

# Distinct Roles for the Hsp40 and Hsp90 Molecular Chaperones during Cystic Fibrosis Transmembrane Conductance Regulator Degradation in Yeast

Robert T. Youker,\* Peter Walsh,† Traude Beilharz,† Trevor Lithgow,† and Jeffrey L. Brodsky\*†

\*Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA 15260; and †Russell Grimwade School of Biochemistry and Molecular Biology, University of Melbourne, Parkville 3010, Australia

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Aberrant secreted proteins can be destroyed by ER-associated protein degradation (ERAD), and a prominent, medically relevant ERAD substrate is the cystic fibrosis transmembrane conductance regulator (CFTR). To better define the chaperone requirements during CFTR maturation, the protein was expressed in yeast. Because Hsp70 function impacts CFTR biogenesis in yeast and mammals, we first sought ER-associated Hsp40 cochaperones involved in CFTR maturation. Ydj1p and Hlj1p enhanced Hsp70 ATP hydrolysis but CFTR degradation was slowed only in yeast mutated for both *YDJ1* and *HLJ1*, suggesting functional redundancy. In contrast, CFTR degradation was accelerated in an Hsp90 mutant strain, suggesting that Hsp90 preserves CFTR in a folded state, and consistent with this hypothesis, Hsp90 maintained the solubility of an aggregation-prone domain (NBD1) in CFTR. Soluble ERAD substrate degradation was unaffected in the Hsp90 or the Ydj1p/Hlj1p mutants, and surprisingly CFTR degradation was unaffected in yeast mutated for Hsp90 cochaperones. These results indicate that Hsp90, but not the Hsp90 complex, maintains CFTR structural integrity, whereas Ydj1p/Hlj1p catalyze CFTR degradation.

## INTRODUCTION

Mutated proteins that are unstable or fold slowly can accumulate in the ER, aggregate, and/or induce apoptosis (Thomas *et al.*, 1995; Kaufman, 1999). However, eukaryotic cells have evolved the ability to identify and degrade these aberrant proteins by a pathway termed ER-associated protein degradation (ERAD). After their identification ERAD substrates are “retro-translocated” (or “dislocated”) to the cytosol, ubiquitinated, and targeted to the 26S proteasome for degradation (Ellgaard *et al.*, 1999; Romisch, 1999; Tsai *et al.*, 2002; Kostova and Wolf, 2003; McCracken and Brodsky, 2003). How ERAD substrates are selected is not completely clear, but a family of proteins, known as molecular chaperones, are involved in this process. Several molecular chaperones bind short, linear arrays of amino acids enriched for hydrophobic residues that normally represent buried regions in folded proteins. As a result chaperones can retain mis-folded proteins in solution and facilitate their refolding and/or retro-translocation.

Previous work indicated unique chaperone requirements for the degradation of soluble and integral membrane proteins in yeast (for review see Fewell *et al.*, 2001). For example, the ER luminal Hsp70 molecular chaperone, BiP (Kar2p in yeast) is required for the efficient degradation of CPY\* (Plempner *et al.*, 1997) and pro- $\alpha$ -factor (Brodsky *et al.*, 1999), which are soluble substrates, but is dispensable for the deg-

radation of some mis-folded yeast membrane proteins (Plempner *et al.*, 1998; Zhang *et al.*, 2001; Taxis *et al.*, 2003). In contrast, cytoplasmic Hsp70 is required for the proteolysis of several ER membrane proteins but is dispensable for CPY\* and pro- $\alpha$ -factor degradation (Hill and Cooper, 2000; Zhang *et al.*, 2001). Because the ATPase activity of Hsp70s—and thus their ability to trap polypeptide substrates—is enhanced by interaction with specific Hsp40 cochaperones (“J-domain”-containing proteins), it was anticipated that luminal and cytoplasmic Hsp40s would also be required for the degradation of soluble and integral membrane ERAD substrates. Indeed, two Hsp40 homologues in the ER lumen, known as Scj1p and Jem1p, function redundantly to facilitate the BiP-dependent solubilization and retro-translocation of soluble ERAD substrates (Nishikawa *et al.*, 2001). However, the cytoplasmic Hsp40s that cooperate with the yeast Hsp70, Ssa1p, to promote the degradation of integral membrane substrates have not been conclusively identified.

Wild-type proteins can also be ERAD substrates, and one prominent example is the cystic fibrosis transmembrane conductance regulator (CFTR): ~80% of wild-type CFTR is degraded by ERAD (Jensen *et al.*, 1995; Ward *et al.*, 1995). Mutations in CFTR can cause cystic fibrosis (CF), the most prevalent fatal inherited disease in Caucasians, and the most common disease-causing allele in CFTR is the deletion of a phenylalanine at position 508 ( $\Delta$ F508). The effect of this mutation is that ~100% of the protein is degraded (Cheng *et al.*, 1990). Not surprisingly, several cytoplasmic molecular chaperones, including Hsp70, Hsp40s, and Hsp90, and the ER luminal quality control lectin, calnexin, interact with immature wild-type and  $\Delta$ F508 CFTR (Yang *et al.*, 1993; Pind *et al.*, 1994; Loo *et al.*, 1998; Meacham *et al.*, 1999; Farinha *et al.*, 2002; Zhang *et al.*, 2002). It is thought that the interaction

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† Corresponding author. E-mail address: jbrodsky@pitt.edu.

of Hsp70 and possibly Hsp90 targets CFTR to an E3 ubiquitin ligase, CHIP, whose activity is required for subsequent proteasome-mediated degradation (Höhfeld *et al.*, 2001; Meacham *et al.*, 2001).

To date, it has been difficult to ascertain whether the interactions between specific chaperones and CFTR result from their attempts to fold the protein and/or to target it for degradation. This fact is highlighted by conflicting studies when the Hsp90 chaperone is disabled and CFTR biogenesis is measured. Hsp90 is one of the most abundant proteins in the eukaryotic cytoplasm, constituting up to ~2% of total protein in nonstressed cells and is required for the folding of a diverse set of client proteins including transcription factors, steroid hormone receptors, and protein tyrosine kinases (Buchner, 1996; Caplan, 1999; Young *et al.*, 2001). Hsp90 action requires the participation of several cochaperones, which constitute the Hsp90 complex and include Hsp70, Hsp40, Hop, Hip, *cis-trans* prolyl isomerases (cyclophilins), and p23. When CFTR biogenesis was examined in BHK and CHO cells treated with geldanamycin (GA), an Hsp90 inhibitor, enhanced CFTR degradation was observed (Loo *et al.*, 1998); however, extended GA treatment might exert pleiotropic effects, including activation of heat shock factor (Zou *et al.*, 1998) and up-regulation of ER chaperones (Lawson *et al.*, 1998). In contrast, CFTR was stabilized when GA was added to a reaction containing dog pancreas microsomes into which CFTR was inserted after *in vitro* transcription/translation (Fuller and Cuthbert, 2000). In neither study was the effect of Hsp90 cochaperones on CFTR degradation investigated.

To better define the roles of molecular chaperones on CFTR biogenesis, and more generally to elucidate how membrane proteins are targeted for ERAD, we expressed CFTR in the budding yeast *Saccharomyces cerevisiae* because of the genetic tools available in this organism and because the trafficking and degradation pathways and participating chaperones are conserved between yeast and humans. We showed previously that CFTR is a bona fide ERAD substrate in yeast as it is in mammals and that Hsp70 facilitates degradation because CFTR stabilization was observed in yeast containing a rapid-onset, temperature-sensitive *SSA1* mutant allele (Zhang *et al.*, 2001). Yeast expression of CFTR has been used by others to purify the protein and obtain single-channel recordings (Huang *et al.*, 1996), to demonstrate that the COPII machinery sorts ERAD substrates to a degradative ER sub-

compartment (Fu and Sztul, 2003) and to further delineate the requirements for its degradation (Kiser *et al.*, 2001; Gnann *et al.*, 2004). For example, a recent report indicates that CFTR degradation is attenuated in *cdc48*, *ufd1*, and *htm1/mnl1* mutant yeast, suggesting a role for the cytosolic Cdc48p-Ufd1p-Npl4p complex and an ER degradation enhancing  $\alpha$ -mannosidase-like protein (EDEEM) in CFTR quality control (Gnann *et al.*, 2004).

In this report, we have again used the yeast system and find that Hsp90, but not the Hsp90 complex, helps maintain CFTR in a folded state. We have also identified two ER-associated, but cytoplasmically disposed Hsp40 cochaperones, Ydj1p and Hlj1p, which function redundantly to facilitate CFTR degradation. Finally, because Hsp90 and Ydj1p/Hlj1p are dispensable for the turnover of a soluble ERAD substrate, our results further define the chaperone requirements for the degradation of membrane and soluble ERAD substrates.

## MATERIALS AND METHODS

### Yeast Strains and Growth Conditions

Yeast strains (Table 1) were grown at 26°C unless indicated otherwise and standard methods for growth, preparation of media, and transformation of yeast cultures were used (Adams *et al.*, 1997). The *hlj1Δ/ydj1-151* and the *hlj1Δ* mutant strain were constructed using PCR-based gene disruption as described previously (Longtine *et al.*, 1998). In brief, the *hlj1Δ/ydj1-151* strain was created by transformation of strain ACY17b with *pMET25-GFP-HLJ1* (Beilharz *et al.*, 2003) followed by direct transformation with an *hlj1::TRP1* disruption cassette, generated by PCR using template vector pFA6a-TRP1 and the primer sequences (forward- GCAATATAACTTGTGGAAGGCTATAATAGGAAG-AACCGGATCCCCGGGTTAATTAA; reverse-CGTTGGAAAAATAATAAAAAAATGACAGTAAAAAATATTGTGTGAATTCGAGCTCGTTTAAAC).

Transformants were selected by plating on minimal medium lacking tryptophan and were counterselected for the *URA3*-marked *pMET25-GFP-HLJ1* plasmid by plating on minimal medium supplemented with amino acids, uracil and 5-fluoro-orotic acid. The resulting strain failed to grow on minimal medium lacking uracil, but grew on medium lacking histidine and tryptophan, confirming the double mutant had been constructed. The *hlj1Δ* strain was constructed by transformation of the isogenic wild-type strain with a *hlj1::HIS3* disruption cassette generated by PCR using template vector pFA6a-HIS3MX6 and the same primers listed above.

### ERAD Assays

Yeast strains expressing HA-CFTR were grown to logarithmic phase ( $OD_{600} = 0.4-0.8$ ) at 26°C in synthetic complete medium lacking uracil, but supplemented with glucose to a final concentration of 2% (SC-ura), and protein synthesis was stopped by the addition of cycloheximide to a final concentration of 50  $\mu$ g/ml. Cells were shifted to 37°C and 2.0–2.5 ODs of cells were removed at the indicated time points. The cells were washed, and total protein was precipitated (Zhang *et al.*, 2002). Proteins were resolved on either

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

Strain name	Genotype	Source
p82a	<i>MAT a, ade2-1, leu2-3, 112, his3-11, 15, trp1-1, ura3-1, can1-100, hsc82::LEU2, hsp82::LEU2, pTGPD-HSP82</i>	Nathan and Lindquist, 1995
G313N	<i>MAT a, ade2-1, leu2-3, 112, his3-11, 15, trp1-1, ura3-1, can1-100, hsc82::LEU2, hsp82::LEU2, pTGPD-HSP82-G313N</i>	Nathan and Lindquist, 1995
<i>STI1/SBA1</i>	<i>MAT a, his3, leu2, met15, ura3</i>	Invitrogen, Carlsbad, CA
<i>sti1Δ</i>	<i>MAT a, his3, leu2, met15, ura3, sti1::KAN<sup>R</sup></i>	Invitrogen, Carlsbad, CA
<i>sba1Δ</i>	<i>MAT a, his3, leu2, met15, ura3, sba1::KAN<sup>R</sup></i>	Invitrogen, Carlsbad, CA
W3031b	<i>MAT α, ade2, his3, leu2, ura3, trp1, can1-100</i>	Shirayama <i>et al.</i> , 1993
ACY17b	<i>MAT α, ade2, his3, leu2, ura3, trp1, can1-100, ydj1-2::HIS3, ydj1-151::LEU2</i>	Caplan <i>et al.</i> , 1992
E0020	<i>MAT α, ura3, leu2, his3, trp1, sse1::HIS3</i>	Shirayama <i>et al.</i> , 1993
<i>HLJ1/YDJ1</i>	<i>MAT a, ade2, his3, leu2, ura3, trp1</i>	This study
<i>hlj1Δ</i>	<i>MAT a, ade2, his3, leu2, ura3, trp1, Δhlj1::HIS3</i>	This study
<i>hlj1Δ/ydj1-151</i>	<i>MAT α, ade2, his3, leu2, ura3, trp1, can1-100 ydj1-2::HIS3, ydj1-151::LEU2, hlj1::TRP1</i>	This study
<i>STI1/SSE1</i>	<i>MAT a, GAL2, his2-11, 15, leu2-3, 112, lys1, lys2, trp1Δ1, ura3-52</i>	Nicolet and Craig, 1989
<i>sti1Δ/sse1Δ</i>	<i>MAT a, GAL2, his2-11, 15, leu2-3, 112, lys1, lys2, trp1Δ1, ura3-52 sti1::HIS3, sse1::KAN<sup>R</sup></i>	Liu <i>et al.</i> , 1999

10 or 12.5% SDS-polyacrylamide gels, transferred to nitrocellulose, and probed with mouse monoclonal anti-HA antibody (12CA5, Roche Molecular Biochemicals, Indianapolis, IN) and polyclonal anti-Sec61p (Stirling *et al.*, 1992). The signals were quantified using <sup>125</sup>I-secondary antibody and phosphorimage analysis (Fuji Medical Systems, Stamford, CT) or horseradish peroxidase-conjugated secondary antiserum and a Kodak 440CF Image Station and the associated Kodak 1D (V. 3.6) software (Rochester, NY). The degradation of a mis-folded, HA-tagged form of carboxypeptidase Y (HA-CPY\*) was measured by <sup>35</sup>S metabolic labeling/pulse-chase analysis as described (Zhang *et al.*, 2001).

### Protein Purifications

The following proteins were purified as described: Ssa1p (McClellan and Brodsky, 2000), Ydj1p (Cyr *et al.*, 1992), NBD1 (G404-L644) of CFTR (Qu and Thomas, 1996), and Sba1p (Fang *et al.*, 1998). Yeast Hsc90 (Hsc82p) was isolated using a modified protocol provided by Dr. David Toft (Mayo Clinic, Rochester, MN) from yeast strain ECUpep4 (Jakob *et al.*, 1995) with deletions in the chromosomal *HSC82* and *HSP82* genes and that encodes *HSC82* on a 2- $\mu$ m plasmid. ECUpep4 cells were grown to logarithmic phase ( $OD_{600}$  = 0.8–1.0) in YPD (1% yeast extract, 2% peptone, and 2% dextrose) at 26°C, and the cells were harvested and frozen in liquid nitrogen. The cell pellets were thawed, resuspended in 3–5 volumes of buffer 1 (20 mM Tris-HCl, pH 7.5, 4 mM EDTA, 1 mM DTT) supplemented with protease inhibitors (1 mM PMSF, 1  $\mu$ g/ml leupeptin, 0.5  $\mu$ g/ml pepstatin A), and subjected to glass bead lysis by vigorous agitation. Unbroken cells were pelleted by centrifugation at  $\sim 2800 \times g$  for 10 min at 4°C, and cell membranes were pelleted by centrifugation at  $\sim 48,000 \times g$  for 40 min at 4°C. The cleared lysate was applied to a DE52 column equilibrated in buffer 1, and the column was washed with 2 volumes of buffer 1 and with 2 volumes of buffer 1 containing 50 mM KCl. Bound protein was eluted with a gradient of buffer up to 1 M KCl, and fractions were collected and peak Hsc82p-containing fractions, as assessed by SDS-PAGE and Coomassie brilliant blue staining, were pooled and dialyzed against buffer 2 (20 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 10% glycerol) overnight at 4°C. The dialysate was loaded onto a high-performance Q-Sepharose column equilibrated in buffer 2, and the column was washed with buffer 2 and then buffer 2 containing 50 mM KCl (flow rate  $\sim 1.0$  ml/min) at 4°C. Bound protein was eluted with a gradient up to 1 M KCl. Peak Hsc82p-containing fractions were pooled, diluted to  $\sim 1.5$  mg/ml protein, and dialyzed against buffer 3 (20 mM Tris-HCl, pH 7.5, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 10% glycerol). The final protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA) with BSA as the standard, and the final purity of Hsc82p was determined to be  $\sim 80\%$ .

To purify the Hlj1p J-domain, a DNA fragment containing the N-terminal 259 base pairs of *HLJ1* was subcloned into pQE60 to add a hexahistidine C-terminal tag, and subsequently this fragment was subcloned into pGEX-KG at the *Bam*HI and *Hind*III restriction sites in order to attach an N-terminal GST tag. The resulting GST-Hlj1p-6His-tagged protein (containing the J-domain) was expressed in M15 *Escherichia coli* and a 100-ml culture was grown in LB + KAN (25  $\mu$ g/ml) to an  $OD_{600}$   $\sim 2.4$ . A total of 10 ml of the culture was diluted into 1 l of LB + KAN (25  $\mu$ g/ml) and grown to  $OD_{600}$   $\sim 0.2$  at 37°C, and then expression was induced by the addition of IPTG to a final concentration of 0.2 mM. Cells were incubated for  $\sim 7$  h at 37°C until an  $OD_{600}$  of  $\sim 0.8$  was reached and were harvested and frozen at  $-80^\circ\text{C}$ . The cells were thawed and resuspended in 10 ml of Buffer 88 (20 mM HEPES, pH 6.8, 150 mM KOAc, 5 mM MgOAc, 250 mM sorbitol) containing 0.1% Triton X-100 and 1 mM EDTA, and protease inhibitors (1 mM PMSF, 1  $\mu$ g/ml leupeptin, 0.5  $\mu$ g/ml pepstatin A), and were broken using a sonic dismembrator (Fisher Scientific, Pittsburgh, PA). The lysate was cleared by centrifugation at 10,000 rpm for 10 min at 4°C in an SS34 rotor (Sorvall, Newton, CT), and the lysate was added to a 1 ml,  $\sim 50\%$  slurry of glutathione-agarose beads (Sigma, St. Louis, MO) and incubated for  $\sim 2$  h with rotation at 4°C. The glutathione beads were washed (10 min, 4°C) with 25 ml of buffer 88 supplemented with 0.1% Triton X-100, 1 mM EDTA, and protease inhibitors, followed by a second wash with the same buffer supplemented with 1 M KCl, and finally washed with buffer 88 plus 0.1% Triton X-100. The bound Hlj1p was eluted by two 1-ml washes of 50 mM Tris, pH 8, 5 mM reduced glutathione. Protein concentration was assessed as described above. Only the GST-Hlj1p-6His fusion protein was evident on a Coomassie Brilliant Blue-stained SDS-PAGE gel.

### Biochemical Assays

Sba1p pull-down assays using purified hexahistidine-tagged Sba1 and NTA resin were performed essentially as described (Fang *et al.*, 1998). Ssa1p-ATP complex formation and single-turnover ATPase assays were performed as published by incubating preformed Ssa1p- $[\alpha^{32}\text{P}]\text{ATP}$  complex with the indicated protein (Hlj1p-J-domain-GST chimera, Ydj1p, and GST) at a final concentration of 0.2  $\mu\text{M}$  (Sullivan *et al.*, 2000).

For luciferase aggregation assays, firefly luciferase (Sigma) at an initial concentration of  $\sim 0.65$   $\mu\text{M}$  was preincubated in the presence or absence of  $\sim 20$   $\mu\text{M}$  purified Hsc82p in 150  $\mu\text{l}$  of refolding buffer (10 mM MOPS/KOH, pH 7.2, 50 mM KCl, 3 mM  $\text{MgCl}_2$ , 3 mM ATP, 2 mM DTT) for 20 min at 25°C

before 500  $\mu\text{l}$  of refolding buffer was added at 45°C to yield a final concentration of 0.15  $\mu\text{M}$  for luciferase and 2.4  $\mu\text{M}$  for Hsc82p. Aggregation was measured by light scattering at a wavelength of 320 nm at 45°C in a 14DS UV-VIS-IR spectrophotometer (AVIV, Lakewood, NJ).

The ability of Hsc82p to prevent the aggregation of NBD1 was investigated as described (Strickland *et al.*, 1997). Our NBD1 construct spans amino acids G404 to L644 from helix H1b to helix H9, which includes the F1-type core ATP-binding subdomain (Lewis *et al.*, 2004). This permits the measurement of early folding intermediates (Strickland *et al.*, 1997). The assay was performed using the purified hexahistidine-tagged NBD1 diluted  $\sim 100$ -fold out of 6 M guanidine-HCl buffer, 20 mM HEPES, pH 7.5, and into 650  $\mu\text{l}$  of refolding buffer (100 mM Tris-HCl, pH 7.4, 0.385 M L-arginine, 10 mM DTT, 200 mM KCl, 20 mM  $\text{MgCl}_2$ ) to a final concentration of 2  $\mu\text{M}$ . Protein aggregation was measured over time at a wavelength of 400 nm at 37°C in a 14DS UV-VIS-IR spectrophotometer (AVIV) in the absence or presence of the indicated concentrations of Hsc82p and other indicated reagents. Results of aggregation experiments were plotted as the relative amount of aggregation, normalized to the NBD1 control at 10 min, vs. time, and fitted to a single exponential using KaleidaGraph software version 3.0.4 (Abelbeck Software, Reading, PA) to determine initial rates. Macbecin I/II was kindly provided by the Drug Synthesis and Chemistry Branch in the Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, at the National Cancer Institute.

For the carbonate extraction and protease sensitivity assays, microsomal membrane fractions were prepared by differential centrifugation as previously described (Burri *et al.*, 2003). Proteins were extracted from membranes by resuspension in 100 mM  $\text{Na}_2\text{CO}_3$  and incubated for 30 min on ice with intermittent agitation on a Vortex mixer (Fujiki *et al.*, 1982). Soluble and insoluble proteins were separated by centrifugation at  $100,000 \times g$ . Trypsin treatments were performed in 100  $\mu\text{l}$  of lysis buffer (250 mM sorbitol, 150 mM potassium acetate, 5 mM magnesium acetate, 0.5 mM PMSF, 1.2  $\mu\text{g}/\text{ml}$  leupeptin, 0.75  $\mu\text{g}/\text{ml}$  antipain, 0.25  $\mu\text{g}/\text{ml}$  chymostatin, 1  $\mu\text{g}/\text{ml}$  pepstatin, 50 mM HEPES, pH 6.8) containing 1.5  $\mu\text{g}$  trypsin and incubated on ice for 30 min, before a 10-fold excess of soybean trypsin inhibitor, or TCA was added to stop the reaction (Beilharz *et al.*, 1998).

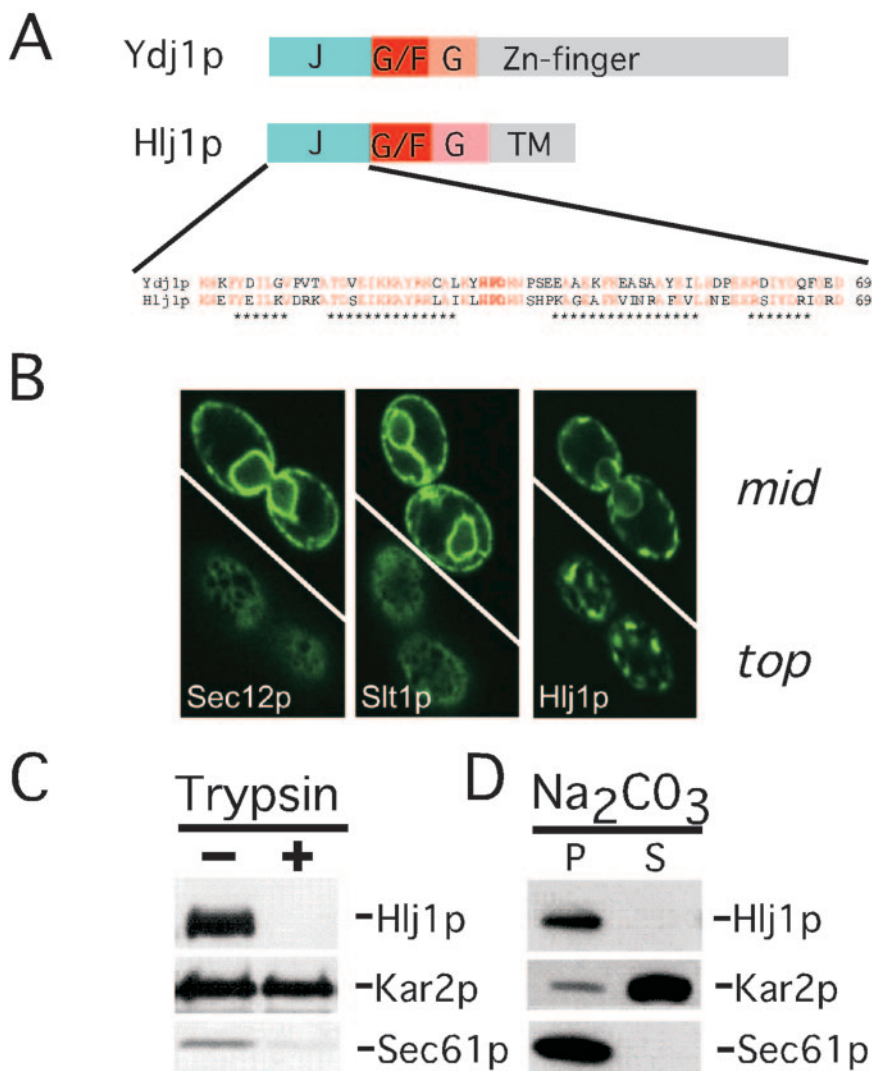
## RESULTS

### *Hsp40* Cochaperones Function Redundantly during CFTR, But Not CPY\* Degradation

We previously reported that a cytoplasmic Hsp70 in yeast, Ssa1p, facilitates CFTR degradation (Zhang *et al.*, 2001) and in mammalian cells Hsc70 cooperates with Hdj2, an Hsp40 homologue, during CFTR biogenesis (Meacham *et al.*, 1999). Ydj1p is the yeast Hdj2p homologue, is tethered to the ER membrane via a farnesyl moiety and interacts with Ssa1p based on genetic and biochemical studies (Caplan *et al.*, 1992; Cyr *et al.*, 1992; Becker *et al.*, 1996). However, CFTR degradation was unaffected in yeast containing a temperature-sensitive allele of *YDJ1* (Zhang *et al.*, 2001), suggesting either that there are inherent differences between CFTR biogenesis in yeast and mammals or that more than one functionally redundant Hsp40 in yeast cooperates with Ssa1p to facilitate CFTR degradation.

There are  $>20$  J-domain-containing proteins in yeast and at least 14 of these reside in the cytoplasm (Costanzo *et al.*, 2001; Walsh *et al.*, 2004). Recently, an Hsp40 homologue, Hlj1p, was revealed in a search for tail-anchored membrane proteins (Beilharz *et al.*, 2003). The N-terminal domain of Hlj1p is 58% identical to the J-domain of Ydj1p (Figure 1A) and the Hlj1p J-domain was predicted to reside in the cytosol (Beilharz *et al.*, 2003). To test this prediction, we expressed GFP-tagged forms of Hlj1p, the tail-anchored SNARE Slt1p (Burri and Lithgow, 2004) and Sec12p, an integral ER membrane protein required for COPII-mediated vesicle budding (Barlowe, 2003). Confocal fluorescence microscopy was performed and the ER residence of Sec12p, Hlj1p, and Slt1p were apparent (Figure 1B). Trypsin digestion of isolated ER microsomes showed that Hlj1p is accessible to exogenous trypsin, as is the polytopic ER membrane protein Sec61p (Figure 1C). In contrast, Kar2p/BiP was protease resistant and protected from trypsin by the microsomal membrane. Moreover, neither Hlj1p nor Sec61p were solu-





**Figure 1.** Hlj1p is a tail-anchored, integral membrane protein localized to the ER membrane. (A) Alignment of Hlj1p with Ydj1p. J, J-domain; G/F, glycine/phenylalanine-rich region; G, glycine-rich region; TM, transmembrane domain. The sequences of the J-domains are depicted and asterisks denote regions corresponding to the four predicted alpha-helices. Amino acids shaded red are identical between Hlj1p and Ydj1p and the functionally essential HPD motif is boldfaced. (B) Yeast expressing GFP-Sec12p (an integral ER membrane protein; Nakano *et al.*, 1988), GFP-Slt1p, a C-terminally anchored protein (Beilharz *et al.*, 2003; Burri *et al.*, 2003) or GFP-Hlj1p were analyzed by confocal fluorescence microscopy and Z-sections are shown from midway through representative cells (mid) and through the periphery of the same cells (top). (C) A crude microsomal membrane fraction was isolated from yeast expressing GFP-Hlj1 and was incubated in the presence (+) or absence (-) of trypsin. The presence of Hlj1p, the luminal protein Kar2p/BiP, and the polytopic ER membrane protein Sec61p were detected by immunoblot analysis. (D) A crude microsomal membrane fraction was isolated from yeast cells expressing GFP-Hlj1, treated with 0.1 M Na<sub>2</sub>CO<sub>3</sub>, and centrifuged to separate soluble proteins (S) from insoluble material (P). Immunoblots for the integral membrane ER protein Sec61p and the luminal protein Kar2p were used to control for carbonate treatment.

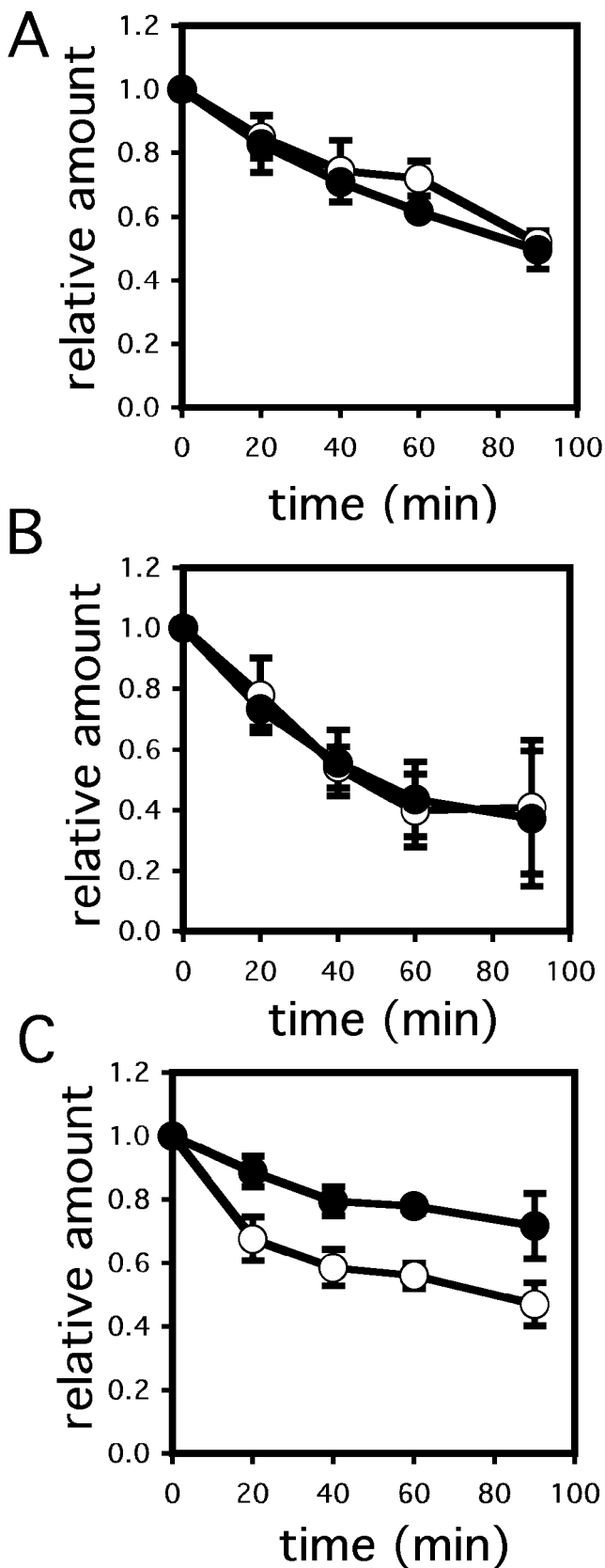
bilized by carbonate (Figure 1D). Together, these results indicate that Hlj1p is tethered to the ER membrane via a C-terminal anchor and, like Ydj1p, contains a cytoplasmically oriented J-domain.

To test whether Hlj1p is involved in CFTR degradation, we expressed an HA-epitope-tagged form of CFTR under the control of a constitutive promoter in an *hlj1* deletion mutant and in an isogenic wild-type yeast strain and performed cycloheximide chase analyses (see *Materials and Methods*). We found that the rates of CFTR degradation were identical (Figure 2A). Consistent with previous results (Zhang *et al.*, 2001), we also found that CFTR degradation was unaffected in the *ydj1-151* strain compared with an isogenic wild-type (Figure 2B). However, in a *hlj1Δ/ydj1-151* double mutant CFTR degradation was slowed relative to the wild-type strain (Figure 2C). The degradation of Ste6p\*, a mis-folded yeast ABC transporter and ERAD substrate, is also attenuated in a *hlj1Δ/ydj1-151* double mutant (Huyer *et al.*, 2004). These data suggest that Ydj1p and Hlj1p function redundantly to facilitate the degradation of CFTR and at least one other integral membrane ERAD substrate.

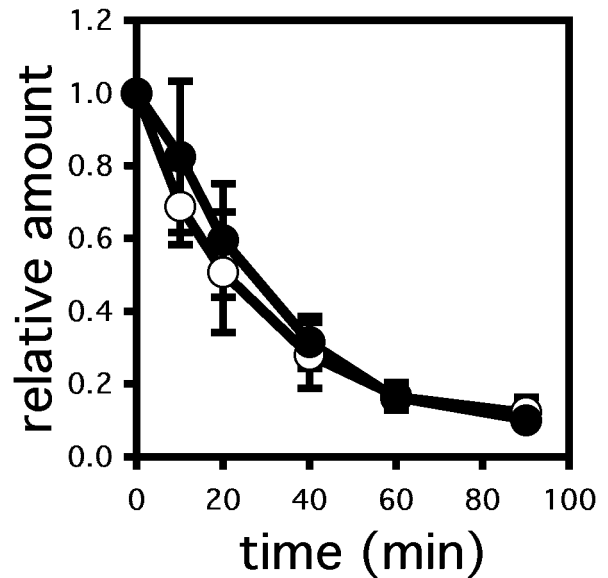
Unique chaperone requirements for the ERAD of soluble vs. integral membrane proteins have been observed (Fewell *et al.*, 2001). To examine this distinction further, we mea-

sured the degradation of CPY\*, a soluble ERAD substrate (Hiller *et al.*, 1996), in the *hlj1Δ/ydj1-151* double mutant and in the isogenic wild-type strains by pulse-chase analysis. We found nearly identical rates of degradation in the two strains (Figure 3), indicating that Ydj1p and Hlj1p are dispensable for the degradation of CPY\*.

On the basis of data presented in Figure 2, we predicted that the effect of Ydj1p/Hlj1p during CFTR degradation is through their interaction with Ssa1p, the Hsp70 that in turn catalyzes CFTR degradation in yeast (Zhang *et al.*, 2001). The functional interaction between J-domain-containing proteins and their cognate Hsp70s is best examined by measurements of Hsp70 ATP hydrolysis in the presence and absence of an Hsp40/J-domain-containing protein. For example, a GST fusion protein containing the J-domain of Sec63p, a membrane protein whose J-domain faces the ER lumen, stimulates BiP's ATPase activity in vitro (Corsi and Schekman, 1997). To examine whether Hlj1p functionally interacts with Ssa1p, we similarly constructed and purified a GST-tagged fusion protein that contains the Hlj1p J-domain and incubated the purified protein with preformed [ $\alpha$ -<sup>32</sup>P]ATP-Ssa1p complex. Hlj1p stimulated Ssa1p in this single-turnover ATPase assay to a similar extent as equimolar amounts of Ydj1p (Figure 4A), indicating that both Hlj1p



**Figure 2.** CFTR degradation is attenuated in an *hlj1/ydj1* double mutant, but not in yeast mutated individually for each gene. Wild-type and mutant yeast strains expressing CFTR were subjected to



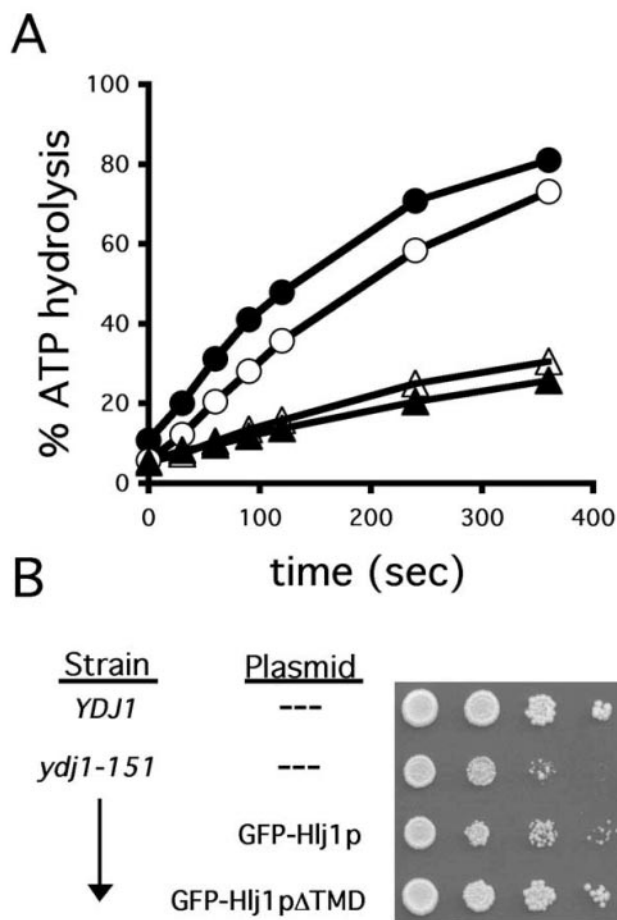
**Figure 3.** CPY\* degradation is unaffected in the *ydj1-151/hlj1*Δ mutant yeast strain. CPY\* degradation was assessed by pulse-chase analysis as described in *Materials and Methods* in wild-type (○) and in *ydj1-151/hlj1*Δ mutant (●) yeast. Data represent the means of three independent experiments  $\pm$  SEM.

and Ydj1p interact with the yeast cytoplasmic Hsp70. Further evidence to support this proposition is shown in Figure 4B in which the abilities of full-length Hlj1p (GFP-Hlj1p) and a form of Hlj1p lacking the transmembrane domain (GFP-Hlj1p $\Delta$ TMD) to suppress a slow-growth phenotype of *ydj1-151* mutant cells were examined. We observed that the viability of the *ydj1-151* mutant increased when Hlj1p was expressed, suggesting that Hlj1p partially supplants Ydj1p function. We also noted in this experiment that the soluble Hlj1p derivative is more effective at improving the growth of the mutant strain than full-length Hlj1p, suggesting that overexpression of the membrane anchor might be somewhat toxic; consistent with this hypothesis, we have observed toxicity derived from the overexpression of other stable, wild-type ER membrane proteins in yeast (our unpublished observations).

#### *Mutations in Hsp90 Enhance CFTR Degradation But Have No Effect on CPY\* Turnover*

The role of the Hsp90 molecular chaperone during CFTR maturation in mammals is controversial (Loo *et al.*, 1998; Fuller and Cuthbert, 2000; see *Introduction*). To better define the action of Hsp90 during CFTR biogenesis we expressed CFTR in yeast deleted for the genes encoding the constitutive (*HSC82*) and heat-inducible (*HSP82*) Hsp90s. Yeast Hsp82 and Hsc82p are  $\sim$ 97% identical at the amino acid level and are functionally interchangeable, but at least one

cycloheximide chase analysis as described in *Materials and Methods*, and the amount of CFTR at time zero was set to 1.0. (A) The relative amounts of CFTR remaining in wild-type (○) and *hlj1*Δ (●) yeast strains over time were plotted. (B) The relative amounts of CFTR remaining in wild-type (○) and *ydj1-151* (●) yeast, and (C) in wild-type (○) and *hlj1*Δ/*ydj1-151* mutant yeast (●) over time were plotted. In each panel the data represent the means of three independent experiments  $\pm$  SEM.



**Figure 4.** Hlj1p and Ydj1p functions partially overlap. (A) The ATPase activity of Ssa1p is enhanced by purified Ydj1p and by a fusion protein containing the Hlj1p J-domain. A preformed [ $\alpha$ - $^{32}$ P]ATP-Ssa1p complex was incubated for the indicated times with equimolar amounts of the Hlj1p J-domain fusion protein (●), Ydj1p (○), GST (▲), or buffer (△) at 30°C, and the extent of ATP hydrolysis was assessed as described in *Materials and Methods*. (B) Wild-type and *ydj1-151* yeast either lacking or containing a MET17-driven GFP-Hlj1p or GFP-Hlj1pΔTMD expression construct were grown overnight and serial dilutions were plated on selective medium containing 500  $\mu$ M methionine at 30°C for 2 d. GFP-Hlj1p and GFP-Hlj1pΔTMD expression was confirmed by fluorescence microscopy (unpublished data).

homologue must be expressed to maintain viability (Borkovich *et al.*, 1989). Therefore, the wild-type strain for this experiment contains a plasmid-borne copy of *HSP82*, and the mutant strain contains a temperature-sensitive allele, *hsp82<sup>ts</sup>* (G313N). The mutant protein is extremely unstable when cells are shifted to the nonpermissive temperature of 37°C and is rapidly degraded (Bohen and Yamamoto, 1993; Fliss *et al.*, 2000). This results in the equivalent of a null phenotype immediately after temperature shift. After cells were grown at a permissive temperature, CFTR degradation in these strains was monitored at 37°C by cycloheximide chase analysis. As shown in Figure 5A, the rate of CFTR degradation was significantly higher in the mutant strain. Similar results were obtained when CFTR degradation in the G170D *hsp82* mutant was examined (unpublished data), which also rapidly loses activity at the nonpermissive temperature (Nathan and Lindquist, 1995). Because only the

immature, unfolded form of CFTR is an ERAD substrate (Gelman *et al.*, 2002), these data suggest that Hsp90 is important to maintain the stability of CFTR.

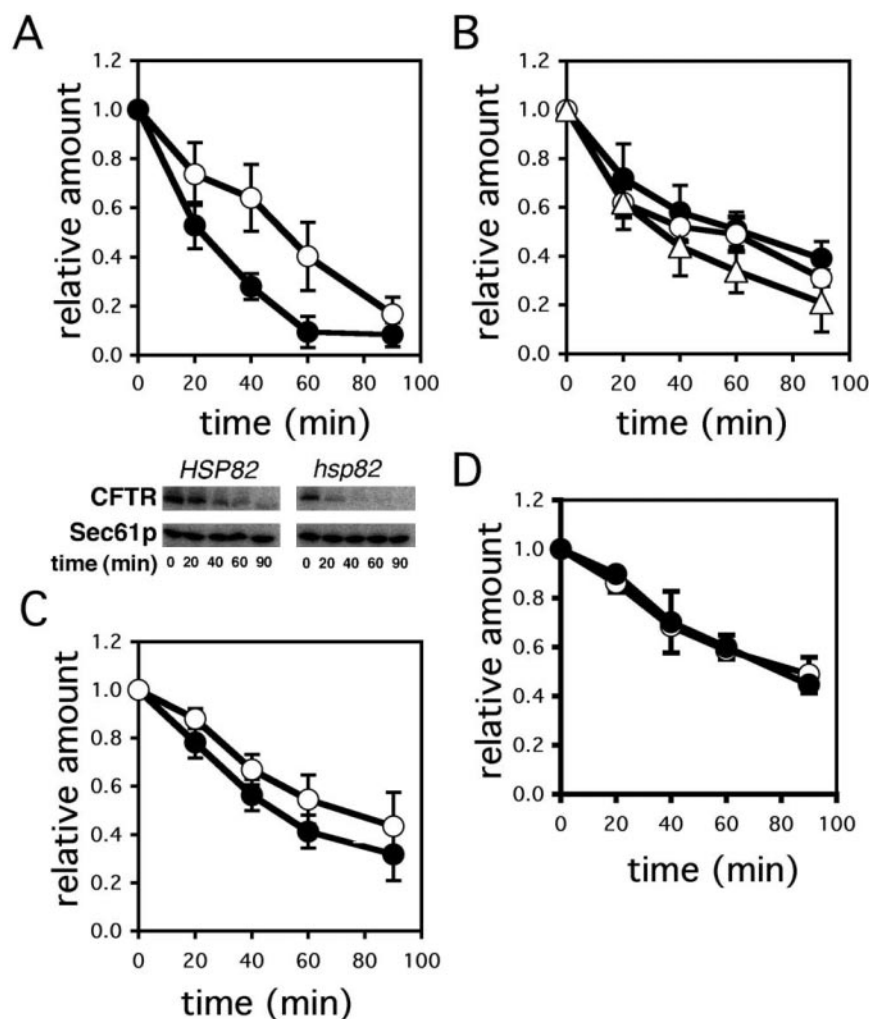
Hsp90 associates with several cochaperones to form a macromolecular complex required for the folding and activation of select client proteins (Caplan, 1999; Richter and Buchner, 2001; Young *et al.*, 2001). Although the role of Hsp90 in mammalian cells can be assessed using ansamycin antibiotics, examining the functions of Hsp90 cochaperones is more challenging. In yeast, however, mutations in Hsp90 cochaperones are readily available. We therefore examined the roles of three well-defined Hsp90 cochaperones (Sti1p, Sba1p, Sse1p) on CFTR stability in yeast. Sti1p is the yeast Hop homologue, and both Hsp70 and Hsp90 can dock onto the TPR domains of Sti1p (Johnson *et al.*, 1998). Sba1p is the p23 homologue that stabilizes Hsp90 substrate binding (Fang *et al.*, 1998). Sse1p is a yeast Hsp110 homologue that resides in the Hsp90 complex (Liu *et al.*, 1999; Goeckeler *et al.*, 2002). Deletion of the genes encoding each of these factors compromises Hsp90 complex-mediated processes in yeast (Chang *et al.*, 1997; Fang *et al.*, 1998; Liu *et al.*, 1999; Cox and Miller, 2002). When CFTR degradation was examined in isogenic wild-type strains and in *sti1Δ*, *sba1Δ*, or *sse1Δ* yeast, we detected no statistically significant differences in the rates of CFTR degradation in the mutants compared with wild-type yeast (Figure 5, B and C). Furthermore, CFTR degradation was unaltered in an *sti1Δ/sse1Δ* double mutant (Figure 5D) that has a severe growth defect at 37°C (Liu *et al.*, 1999), indicating that the accelerated degradation observed in the *hsp82* mutant was not simply the result of shifting temperature-sensitive cells to the nonpermissive temperature. Together, these results demonstrate that Hsp90, but not the Hsp90 complex, participates in CFTR biogenesis in yeast. Moreover, the action of Hsp90 during CFTR biogenesis is not via an indirect effect on cellular signaling pathways because the same signaling pathways are compromised in strains mutated for the cochaperones.

As discussed above, distinct chaperone requirements for the degradation of soluble and membrane proteins have been noted, and this distinction was supported further by the data presented in Figure 3. Therefore, we examined CPY\* degradation in the *hsp82* mutant and in the isogenic wild-type strain but found that there was no statistically significant difference in the rate or extent of degradation (Figure 6). We conclude that the ERAD of CPY\* is Hsp90 independent.

#### Yeast Hsp90 Prevents the Aggregation of the First Nucleotide-binding Domain of CFTR

The data presented in Figure 5 suggest that Hsp90 helps protect CFTR from degradation and might therefore be important to maintain CFTR in its folded conformation. In general, a loss of structural integrity can be accompanied by protein aggregation, and it is well known that the first nucleotide-binding domain (NBD1) in CFTR is aggregation prone; moreover, the low efficiency or slow rate of NBD1 folding directly determines the efficacy of CFTR maturation, and maintaining NBD1 solubility prevents the formation of off-pathway aggregates (Qu and Thomas, 1996; Strickland *et al.*, 1997; Zhang *et al.*, 1998). To test directly whether yeast Hsp90 maintains NBD1 solubility, we isolated Hsc82p (see *Materials and Methods*). First, to confirm that the highly enriched protein was active, we examined the chaperone's ability to suppress the aggregation of firefly luciferase because mammalian Hsp90 was previously shown to slow the aggregation of this substrate (Wiech *et al.*, 1992). Luciferase aggregation was





**Figure 5.** CFTR degradation is accelerated in an Hsp90 mutant yeast strain, but not in yeast strains mutated for Hsp90 cochaperones. Yeast strains expressing CFTR were subjected to a cycloheximide chase and immunoblot analysis as described in *Materials and Methods*. (A) The degradation of CFTR in wild-type (○) and *hsp82* (●) strains is plotted as the relative amount of CFTR remaining over time. Data represent the means of four independent experiments  $\pm$  SEM. Bottom: representative Western blot. Sec61p is an ER integral protein and serves as a loading control. (B) The degradation of CFTR in wild-type (○), *sti1Δ* (●), and *sba1Δ* (△) yeast strains is plotted as the relative amount of CFTR remaining over time. Data represent the means of 3–4 independent experiments  $\pm$  SEM. (C) CFTR degradation in wild-type (○) and *sse1Δ* (●) yeast strains is plotted as the relative amount of CFTR over time. Data represent the means of five independent experiments  $\pm$  SEM. (D) CFTR degradation in wild-type (○) and *sti1Δ/sse1Δ* (●) yeast strains is plotted as the relative amount of CFTR over time. Data represent the means of two independent experiments  $\pm$  range.

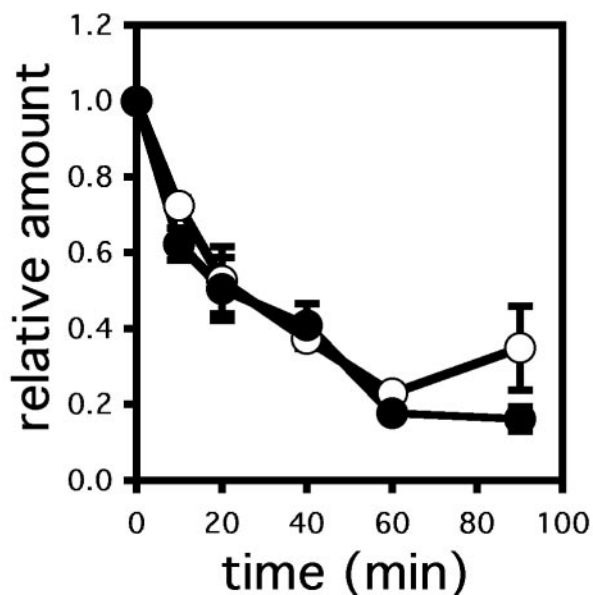
suppressed by  $\sim 65\%$  when a 16:1 M ratio of Hsc82p to luciferase was used (our unpublished observations), consistent with previous data (Minami *et al.*, 2001).

Next, we examined whether Hsc82p associated with the Sba1p cochaperone (Fang *et al.*, 1998). Proficient interaction between Hsc82p and Sba1p was observed by pull-down assay, and importantly the degree of association decreased about sixfold in the presence of Macbecin (Figure 7A), an ansamycin antibiotic that inhibits yeast Hsp90 function both in vivo and in vitro (Bohen, 1998; Fang *et al.*, 1998; Donze and Picard, 1999; Liu *et al.*, 1999). Finally, we assessed Hsc82p prevention of NBD1 aggregation at a 5:1 M ratio and found that aggregation was suppressed by  $\sim 60\%$  (Figure 7B), indicating that yeast Hsp90 maintains NBD1 in solution. The ability of Hsp82p to maintain NBD1 in solution was reduced somewhat if Macbecin was added to a final concentration of 50  $\mu\text{M}$  (our unpublished observations). This partial effect may be due to the fact that Hsp90 contains two polypeptide binding sites, only one of which is sensitive to ansamycin antibiotics (Young *et al.*, 1997; Scheibel *et al.*, 1998). These data are also consistent with previous work in which a partial effect of ansamycin antibiotics on Hsp90-dependent activities was noted (Minami *et al.*, 2001). In any event, these results suggest that Hsp90 stabilizes CFTR by binding NBD1.

## DISCUSSION

Our continued analysis of CFTR degradation in yeast, described in this report, has uncovered several novel aspects of molecular chaperone function and of the ERAD pathway for integral membrane proteins, which we address individually in the following sections.

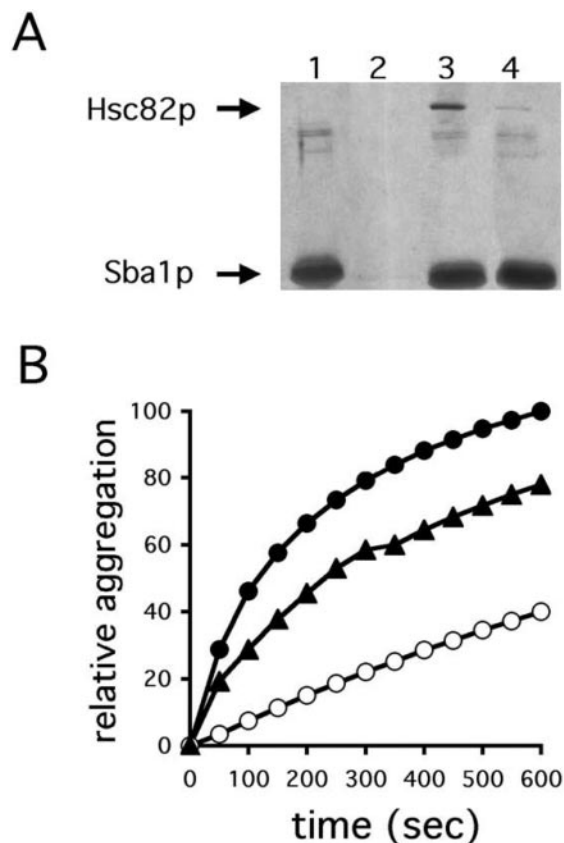
First, we have identified the Hsp40 homologues in yeast that facilitate CFTR degradation—most likely in conjunction with an Hsp70, Ssa1p—and suggest that Hlj1p and Ydj1p function redundantly. Both chaperones enhance Ssa1p ATPase activity and the extent of CFTR stabilization in the *hlj1/ydj1* mutant strain (Figure 2C) is similar to that observed in the *ssa1* mutant (Zhang *et al.*, 2001). These data are reminiscent of the reported functional redundancy and interactions between BiP and the ER luminal Hsp40 chaperones, Scj1p and Jem1p, during the ERAD of soluble proteins (Nishikawa *et al.*, 2001). The functional redundancy displayed by Ydj1p and Hlj1p is not limited to CFTR turnover because yeast mutated for the genes encoding these cochaperones also exhibit slowed degradation of Ste6p\* (Huyer *et al.*, 2004), another integral membrane ERAD substrate. Other recent studies have also hinted at a role for Hlj1p in protein quality control. For example, *hlj1Δ* mutants grew poorly when they express a Huntingtin fragment (HD53Q; Willing-



**Figure 6.** CPY\* degradation is unaffected in the *hsp82* mutant yeast strain. CPY\* degradation was assessed by pulse-chase analysis as described in *Materials and Methods* in wild-type (○) and Hsp90 mutant (●) yeast. Data represent the means of three independent experiments  $\pm$  SEM.

ham *et al.*, 2003) and the degradation of a synthetic, integral membrane ERAD substrate was mildly suppressed in an *hlj1* $\Delta$  strain (Taxis *et al.*, 2003). The functional overlap of *YDJ1* and *HLJ1* reported here might explain the weak phenotype previously observed in the *hlj1* $\Delta$  mutant.

Second, we suggest that the yeast Hsp90 chaperone, Hsp82p, is required to maintain the folded state of CFTR because *hsp82* mutant yeast degrade CFTR faster than isogenic wild-type yeast and because Hsp82p prevents NBD1 aggregation, a domain whose folding is critical for CFTR maturation (Qu and Thomas, 1996; Qu *et al.*, 1997; Zhang *et al.*, 1998). A significant prevention of aggregation was achieved at an Hsp82p:NBD1 M ratio of 5:1, an amount that is not unreasonable given the high concentration of cellular Hsp90 (Buchner, 1996). The more rapid degradation of CFTR in Hsp90 mutant yeast is not due to the well-characterized impact of the Hsp90 complex on cellular signaling pathways because no effect on CFTR degradation was observed in yeast mutated for Hsp90 cochaperones either individually (Sti1p, Sba1p, Sse1p) or in combination (Sti1p/Sse1p). We therefore conclude that CFTR is one of several cellular proteins that require Hsp90 for efficient folding, a list that includes p53, Src and steroid hormone receptors (Richter and Buchner, 2001). In contrast, it is important to note that Hsp90 facilitates the ERAD of apolipoprotein B (Gusarova *et al.*, 2001) and an insulin receptor mutant (Imamura *et al.*, 1998). These data indicate that some substrates utilize Hsp90 for protection, whereas other substrates engage Hsp90 en route to degradation. In some cases, Hsp90 is involved in both events, acting first to promote folding, and then if folding cannot proceed, targeting the substrate to the proteasome (Schneider *et al.*, 1996). A role for Hsp90 in degradation is also supported by connections between Hsp90 and the ubiquitin-proteasome machinery: For example, Hsp90 binds to the 19S cap of the yeast proteasome (Verma *et al.*,



**Figure 7.** Yeast Hsp90 binds the Sba1p/p23 cochaperone and suppresses the aggregation of NBD1 early-folding intermediates. (A) Hexa-histidine-tagged Sba1p was prebound to nickel-linked resin and then incubated in either the absence (lane 1) or presence (lanes 2–4) of highly enriched Hsc82p. Bound protein was eluted, resolved by SDS-PAGE and visualized by silver staining. The reaction shown in lane 2 lacks prebound Sba1p and the reaction shown in lane 4 was supplemented with Macbecin to a final concentration of 50  $\mu$ M. (B) CFTR-NBD1 was diluted out of denaturant into refolding buffer at 37°C in the absence (●) or presence of Hsp90 at a 2.5:1 (▲) and 5:1 (○) molar ratio, and light scattering was measured as described in *Materials and Methods*. Data obtained at 50-s intervals are shown.

2000), and in mammals Hsp90 function is linked to CHIP, an E3 ubiquitin ligase (Connell *et al.*, 2001).

Third, we found that Hsp90 cochaperones do not impact CFTR biogenesis in yeast. To our knowledge, this is the first investigation of the relative contributions of Hsp90 vs. Hsp90 complex members in membrane protein biogenesis. In contrast, a recent study demonstrated that deletion of individual Hsp90 cochaperones had differential effects on the activity of the yeast MAP kinase, Ste11p (Lee *et al.*, 2004), suggesting that interactions between client proteins and Hsp90/Hsp90 cochaperones are likely to be complex. In the best-characterized example, an Hsp90 folding pathway has been proposed based on in-depth studies of the progesterone and estrogen receptor folding pathways. Two distinct Hsp90 complexes are evident in this pathway: an early complex containing Hop (Sti1p), Hsp40, and Hsp70, and a mature complex containing p23 (Sba1p) and cyclophilins. The transition from the early to the late complex involves conformational changes in Hsp90 upon ATP binding and hydrolysis, and upon p23 binding (Smith, 1998; Pratt and



Toft, 2003). It is unknown, however, whether other Hsp90 subcomplexes exist and how additional Hsp90 cochaperones impact this pathway. We therefore cannot rule out the possibility that a novel Hsp90 subcomplex might be important for CFTR biogenesis in yeast.

We also cannot rule out the possibility that Hsp90 cochaperones might be required for CFTR biogenesis in mammals. Formally, the ability of GA to induce more rapid degradation of CFTR in mammalian cells (Loo *et al.*, 1998) might have occurred through direct inhibition of Hsp90 function or through an effect on Hsp90 complex maturation. In fact, it was noted in the published study that p23 associates with CFTR. Until individual Hsp90 cochaperones can be disabled in mammalian cells, this issue cannot be resolved.

Fourth, our data further define the unique chaperone requirements for the degradation of soluble and integral membrane proteins in the ER. Ssa1p impacts the ERAD of integral membrane substrates (Hill and Cooper, 2000; Zhang *et al.*, 2001) and the Ydj1p/Hlj1p pair (this study) acts similarly. Precisely how these chaperones facilitate membrane protein turnover is not completely clear. In mammals the Hsp70-Hsp40 complex is directly linked to the ubiquitin-proteasome degradation machinery through its association with CHIP (Meacham *et al.*, 2001). An Hsp70 cochaperone, BAG-1, might augment Hsp70-catalyzed degradation by transferring substrates from Hsp70 to the proteasome; BAG-1 is an Hsp70 nucleotide exchange factor that promotes substrate release, contains a ubiquitin-like element, and binds to the proteasome (Höhfeld, 1998; Höhfeld *et al.*, 2001). In yeast the cytoplasmic Hsp70-Hsp40 chaperone complex (Ssa1p-Ydj1p/Hlj1p) might similarly link the selection of integral membrane ERAD substrates to one or more of the E3 ligases that play a role in ERAD (Bays *et al.*, 2001; Deak and Wolf, 2001; Swanson *et al.*, 2001). Besides the unique chaperone requirements for the degradation of membrane and soluble proteins, further distinctions in the selection of ERAD substrates were recently uncovered from Ng and colleagues, and it will be interesting to determine how chaperones impact the degradation of substrates through the described ERAD-C and ERAD-L pathways (Vashist and Ng, 2004). Future research efforts will seek to identify how the chaperone machinery is coupled to the action of the ubiquitin-proteasome system during the selection of CFTR and other membrane proteins.

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