

# Small Heat-Shock Proteins Select $\Delta$ F508-CFTR for Endoplasmic Reticulum-associated Degradation<sup>□</sup>

Annette Ahner,<sup>\*†</sup> Kunio Nakatsukasa,<sup>\*</sup> Hui Zhang,<sup>†</sup> Raymond A. Frizzell,<sup>†</sup> and Jeffrey L. Brodsky<sup>\*</sup>

<sup>\*</sup>Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA 15260; and <sup>†</sup>Department of Cell Biology and Physiology, University of Pittsburgh, Pittsburgh, PA 15261

Submitted May 25, 2006; Revised October 25, 2006; Accepted December 5, 2006  
Monitoring Editor: Jonathan Weissman

Secreted proteins that fail to achieve their native conformations, such as cystic fibrosis transmembrane conductance regulator (CFTR) and particularly the  $\Delta$ F508-CFTR variant can be selected for endoplasmic reticulum (ER)-associated degradation (ERAD) by molecular chaperones. Because the message corresponding to *HSP26*, which encodes a small heat-shock protein (sHsp) in yeast was up-regulated in response to CFTR expression, we examined the impact of sHsps on ERAD. First, we observed that CFTR was completely stabilized in cells lacking two partially redundant sHsps, Hsp26p and Hsp42p. Interestingly, the ERAD of a soluble and a related integral membrane protein were unaffected in yeast deleted for the genes encoding these sHsps, and CFTR polyubiquitination was also unaltered, suggesting that Hsp26p/Hsp42p are not essential for polyubiquitination. Next, we discovered that  $\Delta$ F508-CFTR degradation was enhanced when a mammalian sHsp,  $\alpha$ A-crystallin, was overexpressed in human embryonic kidney 293 cells, but wild-type CFTR biogenesis was unchanged. Because  $\alpha$ A-crystallin interacted preferentially with  $\Delta$ F508-CFTR and because purified  $\alpha$ A-crystallin suppressed the aggregation of the first nucleotide-binding domain of CFTR, we suggest that sHsps maintain the solubility of  $\Delta$ F508-CFTR during the ERAD of this polypeptide.

## INTRODUCTION

The first committed step in the secretory pathway is the translocation of newly synthesized polypeptides into the endoplasmic reticulum (ER). Protein maturation and folding in the ER are prerequisites for subsequent transport through this pathway because soluble and integral membrane proteins are subject to ER protein quality control (ERQC) (Ellgaard and Helenius, 2003). Proteins that fail to pass the ERQC checkpoint may be degraded by the proteasome via a process referred to as ER-associated degradation (ERAD) (Tsai *et al.*, 2002; Kostova and Wolf, 2003; McCracken and Brodsky, 2003). This frees the ER from housing aberrant proteins and eliminates the potential formation of toxic protein aggregates. The selection of ERAD substrates and in some cases their polyubiquitination are mediated by molecular chaperones, several of which were first identified as heat-shock proteins (Hsps). Hsps facilitate protein folding or degradation, depending on the conformation of the target protein (Fewell *et al.*, 2001; Ellgaard and Helenius, 2003).

A prominent ERAD substrate is the cystic fibrosis transmembrane conductance regulator (CFTR), and many requirements for its degradation have been established. CFTR is a 1480-amino acid integral membrane glycoprotein that belongs to the ATP-binding cassette (ABC) transporter superfamily and functions as a cAMP-regulated chloride and

bicarbonate channel in the apical membrane of epithelial cells, including those of airways, pancreas, intestine, and other fluid-secreting epithelia (Pilewski and Frizzell, 1999). CFTR is composed of two membrane-spanning domains (MSD1 and MSD2), each comprising six transmembrane segments, two nucleotide-binding domains (NBD1 and NBD2), and a central regulatory (R) domain. Mutations within the gene, particularly the deletion of the phenylalanine at position 508 ( $\Delta$ F508-CFTR), may lead to cystic fibrosis (CF), one of the most common autosomal recessive disorders in individuals of European descent. Although perhaps ~75% of wild-type CFTR is targeted for ERAD, essentially all of the  $\Delta$ F508 variant is destroyed in the ER and thus fails to mature (Cheng *et al.*, 1990).

From studies in mammalian cells and in yeast, it is clear that a large number of cytoplasmic and ER luminal chaperones impact CFTR and  $\Delta$ F508-CFTR biogenesis. For example, Hsp90, Hsp40s, and the Hsp70 nucleotide exchange factor (NEF) HspBP1 facilitate the Hsp70-dependent folding of CFTR (Strickland *et al.*, 1997; Loo *et al.*, 1998; Meacham *et al.*, 1999; Rubenstein and Zeitlin, 2000; Choo-Kang and Zeitlin, 2001; Farinha *et al.*, 2002; Alberti *et al.*, 2004; Youker *et al.*, 2004; Zhang *et al.*, 2006). In addition, the ER chaperone calnexin interacts with the immature form of CFTR and  $\Delta$ F508-CFTR and may also facilitate folding (Pind *et al.*, 1994; Okiyonedo *et al.*, 2004; Farinha and Amaral, 2005). However, when an Hsp70/Hsp90-interacting E3 ubiquitin ligase, known as CHIP, is recruited to chaperone-bound CFTR, there is a shift toward the degradation pathway (Connell *et al.*, 2001; Meacham *et al.*, 2001), and more recent data indicate that CHIP acts after the RMA1 ubiquitin ligase monitors CFTR biogenesis (Younger *et al.*, 2006). Nevertheless, given the complexity of the CFTR-folding pathway (Du *et al.*, 2005; Kleizen *et al.*, 2005; Thibodeau *et al.*, 2005) and the continued

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E06-05-0458>) on December 20, 2006.

<sup>□</sup> The online version of this article contains supplemental material at *MBC Online* (<http://www.molbiolcell.org>).

Address correspondence to: Jeffrey L. Brodsky (jbrodsky@pitt.edu).

**Table 1.** Yeast strains used in this study

Strain	Genotype	Source or reference
JN516 (also referred to as wild type or <i>SSA1</i> )	<i>MAT<math>\alpha</math> leu2-3, 112 his3-1 ura3-52 trp1<math>\Delta</math>1 lys2 ssa2::LEU2 ssa3::TRP1 ssa4::LYS2</i>	Becker <i>et al.</i> (1996)
<i>RSY368</i> (also referred to as wild type or <i>HSP26</i> )	<i>Mata can1-100 leu2-3,112 his3-11, 15 trp1-1 ura3-1 ade2-1</i>	R. Schekman (University of California, Berkeley, CA)
<i>hsp26<math>\Delta</math></i>	<i>Mata can1-100 leu2-3,112 his3-11, 15 trp1-1 ura3-1 ade2-1 hsp26::KanMX4</i>	This study
<i>SEY6211</i> (also referred to as wild type or <i>HSP26HSP42</i> )	<i>Mata ura3-52 leu2-3,112 his3-<math>\Delta</math>200 trp1-<math>\Delta</math>901 ade2-101 suc2-<math>\Delta</math>9 GAL10</i>	Robinson <i>et al.</i> (1988)
<i>hsp26<math>\Delta</math>hsp42<math>\Delta</math></i>	<i>Mata ura3-52 leu2-3,112 his3-<math>\Delta</math>200 trp1-<math>\Delta</math>901 ade2-101 suc2-<math>\Delta</math>9 GAL10 hsp26::HIS3 hsp42::LEU2</i>	Haslbeck <i>et al.</i> (2004)
<i>HRD1DOA10</i> (also referred to as wild type)	<i>MAT<math>\alpha</math>, his3-<math>\Delta</math>200, leu2-3, 112, ura3-52, lys2-801, trp1-1</i>	M. Hochstrasser (Yale University, New Haven, CT)
<i>hrd1<math>\Delta</math>doa10<math>\Delta</math></i>	<i>MAT<math>\alpha</math>, his3-<math>\Delta</math>200, leu2-3, 112, ura3-52, lys2-801, trp1-1, doa10::HIS3, hrd1::LEU2</i>	M. Hochstrasser

identification of factors that facilitate protein folding in the ER and ERAD, it is likely that additional chaperones and chaperone-like proteins that impact CFTR maturation remain to be discovered.

In this report, we show that small heat-shock proteins (sHsps) facilitate CFTR degradation in yeast and that overexpression of a mammalian sHsp selectively accelerates the degradation of  $\Delta$ F508-CFTR. These data suggest that sHsps distinguish terminally misfolded forms of  $\Delta$ F508-CFTR from the wild-type protein and add cystic fibrosis to the growing list of diseases and homeostatic processes that sHsps are thought to affect, a list that includes disorders associated with protein aggregation, ischemia/reperfusion injury, tumorigenesis, progression and metastasis, and apoptosis (Sun and MacRae, 2005).

## MATERIALS AND METHODS

### Yeast Strains, Plasmids, Molecular Methods, and Antisera

The yeast strains used in this study are listed in Table 1. The *hsp26 $\Delta$*  strain in the *RSY368* background was created using polymerase chain reaction (PCR)-based gene disruption (Brachmann *et al.*, 1998). In brief, the *RSY368* wild-type strain was transformed with an *hsp26::KanMX4* disruption cassette generated by PCR from the template vector pRS400 (forward primer: GTGGTATTTTCATAA-CAACGGTCTTTTTACCCCTTATCCCTGTGCGGTATTTCACACCG; reverse primer: TGACATATGTTTCAAGCCATATGCAAGCAACAATGGTCTAGATTGTACTGAGAGTGCAC). Transformants were selected by growth on complete medium supplemented with 2% glucose and Geneticin (G-418) to a final concentration of 250  $\mu$ g/ml (Invitrogen, Carlsbad, CA). Correct insertion of the disruption cassette and the absence of the *HSP26* gene were confirmed by PCR by using primers up- and downstream of the *HSP26* location in combination with primers within the sequence of the disruption cassette and *HSP26* (*KAN* forward: ATTGATGTTGACGAGTCCG; *KAN* reverse: ATCGCAGTGGTGAGTAACCA; *HSP26* upstream, forward: GGA-TCSGGAATAGTAACCGTC; *HSP26* down-stream, reverse: GCCACATCCA-TAGAGATACC; *HSP26* coding, forward: CACCAAGACGTCAGTTAGCA; and *HSP26* coding, reverse: TGTTGTCTGCATCCACACCT).

Cells were grown at the indicated temperatures with vigorous shaking in complete medium (YP) or in synthetic complete medium lacking uracil (Sc-ura) and containing glucose at a final concentration of 2%. Hemagglutinin (HA)-tagged CFTR, CPY\*, and Ste6p\* were expressed from a 2 $\mu$  plasmid containing *URA3* as the selectable marker under the control of the constitutive phosphoglycerate kinase promoter (CFTR and Ste6p\*) or the endogenous promoter (CPY\*) (Ng *et al.*, 2000; Zhang *et al.*, 2002b; Huyer *et al.*, 2004); yeast transformed with the 2 $\mu$  plasmid pRS426 (Christianson *et al.*, 1992) without an insert served as controls. Plasmids were introduced into host strains by using lithium acetate-mediated transformation, and transformants were selected by growth on Sc-ura medium containing glucose. Protein extracts from yeast for immunoblot analysis were prepared as described previously (Brodsky *et al.*, 1998).

The antibodies used in this study were raised against the C and N terminus of CFTR (M3A7 and MM13-4, respectively; Chemicon International, Temecula, CA/Upstate Biotechnology, Lake Placid, NY), the C terminus of

CFTR (R&D Systems, Minneapolis, MN),  $\alpha$ A-crystallin, Hsp70, and BiP (Stressgen Biotechnologies, San Diego, CA), Kar2p and Ssa1p (Brodsky and Schekman, 1993), Sec61p (Stirling *et al.*, 1992), Hsp82p (a gift from A. Caplan, Mount Sinai School of Medicine, New York City, NY), Hsp26p (a gift from J. Buchner, Technical University of Munich, Munich, Germany), Hsp42p (a gift from S. Sandmeyer, University of California, Irvine, Irvine, CA), Hsp104p (a gift from J. Glover, University of Toronto, Toronto, Ontario, Canada), ubiquitin (a gift from C. Pickart and S. Michaelis, Johns Hopkins University School of Medicine, Baltimore, MD), or the HA-epitope (12CA5, Roche Molecular Biochemicals, Indianapolis, IN). To detect the primary antibody, horseradish peroxidase-conjugated secondary antibodies (anti-rabbit or anti-mouse; GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) were used.

### Preparation of RNA and Northern Blot Analysis

A yeast colony was inoculated into 4 ml of Sc-ura supplemented with 2% glucose and incubated overnight at 30°C. The culture was diluted into 250 ml of the same medium and grown to an OD<sub>600</sub> of ~0.3 at 30°C. Cells were harvested by centrifugation at 4000 rpm for 10 min in a Sorvall GSA rotor at 20°C. The pellet was resuspended in 25 ml of sterile water and centrifuged at 4000 rpm for 5 min at 20°C in a Sorvall SS 34 rotor. Pellets were snap frozen in liquid nitrogen and stored at -80°C. Yeast RNA was extracted three times with hot acid-phenol (Sigma-Aldrich, St. Louis, MO) (Ausubel *et al.*, 1988) and twice with chloroform and subjected to the RNeasy Midi kit (QIAGEN, Valencia, CA). Only samples with a ratio of  $A_{260}/A_{280}$  higher than 1.8 were used. RNA integrity was confirmed on a formaldehyde-agarose gel.

For Northern blots, 20  $\mu$ g of RNA was resolved on a formaldehyde-agarose gel, transferred to Gene Screen Plus membranes (PerkinElmer Life Science and Analytical Sciences, Boston, MA), and hybridized using <sup>32</sup>P-labeled, randomly primed probes produced from PCR fragments by using primers against *HSP26* (forward: CACCAAGACGTCAGTTAGCA, reverse: TGTT-GTCTGCAT CCACACCT), *FES1* (forward: CTACAGCAGTTATTCGGTGG, reverse: ACTCTCGT CAGACAAGCACT), *HSP82* (forward: AGAGAGCAC-CATTCGACTTG, reverse: AATTGACCA GTTCTGATAGC), and *SEC61* (forward: CCAACCGTGGTACTTT ACTG, reverse: GTCCAGAAGAGCTTCG-GATA). Filters were processed as described previously (Ausubel *et al.*, 1988), except that the high-temperature wash was performed at 55°C for 10 min. Bound probe was removed from the filters by incubation in 0.1X SSC, supplemented with an additional 15 mM NaCl, and containing 1% SDS for 20 min at 100°C.

### ERAD Assays

The ERAD of HA-tagged CFTR in yeast was performed as described previously (Zhang *et al.*, 2002b) but by using only fresh transformants, and data were quantified with a Kodak 440CF Image Station and Kodak 1D (version 3.6; Eastman Kodak, Rochester, NY) software. The degradation of HA-tagged CPY\* and HA-tagged Ste6p\* were determined by pulse-chase immunoprecipitations from <sup>35</sup>S-metabolically labeled yeast as published previously (Zhang *et al.*, 2001). Wild-type CFTR/ $\Delta$ F508-CFTR degradation and maturation in human embryonic kidney (HEK)293 cells were assessed by pulse-chase immunoprecipitation as reported previously (Zhang *et al.*, 2002a). The immunoprecipitates were resolved by SDS-PAGE and analyzed by PhosphorImager analysis by using Image Gauge software (version 3.45; FujiFilm Science Lab, Stamford, CT). Statistical analyses were performed via the program available at [http://faculty.vassar.edu/lowry/t\\_ind\\_stats.html](http://faculty.vassar.edu/lowry/t_ind_stats.html).

### Detection of CFTR Polyubiquitination

Cells expressing CFTR-HA and containing a vector for the copper-inducible expression of a *myc*-tagged form of ubiquitin (provided by M. Hochstrasser,

Yale University, New Haven, CT) were grown to log phase in 20 ml of selective medium at 30°C before 100  $\mu$ M CuSO<sub>4</sub> was added, and the cells were incubated for 3 h. Pelleted cells were washed with ice-cold 10 mM NaN<sub>3</sub> and disrupted with glass beads in 200  $\mu$ l of 20 mM HEPES, pH 7.4, 50 mM KOAc, 2 mM EDTA, 0.1 M sorbitol, 1 mM dithiothreitol and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1  $\mu$ g/ml leupeptin, and 0.5  $\mu$ g/ml pepstatin A) by agitation on a Vortex mixer five times for 1 min with 1-min intervals on ice between each cycle. The homogenate was collected and pooled with two 500- $\mu$ l rinses of the beads with buffer 88 (20 mM HEPES, pH 6.8, 150 mM KOAc, 250 mM sorbitol, and 5 mM MgOAc). After unbroken cells were removed by centrifugation, the supernatant was centrifuged at 18,000  $\times$  g for 20 min at 4°C. The crude membrane pellet was washed and resuspended in buffer 88 so that the absorbance at 280 nm in 2% SDS was ~40. Membranes (10  $\mu$ l) were mixed with 10  $\mu$ l of buffer 88 and solubilized in 80  $\mu$ l of 50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1.25% SDS, 10 mM N-ethylmaleimide, and protease inhibitors at 37°C for 20 min. Samples were added to 400  $\mu$ l of 50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 2% Triton X-100, 10 mM N-ethylmaleimide, and protease inhibitors and insoluble material was removed by centrifugation at 18,000  $\times$  g for 2 min. CFTR was immunoprecipitated from the supernatants with anti-HA antibody and resolved on a 6% SDS-polyacrylamide gel. CFTR and polyubiquitinated CFTR were decorated on immunoblots with anti-HA antibody and anti-ubiquitin antibody, respectively, after the nitrocellulose filter was incubated in boiling water for 20 min. The bound antibodies were visualized using enhanced chemiluminescence, and the data were quantified as described above.

### Mammalian Cell Culture, Plasmids, and Molecular/Cellular Methods

HEK293 cells were cultured in DMEM (Sigma-Aldrich) with 10% fetal bovine serum (Invitrogen), 4 mM L-glutamine (Sigma-Aldrich), and penicillin-streptomycin (Invitrogen) at 37°C in a humidified environment containing 5% CO<sub>2</sub>. The  $\alpha$ A-crystallin gene was removed from a pCneo vector (provided by U. Andley, Washington University, St. Louis, MO) with Sall (3') and XbaI (5'), and the gene was subcloned into pcDNA3.1 (the Sall site was lost in the cloning process). Construction of CFTR and  $\Delta$ F508-CFTR expression plasmids (pcDNA3.1) and the CFTR C-terminal deletion constructs terminating at residue 588 were described previously (Zhang *et al.*, 2002a; Sun *et al.*, 2006).

HEK293 cells were transiently transfected with the indicated pcDNA3.1 expression plasmids aided by Lipofectamine 2000 (Invitrogen). After 24 h, the cells were subjected to a pulse-chase analysis as described previously (Zhang *et al.*, 2002a) or were lysed in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, and 0.5% NP-40. For coimmunoprecipitation experiments, the cultures were treated with 50  $\mu$ M MG-132 after transfection for 12 h before cell lysis.

### Small Heat-Shock Protein-CFTR Interaction Assays

In total, 500  $\mu$ g of HEK293 cell lysate was added to 13.3 mM HEPES, pH 7.0, 85 mM potassium-glutamate, 3.3 mM NaCl, 4.7 mM MgSO<sub>4</sub>, 13.3 mM EGTA, and 2.2 mM CaCl<sub>2</sub> (vol/vol, 2:1) and incubated with 1  $\mu$ g of anti-CFTR antibody M3A7 (Upstate Biotechnology) and anti-CFTR C-terminal antibody (R&D Systems) or with anti- $\alpha$ A-crystallin antibody (Stressgen Biotechnologies) for 2 h. The immunocomplex was isolated by incubation with protein A and G agarose beads for 2 h. Precipitates were isolated and washed three times with 13.3 mM HEPES, pH 7.0, 85 mM potassium-glutamate, 3.3 mM NaCl, 4.7 mM MgSO<sub>4</sub>, 13.3 mM EGTA, 2.2 mM CaCl<sub>2</sub>, and 0.25% NP-40, and the proteins were resolved on 4–15% SDS-polyacrylamide gels and subjected to Western blot analysis. Data were quantified using ImageJ software (<http://rsb.info.nih.gov/ij/>) and analyzed as described above.

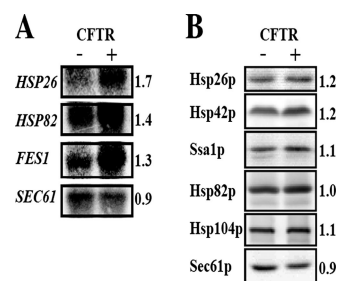
### Purification of $\alpha$ A-Crystallin and the NBD1 Aggregation Assay

$\alpha$ A-crystallin was expressed in *Escherichia coli* BL21 DE from the pAED4 vector (provided by K. P. Das, Bose Institute, Kolkata, India), and the protein was purified as described previously (Biswas and Das, 2004). The molar ratio stated assumes that the sHsp is a monomer. The ability of  $\alpha$ A-crystallin to suppress NBD1 aggregation (provided by R. Youker, University of Pittsburgh, Pittsburgh, PA) was determined as described previously (Strickland *et al.*, 1997; Youker *et al.*, 2004).

## RESULTS

### Induction of sHsps in Yeast Expressing CFTR

Because yeast expressing CFTR and  $\Delta$ F508-CFTR grow as well as cells lacking the CFTR-expression vector, and because CFTR fails to elicit an unfolded protein response (UPR) in yeast (Zhang *et al.*, 2001), we surmised that the expression of specific factors required for the ERAD of CFTR might be induced. To identify these factors, we examined the transcriptional profiles of yeast expressing wild-type

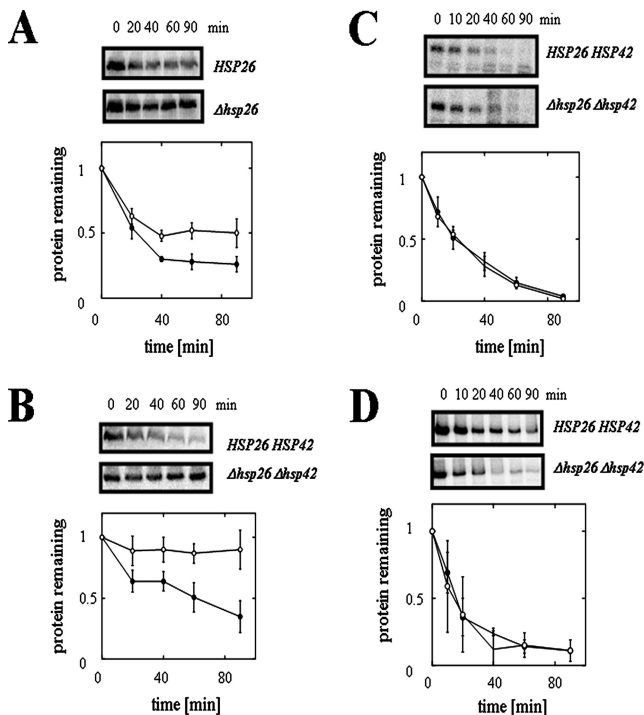


**Figure 1.** HSP26, HSP82, and FES1 mRNA levels are elevated in yeast expressing CFTR. (A) The indicated mRNAs were detected by Northern blot analysis from yeast strain *JN516* transformed with a plasmid expressing CFTR and yeast transformed with a vector control (-; pRS426). The -fold up-regulation upon CFTR expression as determined by Northern blot analysis is indicated, and when standardized to the levels of *SEC61* message, they are *HSP26*, 2.0; *HSP82*, 1.6; and *FES1*, 1.5. By comparison, the average -fold up-regulation from the microarray analyses ( $n = 6$ ) are *HSP26*, 1.4; *HSP82*, 1.3; and *FES1*, 1.4. (B) Total cell extracts were prepared from yeast either lacking or containing the CFTR expression vector, as in A, and proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and analyzed with the indicated antisera. The ratios of protein in each sample from two independent sets of experiments were quantified as described in *Materials and Methods*.

CFTR and control strains by microarray analysis (Ahner and Brodsky, our unpublished data; details on the protocol and statistical analyses of the profiles will be reported elsewhere). Among the ~150 transcripts up-regulated in strains expressing CFTR were *HSP82*, which encodes the yeast Hsp90 homologue, and *FES1*, which is a NEF for a cytosolic Hsp70; both of the corresponding proteins are known to play a role in CFTR biogenesis in yeast and/or mammalian cells (Loo *et al.*, 1998; Zhang *et al.*, 2001; Kabani *et al.*, 2002; Alberti *et al.*, 2004). In addition, we found that the message encoding Hsp26p increased significantly. These results were confirmed by Northern blot analysis (Figure 1A). We also examined the levels of Hsp26p and Hsp42p, two sHsps in yeast (also see below) as well as other chaperones (Figure 1B), some of which were unaffected by CFTR expression as determined by transcriptional profiling. We first noted a reproducible, ~20% increase in the sHsps, whereas little or no change was observed for Ssa1p (a yeast Hsp70) or Hsp104p. Although the *HSP82* message increased, there was no increase in protein levels, suggesting posttranscriptional control. Interestingly, we also noted that the message and protein levels corresponding to Sec61p, a component of the translocon and a UPR target (Casagrande *et al.*, 2000) mildly decreased. These data indicate that CFTR expression does not induce a general heat-shock/stress response in yeast, although the levels of two sHsps rise when CFTR is produced.

### sHsps Facilitate the Degradation of CFTR in Yeast

The expression of Hsp26p is induced by elevated temperature and Hsp26p prevents protein aggregation (Haslbeck *et al.*, 2005). To assess whether Hsp26p depletion affects CFTR turnover, we expressed an HA-epitope-tagged form of wild-type CFTR under the control of a constitutive promoter in wild type yeast and in yeast deleted for *HSP26*. The rate of CFTR degradation was determined by cycloheximide chase analysis and levels of Sec61p were measured to provide a loading control (data not shown). As shown in Figure 2A, we found that the extent of CFTR proteolysis at 90 min was reduced in *hsp26 $\Delta$*  yeast compared with wild-type cells by

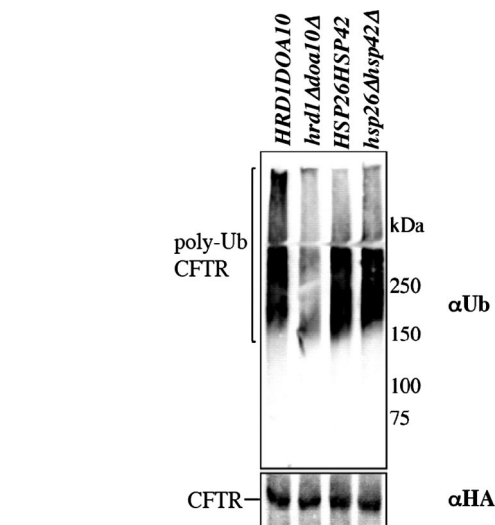


**Figure 2.** Deletion of the small heat-shock protein-encoding genes slows CFTR degradation in yeast. CFTR degradation was assessed in wild-type and  $hsp26\Delta$  yeast (A) and in wild type and  $hsp26\Delta hsp42\Delta$  yeast (B). CFTR protein levels were quantified and averaged from at least three independent sets of experiments and were standardized to the levels detected directly after cycloheximide addition (0 min). Closed circles represent the CFTR protein levels in wild-type yeast, and open circles represent the CFTR protein levels in the mutant strains. Vertical bars indicate the SEs of the mean. *p* values in A at the 40, 60, and 90-min time points are 0.011, 0.022, and 0.062, respectively, and in part B are  $<0.03$ . CPY\* (C) and Ste6p\* (D) degradation rates were determined by pulse-chase immunoprecipitation experiments in  $HSP26HSP42$  wild-type yeast and in the  $hsp26\Delta hsp42\Delta$  double mutant strain after addition of cycloheximide. Protein levels were quantified from two independent sets of experiments and averaged. Standardization of the data and symbols are the same as described above. Vertical bars indicate the range of the data.

~50%, indicating that Hsp26p function is important for maximal CFTR degradation.

sHsps are distinguished by the presence of an  $\alpha$ -crystallin domain (van Montfort *et al.*, 2001), and *Saccharomyces cerevisiae* encodes two  $\alpha$ -crystallin domain-containing proteins, Hsp26p and Hsp42p. Although they recognize ~90% of the same client proteins, only Hsp42p is present constitutively at high levels (Haslbeck *et al.*, 2004). This suggested that the continued expression of Hsp42p might have ameliorated the ERAD defect when  $HSP26$  was deleted. To examine this hypothesis, CFTR was expressed in a different strain deleted for both  $HSP26$  and  $HSP42$ , and indeed a much stronger degree of CFTR stabilization was observed (Figure 2B). For comparison, the deletion of either  $HSP26$  or  $HSP42$  in this strain background led to only subtle effects on CFTR degradation (Supplemental Figure 1S). These results suggest that the sHsps function redundantly during ERAD.

Different ERAD substrates require distinct factors for their degradation, depending on their solubility, membrane association, and location of structural alterations (see Discussion). Thus, it was important to establish whether the proteolysis of other substrates was altered by the loss of Hsp26p



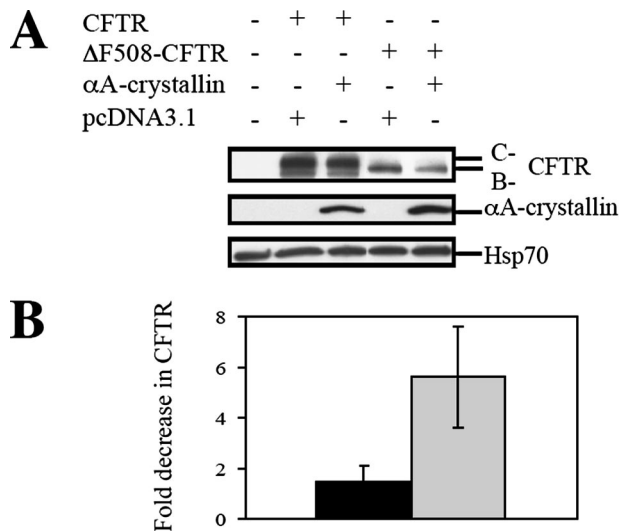
**Figure 3.** Deletion of the small heat-shock protein-encoding genes does not impact CFTR polyubiquitination in yeast. CFTR polyubiquitination was assessed in wild-type and  $hsp26\Delta hsp42\Delta$  yeast and in wild-type and  $hrd1\Delta doa10\Delta$  yeast as described in *Materials and Methods*.

and Hsp42p. To this end, wild-type and  $hsp26\Delta hsp42\Delta$  strains were transformed with a vector for the constitutive expression of CPY\*, a soluble ERAD substrate (Hiller *et al.*, 1996), and Ste6p\*, a truncated form of the yeast  $\alpha$ -mating factor pheromone transporter that, like CFTR, is an ABC transporter and is degraded via ERAD (Loayza *et al.*, 1998). As shown in Figure 2, C and D, we found that the  $hsp26\Delta hsp42\Delta$  mutant proficiently degraded both CPY\* and Ste6p\*, demonstrating that sHsps are important for the proteolysis of only a select class of ER substrates. It is also important to note that these experiments were performed at a permissive temperature for the sHsp mutant strain (30°C) (Petko and Lindquist, 1986). Moreover, Kar2p, a UPR-regulated luminal Hsp70, was not induced in  $hsp26\Delta hsp42\Delta$  cells (our unpublished data). These observations indicate that the observed ERAD defect is due to deletion of the sHsps rather than to a nonspecific up-regulation of stress-inducible genes.

To identify the stage at which the yeast sHsps impact CFTR turnover, CFTR was immunoprecipitated from wild-type and  $hsp26\Delta hsp42\Delta$  cells, and the degree of polyubiquitination was assessed by immunoblot analysis. As a control, CFTR polyubiquitination was also measured in wild-type yeast and in cells lacking  $DOA10$  and  $HRD1$ , two ubiquitin ligases that catalyze CFTR degradation in this organism (Gnann *et al.*, 2004). Surprisingly, we found that CFTR polyubiquitination was unaffected in cells lacking the sHsps, although the degree of modification when the ubiquitin ligases were disabled was  $<50\%$  of the control (Figure 3). We also noted that the steady-state level of total polyubiquitinated proteins in yeast deleted for both  $HSP26$  and  $HSP42$  was unaffected, but was modestly higher when ubiquitin was overexpressed (Supplemental Figure 2S).

#### Overexpression of the Human sHsp $\alpha$ A-Crystallin Accelerates $\Delta F508$ -CFTR Degradation in HEK293 Cells

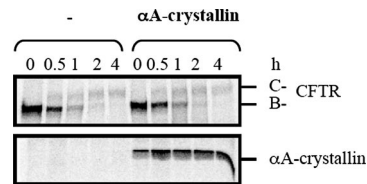
To determine whether sHsps impact CFTR biogenesis in mammals, HEK293 cells were transfected with vectors for the overexpression of wild-type CFTR or  $\Delta F508$ -CFTR and either a vector control or a vector engineered for the overexpression of  $\alpha$ A-crystallin.  $\alpha$ A-crystallin is one of 10 " $\alpha$ -crystallin-like" proteins in humans (Kappe *et al.*, 2003), and



**Figure 4.**  $\alpha$ A-crystallin overexpression decreases the steady state levels of  $\Delta$ F508-CFTR. HEK293 cells were transfected with 1.5  $\mu$ g of pcDNA3.1-CFTR or pcDNA3.1- $\Delta$ F508-CFTR and 0.5  $\mu$ g of pcDNA3.1 or pcDNA3.1- $\alpha$ A-crystallin per 60-mm dish. (A) A representative immunoblot of the indicated proteins is shown after protein concentrations were normalized in all samples. Because Hsp70 levels remained constant, the amount of this chaperone served as an additional loading control. (B) CFTR protein levels were quantified from three independent sets of experiments and averaged. All values were obtained after standardization to the levels of Hsp70. The black bar represents the -fold decrease of wild-type CFTR total levels upon  $\alpha$ A-crystallin overexpression, and the gray bar depicts the -fold decrease of  $\Delta$ F508-CFTR levels upon  $\alpha$ A-crystallin overexpression. Vertical bars indicate the SEs of the means ( $p = 0.013$ ;  $t$  test).

as observed previously for Hsp26p and Hsp42p,  $\alpha$ A-crystallin associates with unfolded proteins and prevents their aggregation (Horwitz, 1992; Jakob *et al.*, 1993; Biswas and Das, 2004). In addition,  $\alpha$ A-crystallin is expressed in human cells that normally synthesize CFTR (our unpublished data). When CFTR expression was examined in extracts from each cell type by Western blot analysis, we noted that  $\alpha$ A-crystallin overexpression did not alter the amount of wild-type CFTR but that  $\Delta$ F508-CFTR steady-state levels decreased (Figure 4). Because Hsp70 levels were unchanged, the observed effect was likely not a result of secondary consequences from enhanced  $\alpha$ A-crystallin expression.

To investigate directly whether  $\alpha$ A-crystallin accelerated CFTR and/or  $\Delta$ F508-CFTR degradation, HEK293 cells were again transfected with the CFTR or  $\Delta$ F508-CFTR expression vector and either the vector control or the  $\alpha$ A-crystallin expression vector. Pulse-chase immunoprecipitation experiments were then performed to follow the stability of core-glycosylated immature CFTR (B-band) and its maturation to the complex-glycosylated form (C-band) (Figure 5);  $\Delta$ F508-CFTR fails to mature and thus only the B-band is evident (Figure 6). We observed that for wild-type CFTR the disappearance of CFTR B-band and appearance of the C-band proceeded at equal rates in cultures transfected with the empty vector and in those overexpressing  $\alpha$ A-crystallin. In contrast, the overexpression of  $\alpha$ A-crystallin enhanced  $\Delta$ F508-CFTR proteolysis by diminishing a reproducible lag in the disappearance of the B-band (Figure 6). This result indicates that  $\alpha$ A-crystallin impacts only the ERAD of  $\Delta$ F508-CFTR and thus might be able to distinguish between the wild type and mutant channels in mammals.

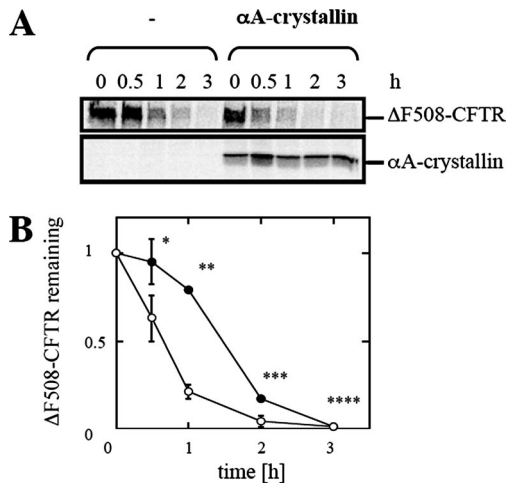


**Figure 5.**  $\alpha$ A-crystallin overexpression has no effect on the biogenesis of wild-type CFTR. HEK293 cells were transfected with 1.5  $\mu$ g of pcDNA3.1-CFTR and 0.5  $\mu$ g of pcDNA3.1 or pcDNA3.1- $\alpha$ A-crystallin per 60-mm dish. Rates of CFTR maturation (C-band) and CFTR B-band degradation as well as  $\alpha$ A-crystallin expression levels were determined by pulse-chase immunoprecipitation in cells transfected with CFTR and containing a vector control or the  $\alpha$ A-crystallin expressing vector.

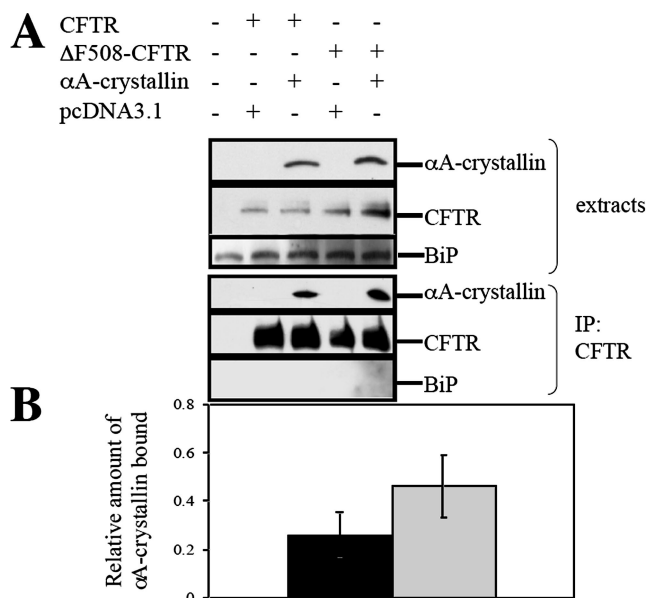
**$\alpha$ A-Crystallin Interacts Preferentially with  $\Delta$ F508-CFTR and Prevents the Aggregation of NBD1**

The interaction between  $\alpha$ A-crystallin and CFTR and  $\Delta$ F508-CFTR was assessed by immunoprecipitation after cotransfection of HEK293 cells with the  $\alpha$ A-crystallin expression vector and expression vectors for either the wild-type or mutant protein. As displayed in Figure 7, the sHsp was isolated in a complex with CFTR or  $\Delta$ F508-CFTR and by quantifying the amount of CFTR/ $\Delta$ F508-CFTR relative to the chaperone, we estimated that approximately twofold more  $\alpha$ A-crystallin bound to  $\Delta$ F508-CFTR than to the wild-type protein.

Eukaryotic sHsps associate with unfolded proteins and prevent their aggregation, which may be important for the proteasome-mediated degradation of some substrates (Horwitz, 1992; Jakob *et al.*, 1993; Ehrnsperger *et al.*, 1997; Lee *et al.*, 1997; Haslbeck *et al.*, 1999, 2004; Biswas and Das, 2004).



**Figure 6.**  $\alpha$ A-crystallin overexpression accelerates the degradation of  $\Delta$ F508-CFTR. (A) HEK293 cells were transfected with 1.5  $\mu$ g of pcDNA3.1- $\Delta$ F508-CFTR and 0.5  $\mu$ g of pcDNA3.1 or pcDNA3.1- $\alpha$ A-crystallin per 60-mm dish. Rates of  $\Delta$ F508-CFTR degradation as well as  $\alpha$ A-crystallin expression levels were determined by pulse-chase immunoprecipitation. (B)  $\Delta$ F508-CFTR degradation was determined from four independent sets of experiments and averaged. All values were obtained after standardization to the levels detected at the beginning of the chase period (0 h). Closed circles represent  $\Delta$ F508-CFTR protein levels in HEK293 cells with the vector control, and open circles represent  $\Delta$ F508-CFTR protein levels in HEK293 cells overexpressing  $\alpha$ A-crystallin. Vertical bars indicate the SEs of the mean. \* $p = 0.076$ , \*\* $p = 0.0001$ , \*\*\* $p = 0.003$ , and \*\*\*\* $p = 0.5$ .

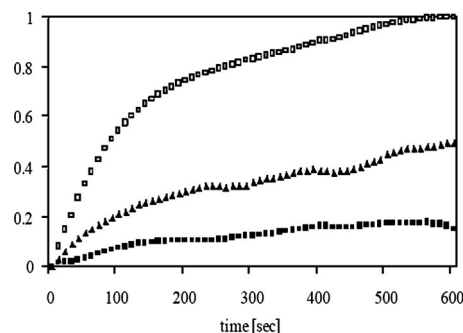


**Figure 7.**  $\alpha A$ -crystallin coprecipitates preferentially with  $\Delta F508$ -CFTR. (A) HEK293 cells were transfected with 6  $\mu g$  of pcDNA3.1-CFTR or pcDNA3.1- $\Delta F508$ -CFTR and 2  $\mu g$  of pcDNA3.1 or pcDNA3.1- $\alpha A$ -crystallin per 100-mm dish. The interaction between CFTR or  $\Delta F508$ -CFTR and  $\alpha A$ -crystallin was assessed by immunoblot analysis for  $\alpha A$ -crystallin after CFTR immunoprecipitation. The ER luminal chaperone BiP served as a negative control. (B) Relative binding efficiency was determined from 3 independent sets of experiments and averaged. All values were obtained after standardization to the amount of CFTR immunoprecipitated. The black bar represents  $\alpha A$ -crystallin protein levels coprecipitated with wild-type CFTR, and the gray bar represents  $\alpha A$ -crystallin protein levels coprecipitated with  $\Delta F508$ -CFTR. Vertical bars indicate the SEs of the mean. \* $p = 0.012$  (one-way analysis of variance for 2 correlated samples).

Because NBD1 in CFTR is aggregation prone and interacts with other chaperones that prevent its aggregation (Qu and Thomas, 1996; Strickland *et al.*, 1997; Meacham *et al.*, 1999; Youker *et al.*, 2004), we examined whether  $\alpha A$ -crystallin also inhibits the formation of high-molecular-weight NBD1 oligomers. Toward this goal,  $\alpha A$ -crystallin was expressed in and purified from *E. coli* and then incubated with NBD1 upon its dilution from denaturant. At a chaperone-to-substrate molar ratio of 0.5:1,  $\alpha A$ -crystallin suppressed NBD1 aggregation by  $\sim 50\%$ , and at a molar ratio of 1:1 aggregation was suppressed by  $\sim 80\%$  (Figure 8). Although most chaperones suppress NBD1 aggregation to a similar extent only when used at higher molar ratios (e.g., Hdj2:NBD1 at 5:1, Hsc70-Hdj2:NBD1 at 2:1, and Hsp90:NBD1 at 5:1; Meacham *et al.*, 1999; Youker *et al.*, 2004), the near complete prevention of aggregation at a 1:1 molar ratio of sHsps to other substrates is common (Horwitz, 1992; Jakob *et al.*, 1993; Chang *et al.*, 1996; Ehrnsperger *et al.*, 1997; Haslbeck *et al.*, 1999; Studer and Narberhaus, 2000). Overall, and combined with the results presented above, we propose that  $\alpha A$ -crystallin plays an important and previously unappreciated role in chaperoning the degradation of  $\Delta F508$ -CFTR.

## DISCUSSION

By profiling which yeast genes are induced upon CFTR expression, we discovered that sHsps catalyze the degradation of CFTR in yeast and  $\Delta F508$ -CFTR in mammalian cells.



**Figure 8.**  $\alpha A$ -crystallin inhibits NBD1 aggregation. NBD1 aggregation was measured as a change in absorbance at 400 nm after its dilution from guanidinium hydrochloride in the absence or presence of  $\alpha A$ -crystallin. Data were standardized to the amount of aggregation in the absence of chaperone at the 600-s time point. Open squares represent endogenous NBD1 aggregation, and closed symbols represent NBD1 aggregation in the presence of  $\alpha A$ -crystallin. Triangles,  $\alpha A$ -crystallin:NBD1 molar ratio of 0.5:1; squares,  $\alpha A$ -crystallin:NBD1 molar ratio of 1:1.

We also found that one sHsp,  $\alpha A$ -crystallin, suppressed NBD1 aggregation and coprecipitated preferentially with  $\Delta F508$ -CFTR. Because CFTR polyubiquitination was robust in yeast deleted for  $\alpha$ -crystallin domain-containing proteins, we suggest that sHsps increase the accessibility of CFTR to the proteasome after polyubiquitination. If this is true, one might have expected an increase in the steady-state level of polyubiquitinated CFTR, but we cannot exclude the possibility that there was a concomitant increase in the activity of a deubiquitination enzyme or in the amount of aggregated protein. It is also formally possible that sHsps increase the accessibility of CFTR to the ubiquitin-conjugating machinery. The simplest model, though, is that sHsps maintain the solubility of a select group of ERAD substrates after ubiquitin modification.

An increasing number of papers implicate sHsps in the ubiquitin-proteasome pathway. Consistent with our findings  $\alpha B$ -crystallin and Hsp27 are up-regulated by and are recruited to aggresomes (Ito *et al.*, 2002), which are large, cytoplasmic inclusions of mis-folded proteins (Johnston *et al.*, 1998). In addition, Hsp27 binds to the 26S proteasome and to a polyubiquitinated substrate (Parcellier *et al.*, 2003). sHsps might directly catalyze the proteolysis of ERAD substrates because  $\alpha$ -crystallins regulate proteasome activity and interact with a subunit in the proteasome core (Wagner and Margolis, 1995; Conconi *et al.*, 1998, 1999; Boelens *et al.*, 2001; Ito *et al.*, 2002). However, other papers suggest a role for sHsps during substrate ubiquitination:  $\alpha B$ -crystallin has been proposed to recruit FBX4, an adaptor molecule for the SCF ubiquitin ligase and promote substrate ubiquitination (den Engelsman *et al.*, 2003; den Engelsman *et al.*, 2004), and in vitro Hsp27 seems to facilitate the ubiquitination reaction (Parcellier *et al.*, 2006). Overall, understanding the precise mechanism of sHsp action during ERAD represents an important, ongoing goal.

The observation that sHsps regulate the half-life of CFTR but not the turnover of a soluble ERAD substrate is in agreement with previous reports demonstrating distinct requirements for the degradation of different classes of ERAD clients (Plemper *et al.*, 1997; Brodsky *et al.*, 1999; Hill and Cooper, 2000; Fewell *et al.*, 2001; Nishikawa *et al.*, 2001; Vashist *et al.*, 2001; Zhang *et al.*, 2001; Kabani *et al.*, 2003; Taxis *et al.*, 2003; Ahner and Brodsky, 2004; Huyer *et al.*, 2004; Vashist and Ng, 2004; Sayeed and Ng, 2005). However,

a recent study reported similar requirements for the ERAD of CFTR and Ste6p\* in yeast, which is in contrast to the results presented in Figure 2D (Huyer *et al.*, 2004). Nevertheless, these newer data are consistent with the exquisite ability of sHsps to recognize subtle conformational differences between substrates (Basha *et al.*, 2006, and see below) and with the fact that CFTR and Ste6p\* are structurally distinct members of the same transporter family. Specifically, the sequence similarity between Ste6p\* and CFTR is low and restricted to the NBDs (Kuchler *et al.*, 1989) and only CFTR contains an R domain. Therefore, it will be important in the future to assess whether other aberrant ABC transporters in yeast require Hsp26p/Hsp42p for their degradation, and combined with the continued structural analysis of ABC transporters (see, for example, Lewis *et al.*, 2004), it is hoped that the determinant(s) for their degradation can be ascertained.

Our observation that  $\alpha$ A-crystallin enhances exclusively the degradation of  $\Delta$ F508-CFTR in mammals implies that this sHsp distinguishes the wild type and the mutant variants of the channel. Although immature CFTR and  $\Delta$ F508-CFTR are often assumed to adopt the same aberrant conformation—one that directs the proteins for ERAD—other evidence indicates discernible differences between immature wild-type and  $\Delta$ F508-CFTR. First, Meacham *et al.* (1999) observed an approximately twofold higher abundance of Hsc70- $\Delta$ F508-CFTR complexes than Hsc70-CFTR complexes. Second, cotransfection of Hsc70 and Hdj-1 stabilized exclusively the immature form of wild-type CFTR (Farinha *et al.*, 2002). Third, coexpression of Csp1 slowed the degradation of immature wild-type CFTR but increased the turnover of  $\Delta$ F508-CFTR (Zhang *et al.*, 2006; our unpublished data). Fourth, complexes between calnexin and  $\Delta$ F508-CFTR were more stable than those between calnexin and wild-type CFTR (Pind *et al.*, 1994). Fifth, the overexpression of RMA1, which may monitor folding defects in  $\Delta$ F508-CFTR, has a greater effect on  $\Delta$ F508-CFTR than on wild-type CFTR degradation (Younger *et al.*, 2006). And finally,  $\Delta$ F508-CFTR seems to be degraded independently of glycan modification, whereas proteolysis of the wild-type protein requires the glycan moiety and calnexin function (Farinha and Amaral, 2005). Moreover, we note that sHsps can distinguish between wild-type protein conformations and structurally identical mutant variants that differ only in their free energies of unfolding; thus, sHsps may be able to recognize transient nonnative states (McHaourab *et al.*, 2002; Koteiche and McHaourab, 2003; Sathish *et al.*, 2004; Shashidharamurthy *et al.*, 2005), and indeed the cytosolic domain of  $\Delta$ F508-CFTR exhibits a distinct conformation as assessed by limited proteolysis (Zhang *et al.*, 1998). Interestingly, however, we found that  $\alpha$ A-crystallin bound with equal efficiencies to an N-terminal, NBD1-containing fragment of wild-type and  $\Delta$ F508-CFTR (Supplemental Figure 3S), suggesting that the chaperone recognizes the  $\Delta$ F508-folding defect only after the full-length protein has been synthesized.

A hint that sHsps might also respond to the long-term expression of  $\Delta$ F508-CFTR in humans was recently provided (Roxo-Rosa *et al.*, 2006). A proteomic analysis of nasal cells from healthy and  $\Delta$ F508-CFTR-expressing individuals uncovered 65 differentially expressed “spots,” and among these polypeptides was Hsp27. Interestingly, Hsp27 levels decreased approximately twofold in the samples from CF patients. Although the nature of this effect is unclear, one hypothesis—consistent with previous data—is that the chaperone negatively regulates the immune response (De *et al.*, 2000). Another hypothesis is that the synthesis of this sHsp responds to the concentration of CFTR: Because the

steady-state level of  $\Delta$ F508-CFTR is low, Hsp27 levels may also drop. However, it is not clear whether the reduction of this sHsp impacts  $\Delta$ F508-CFTR turnover.

If sHsps only affect the degradation of  $\Delta$ F508-CFTR in mammals, why is the wild-type protein stabilized in yeast lacking Hsp26p and Hsp42p? One answer to this question is that CFTR might be unable to assume the native conformation in this organism, and support for this hypothesis derives from the observation that CFTR fails to exit the ER and is quantitatively degraded in yeast (Kiser *et al.*, 2001; Zhang *et al.*, 2001; Fu and Sztul, 2003). We therefore propose that the yeast CFTR expression system better reflects  $\Delta$ F508-CFTR biogenesis than wild-type CFTR biogenesis. Nevertheless, our data suggest that yeast can be used to identify factors that affect the maturation of any mammalian protein, assuming that an expression system has been developed and validated.

## ACKNOWLEDGMENTS

We thank Drs. Fei Sun, Johannes Buchner, Cecile Pickart, Susan Michaelis, Mark Hochstrasser, Robert Youker, Usha Andley, Kali Das, Avrom Caplan, Suzanne Sandmeyer, John Glover, and Rebecca Hughey for providing reagents. This work was supported by grants from the Cystic Fibrosis Foundation to J.L.B. and R.A.F. and by National Institutes of Health Grants DK-075061 and DK-68196 (to J.L.B. and R.A.F., respectively). K.N. acknowledges support from the Uehara Memorial Foundation and a postdoctoral fellowship from the American Heart Association.

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