

Differential effects of Hsc70 and Hsp70 on the intracellular trafficking and functional expression of epithelial sodium channels

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The members of the cytoplasmic 70-kDa heat shock protein family are involved in appropriate folding and trafficking of newly synthesized proteins in the cell. Hsc70, which is expressed constitutively, and Hsp70, the expression of which is stress- and heat shock-induced, are often considered to have similar cellular functions in this regard, but there are suggestions that the intracellular functions of these homologous but not identical proteins may differ. We tested the hypothesis that Hsc70 and Hsp70 would have differential effects on the expression of the epithelial sodium channel (ENaC). In *Xenopus* oocytes, overexpression of human Hsc70 decreased the functional (defined as amiloride-sensitive whole-oocyte current) and surface expression of murine ENaC (mENaC) in a concentration-dependent fashion. In contrast, coinjection of a moderate amount of Hsp70 cRNA (10 ng) increased the functional and surface expression of mENaC, whereas a higher amount of coinjected Hsp70 cRNA (30 ng) decreased mENaC functional and surface expression. The increase in mENaC functional expression with coinjection of 10 ng of Hsp70 cRNA was antagonized by the additional coinjection of Hsc70 cRNA in a concentration-dependent fashion. These data are consistent with Hsc70 and Hsp70 having differential and antagonistic effects with regard to the intracellular trafficking of mENaC in oocytes, which may have an impact on our understanding and potential treatment of diseases of aberrant ion channel trafficking.

chaperone | *Xenopus* oocyte | cystic fibrosis | ENaC | antagonism

The epithelial sodium channel (ENaC) plays a pivotal role in the regulation of blood volume and blood pressure and may have a vital role in the function of the pulmonary epithelia. The ENaC and the cystic fibrosis transmembrane conductance regulator (CFTR) are colocalized at the apical surface of respiratory epithelia, where CFTR can regulate ENaC activity (1, 2). ENaC functional expression appears increased in cystic fibrosis (CF) compared with non-CF airway epithelia (3), which likely leads to enhanced absorption of water from the airway surface liquid, decreased ciliary propulsion of mucus (4), and a compromised ability to clear bacteria from the CF airway. Thus, control of both CFTR and ENaC trafficking and expression is likely to be critical in airway homeostasis.

Some similarities exist in the intracellular processing of the CFTR and ENaC. Wild-type (WT) CFTR is processed inefficiently; only $\approx 25\%$ of newly synthesized WT CFTR reaches the plasma membrane (5), although conflicting data have been published recently (6). The most common mutation of the CFTR is the deletion of phenylalanine-508 ($\Delta F508$); protein with this deletion is retained in the endoplasmic reticulum (ER) (7). The majority of WT CFTR and almost all $\Delta F508$ are targeted for intracellular degradation, at least in part by the ubiquitin/proteasome system (5). Such degradation may depend on Hsc70, the constitutively expressed 70-kDa heat shock protein. Hsc70 associates more avidly with $\Delta F508$ than with WT CFTR (8, 9) and is an essential cofactor for ubiquitination (10). Hsc70 associates with nascent CFTR on the

ribosome (8), and cessation of this interaction correlates with cessation of CFTR ubiquitination (11). Thus, increased association of $\Delta F508$ and Hsc70 may lead to a higher likelihood of ubiquitination and degradation by the proteasome. Interestingly, a number of pharmacologic agents that improve $\Delta F508$ intracellular trafficking, such as sodium 4-phenylbutyrate (12), decrease the expression of Hsc70 and its association with $\Delta F508$ (9).

Intracellular trafficking of ENaC is, in some ways, similar to that of CFTR. In *Xenopus* oocytes, only a small fraction of newly synthesized ENaC reaches the plasma membrane (13). Similarly, the degradation of ENaC is also mediated by the proteasome (13–15). However, little is known regarding the role of 70-kDa cytoplasmic molecular chaperones in this process and how agents that modulate chaperone expression may influence this process.

Hsc70 has a number of other intracellular functions, including catalyzing the ATP-dependent uncoating of clathrin-coated pits (16) and promoting lysosomal degradation of intracellular proteins (16, 17). Although most investigators have treated Hsc70 and Hsp70, the stress-inducible 70-kDa heat shock protein, as equivalent and functionally interchangeable, many functions attributed to Hsc70 have not been associated with Hsp70 (16). In *Escherichia coli*, the Hsp70 homolog DnaK promotes protein folding (16), and overexpression of Hsp70 may improve $\Delta F508$ trafficking in CF epithelial cells (18).

These data suggest that Hsc70 and Hsp70 may affect the intracellular trafficking of ion channels differentially. Decreased Hsc70 expression may decrease the association of an ion channel with Hsc70 and therefore improve its trafficking by decreasing its ubiquitination and degradation. Hsp70 overexpression could decrease the association of an ion channel and Hsc70 by competing with Hsc70 for binding to the ion channel. We therefore tested the specific hypothesis that Hsc70 and Hsp70 overexpression would influence the intracellular trafficking and function of the ENaC differentially. We found that Hsc70 can decrease the functional and surface expression of murine ENaC, whereas Hsp70 has the opposite effect. These data have implications for the treatment of diseases involving aberrant trafficking of ion channels in epithelial cells.

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Abbreviations: $\alpha\beta\gamma$ -mENaC, α , β , and γ subunits of murine epithelial sodium channel; β FLAG, β subunit with external FLAG epitope; β -V5, β subunit with C-terminal V5 epitope; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; ENaC, epithelial sodium channel; ER, endoplasmic reticulum; mENaC, murine epithelial sodium channel; MTSET, [2-(trimethylammonium)ethyl] methanethiosulfonate bromide; NS, not significant; TEV, two-electrode voltage clamp.

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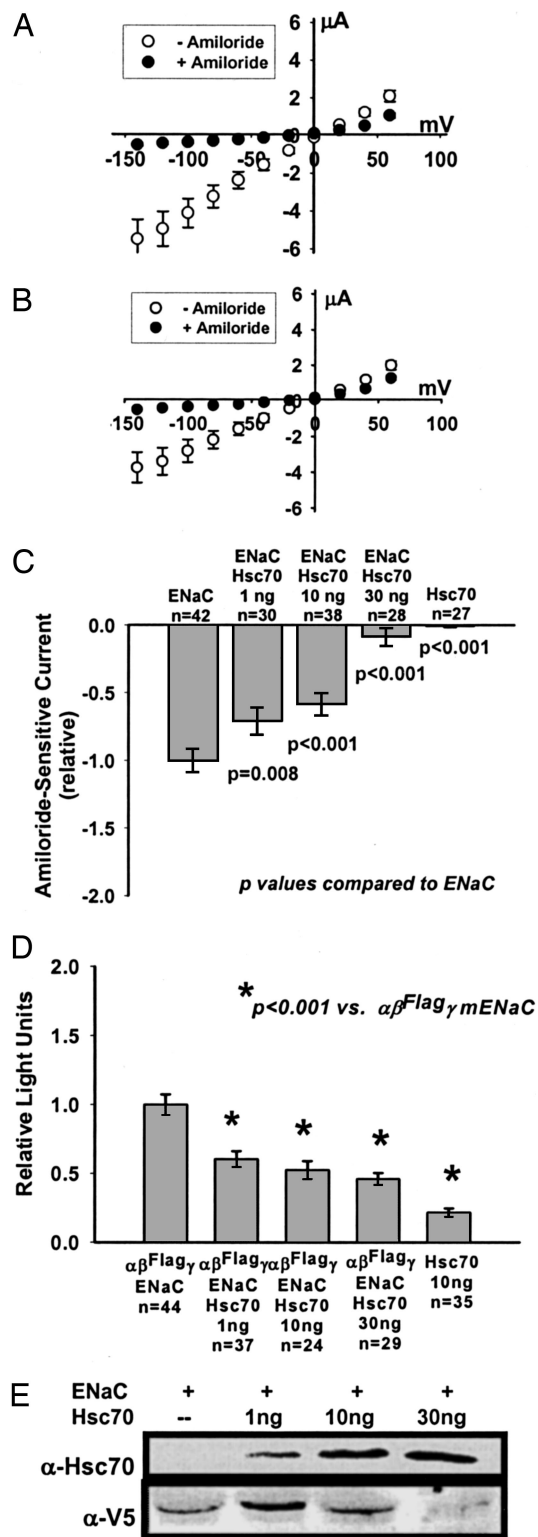


Fig. 1. Effect of Hsc70 on mENaC expression in *Xenopus oocytes*. *Xenopus laevis* oocytes were injected with cRNA for all three subunits of mENaC, α , β , and γ ($\alpha\beta\gamma$ -mENaC) (0.33 ng per subunit), either alone (A) or with 10 ng of cRNA for human Hsc70 (B). TEV was performed 24 h after injection as described in *Materials and Methods*. Shown are the I/V relationships (mean \pm SEM) for $n = 42$ (A) and $n = 38$ (B) oocytes before (open circles) and after (filled circles) the addition of 10 μ M amiloride. (C) Oocytes were injected with cRNA for $\alpha\beta\gamma$ -mENaC (0.33 ng per subunit), Hsc70 (10 or 30 ng), or coinjected with cRNA for $\alpha\beta\gamma$ -mENaC (0.33 ng per subunit) and the indicated amount of Hsc70 cRNA. TEV was performed 24 h after injection, and data are expressed as the relative

Results

Influence of Hsc70 on the Expression of Murine ENaC (mENaC) in *Xenopus Oocytes*. Fig. 1A shows the current/voltage (I/V) relationship by two-electrode voltage clamp (TEV) for oocytes 24 h after injection with mENaC (0.33 ng per subunit) in the absence and presence of 10 μ M amiloride. The linear I/V relationship is characteristic of mENaC expressed in oocytes. The reversal potential of ≈ 0 mV (adjusted for resting membrane potential) suggests that our oocytes are "sodium-loaded" and that sodium feedback inhibition of mENaC (19) is not confounding these observations.

Oocytes coinjected with $\alpha\beta\gamma$ -mENaC cRNA and Hsc70 cRNA (10 ng) also demonstrated a linear whole-oocyte I/V relationship that was inhibited by amiloride, but the slope of the I/V relationship was decreased (Fig. 1B). The reversal potential of this I/V relationship remained at ≈ 0 mV, suggesting that sodium loading was not altered by the presence of Hsc70. These data suggest that Hsc70 decreased the mENaC-mediated whole-oocyte conductance.

We next examined the concentration dependence of Hsc70 overexpression on mENaC functional expression (Fig. 1C). Given the linear I/V relationship, we recorded whole-oocyte amiloride-sensitive currents at -100 mV holding potential (corrected for resting membrane potential) for comparison. Coinjection of 1, 10, or 30 ng of human Hsc70 cRNA resulted in a concentration-dependent decrease in mENaC functional expression relative to injection of mENaC alone (Fig. 1C). Oocytes injected with Hsc70 cRNA alone (10 or 30 ng) did not demonstrate amiloride-sensitive currents.

Alterations in mENaC functional expression may reflect changes in the expression of mENaC at the cell surface (N) or in the mENaC open probability (P_o) or unitary conductance. To assess whether Hsc70 alters N , and therefore mENaC intracellular trafficking, we determined surface expression (Fig. 1D) by a direct antibody binding and chemiluminescence assay (20, 21), where the β -mENaC subunit contains a FLAG epitope in the extracellular loop (β^{FLAG}). Oocytes were injected with $\alpha\beta^{\text{FLAG}}\gamma$ -mENaC (0.33 ng per subunit) alone or coinjected with 1, 10, or 30 ng of human Hsc70 cRNA. Control oocytes were injected with 10 ng of Hsc70 cRNA alone and had a background, nonspecific signal of $\approx 15\%$ of oocytes injected with $\alpha\beta^{\text{FLAG}}\gamma$ -mENaC. Coinjection of with 1, 10, or 30 ng of Hsc70 cRNA resulted in a concentration-dependent decrease in $\alpha\beta^{\text{FLAG}}\gamma$ -mENaC surface expression compared with oocytes injected with $\alpha\beta^{\text{FLAG}}\gamma$ -mENaC alone. This Hsc70 dose-dependent inhibition of surface expression parallels the Hsc70 dose-dependent decrease in mENaC functional expression (Fig. 1C) and is consistent with Hsc70 decreasing mENaC functional expression in oocytes by causing a decrease in surface expression.

We prepared immunoblots to assess whole-oocyte expression of Hsc70 and mENaC (Fig. 1E) and used a β -mENaC fusion protein with a C-terminal V5 epitope (β -V5) to increase sensitivity (22). Oocytes were injected with cRNA for $\alpha\beta$ -V5 γ -mENaC (0.33 ng per subunit) alone or coinjected with increasing amounts of Hsc70

amiloride-sensitive current at -100 mV holding potential (mean \pm SEM), with P values determined by ANOVA as described in *Materials and Methods*. The ENaC and ENaC + 10 ng of Hsc70 data in C correspond to the I/V plots of A and B, respectively. (D) Expression of mENaC at the oocyte surface was assessed 24 h after injection with cRNAs for mENaC (0.33 ng per subunit) or Hsc70 (10 ng) alone or injection with mENaC and the indicated amount of Hsc70 cRNAs. In these experiments, the β -mENaC subunit contained an external FLAG epitope (β^{FLAG}). Relative surface expression is expressed as the mean \pm SEM, with P values determined by ANOVA. (E) Oocytes were injected with cRNA for $\alpha\beta\gamma$ -mENaC (0.33 ng per subunit) where the β subunit contained a C-terminal V5 epitope (β -V5) and the indicated amount of Hsc70 cRNA. Whole-oocyte lysates were prepared 24 h after injection, and expression of Hsc70 and β -V5 was assessed by immunoblot using specific antisera. These data are representative of three or four independent experiments.

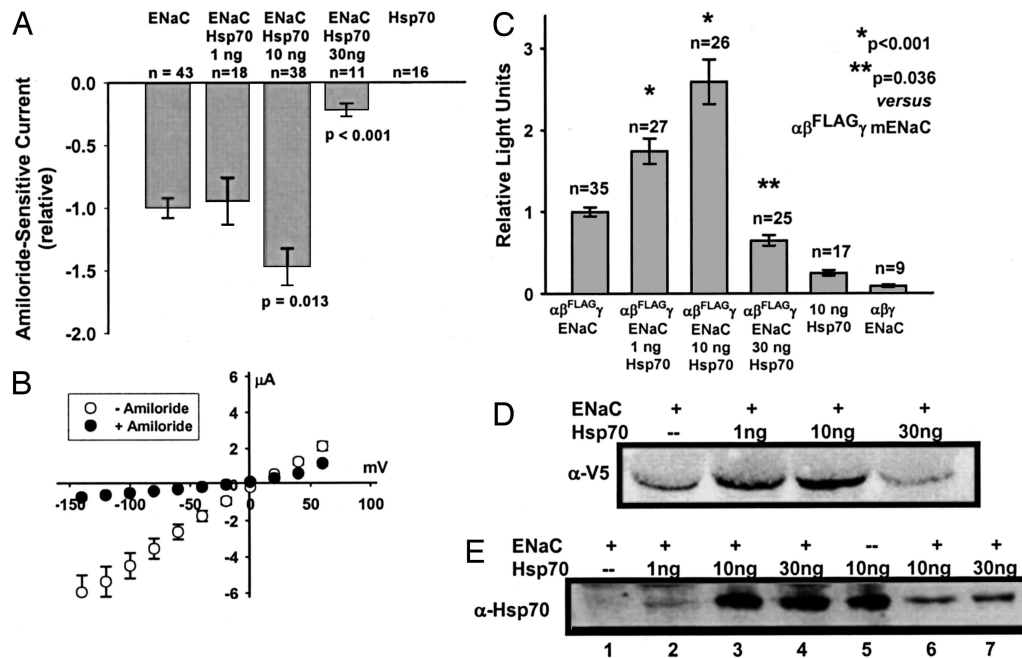


Fig. 2. Effect of Hsp70 on mENaC expression in oocytes. (A) Oocytes were injected with cRNA for $\alpha\beta\gamma$ -mENaC (0.33 ng per subunit) or Hsp70 (10 or 30 ng), or coinjected with cRNA for $\alpha\beta\gamma$ -mENaC (0.33 ng per subunit) and the indicated amount of Hsp70 cRNA. TEV was performed 24 h after injection, and data are expressed as the relative amiloride-sensitive current at -100 mV holding potential (mean \pm SEM), with P values determined by ANOVA. (B) I/V relationship (mean \pm SEM) for oocytes ($n = 38$) coinjected with cRNA for $\alpha\beta\gamma$ -mENaC (0.33 ng per subunit) and 10 ng of cRNA for human Hsp70. TEV was performed 24 h after injection before (open circles) and after (filled circles) the addition of $10 \mu\text{M}$ amiloride. These data correspond to those for ENaC/Hsp70 10 ng in A. (C) Expression of mENaC at the oocyte surface was assessed 24 h after injection with cRNAs for $\alpha\beta^{\text{FLAG}}\gamma$ -mENaC (0.33 ng per subunit) or Hsp70 (10 ng) alone, or injection with $\alpha\beta^{\text{FLAG}}\gamma$ -mENaC and the indicated amount of Hsp70 cRNAs. A separate group of control oocytes were injected with cRNA for WT $\alpha\beta\gamma$ -mENaC (0.33 ng per subunit, β -mENaC lacking the FLAG epitope). Relative surface expression is expressed as the mean \pm SEM, with P values determined by ANOVA. (D) Oocytes were injected with cRNA for $\alpha\beta$ -V5 γ -mENaC (0.33 ng per subunit) and the indicated amount of Hsp70 cRNA. Whole-oocyte lysates were prepared 24 h after injection, and expression of β -V5 was assessed by immunoblotting. (E) Oocytes were injected with cRNA for $\alpha\beta$ -V5 γ -mENaC (0.33 ng per subunit, except lane 5) and the indicated amount of Hsp70 cRNA. Whole-oocyte lysates were prepared 24 h after injection, and expression of Hsp70 was assessed by immunoblotting. Lanes 6 and 7 correspond to lanes 3 and 4 but were loaded with one-fourth the amount of sample. D and E are representative of three or four independent experiments.

cRNA. Coinjection of increasing amounts of Hsc70 cRNA resulted in a corresponding increase in Hsc70 protein expression in whole-oocyte lysates. Interestingly, overexpression of a low amount of Hsc70 (after injection of 1 ng of cRNA) increased the whole-oocyte content of β -V5-mENaC compared with oocytes injected with $\alpha\beta$ -V5 γ -mENaC-alone. Higher amounts of coinjected Hsc70 cRNA decreased the whole-oocyte content of β -V5-mENaC significantly compared with oocytes coinjected with 1 ng of Hsc70 cRNA.

Influence of Hsp70 on the Expression of mENaC in *Xenopus* Oocytes.

We performed similar experiments to assess the effect of Hsp70 on the expression of mENaC in oocytes (Fig. 2). $\alpha\beta\gamma$ -mENaC (0.33 ng per subunit) was injected alone or together with 1, 10, or 30 ng of human Hsp70 cRNA, and whole-cell amiloride-sensitive currents were determined by TEV relative to currents observed for oocytes injected with $\alpha\beta\gamma$ -mENaC alone. In contrast to our observations with Hsc70 in Fig. 1, coinjection of 10 ng of Hsp70 cRNA enhanced mENaC functional expression by $\approx 50\%$, whereas coinjection of 30 ng of Hsp70 cRNA had the opposite effect and inhibited mENaC functional expression significantly. Oocytes injected with Hsp70 cRNA alone (10 or 30 ng) did not demonstrate an amiloride-sensitive current. Coinjection of 10 ng of Hsp70 cRNA did not alter the mENaC-characteristic linear I/V relationship or the reversal potential of ≈ 0 mV of sodium-loaded, mENaC-expressing oocytes (Fig. 2B).

We next assessed the influence of Hsp70 on the surface expression of mENaC (Fig. 2C). Oocytes were injected with cRNA $\alpha\beta^{\text{FLAG}}\gamma$ -mENaC alone or coinjected with 1, 10, or 30 ng of Hsp70 cRNA. Coinjection of 1 or 10 ng of Hsp70 resulted in significantly

increased surface expression of $\alpha\beta^{\text{FLAG}}\gamma$ -mENaC compared with oocytes injected with $\alpha\beta^{\text{FLAG}}\gamma$ -mENaC alone. In contrast, coinjection of 30 ng of Hsp70 cRNA decreased $\alpha\beta^{\text{FLAG}}\gamma$ -mENaC surface expression significantly. Control oocytes injected with 10 ng of Hsp70 cRNA or with cRNAs encoding non-epitope-tagged (WT) $\alpha\beta\gamma$ -mENaC (0.33 ng per subunit) demonstrated nonspecific chemiluminescence of $\leq 15\%$ of oocytes injected with $\alpha\beta^{\text{FLAG}}\gamma$ -mENaC. These data suggest that changes in mENaC functional expression caused by overexpression of Hsp70 at least in part reflect changes in the trafficking of mENaC in oocytes.

We also assessed the whole-oocyte expression of β -V5-mENaC in oocytes injected with $\alpha\beta$ -V5 γ -mENaC either alone or with increasing amounts of Hsp70 cRNA (Fig. 2D). Coinjection of 1 or 10 ng of Hsp70 cRNA increased the whole-oocyte expression of β -V5-mENaC, whereas coinjection of 30 ng of Hsp70 decreased the whole-oocyte expression of β -V5-mENaC. These changes parallel those observed in the surface-expression assay (Fig. 2C). These data are also consistent with the TEV data in which 10 ng of Hsp70 cRNA enhanced and 30 ng of Hsp70 cRNA inhibited mENaC functional expression (Fig. 2A). Fig. 2E demonstrates that injection of increasing amounts of Hsp70 cRNA corresponds to increased Hsp70 protein expression in whole-oocyte lysates.

Antagonistic Effects of Hsc70 and Hsp70 on mENaC Functional Expression.

Our model predicts that Hsc70 and Hsp70 should antagonize each other directly with regard to mENaC trafficking and functional expression. To test this hypothesis, oocytes were injected with cRNA for $\alpha\beta\gamma$ -mENaC alone (0.33 ng per subunit); coinjected with $\alpha\beta\gamma$ -mENaC and an "optimal profunctional expression amount" of Hsp70 cRNA (i.e., 10 ng); or coinjected with $\alpha\beta\gamma$ -mENaC, Hsp70,

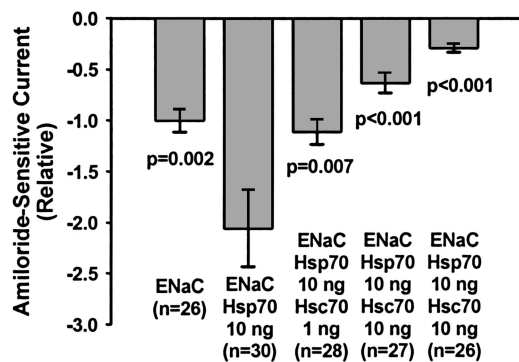


Fig. 3. Antagonistic effects of Hsc70 and Hsp70 on mENaC functional expression. Oocytes were injected with cRNAs encoding $\alpha\beta\gamma$ -mENaC alone (0.33 ng per subunit), coinjected with $\alpha\beta\gamma$ -mENaC (0.33 ng per subunit) and Hsp70 (10 ng) cRNAs, or coinjected with cRNAs for $\alpha\beta\gamma$ -mENaC (0.33 ng per subunit) and Hsp70 (10 ng) and the indicated amount of Hsc70 cRNA. TEV was performed 24 h after injection, and relative amiloride-sensitive currents at -100 mV were determined. *P* values were determined by ANOVA compared with oocytes coinjected with $\alpha\beta\gamma$ -mENaC and Hsp70 cRNAs.

and 1, 10, or 30 ng of Hsc70 cRNA, and mENaC functional expression was then assessed by TEV (Fig. 3). Here, coinjection of 10 ng of Hsp70 cRNA with $\alpha\beta\gamma$ -mENaC yielded a 2-fold increase in relative amiloride-sensitive currents compared with oocytes injected with mENaC alone. Oocytes coinjected with $\alpha\beta\gamma$ -mENaC and Hsp70 and increasing concentrations of Hsc70 (1, 10, and 30 ng) demonstrated a dose-dependent decrease in relative amiloride-sensitive currents compared with $\alpha\beta\gamma$ -mENaC/Hsp70-coinjected oocytes. These data suggest that Hsc70 overexpression can directly antagonize the effect of Hsp70 to increase mENaC functional expression in oocytes and are consistent with the hypothesis that Hsc70 and Hsp70 have differential, nonequivalent, and antagonistic functions with regard to the intracellular trafficking of ion channels such as mENaC.

Influence of Hsc70 and Hsp70 on mENaC Exocytosis and Endocytosis.

To attempt to delineate the mechanism by which Hsc70 and Hsp70 alter the trafficking of mENaC in oocytes, we sought to determine the influence of these chaperones on the rate of delivery of mENaC to the membrane and on its removal from the membrane. To assess the exocytic rate of delivery, we used a mutant of the amiloride binding site in the γ -mENaC subunit (G542C); such mENaC mutants are blocked irreversibly by treatment with [2-(trimethylammonium)ethyl] methanethiosulfonate bromide (MTSET) (23). The initial rate of recovery of the mENaC-mediated current after MTSET blockade is a direct measure of the rate of mENaC exocytosis. In these experiments, the rate of mENaC exocytosis, as determined by the rate of increase of the inward Na^+ current, was -41 ± 6 nA/min (at -100 mV holding potential, $n = 17$). This rate was not significantly altered by the coinjection of 10 ng of cRNA for either Hsc70 [-54 ± 11 nA/min, $n = 18$, $P = \text{not significant (NS)}$] or Hsp70 (-45 ± 8 nA/min, $n = 22$, $P = \text{NS}$).

We also determined the half-life of mENaC at the oocyte membrane in the presence of brefeldin A to block delivery of new channels to the membrane. mENaC was removed from the oocyte membrane with a half-time of 2.6 ± 0.3 h ($n = 7$). This half-time was again not significantly altered by coinjection of 10 ng of cRNA for either Hsc70 ($t_{1/2} = 2.3 \pm 0.2$ h, $n = 7$, $P = \text{NS}$) or Hsp70 ($t_{1/2} = 1.7 \pm 0.3$ h, $n = 7$, $P = \text{NS}$).

Hdj-2 Overexpression Does Not Alter mENaC Functional Expression.

To ensure that the decreased mENaC functional, surface, and whole-oocyte expression when mENaC was coinjected with 30 ng of cDNA for either Hsc70 or Hsp70 was not a nonspecific effect of

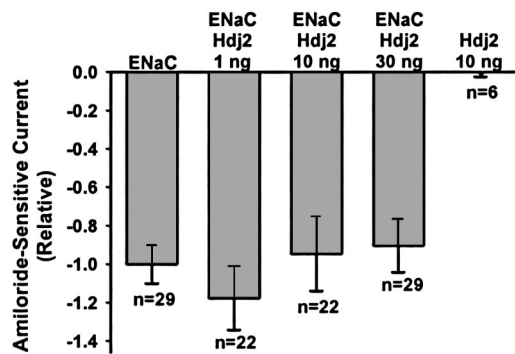


Fig. 4. Hdj-2 overexpression does not alter mENaC functional expression. Oocytes were injected with cRNAs encoding $\alpha\beta\gamma$ -mENaC alone (0.33 ng per subunit) or Hdj2 alone (10 ng), or coinjected with $\alpha\beta\gamma$ -mENaC (0.33 ng per subunit) and the indicated amount of Hdj2 cRNA. TEV was performed 24 h after injection, and relative amiloride-sensitive currents at -100 mV were determined. Statistical analysis was performed by ANOVA compared with oocytes injected with $\alpha\beta\gamma$ -mENaC cRNA alone. There were no statistically significant differences in amiloride-sensitive currents between oocytes coinjected with mENaC and 1, 10, or 30 ng of Hdj2 compared with oocytes injected with mENaC alone.

competition for oocyte translational machinery, we performed a control experiment with coinjection of Hdj2 cRNA. Hdj2 is a cytosolic 40-kDa heat shock protein that regulates the ATPase activity of Hsc70 and may facilitate CFTR trafficking (8). Oocytes were injected with $\alpha\beta\gamma$ -mENaC (0.33 ng per subunit) alone or together with 1, 10, or 30 ng of human Hdj2 cRNA, and whole-cell amiloride-sensitive currents were determined 24 h after injection. Functional expression of mENaC was not altered by coinjection of any amount of Hdj2 cRNA (Fig. 4). These data suggest that the effects on mENaC expression of coinjection of 30 ng of Hsc70 (Fig. 1) or Hsp70 (Fig. 2) cRNA or coinjection of both Hsc70 and Hsp70 cRNA (Fig. 3) were specific and not confounded by competition for translational machinery.

Discussion

With increased recognition of the potential role of ENaC in the pathophysiology of CF airway disease, strategies to bring about repair of mutant CFTRs will require attention to the effect of these interventions on the functional expression of ENaC. Based on our observations that sodium 4-phenylbutyrate improves ΔF508 intracellular trafficking (12) and decreases the expression of Hsc70 (9), which is a necessary cofactor for ubiquitination and degradation of a number of cellular proteins (10), and the suggestion of others that overexpression of Hsp70 may promote repair of ΔF508 trafficking (18), we hypothesized that Hsc70 and Hsp70 may have differential effects on the intracellular trafficking of ion channels, such as CFTR and ENaC. Here, we tested this hypothesis, and we observed that, in fact, Hsc70 and Hsp70 can have the opposite effect on mENaC functional and surface expression in oocytes. These data argue against the presumption that Hsc70 and Hsp70 are functionally interchangeable within the cell.

Interestingly, overexpression of large amounts of either Hsc70 or Hsp70 decreased mENaC functional, surface, and whole-oocyte expression markedly. We were able to discern the differential effects only because we examined multiple amounts of injected chaperone cRNA. The decreases in mENaC functional, surface, and whole-oocyte expression at high amounts of injected chaperone cRNA could be a result of the excess of chaperone cRNA competing for translation machinery and precluding the cell from translating the mENaC cRNA. Our data demonstrating a lack of effect of overexpression of Hdj2 on mENaC functional expression (Fig. 4) argue against such an explanation. Furthermore, competition for translational machinery cannot explain that the overex-

where the β subunit contained a FLAG epitope in the extracellular loop (β^{FLAG}) either alone or coinjected with 1, 10, or 30 ng of either human Hsc70 or human Hsp70 cRNA. For control assays, oocytes were injected with either 10 ng of Hsc70 or Hsp70 cRNA alone or with cRNA encoding non-epitope-tagged WT $\alpha\beta\gamma$ -mENaC (0.33 ng per subunit); 18–24 h after injection, the oocytes were fixed in 4% formaldehyde in MBS for 20 min at 4°C. Nonspecific binding was blocked with MBS containing 10 mg/ml BSA (MBS-BSA) at 4°C overnight. The primary antibody was anti-FLAG M2 mouse monoclonal (0.8 $\mu\text{g}/\text{ml}$, Sigma), and the secondary antibody was peroxidase-conjugated AffiniPure F(ab')₂ fragment goat anti-mouse IgG (0.9 $\mu\text{g}/\text{ml}$, Jackson ImmunoResearch). Both were applied in MBS-BSA for 1–2 h at 4°C. Oocytes were washed extensively (12 times over 2 h) at 4°C and transferred to MBS without BSA. Single-oocyte chemiluminescence was revealed with 100 μl of SuperSignal ELISA Femto (Pierce) and quantified for 1 min in a TD-20/20 luminometer (Turner, Torrance, CA).

Immunoblotting. Oocytes were injected with $\alpha\beta\gamma$ -mENaC (0.33 ng per subunit) and increasing amounts (1, 10, and 30 ng) of cRNA for Hsc70 or Hsp70; in these experiments, the β -mENaC subunit had a V5 epitope at its C terminus (43). Twenty-four hours after injection, oocytes (10 per group) were lysed in equal volumes of 0.15 M NaCl/0.01 M Tris-Cl, pH 8.0/0.01 M EDTA/1.0% Nonidet P-40/0.5% sodium deoxycholate/1.0 mM phenylmethanesulfonyl fluoride/0.1 mM N $^{\alpha}$ -(*p*-tosyl)lysine chloromethyl ketone/0.1 mM L-1-tosylamido-2-phenylethyl chloromethyl ketone/2 $\mu\text{g}/\text{ml}$ aprotinin for 1 h at 4°C and centrifuged at 13,000 $\times g$ for 15 min at 4°C. Equal volumes of protein lysates were resolved on SDS/8% polyacrylamide gels and transferred to nitrocellulose, and nonspecific binding was blocked by overnight incubation with PBS and 5% nonfat dry milk at 4°C. Immunodetections were performed by using specific antisera for the V5 epitope (Invitrogen), Hsp70, or Hsc70 (Stressgen). Immunoreactivity was revealed with horseradish peroxidase-conjugated secondary antibodies and the ECL reagent (Amersham Pharmacia).

Rates of mENaC Exocytosis and Endocytosis. We assessed the rate of mENaC exocytosis as described previously (23, 44). Oocytes were

injected with $\alpha\beta\gamma$ G542C-mENaC either alone or with 10 ng of cRNA for either Hsc70 or Hsp70; 24–36 h after injection, whole-oocyte 100 μM benzamil-sensitive currents were determined by TEV. Whole-oocyte currents were then monitored after a 5-min perfusion of 1 mM MTSET (Toronto Research Chemicals, Downsview, ON, Canada) every minute for 10 min after removal of MTSET from the perfusion buffer and after reapplication of benzamil. The initial rates of reappearance of benzamil-sensitive currents were determined from the linear portion of the current recovery curve (0–2 min).

To assess the rate of mENaC endocytosis, oocytes were injected with cRNAs for $\alpha\beta\gamma$ -mENaC alone or together with 10 ng of cRNA for either Hsc70 or Hsp70. Then 24–36 h after injection, the amiloride-sensitive current was determined by TEV before and after 2, 4, and 6 h of incubation with 5 μM brefeldin A. Amiloride-sensitive currents were expressed relative to the initial amiloride-sensitive current, and half-lives for the decline of amiloride-sensitive current were determined.

Statistical Analyses. Whole-cell amiloride-sensitive current data are expressed relative to those of oocytes injected with mENaC alone. To decrease the influence of batch-to-batch variability in ENaC expression, data were normalized by the mean amiloride-sensitive current within a batch of oocytes before combining data of multiple independent batches for statistical analysis. Cell surface expression data were similarly normalized to the mean $\alpha\beta^{\text{FLAG}}\gamma$ -mENaC luminescence within a batch before combining data of multiple independent batches for statistical analysis. All data are presented as the mean \pm SEM, and *P* values were determined by a one-way ANOVA. A *P* value of ≤ 0.05 was considered significant. All statistical analyses were performed with SigmaStat version 2.03.

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