S-Nitrosylation Regulates Nuclear Translocation of Chloride Intracellular Channel Protein CLIC4**

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Nuclear translocation of chloride intracellular channel protein CLIC4 is essential for its role in Ca2+-induced differentiation, stress-induced apoptosis, and modulating TGF-β signaling in mouse epidermal keratinocytes. However, post-translational modifications on CLIC4 that govern nuclear translocation and thus these activities remain to be elucidated. The structure of CLIC4 is dependent on the redox environment, in vitro, and translocation may depend on reactive oxygen and nitrogen species in the cell. Here we show that NO directly induces nuclear translocation of CLIC4 that is independent of the NO-cGMP pathway. Indeed, CLIC4 is directly modified by NO through S-nitrosylation of a cysteine residue, as measured by the biotin switch assay. NO enhances association of CLIC4 with the nuclear import proteins importin α and Ran. This is likely a result of the conformational change induced by S-nitrosylated CLIC4 that leads to unfolding of the protein, as exhibited by CD spectra analysis and trypsinolysis of the modified protein. Cysteine mutants of CLIC4 exhibit altered nitrosylation, nuclear residence, and stability, compared with the wild type protein likely as a consequence of altered tertiary structure. Moreover, tumor necrosis factor α-induced nuclear translocation of CLIC4 is dependent on nitric-oxide synthase activity. Inhibition of nitric-oxide synthase activity inhibits tumor necrosis factor α-induced nitrosylation and association with importin α and Ran and ablates CLIC4 nuclear translocation. These results suggest that S-nitrosylation governs CLIC4 structure, its association with protein partners, and thus its intracellular distribution.

CLIC4 (chloride intracellular channel protein 4) belongs to a family of differentially expressed chloride channel proteins that are largely conserved from Caenorhabditis elegans to humans.

Various members have been implicated in cell signaling cascades, ranging from stimulation of chloride channel activity by CLIC3 following association with extracellular signal-regulated kinase (ERK) 7 (1) to CLIC5A that is implicated in bone resorption and HCl transport in osteoclasts, in response to phosphorylation by c-Src (2). However, a number of functions of CLIC proteins are independent of channel activity (3, 4).

The best characterized of the CLIC family members is CLIC4, identified in our laboratory as a p53 and TNFα2 response gene that is up-regulated during keratinocyte differentiation. CLIC4 has emerged as a crucial player in many physiological processes, including tubular morphogenesis during angiogenesis (5–7), transdifferentiation of mammary fibroblasts to myofibroblasts in response to TGF-β (8), and adipocyte differentiation (9). CLIC4 has been established as a significant effector of mouse and human keratinocyte differentiation, associated with G1 cell cycle arrest and expression of differentiation markers (10).

An intriguing aspect of CLIC4 biology is its role as an effector of apoptosis, including p53- and c-Myc-induced apoptosis, as well as in response to cytotoxic and genotoxic stress (11, 12). Not surprisingly then, altered CLIC4 expression and distribution has been identified in pathophysiological states like cancer. Progressively decreased CLIC4 expression in the epithelium of many human cancers with a reciprocal increase in the associated tumor stroma are concurrent with increasing tumor grade and poor prognosis (13). Moreover, the subcellular localization of the protein is largely cytoplasmic in tumor epithelium compared with matched normal tissue where it is mainly nuclear in quiescent cells. These data suggest that both the level and the subcellular location of CLIC4 are important to maintain normal cellular homeostasis.

An important aspect of CLIC4 function in apoptosis and differentiation is its ability to translocate to the nucleus upon appropriate stimulus. The protein is present both in the cytosol and within the membranes of the mitochondria and the ER. The

2 The abbreviations used are: TNF, tumor necrosis factor; SNO, S-nitrosylation; NOS, nitric-oxide synthase; TGF, transforming growth factor; NLS, nuclear localization signal; HA, hemagglutinin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; WT, wild type; PMSF, phenylmethylsulfonyl fluoride; RIPA, radiommune precipitation assay; GSNO, nitrosothiol S-nitrosoglutathione; 8-pCPT-cGMP, 8-(4-chlorophenylthio)guanosine-3′,5′-monophosphate.
Soluble, cytosolic portion translocates to the nucleus upon specific stimuli. Nuclear import of CLIC4 involves association of its nuclear localization signal (NLS) with nucleoporins, including importin α and Ran (14). Nuclear targeting of CLIC4 enhances apoptosis in many cell types, whereas inhibiting translocation reduces the apoptotic potential (14). Thus, signaling pathways and post-translational modification(s) modulating CLIC4 nuclear import are extremely attractive targets for cancer therapy.

Soluble CLIC4 shows structural homology with the glutathione S-transferase ω superfamily (15). More importantly, the protein undergoes redox-sensitive structural changes in vitro, showing enhanced membrane association and channel activity under oxidative conditions (15). The NLS lies buried in the structure of the soluble protein, and it has been suggested that the soluble protein would have to unfold and extend for the NLS to associate with nuclear pore proteins like importin α and Ran (15). Thus, we hypothesized that CLIC4 nuclear import may be determined by the cellular redox that may induce unfolding of the protein to expose the NLS.

MATERIALS AND METHODS

Reagents—NO donors lactacystin and YC-1 were purchased from Alexix (San Diego, CA), whereas 8-pCPT-cGMP was from BioLOG. Mouse TNFα was from Peprotech (Rocky Hill, NJ). MG132 was purchased from Calbiochem. All of the reagents used for the biotin switch assay were purchased from Pierce. Anti-HA and β-actin antibodies were purchased from Abcam, lamin A/C antibody was from Santa Cruz Biotechnology, and α-tubulin was from Invitrogen. GAPDH antibody was from Chemicon. Fluorescein isothiocyanate-labeled secondary anti-rabbit antibody was from Vector Laboratories. Rabbit polyclonal CLIC4 N-terminal antibody was prepared by Veritas (Rockville, MD). Trypsin was purchased from Thermo Scientific (Rockford, IL), and A-phosphatase was from New England Biolabs (Ipswich, MA).

Plasmid Construction—Mutagenic primers for cysteine to alanine mutants were as follows: C35A primer, 5′-CTG TGA AAA GGG GGC GTT TTC AAT GC-3′; C100A primer, 5′-GGT ACT TGG GTG GGG CCA AGA CTT CTG CG-3′; and C234A primer, 5′-CGG TGT CGC TGG GAG CGG TGT TGG TGA AC-3′. Wild type CLIC4 cloned into the pALTERMAX plasmid (BD Biosciences), with an HA epitope at the N terminus, was used as the starting vector. An Altered Sites II mutagenesis kit (Promega) was used to generate the mutants. A cysteine 35 to serine mutant and a cysteine 35 and 234 to serine double mutant were generated by GM Biosciences (Rockville, MD). Cys 100 to alanine and Cys 189 to alanine mutants were generated by TOP Gene Technologies (Quebec, Canada). All of the mutant constructs were confirmed by sequencing. For bacterial expression, wild type CLIC4 plasmid preparation has been published previously (15), whereas CLIC4 cysteine to serine (C35S, C234S, and C35S/C234S) mutants for bacterial expression were generated by TOP Gene Technologies.

Purification and in Vitro Nitrosylation of Recombinant CLIC4—CLIC4 was expressed as glutathione S-transferase fusion protein in Escherichia coli BL21 (DE3). The cultures were grown in LB medium containing 100 mg/ml ampicillin at 37 °C until A600 reached 0.6, followed by induction with 1 mM isopropyl β-d-thiogalactopyranoside at 37 °C for 3 h. CLIC4 purification was done using methods described previously (15) with modification. Briefly, cell pellet was resuspended in lysis buffer (phosphate-buffered saline (Amresco), Complete, EDTA-free Protease Inhibitor (Roche Applied Science), and 0.3 mM dithiothreitol) before lysis. After lysis, polyethyleneimine (Sigma) was added to a final concentration of 0.15%, and then lysate was cleared by centrifugation. Supernatant was incubated with glutathione-Sepharose 4B (GE Healthcare) for 1 h at room temperature. The bound fusion protein was cleaved by thrombin (Sigma) at room temperature overnight. Eluted protein was run through Superdex75 gel filtration column (GE Healthcare) at 4 °C using Buffer A (20 mM HEPES, pH 7, 100 mM KC1, 1 mM dithiothreitol).

Synthesis of S-Nitrosothioglutathione (GSNO) for in Vitro Nitrosylation—GSNO was synthesized using 100 mM GSH and 105 mM NaNO2 in the presence of 0.5 mM EDTA. HCl was added into solution until the pH reached 1.5. The solution was then incubated at room temperature for 5 min. Upon this, the pH was adjusted to 7 by the addition of KOH. GSNO was quantified using UV spectroscopy (16).

In Vitro Nitrosylation of CLIC4—After gel filtration, the fractions corresponding to CLIC4 were collected and dialyzed against Buffer B (20 mM HEPES, pH 7, 100 mM KC1, 0.5 mM CaCl2, 0.5 mM EDTA). All of the steps after this were performed in the dark. GSNO was added to protein at a CLIC4:GSNO ratio of 1:200 (1 CLIC4 cysteine to 50 GSNO). Protein/GSNO solution was incubated at room temperature for 15 min. The solution was loaded into Superdex75 gel filtration column, and protein was eluted using Buffer B. Fractions corresponding to CLIC4 were collected and concentrated. The extent of CLIC4 nitrosylation was quantified by UV spectroscopy at 340 nm (17).

CD Analysis of Nitrosylated CLIC4—CD experiments (spectroscopy and thermal denaturation) were performed with a Jasco J-810 CD Spectropolarimeter fitted with Peltier temperature controller. Protein was diluted in 20 mM phosphate buffer, pH 7, to a concentration of ~0.2 mg/ml in all CD experiments. The spectra of CLIC4-WT (untreated CLIC4) and CLIC4-SNO (nitrosylated CLIC4) were collected in the far UV region at 190–260 nm with 50 nm/min scanning rate; the scans were repeated three times and then averaged. The CD spectra were converted to mean residue ellipticity [θ] using the following formula,

\[
[\theta] = \frac{CD-MM}{10lcr}
\]

where [θ] is the mean residue ellipticity (deg cm² dmol⁻¹), CD is observed ellipticity (in millidegree/mdeg), MM is the molecular mass of protein (Dalton), l is the path length (cm), c is the protein concentration (mg/ml), and r is the number of residues in the protein (18).

Thermal denaturation experiments were performed at a scan rate of 1 °C/min between 20 and 80 °C. CLIC4 thermal denaturation is irreversible; hence the data are dependent on the scan rate. A sigmoid curve was fitted to the data using SigmaPlot to
S-Nitrosylation Regulates CLIC4 Nuclear Translocation

obtain an apparent melting temperature ($T_{\text{AM}}$). $T_{\text{AM}}$ is the apparent temperature (because the melts are irreversible and hence dependent on the heating rate), which is defined as the temperature at which half of the protein has unfolded.

**Cell Culture**—Primary mouse keratinocytes were isolated and cultured as previously described (19). Culture of S1, a non-transformed mouse keratinocyte cell line, was the same as for primary mouse keratinocytes. For experiments where high transfection efficiency is required, NIH3T3 cells were utilized and cultured in the Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum, 1 mM sodium pyruvate, penicillin and streptomycin, and a 1:100 dilution of B-27 serum-free supplement (Invitrogen). NIH3T3 cells were transfected with CLIC4 plasmids for 4 h using Lipofectamine 2000 following the manufacturer's recommended protocol.

**Immunofluorescent Cell Staining**—Cell staining was performed as described in Ref. 14, except the cells were fixed with 4% paraformaldehyde for 20 min.

**Protein Lysates and Subcellular Fractionation**—Whole cell lysates were prepared by scraping cells using M-Per (Pierce) with Halt® protease, Halt® phosphatase inhibitors, MG132 (5 μM), and PMSF (1 mM). The cells were fractionated using the NE-Per kit (Pierce) following the manufacturer's recommended protocol. All of the buffers included protease and phosphatase inhibitors and MG132.

**Biotin Switch Assay for S-Nitrosylation**—The assay was performed as outlined in the original protocol (20) with some modifications. Briefly, the cells were washed with phosphate-buffered saline containing 1 mM EDTA and 0.1 mM neocuproine and MG132 (1 μM) before harvesting in the dark. The cells were lysed in HEN buffer containing 100 μM deferoxamine and neocuproine. Protease inhibitors and PMSF were included in all of the buffers throughout the assay.

**Nitric Oxide Assays**—Medium from treatment plates was used to detect nitrite + nitrate concentrations using a colorimetric assay kit (Oxford Biomedical Research).

**cGMP Assays**—cGMP levels were assayed from YC-1-treated lysates using the cyclic GMP (low pH) immunoassay kit (R&D Systems), following the manufacturer's recommended protocol.

**Coimmunoprecipitation Assays**—Exacta Cruz F Matrix (Santa Cruz Biotechnology) was incubated with 7 μg of antibody for 3 h. Immune complexes were washed and used for immunoprecipitation. The cell lysates were prepared with RIPA buffer containing NaF (2 mM), NaVO₃ (200 μM), PMSF (0.2 mM), EGTA (80 μM), mini EDTA-free protease inhibitor tablet (Roche Applied Science), and MG132 (5 μM). The lysates were incubated overnight with the immune complexes. The complexes were washed twice with RIPA buffer (+ PMSF) and once with RIPA + 400 mM NaCl + PMSF, before the addition of 2× Laemmli buffer. The samples were heated and loaded for SDS-PAGE.

**Reverse Transcription-PCR of Exogenous CLIC4**—RNA was extracted using TRIZol (Invitrogen) following the manufacturer's instructions. RNA samples were treated with DNase I (Ambion) followed by reverse transcription reaction using SSRT (Invitrogen) following the manufacturer's instructions. The primers pALTER-T7 (5’-TATACTGACTCAC-TATAGG-3’) and HACLIC4R (5’-GTCCATTCCAGCAG-TGTTGACTCTGGGTG-3’) were used to specifically amplify exogenous CLIC4. PCR conditions involved 94 °C for 5 min and 25 cycles of 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 0.5 min followed by an extension step at 72 °C for 10 min. The primers GAPDH forward (5’-TCCACCAC-CCTGTTGCTGTA-3’) and GAPDH reverse (5’-ACCA-CAGTCCATGCATCAC-3’) were used to amplify endogenous GAPDH under the same PCR conditions.

**Limited Trypsin Digestion**—Whole cell lysates were prepared by scraping cells in RIPA buffer with 1/5 EDTA-free protease inhibitors (Roche Applied Science). Equivalent amounts of lysates were digested in the presence of λ-phosphatase (5 units) with varying amounts of trypsin in RIPA buffer for 5 min at 30 °C. The reactions were stopped by adding 2× Laemmli buffer and heated for 5 min at 99 °C, followed by SDS-PAGE and immunoblotting for HA.

Recombinant wild type and mutant CLIC4 proteins were expressed as previously described and purified using the B-Per GST–Spin purification kit (Thermo Scientific). Recombinant proteins were used for trypsin digestions as detailed above and immunoblotted for CLIC4.

**RESULTS**

**Nitric Oxide Induces Nuclear Translocation of CLIC4 in Normal Mouse Keratinocytes**—The redox sensitivity of the CLIC4 structure suggested that the protein may be sensitive to reactive oxygen and nitrogen species in the cell. Because CLIC4 is highly expressed in skin and much of CLIC4 biology has been defined in keratinocytes, S1 cells, a normal mouse keratinocyte cell line, were treated with the nitric oxide donors, diazeniumdiolates, spermine NONOate, diethylenetriamine NONOate, and the nitrosothiol S-nitrosoglutathione (GSNO). The concentration of various donors used was dependent on their reported rate of NO release such that the rate would be similar for all three donors (21). Immunofluorescence staining for CLIC4 followed by confocal microscopy shows nuclear translocation of the cytoplasmic protein as early as 1 h post-treatment with all three nitric oxide donors (Fig. 1) in all cells visualized by microscopy. Nuclear translocation was significantly or partially diminished by 6 h in cells treated with GSNO and spermine NONOate, respectively, suggesting that this NO-induced nuclear translocation and signaling/modification is reversible and not irreversibly associated with cell death.

**Nuclear Translocation of CLIC4 Is Independent of NO–cGMP Signaling Pathway**—Nitric oxide modulation of many proteins, including numerous ion channels, through stimulation of soluble guanylyl cyclase and the resultant increased levels of cGMP is well established (22). cGMP-mediated signaling can proceed by direct binding of this cyclic nucleotide to the target protein or indirectly through activation of protein kinase G (22). To determine whether CLIC4 nuclear translocation was modulated by cGMP-dependent nitric oxide signaling, keratinocytes were treated with YC-1, a NO-independent activator of soluble guanylyl cyclase, followed by immunostaining for CLIC4. Increasing levels of YC-1 fail to induce nuclear translocation of CLIC4 (Fig. 2B), despite a sustained increase in cGMP levels (Fig. 2A), suggesting that CLIC4 nuclear translocation is independent of the NO–cGMP pathway. Treatment of S1 keratin-
cytes with the cGMP analog 8-pCPT-cGMP also fails to induce nuclear translocation of CLIC4 (Fig. 2 and supplemental Fig. S1), confirming that the pathway is not dependent on the NO-stimulated cGMP-protein kinase G pathway.

**Nuclear Translocation of CLIC4 Is Coincident with Increased S-Nitrosylation of the Protein**—S-Nitrosylation (SNO) has been well established as a cGMP-independent signal transduction pathway of nitric oxide. This reversible, post-translational modification of proteins involves the covalent addition of the NO molecule to specific redox-sensitive cysteine residues. Moreover, CLIC4 has been identified in the S-nitrosoproteome of vascular smooth muscle cells treated with S-nitrosocysteine, a nitrosylating agent (23). S-Nitrosylation has been reported to induce conformational change in blackfin tuna myoglobin (24).

To determine whether NO-induced nuclear translocation is accompanied by S-nitrosylation of CLIC4, lysates from NIH3T3 cells treated with GSNO or left untreated were used in a biotin switch assay to identify the modification (20). Endogenous CLIC4 shows robust S-nitrosylation in response to GSNO compared with untreated cells (Fig. 3A). Lysate from cells stimulated with GSNO was used for a reaction that omitted the SNO-specific reductant ascorbic acid. Moreover, increased SNO-CLIC4 levels are coincident with increased nuclear translocation as evidenced by immunofluorescence staining for endogenous CLIC4 (Fig. 3B) in GSNO-treated cells. This result was confirmed by comparing nuclear levels of CLIC4 following subcellular fractionation of untreated and GSNO-treated cells (Fig. 3C). GSNO induces robust S-nitrosylation of both exogenous and endogenous CLIC4 (Fig. 3D) in NIH3T3 cells, and detection is dependent on reduction by ascorbate. To further establish that CLIC4 nitrosylation was not a phenomenon restricted to GSNO, spermine NONOate and diethylenetriamine NONOate-treated cell lysates were also used in biotin switch assays and show enhanced ascorbate-dependent CLIC4 nitrosylation that is coincident with an increase in nuclear translocation (supplemental Fig. S2). Nitrosylation of CLIC4 is also not a consequence of increased nuclear residence of the protein, because nuclear targeted CLIC4 (14) does not show enhanced levels of modification compared with wild type CLIC4 (data not shown).

**S-Nitrosylation Induces a Conformational Change in CLIC4**—Cysteine 234 in CLIC4 has been previously identified as the site of nitrosylation in vascular smooth muscle cells treated with S-nitrosocysteine (23). To determine whether S-nitrosylation of CLIC4 affected its structure, recombinant CLIC4 was nitrosylated with GSNO in vitro, and CD at 208 nm was used to monitor thermal denaturation of wild type and nitrosylated (SNO) CLIC4 (Fig. 4). For both proteins, the thermal denaturation is irreversible, and hence estimates of the apparent melting temperature ($T_{M}$) are dependent on the scan rate. Under our experimental conditions, wild type CLIC4 showed a $T_{M}$ of 58.1 ± 0.3 °C, whereas CLIC4-SNO exhibits a lower apparent melting temperature of 53.5 ± 0.3 °C.
54.7 ± 0.7 °C (Fig. 4C). Thus, the SNO modification of CLIC4 alters the native structure of the protein.

Limited trypsin digestion of wild type CLIC4 from cells untreated or GSNO-treated was used to further investigate tertiary structure alterations in the modified protein. The amount of trypsin required for 50% degradation of CLIC4 is lower in cells treated with GSNO, suggesting that the protein has undergone subtle structural changes in response to the treatment (Fig. 4D).

Cysteine Mutants of CLIC4 Exhibit Altered S-Nitrosylation and Stability—CLIC4 contains four cysteine residues at positions 35, 100, 189, and 234 (supplemental Fig. S3). CLIC4 shares high homology with CLIC4 and undergoes a structural change under oxidative conditions utilizing a redox-sensitive cysteine residue at position 24 (25, 26). This highly conserved cysteine corresponds to cysteine 35 in CLIC4 (26). All four cysteines were mutated to alanine at positions 35, 100, 189, and 234 to correspond to cysteine 35 in CLIC4 (26). This highly conserved cysteine can be nitrosylated, especially when the native structure of CLIC4 is perturbed.

Cysteine 234 may be the main site for S-nitrosylation in the wild type protein, redox-sensitive cysteine 35 can be nitrosylated, especially when the native structure of CLIC4 is perturbed. These cysteines are proximal to cysteine 234 in the crystal structure (supplemental Fig. S3), are essential for protecting the protein from proteasome-mediated degradation (Fig. 3F), whereas mRNA levels for the mutant and wild type CLIC4 remain similar (Fig. 5B). Mutant protein with serine substituted at cysteine 35 (C35S) is slightly more stable but also shows proteasome-mediated degradation (Fig. 5C); thus, substitution with a charged amino acid like serine is not enough to protect from the subsequent degradation. The C189A mutant also shows decreased stability. These results suggest that cysteines 35 and 189 are vital in maintaining protein structure, possibly for protecting susceptible lysines from ubiquitination.

C35A shows enhanced S-nitrosylation (Fig. 5D) compared with wild type CLIC4 as a percentage of total mutant protein. The C234A also shows higher basal nitrosylation but is not nitrosylated in response to GSNO, suggesting that cysteine 234 may be the target site. This concurs with published results that identify cysteine 234 in CLIC4 as the site for nitrosylation in response to S-nitrosocysteine (23). Although the wild type protein shows an average 2–5-fold increase (or greater where basal nitrosylation cannot be detected) in nitrosylation in response to GSNO, both mutants show high basal levels of the modification that does not increase significantly beyond 2-fold with the treatment. To address the enhanced basal nitrosylation of the C234A mutant, a C35S/C234S double mutant and C35S and C234S single mutants were generated and used for a biotin switch assay (Fig. 5E). The serine mutants were used for these studies because of their improved stability. Even then, the C35S/C234S double mutant shows high levels of degradation by the proteasome (data not shown) and was expressed in the presence of lactacystin. Although C35S and C234S mutants show high basal nitrosylation that is relatively unchanged upon GSNO treatment, the C35S/C234S double mutant fails to show any nitrosylation. These results suggest that whereas cysteine 234 may be the main site for S-nitrosylation in the wild type protein, redox-sensitive cysteine 35 can be nitrosylated, especially when the native structure of CLIC4 is perturbed.

CLIC4 mutant proteins in which cysteine 100 and 189 were substituted to alanine were also analyzed for nitrosylation (Fig. 5F). The C100A mutant shows similar basal levels of nitrosylation to wild type CLIC4, whereas the C189A mutant shows enhanced basal nitrosylation compared with the wild type protein. Neither mutant shows a significant increase in response to GSNO. The enhanced basal nitrosylation of wild type CLIC4, in the presence of lactacystin, is probably a result of enhanced stabilization and activity of NOS enzymes (27, 28). These results taken together suggest that cysteines 35 and 189, which are proximal to cysteine 234 in the crystal structure (supplemental Fig. S3), are essential for protecting the protein from modification under basal and stimulated conditions.

Association with Nuclear Import Proteins and Nuclear Residence Are Enhanced in CLIC4 Hypernitrosylated Mutants—Nuclear translocation of CLIC4 is dependent on the exposure of its NLS and its association with the nuclear import machinery like importin α and Ran in response to diverse proapoptotic...
S-Nitrosylation regulates CLIC4 nuclear translocation.

**Figure 4.** S-Nitrosylation induces unfolding of CLIC4. 

**A.** CD spectra of CLIC4-WT and CLIC4-SNO before and after thermal denaturation. 

**B.** Thermal denaturation curve at 208 nm and 1 °C/min heating rate of CLIC4-WT and CLIC4-SNO. 

**C.** Average apparent melting temperature of both WT and nitrosylated CLIC4 (n = 4). 

**D.** Whole cell lysates from cells expressing wild type CLIC4 untreated or treated with GSNO (1 or 3 h) used for limited trypsin digestion and immunoblotted for HA. The disappearance of the HA band was quantified and plotted against the amount of trypsin used for digestion. The results are representative of three independent experiments.
S-Nitrosylation Regulates CLIC4 Nuclear Translocation

A

B

C

D

E

F

FIGURE 5. CLIC4 cysteine residues are important for protein stability and S-nitrosylation. A–C, cysteine 35 is essential for CLIC4 stability. NIH3T3 cells were transfected with wild type CLIC4 or cysteine → alanine or serine mutants at positions 35 (C35A or C35S), 100 (C100A), 189 (C189A), and 234 (C234A). The cells were treated with lactacystin (5 μM) for 6 h where indicated. Exogenous CLIC4 protein expresses an HA tag. A, lysates from transfected cells were immunoblotted for HA and GAPDH. B, RNA from mutant and wild type transfected plates were used for reverse transcription-PCR to determine mRNA levels of CLIC4 exogenous proteins. Reverse transcription-PCR of untransfected cells were used as a negative control. C, WT, C35A, and C35S expressing lysates were immunoblotted with HA and GAPDH antibodies. D–F, CLIC4 cysteines are important regulators of S-nitrosylation. NIH3T3 cells transfected with wild type, cysteine → alanine or serine mutants of CLIC4 were treated with 100 μM GSNO for 3 h or left untreated. The cells were treated with lactacystin (5 μM) for the last 6 h of expression, where indicated. In the case of D and F, all of the plates were treated with lactacystin for 6 h. The lysates were used to perform biotin switch assays, and GSNO-treated lysates were also used for no ascorbate (no Asc.) control reactions. 5% of lysates were used as input controls. The assays were immunoblotted with HA antibody. The results are representative of two independent experiments.

stimuli like TNFα and etoposide (14). Structural perturbation caused by S-nitrosylation, as indicated by thermal denaturation and tryptic digestion studies, may increase binding of CLIC4 with these nuclear import proteins. Lysates expressing wild type CLIC4, untreated or treated with GSNO, were used for immunoprecipitation of HA-tagged CLIC4 protein and immunoblotted with antibodies to the NLS-binding nuclear receptor importin α and Ran. Wild type CLIC4 shows minimal association with importin α and Ran, but GSNO significantly enhances association as early as 1 h, and this is sustained for 3 h (Fig. 6A).

To investigate whether S-nitrosylation affects this association, lysates from cells expressing cysteine mutants of CLIC4, untreated or treated with GSNO for 1 h, were used for coinmunoprecipitation assays (Fig. 6B). Compared with wild type CLIC4, the hypernitrosylated C35S mutant shows significantly enhanced association with importin α and increased association with Ran, as a percentage of total protein immunoprecipitated. This enhanced association of the mutant is evident even in unstimulated cells and is enhanced ∼2-fold in response to GSNO, compared with the more than 6-fold GSNO-induced enhancement of the wild type protein. Similar enhanced levels of association are seen between the C234A, C234S, and C35S/C234S double mutant and importin α and Ran under basal and stimulated conditions (Fig. 6B and supplemental Fig. S4). Indeed, C35A, C100A, and C189A all show enhanced association with importin α and Ran under basal conditions that is not significantly further enhanced upon treatment compared with wild type CLIC4 (data not shown).

Nitrosylation of endogenous CLIC4 coincides with increased nuclear translocation of the protein in response to GSNO. To determine whether cysteine mutations of CLIC4 alter nuclear residence, lysates from cells expressing wild type and cysteine mutants were used for subcellular fractionation (Fig. 6C–E, and supplemental Fig. S5). Hypernitrosylated C35A and C35S mutants and the un-nitrosylated C35S/C234S mutant show mainly nuclear residence. Forced stabilization by the proteasome inhibitor, lactacystin, specifically enhances the nuclear levels of the mutants compared with wild type CLIC4 (Fig. 6C). The cells were treated with lactacystin followed by GSNO to enhance protein levels of the cysteine 35 mutant and enable comparison with the more stable WT and cysteines 100, 189, and 234 mutants. Subcellular distribution of the cysteine mutants reflects the level of association with importin α and Ran. Wild type CLIC4 shows low constitutive levels of nuclear residence in basal cells that is significantly enhanced in response to GSNO (Fig. 6D and E, and supplemental Fig. S5). The C35A, C234A, and C234S mutants show increased constitutive nuclear residence that is not significantly further enhanced in response to GSNO treatment (Fig. 6D and supplemental Fig. S5). Cysteine 100 and 189 mutants also show enhanced basal nuclear levels that are not substantially increased in response to GSNO (Fig. 6E). These results suggest that the
native folding of CLIC4 is likely important in regulating protein-protein interactions and thus its cellular distribution.

Tryptic digestion of recombinant wild type, C35S, C234S, and the double mutant C35S/C234S were done to characterize possible structural alterations in the CLIC4 mutants (supplemental Fig. S6). All three mutants show enhanced degradation, suggesting an altered tertiary structure compared with wild type protein, perhaps exposing the NLS; this likely accounts for the enhanced association of the mutants with importin α and Ran and thus their enhanced nuclear levels.

TNFα Enhances CLIC4 Nitrosylation, Nuclear Translocation, and Association with Importin α in a NOS-dependent Manner in Primary Mouse Keratinocytes—To examine potential sources of nitrosylation of CLIC4 in an endogenous cellular system, we treated primary mouse keratinocytes with TNFα and examined CLIC4 subcellular distribution (14). Nuclear residence of CLIC4 is enhanced at 5 h (Fig. 7B) and is preceded by increased release of nitric oxide as measured by nitrate and nitrite levels in the media of treated cells (Fig. 7A) (29). These enhanced nuclear levels in response to TNFα are concurrent with the 2–3-fold increased nitrosylation of CLIC4 (Fig. 7C). To determine whether TNFα-induced nitrosylation, association with importin α (14), and the consequent translocation are dependent on NOS activity, primary keratinocytes expressing wild type CLIC4 were pretreated with the general NOS inhibitor L-NAME and analyzed for CLIC4 nitrosylation, nuclear translocation, and association with importin α and Ran (Fig. 7, D–F). Inhibition of NOS activity reduces TNFα-induced nitrosylation by on average 50% and decreases nuclear translocation of CLIC4. This is likely a consequence of reduced association of the protein with importin α and Ran in the presence of the inhibitor. Taken together, these results establish that stimulation-induced nitrosylation of CLIC4 is a dynamically regulated process that governs its association with protein partners, and thus its subcellular distribution, and may in turn regulate its associated proapoptotic and cell growth arrest functions.

DISCUSSION

Nuclear translocation of CLIC4 is essential for its proapoptotic, proiferation-, and cell growth arrest functions (10, 14, 30). In this study we have established that nuclear translocation of CLIC4 is regulated by S-nitrosylation of the protein. This NO-mediated CLIC4 nuclear transport is independent of the NO-stimulated cGMP pathway that has been shown to modify and modulate activities of other channel proteins (22).

Mutant studies suggest that cysteine 234 in CLIC4 is essential for nitrosylation in response to GSNO, whereas cysteines 35 and 189 may act as allosteric regulators and protect the protein from nitrosylation and subsequent nuclear import under basal conditions. In addition, perturbation of the native CLIC4 structure, by a single site mutation, can also make cysteine 35 susceptible to nitrosylation. Coimmunoprecipitation results indicate that nitrosylation of wild type CLIC4 promotes association

FIGURE 6. Cysteine mutants of CLIC4 show altered association with nuclear pore proteins and altered subcellular distribution. NIH3T3 cells transfected with wild type and cysteine mutants of CLIC4 were left untreated or treated with GSNO for 1 h (or 3 h) as indicated. A and B, lysates from 3T3 cells transfected with WT and mutant CLIC4 plasmids, with or without 1 h GSNO treatment, were used for immunoprecipitation (IP) using HA antibody. Coimmunoprecipitated proteins were immunoblotted (IB) for importin α, Ran, and HA antibodies. Lysates from untransfected cells were used for HA immunoprecipitation in a negative control (neg. ctl) reaction. 2.5% of lysates were used as input controls. The numbers below the immunoprecipitation blots represent the ratio of the transport protein that associates with the mutant or WT CLIC4 and has been normalized with the respective amount of exogenous CLIC4 immunoprecipitated. Association with untreated wild type CLIC4 is considered the baseline.

Cysteine mutants of CLIC4 show altered association with nuclear pore proteins and altered subcellular distribution. NIH3T3 cells transfected with wild type and cysteine mutants of CLIC4 were left untreated or treated with GSNO for 3 h. The cells were treated with lactacystin (5 μM) for 6 h where indicated in C, whereas in E and F all of the plates were treated with lactacystin. The cells were fractionated, and nuclear (N) and cytosolic (C) lysates were immunoblotted for HA and lamin A/C and α-tubulin antibodies. The results are representative of two independent experiments.

- TNFα Enhances CLIC4 Nitrosylation, Nuclear Translocation, and Association with Importin α in a NOS-dependent Manner in Primary Mouse Keratinocytes—To examine potential sources of nitrosylation of CLIC4 in an endogenous cellular system, we treated primary mouse keratinocytes with TNFα and examined CLIC4 subcellular distribution (14). Nuclear residence of CLIC4 is enhanced at 5 h (Fig. 7B) and is preceded by increased release of nitric oxide as measured by nitrate and nitrite levels in the media of treated cells (Fig. 7A) (29). These enhanced nuclear levels in response to TNFα are concurrent with the 2–3-fold increased nitrosylation of CLIC4 (Fig. 7C). To determine whether TNFα-induced nitrosylation, association with importin α (14), and the consequent translocation are dependent on NOS activity, primary keratinocytes expressing wild type CLIC4 were pretreated with the general NOS inhibitor L-NAME and analyzed for CLIC4 nitrosylation, nuclear translocation, and association with importin α and Ran (Fig. 7, D–F). Inhibition of NOS activity reduces TNFα-induced nitrosylation by on average 50% and decreases nuclear translocation of CLIC4. This is likely a consequence of reduced association of the protein with importin α and Ran in the presence of the inhibitor. Taken together, these results establish that stimulation-induced nitrosylation of CLIC4 is a dynamically regulated process that governs its association with protein partners, and thus its subcellular distribution, and may in turn regulate its associated proapoptotic and cell growth arrest functions.

DISCUSSION

Nuclear translocation of CLIC4 is essential for its proapoptotic, proliferation-, and cell growth arrest functions (10, 14, 30). In this study we have established that nuclear translocation of CLIC4 is regulated by S-nitrosylation of the protein. This NO-mediated CLIC4 nuclear transport is independent of the NO-stimulated cGMP pathway that has been shown to modify and modulate activities of other channel proteins (22).

Mutant studies suggest that cysteine 234 in CLIC4 is essential for nitrosylation in response to GSNO, whereas cysteines 35 and 189 may act as allosteric regulators and protect the protein from nitrosylation and subsequent nuclear import under basal conditions. In addition, perturbation of the native CLIC4 structure, by a single site mutation, can also make cysteine 35 susceptible to nitrosylation. Coimmunoprecipitation results indicate that nitrosylation of wild type CLIC4 promotes association
S-Nitrosylation Regulates CLIC4 Nuclear Translocation

**FIGURE 7.** TNFα increases CLIC4 nitrosylation, nuclear translocation, and association with importin α in a NOS-dependent manner. A, primary mouse keratinocytes were treated with TNFα (50 ng/ml) for 3 h, and medium was collected and assayed for nitrite + nitrate levels in control and treated cells. B–F, primary mouse keratinocytes overexpressing HA-CLIC4 by adenoviral constructs were treated with TNFα (25 ng/ml) for 5 h or left untreated, as indicated. The cells were pretreated with L-NAME (10 μM) for 22 h in indicated cases, before treatment with TNFα. B and C, cells were used for subcellular fractionation, and nuclear (N) and cytosolic (C) lysates were immunoblotted (IB) for HA and lamin A/C and α-tubulin antibodies. C and D, lysates were used to perform biotin switch assays. 5% of lysates were used as input controls. The assays were immunoblotted for HA. F, lysates were used for HA immunoprecipitation. Coimmunoprecipitated proteins were immunoblotted for importin α, Ran, and HA antibodies. Lysates from cells infected with empty adenoviral constructs were used for HA immunoprecipitation in a negative control (neg. ctrl) reaction. 2.5% of lysates were used as input controls. The results are representative of three independent experiments.

of the protein with the nuclear import machinery and thus its nuclear levels. The crystal structure shows cysteines 35, 189, and 234 in close proximity to each other and close to the NLS at the C terminus of helix 6 (15). Thus, under normal, proliferative conditions cysteines 35 and 189 may protect cysteine 234 from S-nitrosylation and the NLS from exposure to the nuclear pore proteins. Increased NO levels and subsequent nitrosylation of cysteine 234 may increase the accessibility and binding of CLIC4 to the nucleoporins and thus facilitate nuclear translocation. Indeed, the effects of nitrosylation on protein structure have been reported previously (24, 31). Cysteines 35 and 189 are ~12 Å away from cysteine 234, making it unlikely that their regulatory role could be based on any possible catalytic activity. Hypernitrosylation of the unstable cysteine 35 and 189 mutant raises the question of whether nitrosylation protects or targets these mutants for degrada-

dation. Nitrosylation of the antiapoptotic Bcl2 inhibits its proteasome-mediated degradation (32) during Cr(VI)-induced apoptosis, whereas nitrosylation of normoxic hypoxia inducible factor HIF-1α inhibits its degradation in mouse tumors (33).

The increase in nuclear levels of CLIC4 appears to be dynamic and reversible, raising the possibility that nitrosylation of CLIC4 is also a reversible process. Thus, the levels of modified protein may depend on the balance between activities of nitrosylating and denitrosylating enzymes induced upon specific stimulation. This may, in part, explain the multiform effects of CLIC4 translocation on cell growth arrest, cell death, and differentiation. The present study also highlights the importance of the native structure of CLIC4 both for its signaling responses as well as its stability. Indeed, proteasome-mediated degradation of unfolded or “open” CLIC4 may be another mechanism, in addition to denitrosylation, to control nuclear levels of CLIC4.

Nitrosylation of CLIC4 may not be the only mechanism for nuclear translocation. Recently, we have established CLIC4 nuclear translocation as an essential component of TGF-β-mediated cell growth arrest in keratinocytes (30). CLIC4 nuclear translocation under these conditions is mediated by TGF-β-induced interaction of CLIC4 with Schnurri-2, an interaction that is required for the nuclear translocation of the complex and independent of the NLS. Within the nucleus, CLIC4 interacts with phospho-Smad2 and 3, disrupting their interaction with the phosphatase PPM1a and thus their dephosphorylation. This results in sustained signaling of TGF-β. Although stimulation by TGF-β enhances association of Schnurri-2 and CLIC4 within cells, *in vitro* the two proteins fail to show an interaction. This suggests that post-translational modification(s) and/or protein adaptors may play a role in bringing these proteins together. Indeed, an increase of reactive oxygen species and nitrogen species upon stimulation by TGF-β and its importance in downstream signaling has been previously reported in multiple cell types (34–37). It remains to be determined whether nitrosylation of CLIC4 may affect its function in TGF-β-induced cell growth arrest, but chaperone-mediated CLIC4 translocation is another pathway mediating constitutive and stimulus-induced nuclear residence.
The pleiotropic effects of NO as a mediator of cell death and survival are well established and are mainly dependent on its cellular concentration and metabolism in the particular cell type (38, 39). Moreover, protein S-nitrosylation, initially delineated with the GAPDH-Siah1 cell death pathway, has now been well established as a central mechanism for NO-regulated apoptotic signaling (40, 41). In addition, nitrosylation and denitrosylation of proapoptotic proteins like caspase 3 have been reported to control its apoptotic activity (42, 43). This has led to the suggestion that specific levels of NO may activate pro- and anti-apoptotic pathways in response to diverse stimuli, the balance of which may result in cell death or survival (44). Thus, nuclear CLIC4 may induce apoptosis only when there is a sustained and high induction of NO, enough to overcome denitrosylating activities within the cell.

Our previous work established CLIC4 nuclear translocation as a central mechanism for apoptosis and cell growth arrest that is defective in many human cancers, where highly proliferative cancer epithelial cells show exclusion of CLIC4 from the nucleus compared with quiescent cells in normal tissue (13). Our present findings suggest that the altered redox in tumors may account for the mislocalization of CLIC4. Decreased NOS2 expression and activity is associated with malignant progression of many tumor types, including melanoma and colon and breast cancer (45–47). However, others report either little NOS2 expression and activity is associated with malignant progression of many tumor types, including melanoma and colon and breast cancer (45–47). However, others report either little

S-Nitrosylation Regulates CLIC4 Nuclear Translocation

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JULY 30, 2010•VOLUME 285•NUMBER 31
JOURNAL OF BIOLOGICAL CHEMISTRY 23827
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