Evaluation of the membrane-spanning domain of CIC-2

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The CIC family of chloride channels and transporters includes several members in which mutations have been associated with human disease. An understanding of the structure–function relationships of these proteins is essential for defining the molecular mechanisms underlying pathogenesis. To date, the X-ray crystal structures of prokaryotic CIC transporter proteins have been used to model the membrane domains of eukaryotic CIC channel-forming proteins. Clearly, the fidelity of these models must be evaluated empirically. In the present study, biochemical tools were used to define the membrane domain boundaries of the eukaryotic protein, CIC-2, a chloride channel mutated in cases of idiopathic epilepsy. The membrane domain boundaries of purified CIC-2 and accessible cysteine residues were determined after its functional reconstitution into proteoliposomes, labelling using a thiol reagent and proteolytic digestion. Subsequently, the lipid-

embedded and soluble fragments generated by trypsin-mediated proteolysis were studied by MS and coverage of approx. 71 % of the full-length protein was determined. Analysis of these results revealed that the membrane-delimited boundaries of the N- and C-termini of CIC-2 and the position of several extramembrane loops determined by these methods are largely similar to those predicted on the basis of the prokaryotic protein [ecCIC (*Escherichia coli* CIC)] structures. These studies provide direct biochemical evidence supporting the relevance of the prokaryotic CIC protein structures towards understanding the structure of mammalian CIC channel-forming proteins.

Key words: CIC family of chloride channels, membrane domain, planar bilayer, purification, reconstitution, thiol reagent.

INTRODUCTION

CIC-2 is closely related to the skeletal-muscle CIC channel, CIC-1, and the prototypic chloride channel, CIC-0 [1,2]. CIC-2 is expressed in many different tissues and its physiological roles are quite diverse, ranging from a role in transepithelial fluid transport, cell volume regulation and the regulation of neuronal chloride homeostasis [1–3]. While there is some controversy in the literature [4], mutations in CIC-2 have been associated with increased susceptibility to epilepsy, presumably due to a loss of CIC-2 function in the regulation of neuronal chloride homeostasis and altered inhibitory neurotransmission through the GABA_A (where GABA is γ -aminobutyric acid) receptor [5]. An understanding of the molecular basis for its activity will prove invaluable in understanding the primary defect associated with disease-causing mutations.

There has been tremendous progress in our understanding of the structure–function relationships of CIC channels and transporters [6–9]. The X-ray crystal structures of prokaryotic CIC proteins revealed a dimeric structure wherein each monomer possesses a pathway for chloride ion interaction and translocation [7]. Recent functional analyses have revealed that these prokaryotic proteins function as chloride/proton antiporters [10]. Therefore the structural models based on these prokaryotic proteins may be expected to indicate the molecular basis for this shared transporter function in eukaryotic proteins, namely CIC-4 and CIC-5 [11,12]. The fidelity with which these structural models can demonstrate the molecular basis for the chloride channel function exhibited by CIC-0, CIC-1 and CIC-2 is being evaluated in several laboratories [8,13–16]. Interestingly, electrophysiological studies suggest that the above structural models may be useful in predicting the molecular basis for the permeation pathway, the inner and

outer channel vestibules of mammalian channel-forming proteins [6–9,15,16].

Direct evaluation of the validity of the molecular model of the membrane domain of eukaryotic CIC proteins will require the development of methods for the purification and functional reconstitution of these proteins, as well as the development of biochemical and biophysical approaches for their structural analysis. In the present study, we have described novel methods that lead to the functional reconstitution of CIC-2 as a low conductance (2.8 pS) channel, which is regulated by pH and inhibited by ZnCl₂. These methods for reconstitution include the stringent control of the redox state of the protein, a condition we determined to be essential for the reconstitution of the native properties of the channel. Furthermore, the high yield of functionally reconstituted protein permitted analysis of the membrane domain of CIC-2 using proteolysis and MS. These studies provide the first direct biochemical support for certain aspects of the molecular model generated by Dutzler et al. [6–9] for the eukaryotic CIC-2 protein structure. Importantly, the N- and C-termini of the membrane domain have been defined and the boundaries of several putative loops determined. These findings will facilitate progress in our understanding of the molecular basis for CIC channel function.

METHODS

Expression of CIC-2 using the baculovirus expression system

The open reading frame of rat CIC-2 [a gift from T. Jentsch, Institute for Molecular Neuropathology, ZMNH (Zentrum für Molekulare Neurobiologie), Hamburg, Germany] was tagged at the N-terminus with a His₆ tag and introduced into the pBlueBac4

Abbreviations used: CBS, cystathionine β -synthase; DTT, dithiothreitol; ESI, electrospray ionization; MALDI, matrix-assisted laser-desorption ionization; MS/MS, tandem MS; MTSET, [2-(trimethylammonium)ethyl] methanethiosulphonate bromide; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PFO, pentadecafluoro-octanoic acid; PS, phosphatidylserine; Q-TOF, quadrupole–time-of-flight; SCAM, substituted cysteine accessibility modification.

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(Invitrogen, Carlsbad, CA, U.S.A.) vector for generation of baculoviruses containing rat CIC-2. This virus was then employed for high-level expression of CIC-2 in Sf9 cells.

Solubilization and purification of reduced CIC-2

Recombinant CIC-2 was expressed and purified from Sf9 insect cells as previously described, but with some modifications [17]. Briefly, Sf9 insect cells expressing recombinant CIC-2 were harvested from 1 litre of medium by centrifugation at 2000 *g* at 4°C, and the cell pellet was washed once with PBS. The cells resuspended in PBS with 25 mM DTT (dithiothreitol), 10 mM EDTA and protease inhibitors (Roche) were lysed in a French press (Spectronic Instruments, Rochester, NY, U.S.A.), the nuclei and cell debris were pelleted and the supernatant was centrifuged at 100 000 *g* for 90 min to pellet a crude membrane preparation. Integral membrane proteins were solubilized using a detergent solution containing 8% (w/v) PFO (pentadecafluorooctanoic acid; Oakwood Products, West Columbia, SC, U.S.A.) in 25 mM phosphate (pH 8.0) and stirred with a magnetic stirrer at room temperature (25°C) for 2 h. The solubilized sample was filtered through a 0.22 µm filter prior to binding on a freshly regenerated 10 ml Ni²⁺-nitrilotriacetate agarose column (Qiagen, Chatsworth, CA, U.S.A.). An AKTA FPLC column (GE Health Care, Montreal, ON, Canada) was used in all subsequent steps. The column was washed with 100 ml buffer containing 25 mM phosphate, 100 mM NaCl and 4% PFO at pH 8.0 (buffer 1). A pH gradient was applied to the column titrating buffer 1 with buffer 2 containing 20 mM phosphate and 4% PFO at pH 6.0 going from 0% buffer 2 to 100% buffer 2 in 100 ml. Fractions (5 ml) were collected in tubes to which DTT and EDTA were previously added to give 20 mM DTT and 2 mM EDTA final concentrations. Fractions were analysed by Western blotting (10 µl of each fraction) and by silver-stained PAGE (250 µl of each fraction concentrated to 50 µl). Fractions containing CIC-2 protein eluted at pH 6.8 were pooled and concentrated in an Amicon Ultra 50 kDa MWCO (molecular-mass cutoff) concentrator to a final volume of 1 ml. This sample was diluted 10 times using a buffer containing 8 mM Hepes, 0.5 mM EGTA, 10 mM DTT and 0.025% sodium azide at pH 7.2 and reconcentrated to a final volume of 600 µl.

Reconstitution of purified CIC-2

A suspension containing liposomes (3 mg of lipid in 500 µl of a buffer containing 8 mM Hepes and 0.5 mM EGTA, pH 7.0) was mixed with 0.5 mg of purified CIC-2 (see above). The liposomes were composed of PE (phosphatidylethanolamine)/PS (phosphatidylserine)/PC (phosphatidylcholine)/ergosterol (5:2:1:1, by weight). The mixture was dialysed twice (Spectrapor molecular-mass cutoff 50 kDa; Spectrum Laboratories, Rancho Dominguez, CA, U.S.A.), once against 4 litres of 8 mM Hepes and 0.5 mM EGTA, 1 mM DTT and 0.025% sodium azide (pH 7.0) (buffer A) for 18 h, and then against the dialysis buffer without DTT (buffer B).

Cysteine labelling of CIC-2 proteoliposomes

CIC-2 proteoliposomes were treated with 15 mM Alexa Fluor® 488 C₅ maleimide xanthylum (Molecular Probes) for 1 h at room temperature in dialysis buffer B. Unchanged reagent was removed by dialysis against buffer B.

Sample preparation for MS

Purified, reconstituted, Alexa Fluor®-labelled liposomes were spun down in an airfuge at 100 000 *g* and resuspended in ammonium bicarbonate (50 mM) at 1 mg · ml⁻¹ protein. Trypsin (Pro-

teomics Sequencing Grade; Sigma) was added to the resuspended liposomes at a protein/enzyme ratio of 25:1, and after a brief sonication, the sample was incubated at 37°C overnight. The transmembrane fragments were obtained by spinning the sample in an airfuge at 100 000 *g* for 30 min. The pellet that contained the transmembrane fragments was solubilized in 60% (v/v) formic acid. Analyses were carried out on both the supernatant and the formic acid-solubilized pellet. Alexa Fluor®-labelled peptides from the supernatant were enriched by immunoprecipitation with an anti-Alexa Fluor® antibody (Molecular Probes) on Protein A beads. Formic acid (20%) was used to elute the bound peptides from the beads.

Planar lipid bilayer studies of CIC-2

Fusion of proteoliposomes containing purified CIC-2 with planar lipid bilayers was facilitated and detected by the incorporation of nystatin (120 µg · ml⁻¹), a technique originally described in [18]. Planar bilayers were formed by painting a 10 mg · ml⁻¹ solution of phospholipids [PE/PS/POPC (1-palmitoyl-2-oleoyl PC) in the ratio of 4:4:2] in *n*-decane over a 200 µm aperture in a bilayer chamber. The *cis* compartment, the chamber to which liposomes were added, contained 300 mM KCl and the *trans* compartment, connected to the ground, contained 50 mM KCl. As described in our previous study [19], nystatin-mediated fusion of liposomes or proteoliposomes with the bilayer is indicated by a transient conductance spike. Fusion of proteoliposomes led to the appearance of single-channel events. An event is defined as the transition in current from the closed conductance level to the open, and back to the closed conductance level, and PCLAMP 8 software (Molecular Devices) was used for event analyses and fitting of current–amplitude histograms.

Electrodiffusive flux assay of CIC-2

As per the method originally developed by Garty et al. [20] and modified by Goldberg and Miller [21], we assessed the chloride channel function of CIC-2 in proteoliposomes using a concentrative tracer uptake assay. Proteoliposomes were pre-loaded with 150 mM KCl and centrifuged through Sephadex G50 columns equilibrated with glutamate-containing salts: potassium glutamate (125 mM), sodium glutamate (25 mM), glutamic acid (0 mM) and Tris glutamate (20 mM) at pH 7.6, to replace external chloride. Electrodiffusive uptake was initiated and quantified by addition of 1.0 µCi · ml⁻¹ ³⁶Cl⁻. Intravesicular ³⁶Cl⁻ was assessed at various time points after separation of liposomes from the external medium using a mini anion-exchange column (Dowex 1).

MALDI (matrix-assisted laser-desorption ionization) MS and MS/MS (tandem MS) analysis

MALDI mass spectra were acquired on an Applied Biosystems QStar XL Q-TOF (quadrupole–time-of-flight) mass spectrometer equipped with an oMALDI™ 2 source and a nitrogen laser operating at 337 nm. The instrument was controlled by the Analyst® QS 1.1 software, and data acquisition was performed in the positive ionization mode. Typically, MALDI MS and MS/MS experiments were conducted by manual operation at the laser frequency of 20 Hz. 2,5-Dihydroxybenzoic acid (Fluka Chemie) was used as the matrix, and nitrogen and argon were used as the curtaining and collision gases respectively. Theoretically, the instrument is capable of sequencing a peptide up to 6000 Da although its MS/MS fragmentation is limited by difficult breakdown of the large peptide parent ions at low collision energy. Therefore a long acquisition time (more than 30 min for each experiment) is normally required for obtaining a high quality MS/MS spectrum.

NanoLC-ESI (electrospray ionization) MS/MS analysis

The nanoLC MS/MS experiment using the tryptic CIC-2 soluble fraction was carried out on the same type of QStar XL instrument with a nanospray® II source designed by MDS Sciex (Concord, Ontario, Canada). Waters CapLC system was coupled on-line with LC-MS/MS analysis using 5 µl of the sample operating in the microlitre pickup injection mode. Peptides were first trapped by a reversed-phase C18PM column [300 µm i.d. (inner diameter) × 5 mm length; LC Packings] and subsequently separated on a C18 PepMap100 analytical column (75 µm i.d. × 15 cm, particle size 3 µm and pore size 100 Å; 1 Å = 0.1 nm). The composition of mobile phases consisted of: solvent A, 0.1 % formic acid; solvent B, 98 % (v/v) acetonitrile containing 0.1 % formic acid. NanoLC separation was achieved over 100 min on a gradient from 5 to 90 % of solvent B mixed with solvent A at a flow rate of 300 nl · min⁻¹. Information-dependent acquisition was used to obtain MS and MS/MS data using a 1 s survey scan followed by three consecutive 3 s product ion scans.

Data processing

MALDI MS mapping was used initially to examine the trypsin-digested peptides whose masses were recognized by the calculated values derived from *in silico* digestion of the rat CIC-2 protein sequence (SWISS-PROT Protein Sequence database accession no. P35525) using MS-Digest (ProteinProspector; <http://prospector.ucsf.edu/ucsfhtml4.0/msdigest.htm>), and then the sequences were confirmed by MS/MS measurements. In the LC-MS/MS analysis, the MASCOT server (Matrix Science; <http://www.matrixscience.com/>) was employed for peptide and protein sequence identification. To analyse Alexa Fluor® 488-labelled peptides, the data were submitted to the Global Proteome Machine (gpm) search program (<http://www.thegpm.org/>), and a cysteine 'Alexa' modification (monoisotopic mass increment 698.099) was added to the default list of residue modifications. The tolerances of the peptide mass and MS/MS fragment mass were set at ±100 p.p.m. Trypsin was specified as the proteolytic enzyme, and the maximum of three missed cleavages was allowed. Methionine oxidation and de-amidation of asparagine (N) and glutamine (Q) were set as variable modifications in the database search.

RESULTS

Purification and reconstitution of CIC-2 as a 2.8 pS channel

As in our previous purification study of CIC-2 [17], we expressed the protein (engineered to possess a polyhistidine tag at its N-terminus) in Sf9 insect cells using the baculovirus system. High levels of CIC-2 expression were achieved and CIC-2 could be purified after detergent solubilization from a crude membrane preparation to near homogeneity by virtue of the affinity of its polyhistidine tag to a nickel column. Figure 1(A) shows a silver-stained gel of CIC-2 protein analysed by SDS/PAGE. There are two dominant bands corresponding to monomeric and dimeric CIC-2 proteins as confirmed by immunoblotting using a specific anti-CIC-2 antibody [22] and by MS as described below. The physical basis for the appearance of a dimer upon SDS/PAGE is unknown. However, CIC-2 protein was purified using the detergent PFO, which we have shown to be effective in partially preserving the quaternary structure of membrane proteins [23]. Residual amounts of this detergent may be responsible for the appearance of the dimer band upon SDS/PAGE analysis of purified CIC-2.

Figure 1 CIC-2 purified and reconstituted under reducing conditions mediates chloride electrodiffusion

(A) Purified CIC-2 was analysed by SDS/PAGE and silver-stained. Monomeric CIC-2 migrates as a 97 kDa protein and dimeric CIC-2 migrates as a protein close to 220 kDa. (B) Chloride channel function of purified, reconstituted CIC-2 was confirmed by measuring concentrative uptake of ³⁶Cl over time. Uptake by CIC-2-containing proteoliposomes was determined using three different protein preparations (closed square, indicating mean ± S.D., *n* = 4), and uptake by protein-free control liposomes was also determined in three different liposome preparations (open triangle, indicating mean ± S.D., *n* = 4). Uptake by CIC-2-containing proteoliposomes was significantly greater than uptake in empty liposomes at 60 min (*P* < 0.01). (C) Electrodiffusion through purified reconstituted CIC-2 is selective for chloride ion. ³⁶Cl uptake is diminished by addition of 10 mM KCl (Cl) to the extraliposomal solution (*n* = 6, **P* < 0.01) relative to the addition of KBr (Br) (*n* = 6) or potassium glutamate (Glu) (*n* = 10).

Our original methods led to the reconstitution of CIC-2 as an intermediate conductance chloride channel [17], whereas subsequent patch-clamp studies suggested that CIC-2 may function as a low-conductance channel [24,25]. Therefore our original methods were modified in order to enhance correct folding and functional reconstitution. Due to the well-recognized possibility for the formation of non-native disulphide bonds of some purified proteins during refolding under non-reducing conditions [26,27] and the propensity of certain members of the CIC channel family to be modified by oxidation [28], we maintained high concentrations of the reducing agent, DTT, throughout the purification and reconstitution procedures. Figure 1(B) shows that CIC-2 protein, purified and reconstituted in phospholipid liposomes under reducing conditions mediates electrodiffusion of radiolabelled chloride ions (³⁶Cl⁻). The uptake mediated by proteoliposomes containing CIC-2 was significantly greater at 60 min after the initiation of uptake relative to uptake by empty liposomes. Further, as shown in Figure 1(C), this permeation pathway exhibits selectivity for chloride ions relative to bromide and glutamate. The assay employed in the present study demonstrates the relative ability of extra-liposomal salt solutions containing various anions to diffuse through the reconstituted protein and dissipate the electrogenic driving force mediating ³⁶Cl uptake [29]. In the presence of extraliposomal KCl (10 mM), ³⁶Cl uptake is decreased after 60 min relative to uptake in the

Figure 2 CIC-2 purified and reconstituted under reducing conditions exhibits native modulation by pH and inhibition by ZnCl₂

(A) Electrodifusive uptake of ^{36}Cl (normalized for reconstituted CIC-2 protein) is significantly enhanced in proteoliposomes exposed to pH 6.0 (hatched bar) relative to those exposed to pH 7.2 (open bar); * $P < 0.05$ ($n = 10$). (B) Addition of ZnCl₂ (1 mM) caused significant inhibition of ^{36}Cl uptake; * $P < 0.01$ ($n = 10$).

presence of 10 mM KBr or 10 mM potassium glutamate. The selectivity for chloride relative to bromide ions indicated by the flux assay ($\sim 1:0.5$) for reconstituted CIC-2 is somewhat higher than that reported from electrophysiological studies of CIC-2 (1:0.8) and similar to that reported for the related channel, CIC-1 (1:0.4), in biological membranes [30–32]. However, the results obtained using the flux assay are expected to provide only a rough relative estimate of selectivity [29].

Importantly, the function of purified, reconstituted CIC-2 can be modified as in native membranes. It has been documented for CIC-2, as well as several other members of the CIC family, that external acidification from pH 7.2 to 6.0 leads to activation [33,34]. As shown in Figure 2(A), electrodifusive uptake of ^{36}Cl (normalized relative to milligram of reconstituted protein) is significantly enhanced at pH 6 relative to pH 7.2, 60 min after initiation of uptake. Furthermore, ZnCl₂, a well-documented inhibitor of CIC channels, presumably acting through stabilization of the inactivation gate [34,35], led to the significant inhibition of electrodifusive flux. The results of both of these studies point to the reconstitution of the native properties of CIC-2 in this purification system.

The single-channel activity of purified CIC-2 was studied in planar bilayer experiments (Figure 3). A total of five different CIC-2 purification preparations were studied in this assay. For each preparation of proteoliposomes, empty liposomes (no protein), but containing nystatin, were also generated to provide a negative control. The addition of nystatin renders all of the liposomes and proteoliposomes fusogenic and, hence, potential incorporation of protein into the bilayer is reliable [18]. Typically, each empty liposome or proteoliposome preparation was studied in

five to ten different planar bilayers maintained at various holding potentials in the presence of asymmetric KCl solutions (300 versus 50 mM KCl: *cis* versus *trans* compartments). In 35 different bilayer experiments, fusion of empty liposomes as indicated by nystatin-mediated spikes (10–20 per experiment) did not lead to the appearance of clear stepwise changes in current. On the other hand, proteoliposome addition and nystatin-mediated spikes (10–20 per experiment) led to the appearance of stepwise changes in current. In some cases, a relatively high level of background noise prohibited the confident assignment of single-channel steps and the number of those steps. In nine studies, we observed clear steps in current (Figure 3A), consistent with ion channel gating and often there appeared to be multiple similar channels in the bilayer (Figure 3D). Amplitude histograms were generated and the data were fit for single-channel steps occurring at -50 , -100 and -150 mV (Figure 3B). The mean amplitudes at each potential were fit using a straight line that intersected with the zero current axis at approx. $+40$ mV, close to the theoretical prediction for a chloride selective channel (Figure 3C). The slope of the line predicts a unitary conductance of 2.8 pS. Due to the small amplitude of these steps, rigorous channel gating analysis is not possible.

Assessment of purified and functionally reconstituted CIC-2 by MS

MS has been applied to the study of membrane protein structure relatively recently [36–38]. Membrane proteins are notoriously difficult to study using MS due to their hydrophobicity and inherent problems with the use of detergents. Further, as soon as membrane proteins are solubilized in detergents, any information regarding membrane domain boundaries is lost. Some investigators have employed labelling techniques to overcome this problem and identify extramembrane loops of membrane proteins prior to their extraction with detergent [38]. However, in non-purified membrane preparations, the identification of labelled residues originating from the protein of interest using MS is not a trivial undertaking.

To circumvent most of these problems, we studied lipid-reconstituted, purified CIC-2 protein by proteolysis and MS. As described in the Methods section, accessible cysteine residues in liposome-reconstituted CIC-2 were labelled using Alexa Fluor® 488 maleimide, a cysteine-reactive probe employed previously in studies of ion channels [39]. Subsequently, the labelled protein was subjected to trypsin-mediated proteolysis (Figure 4). The proteoliposomes were subjected to sonication in order to permit access by trypsin to both sides of the protein. In this scenario, the lipids will provide protease protection for the regions normally buried in biological membranes and only the naturally accessible regions will be digested, providing the means to distinguish intramembranous from extramembranous regions. Following digestion, the fragments were separated from the lipid-embedded regions by centrifugation for subsequent analysis by MS. The pelleted lipid-embedded fragments were solubilized using formic acid prior to analysis by MS [36].

MS mapping was performed on a high-mass accuracy MALDI QStar XL Q-TOF mass spectrometer at a mass range of m/z 400–20000 to cover all possible tryptic fragments predicted from *in silico* digestion of CIC-2. The identity of the peptide sequences was confirmed by MS/MS, whereby the Alexa Fluor® 488-labelled peptides were recognized by an additional mass increment of 698.1 Da at cysteine residues and two consecutive neutral losses of sulphur trioxide (SO_3 ; 80 Da) from the modified peptide precursor ions ($M + \text{H}^+$). Table 1 shows a detailed list of the peptides identified by MALDI MS and MS/MS, and the sequence coverage accounted for 65 % of the protein CIC-2. To

Figure 3 ClC-2 purified and reconstituted under reducing conditions exhibits low unitary conductance

(A) Single-channel current steps conferred by fusion of a proteoliposome bearing purified ClC-2 at various holding potentials in the presence of asymmetric KCl solutions: 300 mM:50 mM (*cis* versus *trans* compartments). The closed conductance level is indicated by arrowheads. (B) Amplitude histograms generated for single-channel current events at three different holding potentials: -50, -100 and -150 mV. (C) Current-voltage relationship of single-channel activity obtained using data obtained by amplitude histogram analyses. Analysis of the slope of the straight line fitted through data points yields unitary conductance of 2.8 pS, and the line intersects the x-axis at approx. +40 mV, consistent with this conductance pathway being anion selective. (D) Several recordings reveal multiple steps of similar magnitude, consistent with the activity of multiple ClC-2 channel-forming proteins.

detect more of the soluble peptides, we also used high-performance nanoflow LC separation coupled with ESI MS, i.e. on-line LC-MS/MS as a complimentary technique to enhance the detectability of low-abundance peptides. Followed by data interpretation, the peptide sequences retrieved from the Mascot database search were manually validated against each individual MS/MS spectrum. This analysis detected additional residues beyond those identified by the MALDI analysis, and consequently improved the total sequence coverage to 71 % (see Supplementary Figures at <http://www.BiochemJ.org/bj/396/bj3960449add.htm> and Table 1).

Table 2 shows the percentage coverage for each region and reveals that the coverage of the putative cytosolic regions is very high. Therefore, for the first time, the N- and C-terminal

boundaries of the membrane domain of a mammalian ClC protein can be assigned on the basis of experimental data. Fragments up to and including Arg⁹² comprise the solvent-exposed N-terminus. Fragments distal to Lys⁵⁶⁷ probably comprise the solvent-exposed C-terminus. However, further precision in defining the C-terminal boundary was possible by analysis of the lipid-embedded peptides (as described below).

Evaluation of the lipid-associated domain of reconstituted ClC-2

Multiple fragments derived from the lipid-embedded preparation could be fully sequenced by MS/MS (Figure 5). The largest (6550 Da) peptide was identified as residues 500–564 in rat ClC-2, corresponding to the last four helical segments modelled in

Figure 4 Method for study of reconstituted CIC-2 by proteolysis and MS

The diagram shows the primary steps in labelling lipid-reconstituted CIC-2 with Alexa Fluor® 488 maleimide (star) and preparing soluble and lipid-embedded fragments for analysis by proteolysis and MS.

the membrane domain in the X-ray structure of prokaryotic CIC channels (O–R) [7] (Figure 6). Importantly, we found that the putative ‘R’ helix, is partially accessible to trypsin digestion and cleavage occurs at residue Arg⁵⁶⁴. Therefore, together with the previous analyses of soluble fragments (results described above), we can define the C-terminal boundary as lying between Arg⁵⁶⁴ and Lys⁵⁶⁷. According to the model by Dutzler et al. [7], the R helix extends to residue 571, and protrudes into the solvent. Our experimental data support the concept that this helix is partially solvent-exposed.

In addition to O, P, Q and R, several other putative membrane helices were detected in the lipid bilayer (C, D, F, J, K and L) and were sequenced (Figures 7Ai and 7Aii).

Interestingly, the putative E helix, which immediately precedes a chloride-binding site in the prokaryotic protein crystal structure [7] and is predicted to reside in the membrane, was not recovered with the lipid-associated peptides, but rather, it was partially recovered in solution. We suggest that this result is consistent with the idea that this region is proximal to the pore of the CIC-2 channel, as described further in the Discussion section.

The generation of each lipid-associated peptide is dependent on the presence of a solvent-accessible trypsin cleavage site. This limitation, along with the inherent difficulty in analysing hydrophobic membrane helices by MS, may account for the failure to detect other putative helical segments (B, G, H, I, M and N). However, together with the analysis of the soluble fragments and single-trypsin cleavage sites (Figures 7Ai and 7Aii), we confirmed several predictions regarding those regions of CIC-2 that are lipid-embedded versus those that are solvent-exposed.

Five of the eight linkers between helical segments predicted to be localized in the membrane (Figure 7, inset; Table 2), i.e. C–D, K–L, O–P, P–Q and Q–R were detected in the lipid-embedded sample as expected. As the segment encompassing helices G, H and I was not detected, no information about linkers G–H and H–I could be obtained. Similarly, no assignment can be made for linker M–N. The linker between helical segments E–F was found in the solubilized sample, suggesting that it is solvent-exposed. This finding is consistent with the idea that these residues are close to the channel pore.

Importantly, we have also generated data regarding the loops predicted to project out of either side of the membrane. Of the eight loops predicted to project outside of the membrane, we have evidence that all eight are solvent-accessible. A single-trypsin cleavage site was utilized immediately preceding the putative

linker between B–C, supporting the idea that this loop extends out of the membrane (Table 2; Figures 7A and 7B). Further, as predicted, the putative extramembraneous loops between helices D–E, E–F, F–G, I–J, L–M and N–O were either fully or partially recovered in solution, confirming that they reside outside of the lipid bilayer. A trypsin cleavage site, two residues N-terminal to the putative boundary of helix J was utilized, suggesting that this region is exposed to solvent. Furthermore, significant stretches of these loops have been sequenced, providing a solid basis for future mutagenesis studies (Figure 7B).

Endogenous cysteine residues in CIC-2 are labelled by Alexa Fluor® 488 maleimide

Of the 16 cysteine residues in the CIC-2 protein, nine were detected by MS: Cys⁷⁵, Cys⁷⁸, Cys⁸¹, Cys⁸⁵, Cys²⁰⁰, Cys⁶⁰¹, Cys⁷⁵³ and Cys⁹⁰⁶ (Table 1). Seven of these cysteine residues were labelled with Alexa Fluor® 488 maleimide in the folded protein (Figure 8). Peptides bearing this label were enriched in the soluble fragments by immunoprecipitation using an anti-Alexa Fluor® 488 antibody. Six labelled cysteine residues were detected in this immunoprecipitate by MS: Cys⁷⁵, Cys⁷⁸, Cys⁸⁵, Cys²⁰⁰, Cys⁶⁰¹ and Cys⁹⁰⁶. These findings confirm that these cysteine residues are located in solvent-accessible regions in the folded protein. The remaining cysteine residues detected in the soluble fraction, Cys⁸¹ and Cys⁷⁵³, may have failed to be labelled due to inaccessibility to the Alexa Fluor®-labelled probe. Interestingly, one labelled cysteine (Cys²³¹; in the putative F helix) was detected in the lipid-embedded protein, suggesting that it may reside close to the membrane interface of the folded protein.

DISCUSSION

These studies show that purified CIC-2 can be reconstituted in phospholipid liposomes as a very low (2.8 pS) conductance chloride-selective channel that is regulated by pH and inhibited by ZnCl₂. This preparation of functionally reconstituted CIC-2 protein provided an ideal reagent for assessment of its membrane domain boundaries and comparison of experimental data with the model generated by Dutzler et al. [7] based on prokaryotic CIC protein structures. Assessment of this model using direct biochemical tools is particularly important given the difficulty in obtaining structural data regarding eukaryotic ion channels

Table 1 Identification of the tryptic fragments by MALDI QStar XL Q-TOF MS

<i>m/z</i> (measured)	MH ⁺ (calculated)	Δm	Peptide fragment	Peptide sequence	Modification
Fragments in the soluble fraction					
3513.511	3513.539	− 0.028	1–19	(R)DLYDDDDKDPSSRSEMAAATAAA ATVAGEGMEPR(A)	N-terminal tag
1991.912	1991.917	− 0.005	1–19	(R)SEMAAATAAAATVAGEGMEPR(A)	N-terminal tag
1472.719	1472.720	− 0.001	20–31	(R)ALQYEQTLMYGR(Y)	
1683.831	1683.834	− 0.003	32–46	(R)YTQELGAFAKEEAAR(I)	
844.461	844.464	− 0.003	42–48	(K)EEAARIR(L)	
883.460	883.468	− 0.008	49–56	(R)LGGPEPWK(G)	
1438.730	1438.744	− 0.006	49–62	(R)LGGPEPWKGPSAR(A)	
1363.677	1363.685	− 0.008	63–74	(R)ATPELLEYGQSR(C)	
1047.262	349.165	698.097	75–77	(R)CAR(C)	Alexa Fluor®-C75
976.223	278.128	698.095	78–79	(R)CR(I)	Alexa Fluor®-C78
577.320	577.313	0.007	80–84	(R)ICSVR(C)	
1547.833	1547.830	0.003	80–92	(R)ICSVRCHKFLVSR(V)	
1085.280	387.181	698.099	85–87	(R)CHK(F)	Alexa Fluor®-C85
2214.897	1516.823	698.074	195–210	(K)VIGLTICALGSGMPLGK(E)	Alexa Fluor®-C200
1207.623	1207.632	− 0.009	401–410	(K)ETLVTLFDNR(T)	
2440.139	2440.124	0.015	478–499	(R)LVGESMAAWFPDGIHTDSSTYR(I)	
1428.787	1428.800	− 0.013	567–578	(K)KLPYLPELWGR(H)	
1300.698	1300.705	− 0.007	568–578	(K)LPYLPELWGR(H)	
1116.613	1116.620	− 0.007	584–592	(R)VRVEDIMVR(D)	
2042.755	1344.673	698.082	593–604	(R)DVPHVALSCTFR(D)	Alexa Fluor®-C601
2088.115	2088.109	0.006	617–635	(R)MLALVESPEMILLGSIER(S)	
1509.864	1509.875	− 0.011	636–650	(R)SQVVALLGAQLSPAR(R)	
668.230	668.256	− 0.026	750–755	(K)SESCDK(R)	
731.352	731.359	− 0.007	579–583	(R)HQQYR(V)	
2804.432	2804.448	− 0.016	681–706	(R)FQVNTEDSGFPGAHGQTHKPLKALK(R)	
2518.232	2518.232	0.000	707–731	(K)RGPSNATSLQEGTTGNMESAGIALR(S)	
2362.132	2362.131	− 0.001	708–731	(R)GPSNATSLQEGTTGNMESAGIALR(S)	
824.344	824.357	0.013	750–756	(K)SESCDKR(K)	
1284.685	1284.695	− 0.010	799–809	(K)IDPAPFQLVER(T)	
2187.145	2187.156	0.011	815–834	(K)THTIFSLGVDHAYVTSIGR(L)	
1042.606	1042.616	− 0.010	859–867	(K)VRPPLASFR(D)	
2247.013	2247.017	− 0.004	868–888	(R)DSATSSSDTETTEVHALWGPR(S)	
2539.893	1841.808	698.085	891–907	(R)HGLPREGTPSDSDKCK(−)	Alexa Fluor®-C906
Peptides in the insoluble pellet followed by solubilization with formic acid					
4734.547	4734.563	− 0.016	131–174	(R)GLNTNILLQYLAWVTYPVVLITF SAGFTQILAPQAVGSGIPEMK(T)	
4762.535	4734.563	27.972	131–174	(R)GLNTNILLQYLAWVTYPVVLITF SAGFTQILAPQAVGSGIPEMK(T)	Formylation
3386.685	3386.692	− 0.007	211–241	(K)EGPFVHIASMCALLSKFLSLFGG IYENESR(N)	
4084.762	3386.692	698.070	211–241	(K)EGPFVHIASMCALLSKFLSLFGG IYENESR(N)	Alexa Fluor®-C231
3760.993	3760.990	0.003	316–349	(R)FRDLFPFDLQELPAFAVIGIASGFGG ALFVYLNK(K)	
3890.083	3889.084	− 0.001	316–350	(R)FRDLFPFDLQELPAFAVIGIASGFGG ALFVYLNK(I)	
4615.473	4615.506	− 0.033	316–356	(R)FRDLFPFDLQELPAFAVIGIASGFGG ALFVYLNKIVQVMR(K)	
3585.898	3585.915	− 0.017	318–350	(R)LDFFPFDLQELPAFAVIGIASGFGGAL FVYLNK(I)	
4312.346	4312.336	0.010	318–356	(R)LDFFPFDLQELPAFAVIGIASGFGGAL FVYLNKIVQVMR(K)	
3620.030	3620.044	− 0.014	368–400	(K)KRLLFPALVTLLISTLTFPPGFGQFM AGQLSQK(E)	
3491.932	3491.949	− 0.015	369–400	(K)RLLFPALVTLLISTLTFPPGFGQFMA GQLSQK(E)	
3519.936	3491.949	27.987	369–400	(K)RLLFPALVTLLISTLTFPPGFGQFMA GQLSQK(E)	Formylation
3335.844	3335.848	− 0.004	370–400	(R)LLFPALVTLLISTLTFPPGFGQFMAG QLSQK(E)	
3363.845	3335.848	27.997	370–400	(R)LLFPALVTLLISTLTFPPGFGQFMAG QLSQK(E)	Formylation
6550.596	6550.651	− 0.055	500–564	(R)IVPGGYAVVGAAALAGAVTHTVSTA VIVFELTGQIAHILPVMIAVILANAVAQS LQPSLYDSIIR(I)	
6578.638	6550.651	27.987	500–564	(R)IVPGGYAVVGAAALAGAVTHTVSTA VIVFELTGQIAHILPVMIAVILANAVAQS LQPSLYDSIIR(I)	Formylation

Table 2 Coverage of CIC-2 protein obtained by QStar XL Q-TOF MS

Peptide sequences were determined using a combination of MALDI and LC-MS/MS analyses [see Table 1 (MALDI and LC-MS/MS lists)]. Assignment of each region was performed after sequence alignment of rat CIC-2 with human CIC-1 and reference to Dutzler et al. [7], see inset in Figure 7(A). The loops C–D, E–F, G–H, K–L, M–N, O–P, P–Q and Q–R were considered intramembrane. The loops B–C, D–E, F–G, H–I, I–J, J–K, L–M and N–O were considered extramembrane for this analysis.

Region	Subregion	Number of residues	Residues covered	Percentage coverage
Intact protein		907	645	71
N-terminus		93	92	99
C-terminus		333	255	77
Membrane helices		324	186	57
Loops associated with the membrane domain	Intramembrane	42	34	81
	Extramembrane	115	79	69

and the recent evidence that available models demonstrate the structure of antiporters rather than channels [10].

These studies required technique modification and development. In order to reconstitute the native properties of the purified channel, our previous methods [17] were modified to stringently control the redox state of the protein during purification and reconstitution. This requirement highlights the possibility for oxidative damage to this protein, and/or the detrimental effect of non-native disulphide bond formation during folding [26,27]. We

developed a unique strategy with which to analyse the membrane domain of functionally reconstituted CIC-2.

Not surprisingly, analysis of this large membrane protein by MS required the integration of multiple strategies and new technologies. A single-enzyme (trypsin) digestion step of reconstituted CIC-2 led to 71 % coverage of the entire protein and assessment of the boundaries between the membrane-incorporated domains and extramembrane regions. The potential of this approach in defining membrane boundaries is limited by the distribution of trypsin cleavage sites in the sequence of the molecule. However, despite this limitation, several predictions regarding the membrane domain of CIC-2 based on the prokaryotic CIC structures [7] could be evaluated. This success was largely due to the abundance and purity of the protein preparation, the use of lipids rather than denaturants such as detergents to shield and physically separate the membrane domains first from reaction with thiol reagents and secondly from proteolysis. Finally, large membrane-incorporated helical segments could be completely sequenced by the MALDI QStar XL mass spectrometer due to the optimization of collisional energy, focusing potential, collision gas and accumulation time.

Our studies provide direct biochemical evidence supporting the relevance of the prokaryotic CIC protein structures in our understanding of the structure of eukaryotic CIC channel-forming proteins. According to the structural model published by Dutzler et al. [7], the N-terminal boundary of the membrane domain of CIC-2 extends from residue 94 to residue 572 in the rat CIC-2 sequence. The present proteolysis and MS studies are consistent

Figure 5 MALDI MS spectrum of the insoluble CIC-2 pellet, after tryptic digestion and solubilization in 60 % formic acid

Peptide mapping was carried out on an Applied Biosystems QStar XL Q-TOF mass spectrometer with an oMALDI™ 2 source. The principal ions are labelled with the isotopic masses, and the corresponding tryptic peptides are shown above. The formylated products induced by formic acid are indicated by asterisks.

Figure 6 MALDI MS/MS spectrum of a large membrane-embedded peptide

Spectrum of the doubly charged ion at m/z 3275.85, whose single charged ion (MH^+) located at m/z 6550.59 was assigned to the tryptic peptide fragment IVPGGYAVVGAAALAGAVTH-TVSTAVIVFELTGQIAHILPVMIAVILANAVAQSLQPSLYDSIIR at residues 500–564. The m/z values are shown on the fragment ions with isotopic masses, in which the corresponding C-terminal fragments (yn) and N-terminal fragments (bn) are labelled according to nomenclature proposed by Biemann [43].

with this prediction. The N-terminus of the reconstituted protein was solvent-accessible and trypsin-digested to residue 92. A membrane-incorporated peptide corresponding to the C-terminus of the membrane domain (helices O–R) terminated at residue 567. While the experimentally determined C-terminus appears truncated relative to the model, our findings are consistent with the prediction by Dutzler et al. [7] that the R helix extends into the aqueous solution. The solvent accessibility of the ‘R’ helix as determined in the present study is also in agreement with cysteine scanning mutagenesis and electrophysiological studies [13,15]. It has been suggested that the ‘R’ helix extends into the cytosol to convey information regarding conformational change in the cytosolic C-terminus of the protein to the membrane domain during regulated gating.

Recent SCAM (substituted cysteine accessibility modification) studies by Engh and Maduke [15] implicated the solvent accessibility of regions in addition to the ‘R’ helix within the putative cytosolic vestibule of the related CIC-0 channel in the open conductance state, i.e. the putative loop C–D and helices E and J. The present biochemical study fail to indicate accessibility of the C–D loop (reflecting the lack of an accessible trypsin cleavage site) but does suggest accessibility of regions within both helices E and J. The solvent accessibility of helix E is surprising on the basis of predictions from the prokaryotic crystal structure [7] and we cannot exclude the possibility in our present study that the solvent accessibility of helix E may reflect changes in the local structure after trypsin cleavage. However, as previously mentioned, there are consistencies between our biochemical findings and electrophysiological studies mentioned above [15].

Lys¹⁹⁴ in the putative E helix of rat CIC-2 is accessible as a trypsin cleavage site in the present study, and residue Lys¹⁴⁹, the residue with which Lys¹⁹⁴ aligns in CIC-0, is accessible to trypsin cleavage as revealed in SCAM studies [15]. In fact, several of the residues of the putative ‘E’ helix of CIC-2 were soluble according to the present study, which is consistent with their accessibility. Solvent accessibility of the putative ‘E’ helix has also been inferred from homology models of open CIC-0 and CIC-1 channels wherein this region was postulated to contribute to the permeation pathway for chloride ions [40]. Interestingly, trypsin digestion in the putative J helix of CIC-2 occurs at the same juncture as an accessible site in helix J of CIC-0, as determined using SCAM [15]. These findings highlight the potential similarity between the CIC-0 and CIC-2 structures.

Importantly, the present study also serves to provide molecular detail regarding several of the loops projecting from the membrane into the cytosol. This structural information is essential for progress in our understanding of the regulated opening and closing of the channel gate. The cytosolic C-terminus of eukaryotic CIC proteins has been shown to be critical for voltage, and chloride ion and nucleotide-dependent opening and closing of the ‘common’ channel gate [4,14,41,42]. A disease-causing mutation in CIC-2 (G715E), located in the C-terminus between the two highly conserved CBS (cystathionine β -synthase) domains (CBS1 and CBS2) [4,5], induces altered nucleotide-dependent gating [39]. However, there is no information available regarding the structure of the C-terminus or the molecular basis for interaction between the C-terminus and the membrane domain. As a result of the present study, we have provided molecular detail regarding two

Figure 7 Predictions regarding membrane domain based on biochemical studies

The inset in the upper right corner depicts the topology of CIC-2 as depicted by Dutzler et al. [7]. The red highlighted regions indicate the putative chloride ion co-ordination sites. The red stippled box indicates the membrane boundaries used in the analyses below and in Table 2. **(A, i)** Topological model of the membrane domain of CIC-2 annotated to indicate tryptic cleavage sites (blue triangle), segments detected in the supernatant and sequenced (blue + blue hatched) and segments detected in the lipid bilayer and sequenced (red). Each of the putative helical segments has been labelled using the letter code created by Dutzler et al. [7]. Undetected regions are indicated in grey. **(ii)** Rat CIC-2 sequence annotated to indicate predicted intramembrane, helical segments in boxes (derived from model generated for CIC-1 by Dutzler et al. [7]) and alignment of CIC-2 with CIC-1 and CIC-0 using T-Coffee [44]. As described above, soluble fragments are indicated in blue and lipid-embedded fragments, in red. Fragments that have been detected in both the soluble and lipid-embedded samples are indicated in blue with a red underscore. The appearance in both samples may indicate an interfacial location. The orange underscore indicates the region immediately preceding the pore lining residue Glu²¹¹. **(B)** Cartoons show the structure of ecCIC (*Escherichia coli* CIC) (PDB code 1KPK; [7]). Using the alignment described above, we modelled the putative membrane domain of CIC-2. The regions of CIC-2 cleaved by trypsin in proteoliposomes are shown in blue and as expected, these regions lie at the interface between the membrane and the solvent. The membrane-associated helical segments that were sequenced by MALDI MS/MS are shown in red. The region in orange represents the fragment of helix E that was partially detected in the solvent, implicating its proximity to a water-filled vestibule in the context of the mammalian protein. The upper cartoon shows the dimeric structure of the protein. The amino (A helix) and carboxyl (R helix) extend into the solvent. The trypsin-digested extramembraneous loops (L-M and N-O) have been completely sequenced. The predicted linker between B-C was cleaved once by trypsin close to the turn, consistent with its putative solvent accessibility. The lower model clearly shows the larger loops (completely sequenced) which may possibly contribute (with the A and R helices) to the docking of the regulatory C-terminal domain, which is lacking in the prokaryotic structure, but is a large peptide (~35 kDa) in CIC-2.

loops (D-E and F-G) that may extend from the membrane domain of CIC-2 to interact with the C-terminus of the protein during regulated channel gating (Figure 7B).

As another approach to defining the solvent-accessible regions of CIC-2, we labelled the reconstituted protein using thiol-modifying reagents. We reasoned that these smaller reagents may have easier access than trypsin to certain regions of the protein. We employed Alexa Fluor[®]-488 maleimide to label accessible cysteine residues because it had been used previously in studies of ion channel/transporter conformation [39] and the Alexa Fluor[®] moiety permits enrichment by immunoprecipitation for subsequent analysis by MS. We detected labelling of several native cysteine residues in the reconstituted protein, three in the cytosolic N-terminus (Cys⁷⁵, Cys⁷⁸ and Cys⁸⁵), one near the putative

extracellular membrane interface (Cys²⁰⁰), one at the cytosolic membrane interface (Cys²³¹), one in CBS1 (Cys⁶⁰¹) and another at the C-terminus (Cys⁹⁰⁶). Six of these seven labelled residues were detected in analyses of soluble peptides, immunoprecipitated using an anti-Alexa Fluor[®] antibody. The seventh residue (Cys²³¹) was detected in a lipid-embedded fragment. There may be additional cysteine residues that are solvent-accessible in the reconstituted protein, as we could detect only 9 of the 16 native cysteine residues by MS. More extensive coverage may have been achieved using different proteolytic enzymes and/or using smaller, less bulky thiol reagents than Alexa Fluor[®] 488, such as MTSET ([2-(trimethylammonium)ethyl] methanethiosulphonate bromide). Certainly alternative, cysteine-specific reagents with various physical properties will be employed in our future studies

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