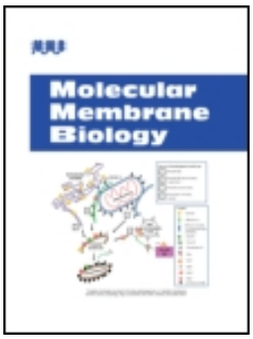

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CLIC4 (p64H1) and its putative transmembrane domain form poorly selective, redox-regulated ion channels

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Abstract
Despite being synthesized in the cytosol without a leader sequence, the soluble 253-residue mammalian protein CLIC4 (Chloride Intracellular Channel 4, or p64H1), a structural homologue of Ω-type glutathione-S-transferase, autoinserts into membranes to form an integral membrane protein with ion channel activity. A predicted transmembrane domain (TMD) near the N-terminus of CLIC4 could mediate membrane insertion, and contribute to oligomeric pores, with minimal reorganization of the soluble protein structure. We tested this idea by reconstituting recombinant CLIC4 in planar bilayers containing phosphatidyethanolamine, phosphatidylserine and cholesterol, recording ion channels with a maximum conductance of ~15 pS in KCl under both oxidizing and reducing conditions. The channels discriminated poorly between anions and cations, incompatible with the current "CLIC" nomenclature, and their conductance was modified by the *trans* (external or luminal) redox potential, as previously observed for CLIC1. We then reconstituted a truncated version of the protein, limited to the first 61 residues containing the predicted TMD. This included a single *trans* cysteine residue in the putative pore-forming subunits, at the external entrance to the pore. The truncated protein formed non-selective channels with a reduced conductance, but they retained their *trans*-redox sensitivity, and could still be blocked or inactivated by *trans* (not *cis*) thiol-reative dithiobisnitrobenzoic acid. We suggest that oligomers containing the putative TMD are essential components of the CLIC4 pore. However, the pore is inherently non-selective, and any ionic selectivity in CLIC4 (and other membrane CLICs) may be attributable to other regions of the protein, including the channel vestibules.

Keywords: *Anion channel, p64, planar bilayer, redox potential*

Introduction

Chloride Intracellular Channel (CLIC) proteins are unique among putative eukaryotic ion channels in being able to assume both soluble and membrane forms. CLICs also bypass the conventional secretory pathway, and "autoinsert" directly into membranes [1,2]. The proteins are related to p64, a putative anion channel purified from bovine kidney by drug affinity chromatography in pioneering work by Al-Awqati and his colleagues [3] before being cloned [4] and characterized in considerable detail (e.g. [5]). However, it has yet to be established (by single-channel recording) that p64 alone is sufficient to form an ion channel, in contrast to the best-studied CLIC protein, CLIC1 [6].

The first CLIC protein to be discovered, rat brain CLIC4 (p64H1, or p64 homologue 1), known in mouse as mitochondrial or mtCLIC [7], was identified and cloned by homology to p64 [8,9] as a potential candidate for an intracellular anion channel previously shown to be co-localized with rat brain ryanodine-sensitive calcium-release channels [10]. CLIC4 isologues are now known to be very widespread, and the proteins are highly conserved in vertebrates ranging from fish to mammals [11]. The structure of a soluble form of human CLIC4 (crystallized with a short random C-terminal extension) is similar [12] to previously crystallized, soluble human CLIC1 [13], as anticipated by modelling CLIC4 onto CLIC1 [2]. Like CLIC1, soluble CLIC4 has an Ω-glutathione-S-transferase (GST) fold, but although Ω-GSTs and CLICs are structurally similar, and distantly related, they do not have overlapping functions. In particular, Ω-GSTs do not autoinsert into membranes and form ion channels [14], and CLIC proteins appear to have little or no enzymatic activity.

In addition to brain and kidney, *CLIC4* mRNA is also expressed in many other mammalian tissues including lung, liver, skeletal muscle, testis and skin

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[7,9]. A putative "cytoplasmic domain" containing most of the protein formed complexes involving brain actin, dynamin I, tubulin and 14-3-3 proteins [15], and cytoplasmic CLIC4 has been shown to co-localize with A-kinase anchoring proteins (AKAPs) in specific cellular microdomains, including centrosomes and the cortical actin cytoskeleton [16]. The much less abundant [15] membrane form of CLIC4 is an integral (not peripheral [9]) membrane protein localized to several intracellular organelles, including the endoplasmic reticulum and outer nuclear membrane [9], (inner) mitochondrial membranes [7], and the membranes of dense core secretory vesicles [17], caveolae and (possibly) the trans-Golgi network [18]. It has also been localized to the plasma membrane [18,19], especially near intercellular junctions [16].

CLIC4 levels are dynamically regulated, and increase with tumour necrosis factor-α and p53 signalling [7]. Interestingly, CLIC4 overexpression induces p53-mediated apoptosis associated with mitochondrial depolarisation, cytochrome C release and caspase activation [20]. Although it is not yet clear whether this involves the soluble or membrane form of the protein, or both, possible implication of a putative mitochondrial ion channel in apoptosis could clearly be very significant. It is however notable that some cells, especially mammalian cells, express many different CLIC proteins, suggesting that some or all of their functions may be redundant. For example, transgenic mice lacking CLIC1 appear to be essentially normal apart from increased weight, splenomegaly and mild thrombocythaemia [21].

In contrast to CLIC1, relatively little is known about CLIC4-associated ion channels. Although the incorporation of microsomal membrane vesicles containing recombinant CLIC4 into planar lipid bilayers gave rise to novel anion channel activity of 10–50 pS [9], this could have been due to the activation of unidentified endogenous channels, especially since another CLIC, CLIC2, has been shown to be a channel modulator rather than an ion channel itself [22]. CLIC4 (possibly with the short C-terminal extension required for crystallisation) gave rise to channel activity in "tip-dip" bilayers [12], but the conductance of the channel was unclear (reported as both 31 pS and 57 pS). Novel ion channels specifically shown to contain FLAG-tagged CLIC4 have also been recorded by patch-clamping the plasma membrane of cells over-expressing the protein [19]. In the presence of large cations (to limit endogenous cation currents), the conductance of individual CLIC4 channels appeared to be very low, of the order of 1 pS. Currents through CLIC1 are now known to be regulated by the external redox potential [23], and if CLIC4 shows a similar mechanism, this may help to explain the very small single-channel currents observed by patch-clamping. Also, as discussed later, the use of large cations may have further reduced the CLIC4 currents.

We set out to investigate the single channel properties of CLIC4 under various redox conditions, because in contrast to a previous report [12], the protein can form channels under both reducing and non-reducing conditions, like CLIC1 [23]. We then tested a simple model for the transmembrane topology of CLIC4 that can be extrapolated to CLIC1 and other membrane CLICs, by reconstituting a truncated protein containing the N terminus of CLIC4 and its single putative transmembrane domain (TMD). We also determined whether CLIC4 is sensitive to the *trans* (extracellular or luminal) redox potential in the presence of a glutathione buffer, like CLIC1 [23], and whether a critical cysteine residue on the *trans* side of membrane CLIC4 subunits, corresponding to the GSH-binding "G-site" of Ω-GSTs, mediates this unusual effect.

Materials and methods

Expression and purification of CLIC4

We cloned rat brain *CLIC4* (*p64H1*, [9]) into pHis8, a modified pET vector encoding an N-terminal octa-His tag and a thrombin cleavage site [24], and inserted a stop codon into one clone by "Quik-Change" PCR to truncate the expressed protein at CLIC4 position 61. The inserts were verified by DNA sequencing (MWG Biotech), and fusion proteins were expressed in *E. coli* BL21 (DE3) cells and recovered from cell lysates by Ni^{2+}-NTA affinity chromatography, with yields for the soluble thrombin-cleaved full-length and truncated proteins of 4.0 ± 0.50 mg/l and 2.0 ± 0.65 mg/l (means $\pm$ SD, $n = 15$ or 3), respectively (determined by the Lowry method using appropriate standards). The preparations were analysed by SDS-PAGE and gel-exclusion FPLC using Superdex 200 (by methods detailed in [25]), and protein aliquots (which had at no stage been exposed to detergents) were stored for up to 3 months at -70°C in the presence of 5 mM DTT. The masses of proteins subjected to gel-exclusion FPLC were determined from a plot of log(Mr) vs. K_{av} (the corrected partition coefficient):

$$K_{av} = (V_e - V_o)/(V_t - V_o)$$

where V_e, V_o and V_t represent the elution volume, the void volume and the packed bed volume, respectively.

Channel incorporation into planar lipid bilayers

Planar bilayers were prepared at room temperature (20°C) using lipids selected from: palmitoyl-oleoyl phosphatidylcholine (POPC), PO-phosphatidylethanolamine (POPE), PO-phosphatidylserine (POPS) and cholesterol (all from Avanti, AL, USA), as detailed in the Results section. Briefly, the lipids were suspended in n-decane (25 μg total lipid/μl), and films were cast across a 0.3 mm hole in a polystyrene partition separating two chambers. The chambers, designated *cis* and *trans*, contained 50 mM KCl with 10 mM Tris-HCl (pH 7.4) and 1 mM DTT, unless otherwise specified, and were connected by agar salt bridges to the headstage input or ground, respectively, of an Axopatch 200-B amplifier, minimising and offsetting liquid junction potentials as previously described [26]. After the lipid film had thinned spontaneously to form a planar bilayer, monitored by measuring a relatively abrupt increase in membrane capacitance from <50 pF to >200 pF, the *cis* chamber was clamped at various holding potentials (HPs) relative to the *trans* chamber, and up to 25 ng/ml purified protein was stirred into the same chamber, followed by small aliquots of 5 M KCl to raise the KCl concentration to 500 mM. Transmembrane currents appeared within 10 min of adding the (full-length) protein, and were digitally recorded. Thereafter, the contents of the chambers were changed by perfusion (at least 10 volumes) as required.

Single-channel analysis

Single-channel currents (labelled following the standard electrophysiological convention, i.e., positive currents represent net cation flux from *cis* to *trans*), were low-pass filtered (8-pole, Bessel type response) at 50 Hz or 25 Hz and analysed with pClamp8 (Axon Instruments) and pStat (SPSS), using amplitude histograms as previously described [23] to measure channel amplitudes and open probabilities (Po). Salt concentrations were converted to activities using standard tables, and (relative) anion permeabilities (P) were calculated under equilibrium conditions using the Nernst equation:

$$P_{\text{anion}}/P_{\text{Cl}} = \text{a[Cl]}_{cis}/\text{a[anion]}_{trans} \times \exp(-\text{zFEr/RT})$$

where a is the activity coefficient of the relevant salt, Er is the reversal or equilibrium potential, and z, F, R and T have their usual significance. Relative anion vs. cation permeabilities (selectivities) were calculated from the following form of the Goldman-Hodgkin-Katz (GHK) voltage equation:

$$P_{\text{anion}}/P_{\text{cation}} = \{\text{n} \times \exp(\text{Er/k})\} - 1/\{\text{n} - \exp(\text{Er/k})\}$$

where n is the *cis*/*trans* salt activity ratio and k = RT/zF (26 mV).

Results

CLIC4 ion channels in fully-reducing conditions

CLIC4 recordings had an inconsistent appearance in experiments using POPC or equimolar POPE and POPS, but like CLIC1 [23], the protein formed highly consistent channels in 80/83 bilayers containing POPE, POPS and cholesterol, 4:1:1 mol/mol, respectively, in the presence of 1 mM DTT, 5 mM GSH or 100 μM H_2O_2. In the latter case, the protein was also exposed to 100 μM H_2O_2 for 5–10 min. before incorporation. Channel activity appeared more rapidly at an acidic *cis* pH of 5.5. However, these recordings were very noisy, and low pH conditions were not pursued further. No channel-like events were seen during prolonged (up to 30 min.) observation of control bilayers in the absence of added protein (15 experiments).

Figure 1A shows examples of recordings obtained in 1 mM DTT, representing "fully-reducing" conditions. The slope conductance of the main open state in a *cis:trans* gradient of 500:50 mM KCl with 1 mM DTT in both chambers was 10.3 ± 1.0 pS (mean ± SD, $n = 15$), calculated (by linear regression) from the linear region of the I/V plot (-100 mV to $+70$ mV) (Figure 1B). We noted several substates, including the prominent ~25% substate shown here. The reversal potential of the main open state was -12.2 ± 3.3 mV (mean + SD, $n = 15$) (Figure 1B), corresponding to a mean Cl^-/K^+ selectivity of 0.54 ± 0.09, i.e., a poorly selective or even mildly cation-selective channel. The Er for the ~25% substate was similar, and we observed direct transitions to and from both states (inset in Figure 2B). The main open state conductance was ohmic in symmetrical KCl solutions (e.g., inset I/Vs in Figure 2), but showed a complicated dependence on KCl activity (Figure 2, main panel). Up to ~350 mM, the relationship could be described as the sum of a hyperbolic and a linear component, but the maximum single-channel conductance was 13.8 ± 0.60 pS (mean ± SD, $n = 5$), and it declined at higher KCl activities, consistent with self-block.

We measured relative anion permeabilities at equilibrium under biionic conditions in the presence of 1 mM DTT by perfusing 50 mM KCl (corrected for activity) into the *cis* chamber, and different potassium salts with the same activity into the *trans* chamber. Relative anion permeabilities calculated from the equilibrium potential were the same for 5 test anions compared to Cl^- (set to 1.0): SCN^- (0.97 ± 0.11); NO_3^- (1.1 ± 0.50); I^- (1.1 ± 0.96);

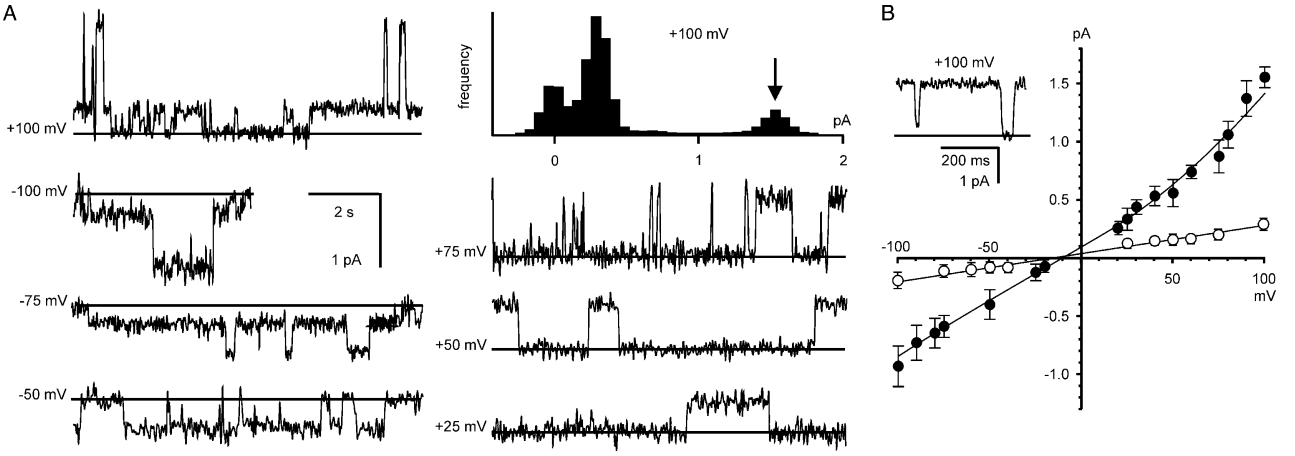

Figure 1. Bilayer reconstitution of CLIC4 in the presence of 1 mM DTT. Part A shows examples of recordings at selected holding potentials, with solid lines to indicate the closed levels, and an example of an all-points amplitude histogram from 30 sec of the +100 mV recording (the arrow indicates the maximum open level). Part B summarizes the I/V relationship of the main open level and a ~25% substate, from 15 independent experiments with 500 mM KCl *cis* vs. 50 mM KCl *trans*. The bars indicate ±1 SD, and the line was fitted by eye (see text for linear regression analysis). The inset shows examples of direct transitions between the ~25% substate, the main open level and the closed level, on an expanded time scale.

Br^- (1.3±0.50); F^- (1.4±0.95). The values in parentheses are the mean±SD for 4 independent comparisons in each case, and as expected they are not significantly different ($p > 0.5$). Single-channel currents were also obtained in 500 mM:50 mM *cis* vs. *trans* TrisCl (pH 7.4) in the presence of 1 mM DTT (Figure 3A). As shown in Figure 3B, the conductance was reduced to 2.6±0.43 pS (mean± SD, $n = 7$), but the reversal potential was +11± 6.1 mV (mean±SD, $n = 7$), giving a mean Cl^-/ $Tris^+$ selectivity of 1.8±0.51 (not corrected for activities). The difference in relative anion vs. cation selectivity compared to KCl is highly significant ($p <$ 0.001).

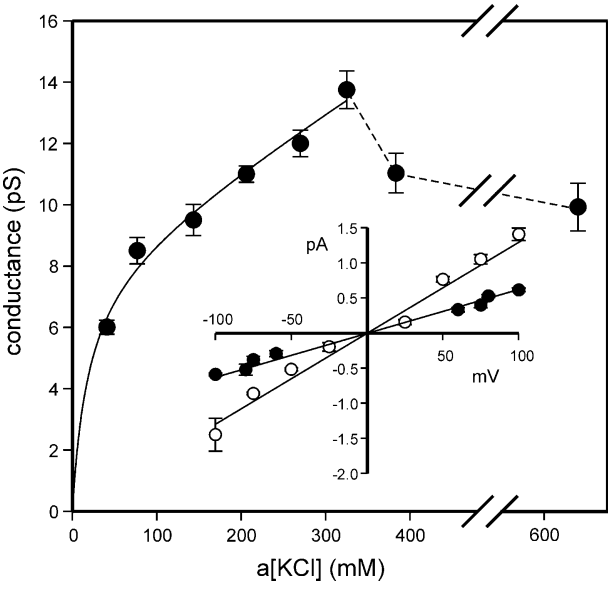

Figure 2. Conductance/activity relationship of CLIC4. The data in the main panel (shown as means±SD from 5 independent experiments) were fitted (by least squares) to the sum of a hyperbolic component with a g_{max} of 8.2 pS and a "K_m" of 19.1 mM plus a non-saturating linear component of 0.017 pS per mM KCl. The relationship breaks down at KCl activities >325 mM (as shown by the dotted lines – note the break in the plot between 400–600 mM KCl). The inset I/V relationships provide examples of the I/V data used to calculate the conductance at low (40 mM, closed circles) and high (325 mM, open circles) activities (points are means±SD, $n = 5$, lines fitted by linear regression).

CLIC4 ion channels in other redox conditions

H_2O_2-oxidized CLIC4 channels were reconstituted in the presence of 100 μM H_2O_2 ("fully-oxidizing" conditions). Ion channels (Figure 4A) appeared more rapidly compared to the reduced protein, and had a slope conductance of 8.9±1.1 pS (mean±SD, $n = 5$) in a *cis:trans* gradient of 500:50 mM KCl, measured (by linear regression) over the linear part of the I/V plot between −100 mV and +25 mV (Figure 4B). This value was indistinguishable from the conductance of channels reconstituted in the presence of DTT ($p > 0.5$). The oxidized channels displayed several substates, and the reversal potential of the main open state was +6.5±6.6 mV (mean± SD, $n = 5$) under fully oxidizing conditions, corresponding to mildly anion-selective channels with a mean Cl^-/K^+ selectivity of 1.4±0.48.

Channels were also obtained in a *cis:trans* gradient of 500:50 mM KCl from (non pre-oxidized) CLIC4 in 5 mM glutathione, corresponding to "physiological" redox conditions [23], e.g., Figure 5. The single-channel currents had higher amplitudes at positive holding potentials (i.e., they were outwardly-rectifying, if *cis* is equivalent to the cell

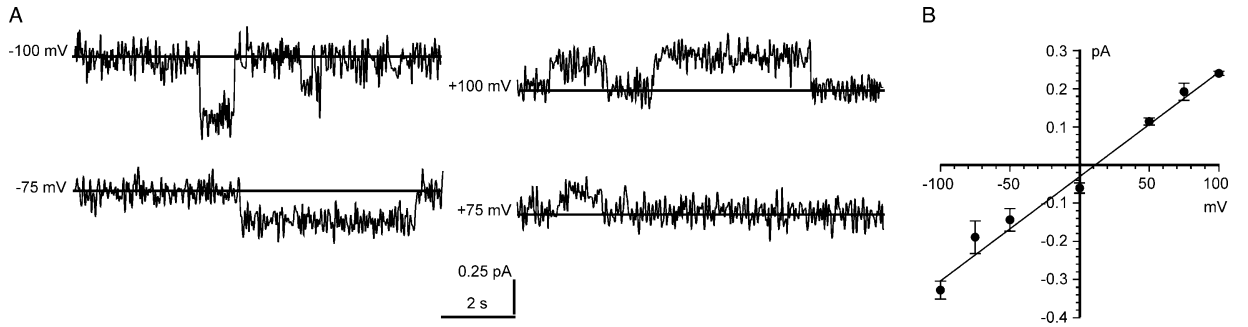

Figure 3. CLIC4 currents in Tris-HCl (pH 7.4). Examples of CLIC4 currents (part A) and a combined I/V relationship from several independent experiments (part B, means ± 1 SD, $n = 7$) in the presence of 1 mM DTT with 500 mM TrisCl *cis* vs. 50 mM TrisCl *trans*. The line was fitted by linear regression.

cytosol), and the maximum slope conductance (with 5 mM GSH/0.5 mM GSSG *cis* and *trans*) was 14.8 ± 1.6 pS (mean ± SD, $n = 5$, calculated by linear regression between −25 mV and +100 mV). The reversal potential of +8.8 ± 4.0 mV (mean ± SD, $n = 5$) (Figure 5B) corresponded to a mean Cl^-/K^+ selectivity of 1.5 ± 0.27. As previously observed for CLIC1 [23], the apparent single-channel slope conductance was modulated by the *trans* redox potential when this was manipulated in a GSH buffer. Sequential additions of GSSG to the *cis* chamber had no effect, but sequential additions of GSSG to the *trans* chamber (e.g., to give [GSH]/[GSSG] ratios of 2:1 or 1:1, as shown in Figure 5A), markedly decreased the apparent single-channel currents. This could be reversed by reverting to a redox potential of −225 mV (5 mM GSH with 0.5 mM GSSG) in the *trans* chamber (also shown in Figure 5A).

Like CLIC1 [23], channel recordings in GSH buffers were less noisy, and contained fewer substates, than recordings under fully-reducing or fully-oxidizing conditions (i.e., in the presence of DTT or H_2O_2, respectively). The excess channel noise we observed in the absence of a glutathione buffer was largely attributable to short-lived or poorly-resolved substates, possibly suggesting that fully-oxidised or fully-reduced membrane CLICs failed to adopt a single, optimally-folded conformation. Consistent with this idea, replacing the *cis* glutathione buffer with 1 mM DTT appeared to induce substates following channel incorporation (e.g., Figure 5C).

Single-channel properties of the putative TMD of CLIC4

As shown in Figure 6, the truncated protein, comprising the first 61 residues of CLIC4 (predicted Mr 6,800), including the N-terminus and the putative TMD [2,11], showed appropriate mobilities on SDS-PAGE, and appropriate retention times during gel-exclusion FPLC (consistent with a monomeric solution, like the full-length protein, which is shown for comparison). We reconstituted truncated CLIC4 into bilayers of the same composition as before (POPE, POPS and cholesterol, 4:1:1 mol/

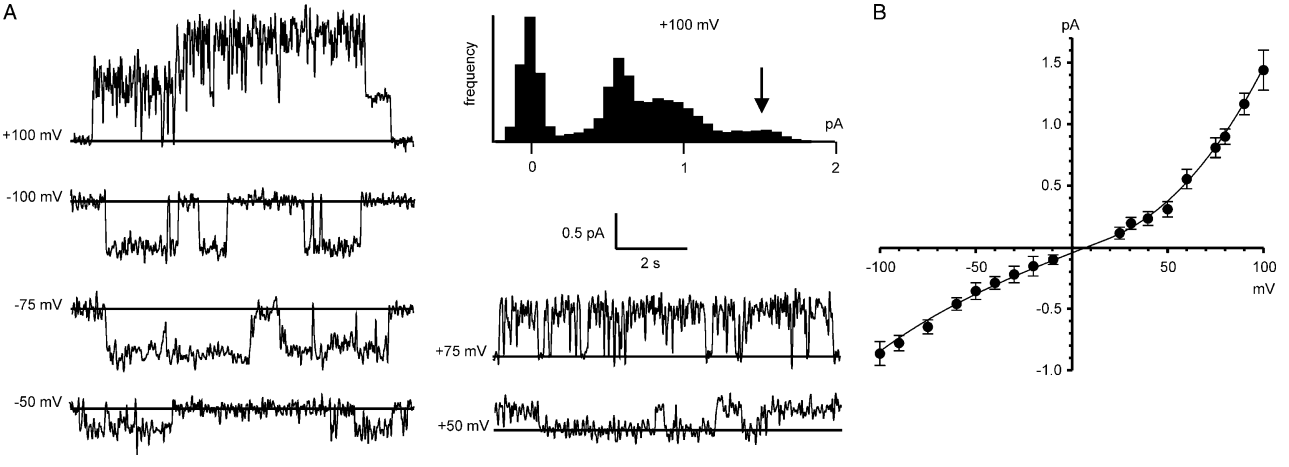

Figure 4. Bilayer reconstitution of CLIC4 in the presence of 100 μM H_2O_2. Part A shows examples of recordings at selected holding potentials, and an example of an all-points amplitude histogram from 30 sec of the +100 mV recording (the arrow indicates the maximum open level). Part B summarizes the I/V relationship obtained from 5 independent experiments (means ± 1 SD, $n = 5$). The line was fitted by eye (see text for linear regression analysis). All the data were obtained with 500 mM KCl *cis* vs. 50 mM KCl *trans*.

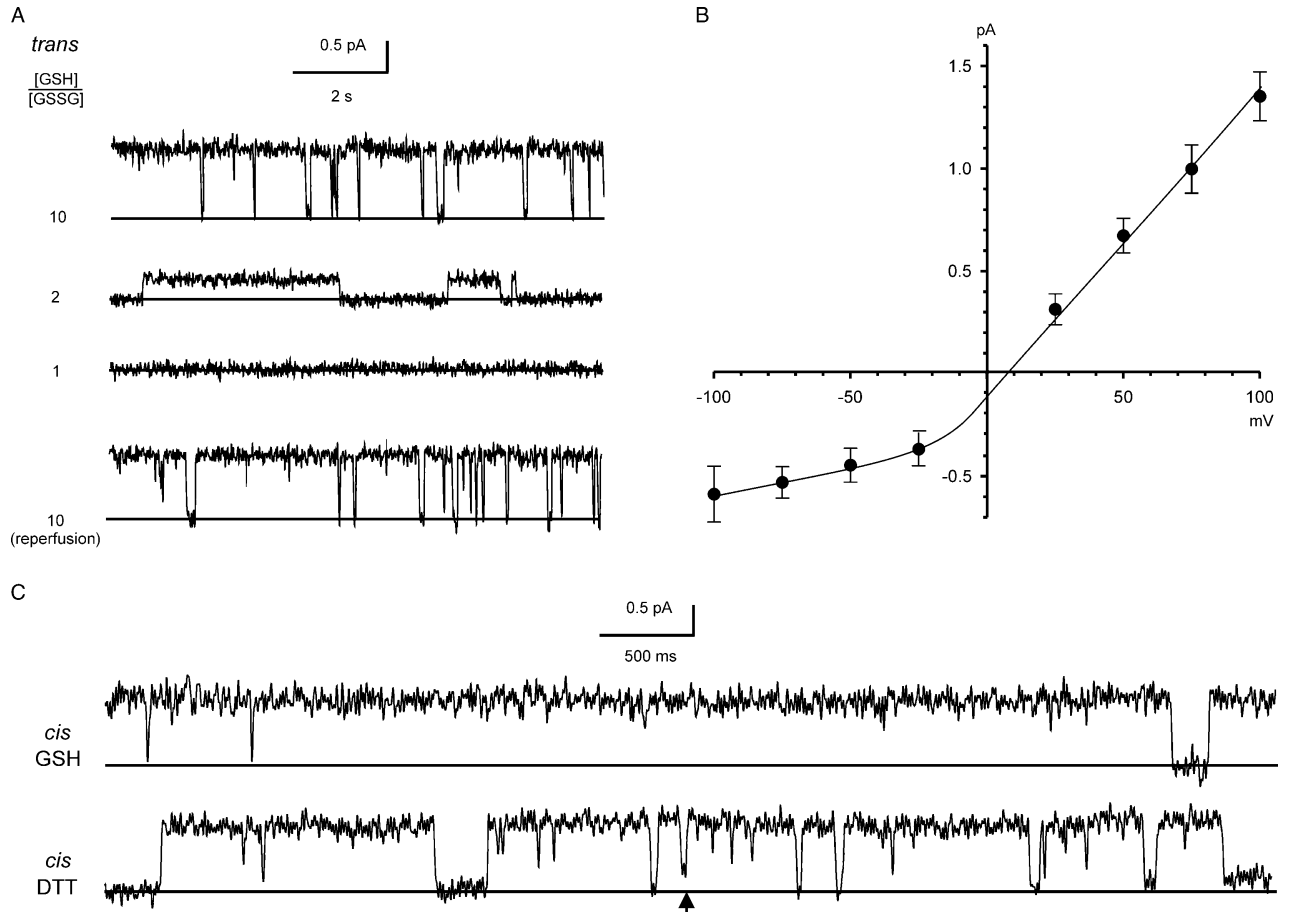

Figure 5. Bilayer reconstitution of CLIC4 in glutathione buffers. Part A shows recordings in 5 mM GSH (*cis* and *trans*), with GSSG added sequentially to the *trans* chamber to give the indicated GSH/GSSG ratios. Part B shows a combined I/V relationship from 5 independent experiments (means ± SD) with 5 mM GSH/0.5 mm GSSG both *cis* and *trans* The line was fitted by eye (see text for results of linear regression analysis). The traces in part C (underfiltered, and on an expanded time scale), illustrate the appearance of substates (best-resolved where indicated by the arrowhead) immediately after replacing the *cis* glutathione buffer (5 mM GSH/0.5 mM GSSG) with 1 mM DTT by perfusion. All the data were obtained with 500 mM KCl *cis* vs. 50 mM KCl *trans*, and the recordings were carried out at a HP of + 100 mV.

mol, respectively) but it formed channels less readily, often taking 20–40 min. compared to less than 10 min for the full-length protein. However, once channels had appeared in the bilayer, "single-channel" recordings were highly consistent between experiments, typically showing infrequent low-amplitude currents of ~0.5 pA at a HP or +100 mV. In some experiments we also observed occasional, large-amplitude currents of up to ~1.5 pA at the same HP, especially in the presence of H_2O_2, including 3 independent experiments using only peak II fractions after FPLC of the truncated protein (Figure 6).

Figure 7A compares the appearance of channels formed by the truncated protein under the different oxidizing and reducing conditions described earlier (all at +100 mV), and Figure 7B summarizes their I/V relationships in a *cis:trans* gradient of 500:50 mM KCl. Currents through the truncated protein remained sensitive to the *trans* (but not the *cis*) redox potential, like the full-length protein (Figure 7A). Overall, the conductances and anion vs. cation selectivities of the truncated protein were very similar in the presence of 1 mM DTT, or 5 mM GSH with 0.5 mM GSSG. From an analysis of several independent experiments, the single-channel slope conductances, calculated by linear regression between +25 mV and +100 mV, were 5.5 ± 0.58 pS and 4.8 ± 0.61 pS (both means ± SD, $n = 5$), respectively, for the two different conditions. The values are not significantly different ($p > 0.5$). The relative anion vs. cation selectivities of the truncated proteins, determined from equilibrium potentials measured under the same conditions, were 1.1 ± 0.27 and 1.0 ± 0.45, respectively (both means ± SD, $n = 5$). Again, these values are indistinguishable ($p > 0.5$), and in Figure 7B all the points are fitted to a single line for both conditions.

As noted earlier, pre-oxidized truncated proteins reconstituted in the presence of 100 μM H_2O_2

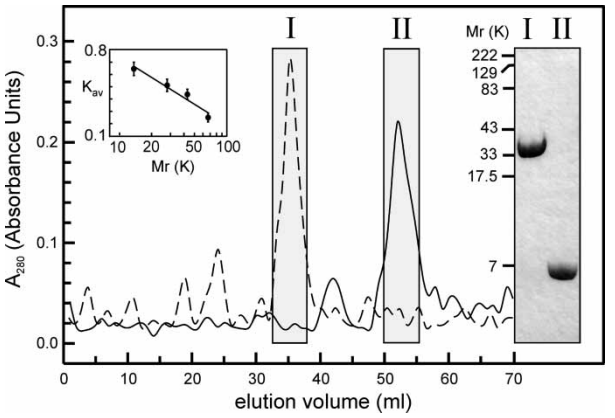

Figure 6. FPLC and SDS-PAGE analysis of full-length and truncated CLIC4. The main panel shows typical gel-exclusion FPLC profiles for full-length (FL) and truncated (TR) proteins in the presence of 5 mM DTT (dashed and solid lines, respectively, shown on the same plot for comparison). Fractions from the boxed peaks (I and II, respectively) were collected and concentrated, and subjected to reducing 10% (w/v) SDS-PAGE and Coomassie-stained as shown. FL and TR proteins show Mr values of ~30 K and ~6–7 K, respectively. The FPLC analysis reveals similar values (V_t was 70 ml, V_o was 4.5 ml, and the K_{av} values for the FL and TR proteins are 0.73 and 0.47, respectively). The column was calibrated (inset plot) with: RNase A (13.7 K), pGEX vector GST (26 K), ovalbumin (43 K) and albumin (67 K).

formed infrequent channels of both "low" and "high" conductance. The former had a slope conductance of 3.8 ± 0.53 pS (mean $\pm$ SD, $n = 5$) measured over the same interval (+25 mV to +100 mV), and a relative anion vs. cation selectivity of 1.2 ± 0.98 (mean $\pm$ SD, $n = 5$). Although these values are not significantly different from those found for the truncated protein in the presence of DTT or glutathione buffers ($p > 0.5$), the experimental points (Figure 7B, open circles) do not appear to align well with the corresponding data obtained using DTT or a glutathione buffer.

Orientation of membrane CLIC4

We previously investigated the bilayer orientation of CLIC1 containing an intact N-terminal His tag by attempting to block or inhibit the channel with 50 μM histidine-reactive Ni^{2+} from the *cis* and *trans* sides in turn, but in similar experiments with CLIC4 the metal ion left some channels unaffected, possibly because the N-terminal region before the putative TMD of CLIC4 is longer and more flexible. As an alternative, we deployed cysteine-specific reagents, also used previously, because the cysteine residues in CLIC4 are located asymmetrically with respect to the putative TMD, just as they are in CLIC1 [2]. As shown later (Figure 9), CLIC4 C35 lies in a cysteine-proline motif on the N-terminal side of the TMD, immediately before the postulated pore entrance. The three remaining cysteines in CLIC4 lie on the other side of the TMD, much further into the primary sequence. C35 is the only cysteine residue in the truncated from of CLIC4.

In contrast to similar experiments with CLIC1 [23], 20 μM NEM had no effect from either side of the bilayer. However, 0.2 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) blocked (or inactivated) CLIC4 channels from the *trans* side, but not the *cis* side (e.g., Figure 8). This applied equally to both full-length CLIC4 and the truncated protein (6/6 experiments in each case), and the effect was quantified by measuring the probability of a given channel being open (Po) over successive periods of 60 sec for each condition. The mean Po for full-length and truncated channels exposed to

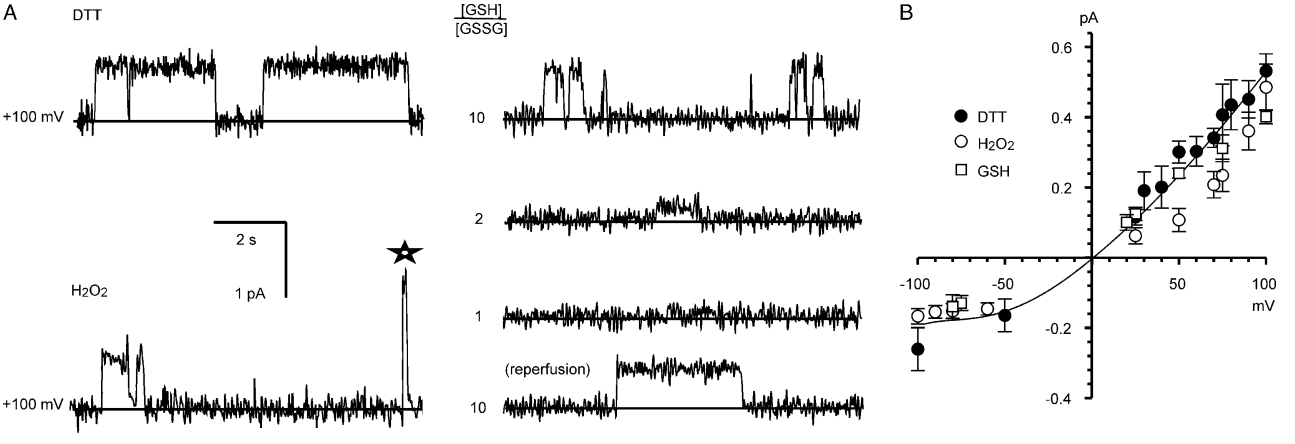

Figure 7. Bilayer reconstitution of truncated CLIC4. Part A compares the appearance of channels at +100 mV in the presence of either 1 mM DTT, 100 μM H_2O_2 (with a star to indicate an unusual large-amplitude opening, see text), or the indicated GSH/GSSG ratios (with [GSH] set to 5 mM), all in a *cis*:*trans* gradient of 500:50 mM KCl. Part B summarizes the corresponding I/V relationships in the presence of 1 mM DTT (closed circles), 100 μM H_2O_2 (open circles) and a 10:1 GSH/GSSG buffer (open squares) (means $\pm$ 1 SD for 5 independent experiments in each case). The line was fitted by eye (see text for results of linear regression analysis).

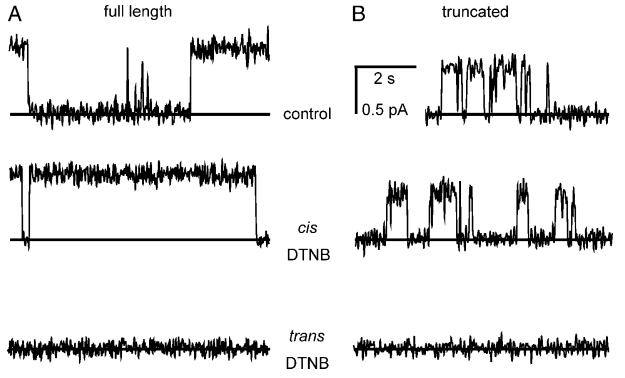

Figure 8. Inhibition of CLIC4 currents by DTNB. Parts A and B show examples of CLIC4 currents from full-length and truncated proteins, respectively, at +100 mV, obtained in the presence of 5 mM GSH (with 500 mM KCl *cis* vs. 50 mM KCl *trans*) before removing the GSH by perfusion with fresh GSH-free solutions. In each case, 0.2 mM DTNB had no effect from the *cis* side, but abolished channel activity when added from the *trans* side. See text for full statistical analysis.

5 mM GSH was 0.63 ± 0.21 and 0.52 ± 0.35, respectively (means $\pm$ SD, $n = 6$). After removing *cis* GSH by perfusion and adding 0.2 mM *cis* DTNB, the Po values were 0.65 ± 0.26 and 0.53 ± 0.34, respectively (means $\pm$ SD, $n = 6$). Following a similar procedure to add 0.2 mM *trans* DTNB, the mean Po values were reduced to 0.05 ± 0.03 and 0.04 ± 0.03, respectively (means $\pm$ SD, $n = 6$).

Although the differences between the mean Po values for control and *trans* DTNB differed significantly, the differences in Po between control and *cis* DTNB, and between control and *trans* DTNB, were also analysed channel by channel by paired *t*-tests, because of the relatively large variation in control Po from channel to channel. This showed no significant difference between control and *cis* DTNB for both full-length and truncated CLIC4 ($p > 0.5$), but significant differences between control and *trans* DTNB in each case ($p < 0.002$ and $p < 0.02$, respectively).

Discussion

Channel activity of CLIC proteins

Despite their structural similarities, Ω-GSTs and CLICs have very distinct functions. In particular, Ω-GSTs remain soluble and have no ion channel activity [14]. However, they are unusual compared to other GSTs in having weak GSH-dependent thioltransferase activities [28]. It has been speculated that this could involve thiol transfer between GSH bound at the G-site (containing the first cysteine residue in the primary sequence), and S-thiolated substrates (including proteins) in an adjacent ligand-binding site (the H-site). In contrast, CLICs appear to have little or no enzymatic activity, but CLIC1 can autoinsert into membranes and form novel ion channels [6]. CLIC proteins contain a single predicted TMD near the N-terminus, which is notable by its absence in Ω-GSTs [2]. In every mammalian CLIC protein, this putative TMD is preceded by an Ω-GST-like "G-site" cysteine residue, in a cysteine-proline motif.

The suggestion that an entire class of soluble eukaryotic proteins, including several mammalian proteins, can bypass the secretory pathway and autoinsert into membranes without any specific insertion machinery, remains highly controversial. In addition, there has been substantial disagreement concerning even the basic properties of the ion channels associated with CLIC1 (the best studied protein) [6,23,29–35]. This includes disagreement over whether the protein can insert into membranes in both oxidized and reduced forms [23], or whether it can only insert as an oxidized protein containing an intrachain disulphide bond [34]. However, the importance of specific membrane lipids, and the sensitivity of reduced membrane CLIC1 to external (or luminal) oxidation, could help to explain many previous inconsistencies. We suggested that membrane CLIC1 oligomers are oxidised and reduced by thiol-disulphide exchange involving GSH, GSSG and external or luminal "G-site"-like subunit cysteines, equivalent to the G-site in Ω-type GSTs. This in turn could regulate channel gating and the overall ion flux through CLIC1 [23]. Thus, channel function may depend on the external (or luminal) redox conditions, which are often poorly-controlled in experiments.

Like CLIC1, proper functional reconstitution of CLIC4 (distinct from simple protein autoinsertion, as shown previously for CLIC1 [23]) required a specific lipid mixture (PE/PS/cholesterol, 4:1:1 mol/mol). Although other, untested, lipid mixtures may also be effective, the presence of cholesterol recalls the specific role of the sterol in cytolysin channel insertion or assembly [36,37], and intracellular membranes contain a similar amount of the free sterol [38]. Alternatively, given that cholesterol and the inverted-cone shaped phospholipid PE are both associated with "curvature stress" in planar membranes, they may promote channel assembly or activity by a physical role similar to the activation of membrane-associated protein kinase C [39].

Channel formation by full-length CLIC4

CLIC4 contains 4 cysteine residues [9] and we reconstituted the membrane protein under a wide range of oxidizing and reducing conditions, including DTT (with a standard redox potential of −330 mV, for "fully-reducing" conditions), GSH/

GSSG (providing "physiological" redox potentials between −225 mV and −195 mV at a pH of 7.4), and 100 μM H_2O_2 ("fully-oxidising" conditions). The channels had a similar maximum conductance of ∼15 pS in a *cis* vs. *trans* gradient of 500 mM: 50 mM KCl, but were noisier and displayed more substates in DTT and H_2O_2 compared to GSH. We speculated that the protein only folds optimally under physiological redox conditions. The single-channel conductance/activity relationship contained both hyperbolic and linear components at a[KCl] values up to ∼350 mM (in 1 mM DTT), with a paradoxical reduction in conductance above 350 mM, consistent with a multi-ion conduction mechanism (i.e., more than one ion at a time in the selectivity filter).

CLIC4 is poorly-selective between anions and cations, inconsistent with the widely-adopted "CLIC" nomenclature proposed by [18] and [40]. It may be necessary to re-evaluate CLIC proteins, especially CLIC4, as "chloride" channels, if they behave like non-selective channels. This could be due to a wide pore lacking specific ion-binding sites. However, a maximum conductance of ∼15 pS in KCl is inconsistent with a wide water-filled pore (as found for example in bacterial porins). Franciolini and Nonner [41,42] noted similar paradoxical behaviour in neuronal "background" Cl^- channels, and suggested that anions and cations crossed the membrane at least partly as counter ions. Based on similar ideas, if the putative pore lining of CLIC4 contains rings of arginine or lysine residues (as suggested in Figure 9), transient binding of permeant anions could provide an opportunity for counter ions (e.g., K^+) to cross the membrane without encountering a prohibitive positive charge.

In a scheme like this, ion permeation requires the presence of anions, and they must be sufficiently small to penetrate the pore. Large, impermeant anions would prevent the passage of both anions and cations (regardless of the latter's size), whereas large, relatively impermeant cations would not prevent all ion permeation, but would make the channel more anion-selective. Consistent with this idea, we noted enhanced anion vs. cation selectivity in the presence of the relatively large cation $Tris^+$ compared to K^+. We also noted that CLIC4 was unable to discriminate between a range of different anions, again consistent with the idea that the pore itself is poorly-selective. However, CLIC1 is slightly but measurably more anion-selective than CLIC4 [23], so if the putative pore-lining is not responsible for this difference (and the sequences are very similar, as shown in Figure 9, suggesting in passing that detailed site-directed mutagenesis studies of putative TMD/pore-lining residues may not be very productive), could other parts of the protein be important for selectivity?

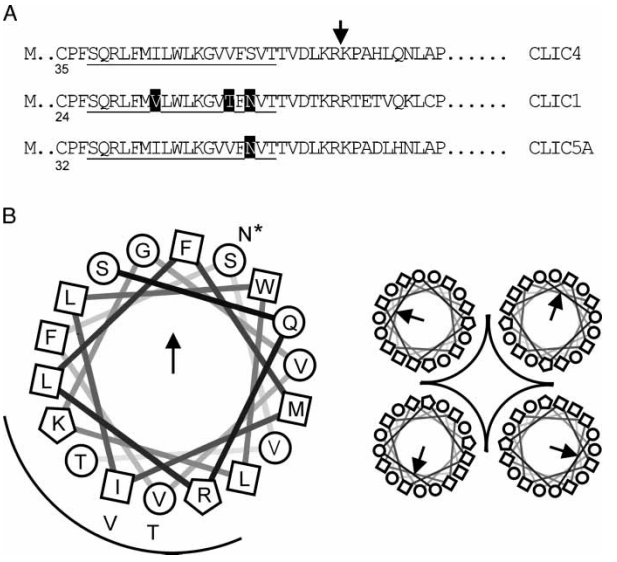

Figure 9. Organization of the putative CLIC TMD. Part A: selected regions of 3 CLICs aligned at the putative 18-residue TMD (underlined). The first cysteine residue in each sequence is numbered, and differences within the TMD are highlighted (rat and human CLIC4 are identical in this region). CLIC4 was truncated where indicated. Part B is a helical wheel projection of the TMD from N to C (D. Armstrong and R. Zidovetzki, http://rzlab.ucr.edu/scripts/wheel/wheel.cgi) with a "tetrameric pore" cartoon. Squares, circles and polygons indicate relatively hydrophobic or hydrophilic residues, or residues with positively charged side chains, respectively. The arrow and the arc indicate the direction of the hydrophobic moment, and suggested pore-lining residues, respectively. The alternative residues in CLIC1 (all 3) and CLIC5A (starred residue only) are also shown.

We also noted enhanced anion vs. cation selectivity in fully-oxidized CLIC4 (exposed to 100 μM H_2O_2) compared to the fully-reduced channel, although the channel remains essentially very poorly-selective. Oxidation of soluble CLIC1 induces a completely new protein conformation involving intrachain disulphide bond formation and non-covalent association into dimers [34], but a similar mechanism has been ruled out for CLIC4 [12], so we speculate that following membrane insertion the pore-lining regions of CLIC4 remain the same irrespective of oxidation or reduction. Instead, we suggest that the difference in selectivity observed here could be due to changes in the charged surface of the protein, away from the putative TMD, that affect the channel vestibule(s) or entrance(s). This suggestion is consistent with experiments involving truncated CLIC4.

Channel formation by truncated CLIC4

Several lines of evidence support the idea that membrane CLICs have a single TMD. Sequence alignments and hydrophobicity plots (e.g., [2,11])

strongly predict a single TMD near the N-terminus, following the first cysteine residue in CLIC1 and CLIC4. This predicted TMD is very obviously absent in Ω-GSTs [2], and it contains a tryptophan residue that could promote membrane autoinsertion. The presence of a single TMD is supported by the positioning of the N- and C-termini of CLIC1 on opposite sides of the membrane [32], by protease digestion studies on membrane CLIC4 [9], by truncation, mutagenesis and membrane localisation studies of the worm protein exc-4 [27,43], and by the presence of potential (and functional) protein interaction sites throughout the "cytosolic" region of, especially, CLIC4 [15]. Based on these findings, we suggest that CLIC4 (and CLIC1) channels are likely to be oligomers comprising at least 4 subunits.

We explored the possibility that a truncated version of CLIC4, comprising the N-terminus and the putative TMD, including a single remaining cysteine residue just before the putative TMD, could form functional ion channels in planar bilayers. Although channel formation proceeded less rapidly, the truncated protein formed ion channels with consistent appearances from experiment to experiment, with a reduced conductance compared to the full-length protein. This reduction is consistent with the idea that the missing parts of the protein form charged channel vestibules that "concentrate" permeant ions near the entrance to the pore [44]. Channels formed from the truncated protein were non-selective, regardless of redox conditions, again consistent with the idea that the missing extramembranous regions of the protein, rather than specific ion binding sites in the pore, confer the (poor) ionic selectivity of full-length CLIC4. Overall, we suggest that under partially- or fully-reducing conditions, despite the absence of much of the protein, including all the potential "cytosolic" domains, the truncated subunits continue to associate (by non-covalent interactions) to form a functional pore.

The truncated protein also formed functional pores under fully-oxidizing conditions, although pore formation occurred much less frequently. Under these conditions, two conductances were recorded, one slightly less than the pores formed under reducing conditions, and another whose conductance was substantially higher. The oxidized proteins presumably insert into bilayers as preformed disulphide-linked dimers, and then associate with other similar dimers in the membrane to form functional channels. However, *trans*-oxidized CLIC4 is normally poorly-conducting (see below), so we speculate that these pores must be assembled from a relatively large number of pre-linked dimers in order to produce a patent pore, compared to fewer pairs of monomers for the reduced protein. Occasional even larger assemblies could explain the infrequent high conductances we observed.

Orientation and redox-regulation of membrane CLIC4

CLIC1 inserted into bilayers with a well-defined orientation, with its N-terminus facing the *trans* ("extracellular" or "luminal") side of the membrane [23]. The same topology was expected for CLIC4, especially given that all the protein interaction sites known to be located between the putative TMD and the C-terminus [27] are cystosolic. This orientation places the first cysteine residue in membrane CLIC monomers in the *trans* chamber, immediately before the predicted TMD. Consistent with this orientation, CLIC4 was sensitive to the *trans* redox potential in glutathione buffers (as discussed below). However, in contrast to CLIC1, NEM had no effect on CLIC4, suggesting that in this case NEM may not have been able to react covalently with the relevant residue, or the reaction had no functional consequences. In contrast, DTNB inhibited the channel, and it only acted from the *trans* side. We speculate that the pore-associated cysteines in CLIC4 oligomers, equivalent to the "G-site" cysteine in Ω-GSTs that forms mixed disulphides with GSH [1], can react with DTNB to form mixed disulphides that block the channel pore or interfere with channel opening.

The maximum slope conductance of full-length CLIC4 showed a substantial and reversible dependence on the *trans* redox potential in glutathione buffers. This is similar to the behaviour of CLIC1, which was analysed in detail [23] and attributed to reversible disulphide bond formation involving pairs of subunits in the presence of GSH and GSSG. The "smooth" reduction in single-channel currents was consistent with rapid reduction and oxidation of the relevant thiol groups in the presence of the glutathione buffer system, at rates too fast to be resolved under the filtering conditions used. The requirement for relatively heavy filtering of CLIC channels in planar bilayers, and their relative long-lived open and closed times, precludes any meaningful channel gating analysis, because recordings would have to be very long in order to collect enough events. Truncated CLIC4 retained its sensitivity to the *trans* redox potential in glutathione buffers, strongly supporting the idea that the effect is mediated by C35, which is the only cysteine left in the truncated protein.

The sensitivity of the channels to extracellular (or luminal) redox conditions could have significant effects on the amplitude of CLIC4 currents recorded *in vitro* (e.g., [9]) and *in vivo* (e.g., [19]), making it difficult to compare recordings unless the *trans*

redox potential is well controlled. In addition, our selectivity data and their interpretation suggest that large cations (e.g., $Tris^+$) may have a dual effect – to reduce single-channel currents (especially with very large cations), and also make the channels more anion-selective. The anion vs. cation selectivities of both CLIC1 and CLIC4 may therefore be "artificially" improved in the presence of relatively large cations like N-methyl-D-glucamine$^+$, often used in patch-clamp experiments to reduce endogenous cation currents.

Conclusion

Our results suggest that mammalian membrane CLIC4 forms poorly-selective, oligomeric ion channels modulated by luminal (or external) GSH-dependent transthiolation. Its redox-sensitivity may shed more light on the role of CLIC4 in apoptosis [20], and at this stage it would be very helpful to establish the stoichiometry of the channel (the tetramer in Figure 9 is entirely speculative), and confirm the topology of its subunits. However, even if CLIC4 monomers contain just a single TMD, other parts of the protein may still contribute in unanticipated ways to the ion channel pore. In common with other transporters, a full understanding of ion permeation through membrane CLICs, and channel regulation by oxidation, awaits detailed structural analysis. As an added complication, it will be especially important to obtain membrane structures relevant to functional channels, with predictable electrophysiological properties, as opposed to the structures of proteins that insert into membranes without forming specific ion channels [23].

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