Hsp 70/Hsp 90 organizing protein as a nitrosylation target in cystic fibrosis therapy

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The endogenous signaling molecule S-nitrosoglutathione (GSNO) and other S-nitrosylating agents can cause full maturation of the abnormal gene product ΔF508 cystic fibrosis (CF) transmembrane conductance regulator (CFTR). However, the molecular mechanism of action is not known. Here we show that Hsp70/Hsp90 organizing protein (Hop) is a critical target of GSNO, and its S-nitrosylation results in ΔF508 CFTR maturation and cell surface expression. S-nitrosylation by GSNO inhibited the association of Hop with CFTR in the endoplasmic reticulum. This effect was necessary and sufficient to mediate GSNO-induced cell-surface expression of ΔF508 CFTR. Hop knockdown using siRNA recapitulated the effect of GSNO on ΔF508 CFTR maturation and expression. Moreover, GSNO acted additively with decreased temperature, which promoted mutant CFTR maturation through a Hop-independent mechanism. We conclude that GSNO corrects ΔF508 CFTR trafficking by inhibiting Hop expression, and that combination therapies—using differing mechanisms of action—may have additive benefits in treating CF.

cystic fibrosis transmembrane conductance regulator | S-nitrosoglutathione corrector | treatment

The most common mutation associated with cystic fibrosis (CF) results in loss of a single phenylalanine (ΔF508) in the CF transmembrane conductance regulator (CFTR) apical membrane chloride channel (1, 2). Posttranslational processing of CFTR requires interactions with chaperones and cochaperones that fold, glycosylate, and screen the protein for defects (1–3). ΔF508 results in a misfolding of this CFTR protein, leading to its degradation. Most wild-type (WT) CFTR—and virtually all ΔF508 CFTR—is degraded before reaching the cell surface; absence of cell-surface ΔF508 CFTR after degradation results in abnormal Cl^- conductance in the lung and other organs. Although there is species variation (1–4), CFTR deficiency-associated lung disease is commonly fatal in humans (2). However, if ΔF508 CFTR can be expressed on the cell surface, it has the potential to be functional (1–3, 5, 6). Therefore, there is an interest in developing compounds that correct ΔF508 CFTR to the cell surface as CF treatments (1, 2, 5–7).

S-nitrosoglutathione (GSNO) is an endogenous signaling molecule that relaxes airway smooth muscle (8–10), enhances ventilation-perfusion matching (9, 10), increases ciliary beat frequency (11), has antimicrobial effects (12, 13), and is one of a class of S-nitrosylating agents that increase expression, maturation, and function of wild-type (WT) and ΔF508 CFTR in primary nasal and monolayer cultures of epithelial cells (7, 14–19). The GSNO effect on CFTR is partly transcriptional (16), but is primarily posttranscriptional (17, 18). Concentration is critical: levels 10- to 100-fold higher than those normally present in airway lining fluid can (*i*) inhibit CFTR transcription (16); (*ii*) cause CFTR degradation through tyrosine nitration (20); and (*iii*) inhibit WT CFTR function through oxidation (21). However, lower concentrations of GSNO and other S-nitrosylating agents augment ΔF508 CFTR expression and maturation. This effect is independent of NO radical or glutathione generation, suggesting that the mechanism of action involves transnitrosation chemistry (10, 18).

We have studied potential transnitrosation targets in the CFTR interactome (3, 22) of relevance to CFTR trafficking. Here, we report that GSNO S-nitrosylates heat shock protein (Hsp)70/Hsp90 organizing protein (Hop)—also known as stress-induced phosphoprotein 1 (Stip-1) (23)—decreasing Hop expression and decreasing the association between Hop and maturing CFTR. This is essential for the effect of GSNO on ΔF508 CFTR correction, but it is independent of—and additive with—the effect of decreased temperature to correct CFTR. Strikingly, Hop siRNA recapitulates the effect of GSNO to increase ΔF508 CFTR expression on the cell surface, representing a unique CF corrector.

Results

GSNO's Effect on ΔF508 CFTR. We first studied the relative ability of low temperature (27 °C) (5, 22) and GSNO to up-regulate ΔF508 CFTR expression in CFBE41o$^-$ cells using immunoblot (IB) analysis. ΔF508 CFTR maturation was enhanced in rank order: 27 °C < GSNO at 37 °C < GSNO at 27 °C (Fig. 1). We also confirmed the dose-dependent effect of GSNO to lead to ΔF508 CFTR cell-surface expression (Fig. 2 *A* and *B*). Additionally, we showed that fully mature ΔF508 CFTR plasma membrane expression increased in a dose-dependent fashion after GSNO treatment (Fig. 2 *C* and *D*). We confirmed the identity of the core glycosylated (Figs. 1*A* and 2*E*, band B) and fully glycosylated mature (Figs. 1*A* and 2*A* and *E*, band C) forms of ΔF508 CFTR following 4 h of 10 μM GSNO (Fig. 2 *A*, *C*, and *E*). Though core glycosylated CFTR was sensitive to endoglycosidase H (Endo H) digestion, the mature, fully glycosylated CFTR (band C) was Endo H resistant, but sensitive to peptide N-glycosidase F (PNGase F; Fig. 2*E*). We also investigated the effects of GSNO on primary human pseudostratified columnar epithelial cell (PCEC) cultures. Although GSNO (100 μM, every 6 h) failed to enhance forskolin-dependent Cl^- transport relative to vehicle control in Ussing chambers, a cell-permeable analog of

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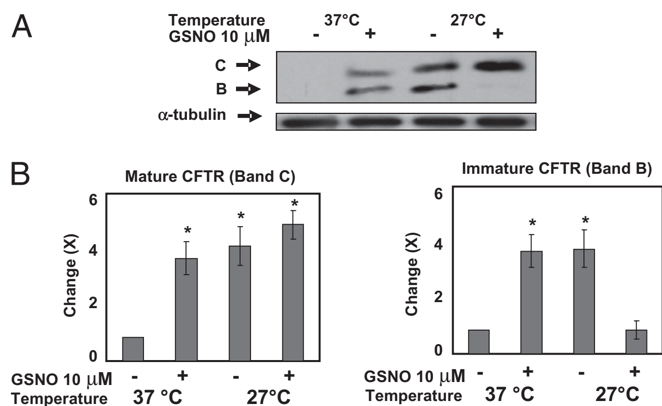

Fig. 1. Combination treatment (low temperature and GSNO) synergistically up-regulates ΔF508 CFTR expression. CFBE41o⁻ cells expressing ΔF508 CFTR were grown at 37 °C to 70% confluence, then incubated for an additional 48 h at 37 °C or 27 °C with GSNO (10 μM) present or absent (4 h). (*A*) Expression of the core glycosylated (band B) and mature, fully glycosylated (band C) forms of ΔF508 CFTR was detected by immunoblotting using mouse monoclonal anti-CFTR antibody (Chemicon). (*B*) Densitometric analysis revealed that the increases in the B and C bands were significant for both GSNO and 27 °C. Of note, combined treatment led to further full maturation of band B. [*$P < 0.001$ by two-way ANOVA for each experiment compared with no treatment/37 °C (C band) and both no treatment/GSNO and GSNO/27 °C (B band)]. Data represent the average fold change for three independent experiments.

GSNO, GSNO diethyl ester (GNODE, 60 μM) (18), increased CFTR current ($P = 0.02$; Fig. 2*F*).

GSNO Decreases Hop Expression in Different Cell Lines. A CFTR cochaperone, Hop, has a reduced cysteine in a potential S-nitrosylation motif (24–26). We found that Hop is expressed in CFBE41o⁻ cells, A549 cells, and CFPAC-1 cells (Fig. 3*A*); 10 μM GSNO (4 h) decreased Hop expression in all cells after 4 h (Fig. 3*A*); and GSNO decreased Hop expression in the cytosol in a dose-dependent fashion (Fig. 3*B*). Exposure at 27 °C did not recapitulate the effect of GSNO to decrease Hop expression (Fig. 3*C*). The GSNO-induced decrease in Hop expression was augmented by cycloheximide (50 μg/mL 15 min before GSNO), but not by 20 μg/mL actinomycin D (2 h before GSNO), suggesting that (*i*) Hop protein is stable; (*ii*) the effect of GSNO is post-transcriptional; and (*iii*) GSNO may lead to augmented Hop degradation (Fig. 3*D*). When CFBE41o⁻ cells were transfected with 50 nM siRNA duplexes specific for Hop (48 h), Hop expression—but not that of Hsp70 or Hsp90—was inhibited (Fig. 3*E*). Further, GSNO at 10 μM for 4 h augmented the inhibition of Hop expression by Hop siRNA (Fig. 3*E*). Taken together, these data suggest that (*i*) Hop protein is normally stable in these cells and (*ii*) GSNO accelerates Hop protein degradation.

Effect of GSNO on Regulation of CFTR Maturation by Hop. Knockdown of endogenous Hop by siRNA in the absence of GSNO increased levels of immature and mature forms of ΔF508 CFTR (Fig. 3*F*). In the presence of GSNO (10 μM, 4 h), expression of immature and mature forms of CFTR increased, but GSNO did not augment the effect of Hop siRNA on ΔF508 CFTR maturation (Fig. 3*F*), suggesting that the effect of GSNO to inhibit Hop expression is important to the maturation effect of GSNO on CFTR.

GSNO Alters the Association of Hop with CFTR in the Cytosol and ER of CFBE41o⁻ Cells. We hypothesized that the effect of GSNO to decrease Hop expression would decrease Hop-CFTR association during CFTR maturation. Cytosol, ER, and Golgi fractions were isolated from CFBE41o⁻ cells (27) with or without GSNO (10 μM, 6 h). We confirmed the ER fraction contained calnexin but not syntaxin 5, and the Golgi fraction contained syntaxin 5 but not calnexin (Fig. 4*A*). From these fractions, we immunoprecipitated (IP'd) CFTR and IB'd for Hop after a pulse of GSNO. Surprisingly, GSNO initially increased the association between CFTR and Hop in the ER (at 2 h; Fig. 4*B*) but then, consistent with inhibition of Hop expression (Figs. 3*A* and 4*C*), GSNO

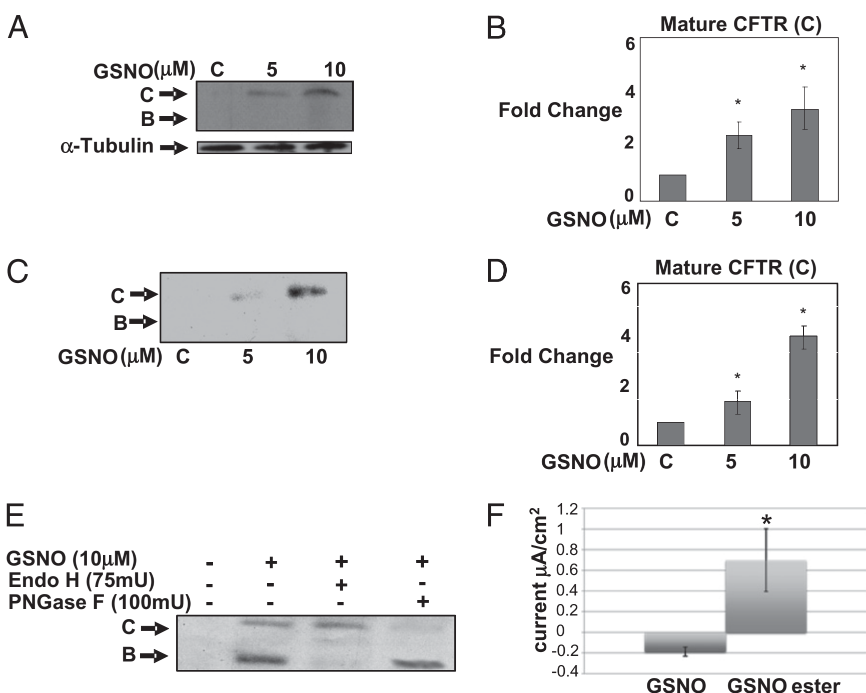

Fig. 2. GSNO cell-surface CFTR expression. (*A* and *B*) Cell-surface expression. CFBE41o⁻ cells were treated with 5 or 10 μM GSNO (4 h). The glycosidic moieties of cell-surface membrane proteins were derivatized by exposing cells to sodium periodate and biotinylation using biotin-LC hydrazide (30 min at 20 °C). After IP and SDS/PAGE, biotinylated CFTR (cell surface C band only) was detected with streptavidin-conjugated HRP. The membrane was reprobed with anti-α-tubulin. (ANOVA: *$P < 0.001$; 5 and 10 μM compared with control). Data represent the average fold change for three experiments. (*C* and *D*) CFBE41o⁻ cells were incubated with or without 5 or 10 μM GSNO (4 h), and cell membranes were isolated on a sucrose gradient before Western blotting for cell-surface CFTR (C band only; ANOVA: *$P < 0.001$, 5 and 10 μM compared with control). Data represent the average of three experiments. (*E*) Whole-cell extracts from CFBE41o⁻ cells incubated with or without 10 μM GSNO (4 h) were Western blotted with anti-CFTR antibody A596 (50 μg protein/lane). Modification of core glycosylated (band B) and fully glycosylated mature (band C) forms of CFTR was confirmed by preincubation of cell lysates with Endo H or PNGase F respectively (2 h, 37 °C). (*F*) Human CF primary pseudostratified columnar epithelia at air-fluid interface were exposed to GSNO (100 μM every 6 h for 72 h to facilitate full-thickness penetration), membrane-permeable GSNO diethyl ester (60 μM every 12 h for 48 h), or vehicle alone (for each) before analysis in Using chambers (42). Relative to vehicle, forskolin-stimulated Cl⁻ current was enhanced by GSNO diethyl ester ($n = 4$ donors, 2–3 wells each) but not by GSNO ($n = 6$ donors, three wells each). *$P = 0.02$.

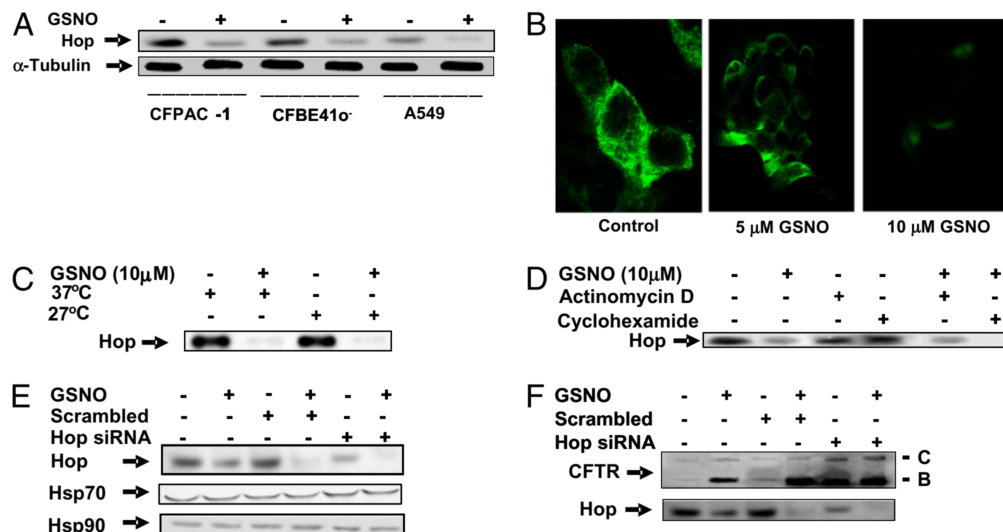

Fig. 3. GSNO, but not decreased temperature, decreases Hop expression to increase CFTR maturation. (*A*) CFPAC-1, CFBE41o$^-$, and A549 cells incubated with or without 10 μM GSNO (4 h) were Western blotted using monoclonal anti-Hop antibody (50 μg of protein per lane). The blot was reprobed with anti-α-tubulin. (*B*) GSNO (4 h) decreased cytosolic Hop in CFBE41o$^-$ cells, as visualized by immunofluorescence. (*C*) Western blot for cells were incubated for 48 h at 37 °C or 27 °C before exposure to GSNO; GSNO, but not 27 °C, decreased Hop expression. (*D*) IB of Hop from CFBE41o$^-$ cells treated for 4 h with GSNO with or without pretreatment with actinomycin D or cycloheximide. (*E*) Hop Western blot from CFBE41o$^-$ cells treated with GSNO (10 μM, 4 h) after transfection with Hop siRNA or scrambled sequence DNA; Hsp 70 and Hsp 90 were Western blotted as controls. (*F*) Western blot of Hop and CFTR from CFBE41o$^-$ cells transfected with 50 nM of Hop siRNA (or control RNA) and analyzed after 48 h (50 μg protein per lane).

decreased CFTR-associated Hop in all fractions (Fig. 4*B*). Although a GSNO pulse increases S-nitrosylation of proteins in the ER, Golgi, and cytosol (18), CFTR-associated Hop S-nitrosylation was only observed on the cytosol and ER at baseline (Fig. 4*C*), and no S-nitrosylated CFTR-associated Hop at all was observed after a pulse of GSNO. Thus, S-nitrosylation of Hop may prevent Hop association with CFTR, likely by augmenting Hop degradation (see following). Figure 4*D* shows time-dependent loss of CFTR-associated Hop (IP followed by IB) following treatment with 10 μM GSNO in whole-cell extract. Additionally, we used more sensitive LC-MS techniques to show that cytosolic Hop was S-nitrosylated by 10 μM GSNO (2 h) in CFBE41o$^-$ cells in which biotin-substituted proteins were isolated by streptavidin. Figure 4*E* shows the relative intensity of one of the tryptic peptides that was sequenced using tandem MS from the treated and untreated extracts. Table S1 lists the tryptic peptides from Hop and their MS data. These findings confirm that Hop is S-nitrosylated; however, S-nitrosylation appears to target Hop for degradation.

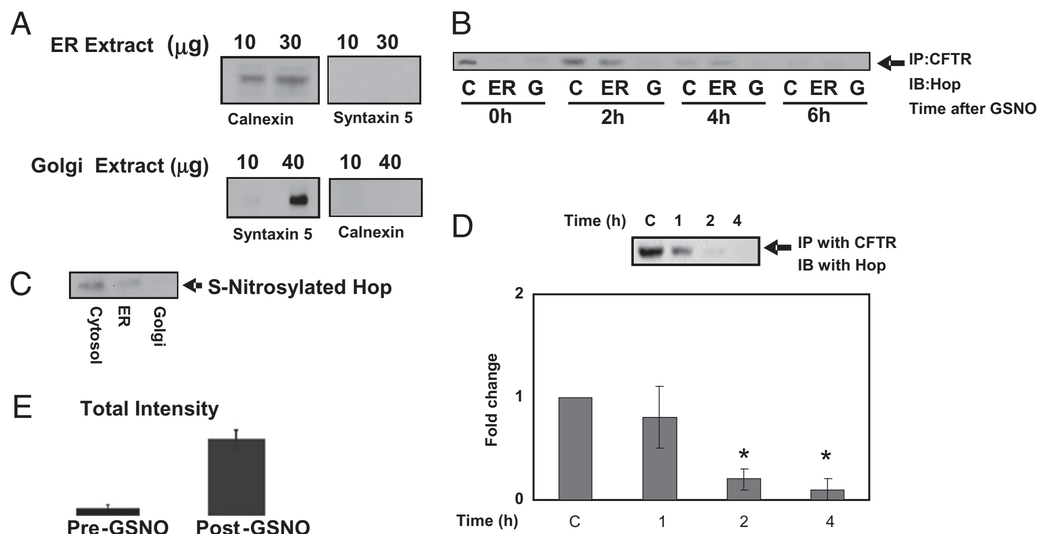

Fig. 4. Hop S-nitrosylation decreases Hop expression and Hop-CFTR association. (*A*) Western blotting for calnexin and syntaxin 5 in the ER and Golgi fractions of CFBE41o$^-$ cells. (*B*) IP with anti-CFTR followed by Western blotting with anti-Hop revealed CFTR-associated Hop exclusively in the cytosol at baseline. Exposure to GSNO (10 μM) resulted in a translocation of CFTR-associated Hop to the ER at 2 h, with a subsequent loss of CFTR-associated Hop from all fractions thereafter. (*C*) At baseline, some Hop associated with the CFTR was S-nitrosylated (IP for CFTR followed by biotin switch, streptavidin isolation, and Western blot for Hop on each fraction). After 2 h GSNO treatment (10 μM), no CFTR-associated S-nitrosylated Hop was present. (*D*) In a time-course analysis, GSNO (10 μM) decreased CFTR-associated Hop (*$P < 0.001$ relative to baseline at 2 and 4 h, ANOVA). (*E*) Extracts from CFBE41o$^-$ cells treated with GSNO (10 μM, 2 h) underwent biotin switch followed by avidin affinity purification, proteolysis, and LC-MS. The relative intensity of a representative peptide from S-nitrosylated Hop (LAYINPDLALEEK) from treated and untreated cells is shown.

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Role of Hop Cysteine 403 in Hop Degradation. Hop has a cysteine (C403) in a motif that can be associated with S-nitrosylation, particularly under conditions of inflammation (26, 28). Overexpression of C→S mutation of Hop Cys-403 resulted in (*i*) protection from GSNO-induced Hop degradation and (*ii*) decreased Hop S-nitrosylation C403S relative to expression by GSNO (Fig. 5*A*).

Hop S-Nitrosylation Promotes Its Proteasomal Degradation. The proteasome inhibitor MG132 prevented depletion of total cellular Hop after GSNO treatment (Fig. 5*B*). Further, S-nitrosylation (Fig. 5*C*) and ubiquitination (Fig. 5*D*) of Hop increased after GSNO; these effects were increased by MG132.

Discussion

CF is caused by defective function of the *cftr* gene product. The most common mutation, ΔF508 CFTR, results in the production of an immature protein that is degraded, preventing correct localization to the plasma membrane (1, 2). However, if ΔF508 CFTR reaches the plasma membrane, it can function as a cAMP-activated chloride channel (4, 7). Thus, there is interest in finding corrector therapies to direct ΔF508 CFTR protein to the plasma membrane. In vitro strategies have included low temperature and chemical agents such as glycerol and 4-phenylbutyrate (22), as well as GSNO and other S-nitrosylating agents (18). Strikingly, previous studies (7, 14–19) and the current work suggest that low micromolar GSNO concentrations increase maturation and cell-surface expression of ΔF508 CFTR. Here, we show that GSNO appears to act through a mechanism independent of—and additive with—low temperature to increase ΔF508 CFTR maturation and cell-surface expression. Inhibition of Hop expression (and therefore of Hop association with CFTR) appears to be required. Indeed, we report that Hop siRNA increases the expression and maturation of ΔF508 CFTR in human airway epithelial cells.

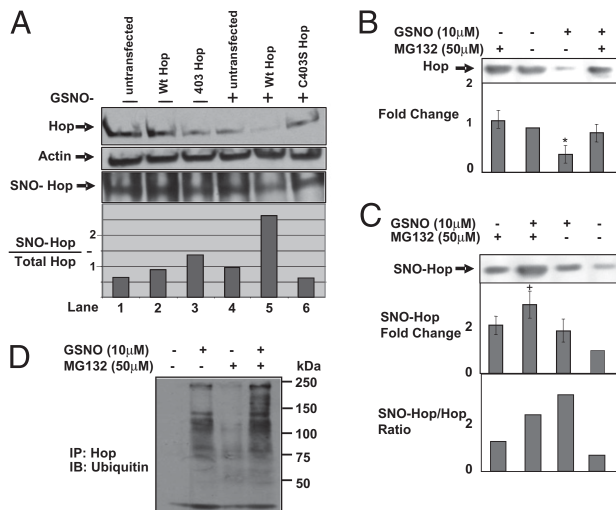

Fig. 5. Hop degradation is prevented by C403S mutation and proteasomal inhibition. (*A*) CFBE41o⁻ cells untransfected or overexpressing either WT Hop or C403S mutant Hop were treated for 2 h with 10 μM GSNO and Western blotted for Hop (*Upper*) and actin (representative of two experiments). Additionally, S-nitrosylated proteins from these samples underwent biotin substitution, followed by streptavidin isolation and Western blotting for Hop. C403S mutation inhibited Hop S-nitrosylation in Hop-overexpressing cells. (*B*) The proteasome inhibitor MG132 completely inhibited the ability of GSNO (10 μM, 2 h) to decrease Hop expression. (*C*) MG132 also preserved S-nitrosylated Hop (*C*) and Hop ubiquitination (*D*) in CFBE41o⁻ cells treated with MG132 and/or GSNO. **P* < 0.002; ⁺*P* < 0.05.

GSNO itself has several features that could make it appealing as a potential therapy for CF. (*i*) It is an endogenous compound in the airways (8). (*ii*) Airway levels are low both in CF and in asthma (29, 30). (*iii*) It has been used safely in human trials (9, 31). (*iv*) It has CFTR-independent beneficial effects that include relaxing airways smooth muscle, augmenting ventilation/perfusion matching, increasing ciliary beat frequency, inhibiting amiloride-sensitive Na^+ transport, promoting inflammatory cell apoptosis, and antimicrobial effects (8–13, 32). (*v*) It can increase wild-type CFTR function (17). (*vi*) Last, it can increase ΔF508 expression and maturation (7, 14–19), and the corrected protein is functional in monolayer and primary nasal cells (4, 7). Dose–response experiments in monolayer cell cultures suggest that concentrations of 5–10 μM may be optimal (15, 16, 18) (Fig. 2). These levels of GSNO appear to affect CFTR primarily through posttranscriptional mechanisms (16). In the current study, GSNO was ineffective in human airway pseudostratified columnar epithelium. The culture system may not allow for S-nitrosothiol uptake in all cell layers, preventing full thickness effect. GSNO tissue effects require peptidases—such as γ glutamyl transpeptidase—and active transport (10). These systems may be variably expressed in cells at different depths of pseudostratified epithelium, preventing a full-thickness effect on overall Cl^- transport. In support of this notion, a cell-permeable GSNO analog, used at lower concentrations, modestly increased functional ΔF508 CFTR expression in full-thickness cultures. Thus, the effect of S-nitrosylating agents to increase ΔF508 CFTR maturation can be achieved in complex epithelial systems if a cell membrane permeable ester is used.

Note that GNODE and GSNO release NO radical at roughly the same rate (18), suggesting that the effect of GNODE on ΔF508 CFTR function is independent of the conventional biology of NO radical. Indeed, biochemical data to date suggest that NO^+ transfer (transnitrosation) increases CFTR maturation. (*i*) Several different S-nitrosylating agents have this effect, and the activity of these agents is not related to either the rate at which they form NO or their ability to serve as thiol donors (18) (Fig. 2). (*ii*) The only common feature of all of these agents is that they each can cause cysteine S-nitrosylation (18). (*iii*) The effect is cGMP independent, inhibited by acivicin, and reversed by DTT (15, 16, 18). Transnitrosation is important in several signaling pathways relevant to health and disease in a variety of cell and organ systems (10, 33).

Several proteins in the complex CFTR interactome (3) contain reduced thiols that may be targets for transnitrosation (33). Previously, we found that GSNO increases cysteine string protein (Csp) expression, and that this effect is relevant to the effect on CFTR trafficking (18). We have also shown that GSNO S-nitrosylates Hsc70, which may also be permissive for increased CFTR maturation (18).

Here, we hypothesized that the effect of GSNO to increase CFTR trafficking to the cell surface in monolayer cultures would be through an effect on Hop (also known as Stip 1). Hop is a 60-kDa homodimer that links Hsp70 and Hsp90, central components of the regulation of CFTR trafficking, and it Co-IP's with CFTR (3). In addition, Hop is involved in cell-cycle regulation and steroid receptor maturation (24, 34, 35), and it is homologous to the CHIP complex, of importance to CFTR degradation (36). Hop has a conserved cysteine (C403) (23, 24) located in an S-nitrosylation motif believed to be relevant to S-nitrosylation (26). We found that GSNO inhibited Hop expression in all cell lines. Suppression of Hop expression appears central to the effect of GSNO on CFTR maturation: Hop siRNA itself increased ΔF508 CFTR maturation.

Interestingly, Hop does not appear to be involved in the mechanism by which decreased temperature increases ΔF508 CFTR maturation: (*i*) the effects of GSNO and hypothermia were additive, and (*ii*) hypothermia did not inhibit Hop expres-

sion. This suggests that there is more than one pathway by which ΔF508 CFTR can be induced to mature, and that the beneficial effects of these pathways may be additive. Temperature-sensitive proteins other than Hop may be responsible.

The GSNO effect to decrease Hop expression was augmented by cycloheximide pretreatment, but unaffected by actinomycin D, suggesting both that Hop is normally stable and that there is a posttranscriptional effect of GSNO on Hop expression. Consistent with a previous report (28), we found that cytosolic Hop is S-nitrosylated, but that GSNO treatment leads to loss of total cellular Hop. Further, GSNO increases CFTR-associated Hop in the ER transiently, then completely depletes all CFTR-associated Hop and S-nitrosylated Hop. This would suggest that rapid Hop degradation is favored by S-nitrosylation, preventing its association with CFTR. Indeed, mutation of C403 both prevented GSNO-induced Hop degradation in an overexpressing system and decreased S-nitrosylation of overexpressed Hop. Note that it is also possible that S-nitrosylation of a Hop-interacting protein, such as Hsp90 (37), is also involved in signaling Hop degradation, and cysteines other than C403 are S-nitrosylated (28). However, C403 appears necessary to target Hop for degradation. Further, this S-nitrosylation-induced degradation is proteasomal and appears to be signaled by ubiquitination: recovery of Hop (Fig. 5*B*), S-nitrosylated Hop (Fig. 5*C*), and ubiquitinated Hop (Fig. 5*D*) following GSNO treatment is augmented by proteasomal inhibition.

The effects of GSNO to signal the degradation of Hop may have relevance beyond its effect on CFTR trafficking. Transnitrosation reactions are increasingly recognized to be important for cell signaling, and Hop interacts with and regulates the expression and function of a number of proteins that, themselves, are functionally S-nitrosylated. These include Hsp90 itself, protein von Hippel Lindau, S100A class proteins, and others (37–40). Hop is believed to have an array of important cellular functions that could be affected by GSNO (23, 24), an important consideration with regard to potential toxicities of S-nitrosothiol therapies.

In summary, GSNO corrects ΔF508 CFTR trafficking—permitting cell-surface expression—by inhibiting Hop expression and CFTR-Hop interaction. Indeed, Hop siRNA causes ΔF508 CFTR maturation, and it could be of interest to study administration of Hop siRNA in animal models of CF. This effect is additive with the Hop-independent effect of low temperature to augment CFTR maturation, suggesting that different corrector therapies could prove to be of synergistic benefit for patients with CF.

Materials and Methods

Cell Culture. CFBE 41o$^-$ cells were provided by Dieter Gruenart (San Francisco, CA). CFPAC-1 and A549 cells were from ATCC. Cells were grown as previously described (see details in *SI Text*).

Reagents. Reagents were from Bio-Rad and Sigma unless otherwise stated. GSNO and GNODE were prepared as previously described (9, 18).

Immunoblotting. Immunoblotting was performed as previously described (15, 16, 18) (*SI Text*). Antibodies were as follows: mouse monoclonal anti-CFTR R-domain-specific (R&D Systems); monoclonal anti-CFTR (Chemicon) or monoclonal antibody 596 (provided by J. Riordan); monoclonal anti-Hop (Stressgen); monoclonal anti-Hsp70 (Abcam Inc.); monoclonal anti-Hsp90 and calnexin (Abcam Inc.); and rabbit polyclonal anti-syntaxin 5 and anti-tubulin, as well as rabbit polyclonal anti-ubiquitin (Stressgen). Modification of core glycosylated and fully glycosylated forms of CFTR was confirmed by preincubation of cell lysates with endoglycosidase H or peptide N-glycosidase F (New England Biolabs).

Isolation of Cytosol, ER, and Golgi. Cells were rinsed twice with ice-cold PBS, trypsinized, and pelleted by centrifugation (5,000 rpm, 10 min). The pellet was washed (×2) in ice-cold PBS and then suspended in 50 mM Tris·HCl (pH 8.0) with 0.5 M sucrose. After undergoing centrifugation (5,000 rpm, 10 min at 4 °C), the supernatant was transferred to ultracentrifuge tubes. The cytosol was clarified (48,000 rpm, 60 min at 4 °C). A total of 2.3 M sucrose stock was added to a concentration of 1.25 M sucrose. A sucrose density step gradient was performed in an ultracentrifuge tube using a paired gradient (by weight) with a balance tube. Organelles were separated by density gradient (48,000 rpm, 2 h at 4 °C). Off-white bands of Golgi membrane and endoplasmic reticulum membrane were harvested (4 °C) with a Pasteur pipette (18, 27).

Immunoprecipitation. Cytosolic, ER, and Golgi fractions were transferred to chilled Eppendorf tubes. Ten microliters of primary CFTR antibody (anti-CFTR clone L12B4 and anti-CFTR clone M3A7, mouse monoclonal antibodies; Upstate) was added to each fraction and incubated overnight at 4 °C with gentle shaking. Supernatant antibody mixtures were treated with 70 μL of Protein A (Boehringer Mannheim) and incubated for another 4 h. Then, samples underwent centrifugation (1 min), and proteins not bound to the beads were removed by washing beads twice with RIPA buffer. Proteins were then eluted from beads by incubation with 100 μL sample buffer at room temperature with continuous mixing for 1 h. Samples were divided into two aliquots: one for S-nitrosothiol assay and the other for IB.

Immunofluorescence. CFBE41o$^-$ cells grown on glass coverslips to confluence were treated, washed (×2) with 5 mL of complete Dulbecco's PBS (OW-PBS), fixed with 3.7% paraformaldehyde (5 min), washed two times with 1× OW-PBS, permeabilized by 0.1% Triton X-100 in OW-PBS (vol/vol, 10 min), washed two times with 1% PBS then with 1% BSA in OW-PBS, incubated in 1% BSA in OW-PBS for 45 min at RT, then incubated with anti-Hop antibody (1:100, 4 °C, overnight; Stressgen). Cells were then washed (×2 for 5 min) with 0.1% BSA in PBS, incubated with secondary antibody for 30 min at room temperature (1:500, Alexa Fluor 488; Invitrogen), mounted, and visualized using confocal microscopy (customized Zeiss equipment).

Cell-Surface Biotinylation. CFBE41o$^-$ cells were treated for 4 h with or without GSNO. The cells were washed (×3) with ice-cold PBS (pH 7.4) containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBSCM) and then treated in the dark with PBSCM buffer containing 10 mM sodium periodate for 30 min at 20 °C . The cells were washed (×3) with PBSCM and biotinylated by treating with sodium acetate buffer [100 mM sodium acetate buffer (pH 5.5), 0.1 mM $CaCl_2$,and 1 mM $MgCl_2$] containing 2 mM biotin-LC hydrazide (Pierce) for 30 min at 20 °C in the dark. The cells were then washed (×3) with sodium acetate buffer and solubilized with lysis buffer containing Triton X-100 and protease inhibitors (26). CFTR was IP'd as described and subjected to SDS/PAGE on 6% gels (27); biotinylated CFTR was detected with streptavidin-conjugated HRP.

Cell Membrane Preparation. CFBE41o$^-$ cells stably expressing ΔF508 CFTR were grown to confluence, then were treated for 4 h in the absence or presence of 5 and 10 μM GSNO. The cells were harvested in ice-cold PBS and homogenized with a Dounce homogenizer in buffer containing 0.5 M sucrose, 2 mM EDTA, 0.1 mM PMSF, 10 mM Hepes, and protease inhibitors (pH 7.4). Nuclei, mitochondria, and cell debris were pelleted (15 min, 10,000 rpm). The supernatant was centrifuged at 48,000 rpm for 2 h. The resulting supernatant was recovered as cytosol, and the membrane as a pellet. Total protein was assayed on each fraction (Bradford).

Ussing Chamber Experiments. Under the auspices of the University of North Carolina at Chapel Hill Institutional Review Board-approved protocol, primary human bronchial epithelial cells were obtained from lungs explanted during transplantation. Passage 2 cells from six different ΔF508 CFTR homozygous individuals were cultured at an air-liquid interface for study in Ussing chambers as described in detail previously (42). CFTR function was assayed as forskolin-stimulated Cl^- current in PCECs that had been treated with amiloride; 10 μM CFTRinh-172 (Sigma) was added after forskolin. ΔF508 CFTR homozygous PCECs were exposed (blind) to GSNO (100 μM every 6 h × 72 h) or (GNODE 60 μM every 12 h × 48 h) before assay.

S-Nitrosothiol Assays. S-nitrosylated proteins were assayed by the biotin substitution as previously described (28). CFTR IP (noted previously) was carried out on each fraction at each time, followed by biotin substitution, streptavidin isolation, and IB for Hop. Additionally, biotin-substituted S-nitrosylated proteins were assayed by LC-MS, as follows.

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Liquid Chromatography/Mass Spectrometry Assay for S-Nitrosylated Proteins. Biotin substitution proteins were analyzed by LC-MS as previously described (43) with modifications as noted in *SI Text*.

Small Interfering RNA Knockdown of Hop. To confirm the role of Hop in ΔF508 CFTR maturation, siRNA sequences were synthesized and then identified (using matrix-assisted laser desorption ionization/time-of-flight spectrometric analysis; QIAGEN Inc.). Hop siRNA duplexes were >90% pure as measured by HPLC analysis with sense r(CAA UGA UGG UUG AGU GAA)dTdT and antisense r(UUU CAC UCA ACC AUC AUU G)dTdA. Scrambled Hop siRNA was used as a control. For transfection, CFBE41o$^-$ cells were transfected with 50 nM of Hop construct using Lipofectamine 2000 (Invitrogen). After 48 h of transfection, with or without preincubation with GSNO (10 μM, 4 h), cells were rinsed three times with PBS and lysed directly on plates using lysis buffer containing protease inhibitors.

Site-Directed Mutagenesis. The C403S mutant was made using the Quik-Change Multi Site-Directed Mutagenesis Kit from Stratagene according to the manufacturer's instructions. Primers were as follows: Cys403-Ser-5′ CGAGCTGCCTCCTACACC-3′ and Cys403-Ser-3′ GGTGTAGGAGGCAGCTCG-5′.

Primers (100 ng) were incubated with DNA template (100 ng) in reaction buffer (25 μL), dNTP mix (1 μL), and Quick change multienzyme blend (1 μL) for 95 °C × 2 min (one cycle), then for 65 °C × 8 min (25 cycles). After incubation, the mixture was digested with Dpn 1 enzyme for 1 h, followed by transformation in XL10-Gold Ultracompetent Cells (Stratagene). Positive colonies (blue) were selected for transfection in LB-agar plates. DNA was purified using QIAprep Spin Miniprep Kit (QIAGEN Inc.). For transfection, 1×10^6 cells per well were plated on six-well plates and transfected with 50 nM of Hop construct using Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer. After 48 h of transfection, and with or without preincubation with GSNO (10 μM, 4 h), cells were studied by IB.

Statistical Analyses. We conducted two-way ANOVAs for each experiment. In each model, we included the main effects of treatment and band, and their interaction. The statistical analyses were carried out in SAS 9.1 (SAS Institute Inc.). Multiple comparisons were adjusted by Dunnett's method.

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