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Challenging accepted ion channel biology: p64 and the CLIC family of putative intracellular anion channel proteins (Review)

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Summary

Parchorin, p64 and the related chloride intracellular channel (CLIC) proteins are widely expressed in multicellular organisms and have emerged as candidates for novel, auto-inserting, self-assembling intracellular anion channels involved in a wide variety of fundamental cellular events including regulated secretion, cell division and apoptosis. Although the mammalian phosphoproteins p64 and parchorin (49 and 65K, respectively) have only been indirectly implicated in anion channel activity, two CLIC proteins (CLIC1 and CLIC4, 27 and 29K, respectively) appear to be essential molecular components of anion channels, and CLIC1 can form anion channels in planar lipid bilayers in the absence of other cellular proteins. However, these putative ion channel proteins are controversial because they exist in both soluble and membrane forms, with at least one transmembrane domain. Even more surprisingly, soluble CLICs share the same glutaredoxin fold as soluble omega class glutathione-*S*-transferases. Working out how these ubiquitous, soluble proteins unfold, insert into membranes and then refold to form integral membrane proteins, and how cells control this potentially dangerous process and make use of the associated ion channels, are challenging prospects. Critical to this future work is the need for better characterization of membrane topology, careful functional analysis of reconstituted and native channels, including their conductances and selectivities, and detailed structure/function studies including targeted mutagenesis to investigate the structure of the putative pore, the role of protein phosphorylation and the role of conserved cysteine residues.

Keywords: auto-insertion, chloride channel, patch clamp, planar lipid bilayer.

Introduction

Anion channels are present in many prokaryotes and every eukaryotic cell. Cl^-, the major physiological anion, is often distributed across cell membranes at electrochemical equilibrium, and congenital human myotonias can be a devastating consequence of defects in plasma membrane anion channels deployed by excitable mammalian cells as passive or active regulators of their membrane potential (Koch *et al.* 1992). The more common 'anion channelopathy', cystic fibrosis (CF), reflects a lack of functional CF-associated transmembrane conductance regulator (CFTR) Cl^- channels (Riordan *et al.* 1989) essential for normal salt and water movement across secretory epithelia. This review will describe p64, parchorin and chloride intracellular channel (CLIC) proteins, putative anion channel proteins which, very unusually, often coexist in both soluble and membrane forms.

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Many anion channels, including, for example, volume-regulated anion channels (VRAC; Eggermont *et al.* 2001), have yet to be identified at the molecular level (Jentsch *et al.* 2002). Apart from the p64/CLIC family, several other novel anion channel proteins have been proposed, including P-glycoprotein (Valverde *et al.* 1992), pI_{Cln} (Paulmichl *et al.* 1992), phospholemman (Moorman *et al.* 1992) and the related protein mat-8 (Morrison *et al.* 1995), but only with limited success. This is because proteins that are able to form a transmembrane pore from a single subunit or homo-oligomers must:

- be able to adopt a structure compatible with a transmembrane, channel-forming protein;
- form ion channels when expressed in cells (especially cells that normally lack the protein);
- form identical channels when incorporated as a pure protein into liposomes or planar bilayers;
- show altered function (e.g. differences in ionic selectivity) when critical pore-forming regions are modified (e.g. by mutagenesis).

A protein may fail one or more of these tests because it is only one component of a channel, but at least one member of the CLIC family of putative anion channels is close to satisfying these stringent criteria. CLIC proteins would certainly be stimulating new members of the anion channel family, challenging many accepted views of ion channel biochemistry. After briefly describing well-established anion channels with distinct molecular identities, CLIC proteins will be discussed in detail, highlighting outstanding questions concerning their biochemistry and cellular mechanisms.

Structurally, what do we expect of a 'conventional' anion channel protein? Native and recombinant plasma membrane anion channels are readily accessible for electrophysiological recording, and by combining detailed functional analysis with powerful molecular techniques, many well-established anion channels can be classified into three or possibly four distinct structural groups:

- Chloride channel (CLC) proteins were originally discovered in *Torpedo* electroplax (Miller 1982), and the *Torpedo* cDNA (*ClC-0*) was subsequently expression cloned (Jentsch *et al.* 1990, Bauer *et al.* 1991). Mammalian CLCs include nine proteins and the crystal structures of two bacterial CLC channels have been solved (Dutzler *et al.* 2002). The channels are homodimers, and each monomer contains its own pore.
- CFTR, identified by positional cloning (Riordan *et al.* 1989) and established by comprehensive experimentation (reviewed in Sheppard and Welsh 1999) to be a channel protein, can also form channels from a single subunit (Ramjeesingh *et al.* 2001). Although CFTR is normally

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localized (via a PDZ-interacting domain) to the apical membranes of polarized secretory epithelial cells, it has (together with some CLC channels) additional intracellular roles.
- Ligand-gated anion channels appear to be oligomers (Langosch *et al.* 1990, Hevers and Luddens 1998) in which individual subunits surround and contribute to a single pore.
- The molecular identity of Ca^{2+}-activated Cl^- channels is debated. They may be CLCA (or calcium-activated chloride channel, CaCC) proteins (Pauli *et al.* 2000) that undergo complex post-translational modification, including proteolytic processing and disulphide bond formation (Ran *et al.* 1992).

Atypical VDAC (the mitochondrial voltage-dependent anion-selective channel; Schein *et al.* 1976) is poorly selective for anions over cations and may form a transmembrane β-barrel, but otherwise all these channel proteins appear to have multiple α-helical transmembrane domains (TMDs). The crystal structures of bacterial CLC (Dutzler *et al.* 2002) (and K^+; Doyle *et al.* 1998, Zhou *et al.* 2001) channels also reveal novel pore architectures that focus the charged dipoles of intraprotein α-helices onto permeating ions. pI_{Cln} and phospholemman are small proteins that would be hard pressed to form a similar mixture of transmembrane helices and short, internal α-helices. Members of the p64/CLIC family could conceivably do so, and as discussed later, the crystal structure of the soluble form of CLIC1 (Harrop *et al.* 2001) may offer some insights in this respect.

Intracellular anion channels

Apart from 'metabolite' (e.g. phosphate; Laver *et al.* 2001) channels, most intracellular anion channels (including putative CLIC channels) are likely to be relatively mundane but essential 'charge compensators', balancing the uptake and release of endoplasmic reticulum (ER) Ca^{2+} and positively charged neurotransmitters or neurohormones in synaptic or secretory vesicles, or proton movements in more acidic organelles (voltage-activated Cl^- channels seem to be essential for a compartment to reduce its pH below about 5.5 without developing a limiting membrane potential; Rybak *et al.* 1997). Although it is sometimes possible to 'drive' intracellular channels to the plasma membrane by over-expressing them, in general they have to be reconstituted in planar lipid bilayers for functional analysis. Listing just a few examples, native anion channels have been reconstituted from: muscle sarcoplasmic reticulum (Smith *et al.* 1985, Sukhareva *et al.* 1994, Kourie *et al.* 1996), mammalian ER (Clark *et al.* 1997), plant vacuoles (Klughammer *et al.* 1992), and Golgi (Nordeen *et al.* 2000), inner mitochondrial (Hayman *et al.* 1993), lysosomal (Tilly *et al.* 1992) and secretory (Stanley *et al.* 1998) vesicle membranes.

Unfortunately, all membrane preparations are impure, and channels will be 'selected' from particularly 'fusogenic' vesicles. These could be minor, even plasma membrane, contaminants. Bilayering is also liable to contamination with impure lipids (including studies with purified proteins). Doping vesicles or liposomes with nystatin and sterols (Woodbury and Miller 1990) 'randomizes' fusion and minimizes operator bias, but requires rather precise control of sterol and nystatin concentrations to be successful. Though used for CFTR (Bear *et al.* 1992), this has not found general applicability. Patch clamping internal (e.g. nuclear; Tabares *et al.* 1991, Prat and Cantiello 1996) membranes does not entirely solve the localization problem either, because patch-pipettes can pick up other membranes (even from 'isolated' nuclei). As a result, specific molecular or functional markers are invaluable, as shown in the discovery of protein translocation channels (Simon and Blobel 1991) and intracellular Ca^{2+} channels (reviewed in Mikoshiba 1997, Shoshan-Barmatz and Ashley 1998), and bilayering continues to be a useful tool to help verify the channel function of purified proteins.

The possibility that 'intracellular' anion channels are known plasma membrane channels, unrecognized in the absence of 'normal' regulation, has to be taken seriously. The role of accessory subunits remains particularly poorly explored (ClC-Kb channels, for example, are regulated by an essential β-subunit; Estevez *et al.* 2001). Plasma membrane Kv channel subunits expressed *in vitro* are processed into ER membranes to form functional, homotetrameric channels (Rosenberg and East 1992). CFTR and CLC proteins also have very minimal assembly requirements, and CFTR (Biwersi and Verkman 1994), ClC-3 (Stobrawa *et al.* 2001) and ClC-5 (Gunther *et al.* 1998) have all been implicated as charge compensators in the acidification of intracellular organelles. It is probably more relevant to ask what *prevents* proteins from operating as intracellular channels (e.g. membrane potential, endogenous inhibitors or phosphorylation status). Intriguingly, CLIC proteins may target subcellular membranes directly. This circumvents the need to regulate channels processed through the secretory pathway, but calls for novel mechanisms to control membrane insertion and channel formation.

Reconstituted 'intracellular' anion channels tend to discriminate poorly between anions and cations, with Cl^-:K^+ selectivity ratios as low as 2:1 (Clark *et al.* 1997), and frequently have prominent substate behaviour. The substate amplitudes are often simple fractions (typically one-half or one-quarter) of the main open state amplitude (Hayman and Ashley 1993, Hayman *et al.* 1993, Morier and Sauve 1994, Clark *et al.* 1997), recalling similar findings with CLC channels (Miller 1982) (attributed in part to their dimeric quaternary structure; Weinreich and Jentsch 2001). The gating of intracellular anion channel 'protomers' is often cooperative (Hayman and Ashley 1993, Clark *et al.* 1997) and, as discussed later, recombinant CLIC1 channels display similar behaviour in 'tip-dip' bilayers (Warton *et al.* 2002).

p64, founder member of the p64/CLIC family

The discovery of p64 has been extensively reviewed (Landry *et al.* 1990, Al-Awqati *et al.* 1992, Al-Awqati 1994, 1995). Briefly, proteins were isolated from octyl glucoside (OG) solubilisates of bovine tracheal apical epithelium and kidney cortex microsomal membrane vesicles previously shown to contain indanyloxy acetic acid (IAA)-sensitive Cl^- transpor-

ters with properties typical of channels rather than carriers or pumps (Landry *et al.* 1987), using an IAA derivative as an affinity ligand. The drug affinity column bound proteins of 97, 64 ('p64'), 40 and 27K, identified by denaturing silver-stained SDS-PAGE (Landry *et al.* 1989). Because the microsomes were pretreated with 1 M thiocyanate, these were all presumably intrinsic membrane proteins containing at least one TMD. A similar procedure was used to isolate IAA-binding proteins from solubilized skeletal muscle sarcolemma (Weber-Schurholz *et al.* 1993). In this case, the affinity matrix specifically bound two polypeptides of 110-120 and 60K.

Reconstitution of the microsomal proteins into asolectin (partially purified soybean phosphatidylcholine, PC) bilayers revealed three distinct types of anion channel with increasing conductances and decreasing Cl^-:K^+ selectivities: 26 pS and $>$20:1, about 100 pS and 13:1, and about 400 pS and between 5:1 and 2:1, under the same (150 mM KCl) or similar conditions (Landry *et al.* 1989). The sarcolemmal proteins (assayed by patch-clamping reconstituted proteoliposomes) formed heterogeneous channels of 100–150 pS in 135 mM NaCl (Weber-Schurholz *et al.* 1993). Surprisingly, only these channels, and not the microsomal channels, were inhibited by IAA, and there was no direct evidence to implicate any specific protein as a molecular component of an anion channel. Microsequencing (Redhead *et al.* 1992) eliminated the 96 and 29K proteins as an IAA-binding Na^+, K^+ ATPase α-subunit, and an unspecified glutathione-*S*-transferase (GST), respectively (Al-Awqati 1994). Presumably the GST was a membrane-bound rather than a soluble GST. As discussed later, the p64-related CLIC proteins are related to the GSTs, and share a possible IAA binding site (Harrop *et al.* 2001). The 40K band does not appear to have been pursued further, but an antiserum was raised to the 64K band (p64).

In a crucial experiment, the anti-p64 antiserum was shown to immunodeplete Cl^- transporting activity present in a high molecular weight complex of 250–600K (depending on the presence or absence of associated detergent or lipid) from IAA affinity-purified kidney microsomal membrane proteins (Redhead *et al.* 1992). While supporting an ion channel role for p64, this raised new questions. Do p64-associated channels contain additional cellular proteins, e.g. the unidentified 40K protein? Against this, no extra proteins were co-immunoprecipitated by anti-p64 antibodies (Redhead *et al.* 1992). Alternatively, are homo-oligomers of p64 alone sufficient to form a Cl^- channel? Against this, immunopurified p64 was inactive. If p64 alone is sufficient to form an ion channel, it must be partially denatured during affinity purification (losing its ability to bind IAA), and irreversibly denatured during immunoaffinity purification (completely losing any ability to form an ion channel).

Molecular identification of p64 and parchorin

Other lines of evidence emphasized the importance of protein phosphorylation. Protein kinase A (PKA)-dependent phosphorylation of a 64K protein in brain clathrin-coated vesicles and kidney endosomes paralleled phosphorylation-induced Cl^- channel activity (Bae and Verkman 1990, Reenstra *et al.* 1992, Mulberg *et al.* 1995), and immunoreactive p64 was localized to secretory vesicle membranes in sheep thyroid parafollicular cells, where vesicle acidification was correlated with changes in the phosphorylation of p64 (Tamir *et al.* 1994). Meanwhile, efforts continued to identify p64 at the molecular level, and its cDNA was cloned from a bovine renal cortex cDNA library (Landry *et al.* 1993). The encoded acidic (pI 4.34) protein was predicted to contain only 437 residues, with a calculated mass of 49K (figure 1).

Despite its disconcertingly small size, the expressed protein was immunologically confirmed to be p64, which migrates anomalously slowly on denaturing gel electrophoresis (probably because of its low pI). Endogenous p64 was localized to apical membrane vesicles in CFPAC-1 cells (pancreatic duct adenocarcinoma cells from a patient with CF). The predicted protein has no signal sequence, and we now know that the related CLIC proteins (discussed below) have an unusual dual cytosolic and membrane localization, and do not require a signal sequence for their N-terminal region to cross membranes. It remains unclear whether endogenous p64 has a similar dual localization, although the recombinant protein does appear in the cytosol of HeLa cells (Edwards *et al.* 1998). When *p64* mRNA was expressed in *Xenopus* oocytes, the protein remained defiantly confined to intracellular membranes (Landry *et al.* 1993) and did not reach the plasma membrane.

The p64 homologue parchorin was originally identified as a secretory epithelium phosphoprotein in gastric *par*ietal and airway epithelia cells. Its cDNA (first cloned from rabbit *chor*oid plexus; Nishizawa *et al.* 2000) encodes a 637 residue, 65K predicted that also has a very low pI (4.05), and migrates anomalously slowly during SDS-PAGE (about 120K). In contrast to p64, parchorin is mainly cytosolic, but a GFP (green fluorescent protein) parchorin fusion protein associated with the plasma membrane of clonal pig proximal renal tubule epithelium cells in Cl^--depleted medium, indirectly implicating parchorin in membrane transport. The

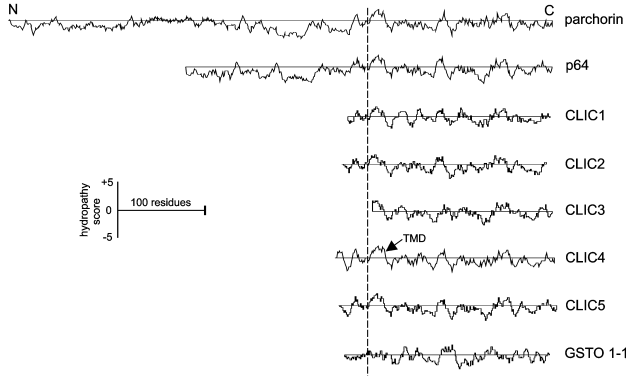

Figure 1. p64/CLIC protein family and GSTO 1-1. Kyte–Doolittle hydropathy plots (nine residue windows) of human CLIC and GSTO sequences, with rabbit parchorin and bovine p64, aligned (vertical dotted line) against the well-conserved cysteine sited N-terminally of the most convincing predicted TMD of CLIC4 (arrowed). Recent entries of predicted human homologues of parchorin and p64 (GenBank AK092733 and AK097098, respectively) show moderate to high similarity to the rabbit and bovine proteins, and AK097098 and CLIC5 appear to be splice variants.

relocalization of cytosolic CLIC proteins to membranes appears to be common in this protein family, suggesting this process may be central to their cellular functions.

Expression, localization and function of p64

The subcellular localization of p64 was investigated in detail in kidney tissues and clonal cell lines (including PancI cells, devoid of endogenous p64, in contrast to CFPAC-1 cells; Redhead *et al.* 1992, Landry *et al.* 1993), and in *Xenopus* oocytes (Redhead *et al.* 1997). Serum supplementation increased levels of endogenous membrane-associated p64 in T84 intestinal epithelial colon cancer cells, consistent with the idea that p64 can actively relocalize from a cytosolic pool to newly synthesized or recycling membranes (like CLIC4; Suginta *et al.* 2001). p64 was localized by confocal microscopy and immuno-EM to the membranes of large (0.5–1.0 μm) dense-core secretory vesicles. Rat brain CLIC4 localizes to similar (possibly neurohormone) secretory vesicles (Chuang *et al.* 1999). Interestingly, although the vesicles moved towards the plasma membrane after treatment with secretogues, they were not exocytosed. Perhaps they are immature and not in a releasable pool. As previously noted for *Xenopus* oocytes (Landry *et al.* 1993), p64 never appears to enter the plasma membrane of cells.

p64 was expressed as a series of truncation mutants, and with part of its N-terminal domain fused to N-terminally truncated CD4, to investigate molecular controls governing membrane targeting. The N-terminus contains 'positive' signals directing p64 to its normal location, whereas the C-terminus contains 'negative' or prohibitory signals, confined to the last few C-terminal residues, preventing default-like localization to the plasma membrane. However, CLIC1 (Valenzuela *et al.* 1997), CLIC4 (Proutski *et al.* 2002) and CLIC5 (Berryman and Bretscher 2000), with similar or identical C-termini to p64, can all localize to the plasma membrane (discussed below), suggesting these signals can be overridden. As discussed later, CLIC1 can also auto-insert into artificial membranes, somewhat undermining the importance of sequence-specific controls on membrane targeting.

Readdressing the crucial question of whether p64 contributes to specific anion channels, membrane vesicles containing highly overexpressed p64 from a vaccinia/HeLa cell system (Edwards *et al.* 1998) were incorporated into asolectin bilayers. p64-expressing cells contained novel strongly rectifying channels of 42 pS in 140 mM KCl. Their selectivity was not examined, but notably this conductance does not correspond to any of the wide range of channels originally associated with p64 (Landry *et al.* 1989). The channels could be induced by adding alkaline phosphatase to the side of vesicle addition. Assuming the phosphatase acts on cytoplasmic sites, similar plasma membrane channels would be orientated as 'outward' rectifiers (like VRAC currents; Eggermont *et al.* 2001). They were unaffected by PKA-mediated phosphorylation, and the effect of IAA was not reported. OG-solubilized vesicular proteins reconstituted into asolectin liposomes showed enhanced phosphatase-*insensitive* Cl^- efflux, and metabolically labelled, recombinant, immunoprecipitated p64 was shown to be phosphorylated. Although these p64-associated channels are 'tonically inhibited' by phosphorylation, consistent with earlier studies (Landry *et al.* 1987), thyroid parafollicular cell p64-associated Cl^- channels are *opened* by phosphorylating p64 (Tamir *et al.* 1994). p64 may be phosphorylated on multiple sites by several kinases and phosphatases, with different functional consequences, and the enzymes themselves may be regulated by phosphorylation, so these data may not be inconsistent.

p64 (and the related CLIC proteins) contain consensus sequences for tyrosine phosphorylation and Src-homology domain type 2 (SH2) binding domains. In a follow-up study, co-expression of p64 with the Src kinase p59fyn was correlated with Cl^- transporting activity reconstituted from solubilized cellular proteins (Edwards and Kapadia 2000). In these experiments, Cl^- fluxes were not enhanced when p64 was expressed alone. By making site-directed mutants, p64 Tyr 33 was shown to be phosphorylated (by Fyn kinase itself or by another, unidentified tyrosine kinase), and phosphorylated (but not dephosphorylated) p64 bound to the SH2 domain of the Src kinase. Native (as opposed to recombinant, overexpressed) bovine p64 also bound to the kinase SH2 domain *in vitro*, and a tyrosine kinase (probably Fyn kinase) was co-immunoprecipitated with p64 from solubilized microsomal membranes.

However, purified p64 has yet to be functionally reconstituted *in vitro*, and its contribution to intracellular ion channels, and the role of protein phosphorylation, remain unclear. While these questions still need to be addressed, investigators have recently directed more effort to the p64-related CLIC proteins, in part because these have been directly linked to anion channel activity.

CLIC1 (NCC27), a putative nuclear membrane channel

Although rat brain p64H1 was the first homologue of p64 to be described (Howell *et al.* 1996), its human (Edwards 1999) and murine (Fernandez-Salas *et al.* 1999) homologues were named CLIC4 (rather than CLIC0). Human *CLIC1* was cloned serendipitously after screening a monocytoid blood cell line for PKC-activated genes (Valenzuela *et al.* 1997). The predicted protein contains 241 residues with a theoretical mass of 27K and a pI of 4.85, and is about 60% identical to the C-terminal region of bovine p64 (figure 1). A FLAG-tagged version localizes to nucleoplasm and the cytosol in Chinese hamster ovary (CHO)-K1 cells, as well to nuclear and plasma membranes. Indirect immunofluorescence and immunoblotting (using antibodies raised to a GST CLIC1 fusion protein, controlled with anti-GST antibodies) confirmed more limited distribution of the native protein to the nucleoplasm and nuclear membrane (Valenzuela *et al.* 1997). However, a highly specific anti-CLIC1 antibody failed to detect nuclear CLIC1 in placental trophoblast cells (Berryman and Bretscher 2000) (which showed a cytosolic distribution).

CLIC1-transfected cells examined by cell and nuclear membrane patch-clamp recording, the first application of patch clamping to this putative channel family (Valenzuela *et al.* 1997), revealed enhanced whole cell currents with relative

anion selectivities: $SCN^- > F^- > Cl^- > NO_3- \sim I^- = HCO_3- >$ acetate$^-$. These data (from a single experiment) are currently the sole account of the relative anionic selectivity of CLIC1-associated channels in cells, and their precise anion versus cation selectivity remains uncertain. Selectivity is important to assign an identity to cellular anion channels, but on this evidence, the relative Cl^-:I^- permeability of the channels is distinct from VRAC currents (Eggermont *et al.* 2001, Jentsch *et al.* 2002). The conductance of single CLIC1-associated channels was 22 pS in the plasma membrane, and 33 pS in nuclear membrane patches (Valenzuela *et al.* 1997). Because of the nuclear membrane localization of the protein, and the functional localization of the novel channel activity, CLIC1 was called NCC27 (nuclear membrane chloride channel of 27K).

The conductance of CLIC1-associated channels in transfected CHO-K1 cells (Tonini *et al.* 2000) is [Cl^-]-dependent, and a single site model predicts a maximum, saturating conductance of 21 pS and a K_m of about 40 mM. In symmetric 140 mM Cl^- the single-channel conductance is 17 pS. A similar channel was observed in 1/30 control cell patches (later revised to 3/80 patches; Valenzuela *et al.* 2000). In an important series of experiments, antibodies recognizing FLAG epitopes at either the N- or C-terminus of CLIC1 inhibited CLIC1-associated plasma membrane channels only when the antibody was added to the external surface for the N-terminally tagged protein, or the cytoplasmic face for the C-terminally tagged protein (Tonini *et al.* 2000). Thus, membrane CLIC1 is an integral membrane protein that crosses the membrane an odd number of times, and at least some of its C-terminal sequence (containing putative phosphorylation sites and protein interaction domains) lies in the cytoplasm.

Additional experiments (Valenzuela *et al.* 2000) showed, first, that similar channels could be recorded from the plasma membrane of non-transfected cells following nuclear membrane fragmentation during the G2/M phase of mitosis. Second, CLIC1-associated channels were reversibly inhibited by 10 μM IAA-94, unlike IAA affinity-purified p64-related channels, suggesting that the IAA binding site of p64 is formed by the C-terminal half of the molecule. If IAA is, for example, a voltage-dependent blocker that enters channels formed by CLIC1 to reach a binding site located within the membrane electric field (cf. Clark *et al.* 1997), further analysis together with mutagenesis studies, as for (cationic) blockade in K^+ channels (e.g. del Camino *et al.* 2000), could be very revealing. Finally, the channels were compared with endogenous plasma membrane channels recorded from three non-transfected cells. They appeared to be generally similar in terms of conductance, ionic selectivity and gating behaviour, raising the possibility that CLIC1 may contribute to an endogenous cellular anion channel.

To summarize so far, CLIC1 is known to be present in cells at least in part as a soluble protein and in part as an integral membrane protein, and over-expression in cells (that also contain endogenous CLIC1) gives rise to specific ion channels. The third important test introduced earlier for a putative ion channel is whether the purified protein can form identical channels *in vitro* in the absence of other cellular proteins. This issue has recently been addressed by two independent groups (Tulk *et al.* 2000, 2002, Harrop *et al.* 2001, Warton *et al.* 2002), and one of the groups has also solved the crystal structure of the soluble form of CLIC1 (Harrop *et al.* 2001).

Soluble CLIC1: structure and membrane 'auto-insertion'

The crystal structure of the soluble form of CLIC1 (resolved to 1.4 Å, PDB file 1K0O; Harrop *et al.* 2001) reveals a glutaredoxin fold typical of the omega class GSTs (Board *et al.* 2000, Dulhunty *et al.* 2001), with a putative GSH α/β N-terminal binding domain and a compact, mainly α-helical, C-terminal domain (figure 2). The omega class GST, GSTO 1-1, does not form ion channels (Dulhunty *et al.* 2001), and from being a relatively compact, soluble, globular protein, CLIC1 must undergo major structural rearrangements to insert into a bilayer. However, the evidence that it can cross the membrane at least once is compelling (Tonini *et al.* 2000), and this is complemented by supporting biochemical evidence for CLIC4 (Duncan *et al.* 1997), as discussed below.

What do we know about membrane insertion, and the resulting ion channels? Purified, solubilized CLIC1 is monomeric and (partly) co-reconstitutes into asolectin liposomes as the detergent is removed (Tulk *et al.* 2000). It can also insert into membranes spontaneously in the absence of detergent (Warton *et al.* 2002, Tulk *et al.* 2002). It cannot insert, or inserts but cannot function as a channel, into PC liposomes, unless they are supplemented with acidic lipids (e.g. phosphatidylserine), or replaced by liposomes containing 7:3 (w/w) phosphatidylserine:phosphatidylethanolamine (Tulk *et al.* 2002). Protein partitioning is pH-dependent (Tulk *et al.* 2002, Warton *et al.* 2002), with more insertion at low pH (and also at high pH, suggesting the effect may be rather non-specific; Tulk *et al.* 2002). More information on partitioning between the aqueous and membrane phases as a function of lipid composition and pH would be useful. The equilibrium appears to be strongly in favour of the soluble protein in cells, but achieving higher membrane concentrations might facilitate structural analysis of the membrane form of CLIC1.

Cl^- flux in CLIC1-containing liposomes is [protein] dependent (Tulk *et al.* 2000) and sensitive to IAA (IC_{50} 8.6 μM when the inhibitor is added before reconstitution). Vesicles incorporated into asolectin bilayers revealed Cl^- (and, surprisingly, K^+) channels (Tulk *et al.* 2000). The anion channels had Cl^-:K^+ selectivities of 4:1 to 6:1, and relative anion selectivities of $Br^- \sim Cl^- > I^-$ (determined from a single experiment). Their single-channel conductance, 67 or 160 pS in symmetrical 150 or 300 mM KCl, respectively, is well above the limiting conductance of about 20 pS in CLIC1-transfected cells (Tonini *et al.* 2000), and the IC_{50} for IAA (added to the *trans* chamber) is 86 μM, an order of magnitude higher than the value in reconstituted vesicles. When reconstituted in the absence of detergents (Tulk *et al.* 2002), but still in reducing conditions (Tulk *et al.* 2000), the Cl^-:K^+ permeability ratio of the channels was 8:1, with conductances that remained relatively high. 50 μM IAA (added on this occasion to the *cis* chamber) completely abolished channel activity. Pretreatment with reduced or

oxidized glutathione, or alkylation with N-ethyl maleimide (NEM), also inhibited the channels (Tulk *et al.* 2002).

In a different approach with greater resolving power, purified CLIC1 was added to the subphase below nominally solvent free PC/cholesterol 9:1 (w/w) bilayers formed across the tips of patch pipettes ('tip-dip' bilayers) (Warton *et al.* 2002). Despite the absence of acidic phospholipids, 8 pS channels assembled in discrete steps in 140 mM KCl to form 31 pS channels equivalent to four 'subchannels', consistent with the 30 pS channels seen in CLIC1-transfected cells (Tonini *et al.* 2000, Valenzuela *et al.* 2000). These were compared to an example of CLIC1-associated channel activity obtained from a single patch on one transfected CHO-K1 cell (Warton *et al.* 2002). Channel conductance and selectivity were similar, but the analysis of open and closed lifetimes was limited to fewer than 100 events, statistically insufficient to determine (let alone compare) single component open or closed time constants, and certainly insufficient to reveal multiple components (Colquhoun and Sigworth 1983). However, the tip-dip channels were sensitive to the same blockers as CLIC1-associated channels in CHO-K1 cells, and had an IC_{50} for IAA of 25 μM.

It is unclear why the conductance of reconstituted CLIC1 channels differs between studies from different laboratories (Tulk *et al.* 2000, Warton *et al.* 2002), and whether these findings are related to different numbers of cooperating 'subchannels' or protomers (Hayman and Ashley 1993, Clark *et al.* 1997, Warton *et al.* 2002). More experiments are needed to compare recombinant CLIC1 channels with recombinant (and 'native'; Tonini *et al.* 2000, Valenzuela *et al.* 2000) channels in cells. This is especially important because GSTO 1-1, while unable to form ion channels itself, can modulate ryanodine-sensitive Ca^{2+}-release channels (Dulhunty *et al.* 2001), suggesting that the soluble form of its structural homologue, CLIC1, could also be an ion channel modulator. NEM abolishes this activity of the GST (Dulhunty *et al.* 2001), as well as the ion channel activity of CLIC1 (Tulk *et al.* 2002). Finally, no studies are yet available to show that modifying CLIC1 (e.g. by site-directed mutagenesis) modifies the properties of CLIC1-associated channels (the final 'test' for a pore-forming protein outlined in the Introduction).

CLIC2 and MAP kinase-associated CLIC3

CLIC2 in the telomeric region Xq28 of the human X chromosome encodes a hypothetical protein of 243 residues with a calculated mass of 28K and a pI of 5.24 (Heiss and Poustka 1997). Its mRNA is expressed at low levels in adult skeletal muscle, but a corresponding protein has yet to be reported. This region of the X chromosome has been linked to several hereditary diseases, including X-linked mental retardation and X-linked epilepsy, and if CLIC2 is expressed at the protein level in the CNS, it could be of substantial medical significance. Human CLIC3, isolated during a yeast two-hybrid (YTH) screen using the cytoplasmic tail of the atypical MAP kinase ERK-7 as the bait (Qian *et al.* 1999), is predicted to contain 207 residues, with a calculated mass of 23.5K and a pI of 6.34. It co-localizes with ERK-7 to the nucleus, and CLIC3 overexpression in murine fibroblast cells is associated with enhanced whole cell anionic currents. Unfortunately, the presumed underlying channel activity has not been examined in detail, and despite a proven association in cells, CLIC3 is not a substrate for ERK-7 (Qian *et al.* 1999). CLIC3 lacks significant N-terminal sequence compared with CLIC1, and as a potential nuclear membrane Cl^- channel protein it should be compared directly with CLIC1 in future studies.

CLIC4, exo/endocytosis and apoptosis

Northern blots suggested that bovine *p64* was a member of a multigene family (Landry *et al.* 1993), and CLIC4 was cloned from rat brain (Howell *et al.* 1996, Duncan *et al.* 1997) as a potential p64 homologue and candidate for the intracellular anion channel co-localized with brain ER ryanodine sensitive Ca^{2+}-release channels (Ashley 1989). Mammalian *CLIC4* is very widely expressed (Duncan *et al.* 1997). It encodes a predicted protein of 253 residues, with a calculated mass of 29K and a pI of 5.42 (for the human isoform). Like other CLIC proteins, CLIC4 lacks a signal sequence, and is distributed as both a soluble and intracellular membrane protein (Duncan *et al.* 1997, Edwards 1999, Fernandez-Salas *et al.* 1999). The membrane form is an integral membrane protein resistant to alkaline carbonate extraction and incompletely removed by protease digestion (Duncan *et al.* 1997), and its C-terminus is cytoplasmic. It can be 'driven' to the plasma membrane in CLIC4-overexpressing human embryonic kidney (HEK)-293 cells, where it is associated with anion channel activity (Proutski *et al.* 2002). This activity is abolished by an antibody directed against an epitope fused to the C-terminus of CLIC4, or by an anti-CLIC4 antiserum that cannot recognize the N-terminus, but only when these are added to the cytoplasmic side of the membrane (Proutski *et al.* 2002). However, this does not prove that CLIC4 is a pore-forming protein, and further experiments will be required to identify mutations in CLIC4 that alter specific channel properties.

Nonetheless, the demonstration of a direct molecular link between specific cell currents and channels and membrane CLIC4 (Proutski *et al.* 2002) suggests that like CLIC1, CLIC4 is a closely associated accessory protein, if not a novel pore-forming protein. The channels have a low conductance of about 1 pS in 140 mM Cl^-, difficult to resolve even by patch clamping. Whole cell recording suggests that $P_I > P_{Cl}$, but further detailed selectivity studies are required. Bilayer incorporation of microsomal (mainly ER) membrane vesicles containing recombinant CLIC4 revealed novel anion channel activity of much higher conductance (at least 10 pS in equivalent conditions; Duncan *et al.* 1997). It is unclear whether the reconstituted channels are directly or indirectly related to CLIC4, or whether the conductance of CLIC4-associated channels varies under different reconstitution or recording conditions, like CLIC1. As already noted, the definitive identification of channels incorporated into bilayers from membrane vesicles often requires specific markers. It is not known whether CLIC4 can auto-insert into membranes like CLIC1 and form specific anion-selective ion channels in the absence of any other protein, and it is possible that such channels may only be resolved by tip-dip recording.

In an effort to shed more light on the cellular role of CLIC4 and explore alternative explanations for CLIC4-associated channel activity, rat brain proteins binding to the major region of soluble CLIC4, extending from the C-terminus to the predicted TMD (figures 1 and 2), were identified by GST pull down assays, MS analysis and immunoprecipitation (Suginta *et al.* 2001). CLIC4 forms complexes containing β-actin, tubulin, dynamin I, 14-3-3 isoforms, and other unidentified proteins, in line with the partial localization of cellular CLIC4 to caveolar membranes (Edwards 1999, Suginta *et al.* 2001) and brain dense core vesicles (Chuang *et al.* 1999). What could be the roles of these protein complexes? Although it is not known whether soluble CLICs have any GST-like enzymatic activities, a thiol transferase could be useful to reverse oxidative stress-induced thiolation of actin or dynamin. On the other hand, if membrane CLIC4 contributes to a functional ion channel, the association of CLIC4 with cytoskeletal actin and dynamin could dock it to relevant membranes (e.g. secretory vesicles; Chuang *et al.* 1999) but stop it inserting until activated by local pH changes, or by specific kinases or phosphatases.

CLIC4 is tyrosine phosphorylated *in vivo* and is phosphorylated *in vitro* by PKA and casein kinase II (all N. Karoulias and R. H. Ashley, unpublished data), as well as by PKC (Duncan *et al.* 1997). In contrast to p64, the relationship between protein localization, ion channel activity and cellular CLIC4 (or CLIC1) phosphorylation has not yet been investigated. Examining the differential localization of soluble and membrane CLIC4 (including phosphorylated CLIC4) in activated or stimulated cells, particularly cells undergoing extensive membrane remodelling (e.g. growth, membrane recycling or apoptosis), may shed further light on the cellular role of CLIC4. PKA-mediated phosphorylation (at the predicted N-terminal consensus PKA phosphorylation site) (figure 2b), and the associated possibility that CLIC4 is anchored along with other proteins in defined signalling microdomains by specific AKAPs (A kinase anchoring proteins) requires further investigation. Interestingly, tyrosine phosphorylation at a nearby consensus site in CLIC4 could create a potential SH2 binding domain (figure 2b), leading to the possibility of dual or opposed regulation (of binding to protein partners or cellular activity) by alternative phosphorylation. Other CLIC proteins could also be anchored to AKAPs or similar scaffolds (see CLIC5 below), and like CLIC4, both p64 and CLIC5 (discussed below) have a similar N-terminal PKA consensus phosphorylation site.

Murine keratinocyte CLIC4 (cloned during differential display analysis of p53 null versus p53+/+ mice, Fernandez-Salas *et al.* 1999) appears to be a proapoptotic protein (Fernandez-Salas *et al.* 1999, 2002). Again, this version of CLIC4 is localized to both the cytosol and intracellular membranes, particularly the inner mitochondrial membrane. Expression is reduced in p53 knockout mice, and enhanced by expressing recombinant p53, or by adding α-tumour necrosis factor (TNF) to induce apotosis. High natural turnover and apoptosis in skin cells obviates UV-induced DNA damage and malignancy, and murine keratinocyte CLIC4 could conceivably function in parallel with the well-

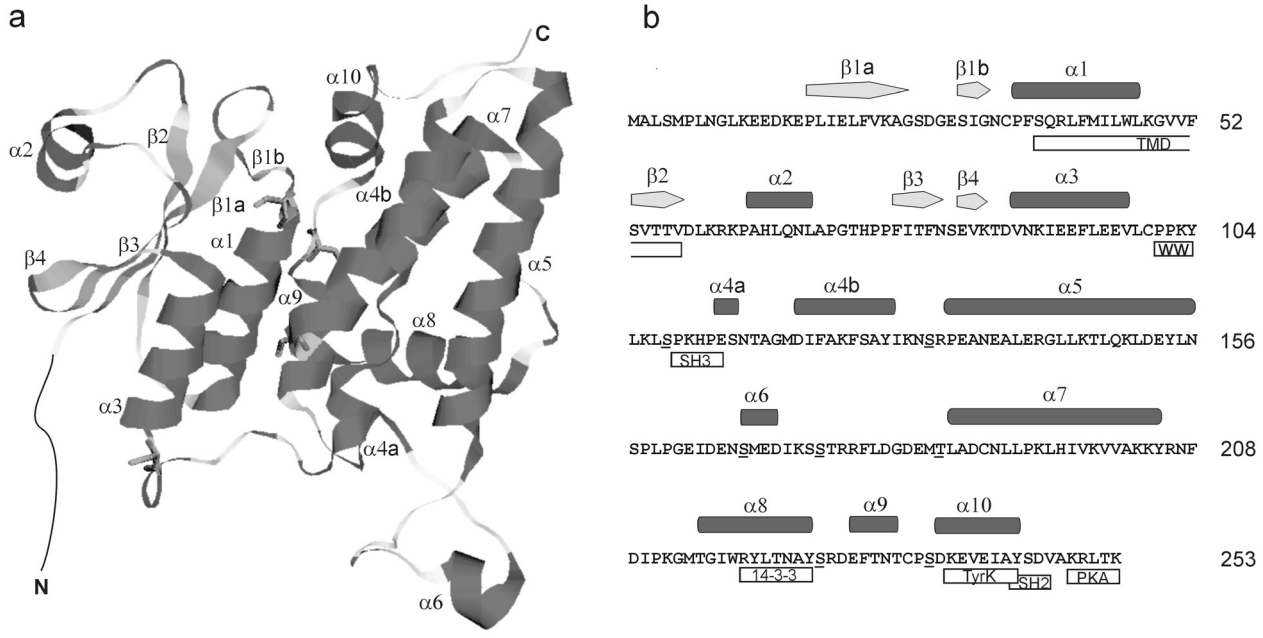

Figure 2. Structure of soluble CLIC4. (a) Rat CLIC4 modelled onto the soluble form of CLIC1 using SWISS-MODEL, rmsd between target and template (1K0M.pdb) Cα atoms = 0.08 Å (D. Soares, N. Karoulias and R. H. Ashley, unpublished data). The four cysteine residues are shown. CLIC1 has two additional cysteines, in α2 and α7 (labelled h6 in Harrop *et al.* 2001, from 1KO0.pdb). The 11 N-terminal residues of CLIC4 remain unmodelled in this RASMOL ribbon diagram. (b) Secondary structure summary of CLIC4 with regions of interest including: predicted transmembrane domain (TMD) (see figure 1); consensus WW, SH3 and 14-3-3 binding domains; consensus tyrosine kinase phosphorylation site and potential SH2 binding domain; PKA consensus phosphorylation site. Potential PKC and casein kinase II phosphorylation sites are underlined.

established Bax-related pathway to permeabilize the inner mitochondrial membrane (Fernandez-Salas *et al.* 2002). Although this is potentially a very exciting role for CLIC4, and there is circumstantial evidence for this model, it is not yet clear that all the details are correct. In particular, the ion channels associated with CLIC4 appear to be far too small to allow the passage of molecules as large as the cytochromes and flavoproteins, characteristic of mitochondrial involvement in apoptosis (Proutski *et al.* 2002).

CLIC5 and other CLIC homologues

CLIC4 is not the only CLIC protein to associate with cytoskeletal proteins. Human CLIC5 was pulled down with actin and other proteins from human placenta by actin-binding regions of the C-terminal domain of the AKAP ezrin. *CLIC5* cDNA encodes a predicted protein of 251 residues with a calculated mass of 28K and a pI of 5.44. The protein associates tightly with the microvillus cytoskeleton, binding either to ezrin itself or to ezrin-associated actin or other ezrin-bound proteins. CLIC1 did not associate with the cytoskeleton, though CLIC4 associated weakly (Berryman and Bretscher 2000). CLIC5 itself has not yet been subjected to any functional characterization, and another recent GenBank entry (AF426169, 'CLIC6') also remains to be characterized.

Chick osteoclasts contain a 62K protein immunologically related to bovine p64 (Schlesinger *et al.* 1997). Incorporation of the partially purified protein into bilayers revealed 17 pS channels in 140 mM Cl^-, but there is no additional molecular or functional information on this possible p64 homologue. A number of other non-mammalian species express CLIC homologues, including *Drosophila*, *Zebrafish* and possibly *C. elegans*, and two CLIC-related genes may be expressed during *Xenopus* development (D. Wilson, B. Shorning, R. R. Meehan and R. H. Ashley, unpublished data). The widespread expression of *CLIC*-related genes in multicellular organisms is consistent with a fundamental cellular role, and knockout studies in model organisms, or RNA knockdown at the cellular level, could be particularly revealing.

Outstanding questions

Although purified CLIC proteins appear to form transmembrane anion channels in artificial membranes, whether they contribute to transmembrane pores in cells, and their precise cellular roles, remain largely open questions that need to be tackled by expressing the proteins in cells where they are normally absent, and by knockout studies at the cellular and organismal level. Meanwhile, a major structural puzzle needs to be resolved. The conformation of the membrane form of CLIC1 must differ radically from the soluble form of the protein because of the need to reverse 'inward facing' hydrophobic residues and 'outward facing' hydrophilic residues, apart from the pore lining. Effectively, the soluble protein has to unfold (possibly promoted by pH changes) and turn itself 'inside out'.

The omega class GSTs (Board *et al.* 2000) that share the same fold as soluble CLICs (Harrap *et al.* 2001) are also abundant in nucleoplasm (Yin *et al.* 2001). How do these proteins differ from auto-inserting CLIC channels and what do the proteins retain in common? Despite their low overall identities (15% between CLIC1 and GSTO 1-1), the cysteine near the N-terminus of the predicted TMD of CLIC1 (Cys24), corresponding to the active site cysteine of GSTO 1-1, is conserved in p64, parchorin and all the CLIC proteins, apart from CLIC3 (figure 1). This cysteine would be in a very unusual position, at the top of a transmembrane helix, if α1 and β2 do refold to form a TMD (figure 2). CLIC3 also lacks much of the α1 helix (figure 2). (Note that the partial CLIC3 sequence shown in Harrop *et al.* (2001) is incorrect.) Further studies of the ion channel activity associated with CLIC3 (Qian *et al.* 1999) will help test the hypothesis that a predicted membrane-spanning domain in this region is essential to form a channel. CLIC proteins also retain four unique hydrophobic residues (V-WL-G) in α1 compared with the same region of enzymatically active homologues (Harrop *et al.* 2001). A conserved hydrophobic patch on the surface of a protein, surrounded by charged residues, is more likely to be a hydrophobic protein interaction domain than a TMD.

It has previously been noted (Tulk *et al.* 2002) that α4a/b and α5 have the potential to form amphipathic, antiparallel membrane-inserting α-helices, similar to other 'auto-inserting' channel proteins (Zhou *et al.* 1997). Could the membrane form of CLIC1 then refold to form one or more intrapore anion-coordination sites, somewhat similar to the CLC structures (Dutzler *et al.* 2002)? The relatively well-conserved cysteines may be important, and CLIC1 inactivation by NEM (Tulk *et al.* 2002) suggests that mutagenesis of the cysteine residues in CLIC1 (and CLIC4) should assume a high priority. In CLIC4, Cys 35 can also be brought within disulphide bonding distance of Cys 234 (just after α9) by movement about the hinge region between α3 and α4a (figure 2), and this may also be significant. Unfortunately, if cysteine residues are critical for channel formation and function, the powerful technique of cysteine scanning mutagenesis (Frillingos *et al.* 1998) may be unusable. Overall, given the uncertainties surrounding the membrane structure of the CLIC proteins, it is currently difficult to predict putative 'pore-lining' residues. Membrane crossings need to be firmly established, preferably by two or more techniques, although systematic mutagenesis of residues in α1 and β2, strongly predicted to form a TMD (figure 1), is an obvious first approach.

The properties of 'wild-type' CLIC1 (and CLIC4)-associated channels also need to be clarified, including their ionic selectivities, before they are compared with mutagenized proteins. The identification of potent and specific blockers would also be helpful, and might help elucidate the cell biology of native CLIC-associated channels. Supporting experiments are also needed to find out how putative CLIC1 'subchannel' pores combine to form larger conductances (Warton *et al.* 2002). The biophysical properties of the 'summed' conductance should remain the same as the constituent protochannels in a 'cooperative' model, but may be different if the protochannels combine to surround a single, larger pore. Pore blockers may also block any protochannels one by one conformational changes. Finally, contingent on phosphorylation or dephosphorylation might trigger refolding and membrane insertion, modify protein:-

protein associations in signalling microdomains, or control the function of associated ion channels.

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Note added in proof

It has recently been confirmed that AKAP350 associates with several CLIC proteins and also with p64, and localizes p64 to Golgi membranes and the centrosome (Shanks, R. A., Larocca, M. C., Berryman, M., Edwards, J. C., Urushidani, T., Navarre, J. & Goldenring, J. R., 2002, AKAP350 at the Golgi apparatus: II. Association of AKAP350 with a novel CLIC family member. *Journal of Biological Chemistry*, in press.

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