Reversible Silencing of CFTR Chloride Channels by Glutathionylation

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ABSTRACT The cystic fibrosis transmembrane conductance regulator (CFTR) is a phosphorylation- and ATP-dependent chloride channel that modulates salt and water transport across lung and gut epithelia. The relationship between CFTR and oxidized forms of glutathione is of potential interest because reactive glutathione species are produced in inflamed epithelia where they may be modulators or substrates of CFTR. Here we show that CFTR channel activity in excised membrane patches is markedly inhibited by several oxidized forms of glutathione (i.e., GSSG, GSNO, and glutathione treated with diamide, a strong thiol oxidizer). Three lines of evidence indicate that the likely mechanism for this inhibitory effect is glutathionylation of a CFTR cysteine (i.e., formation of a mixed disulfide with glutathione): (a) channels could be protected from inhibition by pretreating the patch with NEM (a thiol alkylating agent) or by lowering the bath pH; (b) inhibited channels could be rescued by reducing agents (e.g., DTT) or by purified glutaredoxins (Grxs; thiol disulfide oxidoreductases) including a mutant Grx that specifically reduces mixed disulfides between glutathione and cysteines within proteins; and (c) reversible glutathionylation of CFTR polypeptides in microsomes could be detected biochemically under the same conditions. At the single channel level, the primary effect of reactive glutathione species was to markedly inhibit the opening rates of individual CFTR channels. CFTR channel inhibition was not obviously dependent on phosphorylation state but was markedly slowed when channels were first "locked open" by a poorly hydrolyzable ATP analogue (AMP-PNP). Consistent with the latter finding, we show that the major site of inhibition is cys-1344, a poorly conserved cysteine that lies proximal to the signature sequence in the second nucleotide binding domain (NBD2) of human CFTR. This region is predicted to participate in ATP-dependent channel opening and to be occluded in the nucleotide-bound state of the channel based on structural comparisons to related ATP binding cassette transporters. Our results demonstrate that human CFTR channels are reversibly inhibited by reactive glutathione species, and support an important role of the region proximal to the NBD2 signature sequence in ATP-dependent channel opening.

KEY WORDS: cystic fibrosis transmembrane conductance regulator • ABC transporter • glutathione • glutaredoxin • redox

INTRODUCTION

The cystic fibrosis transmembrane conductance regulator (CFTR) is a phosphorylation- and ATP-dependent chloride channel that controls salt and water transport across epithelial tissues (for reviews see Gadsby and Nairn, 1999; Sheppard and Welsh, 1999). The CFTR channel is essential for normal lung and gut physiology in humans (Welsh and Smith, 1993; Gabriel et al., 1994). CFTR belongs to the large family of ATP binding cassette (ABC) transporters on the basis of its two cytoplasmic nucleotide binding domains (NBDs) and two membrane spanning domains (Riordan et al., 1989). The NBDs bind MgATP and promote channel opening provided that sites within the centrally positioned regulatory (R) domain are phosphorylated (typically by cAMP-dependent protein kinase; Gadsby and Nairn, 1999). The major elements within the NBDs that mediate ATP binding include the Walker A and B motifs and the signature sequences, which are well conserved among ABC transporters (Higgins, 1992). Both CFTR NBDs participate in ATP-dependent channel gating; a conclusion that is supported by a large number of studies of CFTR NBD mutants (Gregory et al., 1991; Carson et al., 1995; Gunderson and Kopito, 1995; Cotton and Welsh, 1998). How the Walker motifs and signature sequences within the two NBDs work together to bind MgATP and to signal pore opening at

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Abbreviations used in this paper: ABC, ATP binding cassette; BHK, baby hamster kidney; CFTR, cystic fibrosis transmembrane conductance regulator; DTT, dithiothreitol; Grx, glutaredoxin; GSH, reduced glutathione; GSNO, nitrosylated glutathione; GS(O)SG, glutathione disulfide S-oxide; GSSG, glutathione disulfide; NBD, nucleotide binding domain; NEM, *N*-ethylmaleimide; NHS, *N*-hydroxysulfosuccinimide; PKI, PKA inhibitory peptide; R, regulatory; Trx, thioredoxin; WT, wild type.

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the transmembrane domains is unclear. Current models of CFTR channel gating (e.g., Berger et al., 2002; Vergani et al., 2003) are based in large part on structural information from bacterial ABC transporters such as the *Escherichia coli* vitamin B_{12} and maltose transporters and from the crystal structure of Rad50, a related ATPase (Hopfner et al., 2000; Locher et al., 2002; Chen et al., 2003). The NBDs within these proteins dimerize to form two composite ATP binding sites. Residues from the Walker A motifs and signature sequences of the opposing NBDs line each ATP binding pocket in the crystal structures of Rad50 and the NBD dimer of the maltose transporter (Hopfner et al., 2000; Chen et al., 2003). Whether the two CFTR NBDs also associate to form composite ATP binding sites, and how nucleotide binding subsequently promotes CFTR channel opening, are unresolved issues.

CFTR channels potentially can be modulated by oxidants in addition to the more well-studied physiologic activators (e.g., PKA and ATP). The CFTR polypeptide possesses 18 cysteine residues that are possible targets for oxidation, many of which reside in the NBDs and R domain. Identifying reactive species that modulate CFTR channel activity and mapping the target cysteines could provide insights into the structural basis of CFTR gating. In addition, if CFTR channels are regulated substantially by reactive species, such regulation may have pathophysiologic significance since the lung and gut are rich in oxidants under inflammatory conditions such as asthma and colitis (Montuschi and Barnes, 2002; Pavlick et al., 2002). Harrington et al. (1999) first reported that several conventional thiol oxidizers (e.g., $KMnO_4$ and NO) can affect the dynamics of CFTR channel gating in synthetic lipid bilayers and excised inside-out membrane patches. The oxidizers that were tested in these initial studies had interesting effects on gating kinetics (i.e., they slowed both the open and close rates), but appeared to have only modest effects on net channel activity (i.e., single channel open probability).

We were intrigued by the possibility that CFTR channels might be regulated more substantially by oxidized forms of glutathione for several reasons. First, glutathione is abundant within cells (i.e., millimolar concentrations), and can be oxidized by a variety of reactive oxygen and nitrogen species and other thiol-modifying reagents in vitro and in vivo (Thomas et al., 1995; Pompella et al., 2003; Schrammel et al., 2003). In turn, oxidized forms of glutathione (i.e., glutathione disulfide S-oxide, GS(O)SG, nitrosylated glutathione, GSNO, and glutathione disulfide, GSSG) can promote the formation of mixed disulfides with cysteines on proteins in vitro and in vivo; a process that is termed protein glutathionylation (Li et al., 2001; Aracena et al., 2003; Taylor et al., 2003). Glutathionylation has been shown to have profound stimulatory or inhibitory effects on the functions of several proteins (Barrett et al., 1999; Wang et al., 2001; Humphries et al., 2002; Aracena et al., 2003; Caplan et al., 2004), although its role in regulating ABC transporters is unknown. This covalent modification can be reversed in vitro and in vivo by glutaredoxins (thiol disulfide oxidoreductases), which has led several groups to propose that glutationylation is a dynamic posttranslational modification that can reversibly influence protein function during oxidative stress (Huang and Huang, 2002; Lind et al., 2002; Fernandes and Holmgren, 2004). Finally, the relationship between glutathione and CFTR is of added interest because of recent reports that (a) CFTR can transport reduced and oxidized glutathione in addition to small anions by an unknown mechanism (Linsdell and Hanrahan, 1998; Kogan et al., 2003) and (b) GSNO can enhance the biosynthetic maturation of CFTR processing mutants that associate with cystic fibrosis (Zaman et al., 2001). Based on this latter result, Zaman et al. (2001) proposed that GSNO might be a useful therapeutic agent for CF. Given this growing interest in the connections between CFTR and glutathione, it seemed important to clarify the effects of reactive forms of glutathione on the most well-accepted property of CFTR; namely, its chloride channel function.

Here we provide functional and biochemical evidence for the reversible glutathionylation of human CFTR channels by several oxidized forms of glutathione. Glutathionylated channels exhibit very low open rates in the presence of normally saturating concentrations of ATP, but can be protected from this inhibition if they are first locked open with a poorly hydrolyzable ATP analogue. We provide evidence that the major site of inhibition is cys-1344, which is proximal to the ABC signature sequence in NBD2. Based on our data and on comparisons to the crystal structures of other ABC transporters, we propose that glutathionylation at this site disrupts ATP binding or the link between ATP binding and pore opening (the preliminary results of this study were presented in abstract form at the 2003 Biophysical Society meeting).

MATERIALS AND METHODS

Cell Culture, DNA Constructs, and Transfections

Baby hamster kidney (BHK) cells stably expressing wild-type (WT) human CFTR (BHK-CFTR) were provided by J. Hanrahan (McGill University, Montreal, Quebec, Canada). BHK-CFTR cells and Calu-3 human airway epithelial cells expressing native CFTR were cultured in Dulbecco's modified Eagle's medium (DMEM; Mediatech) supplemented with 5% or 10% FBS and 1 mM penicillin–streptomycin. The growth media for the BHK-CFTR cells also contained 0.5 mM methotrexate to maintain selection for CFTR-expressing cells (Chappe et al., 2003). The S660A/ΔR-CFTR mutant and all but one of the alanine-substituted cysteine mutants were provided by M. Welsh (University of Iowa, Iowa City, IA) (Rich et al., 1991; Cotton and Welsh, 1997) and were subcloned into the pCDNA3 expression vector (Invitrogen). C1458A-CFTR was gener-

ated by PCR mutagenesis and subcloned into pCDNA3. A CFTR construct lacking all 18 cysteines (16CS C590L/592L) was provided by D. Gadsby (Rockefeller University, New York, NY) (Mense et al., 2004). Cys-1344 was reintroduced into the latter construct by PCR mutagenesis and both constructs (+/− cys-1344) were subcloned into the pIRESneo2 expression vector (CLONTECH Laboratories, Inc.). All mutations were confirmed by DNA sequencing.

HEK-293T cells were transiently transfected with WT or mutant CFTR cDNA using the Lipofectamine transfection kit following manufacturer's recommendations (Invitrogen). HEK-293T cells were cultured in DMEM. All cells were grown on plastic coverslips for patch clamp recording and were used 1–4 d post-seeding. HEK-293T cells that were transfected with S660A/ΔR-CFTR or with the cys-free constructs were grown overnight at 27°C because these mutants are temperature-sensitive ER processing mutants as determined by immunoblot analysis.

Electrophysiology and Data Analysis

Macroscopic and single channel currents were recorded in the excised, inside-out configuration. Patch pipettes were pulled from Corning 8161 glass to tip resistances of 1.5–4.0 mOhm (macroscopic recordings) or 15–18 mOhm (single channel studies). CFTR channels were activated following patch excision by exposure of the cytoplasmic face of the patch to catalytic subunit of PKA (110 U/ml; Promega) and MgATP (1.5 mM). CFTR currents were recorded in symmetrical solution containing (in mM) 140 *N*-methyl-D-glucamine-Cl, 3 $MgCl_2$, 1 EGTA, and 10 TES. The pH was adjusted to 7.3 unless otherwise noted. Macroscopic currents were evoked using a ramp protocol from +80 to −80 mV with a 10-s time period. Patches were held at −80 mV for single channel recordings. All patch clamp experiments were performed at 21–23°C. Signals from macroscopic and single channel recording were filtered at 20 and 200 Hz, respectively. Data acquisition and analysis were performed using pCLAMP8 software (Axon Instruments). Curve fitting for kinetic analysis was performed using Microcal Origin software. Averaged data are presented as mean ± SEM. Statistical comparisons were made by performing unpaired *t* tests unless otherwise indicated.

Biochemical Detection of CFTR Glutathionylation in Microsomal Membrane Vesicles

Reduced glutathione (GSH) was labeled with biotin (EZ-Link Sulfo-NHS-LC-Biotin; Pierce Chemical Co.) by reaction of the primary amine of GSH with *N*-hydroxysulfosuccinimide (NHS)-biotin. The reaction was performed by adding stoichiometric amounts (10 mM) of NHS-biotin and GSH to PBS (pH 7.4) for 1 h at 21–23°C. Unreacted NHS-biotin was quenched by the addition of 50 mM ethanolamine. The isolation of microsomal membrane vesicles from BHK-CFTR cells followed exactly a previously published protocol (Aleksandrov et al., 2001). Glutathionylation of CFTR in microsomes was performed by incubating the microsomes (200 μg) with 100 μM diamide and 125 μM biotin-GSH for 10 min at 21–23°C in PBS. Some samples were then treated with 4 μM *E. coli* glutaredoxin (Grx1) (plus 1 mM GSH), 20 mM GSH, or 20 mM dithiothreitol (DTT) for an additional 15 min at 21–23°C. Microsomes were then solubilized in 0.2% Triton X-100 in PBS, and biotinylated CFTR was mixed with Steptavidin beads (Novagen) in this buffer for 2 h at 21–23°C. Beads were then pelleted by a brief spin and then washed three times in 1 ml of PBS containing 0.2% Triton X-100. CFTR was eluted in SDS sample buffer containing 50 mM DTT, resolved by SDS-PAGE (4–15% polyacrylamide, Bio-Rad Laboratories), transferred to PVDF membranes, blocked for 1 h at 21–23°C with 5% milk in Tris (25 mM) buffered saline (TBS), and then incubated for 1 h with CFTR monoclonal antibody (R&D Systems; 24-1). Blots were then washed extensively in TBS and incubated with goat anti-mouse HRP-conjugated antibody (Amersham Biosci-

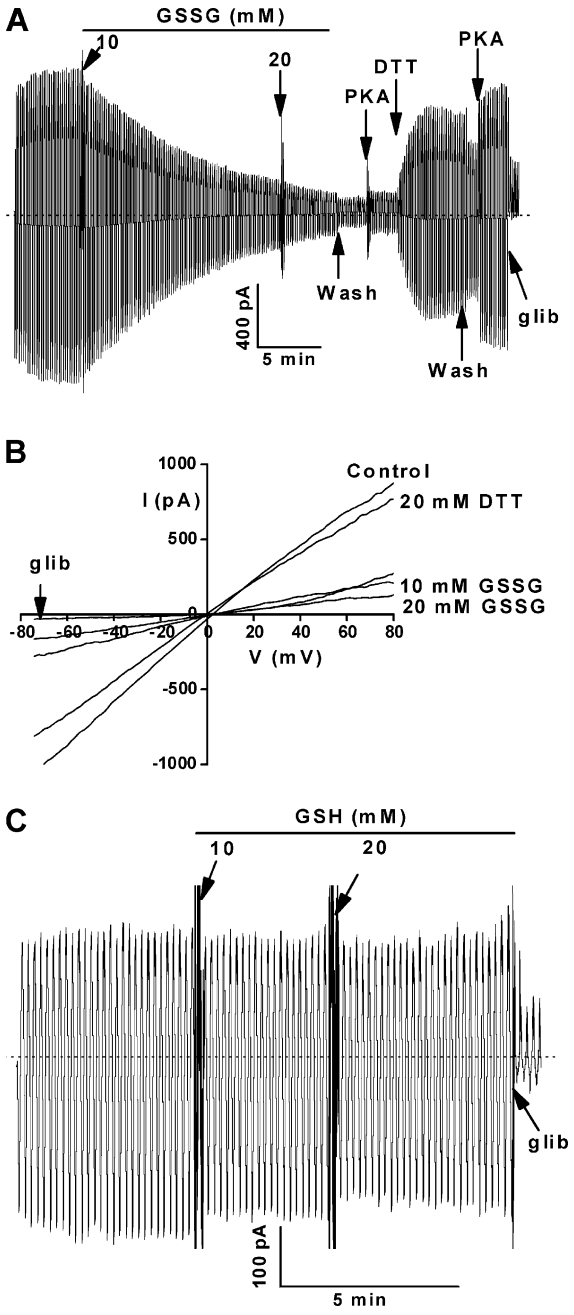

FIGURE 1. Reversible inhibition of CFTR channel activity by GSSG. Macroscopic currents were recorded for membrane patches excised from BHK-CFTR cells as described in MATERIALS AND METHODS. (A) GSSG was added at the indicated final bath concentrations. The bath chamber was washed free of PKA and GSSG and then PKA (110 U/ml each addition), and 20 mM DTT was added as indicated. Glibenclamide (300 μM), a voltage-dependent blocker of CFTR current, was added at the end of the experiment. (B) Corresponding I–V curves showing voltage-independent inhibition of CFTR current by GSSG. (C) Modest effect of GSH on CFTR current. Dotted lines indicate zero current levels.

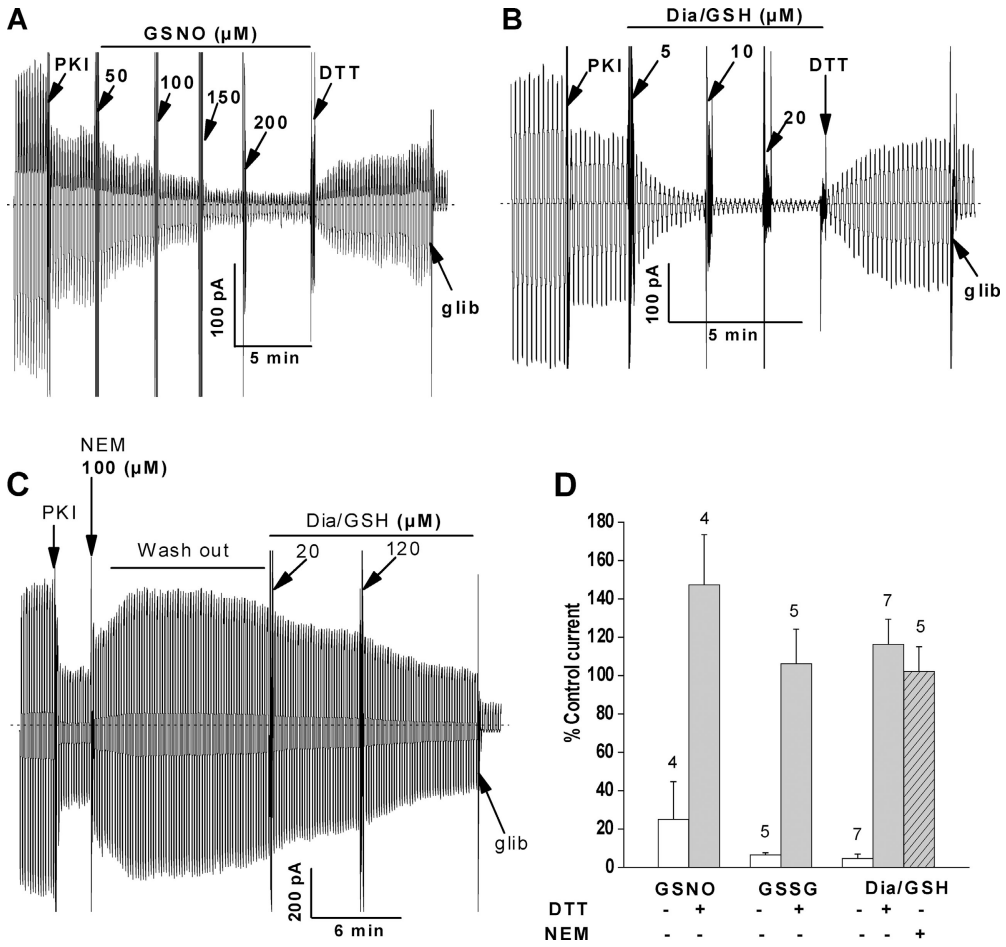

FIGURE 2. CFTR inhibition by GSNO and diamide/GSH. (A) GSNO inhibition of CFTR currents. PKA inhibitory peptide (1.4 μg/ml PKI) was added at the first arrow. (B) Reversible inhibition of CFTR current by equimolar diamide/GSH. (C) CFTR channels are protected from diamide/GSH inhibition by pretreating the patch with NEM. NEM by itself stimulated the current as previously reported for CFTR (Cotton and Welsh, 1997). NEM, PKA, and PKI (but not MgATP) were washed out from the bath where indicated. (D) Mean data showing the inhibitory effects of GSNO (200 μM), GSSG (20 mM), and diamide/GSH (20 μM) on CFTR current and the rescue by subsequent addition of 20 mM DTT. Also shown is the protective effect of NEM pretreatment (0.1 mM) on the inhibition by diamide/GSH (20 μM). The results shown in Fig. 1 D were obtained following PKA inhibition with PKI (1.4 μg/ml). All data are normalized to the current recorded before the addition of the glutathione species. The number of experiments is shown above each bar.

ences; 1:5,000 dilution) for 1 h. Following extensive washing in TBS, blots were exposed to SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical Co.) for 5 min and then developed on HXR film (Hawkins X-Ray Supply) for 0–5 min.

Materials

GSH, GSSG, *S*-nitrosoglutathione (GSNO), and purified *E. coli* glutaredoxin (Grx1) and thioredoxin were purchased from Calbiochem. Recombinant human glutaredoxin1 was purchased from American Diagnostica Inc. The monothiol mutant of *E. coli* Grx3 (C14S/C65Y) was expressed and purified as previously described (Nordstrand et al., 1999). All other reagents were from Sigma-Aldrich. Glutaredoxins were reconstituted in sterile distilled H_2O. Glibenclamide and *N*-ethylmaleimide (NEM) were dissolved and stored in DMSO. Mixtures of diamide and reduced glutathione (GSH) and GSNO stocks were made fresh daily in H_2O.

RESULTS

CFTR Channel Activity Is Inhibited by Oxidized Forms of Glutathione

Fig. 1 shows that glutathione disulfide (GSSG) gradually inhibited macroscopic CFTR current ($t_{1/2}$ of 5–10 min) when added to the cytosolic face of an inside-out patch excised from a BHK-CFTR cell. The inhibition by GSSG was voltage independent (Fig. 1 B) and could be reversed by adding a high concentration (20 mM) of the reducing agent DTT (Fig. 1 A; see mean data in Fig. 2 D). Conversely, reduced glutathione (GSH; 10-20 mM) had only modest effects on macroscopic CFTR currents (Fig. 1 C). Although it had been reported previously that GSH blocks the CFTR pore (Linsdell and Hanrahan, 1998), this blocking effect apparently was rather weak under the conditions of our experiments.

GSSG promotes the glutathionylation of proteins at high concentrations such as those required to inhibit CFTR currents (Aracena et al., 2003). Accordingly, we tested the effects of two other oxidized forms of glutathione that are more potent thiolating reagents; (1) GSNO, a naturally occurring form of glutathione that promotes glutathionylation either directly or via metabolites such as GS(O)SG (Li et al., 2001; Huang and Huang, 2002), and (2) GSH together with diamide, a strong thiol oxidizer (Fig. 2). Diamide is an exception-

ally potent oxidizer of GSH (Kosower and Kosower, 1995), and mixtures of diamide and GSH (diamide/GSH) have been reported to be effective glutathionylating reagents (Humphries et al., 2002). To rule out a trivial effect of oxidized forms of glutathione on the PKA in the bath, these experiments were performed following PKA inhibition with excess inhibitory peptide (PKI; 1.4 μg/ml). Inactivating the PKA inhibited the current by ~50% presumably because of phosphatase activity within the membrane patch (Fig. 2, A–C). The subsequent addition of GSNO nearly abolished the CFTR currents but with a faster time course and at much lower concentrations than observed for GSSG (50–200 μM; see Fig. 2 A for time course and Fig. 2 D for mean data). The inhibition by GSNO was reversed by the addition of DTT (20 mM). The effect of GSNO was unlikely due to NO that had been released from GSNO since the addition of 2 mM SNAP (an NO donor) had no effect on the CFTR current (unpublished data).

CFTR currents also were rapidly inhibited by equimolar diamide/GSH at very low concentrations (5–20 μM; Fig. 2, B and D). The combination of diamide and GSH, when premixed for 2–5 min, had the most potent effects on CFTR channel activity of the various forms of glutathione that were tested. Diamide alone, which can promote disulfide bond formation in proteins, had no effects at these low concentrations. In addition, CFTR currents were not inhibited by adding GSH alone to patches that had been pretreated with diamide (unpublished data). Thus, the key reaction in the diamide/GSH mixture is oxidation by glutathione. As with GSSG and GSNO, the inhibition of CFTR currents by GSNO or diamide/GSH was voltage independent and completely reversible by DTT. The inhibition by diamide/GSH also could be blunted by prior incubation with 0.1 mM NEM (a thiol alkylating agent; Fig. 2, C and D), which by itself moderately stimulates CFTR current as reported previously (Cotton and Welsh, 1997).

The protection by NEM and the rescue by DTT indicate that reactive forms of glutathione inhibit CFTR currents by oxidizing a cysteine or cysteines on the CFTR polypeptide. If so, this inhibition should be sensitive to ambient pH since the thiolate form of cysteine typically is the preferred target for oxidation (Griffith et al., 2002). Fig. 3 shows that the inhibitory effect of diamide/GSH on CFTR currents was blunted at low pH (6.3) and was accelerated at high pH (8.3). This pH dependence is consistent with oxidation of a titratable cysteine by reactive forms of glutathione possibly by a glutathionylation mechanism.

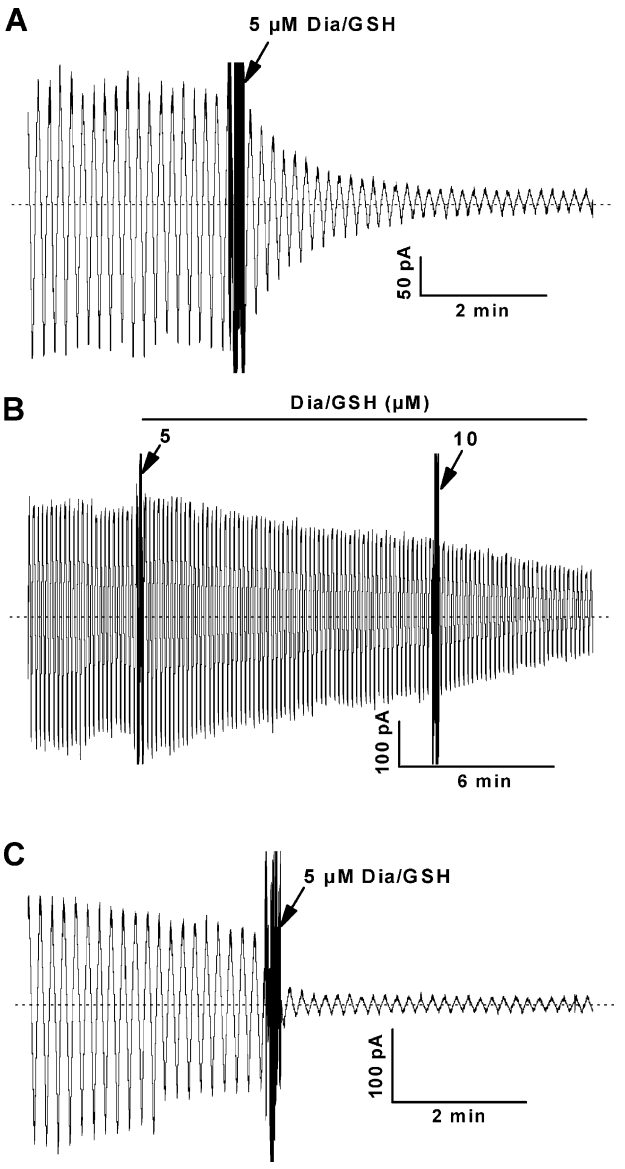

FIGURE 3. pH dependence of the inhibition of CFTR current by diamide/GSH. Macroscopic currents were recorded for membrane patches excised from BHK-CFTR cells at pH 7.3 (A), pH 6.3 (B), and pH 8.3 (C). Diamide and GSH were premixed at pH 7.3 and then added to the bath where indicated. Results are representative of four experiments. CFTR currents per se were only modestly affected by changing the pH over this range; e.g., changing the bath pH from 7.3 to 8.3 resulted in a 10–20% inhibition of macroscopic current (unpublished data).

Glutaredoxin Rescues CFTR Channels from Inhibition by Oxidized Forms of Glutathione

If reactive forms of glutathione inhibit CFTR currents by a glutathionylation mechanism, then this effect should be reversed by an enzyme with reductase activity toward mixed disulfides. To explore this point, we tested two enzymes (thioredoxin [Trx] and glutaredoxin [Grx]) for their abilities to rescue CFTR currents from inhibition by oxidized glutathione. These enzymes have overlapping specificities but have different preferences for mixed disulfides (i.e., Grx has greater activity as a mixed disulfide reductase provided that GSH is present as a cofactor, or electron

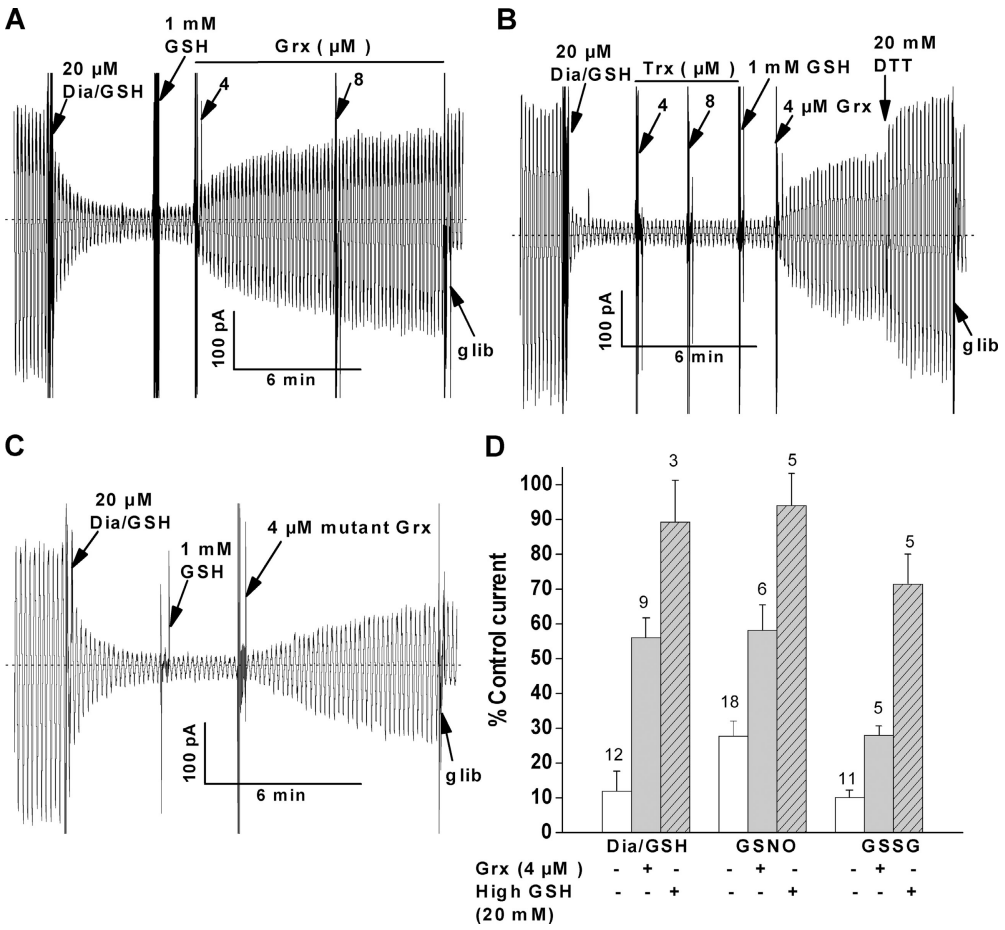

FIGURE 4. Glutaredoxin rescues CFTR channels from inhibition by reactive glutathione species. (A) Purified *E. coli* Grx1 (4 and 8 μM) reversed the inhibition of CFTR activity induced by diamide/GSH in an excised BHK-CFTR patch. (B) Purified *E. coli* Trx was unable to rescue CFTR activity following the addition of diamide/GSH. (C) CFTR activity also was rescued by a mutant Grx that is a specific mixed disulfide reductase (Grx3 C14S/C65Y). (D) Mean data showing the inhibitory effects of the indicated glutathione species (20 μM diamide/GSH; 200 μM GSNO; 20 mM GSSG) and the rescue effects of *E. coli* Grx1 (4 μM Grx1 plus 1 mM GSH) and GSH alone at high dose (20 mM). CFTR channels were preactivated with PKA and MgATP as described in MATERIALS AND METHODS, and all additions were made after inhibiting the bath PKA with excess PKI inhibitory peptide (1.4 μg/ml). Results are normalized to the CFTR currents recorded before addition of the reactive glutathione species. The number of experiments is shown above each bar. For each glutathione species, the rescue by Grx or high GSH was statistically significant ($P < 0.05$) when compared with the current before Grx or GSH addition.

donor; Fernandes and Holmgren, 2004). Recombinant *E. coli* Grx1 at a concentration of 4 μM restored the CFTR currents following inhibition with diamide/GSH to >50% of control (prediamide/GSH) levels (Fig. 4 A; see also Fig. 4 D for Grx effects on channels inhibited by GSNO and GSSG). Similar results were obtained for purified human Grx1 (unpublished data). Conversely, Trx was unable to rescue CFTR channels that had been inhibited by diamide/GSH when added either before or after washout of the diamide and GSH (Fig. 4 B). Grx required GSH (1 mM) as an electron donor to rescue the CFTR currents (unpublished data). At these low concentrations, GSH by itself had negligible effects on CFTR currents. However, at much higher concentrations (>10 mM), GSH alone could slowly recover the currents following prior inhibition with diamide/GSH, GSNO, or GSSG presumably because of its antioxidant activity (Fig. 4 D).

Grx has two cysteines in its active site that enable it to reduce intramolecular disulfide bonds by a dithiol mechanism as well as to reduce mixed disulfides by a monothiol mechanism (Bushweller et al., 1992; Fernandes and Holmgren, 2004). To distinguish between these two possible mechanisms by which Grx could rescue oxidized CFTR channels, we also tested a Grx mutant that lacks the second cysteine in the active site (*E. coli* Grx3 C14S/C65Y). This Grx mutant is a highly specific mixed disulfide reductase that lacks protein disulfide reductase activity (Bushweller et al., 1992; Fernandes and Holmgren, 2004). Fig. 4 C shows that the Grx mutant also rescued CFTR currents from prior inhibition by diamide/GSH, which supports the idea that reactive forms of glutathione inhibit CFTR channels by a glutathionylation mechanism. Like for the WT enzyme, the current recovery was substantial but incomplete. The incomplete recovery by Grx may indicate that reactive glutathione species also inhibit channel function by an additional mechanism that does not involve glutathionylation. In this regard, we show below that Grx completely reverses the inhibition by diamide/GSH of a mutant CFTR channel that lacks most of the R domain (see Fig. 9).

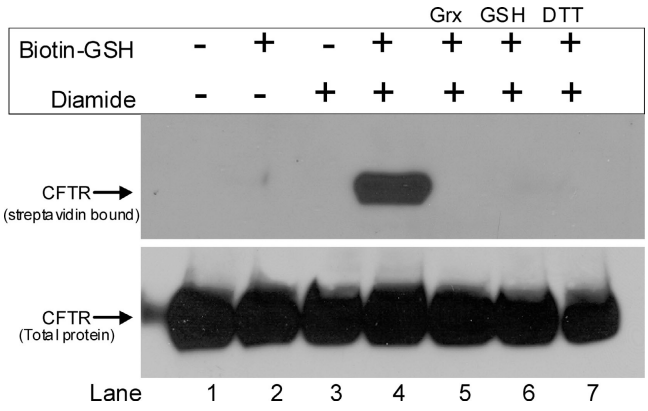

FIGURE 5. Reversible glutathionylation of CFTR in isolated membrane vesicles. Microsomal membranes were prepared from BHK-CFTR cells as described in MATERIALS AND METHODS. Microsomes (200 mg total protein) were incubated in PBS in the absence (lane 1) or presence of 125 μM biotin GSH and/or 100 μM diamide for 10 min at 21–23°C (lanes 2–7). Where indicated, 4 μM *E. coli* Grx1 (plus 1 mM GSH) (lane 5), 20 mM GSH (lane 6), or 20 mM DTT (lane 7) was added for an additional 15 min at 21–23°C. Microsomes were then solubilized, and biotin-containing proteins were isolated by pull-down with streptavidin-agarose and probed for CFTR by immunoblotting as described in MATERIALS AND METHODS. Total CFTR protein was assayed by immunoprecipitating CFTR from 3% of the total crude membrane lysate and blotting the immunoprecipitate with the same CFTR monoclonal antibody. This experiment was repeated four times with similar results.

Biochemical Confirmation of Reversible CFTR Glutathionylation

As a biochemical test for CFTR glutathionylation under these conditions, microsomes containing CFTR channels were incubated with biotinylated glutathione (biotin-GSH) with or without diamide at concentrations that inhibit CFTR channel activity. Microsomes were then solubilized and biotinylated proteins were captured on streptavidin beads followed by immunoblotting with a CFTR antibody. Fig. 5 shows that CFTR channels were modified by biotin-GSH (i.e., captured on streptavidin beads) following treatment with diamide and biotin-GSH together but not with either alone. In addition, this modification could be reversed by the same factors that rescued CFTR currents from inhibition by oxidized forms of glutathione; namely, Grx (with 1 mM GSH as a cofactor), DTT, and high concentrations of GSH alone. These biochemical results confirm that membrane-associated CFTR polypeptides can be reversibly glutathionylated under the same conditions that promote channel inhibition.

Glutathionylation Virtually Abolishes the Opening of Individual CFTR Channels

The nearly complete inhibition of macroscopic CFTR currents by oxidized glutathione implies that glutathionylation dramatically affects the gating of CFTR channels. To examine this point, we performed single channel experiments to assess the effect of thiolation on the gating properties of individual CFTR channels in excised inside-out patches (Fig. 6). Calu-3 airway epithelial cells were used for these experiments, since the levels of native CFTR protein in these cells (at least 5–10-fold lower than for recombinant CFTR in BHK-CFTR cells) are optimal for obtaining patches with one or a few detectable channels. In pilot experiments, we verified that macroscopic CFTR currents in patches excised from Calu-3 cells were reversibly inhibited by diamide/GSH as in BHK-CFTR patches (Fig. 6 A). In single channel experiments, we observed a nearly complete inhibition of open probability (P_o) that was due primarily to a dramatic reduction in the frequency of channel openings (Fig. 6, B–D). This marked inhibition of channel opening rate was observed in the presence of a normally maximally activating concentration of MgATP (1.5 mM). Increasing the MgATP concentration up to 10 mM had no stimulatory effect on the activities of glutathionylated CFTR channels (unpublished data). However, channels that were silenced by glutathionylation could be stimulated by the subsequent addition of Grx and GSH. These results indicate that glutathionylation of CFTR reversibly inhibits channel opening in the presence of a normally saturating concentration of MgATP.

Cys-1344 Is the Major Site of CFTR Channel Inhibition by Oxidized Forms of Glutathione

The simplest explanation of our functional and biochemical results is that reactive glutathione species inhibit CFTR channel gating by modifying a cysteine residue in the CFTR polypeptide. To identify cysteine residues in CFTR channels that may be targets for glutathionylation, we screened a panel of alanine-substituted cysteine mutants for inhibition by oxidized forms of glutathione (Fig. 7). Each mutant (as well as WT-CFTR) was transfected into HEK-293T cells and tested for inhibition by diamide/GSH, GSNO, or GSSG in excised membrane patches. All CFTR constructs, with the exception of C1344A-CFTR, were markedly inhibited by diamide/GSH (Fig. 7, B and C). C1344A-CFTR was largely, although not completely, resistant to each of the three glutathione species at the indicated concentrations (Fig. 7, B and D). The fact that this mutant is not completely protected may indicate that other cysteines can be modified by oxidized glutathione, but that such modifications have less dramatic effects on channel function than modification at cys-1344.

To determine if cys-1344 alone is sufficient for CFTR inhibition by reactive glutathione species, we tested the effects of diamide/GSH on two additional constructs:

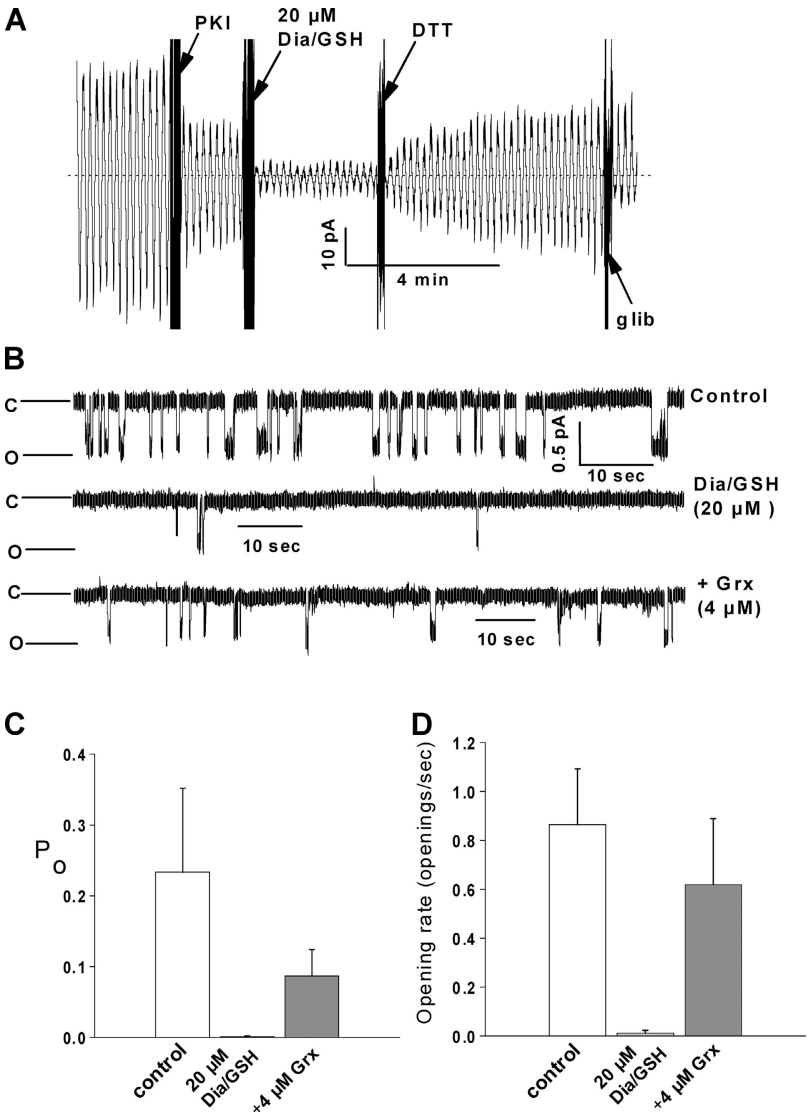

FIGURE 6. Glutathionylated CFTR channels exhibit markedly reduced open rates. (A) Macroscopic current trace showing inhibition of CFTR current by diamide/GSH in membrane patch excised from Calu-3 epithelial cell. PKI (1.4 μg/ml) was added at the arrow. (B) Single channel record obtained from Calu-3 patch showing marked inhibition of channel opening by diamide/GSH (20 μM) and partial recovery by *E. coli* Grx1 (4 μM Grx1 plus 1 mM GSH). Holding potential was −80 mV. (C) Mean data showing inhibitory effects of diamide/GSH (20 μM) and rescue effects of Grx1 (+1 mM GSH) on single channel open probability (P_o) and channel opening rate (n = 3 patches). The results shown in B and C were obtained without PKI addition. Nearly identical results were obtained for two additional patches that were treated with diamide/GSH after PKI addition (unpublished data).

(1) a CFTR construct that lacks all cysteine residues (16CS C540L/C542L; Mense et al., 2004) and (2) a CFTR construct that contains cys-1344 as its only cysteine (cys-1344-only). We were able to obtain a limited amount of patch clamp data for each (Fig. 8), although these constructs are severe ER processing mutants that express poorly in mammalian cells (not depicted). Cys-free CFTR channels were insensitive to diamide/GSH (Fig. 8, A and C), which confirms that channel inhibition is due to oxidation of a CFTR cysteine. The cys-1344-only construct was inhibited ~60% by a dose of diamide/GSH that caused an 80–90% decrease in WT currents (compare Fig. 8 C, Fig. 2 D, and Fig. 7 C). Thus, oxidation of cys-1344 alone can account for much of the inhibition by diamide/GSH. The lack of complete recovery of the inhibition by diamide/GSH might reflect the involvement of other cysteines in the inhibitory response (see above). Alternatively, the cys-1344-only construct may be less sensitive to diamide/GSH than the WT channel because of altered structural and/or functional properties (evidenced by its inefficient biosynthetic processing).

Cys-1344 is located near the signature sequence in NBD2 (Fig. 7 A). The signature sequences in other ABC transporters have been shown to participate in NBD dimerization and ATP binding as well as to associate closely with structural elements that link the NBDs to the transmembrane domains (Locher et al., 2002; Chen et al., 2003). The local sequence surrounding cys-1344 lacks basic residues that might otherwise favor the thiolate form of this cysteine (i.e., the form that is the presumed substrate for oxidation). However, by mass spectrometry, we verified that a synthetic peptide containing cys-1344 (a.a. 1340–1352) could be glutathionylated at this position when incubated with 20 mM GSSG for 30 min at 22°C (pH 7.4; unpublished data). We could not purify enough full-length CFTR protein for similar analysis by mass spectrometry. In addition,

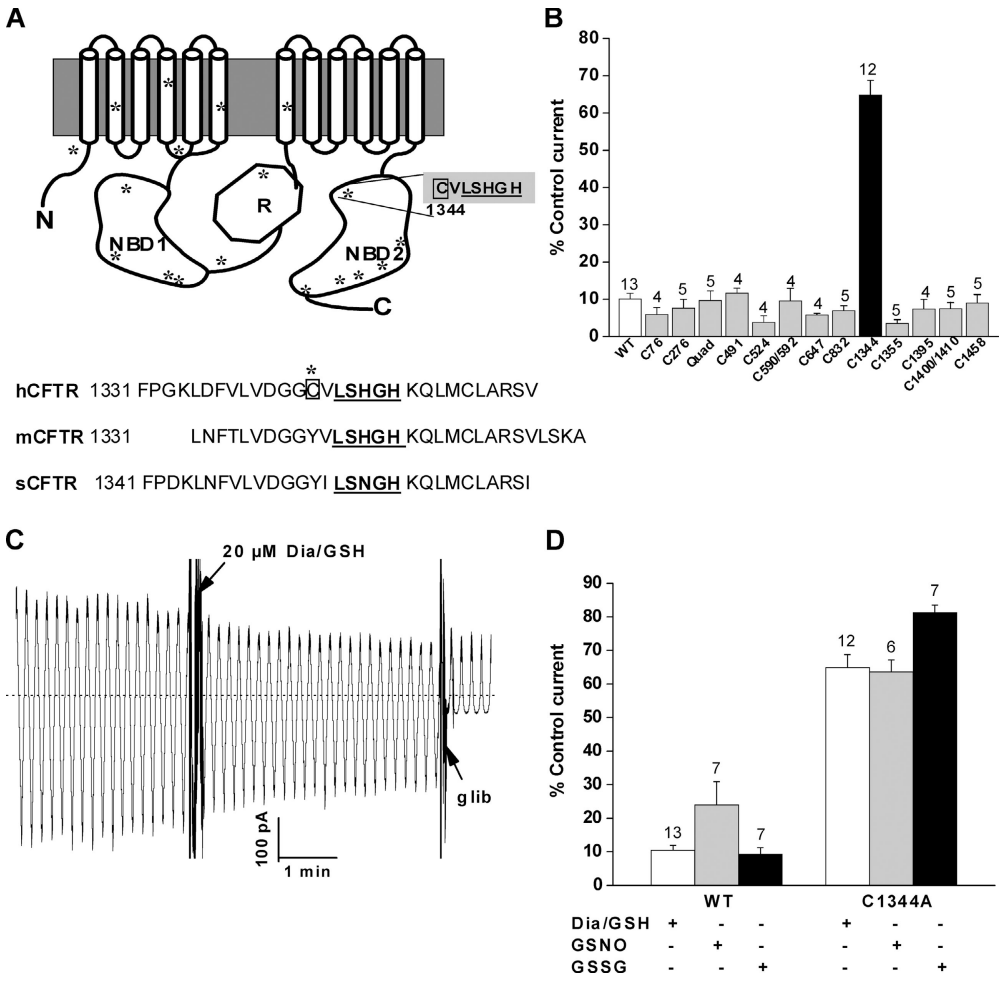

FIGURE 7. CFTR channels that lack cys-1344 (C1344A-CFTR) are largely resistant to inhibition by reactive glutathione species. (A) Schematic view of CFTR depicting the 18 cysteines within the polypeptide and the specific location of cys-1344 near the ABC signature motif in NBD2. Bottom, sequences surrounding the NBD2 signature sequences in CFTR from human (h), mouse (m), and shark(s). (B) Mean data showing the inhibitory effects of diamide/GSH (20 μM) on the indicated alanine-substituted mutants and WT CFTR expressed in HEK-293T cells. Quad mutant is C128/225/343/866. Diamide/GSH was added after inhibiting the bath PKA with PKI. Data are normalized to currents measured just before the addition of diamide/GSH. (C) Representative current trace showing resistance of C1344A-CFTR to inhibition by diamide/GSH. (D) Mean data comparing the sensitivities of WT CFTR and C1344A-CFTR to inhibition by diamide/GSH (20 μM), GSNO (200 μM), and GSSG (20 mM).

structural elements from other regions of the polypeptide may influence the susceptibility of this residue to oxidation (see below and DISCUSSION).

CFTR Channels That Are First "Locked Open" by a Poorly Hydrolyzable ATP Analogue Are Protected from Inhibition by Glutathionylation

The major cytoplasmic domains that control CFTR channel gating are the NBDs and the R domain. If glutathionylation occurs at a site near one of the ATP binding pockets in an NBD rather than near one of the phosphorylation sites in the R domain, one would predict that (a) an R domain deletion mutant that does not require PKA phosphorylation for channel activation would also be inhibited by glutathionylation, and (b) channels that are locked upon with a poorly hydrolyzable ATP analogue (AMP-PNP) that binds stably to the channel would be protected from this inhibition. Regarding the latter prediction, the signature sequences of Rad50 are buried in the AMP-PNP–bound form of this ATPase (Hopfner et al., 2000). Fig. 9 (A and B) shows that S660A/ΔR-CFTR, which was previously shown to have moderate channel activity in the absence of PKA (Rich et al., 1991), was substantially and reversibly inhibited by diamide/GSH in the absence of added kinase. This result argues against a primary effect of thiolation on CFTR phosphorylation. Interestingly, Grx could rescue completely the currents mediated by S660A/ΔR-CFTR, which indicates that the Grx-insensitive oxidation previously observed for the WT channel (Fig. 4) requires an intact R domain. Fig. 9 (C and D) shows that the rate of channel inhibition by diamide/GSH was slowed ~10-fold in the presence of AMP-PNP. This poorly hydrolyzable analogue greatly prolongs CFTR channel openings apparently by stably binding to one or both NBDs (Gunderson and Kopito, 1994; Aleksandrov et al., 2001). The time constant for current inhibition in the presence of AMP-PNP (ca. 250 s) is similar to the "off rate" for AMP-PNP observed in previous washout or deactivation experiments (Powe et al., 2002). We interpret the AMP-PNP results to indicate that open channels are protected from inhibition by thiolation. The gradual inhibition that is observed in the presence of AMP-PNP likely reflects the modification of channels that have closed at a very slow rate due to the slow dissociation and/or hydrolysis of AMP-PNP.

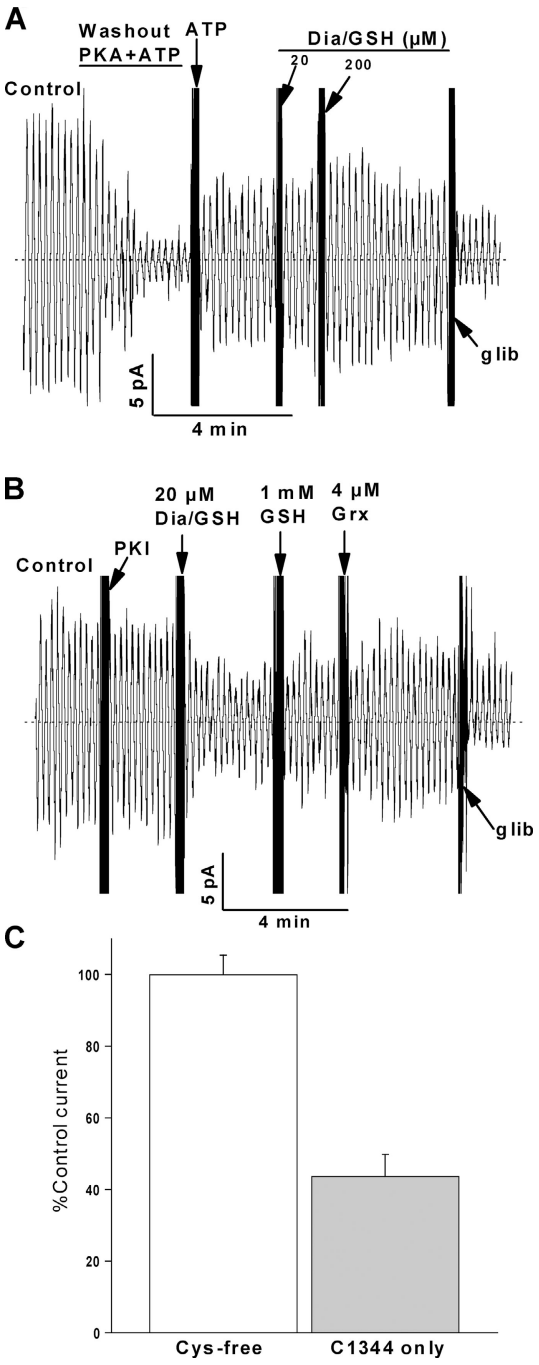

FIGURE 8. Cys-1344 is sufficient for reactive glutathione species to inhibit CFTR currents. Cys-free CFTR and cys-1344-only CFTR were expressed in HEK-293T cells as described in the text. (A) Representative current trace showing lack of inhibition of cys-free CFTR by diamide/GSH. To confirm that the observed currents were CFTR-mediated, PKA and MgATP were washed out of the bath and 1.5 mM MgATP was readded where indicated. (B) Representative current trace showing inhibition of the current mediated by the cys-1344-only construct by diamide/GSH. The currents mediated by both constructs were blocked by glibenclamide in a voltage-dependent manner, although they were inhibited by PKI to a smaller degree than that observed for WT channels (possibly because of the altered conformations of these ER processing mutants). (C) Mean data showing percent current remaining after the addition of 20 μM diamide/GSH (cys-free, $n = 7$; cys-1344-only, $n = 8$).

This protective effect of the poorly hydrolyzable AMP-PNP is consistent with modification at cys-1344, since the signature sequence in NBD2 likely is occluded in the nucleotide-bound state of the channel (see schematic in Fig. 10).

DISCUSSION

The present results indicate that human CFTR channels are reversibly inhibited by several reactive forms of glutathione. The underlying mechanism appears to involve the glutathionylation of cys-1344 near the signature sequence in NBD2. Inhibited CFTR channels exhibit very low open rates, but can be rescued by several glutaredoxins, including a mutant form that is a selective mixed disulfide reductase. This, coupled with biochemical evidence for the reversible glutathionylation of CFTR protein in microsomes, supports the argument that channel activity is inhibited by glutathionylation of a cysteine or cysteines within the CFTR polypeptide. We were unable to directly detect the glutathionylation of cys-1344 within the intact CFTR protein due to technical reasons. However, the fact that C1344A-CFTR was the only cysteine mutant that was highly resistant to inhibition by all three reactive glutathione species (GSNO, GSSG, and diamide/GSH) indicates that this cysteine likely is the functionally important site for glutathionylation. To our knowledge, this is the first evidence for the modulation of an ABC transporter by glutathionylation. The present findings provide insights into the structural basis of CFTR channel gating by supporting an important role of NBD2 (in particular, the region surrounding cys-1344) in controlling the opening of CFTR channels. Our results also raise the possibility that glutathionylation at this site may reversibly silence CFTR channels under strongly oxidizing conditions (e.g., in inflamed lung or gut). These implications are discussed in more detail below.

How Does CFTR Glutathionylation Disrupt Channel Gating?

CFTR channel opening is controlled primarily by two factors: MgATP binding to both NBDs and phosphorylation of the R domain (Gadsby and Nairn, 1999). Two observations rule out the possibility that the markedly reduced open rate of the glutathionylated CFTR channel is due to altered phosphorylation. First, CFTR channels could be inhibited by glutathionylation and subsequently rescued by Grx or DTT after PKA inhibition or removal. Second, an R domain deletion construct that does not require phosphorylation for its activity also was inhibited by glutathionylation. These results point to an effect of glutathionylation on nucleotide binding or a downstream event that couples ATP binding to channel opening.

The location of the apparent site of modification (cys-1344) near the signature sequence in NBD2 is con-

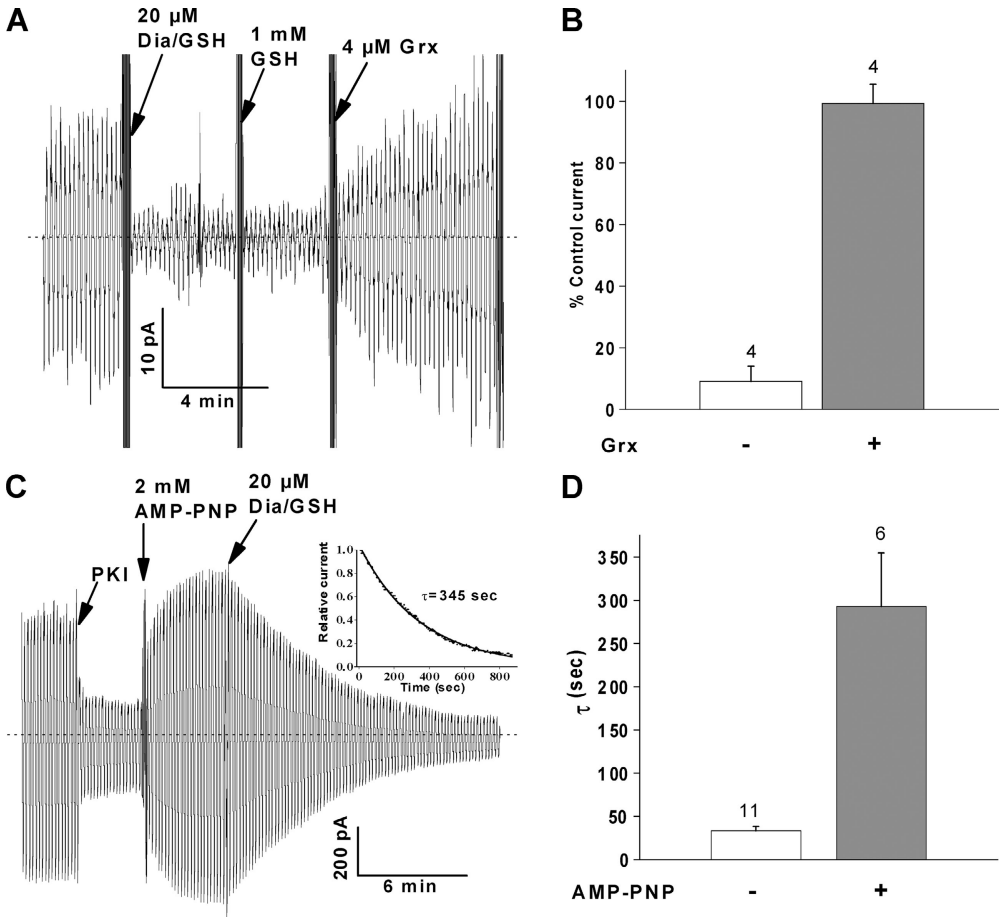

FIGURE 9. An R domain deletion mutant is highly sensitive to diamide/GSH inhibition, whereas channels that are locked open by AMP-PNP are protected. (A) Inhibitory effect of 20 μM diamide/GSH on macroscopic current mediated by ΔR-S660A-CFTR in patch excised from transfected HEK-293T cell. (B) Mean data for ΔR-S660A-CFTR showing inhibition by diamide/GSH (20 μM) and recovery by *E. coli* Grx1 (4 μM) plus 1 mM GSH. Currents are normalized to control currents before diamide/GSH. Currents were activated with 1.5 mM MgATP in the absence of PKA for the experiments summarized in A and B. (C) Slow inhibition of CFTR currents by diamide/GSH following addition of 2 mM AMP-PNP to BHK-CFTR patch. Inset, exponential fit of current trace following addition of diamide/GSH to this patch. (D) Mean data comparing the exponential time constants for current inhibition by 20 μM diamide/GSH in the presence and absence of 2 mM AMP-PNP. Note that the time constants in the absence of AMP-PNP are overestimates due to the very rapid inhibition observed under the control condition.

sistent with an effect of glutathionylation on ATP-dependent channel opening. Current structural models of other ABC transporters (e.g., the bacterial BtuCD and maltose transporters) place their signature sequences at an interface between the two NBDs, which associate as dimers in these transporters (Locher et al., 2002; Chen et al., 2003). The signature sequence of one NBD opposes the Walker A motif (or P loop) of the other NBD to form a composite nucleotide binding pocket. Residues within each of these sequences or motifs interact directly with ATP (Fig. 10, A and B). It is likely that some or all of the basic elements of this model apply to the CFTR NBDs as well. Although there is little structural or physical evidence for dimerization of the CFTR NBDs (due perhaps to technical reasons), there is considerable functional evidence for cooperative interactions between the two CFTR NBDs regarding both channel gating and nucleotide binding/hydrolysis (e.g., Ramjeesingh et al., 1999; Powe et al., 2002; Vergani et al., 2003; Kidd et al., 2004). And, there is good evidence that the signature sequences of the two CFTR NBDs play important roles in channel gating. For example, disease-associated mutations within the NBD1 signature sequence (e.g., G551D) dramatically inhibit the rate of CFTR channel opening (Li et al., 1996). In addition, Cotton and Welsh (1998) reported that introducing an engineered cysteine into the signature sequence of NBD2 sensitized CFTR channels to inhibition by NEM. The apparent location of glutathionylation to cys-1344 is consistent with the observation that CFTR channels that are first locked open with the poorly hydrolyzable AMP-PNP are protected from inhibition. AMP-PNP locks open CFTR channels by stably associating with one or both NBDs (Gunderson and Kopito, 1994; Aleksandrov et al., 2001), which, according to the structural models described above, would be expected to occlude residues within and near the signature sequences.

Given these considerations, it is perhaps not surprising that the glutathionylation of a cysteine near one of

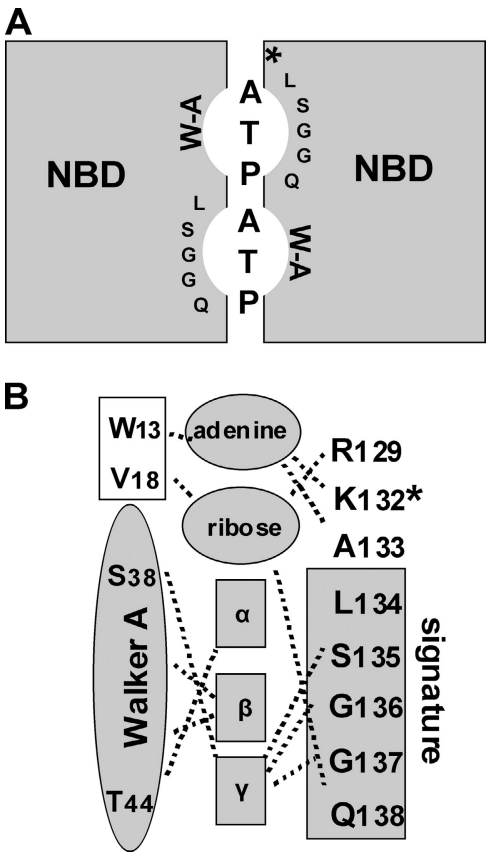

FIGURE 10. Schematic views of the regions proximal to the signature sequences in related ABC transporters. (A) Schematic view of ATP binding pockets sandwiched between NBD dimers based on structures of Rad50 (Hopfner et al., 2000) and MalK (Chen et al., 2003). (B) ATP binding pocket in the MalK dimer of the bacterial maltose transporter (adapted from Chen et al., 2003). Asterisks denote the positions corresponding to cys-1344 in CFTR NBD2.

the signature sequences could disrupt ATP-dependent channel opening. However, it should be emphasized that the cysteine at this position (cys-1344) is not essential for CFTR channel activity per se. CFTR mutants that lack this residue exhibit robust channel activity (Fig. 7), and the apparent modification of cys-1344 by NEM (which protects against modification of this site) only modestly affects the channel activity of WT CFTR. Furthermore, this cysteine is poorly conserved among CFTR polypeptides across species or among other ABC transporters (Fig. 7 A). Based on these observations, it seems likely that glutathionylation at this site affects channel gating because of the bulk and/or hydrophilicity of the covalently linked adduct (i.e., glutathione tripeptide). Such a substantial modification could have large effects on the local structure of the CFTR polypeptide within the vicinity of the NBD2 signature sequence.

This putative structural perturbation in NBD2 could inhibit the opening of CFTR channels in one of two ways: (1) by reducing the affinity of the NBDs for MgATP or (2) by disrupting the link between ATP binding at the NBDs and subsequent pore opening. The first possibility is worth considering given the apparent roles of the signature sequences of ABC transporters in ATP binding, as discussed above. Indeed, within the crystal structure of the ATP-bound MalK dimer of the bacterial maltose transporter (Chen et al., 2003), the residue that corresponds in position to cys-1344 of CFTR (K132 in *E. coli* MalK) makes direct contact with the adenine of the bound ATP (Fig. 10 B). Given this comparison, it seems possible that the glutathionylated CFTR channel would have a lower affinity for ATP especially with respect to nucleotide binding to the Walker A motif in NBD1 (which would oppose the NBD2 signature sequence in current models). However, there are several arguments against a primary effect of CFTR glutathionylation on ATP binding. First, we could not stimulate the low activity of glutathionylated CFTR channels by elevating the bath MgATP to concentrations above those that normally saturate CFTR (i.e., from 1.5 to 10 mM; unpublished data). Thus, if the primary effect of glutathionylation is to reduce the affinity for MgATP, then this effect must be sufficiently great to prevent a detectable increase in channel activity in response to a greater than sixfold increase in MgATP concentration. Second, the binding interaction with ATP is dominated by the highly conserved Walker A motifs and signature sequences that are found in all ABC transporters (Fig. 10, A and B). The region proximal to the signature sequence that includes cys-1344 in CFTR is not well conserved among ABC transporters. This lack of conservation of primary sequence would seem to be more consistent with a role for this region as a linker or "relay" rather than in nucleotide binding per se.

On the basis of the latter considerations and the arguments below we favor the second possible mechanism, i.e., disruption of the link between ATP binding and pore opening in the glutathionylated channel. The region proximal to the signature sequence of the nucleotide-free form of the bacterial vitamin B12 transporter (BtuCD) lies adjacent to the Q loop, which is the major structural element linking the NBDs to the transmembrane domains (Locher et al., 2002). Although currently there is no crystal structure of the ATP-bound form of a complete ABC transporter, Chen et al. (2003) have generated a structural model of the ATP-bound form of the bacterial maltose transporter in which the region proximal to the signature motif (corresponding to the location of cys-1344) similarly is positioned close to the Q loops. Thus, the residues in the region proximal to the signature sequence seem to be well positioned both to sense the binding of ATP and to relay this information to downstream elements (e.g., Q

loops) that presumably mediate channel opening. That the putative Q loops in CFTR participate in channel gating is supported by the observations of Berger et al. (2002), who reported that mutations within the region of CFTR NBD2 that corresponds to the Q loop of BtuCD disrupt channel gating. Glutathionylation of cys-1344 within CFTR could perturb the ability of this residue and neighboring residues to interact with the bound ATP (i.e., to sense ATP binding) and/or to interact with the Q loops. This hypothetical mechanism is consistent with our functional data and with the available structural data for other ABC transporters as noted above. However, without additional biochemical data we cannot completely rule out other possible mechanisms such as reduced ATP binding (see above) and/or inhibitory effects of glutathionylation on NBD dimerization.

Physiologic Implications

The CFTR channel is expressed in lung and gut; tissues that are continually exposed to thiol oxidizers under a variety of inflammatory conditions (e.g., asthma and colitis; Montuschi and Barnes, 2002; Pavlick et al., 2002). Reactive glutathione species are formed during inflammation, some of which have the potential to glutathionylate target proteins within these tissues. Although intracellular levels of glutathione disulfide (GSSG) increase in inflamed tissues (Sido et al., 1998), this form of glutathione is not a particularly potent mediator of protein glutathionylation (e.g., Fig. 1) and probably would have little or no effect on CFTR glutathionylation in vivo. However, other more reactive forms of glutathione (e.g., GSNO or GS(O)SG) can be produced during inflammation, some of which have the potential to glutathionylate CFTR channels (Huang and Huang, 2002). CFTR channels are protected to a large extent by the actions of GSH and glutaredoxin; this was one of the findings of the present study. Presumably these factors help prevent inhibition of CFTR channel activity by weak oxidizers or during short term exposure to oxidants. In this regard, the acute treatment of intact Calu-3 epithelial monolayers with H_2O_2 reportedly increases rather than decreases cAMP-dependent anion secretion (Cowley and Linsdell, 2002). However, it is possible that the protective effects of Grx and GSH are overwhelmed under highly oxidizing conditions and/or during chronic exposure to oxidants. Human Grx1 itself can be inhibited by oxidation (Starke et al., 1997) such that its protective effects may become limiting during chronic exposure to strong oxidants. Further studies will be required to determine the extent to which CFTR channels are modulated by glutathionylation in vivo (e.g., using animal models of inflammation). This is a potentially significant issue, since the silencing of CFTR channels by glutathionylation could influence the physiology of the inflamed lung or gut (e.g., by minimizing CFTR-mediated fluid secretion as a mechanism to counteract diarrhea in colitis).

The present findings have additional implications with respect to the recent interest in glutathione species as modulators of CFTR biogenesis and as transported substrates of CFTR. Our observation that GSNO (or one of its metabolites) can inhibit CFTR activity raises a note of caution about using this glutathione species to treat CF patients (Zaman et al., 2001). On the positive side, the concentrations of GSNO that reportedly are effective in promoting the biogenesis of mutant CFTR are quite low and may be below those GSNO concentrations that would lead to appreciable CFTR glutathionylation. Regarding the intriguing possibility that CFTR may itself transport various forms of glutathione out of cells (Kogan et al., 2003), it would seem important to determine the extent to which glutathionylation influences this putative functional attribute of CFTR. Perhaps oxidized forms of glutathione inhibit GSH export by CFTR as a negative feedback mechanism to preserve the reducing environment of the epithelial cell cytoplasm.

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REFERENCES

Aleksandrov, L., A. Mengos, X.-B. Chang, A. Aleksandrov, and J.R. Riordan. 2001. Differential interactions of nucleotides at the two nucleotide binding domains of the cystic fibrosis transmembrane conductance regulator. *J. Biol. Chem.* 276(16):12918–12923.

Aracena, P., G. Sanchez, P. Donoso, S.L. Hamilton, and C. Hidalgo. 2003. *S*-glutathionylation decreases Mg^{2+} inhibition and *S*-nitrosylation enhances Ca^{2+} activation of RyR1 channels. *J. Biol. Chem.* 278(44):42927–42935.

Barrett, W.C., J.P. DeGnore, S. König, H.M. Fales, Y.-F. Keng, Z.-Y. Zhang, M.B. Yim, and P.B. Chock. 1999. Regulation of PTP1B via glutathionylation of the active site cysteine 215. *Biochemistry.* 38: 6699–6705.

Berger, A.L., M. Ikuma, J.F. Hunt, P.J. Thomas, and M.J. Welsh. 2002. Mutations that change the position of the putative γ-phosphate linker in the nucleotide binding domains of CFTR alter channel gating. *J. Biol. Chem.* 277(3):2125–2131.

Bushweller, J.H., F. Åslund, K. Wüthrich, and A. Holmgren. 1992. Structural and functional characterization of the mutant *Escherichia coli* glutaredoxin (C14S) and its mixed disulfide with glutathione. *Biochemistry.* 31:9288–9293.

Caplan, J.F., N.R. Filipenko, S.L. Fitzpatrick, and D.M. Waisman. 2004. Regulation of annexin A2 by reversible glutathionylation. *J. Biol. Chem.* 279(9):7740–7750.

Carson, M.R., S.M. Travis, and M.J. Welsh. 1995. The two nucleotide-binding domains of cystic fibrosis transmembrane conductance regulator (CFTR) have distinct functions in controlling channel activity. *J. Biol. Chem.* 270(4):1711–1717.

Chappe, V., D.A. Hinkson, T. Zhu, X.-B. Chang, J.R. Riordan, and J.W. Hanrahan. 2003. Phosphorylation of protein kinase C sites in NBD1 and the R domain control CFTR channel activation by PKA. *J. Physiol.* 548(1):39–52.

Chen, J., G. Lu, J. Lin, A.L. Davidson, and F.A. Quiocho. 2003. A tweezers-like motion of the ATP-binding cassette dimer in an ABC transport cycle. *Mol. Cell.* 12:651–661.

Cotton, J.F., and M.J. Welsh. 1997. Covalent modification of the regulatory domain irreversibly stimulates cystic fibrosis transmembrane conductance regulator. *J. Biol. Chem.* 272(41):25617–25622.

Cotton, J.F., and M.J. Welsh. 1998. Covalent modification of the nucleotide binding domains of cystic fibrosis transmembrane conductance regulator. *J. Biol. Chem.* 273(48):31873–31879.

Cowley, E.A., and P. Linsdell. 2002. Oxidant stress stimulates anion secretion from the human airway epithelial cell line Calu-3: implications for cystic fibrosis lung disease. *J. Physiol.* 543(1):201–209.

Fernandes, A.P., and A. Holmgren. 2004. Glutaredoxins: glutathione-dependent redox enzymes with functions far beyond a simple thioredoxin backup system. *Antioxid. Redox Signal.* 6(1): 63–74.

Gabriel, S.E., K.N. Brigman, B.H. Koller, R.C. Boucher, and M.J. Stutts. 1994. Cystic fibrosis heterozygote resistance to cholera toxin in the cystic fibrosis mouse model. *Science.* 266(5182):107–109.

Gadsby, D.C., and A.C. Nairn. 1999. Control of CFTR channel gating by phosphorylation and nucleotide hydrolysis. *Physiol. Rev.* 79:S77–S107.

Gregory, R.J., D.P. Rich, S.H. Cheng, D.W. Souza, S. Paul, P. Manavalan, M.P. Anderson, M.J. Welsh, and A.E. Smith. 1991. Maturation and function of cystic fibrosis transmembrane conductance regulator variants bearing mutations in putative nucleotide-binding domains 1 and 2. *Mol. Cell. Biol.* 11:3886–3893.

Griffith, S.W., J. King, and C.L. Cooney. 2002. The reactivity and oxidation pathway of cysteine 232 in recombinant human α1-antitrypsin. *J. Biol. Chem.* 277:25486–25492.

Gunderson, K.L., and R.R. Kopito. 1994. Effects of pyrophosphates and nucleotide analogs suggest a role for ATP hydrolysis in cystic fibrosis transmembrane regulator channel gating. *J. Biol. Chem.* 269:19349–19353.

Gunderson, K.L., and R.R. Kopito. 1995. Conformational states of CFTR associated with channel gating: the role of ATP binding and hydrolysis. *Cell.* 82(2):231–239.

Harrington, M.A., K.L. Gunderson, and R.R. Kopito. 1999. Redox reagents and divalent cations alter the kinetics of cystic fibrosis transmembrane conductance regulator channel gating. *J. Biol. Chem.* 274:27536–27544.

Higgins, C.F. 1992. ABC transporters: from microorganisms to man. *Annu. Rev. Cell Biol.* 8:67–113.

Hopfner, K.-P., A. Karcher, D.S. Shin, L. Craig, L.M. Arthur, J.P. Carney, and J.A. Tainer. 2000. Structural biology of Rad50 ATPase: ATP-driven conformational control in DNA double-strand break repair and the ABC-ATPase superfamily. *Cell.* 101:789–800.

Huang, K.-P., and F.L. Huang. 2002. Glutathionylation of proteins by glutathione disulfide S-oxide. *Biochem. Pharmacol.* 64:1049–1056.

Humphries, K.M., C. Juliano, and S.S. Taylor. 2002. Regulation of cAMP-dependent protein kinase activity by glutathionylation. *J. Biol. Chem.* 277(45):43505–43511.

Kidd, J.F., M. Ramjeesingh, F. Stratford, L.-J. Huan, and C.E. Bear. 2004. A heteromeric complex of the two nucleotide binding domains of cystic fibrosis transmembrane conductance regulator (CFTR) mediates ATPase activity. *J. Biol. Chem.* 279(40):41664–41669.

Kogan, I., M. Ramjeesingh, C. Li, J.F. Kidd, Y. Wang, E.M. Leslie, S.P. Cole, and C.E. Bear. 2003. CFTR directly mediates nucleotide-regulated glutathione flux. *EMBO J.* 22(9):1981–1989.

Kosower, N.M., and E.M. Kosower. 1995. Diamide: an oxidant probe for thiols. *Methods Enzymol.* 251:123–133.

Li, C., M. Ramjeesingh, W. Wang, E. Garami, M. Hewryk, D. Lee, J.M. Rommen, K. Galley, and C.E. Bear. 1996. ATPase activity of the cystic fibrosis transmembrane conductance regulator. *J. Biol. Chem.* 271(45):28463–28468.

Li, J., F.L. Huang, and K.-P. Huang. 2001. Glutathiolation of proteins by glutathione disulfide *S*-oxide derived from *S*-nitrosoglutathione. *J. Biol. Chem.* 276:3098–3105.

Lind, C., R. Gerdes, Y. Hamnell, I. Schuppe-Koistinen, H. Brockenhuus, H.B. von Lowenhielm, A. Holmgren and I.A. Cotgreave. 2002. Identification of S-glutathionylated cellular proteins during oxidative stress and constitutive metabolism by affinity purification and proteomic analysis. *Arch. Biochem. Biophys.* 406:229–240.

Linsdell, P., and J.W. Hanrahan. 1998. Glutathione permeability of CFTR. *Am. J. Physiol.* 275:C323–C326.

Locher, K.P., A.T. Lee, and D.C. Rees. 2002. The *E. coli* BtuCD structure: a framework for ABC transporter architecture and mechanism. *Science.* 296:1091–1098.

Mense, M., P. Vergani, D.M. White, G. Altberg, A.C. Nairn, and D.C. Gadsby. 2004. Sulfhydryl-specific cross-linking establishes Rad50-like interactions between NBD1 and NBD2 in CFTR chloride channels. *Biophys. J.* 86:332a.

Montuschi, P., and P.J. Barnes. 2002. Analysis of exhaled breath condensate for monitoring airway inflammation. *Trends Pharmacol. Sci.* 23:232–237.

Nordstrand, K., F. Åslund, A. Holmgren, G. Otting, and K.D. Berndt. 1999. NMR structure of *Escherichia coli* glutaredoxin 3-mixed disulfide complex: implications for the enzymatic mechanism. *J. Mol. Biol.* 286:541–552.

Pavlick, K.P., F.S. Laroux, J. Fuseler, R.E. Wolf, L. Gray, J. Hoffman, and M.B. Grisha. 2002. Role of reactive metabolites of oxygen and nitrogen in inflammatory bowel disease. *Free Radic. Biol. Med.* 33:311–322.

Pompella, A., A. Visvikis, A. Paolicchi, V. DeTata, and A.F. Casini. 2003. The changing faces of glutathione, a cellular protagonist. *Biochem. Pharmacol.* 66:1499–1503.

Powe, A.C., L. Al-Nakkash, M. Li, and T.-C. Hwang. 2002. Mutation of Walker-A lysine 464 in cystic fibrosis transmembrane conductance regulator reveals functional interaction between its nucleotide-binding domains. *J. Physiol.* 539:333–346.

Ramjeesingh, M., C. Li, E. Garami, L.J. Huan, K. Galley, Y. Wang, and C.E. Bear. 1999. Walker mutations reveal loose relationships between catalytic and channel-gating activities of purified CFTR (cystic fibrosis transmembrane conductance regulator). *Biochemistry.* 38(5):1463–1468.

Rich, D.P., R.J. Gregory, M.P. Anderson, P. Manavalan, A.E. Smith, and M.J. Welsh. 1991. Effect of deleting the R domain on CFTR-generated chloride channels. *Science.* 253:205–207.

Riordan, J.R., J.M. Rommens, B.-S. Kerem, N. Alon, R. Rozmahel, Z. Grzelczak, J. Zielenski, S. Lok, N. Plavsic, J.-L. Chou, et al. 1989. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science.* 245:1066–1073.

Schrammel, A., A.C. Gorren, K. Schmidt, S. Pfeiffer, and B. Mayer.

2003. S-nitrosation of glutathione by nitric oxide, peroxynitrite, and NO/O_2^-. *Free Radic. Biol. Med.* 34:1078–1088.

Sheppard, D.N., and M.J. Welsh. 1999. Structure and function of the CFTR chloride channel. *Physiol. Rev.* 79:S23–S45.

Sido, B., V. Hack, A. Hochlehnert, H. Lipps, C. Herfarth, and W. Droge. 1998. Impairment of intestinal glutathione synthesis in patients with inflammatory bowel disease. *Gut.* 42(4):485–492.

Starke, D.W., Y. Chen, C.P. Bapna, E.J. Lesnefsky, and J.J. Mieyal. 1997. Sensitivity of protein sulfhydryl repair enzymes to oxidative stress. *Free Rad. Biol. Med.* 23:373–384.

Taylor, E.R., F. Hurrell, R.J. Shannon, T.-K. Lin, J. Hirst, and M.P. Murphy. 2003. Reversible glutathionylation of Complex I increases mitochondrial superoxide formation. *J. Biol. Chem.* 278: 19603–19610.

Thomas, J.A., B. Poland, and R. Honzatko. 1995. Protein sulfhydryls and their role in the antioxidant function of protein S-thiolation. *Arch. Biochem. Biophys.* 319:1–9.

Vergani, P., A.C. Nairn, and D.C. Gadsby. 2003. On the mechanism of MgATP-dependent gating of CFTR Cl^- channels. *J. Gen. Physiol.* 121(1):17–36.

Wang, J., E.S. Boja, W. Tan, E. Tekle, H.M. Fales, S. English, J.J. Mieyal, and P.B. Chock. 2001. Reversible glutathionylation regulates actin polymerization in A431 cells. *J. Biol. Chem.* 276(51): 47763–47766.

Welsh, M.J., and A.E. Smith. 1993. Molecular mechanisms of CFTR chloride channel dysfunction in cystic fibrosis. *Cell.* 73:1251–1254.

Zaman, K., M. McPherson, J. Vaughan, J. Hunt, F. Mendes, B. Gaston, and L.A. Palmer. 2001. S-nitrosoglutathione increases cystic fibrosis conductance regulator maturation. *Biochem. Biophys. Res. Commun.* 284:65–70.