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ORIGINAL PAPER

Oxidation promotes insertion of the CLIC1 chloride intracellular channel into the membrane

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Abstract Members of the chloride intracellular channel (CLIC) family exist primarily as soluble proteins but can also auto-insert into cellular membranes to form ion channels. While little is known about the process of CLIC membrane insertion, a unique feature of mammalian CLIC1 is its ability to undergo a dramatic structural metamorphosis between a monomeric glutathione-*S*-transferase homolog and an all-helical dimer upon oxidation in solution. Whether this oxidation-induced metamorphosis facilitates CLIC1 membrane insertion is unclear. In this work, we have sought to characterise the role of oxidation in the process of CLIC1 membrane insertion. We examined how redox conditions modify the ability of CLIC1 to associate with and insert into the membrane using fluorescence quenching studies and a sucrose-loaded vesicle sedimentation assay to measure membrane binding. Our results suggest that oxidation of monomeric CLIC1, *in the presence of membranes*, promotes insertion into the bilayer more effectively than the oxidised CLIC1 dimer.

Keywords CLIC1 · Fluorescence quenching · Membrane protein · Ion channel

Abbreviations

CLIC	Chloride intracellular channel
GST	Glutathione-*S*-transferase
SLV	Sucrose-loaded vesicles
PTM	Putative transmembrane region encompassing Cys24 through Val46 in CLIC1

Introduction

Conventionally, it is thought that proteins adopt a unique, evolutionarily conserved three-dimensional structure. More recently, a small but growing number of "metamorphic" proteins, capable of reversibly transiting between two or more different-folded conformations as a result of environmental triggers, have been identified (Murzin 2008). One such family of these "metamorphic" proteins, capable of undergoing such reversible conformational changes, are the chloride intracellular channels (CLICs). It is now well-established that the CLICs are able to undergo a transition between soluble and integral membrane ion channel forms, but the environmental triggers and process by which this transition proceeds are unclear. Of further intrigue, human CLIC1 is also able to undergo a large-scale structural transition between a monomer possessing a conserved

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glutathione-*S*-transferase (GST) like fold and a non-covalent, all helical soluble dimer, upon oxidation (Littler et al. 2004). The biological relevance of this dramatic metamorphic transition controlled by oxidation is still not clear.

The CLICs are a recently identified family of highly conserved proteins that have a wide tissue and cellular distribution. Six CLICs have been identified in vertebrates (CLIC1-6) with homologues also identified in invertebrates (Berry et al. 2003; Littler et al. 2008) and plants (Elter et al. 2007). All CLICs comprise a highly conserved C-terminal "CLIC-module" of approximately 240 amino acids constituting the conserved GST-like fold (Harrop et al. 2001; Littler et al. 2005, 2008; Cromer et al. 2007), with some members also possessing an additional, unrelated N-terminal domain often divergent in both sequence and size (Nishizawa et al. 2000). While the maintenance of a fixed set of CLIC paralogues in vertebrate evolution and high sequence conservation of the CLIC family attest to their functional importance, the precise function of the CLIC proteins remains elusive. Nonetheless, members of the CLIC family have been implicated in many fundamental physiological processes such as cellular division (Valenzuela et al. 1997; Berryman and Goldenring 2003), cell cycle and apoptosis (Shiio et al. 2006; Suh and Yuspa 2005), cellular differentiation (Suh et al. 2007), immune function (Valenzuela et al. 1997; Valenzuela et al. 2000), bone reabsorption (Schlesinger et al. 1997) and kidney function (Berry et al. 2003), and disease states such as pulmonary artery hypotension (Laudi et al. 2007), neurodegenerative diseases (Novarino et al. 2004) and various cancers (Fernandez-Salas et al. 1999; Rønnov-Jessen et al. 2002; Suh and Yuspa 2005; Shiio et al. 2006).

Unlike most other ion channel proteins, the mammalian CLICs are expressed as soluble proteins without a leader sequence for membrane targeting and exist almost entirely in the cytosol from where they appear to auto-insert into the membrane (Tulk et al. 2002). Strong evidence demonstrates that the membrane inserted form of CLIC1 spans the bilayer an odd number of times with the N-terminus appearing intraluminal (Duncan et al. 1997) or extracellular (Tonini et al. 2000). There is also a growing body of evidence for the existence of only a single N-terminal putative transmembrane (PTM) region encompassing Cys24 through Val46, roughly corresponding to helix 'α1' and strand 'β2' of the CLIC1 monomer (Fig. 1a) (Duncan et al. 1997; Warton et al. 2002; Berry et al. 2003; Berry and Hobert 2006; Singh and Ashley 2007). Despite inconsistencies existing in the literature for channel recording properties, the CLICs can form ion channels under specific conditions (Tulk et al. 2000, 2002; Harrop et al. 2001; Warton et al. 2002; Littler et al. 2005; Singh and Ashley 2007; Cromer et al. 2007), which are influenced by factors such as the stage of the cell cycle (Valenzuela et al. 2000). Although, the biochemical basis of this novel autonomous mechanism of membrane insertion and channel activity are still unclear, environmental triggers such as oxidation and pH are suggested to play a role (Warton et al. 2002; Littler et al. 2004; Singh and Ashley 2006; Berry and Hobert 2006; Cromer et al. 2007; Fanucchi et al. 2008).

The presence of the conserved glutathione binding site in the soluble GST-like monomer crystal structure of CLIC1 first suggested that the chloride ion channel activity of the CLICs may be under the control of redox-active signalling molecules (Harrop et al. 2001). Indeed, in 2004 it was demonstrated that CLIC1 undergoes a significant structural metamorphosis between the soluble GST-like monomer and a soluble, all α-helical, non-covalent dimer upon oxidation (Littler et al. 2004). This reversible structural transition involves a large scale rearrangement of the N-terminal domain, exposing a large hydrophobic surface, which forms the interface between the two indistinguishable dimer subunits. The conformation of the dimer subunit appears to be stabilised by the formation of an intra-domain disulphide between Cys24 and Cys59, where each of these residues also appears to be essential for optimal ion channel activity (Littler et al. 2004).

This propensity for CLIC1 to undergo such a dramatic redox-induced structural transition in the absence of membrane, leads to the notion that the process of CLIC1 membrane insertion is also likely to be under redox control. Surface plasmon resonance experiments have shown that oxidation significantly enhances the binding of human CLIC1, CLIC4 and *Drosophila melanogaster* CLIC to an artificial membrane bilayer (Littler et al. 2005, 2008). Although the mechanism by which oxidation effects the interaction between CLIC and the membrane is poorly understood, a model for the transition between the monomeric, soluble CLIC1 and the integral membrane ion form has been proposed (Fig. 1b) (Littler et al. 2004). It is suggested that the hydrophobic surface that forms the dimer-interface may also represent a membrane-docking surface which, in solution, becomes masked by non-covalent dimerisation. However, it is not clear whether formation of the discrete oxidised CLIC1 dimer in solution is required for membrane docking or whether oxidation of only a single CLIC1 triggers/stabilises the conformational change needed for membrane docking, thereby bypassing the dimer form.

In this study, we examine the role of oxidation in the processes leading to the CLIC1 pore forming transmembrane state. We have investigated two stages of the mechanism through which the formation of the CLIC1 ion channel must proceed: membrane association and membrane insertion. Our results show that monomeric CLIC1 under reducing conditions does not integrate into the membrane while the same monomer under oxidising conditions shows maximal membrane association and

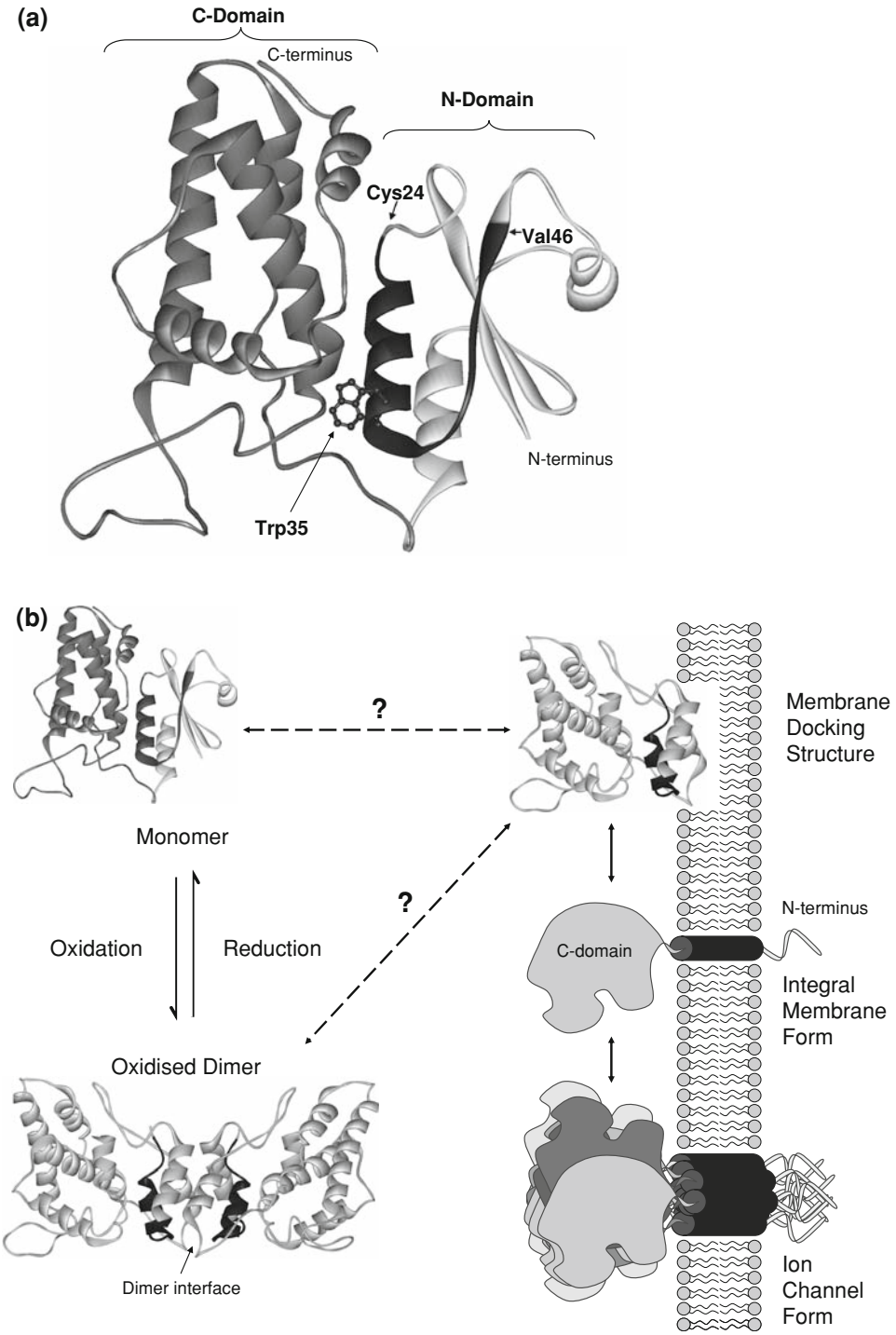

Fig. 1 **a** Crystal structure of CLIC1 monomer. The N-terminal domain containing the thioredoxin fold is *shaded white* and the all α-helical C-terminal domain is *shaded grey*. The single tryptophan residue, Trp35, is shown. The putative transmembrane domain (Cys24–Val46) is *shaded black*. The figure was rendered with WebLabViewerPro 3.7 using PDB entry 1k0m (Harrop et al. 2001). **b** Current model of CLIC1 membrane insertion. Upon oxidation, the soluble, GST-like CLIC1 monomer undergoes a reversible structural transition to an all α-helical, non-covalent dimer in solution. It has been proposed that in the presence of the membrane, the hydrophobic surface that forms the dimer-interface may dock to the bilayer as an initial step in the process of membrane insertion (Littler et al. 2004). However, it is unclear whether formation of the discrete oxidised dimer in solution is required or whether oxidation of a single CLIC1 monomer can trigger the conformational changes needed for membrane docking. Following membrane docking, the second stage in the process of membrane insertion involves insertion of the N-terminus and PTM (Cys24–Val46, black in all structures) across the lipid bilayer, while the C-domain remains in the cytosol. This results in the CLIC1 integral membrane form, while a number of integral membrane CLIC1 subunits then converge to form the active CLIC1 ion channel form, most likely oligomeric in nature

integration. Fluorescence quenching of Trp35 in the PTM region by brominated phospholipids locate this residue within the bilayer, supporting the current model that the PTM is, in fact, the transmembrane domain of the CLIC1 channel. Finally, our results indicate that the soluble oxidised dimer form of CLIC1 is unlikely to be on the membrane insertion pathway for CLIC1 per se, although the conformation of the oxidised dimer subunit may.

Methods

CLIC1 expression and purification

Recombinant GST-CLIC1 fusion protein (wild type) was expressed in *Escherichia coli* BL21(DE3)pLysS using the pGEX-4T-1 vector (Valenzuela et al. 1997). Cells were grown in 2× YT media at 20°C and expression of the

soluble GST-CLIC1 fusion construct in *E. coli* cells was induced by the addition 1 mM IPTG at mid-log growth phase. Cells were harvested after 16 h and the CLIC1 protein isolated as previously described (Littler et al. 2004). In brief, the soluble CLIC1 fusion protein was purified from the soluble *E. coli* fraction by batch-binding to glutathione-*S*-Sepharose 4B resin (GE Healthcare, Piscataway, USA). The GST tag was cleaved overnight using 30 NIH units of bovine plasma thrombin (Sigma, St Louis, USA) per litre of *E. coli* culture. The CLIC1 was further purified at 4°C by size exclusion chromatography on a Superdex 75 prep grade high performance column (GE Healthcare, Piscataway, USA) pre-equilibrated in 100 mM KCl, 1 mM NaN_3, 20 mM HEPES pH 7.5, to an estimated purity of greater than 99%. Reduced CLIC1 monomer was obtained by purification in the presence of 1 mM DTT. CLIC1 oxidised dimer was obtained by treatment of CLIC1 with 2 mM H_2O_2 for 2 h at 4°C prior to injection onto the size exclusion column where it is well-resolved from the monomeric form.

Liposome vesicle preparation

Soybean phosphatidylcholine (Sigma, St Louis, USA P-5638) and cholesterol (Sigma, St Louis, USA C-8662) were dissolved in chloroform and mixed at a ratio of 10:1 (w/w), respectively. The solvent was evaporated under a N_2 stream to form a thin lipid film over the bottom of a glass tube and the mixture was further dried under vacuum for 3 h. Lipids were rehydrated at 40 mg/mL for 30 min on ice with required buffers as specified below, whilst vortexing intermittently in the presence of glass beads. The resulting dispersions were extruded through polycarbonate filters (100-nm pore size) using a Liposofast extrusion apparatus (Avestin, Ottawa, Canada) to obtain large unilamellar vesicles.

Fluorescence spectroscopy

All fluorescence measurements were recorded at room temperature using a Perkin Elmer LS50B fluorimeter operating in ratio mode. Spectra were collected using the FL WinLab 3.0 software package. Samples were prepared in 'fluorescence buffer' (100 mM KCl, 50 mM phosphate buffer pH 6.0) at a protein to lipid ratio of 1:250, using samples containing 10 μM CLIC1 protein and 2.5 mM liposome vesicles (extruded into fluorescence buffer). The intrinsic fluorescence of CLIC1 was monitored from a single tryptophan residue of CLIC1 (Trp35) excited at 290 nm and the fluorescence emission collected from 300 to 500 nm. Slit widths of ~3–4 nm were used. Where appropriate, measurements were corrected for background fluorescence using fluorescence buffer or suspensions of liposomes in fluorescence buffer.

Quenching of Trp35 fluorescence by acrylamide

Acrylamide, an effective quenching agent of tryptophan fluorescence when exposed to the soluble phase, was used to probe the solution structure of CLIC1 and structural changes occurring upon interaction with the membrane. Acrylamide quenching of the CLIC1-Trp35 residue was measured for reduced CLIC1 monomer in 5 mM DTT, purified oxidised CLIC1 dimer and reduced CLIC1 monomer oxidised with 2 mM H_2O_2. Quenching profiles were measured for all three samples both in the absence and presence of liposomes. Ultrapure acrylamide (Sigma, St Louis, USA) was added from an aqueous 5 M stock solution, resulting in concentrations between 10 and 250 mM [Q]. Quenching efficiency (F_0/F) was calculated by dividing the Trp fluorescence intensity at 340 nm of the CLIC1 sample in the absence of quencher (F_0) by the fluorescence intensity of the solution in the presence of different concentrations of quenching agent (F). Values obtained were corrected for dilution, and the scatter contribution was derived from acrylamide titration of a vesicle blank sample. Linear regression was performed using the Stern–Volmer equation for a dynamic process:

$$\frac{F_0}{F} = 1 + K_{SV}[Q] \quad (1)$$

where K_{SV} is the Stern–Volmer quenching constant which is a measure of the accessibility of the Trp to the quenching agent. Acrylamide does not readily partition into the membrane bilayer and thus the K_{SV} value is considered to be a reliable reflection of the bimolecular rate constant for dynamic quenching of the Trp residue present in the aqueous phase.

Quenching of Trp35 fluorescence by brominated phospholipids

Collisional quenching of the CLIC1-Trp35 residue by brominated phospholipids (Br_2PC) was used to examine the localisation of this residue in the lipid bilayer. Trp35 is proposed to lie within the putative transmembrane region. The three brominated phospholipids: 1-palmitoyl-2-stearoyl (6,7-dibromo)-*sn*-glycero-3-phosphocholine (6,7-BrPC), 1-palmitoyl-2-stearoyl (9,10-dibromo)-*sn*-glycero-3-phosphocholine (9,10-BrPC), and 1-palmitoyl-2-stearoyl (11,12-dibromo)-*sn*-glycero-3-phosphocholine (11,12-BrPC) were obtained from Avanti Polar Lipids (Alabaster, USA). Liposome vesicles containing 30% (w/w) Br_2PCs and 70% soybean phosphatidylcholine were prepared as described above.

The Trp fluorescence intensity of the reduced CLIC1 monomer and oxidised dimer were measured immediately upon addition of the Br_2PCs liposomes and following 24 h incubation at 4°C, by excitation at 290 nm. Measurements were further performed 24 h after the subsequent addition of 2 mM H_2O_2 to the reduced monomer sample. Likewise for the acrylamide quenching studies, the fluorescence intensity of Trp35 in the absence (F_0) and presence (F) of the bromine quencher agent was measured at 340 nm and the quenching efficiency (F_0/F) calculated for each brominated lipid chain length.

Membrane binding sedimentation assay

The binding of CLIC1 to lipid vesicles was measured using sucrose-loaded vesicles (SLVs) which are easily sedimented by centrifugation to separate soluble protein from membrane-bound protein (Rebecchi et al. 1992; Mosior and Epand 1993). Liposome vesicles were extruded in 'sucrose buffer' (176 mM sucrose, 1 mM MOPS pH 6.5) and mixed with 'KCl buffer' (100 mM KCl, 1 mM MOPS pH 6.5), which is iso-osmotic with the entrapped sucrose buffer, in a ratio 1:5. After centrifugation at 100,000*g* for 30 min at 20°C (Sorvall RC M120, RP80AT rotor), the supernatant was removed and the lipid pellet resuspended in KCl buffer. Samples containing 34 μM CLIC1 protein (clarified by centrifuging at 100,000*g* for 60 min at 10°C) and 8.5 mM SLVs were prepared in KCl buffer to a volume of 1 mL (protein to lipid ratio of 1:250). For binding experiments, unbound CLIC1 was separated from membrane bound CLIC1 by sedimentation at 100,000*g* for 60 min at 10°C, either immediately upon addition of the SLVs or following a 24 h incubation at 4°C. Following removal of the supernatant, the lipid pellet was resuspended in KCl buffer.

To determine the percentage of membrane bound-CLIC1, the concentration of CLIC1 in the supernatant after centrifugation was measured using UV–Vis using an extinction coefficient of 16,200 M^{-1} cm^{-1}. The amount of CLIC1 bound to the vesicles was estimated using the difference between the amount of protein used in the reaction and the amount of protein remaining in the supernatant. Additionally, the proportion of soluble CLIC1 was confirmed using an amido-black protein assay which could also directly quantitate the membrane bound CLIC1 fraction (Kaplan and Pedersen 1989).

The binding assay was repeated at 0 and 24 h for both CLIC1 reduced monomer and oxidised dimer in the absence of any further oxidising agent and upon oxidation of the reduced monomer with 2 mM H_2O_2 in the presence of the SLVs. These conditions reflect the same used for the lipid fluorescence quenching measurements.

Results

CLIC1 membrane association: quenching of Trp35 by acrylamide

To investigate the effect of oxidation and formation of the dimer species on the interaction between CLIC1 and the lipid bilayer, acrylamide, a neutral water-soluble quencher of tryptophan fluorescence, was used to probe the accessibility of CLIC1 Trp35 located in the PTM region, to the aqueous phase (Fig. 1a). The relative Trp35 accessibilities of purified reduced CLIC1 monomer, CLIC1 monomer treated with oxidising agent (2 mM H_2O_2) and purified oxidised dimer were measured both in solution and in the presence of lipid vesicles. Stern–Volmer plots of the quenching of Trp35 by acrylamide for each of the three protein samples, recorded both in the absence and presence of lipid vesicles, are shown in Fig. 2. The Trp fluorescence decreased in a concentration-dependent manner upon titration with acrylamide for all samples. Samples displaying a smaller decrement in fluorescence intensity during the acrylamide titration reveal a Trp residue, which is less accessible to the quencher and thus soluble phase, reflected by lower K_{SV} values (Table 1). The Stern–Volmer plots clearly showed that the Trp35 residue of the CLIC1 monomer form in solution is more highly accessible to the aqueous phase than the oxidised dimer in solution. This is

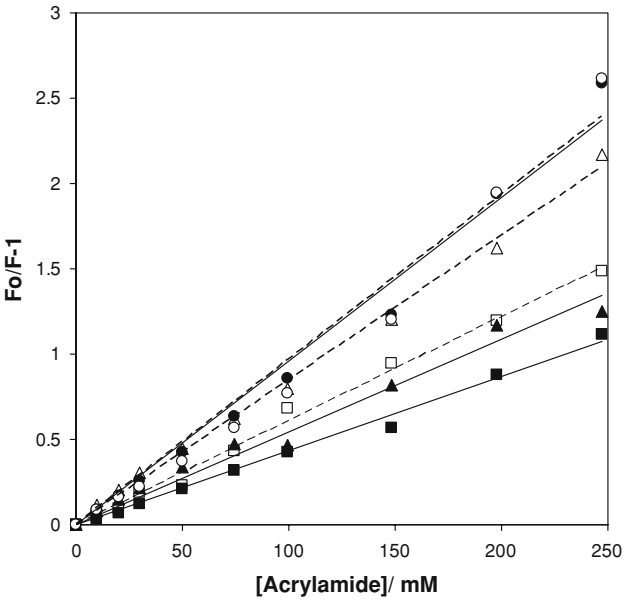

Fig. 2 Stern–Volmer plots for CLIC1 Trp35 fluorescence quenching by acrylamide in both the absence (*open symbols*) and presence of lipid (*filled symbols*). Trp35 was excited at 290 nm and fluorescence emission was monitored at 340 nm for: *filled circle* reduced monomer in the presence of 5 mM DTT (reducing conditions); *filled triangle* reduced monomer in the presence of 2 mM H_2O_2 (oxidising conditions); and, *filled square* oxidised dimer

Table 1 Stern–Volmer quenching constants determined for acrylamide quenching of CLIC1 Trp35 fluorescence both in the absence$_{(-lip)}$ and presence$_{(+lip)}$ of liposome vesicles

	$K_{sv(-lip)}$ (M^{-1})	$K_{sv(+lip)}$ (M^{-1})
Monomer reducing conditions	9.5 ± 0.7	9.6 ± 0.6
Monomer oxidising conditions	8.4 ± 0.2	5.4 ± 0.3
Dimer	6.1 ± 0.2	4.3 ± 0.2

Error of all values is 95% confidence interval determined from linear regression

demonstrated by the significantly higher K_{SV} values corresponding to both reduced monomer (9.6 ± 0.6 M^{-1}) and, to a lesser extent, monomer under oxidising conditions (8.4 ± 0.2 M^{-1}) than the value obtained for oxidised dimer (6.1 ± 0.2 M^{-1}) (Table 1). These values are consistent with the more buried environment observed for Trp35 in the dimer crystal structure (Littler et al. 2004) compared with the monomer structure (Harrop et al. 2001).

In the presence of lipid vesicles, there was essentially no change in the accessibility of the Trp residue of the reduced monomer, with similar measured K_{SV} values found both in the absence and presence of membranes (9.5 ± 0.7 M^{-1}). However, for both the monomer CLIC1 sample treated under oxidising conditions and the oxidised dimer, the slopes of the Stern–Volmer plots were significantly lower in the presence of the lipid vesicles than for the corresponding sample in solution. This suggests that there was a reduction in the accessibility of Trp35 to the aqueous phase in the presence of the lipid bilayer, most likely due to association with and/or insertion into the membrane bilayer.

CLIC1 membrane-binding sucrose-loaded vesicle sedimentation assay

Binding of CLIC1 to lipid vesicles was measured by physical separation of soluble and membrane bound protein via a centrifugation assay using sucrose-loaded vesicles. For reduced CLIC1 monomer, we observe ~32% binding of the CLIC1 to vesicles upon the initial addition of the protein with the liposomes. To investigate the effect of oxidation on binding of the reduced CLIC1 monomer to the lipid bilayer, the SLV assay was repeated at 24 h using two samples of reduced CLIC1 monomer, where one of the samples was oxidised at 0 h by the addition of 2 mM H_2O_2 (Fig. 3a). In the absence of hydrogen peroxide, the binding to the SLVs increased slightly over time (46%). In contrast, the addition of oxidising hydrogen peroxide resulted in increased binding of CLIC1 to the vesicles of up to ~75%, which was the highest observed level of binding.

The membrane binding assay was also performed with purified CLIC1 oxidised dimer. Membrane binding of CLIC1 dimer upon the initial addition of SLVs (0 h) was negligible (<1%) but increased significantly to 32% following a 24 h incubation period (Fig. 3b). No additional oxidising agents were added to this sample; hence the solution was non-oxidising (since the H_2O_2 used to generate the dimer was removed during purification by SEC). Although the proportion of membrane-bound CLIC1 increases over time, the proportion of interacting CLIC1 dimer is still considerably less than that observed for the reduced CLIC1 monomer sample when oxidised in the presence of the membrane over the same time period.

CLIC1 membrane insertion: quenching of Trp35 by brominated lipids

To establish whether the decreased accessibility of the Trp35 residue of the oxidised CLIC1 samples to acrylamide in the presence of the lipid vesicles is due to membrane association or insertion, quenching with lipid vesicles

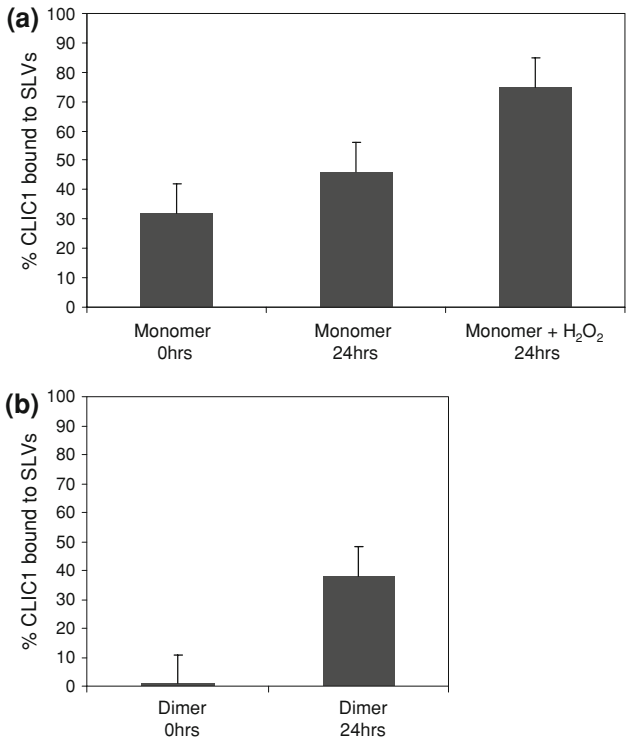

Fig. 3 Percentage of membrane-bound CLIC1 protein as determined using the sucrose-loaded vesicle sedimentation assay. CLIC1 associates with lipid vesicles as a function of time and according to its initial conformation and redox buffer condition. Optimal binding to membranes is observed with reduced CLIC1 incubated under oxidative conditions (2 mM H_2O_2) for 24 h **a** reduced monomer initially upon addition of the SLVs (Monomer 0 h), following 24 h incubation with no additional redox reagents (Monomer 24 h) and 24 h incubation of monomer with 2 mM H_2O_2 (Monomer + H_2O_2 24 h) **b** oxidised dimer initially upon addition of SLVs (Dimer 0 h) and following 24 h incubation in the absence of additional redox agents (Dimer 24 h). *Error bars* represent standard deviation ($n = 3$)

containing Br_2PC liposomes was performed. Insertion of the CLIC1 protein across the lipid bilayer should be accompanied by an increased accessibility to the brominated quencher group within the hydrocarbon phase of the bilayer. The location of the Trp residues within the bilayer was assessed by using three different brominated phospholipids where the depth of the bromine quencher agent varied within the bilayer. The pattern of insertion resulting from membrane-bound CLIC1 was therefore assessed from the quenching profiles of the following three lipid derivatives, 6,7-BrPC, 9,10-BrPC and 11,12-BrPC, where the brominated group is positioned at an increasing chain depth.

For reduced CLIC1 monomer, the greatest quenching occurred with the two brominated lipids where the bromine groups are closest to the membrane surface (6,7-BrPC and 9,10-BrPC) (Fig. 4a). Without the addition of any redox-active reagents, the degree of quenching increased slightly after 24 h but the quenching profile remained similar, with the highest quenching by the same brominated lipids. Noticeably, oxidation of the reduced CLIC1 monomer sample with 2 mM H_2O_2 over a 24 h time period significantly increased the observed quenching of Trp35 by all three brominated chain depths and dramatically changed the quenching profile. The greatest quenching is observed by 9,10-BrPC, followed by the innermost brominated lipid chain in the bilayer, 11,12-BrPC, and then the shallowest brominated quencher, 6,7-BrPC.

Surprisingly, no significant quenching of Trp35 is seen upon the initial addition of purified oxidised dimer to the brominated liposomes (Fig. 4b). However, after 24 h, a small amount of quenching by the two deepest brominated lipid chain lengths, 9,10-BrPC and 11,12-BrPC, is observed. These data suggest that partitioning of the CLIC1 into the bilayer is driven by oxidative conditions in the presence of membrane, with the Trp35 residue located deep within the lipid bilayer. From the sedimentation and acrylamide quenching studies, it appears that although the oxidised dimer is able to bind effectively to vesicles, it can only partition slowly into the bilayer over time.

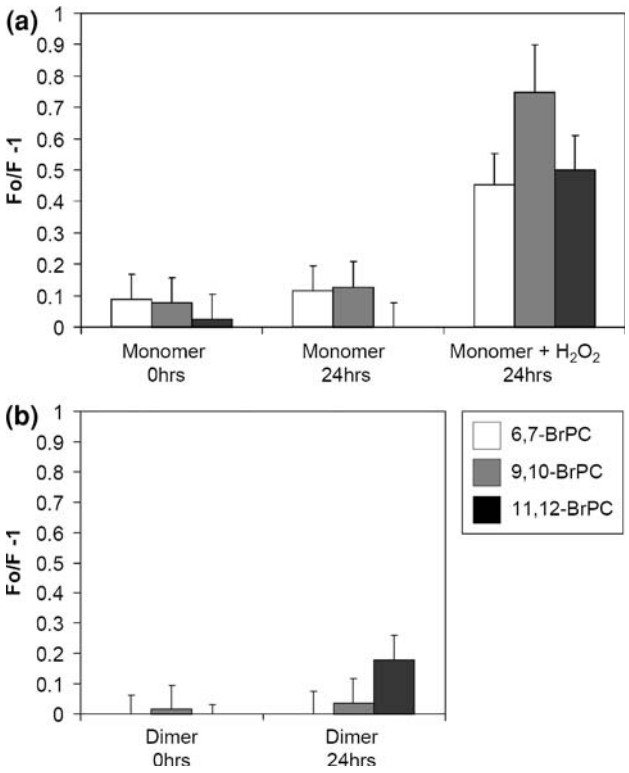

Fig. 4 Fluorescence quenching of CLIC1 Trp35 by brominated lipids. Membrane insertion was monitored using a single fixed brominated lipid concentration (30% w/w) of three lipid derivatives, 6,7-BrPC, 9,10-BrPC, 11,12-BrPC brominated at increasing chain depths. Trp35 was excited at 290 nm and fluorescence emission was monitored at 340 nm: **a** reduced monomer initially upon addition of the SLVs (Monomer 0 h), following 24 h incubation with no additional redox reagents (Monomer 24 h) and 24 h incubation with 2 mM H_2O_2 (Monomer + H_2O_2 24 h) **b** oxidised dimer initially upon addition of SLVs (Dimer 0 h) and following 24 h incubation in the absence of additional redox agents (Dimer 24 h). *Error bars* represent standard deviation

Discussion

The CLICs are a family of metamorphic proteins capable of transiting between soluble and integral membrane forms. However, little is known about how the soluble CLIC form can switch to an integral membrane form and thus facilitate ion channel conductance. It is most likely that these ion conductance properties of the CLICs arise from a membrane conformation that is significantly different from the known solution conformations. There is no clear picture of how this transition from a soluble globular protein to an integral membrane protein occurs but one recent proposal suggests that the acidic environment encountered by the CLICs at the surface of the membrane primes the transmembrane region in the N-domain, enabling it to interact and insert into the membrane (Fanucchi et al. 2008). It is unclear what roles other environmental triggers such as membrane potential, membrane curvature or phosphorylation may play in aiding this process.

A unique feature of CLIC1 is that it possesses the ability to undergo a dramatic structural metamorphosis between a GST-like monomer and non-covalent dimer upon oxidation in solution (Littler et al. 2004). The propensity for CLIC1 to undergo this structural rearrangement in the absence of the lipid bilayer suggests that oxidation, disulphide bond formation and/or formation of the non-covalent dimer may play a role in membrane insertion and formation of the CLIC ion channel form. To investigate this, we have examined the role of oxidation of the CLIC1 monomer in

the presence of membranes to determine if oxidation plays a significant role in the process of membrane insertion. This was compared to results obtained for the pre-formed and purified oxidised dimer conformation where no oxidising reagents were added in the presence of the membranes.

In order to obtain an initial indication of the effect of oxidation on the interaction between CLIC1 and the membrane, the relative accessibility of Trp35 to the aqueous phase was probed using Stern–Volmer quenching with acrylamide. Trp35 in the soluble reduced monomer form of CLIC1 was found to be more accessible to the aqueous phase than in the oxidised dimer form, consistent with the positioning of this residue in their respective crystal structures [CLIC1 oxidised dimer: PDB #1RK4 (Littler et al. 2004) and CLIC1 monomer PDB #1K0M (Harrop et al. 2001)]. The K_{sv} value measured for the CLIC1 monomer under oxidising conditions in solution lies between the values obtained for CLIC1 under reducing conditions and the oxidised dimer. It is likely that this results from contributions of both monomer and dimer CLIC1 conformations as a mixed population of both states is likely to be present upon treatment of CLIC1 with H_2O_2 (Littler et al. 2004). These results affirmed the success of this approach to probe the local environment of Trp35 in solution. Thus, further changes in Trp accessibility upon association with the lipid bilayer are likely to be due to a conformational rearrangement of the CLIC1 protein, most likely due to its interaction with the membrane.

In the presence of lipid vesicles, and maintaining the sample under reducing conditions, there was no change in the accessibility of Trp35 for reduced CLIC1 monomer. As the local Trp35 environment of reduced monomer appears unchanged, this suggested that no interaction occurred between the N-domain of CLIC1 (which includes the PTM region) with the lipid bilayer. Without rearrangement of the CLIC1 PTM domain, the CLIC1 protein would be unable to form the pore required for ion channel conductance and thus, under reducing conditions, we would not expect ion channel activity to occur, as has previously been reported (Littler et al. 2004).

For purified CLIC1 dimer in the presence of lipid, a reduction in the accessibility of Trp35 to the aqueous solution is observed. This decrease in Trp35 likely represents rearrangement of the PTM domain as it interacts with the lipid vesicles and possibly becomes embedded in the lipid bilayer and thus inaccessible to acrylamide quenching. Although the lower accessibility of Trp35 in CLIC1 monomer under oxidising conditions (as opposed to reduced monomer) is likely to be due to formation of the dimer in solution, the K_{SV} of Trp35 in the presence of lipid for the hydrogen peroxide treated monomer is still significantly lower than that of the dimer in solution (Table 1). Thus, this decrease in Trp35 accessibility upon oxidation in the presence of membranes cannot be equated solely to the formation of dimer in solution, which is known to account for up to a maximum of two-thirds of the total CLIC1 protein yield under equivalent oxidising conditions (Littler et al. 2004). This result implies that under oxidising conditions, the PTM domain of the monomer also interacts with the lipid bilayer to become inaccessible to acrylamide quenching. However, relating K_{sv} values to protein conformation is difficult to do, especially with mixed populations.

Having established that oxidation appears to play a role for the interaction between the CLIC1 PTM domain and the membrane, we sought to determine whether this membrane association is the result of weak interactions or 'docking' with the surface of the lipid bilayer or whether the membrane binding is due to insertion to form the ion channel. The decreased access of the acrylamide quencher to Trp35 upon interaction of the PTM domain with the lipid bilayer should be accompanied by an increased accessibility to quenchers present in the hydrocarbon side chains of the bilayer. The insertion of tryptophan into the lipid bilayer was assessed from the quenching profile of three phospholipids derivatives brominated at increasing chain depths, 6,7-BrPC, 9,10-BrPC and 11,12-BrPC, in conjunction with a SLV sedimentation assay to measure membrane binding. When tryptophan is inserted into the lipid bilayer, its fluorescence emission is quenched to the greatest degree by the brominated chain depth closest to its depth of insertion. Thus, the quenching profile of the three brominated lipid derivatives at varying chain depths within the vicinity of the inserted tryptophan allows an approximation of the depth of insertion into the membrane. However, in comparing data from different samples, it is also essential to obtain a quantitative measure of the proportion of protein bound to the membrane thus giving rise to the observed quenching.

We observed initially upon the addition of reduced CLIC1 monomer to the brominated vesicles to that a moderate proportion of $\sim$30% of the protein bound to the lipid bilayer and was weakly quenched by the brominated lipid groups closest to the membrane surface (6,7-BrPC9 and 9,10-BrPC). This more likely represents association of the majority of the CLIC1 monomer with the bilayer rather than insertion of the PTM domain into the interior of the bilayer as no change in the accessibility of Trp35 was observed by acrylamide quenching. In the absence of oxidative treatment of this sample, both membrane binding and quenching by the Br groups increased slightly over time. However, a similar surface proximal quenching profile was observed, suggesting no considerable membrane insertion had occurred.

Upon oxidation of the monomer with H_2O_2, both membrane binding and quenching by brominated lipid groups increased dramatically. This was also the only CLIC1 sample which differed significantly in its quenching profile with the greatest quenching caused by 9,10-BrPC followed by 11,12-BrPC and 6,7-BrPC. This change in profile and the higher values of quenching by each lipid chain suggested that the tryptophan is largely inserted in the interior of the bilayer and lies between the location depth of the 9,10-BrPC and 11,12-BrPC bromine groups. However, this result may contain a bias toward a shallower depth if quenching results from a mixed population of both CLIC1 monomers associated with the surface of the membrane and the membrane-inserted CLIC1. Further studies to measure the value of the ratio of such a mixed population or the existence of additional intermediate structures are currently being explored.

In contrast to reduced CLIC1 which does not appear to insert into the bilayer and thus cannot form an ion-conducting pore, a CLIC1 oxidised dimer form isolated in solution was shown to form effective chloride ion channels when added to artificial bilayers and vesicles (Littler et al. 2004). The structural change that occurs on oxidation results in a dramatic rearrangement of the N-domain (including the PTM) to form a large hydrophobic surface, which is the dimerisation interface. It has been postulated that this hydrophobic surface acts as a membrane docking surface in the presence of membranes (Littler et al. 2004).

We therefore sought to compare the binding and insertion properties of the solution formed dimer with our oxidised monomer results to determine if the dimer could effectively dissociate in the presence of the membrane. Initially, upon the addition of the purified oxidised dimer to the membranes, no binding was observed. This was mirrored by the absence of significant quenching by any of the brominated lipid derivatives. The inability of the CLIC1 dimer to effectively associate or insert into the membrane suggests that the two subunits of the dimer are 'trapped' within their discrete dimer form by the strong interacting hydrophobic surface which is stabilised by the formation of the Cys24–Cys59 disulphide within each subunit. However, over time, some dimers revert to monomers that bind to vesicles and (possibly) insert into the bilayer. This accounts for the weak binding and quenching observed by the brominated lipid derivatives over the 24 h time period.

In order for the PTM domain to span the bilayer, it seems impossible for the Cys24–59 disulphide to remain intact and thus, disulphide bond formation may not be necessary for membrane insertion. Although it has been reported that both residues Cys24 and Cys59 are necessary for ion channel conductance (Littler et al. 2004), it is not possible to distinguish between whether they play a role in membrane association and thus formation of the pore or whether they have a more direct role in channel conductance. It has been suggested that Cys24, which is conserved throughout all mammalian CLICs, is not required for membrane insertion but is involved in regulation of ion channel conductance (Singh and Ashley 2006). As the redox active Cys59 is not conserved in other mammalian CLICs, this precludes formation of an oxidised dimer form equivalent to that of CLIC1 for the other family members and thus further questions its role in this channel family. However, if formation of the Cys24–Cys59 is not necessary for membrane insertion as we propose, oxidation may promote membrane insertion of other CLIC family members. Thus, the CLIC1 dimer subunit structure could resemble a membrane insertion 'intermediate state' that otherwise forms upon oxidation of the monomer. This intermediate state becomes stabilised by association with the membrane surface but is trapped by formation of a disulphide in the absence of the membrane. The suggestion of a similar intermediate state of CLIC1 was recently suggested by Fanucchi et al. (2008) who described a pH-induced CLIC1 structure form in solution that, with the exception of the disulphide, bears several marked similarities to the dimer subunit including a large exposed hydrophobic surface encompassing Trp35 and high structural similarity to the reduced monomer. Formation of this CLIC1 structure may represent similar structural changes to those required for membrane insertion as a result of environmental triggers other than oxidation in the presence of the membrane.

In conclusion, our results imply that oxidation in the presence of the lipid bilayer promotes membrane insertion via an intermediate state, while oxidation to the dimer species formed in solution, prior to encountering the membrane, is unlikely to play a role in the process of membrane insertion. From these results, we propose that CLIC1 membrane insertion proceeds through an initial association between the CLIC1 monomer and the surface of the lipid bilayer either concomitant with or followed by oxidation of the CLIC1 bound to the surface of the membrane which then induces the structural metamorphosis required to insert across the bilayer. Although formation of the discrete CLIC1 dimer form in solution is unlikely to play a direct role in the process of membrane insertion, oxidation of the reduced monomer on the surface of the membrane may induce an intermediate structure that resembles the dimer subunit. To further understand the role of oxidation in the process of CLIC membrane insertion we need to clarify the nature of any CLIC1 membrane binding intermediates and whether oxidation also promotes the membrane insertion of other CLIC family members.

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References

Berry KL, Hobert O (2006) Mapping functional domains of chloride intracellular channel (CLIC) proteins in vivo. J Mol Biol 359:1316–1333. doi:10.1016/j.jmb.2006.04.046

Berry KL, Bulow HE, Hall DH et al (2003) A *C. elegans* CLIC-like protein required for intracellular tube formation and maintenance. Science 302:2134–2137. doi:10.1126/science.1087667

Berryman MA, Goldenring JR (2003) CLIC4 is enriched at cell–cell junctions and colocalizes with AKAP350 at the centrosome and midbody of cultured mammalian cells. Cell Motil Cytoskeleton 56:159–172. doi:10.1002/cm.10141

Cromer BA, Gorman MA, Hansen G et al (2007) Structure of the Janus protein human CLIC2. J Mol Biol 374:719–731. doi:10.1016/j.jmb.2007.09.041

Duncan RR, Westwood PK, Boyd A et al (1997) Rat brain p64H1, expression of a new member of the p64 chloride channel protein family in endoplasmic reticulum. J Biol Chem 272:23880–23886. doi:10.1074/jbc.272.38.23880

Elter A, Hartel A, Sieben C et al (2007) A plant homolog of animal CLICs generates an ion conductance in heterologous systems. J Biol Chem 282:8786–8792. doi:10.1074/jbc.M607241200

Fanucchi S, Adamson RJ, Dirr HW (2008) Formation of an unfolding intermediate state of soluble chloride intracellular channel protein CLIC1 at acidic pH. Biochemistry 47:11674–11681. doi:10.1021/bi801147r

Fernandez-Salas E, Sagar M, Cheng C et al (1999) p53 and tumor necrosis factor alpha regulate the expression of a mitochondrial chloride channel protein. J Biol Chem 274:36488–36497. doi:10.1074/jbc.274.51.36488

Harrop SJ, DeMaere MZ, Fairlie WD et al (2001) Crystal structure of a soluble form of the intracellular chloride ion channel CLIC1 (NCC27) at 1.4-A resolution. J Biol Chem 276:44993–45000. doi:10.1074/jbc.M107804200

Kaplan RS, Pedersen PL (1989) Sensitive protein assay in presence of high levels of lipid. Methods Enzymol 172:393–399. doi:10.1016/S0076-6879(89)72025-5

Laudi S, Steudel W, Jonscher K et al (2007) Comparison of lung proteome profiles in two rodent models of pulmonary arterial hypertension. Proteomics 7:2469–2478. doi:10.1002/pmic.200600848

Littler DR, Harrop SJ, Fairlie WD et al (2004) The intracellular chloride ion channel protein CLIC1 undergoes a redox-controlled structural transition. J Biol Chem 279:9298–9305. doi:10.1074/jbc.M308444200

Littler DR, Assaad NN, Harrop SJ et al (2005) Crystal structure of the soluble form of the redox-regulated chloride ion channel protein CLIC4. FEBS J 272:4996–5007. doi:10.1111/j.1742-4658.2005.04909.x

Littler DR, Harrop SJ, Brown LJ et al (2008) Comparison of vertebrate and invertebrate CLIC proteins: the crystal structures of Caenorhabditis elegans EXC-4 and *Drosophila melanogaster* DmCLIC. Proteins 71:364–378. doi:10.1002/prot.21704

Mosior M, Epand RM (1993) Mechanism of activation of protein kinase C: roles of diolein and phosphatidylserine. Biochemistry 32:66–75. doi:10.1021/bi00052a010

Murzin AG (2008) Biochemistry. Metamorphic proteins. Science 320:1725–1726. doi:10.1126/science.1158868

Nishizawa T, Nagao T, Iwatsubo T et al (2000) Molecular cloning and characterization of a novel chloride intracellular channel-related protein, parchorin, expressed in water-secreting cells. J Biol Chem 275:11164–11173. doi:10.1074/jbc.275.15.11164

Novarino G, Fabrizi C, Tonini R et al (2004) Involvement of the intracellular ion channel CLIC1 in microglia-mediated beta-amyloid-induced neurotoxicity. J Neurosci 24:5322–5330. doi:10.1523/JNEUROSCI.1170-04.2004

Rebecchi M, Peterson A, McLaughlin S (1992) Phosphoinositide-specific phospholipase C-delta 1 binds with high affinity to phospholipid vesicles containing phosphatidylinositol 4, 5-bisphosphate. Biochemistry 31:12742–12747. doi:10.1021/bi00166a005

Rønnov-Jessen L, Villadsen R, Edwards JC et al (2002) Differential expression of a chloride intracellular channel gene, CLIC4, in transforming growth factor-beta1-mediated conversion of fibroblasts to myofibroblasts. Am J Pathol 161:471–480

Schlesinger PH, Blair HC, Teitelbaum SL et al (1997) Characterization of the osteoclast ruffled border chloride channel and its role in bone resorption. J Biol Chem 272:18636–18643. doi:10.1074/jbc.272.30.18636

Shiio Y, Suh KS, Lee H et al (2006) Quantitative proteomic analysis of myc-induced apoptosis: a direct role for Myc induction of the mitochondrial chloride ion channel, mtCLIC/CLIC4. J Biol Chem 281:2750–2756. doi:10.1074/jbc.M509349200

Singh H, Ashley RH (2006) Redox regulation of CLIC1 by cysteine residues associated with the putative channel pore. Biophys J 90:1628–1638. doi:10.1529/biophysj.105.072678

Singh H, Ashley RH (2007) CLIC4 (p64H1) and its putative transmembrane domain form poorly selective, redox-regulated ion channels. Mol Membr Biol 24:41–52. doi:10.1080/09687860600927907

Suh KS, Yuspa SH (2005) Intracellular chloride channels: critical mediators of cell viability and potential targets for cancer therapy. Curr Pharm Des 11:2753–2764. doi:10.2174/1381612054546806

Suh KS, Mutoh M, Mutoh T et al (2007) CLIC4 mediates and is required for Ca_2^+-induced keratinocyte differentiation. J Cell Sci 120:2631–2640. doi:10.1242/jcs.002741

Tonini R, Ferroni A, Valenzuela SM et al (2000) Functional characterization of the NCC27 nuclear protein in stable transfected CHO-K1 cells. FASEB J 14:1171–1178

Tulk BM, Schlesinger PH, Kapadia SA et al (2000) CLIC-1 functions as a chloride channel when expressed and purified from bacteria. J Biol Chem 275:26986–26993

Tulk BM, Kapadia S, Edwards JC (2002) CLIC1 inserts from the aqueous phase into phospholipid membranes, where it functions as an anion channel. Am J Physiol Cell Physiol 282:C1103–C1112

Valenzuela SM, Martin DK, Por SB et al (1997) Molecular cloning and expression of a chloride ion channel of cell nuclei. J Biol Chem 272:12575–12582. doi:10.1074/jbc.272.19.12575

Valenzuela SM, Mazzanti M, Tonini R et al (2000) The nuclear chloride ion channel NCC27 is involved in regulation of the cell cycle. J Physiol 529:541–552. doi:10.1111/j.1469-7793.2000.00541.x

Warton K, Tonini R, Fairlie WD et al (2002) Recombinant CLIC1 (NCC27) assembles in lipid bilayers via a pH-dependent two-state process to form chloride ion channels with identical characteristics to those observed in Chinese hamster ovary cells expressing CLIC1. J Biol Chem 277:26003–26011. doi:10.1074/jbc.M203666200