modulate the expression or activity of Hsp105 or its functional partners can be designed that will potentially improve the cell surface functional expression of ΔF508 CFTR. Using cell-based assays, a number of small molecule CFTR correctors have been identified that promote the maturation and cell surface functional expression of ΔF508 CFTR (73–75). In particular, VX-809, currently under clinical trial for treating CF (76), potently enhances the accumulation and functioning of ΔF508 CFTR on the apical membrane of primary HBE cells from CF patients homozygous for the ΔF508 mutation (77). Although the underlying mechanism is not entirely clear, recent data suggest that it acts at the level of the ER and stabilizes ΔF508 CFTR (77). We found that Hsp105 promotes conformational stabilization of ΔF508 CFTR at both the ER and the peripheral levels. Although both VX-809 and Hsp105 stabilize ΔF508 CFTR conformation, the molecular mechanisms might be different, and hence the two might be synergistic when combined.

Given its high affinity for misfolded proteins, Hsp105 can serve as a potential molecular target for the treatment of other protein misfolding diseases such as congenital long QT syndrome (78), diabetes, and neurodegenerative diseases (79). The Hsp105 holdase activity can prevent aggregation of misfolded proteins and allow them to refold or be degraded. Hsp105 can also collaborate with Hsp70 and Hsp90 to further promote the refolding and rescue of misfolded intracellular proteins (80). A systematic functional characterization of Hsp105 in the context of the disease-related misfolding and rescue will provide valuable information for the successful design of relevant molecular interventions.

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