Hsp70 and Hsp90 Multichaperone Complexes Sequentially Regulate Thiazide-sensitive Cotransporter Endoplasmic Reticulum-associated Degradation and Biogenesis*

The thiazide-sensitive NaCl cotransporter (NCC) is the primary mediator of salt reabsorption in the distal convoluted tubule and is a key determinant of the blood pressure set point. Given its complex topology, NCC is inefficiently processed and prone to endoplasmic reticulum (ER)-associated degradation (ERAD), although the mechanisms governing this process remain obscure. Here, we identify factors that impact the ER quality control of NCC. Analyses of NCC immunoprecipitates revealed that the cotransporter formed complexes with the core chaperones Hsp90, Hsp70, and Hsp40. Disruption of Hsp90 function accelerated NCC degradation, suggesting that Hsp90 promotes NCC folding. In addition, two chaperones, the C terminus of Hsp70-interacting protein (CHIP) and the Hsp70/Hsp90 organizer protein, were associated with NCC. Although CHIP, an E3 ubiquitin ligase, promoted NCC ubiquitination and ERAD, the Hsp70/Hsp90 organizer protein stabilized NCC turnover, indicating that these two proteins differentially remodel the core chaperone systems to favor cotransporter degradation and biogenesis, respectively. Adjusting the folding environment in mammalian cells via reduced temperature enhanced NCC biosynthetic trafficking, increased Hsp90-NCC interaction, and diminished binding to Hsp70. In contrast, cotransporters harboring disease-causing mutations that impair NCC biogenesis failed to escape ERAD as efficiently as the wild type protein when cells were incubated at a lower temperature. Instead, these mutants interacted more strongly with the wild type protein when cells were incubated at a lower temperature. Thus, factors that regulate NCC function and synthesis play an important role in the pathogenesis of hypertension in the general population. Like other SLC12 cation cotransporters, NCC possesses a complex topology, consisting of 12 transmembrane helices, a glycosylated extracellular loop, and large intracellular N and C termini. As is evident for nearly all membrane proteins in eukaryotes, NCC folds and assembles in the endoplasmic reticulum (ER) during or soon after it is translated. Consequently, an extensive ER quality control system must be in place to ensure that NCC adopts its correct conformation. Indeed, loss of function NCC mutations that cause Gitelman syndrome

Background: An incompletely defined system of cytoplasmic chaperones mediates thiazide-sensitive cotransporter (NCC) ER quality control.

Results: Hsp70 and Hsp90 select NCC for cochaperone-regulated ERAD or biosynthetic maturation.

Conclusion: Hsp70 and Hsp90 sequentially monitor early stages of NCC biogenesis.

Significance: Differential interaction of aberrantly folded NCC with these chaperones likely contributes to the molecular basis of hereditary salt wasting and hypertension resistance.

The thiazide-sensitive NaCl cotransporter (NCC)2 is a member of the SLC12 family of electroneutral cation chloride cotransporters that regulate cell volume and ion homeostasis in diverse tissues (1). Other members of this family include the Na-K-2Cl cotransporters NKCC1 and NKCC2 and four K-Cl cotransporters (KCC1–4). NCC is expressed at the apical surface of the renal distal convoluted tubule, where it mediates the reabsorption of 5–10% of filtered sodium chloride and regulates blood pressure. The medical relevance of this process is evidenced by the utility of thiazide diuretics, which inhibit NCC and reduce blood pressure (2). Moreover, loss of function mutations in NCC cause Gitelman syndrome, an autosomal recessive salt wasting disorder (3). Population-based genetic studies indicate that the carrier state for this Mendelian disease confers lifelong protection from the development of hypertension and its associated complications (4). Thus, factors that regulate NCC function and synthesis play an important role in the pathogenesis of hypertension in the general population.

2 The abbreviations used are: NCC, thiazide-sensitive NaCl cotransporter; ER, endoplasmic reticulum; CFTR, cystic fibrosis transmembrane conductance regulator; ERAD, endoplasmic reticulum-associated degradation; Hsp, heat shock protein; MDCK, Madin-Darby canine kidney; TPR, tetratricopeptide; HOP, Hsp70/Hsp90 organizer protein; CHIP, C terminus of Hsp70-interacting protein; GR, glucocorticoid receptor; 17-AAG, 17-N-allylamino-17-demethoxy-geldanamycin.
or carrier state hypertension resistance frequently lead to impaired biosynthetic trafficking and plasma membrane expression (5–7), and many of these variants shift the steady state balance of NCC expression toward the immature, ER-localized, and core-glycosylated state. These data strongly suggest that misfolded forms of the cotransporter are recognized by the biosynthetic machinery that targets NCC for ER-associated degradation (ERAD). Generally, this selection process is mediated by molecular chaperones, which interact in a substrate-specific manner to facilitate the quality control of aberrant proteins (8, 9).

Based on the importance of defining how NCC is subject to quality control and regulated, we sought to define the mechanisms underlying the ERAD of this protein. As an initial approach, we employed the yeast Saccharomyces cerevisiae as a model eukaryotic expression system to identify evolutionarily conserved factors that regulate NCC biogenesis. These findings were then verified in mammalian expression systems. This analysis revealed that NCC is subject to an ER quality control process that is primarily dependent on cytoplasmic rather than ER luminal chaperones (10). Specifically, we found that a cytoplasmic heat shock protein, the stress-inducible isoform of Hsp70 (Hsp72), binds to NCC and selects it for ubiquitination and proteasomal degradation. In contrast, chaperones residing in the ER lumen, such as the Hsp70 Kar2/Bip, had no effect on NCC turnover. These findings are reminiscent of other membrane proteins that undergo inefficient biosynthetic processing because of their complex fold, such as the cystic fibrosis transmembrane conductance regulator (CFTR) (11).

Hsp70 is a ubiquitously expressed multifunctional chaperone that mediates a variety of biological processes, including protein degradation, folding, targeting, and protein-protein interactions between its clients and other regulators (12–14). The capacity of cytoplasmic Hsp70 to perform these diverse tasks requires ATP binding and hydrolysis, which is facilitated by other chaperones or cofactors (i.e., “cochaperones”). According to current models, an unfolded polypeptide is captured by a chaperone that recognizes exposed hydrophobic patches. This chaperone could be Hsp70 itself or a J-domain cochaperone that forms a complex with Hsp70, such as an Hsp40. The Hsp40/Hsp70 machinery then guides the client through cycles of binding and release, in which successive rounds of folding are coupled to Hsp70-mediated ATP hydrolysis. Although this process most commonly results in the formation of a fully folded client, a terminally misfolded or slowly folding species may be generated, which can be targeted for degradation to clear the cell of potentially toxic aggregates. In contrast, if the folding pathway is followed, the Hsp70-client complex can recruit Hsp90, a central regulator of protein homeostasis that also chaperones protein folding via an ATP-dependent hydrolytic cycle (15–17). Formation of these “intermediate complexes” may be augmented by the Hsp70/Hsp90 organizer protein (HOP), which connects the C termini of Hsp70 and Hsp90 via tetratricopeptide repeat (TPR) domains to promote productive folding (18). On the other hand, quality control of misfolded Hsp70- and Hsp90-bound clients can be managed by a different TPR domain-containing cochaperone, the C terminus of Hsc70 interacting protein (CHIP). CHIP is a Ubox E3 ubiquitin ligase that associates with Hsp70 or Hsp90 via its TPR domain and ubiquitinates misfolded substrates, targeting them for ERAD (19).

In this study, we employed a combination of proteomic and biochemical strategies to identify additional factors that mediate NCC quality control. We found that the cotransporter resided in a cytoplasmic chaperone-containing complex that included Hsp90, Hsp70, and Hsp40. We also found that NCC degradation was sensitive to drugs that inhibit Hsp90 function and that CHIP and HOP influence NCC turnover in an opposing manner; namely, CHIP promoted NCC degradation, whereas HOP favored NCC folding. Finally, we established that the quality control of NCC is a temperature-dependent process. Low temperature incubation enhanced wild type NCC maturation. In contrast, two Gitelman syndrome mutants were insensitive to temperature downshift and instead interacted more strongly with the Hsp70/Hsp40 system, likely resulting in persistent ER trapping and selection for ERAD. Combined with other data, our results indicate that Hsp70 and Hsp90 comprise functionally distinct ER quality control checkpoints that sequentially monitor the early stages during cation chloride cotransporter biogenesis.

EXPERIMENTAL PROCEDURES

Molecular Methods—All of the NCC clones used in these studies were derived from previously described and characterized cDNAs, including untagged mouse NCC (6), N-terminal hemagglutinin-tagged wild type mouse NCC (HA-NCC) in pcDNA3.1 (20), and two previously studied N-terminal HA-tagged Gitelman mutants, R948X and R989X (10). Myc-tagged wild type, ΔUBox, and ΔTPR C terminus of Hsc70 interacting protein (CHIP/STUB1) were gifts of Ray Frizzell (University of Pittsburgh, Pittsburgh, PA). The untagged Hsp70/Hsp90 organizer protein (HOP/STIP1; NM_006819) was purchased as a complete cDNA in pNiCY from Open Biosystems, excised with EcoRI and NotI, and ligated into the same sites in pcDNA3.1. All of the sequences were verified by automated DNA sequence analysis performed at the University of Pittsburgh Genomics and Proteomics Core Laboratories prior to use.

Antibodies and Reagents—The following commercial antibodies were used: mouse monoclonal anti-Hsp70 (Hsp72) (C92F3A-5; Enzo), mouse monoclonal anti-Hsp90 (16F1; Enzo), rabbit polyclonal anti-Hsp40 (Hdj1; Cell Signaling Technology), rabbit polyclonal anti-HOP (Cell Signaling Technology), mouse monoclonal anti-p23 (JJ3; Thermo), rabbit polyclonal anti-CHIP (C-terminal; Sigma), rabbit polyclonal anti-ubiquitin antibody (FL-76; Santa Cruz), mouse monoclonal anti-HA (HA-11; Covance), mouse monoclonal anti-Myc (4A6 Millipore), rabbit polyclonal anti-c-Myc (A-14; Santa Cruz), HRP-conjugated goat anti-mouse and goat anti-rabbit antibodies (Jackson ImmunoResearch), and HRP-conjugated rat monoclonal anti-HA high affinity (3F10; Roche Applied Science). Polyclonal rabbit anti-mouse NCC antibody was purchased from Stressmarq. 17-N-Allylamino-17-demethoxygeldanamycin (17-AAG), MG-132, and cycloheximide were purchased from Sigma. 17-Dimethylaminoethylamino-17-demethoxygeldana-mycin was purchased from Tocris.
Sequential Regulation of NCC by Hsp70 and Hsp90

Cell Culture and Transfection—Madin Darby Canine Kidney (MDCK) cells and HEK293T cells were cultured in high glucose DMEM supplemented with 10% FBS, l-glutamine, and penicillin/streptomycin at 37 °C. Transient transfections were performed using either FuGENE 6 (Roche Applied Science) or Lipofectamine 2000 (Invitrogen) per the manufacturers’ recommendations, and cells were subjected to analysis 24–48 h post-transfection.

Preparation of Mammalian Cell Lysates and Immunoblot Analysis—The cells were washed twice with PBS, scraped, collected, and isolated by centrifugation at 1000 × g for 5 min. Postnuclear lysate supernatants were obtained by passing the pellets 25 times through a 20–200-μl pipette tip in one of two lysis buffers, depending on the experiment: cell lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 μg/ml leupeptin, 1 mM PMSF, and 10 μg/ml pepstatin) or detergent solution (50 mM Tris-HCl, pH 8.5, 1% Nonidet P-40, 0.4% sodium deoxycholate, and 62.5 mM EDTA, supplemented with 1 tablet of Roche Complete Protease Inhibitor Mixture, 1 mM PMSF, and 10 μg/ml pepstatin). The samples were incubated on ice for 15 min, and insoluble material was removed by centrifugation at 16,000 × g for 5 min. The protein concentrations were determined by the Bradford method (Bio-Rad protein assay kit). The lysates were diluted according to the instructions of the manufacturer, so that the detergents in the lysis buffers would not interfere with the protein assay. For samples subjected to SDS-PAGE, the lysates were denatured in Laemmli buffer, maintained at room temperature for 30 min, and loaded onto 10% polyacrylamide gels preincubated with chilled SDS buffer, and the proteins were resolved at 4 °C on the polyacrylamide gels. Immunoblot analysis was performed as described previously (20), with the exception of the anti-ubiquitin immunoblots. For those studies, following transfer to nitrocellulose, the membranes were boiled in distilled water for 10 min and cooled to room temperature prior to the blocking step.

Mass Spectrometry and Analysis—HEK293T cells transiently expressing HA-tagged NCC or untagged NCC (negative control) were lysed in detergent solution, described above. A total of 300 μg of the whole cell lysate was diluted to 300 μl, pre-cleared with 30 μl of Sepharose CL6B slurry (Sigma), and subjected to immunoprecipitation with 30 μl of anti-HA-conjugated agarose resin (Sigma) overnight at 4 °C. The next day, the resin was washed four times with PBS, immunoprecipitated proteins were eluted by incubating the beads at room temperature for 30 min in 5× Laemmli buffer, and proteins were resolved by SDS-PAGE on 12% polyacrylamide gels. The proteins were visualized using a MALDI-safe silver staining kit (Sigma) per the manufacturer’s recommendations. Gel bands of interest were excised as 1-mm² pieces, and corresponding molecular weight gel pieces in the negative control samples were processed for comparison. Protein in-gel trypsin digestion was carried out as described previously, with minor modifications (21). The digests were analyzed by LC-MS/MS using a linear trap quadrupole Velos Orbitrap (Thermo Fisher, San Jose, CA) using a 90-min LC gradient. Thermo Fisher Discoverer Daemon 1.3 was used for batch submissions of acquired .raw files from the linear trap quadrupole Velos Orbitrap to Thermo Fisher Proteome Discoverer 1.3. Database searches were performed against the Uniprot human complete canonical isoform database downloaded November 2012 (84,874 sequences, 34,774,549 residues) appended to a contaminant database from ABScies (Foster City, CA) with the Sequest search engine, for a trypsin digest with two missed cleavages and two dynamic modifications (carbamidomethylation of cysteines and oxidation of methionines). The mass tolerance was set at 10 ppm for precursor mass and 0.5 Da for collision-induced dissociation fragment ion masses. Peptide identifications were filtered with a q value cutoff of 0.05 (5% global false discovery rate) using Percolator.

Assays to Monitor NCC ER-associated Degradation—Yeast cycloheximide chase assays were performed as described previously (10). For cycloheximide chase studies in mammalian cells, MDCK cells transiently expressing HA-tagged NCC in 6-well plates were assayed 24 h post-transfection. To stop protein translation, the cells were placed in fresh high glucose DMEM containing 10% FBS, l-glutamine, and cycloheximide (Sigma) to a final concentration of 100 μg/ml. The cells were then chased in a 37 °C 5% CO₂ incubator for the indicated times. The cycloheximide-containing medium was aspirated, and the samples were washed on ice twice with ice-cold PBS. The cells were then lysed, protein was quantified, and HA-NCC turnover was analyzed by SDS-PAGE and immunoblotting as described above. For chase assays performed in the presence of proteasome inhibitors, the samples were first incubated with MG-132 (10 μM) or dimethyl sulfoxide (DMSO) control for 4 h prior to initiation of the chase, followed by the addition of 100 μg/ml cycloheximide for the indicated chase times.

Coimmunoprecipitation Analysis—Cells transiently expressing the indicated constructs were lysed in cell lysis buffer as described above. A total of 250–300 μg of the lysate was diluted to a total of 300 μl and used in the immunoprecipitation assay. The lysate was then pre-cleared with 50 μl of Sepharose CL6B slurry by end over end rotation at 4 °C for 2 h. For HA-NCC immunoprecipitations, the cleared lysates were rotated in fresh 20–30-μl aliquots of anti-HA-conjugated agarose resin (Sigma) overnight at 4 °C. For CHIP immunoprecipitations, 1 μg of anti-Myc antibody was added to the cleared lysates, and the samples were rotated in fresh 30-μl aliquots of protein A/G beads overnight at 4 °C. The samples were centrifuged at low speed, and the beads were washed once in 500 μl of PBS, twice in 500 μl of PBS containing 0.5% Triton X-100, and once more in 500 μl of PBS. Immunoprecipitated proteins were eluted by incubating the beads at room temperature for 30 min in 5× Laemmli buffer, separated by SDS-PAGE, and analyzed by immunoblotting as described above.

Data Analysis—For Western blot quantification, densitometry was carried out with NIH ImageJ software. The data from yeast cycloheximide chase analyses were analyzed on a Kodak 440CF Image Station and the associated Kodak 1D software. GraphPad Prism software was used for statistical analyses. Comparisons between two groups were determined by a Student’s t test.
RESULTS

A Cytoplasmic Multichaperone Complex Coimmunoprecipitates with NCC in Mammalian Cells—We recently identified a role for cytoplasmic Hsp70 during NCC selection for ERAD in yeast and in mammalian epithelial cells (10). Because Hsp70 rarely works alone to facilitate client degradation or folding (12–14), we reasoned that other cytoplasmic chaperones or cochaperones function with Hsp70 during the NCC ER quality control process. To begin to evaluate this possibility, we employed coimmunoprecipitation and mass spectrometry to perform an unbiased assessment of proteins that form complexes with NCC. Given the high abundance of heat shock proteins in cytosol and the significant effect of Hsp70 in targeting NCC for degradation via the ERAD pathway (10), we hypothesized that this approach would identify additional ER quality control components that interact with NCC. HA-tagged NCC was expressed in HEK293T cells, the cells were lysed, NCC was immunoprecipitated on anti-HA-conjugated agarose resin, and the bound proteins were fractionated by SDS-PAGE. Coimmunoprecipitating proteins were then visualized by silver staining. As shown in Fig. 1A, we identified several proteins that coimmunoprecipitated with HA-NCC; these species were absent in control HA-agarose immunoprecipitations from cell lysates that contained the untagged cotransporter. Of these proteins, four major signals at ~85, 70, 50, and 40 kDa were selected for further analysis. These gel bands were excised, the protein was extracted, and the identities of the potential NCC binding partners were determined by peptide mass fingerprinting of tryptic digests by LC-MS/MS. Using this shotgun proteomics approach, we identified cytoplasmic Hsp90 (Hsp90) and the two cytoplasmic Hsp70s, Hsp70 and Hsc70, as the major species migrating at 85 and 70 kDa, respectively. Although β-actin was identified at ~40 kDa, the Hsp40 DNAJA1 was also detected with high confidence. Several tubulin isoforms were also identified in the ~50-kDa range, consistent with prior reports that cation chloride cotransporters undergo microtubule-dependent protein trafficking (22). The mass spectrometry protein identification data for Hsp90, Hsp70/Hsc70, and Hsp40 are provided in Table 1. As a second approach, we used a previously validated panel of antibodies to probe HA-NCC immunoprecipitates for the same chaperones that were identified by mass spectrometry. As shown in Fig. 1B, Hsp90, Hsp70, and Hsp40 were specifically detected in anti-HA immunoprecipitates from HEK293T cell lysates expressing the tagged cotransporter. Together, these observations suggested that NCC associates with several cytoplasmic chaperones, including Hsp90, Hsp70, and Hsp40.

Hsp70 and Hsp90 Cochaperones CHIP and HOP Coimmunoprecipitate with NCC—Depending on the substrate, Hsp70 and Hsp90 have the capacity to facilitate either client folding or degradation during ERAD (23). The tendency of these core chaperone systems to perform one function versus the other may be influenced by cochaperones. Because both Hsp90 and Hsp70 coimmunoprecipitated with NCC, we reasoned that their associated cochaperones might also interact with NCC to regulate its maturation or ERAD. Cochaperones containing TPR domains would be ideal candidates to carry out such a function, because all members of this protein family have the capacity to interact with both Hsp70 and Hsp90. Among the best studied of these ubiquitously expressed cochaperones are the E3 ubiquitin ligase CHIP, which is required for the ERAD of some substrates (24–27), and HOP, which transitions clients to the Hsp90 chaperone cycle by connecting Hsp70-bound sub-
stratifies to Hsp90 (28, 29). As predicted, coimmunoprecipitation studies indicated that both of these TPR cochaperones associated with NCC in protein complexes that also contained Hsp90, Hsp70, and Hsp40 (Fig. 1B). In contrast, p23, a well characterized non-TPR cochaperone that facilitates the biogenesis of a number of Hsp90 clients (30), was abundantly expressed in HEK293T cells but did not coimmunoprecipitate with NCC.

**Hsp90 Facilitates NCC Biogenesis in Mammalian Cells**—The finding that a complex containing Hsp90, CHIP, and HOP associates with NCC suggests that the Hsp90 system may regulate NCC quality control. To address this hypothesis, MDCK cells expressing the cotransporter were treated with a geldanamycin analog, 17-AAG, which blocks Hsp90 chaperone activity by interfering with its ATP hydrolysis cycle (31). As shown in Fig. 2A, 4 h of 17-AAG treatment decreased the abundance of both the high mannose core and mature glycosylated forms of NCC (10) in a dose-dependent manner; treatment of cells with 10 μM 17-AAG resulted in a 53% decrease in total NCC protein abundance (Fig. 2B; *n* = 4, *p* = 0.0032). Consistent with prior reports, 17-AAG also increased Hsp70 abundance, a molecular signature of effective Hsp90 inhibition (Ref. 32 and Fig. 2A). Similar effects on both the core and mature NCC glycoforms were seen when HA-NCC-expressing MDCK cells were incubated with lower doses of 17-AAG (0.5 μM) over a longer (12 h) time course, compared with the Me2SO vehicle-treated controls (Fig. 2, C and D). In addition, treatment of NCC expressing cells with a different Hsp90 inhibitor, 17-dimethylaminoethylamino-17-demethoxygeldanamycin, resulted in a similar dose-dependent decrease in the steady state amount of NCC (data not shown). Collectively, these data indicate that Hsp90 augments NCC biogenesis and that this effect requires an intact ATP-dependent Hsp90 chaperone cycle.

**CHIP Stimulates the Chaperone-dependent ERAD of NCC**—CHIP is an E3 ubiquitin ligase that converts Hsp70 and Hsp90 from folding machines into degradation factors that target misfolded clients for ERAD (24). Because NCC interacts with CHIP, we reasoned that it might stimulate NCC ubiquitination and degradation by the ubiquitin proteasome pathway. To test this hypothesis, we determined whether wild type CHIP and CHIP mutants lacking critical functional domains differentially affect NCC turnover. The cDNAs employed for this experiment encode full-length Myc epitope-tagged CHIP, Myc-CHIP-DTPR (a mutant lacking the TPR domain that binds the extreme C termini of Hsp70 and Hsp90 (33)), or Myc-CHIP-ΔUBox (a deletion mutant lacking the E3-ligase domain that catalyzes substrate ubiquitination) (Fig. 3A). In analyses of steady state NCC expression in HEK293T cells, coexpression of full-length wild type CHIP decreased the levels of both the

**TABLE 1** Identification of NCC-interacting chaperones by mass spectrometry

| Band molecular mass | Protein | IP | Peptides | PSMs | Coverage | Score |
|---------------------|---------|----|----------|------|----------|-------|
| 85 kDa              | Hsp90a  | HA-NCC | 54 | 215 | 58.47 | 647.68 |
|                     | Control | 19  | 21      | 23.50 | 62.67 |
| 70 kDa              | Hsp70   | HA-NCC | 43 | 191 | 68.02 | 662.24 |
|                     | Control | 24  | 43      | 42.28 | 147.08 |
| 40 kDa              | Hsp40   | HA-NCC | 36 | 149 | 65.02 | 543.05 |
|                     | Control | 23  | 32      | 41.02 | 105.66 |

**FIGURE 2.** Hsp90 facilitates NCC biogenesis in mammalian cells. A, MDCK cells were transiently transfected with HA-NCC and 24 h post-transfection were treated with increasing doses of 17-AAG from 0.1 to 10 μM for 4 h. The cell lysates were analyzed by immunoblotting with anti-HA antibodies. Parallel immunoblots for Hsp90 and Hsp70 were performed to evaluate the effects of Hsp90 inhibition, and immunoblots for tubulin were performed as a loading control. Core, high mannose-glycosylated (immature) NCC is indicated with an asterisk, maturely glycosylated NCC is indicated with an arrowhead, and a NCC oligomer is shown with a bracket. The figure is representative of four independent experiments. B, quantification of the change in total NCC protein abundance following treatment with 10 μM 17-AAG for 4 h (*n* = 4, *p* = 0.0032). C, effect of 0.5 μM 17-AAG treatment over a 12 h time course on HA-NCC protein abundance in MDCK cells. The cells were incubated with 17-AAG 24 h following transient transfection with HA-NCC and 24 h post-transfection were performed to evaluate the effects of Hsp90 inhibition, and immunoblots for tubulin were performed as a loading control. Core, high mannose-glycosylated (immature) NCC is indicated with an asterisk, maturely glycosylated NCC is indicated with an arrowhead, and a NCC oligomer is shown with a bracket. The figure is representative of four independent experiments. D, quantification in the change in NCC protein abundance during the time course, compared with dimethyl sulfoxide-treated controls (DMSO), *p* < 0.011 for each time point comparison by Student’s t test (*n* = 3 dimethyl sulfoxide-treated controls and seven experimental replicates).
mature and core-glycosylated forms of NCC; this effect was absent when the ΔUBox mutant was expressed (Fig. 3B). In cycloheximide chase assays, CHIP coexpression accelerated the disappearance of core-glycosylated NCC, indicating that this effect was due to enhanced post-translational turnover (Fig. 3C). Consistent with this finding, NCC degradation in the presence of extra copies of CHIP was attenuated by the proteasome inhibitor MG-132 (Fig. 3D).

**FIGURE 3.** The TPR cochaperone/E3 ubiquitin ligase CHIP stimulates the chaperone-dependent ERAD of NCC. A, domain architecture of CHIP and the ΔUBox and ΔTPR deletion mutants used for these studies. B, analysis of steady state HA-NCC expression by immunoblotting in HEK293T cells coexpressing either full-length Myc-tagged CHIP, the ΔUBox Myc-CHIP mutant, or empty vector (pcDNA3.1). Mature and core NCC glycoforms are indicated with arrowheads. Parallel immunoblots of Hsc70 were performed as a loading control. C, cycloheximide chase analysis of NCC degradation. MDCK cells transiently expressing HA-NCC with either empty pcDNA3.1 vector or Myc-CHIP were treated for 3 h with 100 μg/ml cycloheximide as described under “Experimental Procedures.” At the indicated time points, cells were lysed, and whole cell lysates were subsequently probed for HA-NCC using anti-HA antibodies. Top panel, quantification of core-glycosylated NCC degradation in the absence and presence of CHIP. *, p < 0.012 by Student’s t test (n = 3). Representative immunoblots for both experimental groups are shown below the graph. D, effect of the proteasome inhibitor MG-132 on NCC degradation. MDCK cells transiently expressing HA-NCC and full-length CHIP were incubated in either 10 μM MG-132 or dimethyl sulfoxide (DMSO) vehicle control. 4 h later, 100 μg/ml cycloheximide was added, and the samples were chased for 90 min. On the left are anti-HA immunoblots for NCC and anti-Myc immunoblots for CHIP; quantification of NCC degradation observed during the chase period is shown on the right. E, analysis of NCC polyubiquitination by CHIP. HEK293T cells transiently expressing HA-NCC with either full-length Myc-tagged CHIP or the Myc-tagged ΔUBox CHIP mutant were assayed 24 h post-transfection. Following 4 h of pretreatment with 10 μM MG-132, HA-NCC was immunoprecipitated from the lysates with anti-HA-conjugated agarose resin, and the immunoprecipitates were subjected to SDS-PAGE and immunoblotting (IB) with the indicated antibodies. In the total ubiquitin immunoblots, the immunoreactive signal corresponding to polyubiquitinated NCC is indicated in brackets. In both the ubiquitin and NCC immunoblots, a lower intensity signal corresponding to an NCC proteolytic fragment is indicated with an asterisk. An anti-Myc immunoblot from whole cell lysates (WCL, 15 μg; 5% input) is presented below the immunoprecipitations, demonstrating equivalent expression of both the full-length and mutant CHIP constructs. The figure is representative of four independent experiments. F, reciprocal TPR domain-dependent communoprecipitation of NCC and CHIP. Left panels, whole cell lysates of HEK293T cells transiently expressing HA-NCC with Myc-CHIP, Myc-CHIP ΔTPR, or Myc-CHIP ΔUBox were immunoprecipitated with anti-HA-conjugated agarose resin and immunoblotted (IB) with the indicated antibodies. Middle panels, the same whole cell lysates were immunoprecipitated with polyclonal anti-Myc antibody and probed with anti-HA antibody (top panel) or monoclonal anti-Myc antibodies (bottom panel). Right panels, 5% of the whole cell lysate inputs (15 μg/sample) for the immunoprecipitations were immunoblotted for HA-NCC (top panel) and Myc-CHIP (bottom panel) with the indicated antibodies.
Sequential Regulation of NCC by Hsp70 and Hsp90

To confirm that CHIP polyubiquitinates NCC, we performed a coimmunoprecipitation experiment in HEK293T cells transfected with HA-tagged NCC and a second plasmid encoding either Myc-tagged wild type CHIP or the E3 ligase deficient ΔUBox mutant. For these studies, both sets of transfected cells were pretreated with 10 μM MG-132 for 4 h prior to lysis to preserve the polyubiquitinated pool of cotransporters that would normally be targeted for ERAD. Whole cell lysates from the cells were then immunoprecipitated with anti-HA-conjugated agarose resin, and the bound protein was fractionated by SDS-PAGE and subjected to immunoblotting with anti-ubiquitin antibody. In HA-NCC expressing cells cotransfected with wild type CHIP, anti-HA immunoprecipitation yielded a robust immunoreactive smear that was strongest at a molecular mass of 110 kDa and higher (Fig. 3F). An additional lower intensity signal was seen at ~75 kDa (Fig. 3E, asterisks) that, based on the NCC migration pattern in the whole cell lysates, most likely corresponded to the ubiquitination of a low abundance NCC proteolytic fragment. In contrast, the intensity of this smear was significantly reduced in cells cotransfected with HA-NCC and the ΔUBox mutant. Collectively, these observations indicate that CHIP stimulates NCC polyubiquitination and ERAD in mammalian cells.

To determine whether chaperone binding is required for CHIP to associate with NCC, we performed coimmunoprecipitation experiments in HEK293T cells expressing HA-NCC and either wild type CHIP or the aforementioned ΔUBox or ΔTPR deletion mutants. As shown in Fig. 3F (left panels), an anti-HA antibody coimmunoprecipitated the Myc-tagged CHIP protein only in cell lysates where either wild type or UBox-deficient CHIP were coexpressed. Similar results were seen in the reciprocal direction (Fig. 3F, middle panels). We also noted that CHIP preferentially interacted with the 110-kDa immature core-glycosylated form of NCC. This pattern of immunoreactivity is compatible with previous observations that Hsp70 interacts primarily with immature NCC species (10). Together, these observations provide strong evidence that NCC associates with CHIP in a TPR domain-dependent manner, most likely via the Hsp70 and/or Hsp90 chaperones.

**HOP Stabilizes NCC—**We next asked whether HOP, the other TPR cochaperone that we identified in NCC coimmunoprecipitation assays, also regulates NCC protein abundance. Unlike CHIP, HOP consists of three TPR domains. These domains have different binding affinities for the C termini of Hsp70 and Hsp90, and this allows HOP to link the chaperones, providing a mechanism for client transfer from one chaperone system to the other (28, 34). Because Hsp70 stimulates NCC ERAD (10), whereas Hsp90 facilitates productive NCC folding (Fig. 2), we reasoned that HOP might divert NCC away from the Hsp70 ERAD pathway, increase steady state NCC abundance, and attenuate its post-translational turnover. To test the hypothesis that HOP stabilizes NCC, we coexpressed the HOP cochaperone with NCC in HEK293T cells to evaluate its effects on NCC abundance. We observed a 38% increase in steady state NCC expression when the levels of HOP were increased (n = 4, p = 0.0029; Fig. 4A). Both the mature and core forms of the cotransporter (which tend to migrate closely together in HEK293T cells (10)) were equally increased upon HOP overexpression, suggesting that NCC export beyond the cis-Golgi rose because of decreased ERAD. Consistent with an increase in NCC levels when HOP was overexpressed, HOP coexpression significantly attenuated NCC turnover in cycloheximide chase assays in MDCK cells (Fig. 4B). Collectively, the data implicate HOP in stabilizing NCC, likely by attenuating Hsp70-dependent ERAD.

**Low Temperature Enhances NCC Association with Hsp90—**Protein folding is a temperature-sensitive process. For example, reduced temperature rescues both wild type and mutant forms of immature CFTR, resulting in increased steady state CFTR abundance, enhanced ER export, and delivery to the cell surface (35). A recent study found that incubation of cells at 25 °C also increased wild type NKCC2 protein abundance, indicating that, like CFTR, members of the cation chloride cotransporter family are processed in a temperature-sensitive manner (35, 36). Current evidence suggests that altered client-chaperone interactions are partly responsible for the beneficial effect of low temperature on protein biogenesis (37). Thus, we reasoned that we...
could temperature shift NCC-expressing cells to detect functional interactions between NCC and biologically relevant chaperones that facilitate NCC folding and/or ERAD. To begin to test this idea, we first sought to determine whether NCC processing is temperature-sensitive. When NCC-expressing HEK293T cells were incubated for 14 h at 25 °C, we observed a statistically significant increase in steady state NCC levels (p = 0.0066, n = 3; Fig. 5A). Comparable results were noted in MDCK cells (data not shown). These data strongly suggest that reduced temperature shunts wild type NCC away from a degradation pathway and thus increases its abundance.

Next, we asked whether low temperature correction is associated with changes in chaperone or cochaperone binding. Cells transiently expressing HA-tagged NCC were incubated for 14 h at either 37 or 25 °C, and the association of the core Hsp90/70/40 chaperones and the cochaperones CHIP and HOP was measured. In these experiments, higher amounts of protein lysate and lower amounts of anti-HA-agarose affinity resin were used to ensure that the HA-conjugated beads were equally saturated with the HA-tagged cotransporter under both conditions. Using this approach, equal immunoprecipitation of the HA-tagged cotransporter was achieved, despite higher steady state NCC expression in whole cell lysates that were obtained from cells incubated at 25 °C (Fig. 5B, top panel). As shown in Fig. 5 (B and C), the low temperature shift was associated with decreased NCC coimmunoprecipitation with Hsp40 and Hsp70. In contrast, more Hsp90 associated with NCC at 25 °C. Because the low temperature shift enhanced the steady state levels of NCC protein, these data indicate that the stabilizing effects of reduced temperature are most likely due to diminished NCC ERAD via the Hsp70/Hsp40-based chaperone system and via enhanced NCC folding, which is mediated by Hsp90. We also observed decreased association between NCC and HOP at low temperatures. Because these same NCC coimmunoprecipitates were relatively enriched in Hsp90, we suggest that the majority of NCC in temperature-shifted cells was present in chaperone-bound conformers that had advanced beyond the HOP-mediated, Hsp70 to Hsp90 transfer step. The outcome of this phenomenon results in enhanced binding to Hsp90, productive folding, and protection from ERAD.

Gitelman Mutants That Are Resistant to Temperature Shifting Exhibit Enhanced Association with the Hsp70/Hsp40 System and CHIP—In Gitelman syndrome, loss of function mutations of NCC commonly result in reduced cotransporter plasma membrane expression and a shift of the equilibrium of NCC expression toward its core-glycosylated form (6). This implies that most Gitelman syndrome mutations instigate NCC misfolding, resulting in enhanced recognition by ER quality control mechanisms and ERAD. Because our data indicate that Hsp70 and Hsp90 select NCC for either disposal or folding, we reasoned that mutant forms of NCC might interact differently with these systems. To test this hypothesis, we chose to study two Gitelman sequence variants harboring nonsense mutations at the C terminus of the NCC protein: R948X and R989X (Fig. 6A). Both of these mutations likely alter NCC protein conformation and promote chaperone-dependent ERAD, because they introduce a premature stop codon within a structurally compact and phylogenetically conserved cytosolic domain (38).

The mutations also reduce NCC plasma membrane delivery, thiazide-sensitive sodium transport activity, and steady state mature glycosylation (6, 10, 39). In initial tests of the severity of these mutations on NCC processing in HEK293T cells, we
found that both mutations reduced steady state NCC protein expression and mature glycosylation, both at physiologic temperature and at 25 °C (Fig. 6B). As shown in the graph in Fig. 6B, low temperature incubation increased wild type NCC abundance by 75%, but the same maneuver had little effect on the biosynthetic processing of the two nonsense mutants (n/H11005 = 5, p < 0.01 for each mutant compared with wild type NCC by one-way analysis of variance, Dunnett’s post hoc test). These data suggest that the R948X and R989X Gitelman mutations introduce severe folding defects into the cotransporter that result in obdurate selection for chaperone-dependent ERAD and resistance to low temperature rescue.

Consistent with this interpretation, communoprecipitation studies performed in HEK293T cells expressing NCC at 25 °C revealed that both Gitelman mutants exhibited enhanced association with Hsp70 and Hsp40 compared with the wild type protein. Relative to the wild type cotransporter, the R948X and R989X mutants exhibited a 100% increase in association with Hsp70 and an even greater increase in coimmunoprecipitation with Hsp40 (Fig. 6, C and D). We also found that the two mutants associated with CHIP more strongly than wild type NCC (n/H11005 = 3, p < 0.05 for both mutants by one-way analysis of variance, Dunnett’s post hoc test). Collectively, these findings strongly suggest that disease-causing mutant forms of NCC that exhibit impaired biosynthetic trafficking from the ER are selected by the Hsp70/Hsp40 system for disposal. Moreover, these defective cotransporters appear to be more highly associated with CHIP, a cochaperone that triages misfolded Hsp70-bound substrates for ERAD.

**DISCUSSION**

In this study, we identified several molecular chaperones and cochaperones that participate in the ER quality control of NCC, a protein that is closely linked to blood pressure homeostasis.
To this end, we used a proteomic approach to establish that the cotransporter associates with cytoplasmic Hsp90, Hsp70, and Hsp40 and with the TPR cochaperones CHIP and HOP. In contrast to Hsp70, which stimulates NCC ERAD (10), we find here that Hsp90 facilitates NCC maturation, most likely by enhancing protein folding. This conclusion is supported by studies in mammalian cells subjected to pharmacologic Hsp90 inhibition, which enhances NCC turnover. Moreover, we find that reduced temperature stabilizes NCC, an effect that is associated with increased Hsp90 binding and diminished Hsp70 interaction. In contrast to the wild type protein, two Gitelman mutants were resistant to temperature shifting and exhibited increased association with the Hsp70/Hsp40 system. Finally, we report that CHIP and HOP exert opposite effects on NCC protein abundance. Specifically, CHIP stimulates NCC ERAD via a mechanism that requires intact E3 ligase activity and the binding of its TPR to Hsp70 and/or Hsp90. In contrast, HOP increases NCC protein abundance, suggesting that it helps NCC transition from an Hsp70-dependent degradation complex to Hsp90-dependent folding complex.

According to the current paradigm, Hsp70/Hsp40 and Hsp90 act sequentially to facilitate client folding or degradation. Among the most extensively studied proteins that adhere to such a model is the glucocorticoid receptor (GR) (18). During GR biogenesis, the nascent, unfolded polypeptide contains hydrophobic regions that become cytosol-exposed. Hsp40 recognizes these motifs and prevents them from aggregating. Hsp40 then associates with Hsp70 to form a preliminary chaperone-client complex, which augments GR folding. HOP mediates the subsequent transfer of GR to Hsp90 for additional folding; prior to doing so, its TPR domains link Hsp40/Hsp70 and Hsp90 to form an "intermediate" heterocomplex (28, 40). Once the GR nears its native steroid binding-competent conformation, other cochaperones associate with the complex, including p23 and certain immunophilins (41). Some cytosolic proteins adhere to a similar model of chaperone-dependent protein folding (for example, see Ref. 18). Indeed, the Hsp90 clientele includes a large number of proteins (42), but only a few of them are ERAD substrates (43–46). The data presented here add a new member of the SLC12 cation chloride cotransporter family to this list of Hsp90-regulated ERAD substrates.

Several observations strongly suggest that Hsp90, Hsp70, and Hsp40 do not necessarily regulate NCC biogenesis in precisely the same manner as the GR or CFTR. For example, unlike polytopic membrane proteins such as CFTR (45, 47) or the epithelial sodium channel (48), we have found that both the cytoplasmic and ER luminal Hsp40s are dispensable for NCC quality control (10). On the other hand, cytoplasmic Hsp70 strongly influences NCC turnover in both yeast and mammalian renal epithelial cells, but this default role appears to be opposite to its role in the selection of clients such as the GR, because it stimulates NCC ERAD (10). Taken together, these observations support a model in which the Hsp70 and Hsp90 chaperone systems play key roles in the ER quality control of a variety of substrates, but their default functions may vary drastically depending upon the client with which they interact (23).

How can the Hsp40/Hsp70 and Hsp90 chaperone systems exert such a broad range of effects on a diversity of clients? The specificity of these core systems to mediate folding or degradation is defined by cochaperones, such as CHIP and HOP (24). Indeed, once CHIP was overexpressed with NCC, it accelerated NCC turnover through TPR-dependent interactions with Hsp70 and/or Hsp90, indicating that it remodeled both chaperone systems to favor NCC ERAD. Conversely, HOP protected NCC from degradation, likely by catalyzing the transfer of non-native forms of NCC to Hsp90 for additional folding attempts. In all likelihood, complex membrane proteins with large cytosolic and transmembrane domains that are difficult to fold, such as NCC, are intimately monitored by the Hsp70 and Hsp90 chaperone systems, and during ATP-dependent cycling they are bound to cochaperones that either try to facilitate a productive fold (such as HOP) or triage misfolded conformers for degradation (such as CHIP). Thus, we propose that each Hsp70 and Hsp90 substrate possesses an individualized balance of cochaperones that differs at steady state and that these cochaperone profiles vary depending on the structure and intrinsic folding properties of the client.

Despite the aforementioned differences between NCC and other substrates, our data indicate that NCC is similar to GR in that it undergoes an Hsp70-dependent quality control step prior to Hsp90 engagement (18). Three pieces of evidence support this sequential model. First, we found that the rapid rate of NCC turnover in yeast was strongly stabilized in strains deficient in cytoplasmic Hsp70 (10); in contrast, the rate of NCC degradation was only slightly accelerated in yeast lacking functional Hsp90 (data not shown). This suggests that most of the overexpressed cotransporter becomes terminally misfolded shortly after translation in yeast and that Hsp70 immediately triages this defective NCC pool for ERAD via the E3 ubiquitin ligases Hrd1 and to a lesser degree Doa10 (10). Because only a small fraction of the total NCC pool progresses to the Hsp90-dependent checkpoint, we observed only a mild effect of Hsp90 deletion on NCC turnover. Second, HOP, which mediates the sequential transfer of substrates from Hsp70 to Hsp90, increased NCC protein abundance and protected the cotransporter from ERAD. Finally, we found that reduced temperature incubation in HEK293T cells diminished wild type NCC interaction with Hsp90 and simultaneously enhanced NCC interaction with Hsp90. This suggests that a low temperature shift increases NCC maturation beyond steps that require Hsp70, including HOP-mediated Hsp70 to Hsp90 transfer. Because low temperature incubation diminishes the interaction of HOP with NCC, our findings suggest that this cochaperone interacts to a lower degree with wild type cotransporters that have advanced further along the Hsp90-dependent folding pathway.

Taken together, our data suggest that the early stages of NCC quality control require sequential Hsp70- and Hsp90-dependent checkpoints (Fig. 7). We propose that shortly after membrane integration, Hsp70 associates with NCC in an “early recognition complex” that targets cotranslationally misfolded forms of the cotransporter for ERAD via CHIP, and perhaps other E3 ligases, including HRD1 and the human Doa10 homolog TEB4 (49). Although Hsp40 is bound to Hsp70 within this complex, our data suggest that it does not influence NCC ERAD, because we have found that the cytoplasmic Hsp40s do not alter the rate of NCC degradation (10). The Hsp40/Hsp70 complex then binds to HOP, which assembles an “intermediate
Sequential Regulation of NCC by Hsp70 and Hsp90

FIGURE 7. Sequential model of chaperone-dependent ER quality control steps during early NCC biogenesis. Hsp70 interacts with ER-associated NCC, most likely soon after translation. At this early stage, Hsp70 targets misfolded NCC conformers for ERAD via E3 ligases. Although Hsp40 is a part of this complex, current data suggest that it does not regulate NCC turnover (10). HOP promotes non-native NCC transfer from Hsp70 to Hsp90. Upon Hsp90 binding, NCC may either undergo chaperone-dependent folding via cochaperones that promote NCC biogenesis, or ERAD via cochaperones that remodel Hsp90 function to promote substrate disposal, such as CHIP.

complex” that includes Hsp90. From this intermediate state, NCC can either advance into an exclusively Hsp90-bound “folding complex” that is relatively shielded from ERAD, possibly because of currently unidentified cochaperones that promote NCC biogenesis. Alternatively, unsuccessful folding attempts at this stage may be subject to CHIP-dependent ERAD as well, because its TPR domain has been shown to interact with Hsp90 and regulate Hsp90-bound substrates (24, 50).

By adding to the list of factors that regulate NCC ER quality control, the work presented here provides a new and deeper understanding of the NCC quality control pathway. In Gitelman syndrome and carrier state hypertension resistance, mutations that alter the NCC coding sequence cause the cotransporter to misfold, resulting in enhanced recognition by quality control machinery that targets the cotransporter for ERAD (6, 7, 10, 39). Our data indicate that the cytoplasmic Hsp70 and Hsp90 systems, and their associated cochaperones, are directly involved in selecting NCC for either ERAD or productive folding during the early stages of NCC biogenesis. Thus, we anticipate that our efforts will begin to lay the groundwork for future studies to identify the molecular basis of hereditary salt wasting conditions that cause hypotension, or, in a milder phenotype, protect patients from the deleterious consequences of high blood pressure. In addition, our observations that Hsp70/Hsp40 interact with mutant misfolded forms of NCC differently suggest that conformationally defective cotransporters selected for ERAD may be responsive to small molecule “correctors” that can enhance cellular folding machineries (51). These agents may ultimately serve as a therapy for severe salt-wasting diseases associated with defects in cation chloride cotransporter processing, such as Gitelman syndrome.

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REFERENCES
1. Gamba, G. (2005) Molecular physiology and pathophysiology of electro-neutral cation-chloride cotransporters. Physiol. Rev. 85, 423–493
2. ALLHAT Officers and Coordinators for the ALLHAT Collaborative Research Group (2002) Major outcomes in high-risk hypertensive patients randomized to angiotensin-converting enzyme inhibitor or calcium channel blocker vs diuretic. The Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial (ALLHAT). JAMA 288, 2981–2997
3. Simon, D. B., Nelson-Williams, C., Bia, M. J., Ellison, D., Karat, F. E., Molina, A. M., Vaara, I., Iwata, F., Cusnehmer, H. M., Koolen, M., Gainza, F. J., Gitelman, H. J., and Litton, R. P. (1996) Gitelman’s variant of Bartter’s syndrome, inherited hypokalemic alkalosis, is caused by mutations in the thiazide-sensitive NaCl cotransporter. Nat. Genet. 12, 24–30
4. Ji, W., Foo, J. N., O’Roak, B. J., Zhao, H., Larson, M. G., Simon, D. B., Newton-Cheh, C., State, M. W., Levy, D., and Litton, R. P. (2008) Rare independent mutations in renal salt handling genes contribute to blood pressure variation. Nat. Genet. 40, 592–599
5. Acuña, R., Martínez-de-la-Maza, L., Ponce-Coria, J., Vázquez, N., Ortal-Vite, P., Pacheco-Alvarez, D., Bobadilla, N. A., and Gamba, G. (2011) Rare mutations in SLC12A1 and SLC12A3 protect against hypertension by reducing the activity of renal salt cotransporters. J. Hypertens. 29, 475–483
6. Kunchaparty, S., Palcsó, M., Berkmann, J., Velázquez, H., Desir, G. V., Bernstein, P., Reilly, R. F., and Ellison, D. H. (1999) Defective processing and expression of thiazide-sensitive NaCl cotransporter as a cause of Gitelman’s syndrome. Am. J. Physiol. 277, F643–F649
7. De Jong, J. C., Van Der Vliet, W. A., Van Den Heuvel, L. P., Willems, P. H., Knoers, N. V., and Bindels, R. J. (2002) Functional expression of mutations in the human NaCl cotransporter. Evidence for impaired routing mechanisms in Gitelman’s syndrome. J. Am. Soc. Nephrol. 13, 1442–1448
8. Brodsky, J. L., and Skach, W. R. (2011) Protein folding and quality control in the endoplasmic reticulum. Recent lessons from yeast and mammalian cell systems. Curr. Opin. Cell Biol. 23, 464–475
9. Braakman, I., and Bulleid, N. J. (2011) Protein folding and modification in the mammalian endoplasmic reticulum. Annu. Rev. Biochem. 80, 71–99
10. Needham, P. G., Mikoluk, K., Dhakarwal, P., Khadem, S., Snyder, A. C., Subramanya, A. R., and Brodsky, J. L. (2011) The thiazide-sensitive NaCl cotransporter is targeted for chaperone-dependent endoplasmic reticulum-associated degradation. J. Biol. Chem. 286, 43611–43621
11. Zhang, Y., Nijbroek, G., Sullivan, M. L., Willems, P. H., Watkins, S. C., Michaelis, S., and Brodsky, J. L. (2001) Hsp70 molecular chaperone facilitates endoplasmic reticulum-associated protein degradation of cystic fibrosis transmembrane conductance regulator in yeast. Mol. Biol. Cell 12, 1303–1314
12. Mayer, M. P., and Bukau, B. (2005) Hsp70 chaperones. Cellular functions and molecular mechanism. Cell Mol. Life Sci. 62, 670–684
13. Shaker, L., and Morano, K. A. (2007) All in the family. Atypical Hsp70 chaperones are conserved modulators of Hsp70 activity. Cell Stress Chaperones 12, 1–8
14. Kampinka, H. H., and Craig, E. A. (2010) The Hsp70 chaperone machinery: proteins as drivers of functional specificity. Nat. Rev. Mol. Cell Biol. 11, 579–592
15. Pearl, L. H., and Prodomou, C. (2006) Structure and mechanism of the Hsp90 molecular chaperone machinery. Annu. Rev. Biochem. 75, 271–294
16. Waddington, S. K., Richter, K., and Buchner, J. (2008) The Hsp90 chaperone machinery. J. Biol. Chem. 283, 18473–18477
17. Krueckenberg, K. A., Street, T. O., Lavery, L. A., and Agard, D. A. (2011) Conformational dynamics of the molecular chaperone Hsp90. Q. Rev. Biophys. 44, 229–255
18. Pratt, W. B., and Toft, D. O. (2003) Regulation of signaling protein function and trafficking by the Hsp90/Hsp70-based chaperone machinery. Exp. Biol. Med. (Maywood) 228, 111–133
19. Cyr, D. M., Höhfeld, J., and Patterson, C. (2002) Protein quality control. U-box-containing E3 ubiquitin ligases join the fold. Trends Biochem. Sci. 27, 368–375
20. Subramanya, A. R., Liu, J., Ellison, D. H., Wade, J. B., and Welling, P. A. (2009) WNK4 diverts the thiazide-sensitive NaCl cotransporter to the
lysosome and stimulates AP-3 interaction. J. Biol. Chem. 284, 18471–18480
21. Shevchenko, A., Tomas, H., Havlis, J., Olsen, J. V., and Mann, M. (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat. Protoc. 1, 2856–2860
22. Meade, P., Hoover, R. S., Plata, C., Vázquez, N., Bobadilla, N. A., Gamba, G., and Hebert, S. C. (2003) cAMP-dependent activation of the renal-specific Na⁺-K⁺-2Cl⁻ cotransporter is mediated by regulation of cotransporter trafficking. Am. J. Physiol. Renal Physiol. 284, F1145–F1154
23. Brodsky, I. L. (2007) The protective and destructive roles played by molecular chaperones during ERAD (endoplasmic reticulum-associated degradation). Biochem. J. 404, 353–363
24. Connell, P., Ballinger, C. A., Jiang, J., Wu, Y., Thompson, L. J., Höhfeld, J., and Patterson, C. (2001) The co-chaperone CHIP regulates protein triage decisions mediated by heat-shock proteins. Nat. Cell Biol. 3, 93–96
25. Younger, J. M., Ren, H. Y., Chen, L., Fan, C. Y., Fields, A., Patterson, C., and Cyr, D. M. (2004) A foldable CFRΔF508 biogenic intermediate accumulates upon inhibition of the Hsc70-CHIP E3 ubiquitin ligase. J. Cell Biol. 167, 1075–1085
26. Meacham, G. C., Patterson, C., Zhang, W., Younger, J. M., and Cyr, D. M. (2001) The Hsc70 co-chaperone CHIP targets immature CFTR for posttranslational degradation. Nat. Cell Biol. 3, 100–105
27. Faresse, N., Ruffieux-Daidie, D., Salamin, M., Gomez-Sanchez, C. E., and Staub, O. (2010) Mineralocorticoid receptor degradation is promoted by Hsp90 inhibition and the ubiquitin-protein ligase CHIP. Am. J. Physiol. Renal Physiol. 299, F1462–F1472
28. Scheufler, C., Brinker, A., Bourenkov, G., Pegoraro, S., Moroder, L., Bartunik, H., Hartl, F. U., and Moarefi, I. (2000) Structure of TPR domain-peptide complexes. Critical elements in the assembly of the Hsp70-Hsp90 multichaperone machine. Cell 101, 199–210
29. McClellan, A. J., Scott, M. D., and Frydman, J. (2005) Folding and quality control of the VHL tumor suppressor proceed through distinct chaperone pathways. Cell 121, 739–748
30. Felts, S. J., and Toft, D. O. (2003) p23, a simple protein with complex activities. Cell Stress Chaperones 8, 108–113
31. Whitesell, L., Santagata, S., and Lin, N. U. (2012) Inhibiting Hsp90 to treat cancer: A strategy in evolution. Curr. Med. Mol. 12, 1108–1124
32. Bagatell, R., Paine-Murrieta, G. D., Taylor, C. W., Pulcini, E. J., Akinaga, S., Whitesell, L., and Santagata, S. (2007) The protective and destructive roles played by molecular chaperones during ERAD (endoplasmic reticulum-associated degradation). Biochem. J. 404, 353–363
33. Brodsky, I. L. (2007) The protective and destructive roles played by molecular chaperones during ERAD (endoplasmic reticulum-associated degradation). Biochem. J. 404, 353–363
34. Bagatell, R., Paine-Murrieta, G. D., Taylor, C. W., Pulcini, E. J., Akinaga, S., Whitesell, L., and Santagata, S. (2007) The protective and destructive roles played by molecular chaperones during ERAD (endoplasmic reticulum-associated degradation). Biochem. J. 404, 353–363
35. Denning, G. M., Anderson, M. P., Amara, J. F., Marshall, J., Smith, A. E., and Welsh, M. J. (1992) Processing of mutant cystic fibrosis transmembrane conductance regulator is temperature-sensitive. Nature 358, 761–764
36. Monette, M. Y., Rinehart, J., Lifton, R. P., and Forbush, B. (2011) Rare mutations in the human Na⁺-K⁺-Cl⁻ cotransporter (NKCC2) associated with lower blood pressure exhibit impaired processing and transport function. Am. J. Physiol. Renal Physiol. 300, F840–F847
37. Wang, X., Koulou, A. V., Kellner, W. A., Riordain, J. R., and Balch, W. E. (2008) Chemical and biological folding contribute to temperature-sensitive ΔF508 CFTR trafficking. Traffic 9, 1878–1893
38. Warmuth, S., Zimmermann, I., and Dutzler, R. (2009) X-ray structure of the C-terminal domain of a prokaryotic cation-chloride cotransporter. Structure 17, 538–546
39. Sabath, E., Meade, P., Berkman, J., de los Heros, P., Moreno, E., Bobadilla, N. A., Vázquez, N., Ellison, D. H., and Gamba, G. (2004) Pathophysiology of functional mutations of the thiazide-sensitive Na Cl cotransporter in Gitelman disease. Am. J. Physiol. Renal Physiol. 287, F195–F203
40. Kosano, H., Stensgard, B., Charlesworth, M. C., McMahon, N., and Toft, D. (1998) The assembly of progesterone receptor-Hsp90 complexes using purified proteins. J. Biol. Chem. 273, 32973–32979
41. Freeman, B. C., Toft, D. O., and Morimoto, R. I. (1996) Molecular chaperone machines. Chaperone activities of the cyclophilin Cyp-40 and the steroid aporeceptor-associated protein p23. Science 274, 1718–1720
42. Echeverria, P. C., Bemthaler, A., Dupuis, P., Mayer, B., and Picard, D. (2011) An interaction network predicted from public data as a discovery tool. Application to the Hsp90 molecular chaperone machine. PLoS One 6, e26044
43. Yan, F. F., Pratt, E. B., Chen, P. C., Wang, F., Skach, W. R., David, L. L., and Shyng, S. L. (2010) Role of Hsp90 in biogenesis of the β-cell ATP-sensitive potassium channel complex. Mol. Biol. Cell 21, 1945–1954
44. Loo, M. A., Jensen, T. J., Cui, L., Hou, Y., Chang, X. B., and Riordan, J. R. (1998) Perturbation of Hsp90 interaction with nascent CFTR prevents its maturation and accelerates its degradation by the proteasome. EMBO J. 17, 6879–6887
45. Youker, R. T., Walsh, P., Beilharz, T., Lithgow, T., and Brodsky, J. L. (2004) Distinct roles for the Hsp40 and Hsp90 molecular chaperones during cystic fibrosis transmembrane conductance regulator degradation in yeast. Mol. Biol. Cell 15, 4787–4797
46. Ficker, E., Dennis, A. T., Wang, L., and Brown, A. M. (2003) Role of the cytosolic chaperones Hsp70 and Hsp90 in maturation of the cardiac potassium channel HERG. Circ. Res. 92, 890–1000
47. Zhang, H., Schmidt, B. Z., Sun, F., Condiffe, S. B., Butterworth, M. B., Youker, R. T., Brodsky, J. L., Aridor, M., and Frizzell, R. A. (2006) Cysteine string protein monitors late steps in cystic fibrosis transmembrane conductance regulator biogenesis. J. Biol. Chem. 281, 11312–11321
48. Buck, T. M., Kolb, A. R., Boyd, C. R., Kleyman, T. R., and Brodsky, J. L. (2010) The endoplasmic reticulum-associated degradation of the epithelial sodium channel requires a unique complement of molecular chaperones. Mol. Biol. Cell 21, 1047–1058
49. Claessen, J. H., Kundrat, L., and Ploegh, H. L. (2012) Protein quality control in the ER. Balancing the ubiquitin checkbook. Trends Cell Biol. 22, 22–32
50. Kundrat, L., and Regan, L. (2010) Balance between folding and degradation for Hsp90-dependent client proteins. A key role for CHIP. Biochemistry 49, 7428–7438
51. Luo, T. W., Bartlet, M. C., and Clarke, D. M. (2008) Correctors promote folding of the CFTR in the endoplasmic reticulum. Biochem. J. 413, 29–36
52. Krishnamurthy, H., Piscitelli, C. L., and Gouaux, E. (2009) Unlocking the molecular secrets of sodium-coupled transporters. Nature 459, 347–355