

# High-Level Expression, Functional Reconstitution, and Quaternary Structure of a Prokaryotic ClC-type Chloride Channel

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**abstract** ClC-type anion-selective channels are widespread throughout eukaryotic organisms. BLAST homology searches reveal that many microbial genomes also contain members of the ClC family. An *Escherichia coli*-derived ClC Cl<sup>-</sup> channel homologue, "EriC," the product of the *yadQ* gene, was overexpressed in *E. coli* and purified in milligram quantities in a single-step procedure. Reconstitution of purified EriC into liposomes confers on these membranes permeability to anions with selectivity similar to that observed electrophysiologically in mammalian ClC channels. Cross-linking studies argue that EriC is a homodimer in both detergent micelles and reconstituted liposomes, a conclusion corroborated by gel filtration and analytical sedimentation experiments.

**key words:** *yadQ* • liposome • chromatography • glutaraldehyde • ultracentrifugation

## INTRODUCTION

The phylogenetically ubiquitous Cl<sup>-</sup> channel proteins of the ClC family are responsible for a multitude of physiological functions in organisms as varied as mammals, elasmobranchs, yeast, and green plants (Jentsch et al., 1995, 1999). In humans, these channels are intimately involved in electrical excitability of skeletal muscle and neurons, in epithelial electrolyte homeostasis, and in cellular volume regulation. The ClC channels make up the only known molecular family of voltage-gated Cl<sup>-</sup> channels, but very little is understood about their molecular architecture or about the roles their conserved sequences play in gating or permeation. Our ignorance of ClC mechanisms stems from the molecular asymmetry of the Cl<sup>-</sup> permeation pathway, a necessary consequence of the unusual construction of these channels, in which each subunit of a homodimeric complex forms an independent pore (Middleton et al., 1994a, 1996; Ludewig et al., 1996). This architecture demands that the pore is lined by protein residues scattered throughout the primary sequence rather than in localized "hot spots" (Middleton et al., 1996). In this sense, ClC channels differ fundamentally from many well-studied ion channels, such as voltage-gated Na<sup>+</sup>, K<sup>+</sup>, and Ca<sup>2+</sup> channels, cyclic nucleotide-gated channels, inward rectifiers, neurotransmitter-activated channels, and connexins, which are all built on a "barrel-stave" plan with the ion conduction pore formed by repetition of a transmembrane subunit around an axis of four-, five-, or sixfold symmetry (Hille, 1992). Just as the symmetry of these familiar channels has contrib-

uted greatly to their tractability in sequence-function studies, its absence seriously hinders efforts to discern molecular features of ClC channels by the classical, but indirect, means of mutagenesis and functional analysis.

Recently emergent genome sequences have revealed in prokaryotes many homologues of ion channels hitherto considered strictly eukaryotic (MacKinnon and Doyle, 1997; Jentsch et al., 1999). The biological functions of these bacterial genes are entirely unknown. Nevertheless, membrane proteins from prokaryotes have been uniquely successful in heterologous overexpression, and so we were inspired to search for prokaryotic members of the ClC family. This study examines sequences of nine putative ClC channel genes from bacteria and archaea and pursues one of these from *Escherichia coli*, which can be overexpressed and functionally reconstituted as a Cl<sup>-</sup> channel. This prokaryotic ClC channel, which we name "EriC," forms a functional homodimer, a result implying that dimeric quaternary structure is general to the ClC family.

## MATERIALS AND METHODS

### Materials

All chemicals were reagent grade. <sup>36</sup>Cl was purchased from DuPont NEN as a 0.5–1 M HCl solution and was neutralized with NaOH. Working stock solutions were 50–60 mM NaCl, 26–31 μCi/ml. <sup>3</sup>H-Glutamic acid was also obtained from DuPont NEN. Dowex 1×4–100, from Sigma Chemical Co. was converted to the glutamate form and stored in water. *E. coli* lipids (polar lipid extract) were obtained from Avanti Polar Lipids. Glutaraldehyde was Grade I from Sigma Chemical Co.

### ClC Searches

Searches for ClC homologues were performed using BLAST 2.0 (Altschul et al., 1997) or WU-BLAST2 at the following sites on the indicated protein database: National Center for Biotechnology In-

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formation (NCBI; nonredundant data base), Swiss Institute of Bioinformatics (SIB; completed bacterial genomes and EMBL prokaryotes), The Institute for Genomic Research (TIGR; microbial genomes). Initial searches using CIC-0, CIC-3, or CIC-7 as query retrieved eight prokaryotic sequences with high statistical scores for similarity to CICs; further searches using prokaryotic sequences as query retrieved one additional sequence (*Methanococcus*).

### Recombinant DNA and General Biochemical Methods

Oligonucleotides corresponding to flanking regions of the *E. coli* CIC gene *yadQ* (Blattner et al., 1997) were used to PCR-amplify the gene from *E. coli* genomic DNA. The PCR product was blunt-end ligated into the ZeroBlunt vector (Invitrogen Corp.) and subcloned into pASK90 (Skerra, 1994), downstream from the tetracycline promoter. The sequence of the open reading frames of the products of two independent PCR reactions matched the sequence in the *E. coli* TIGR database. A hexahistidine tag was added immediately after the initiating methionine codon using PCR mutagenesis, and the resulting construct, pEriC, was used in all experiments.

All SDS-PAGE experiments were run under reducing conditions (2%  $\beta$ -mercaptoethanol) using standard Laemmli solutions (Laemmli, 1970). Proteins were visualized using Coomassie Brilliant Blue R-250.

### Expression and Purification

*E. coli* JM83 cells were transformed with pEriC, grown overnight at 37°C, and diluted 50-fold into room-temperature Terrific Broth (Sambrook et al., 1989), containing 100  $\mu$ g/ml ampicillin. Cells were grown at room temperature (23–25°C) to  $A_{550}$  of 1.0, and then induced overnight by addition of 0.2 mg/liter anhydrotetracycline (added from a 0.2 mg/ml stock in dimethylformamide). Cells were harvested and kept at 4°C during all subsequent steps. After washing with Buffer A (95 mM NaCl, 5 mM KCl, 50 mM MOPS-NaOH, pH 7.0) and resuspension at 50 ml per liter culture, the cells were disrupted by sonication in the presence of leupeptin (1  $\mu$ g/ml), pepstatin (1.4  $\mu$ g/ml), PMSF (0.17 mg/ml), and  $\beta$ -mercaptoethanol (6 mM). Debris was discarded after centrifugation (8,000 *g*, 25 min), and membranes were collected from the resulting supernatant (110,000 *g*, 45 min). The membranes were resuspended in Buffer B (95 mM Na-P<sub>i</sub>, 5 mM KCl, pH 7.0), supplemented with 250 mM sucrose and then stored at –80°C.

Membranes were extracted for 2 h (at a concentration equivalent to  $A_{280} = 20$  in 0.5% SDS) in Buffer BK (95 mM K-P<sub>i</sub>, 5 mM KCl, pH 7.0) containing 15 mM dodecylmaltoside (DDM)<sup>1</sup>. Insoluble material was removed by centrifugation (110,000 *g*, 45 min). For binding, 1/10 volume of Buffer E (400 mM imidazole-HCl in Buffer BK, 1 mM DDM) was added and the pH was adjusted to 7.8. This extract was incubated overnight with Ni-NTA beads (0.25 ml beads/liter culture; QIAGEN Inc.). The beads were transferred into a column and washed with ~40 vol wash buffer (40 mM imidazole-HCl, 95 mM K-P<sub>i</sub>, 5 mM KCl, 1 mM DDM, pH 7.8) until  $A_{280} < 0.02$ . EriC was then eluted with Buffer E, pH 7.0. The 40-mM imidazole present during binding and wash steps suppresses the binding of contaminating *E. coli* proteins and thereby increases the purity (while decreasing the final yield) of the EriC preparations.

Protein concentration was measured from the absorbance at 280 nm, using a mass extinction coefficient ( $\epsilon = 0.85$  cm<sup>2</sup>/mg) calculated from the sequence (Creighton, 1984), or with the Coomassie Plus protein microassay (Pierce), calibrated using purified

EriC dialyzed free of imidazole. Some experiments used the bacterial K<sup>+</sup> channel protein KcsA, for which expression and purification were carried out as described (Heginbotham et al., 1997).

### Protein Reconstitution

Reconstituted vesicles were formed by combining EriC with lipid-detergent-mixed micelles, and then removing detergent by gel filtration. Except where noted, all reconstitution procedures and flux assays were performed at room temperature. *E. coli* lipid was dried under N<sub>2</sub> in a glass tube, resuspended in pentane, and redried. The lipid was suspended at 20 mg/ml by sonication in Buffer R [450 mM KCl, 20 mM morpholino ethanesulfonic acid (MES)-NaOH, pH 6.2], 34 mM 3-[3-cholamido-propyl]dimethylammonio]-1-propanesulfonate (CHAPS) was added, and the suspension was sonicated again. After a 2-h incubation, EriC was added to the desired concentration (0.09–5  $\mu$ g/mg lipid), along with enough Buffer R to bring the lipid concentration to 10 mg/ml. In control samples, equivalent volumes of Buffer E were added in place of EriC. After 20 min, detergent was removed and vesicles were retrieved using Sephadex G-50 spin columns as follows. Columns (1.5-ml bed volume) equilibrated with Buffer R were prespun in a clinical centrifuge at 1,000 *g*, 15 s to remove excess solution. A 95- $\mu$ l sample of the reconstitution mix was applied to each column, and vesicles were collected by centrifuging 700 *g*, 1 min. Recovery from the spin varied in the range 100–150  $\mu$ l. The samples were frozen in a dry ice/acetone bath and stored overnight in a frost-free freezer at –20°C. Before use, samples were removed from the freezer, cooled in a dry ice-acetone bath, thawed at room temperature, and then sonicated 5–10 s in a cylindrical bath sonicator.

### Concentrative <sup>36</sup>Cl<sup>-</sup> Uptake Assay

Influx of <sup>36</sup>Cl<sup>-</sup> against a concentration gradient was assayed by a three-step procedure, essentially as described (Goldberg and Miller, 1991; Middleton et al., 1994a,b). First, extravesicular Cl<sup>-</sup> was removed by spinning reconstituted vesicles (100  $\mu$ l) through Sephadex G-50 columns equilibrated in Buffer F (400 mM sorbitol, 20 mM MES-NaOH, pH 6.2). Second, <sup>36</sup>Cl<sup>-</sup> uptake was initiated by adding 5  $\mu$ l of the working <sup>36</sup>Cl<sup>-</sup> stock to 100  $\mu$ l of each vesicle sample (final extravesicular concentrations: 2.4–2.8 mM NaCl, 1.2–1.5  $\mu$ Ci/ml <sup>36</sup>Cl<sup>-</sup>). Finally, after the desired time of uptake, <sup>36</sup>Cl<sup>-</sup> trapped inside the liposomes was measured after applying the sample to a Dowex-glutamate column (1.5-ml bed volume) and eluting into a scintillation vial with of 1.5 ml 400 mM sorbitol. Just before the assay, Dowex columns were prewashed with 400 mM sorbitol–5 mg/ml bovine serum albumin (2 ml) followed by 400 mM sorbitol (2 ml). To test for conductive release of <sup>36</sup>Cl<sup>-</sup>, valinomycin (1  $\mu$ g/ml) was added after uptake had reached steady state. In some experiments, the internal anion was varied by replacing KCl with the potassium salt of the desired anion in Buffer R. In other experiments, external test anions were added from stock solutions in Buffer F immediately before addition of <sup>36</sup>Cl<sup>-</sup>.

### <sup>3</sup>H-Glutamate Trapped-Volume Assay

EriC-reconstituted liposomes were prepared as described above with the following variations. Proteoliposomes were formed in Buffer L (20 mM KCl, 20 mM K-glutamate, 20 mM MES-KOH, pH 6.0), and stock solutions of <sup>36</sup>Cl<sup>-</sup> and <sup>3</sup>H-glutamate were added to the vesicles (final concentrations of 2 and 7  $\mu$ Ci/ml, respectively). Intra- and extra-vesicular solutions were equalized by freezing and thawing the samples twice, and then sonicating the suspension 5–10 s. Passive equilibrium-exchange efflux was initiated by diluting the loaded vesicles into Buffer L lacking radioac-

<sup>1</sup>Abbreviations used in this paper: DDM, dodecylmaltoside; MES, morpholino ethanesulfonic acid; SDS, sodium dodecylsulfate.

tive tracers. After 40–60 min, intravesicular content was determined by spinning through Buffer L–equilibrated G-50 columns as above. Separate experiments under these conditions demonstrated: (a) that insignificant efflux of  $\text{Cl}^-$  occurs in protein-free liposomes, (b) that full equilibration of  $\text{Cl}^-$  is achieved with EriC-containing liposomes, and (c) that negligible efflux of glutamate occurs for liposomes with or without EriC. The crucial measurement in this assay is the fraction of the intraliposomal volume that is inaccessible to external  $\text{Cl}^-$ . This parameter is determined from the fractional trapped  $\text{Cl}^-$  space,  $f$ , measured by double-label counting of internal and external tracer concentrations (dpm per gram lipid and per cubic-centimeter solution, respectively, shown in Eq. 1a):

$$f = \frac{[\text{}^{36}\text{Cl}^-]_{\text{in}}}{\theta[\text{}^{36}\text{Cl}^-]_{\text{ex}}}, \quad (1a)$$

where  $\theta$ , the total intravesicular volume ( $\text{cm}^3/\text{g}$  lipid), is measured from  $^3\text{H}$ -glutamate (Eq. 1b):

$$\theta = [\text{}^3\text{H}]_{\text{in}}/[\text{}^3\text{H}]_{\text{ex}}. \quad (1b)$$

For vesicles that have been diluted infinitely, the  $\text{Cl}^-$ -inaccessible fraction,  $f_0$  is (Eq. 2a):

$$f_0 = f. \quad (2a)$$

For practical purposes, the samples were diluted not infinitely, but 10-fold, and  $f_0$  was correspondingly normalized, by Eq. 2b:

$$f_0 = (f - 0.1)/0.9. \quad (2b)$$

This assay can be used to estimate the fraction,  $s$ , of EriC protein that is functionally active as a  $\text{Cl}^-$  channel. The analysis assumes that the protein distributes randomly according to a Poisson distribution into spherical liposomes of uniform size, and that  $\text{Cl}^-$  is retained only in those liposomes that contain no EriC molecules. As the mass,  $m_E$ , of EriC reconstituted in a fixed mass,  $m_L$ , of liposomes increases, this  $\text{Cl}^-$ -inaccessible fraction will decrease according to Goldberg and Miller (1991):

$$\ln f_0 = -36\pi N_0 \theta^2 s m_E / M_E m_L \sigma^3 \quad (3)$$

where  $N_0$  is Avogadro's number,  $\theta$  is the intravesicular volume ( $\text{cm}^3/\text{g}$  lipid),  $M_E$  is the molecular mass of the EriC channel (51,000 g/mol per subunit), and  $\sigma$  is the surface area of lipid in a bilayer ( $\text{cm}^2/\text{g}$ ).

### Chemical Cross-linking

Glutaraldehyde-mediated cross-linking of EriC was performed at room temperature. EriC (6  $\mu\text{g}$ ) was diluted to 27.5  $\mu\text{l}$  in Buffer X (150 mM NaCl, 50 mM Na-P<sub>i</sub>, 1 mM DDM, pH 7.0), and glutaraldehyde (0.5  $\mu\text{l}$ , 18 mM final concentration) was added. The reaction was quenched with 19 mM Tris for 10 min. Sodium dodecyl-sulfate (SDS)-loading buffer was added, and the sample was analyzed by SDS-PAGE. In some experiments, we varied concentrations of protein (5–500  $\mu\text{g}/\text{ml}$ ), DDM (1–5 mM), or glutaraldehyde (14–160 mM).

### Gel Filtration

Samples were chromatographed at 1 ml/min on a Superdex 200 column (10  $\times$  300 mm, 24 ml bed volume; Pharmacia LKB Biotech-

nology Inc.) equilibrated in buffer G (100 mM KCl, 50 mM Na-P<sub>i</sub>, 1 mM DDM, pH 7.0). Elution profiles were monitored at 280 nm.

### Analytical Ultracentrifugation

EriC was dialyzed overnight against Buffer U (155 mM KCl, 95 mM K-P<sub>i</sub>, 1 mM DDM, pH 7.0) containing 5 mM dithiothreitol (DTT). For control experiments, KcsA protein was dialyzed into Buffer U lacking DTT. To ensure proper blank subtraction, for each dialyzed sample, the solution outside the dialysis cell at equilibrium was collected as the blank. Samples and buffer blanks were loaded into charcoal-Epon two-sector cells. Sedimentation velocity was analyzed using an Optima XL-A analytical ultracentrifuge (Beckman Instruments, Inc.) at 44,000 rpm, 20°C; scans at 280 nm were taken every 5 min. Data were fit to the modified Fujita-Macosham function (Philo, 1997) using Svedberg 5.01 software. Protein partial-specific volumes, calculated from amino acid composition (Cohn and Edsall, 1943; McMeekin and Marshall, 1952), were 0.743 and 0.724  $\text{cm}^3/\text{g}$  for EriC and KcsA, respectively.

## RESULTS

For the past few years, it has been appreciated that CIC channels are represented in microbial genomes (Jentsch et al., 1999). By sequence identity, the prokaryotic CICs lie in the twilight zone for homology modeling (~15–25%), but the statistical scores obtained from BLAST alignments indicate a high probability that the prokaryotic proteins are true CIC homologues (Abagyan and Batalov, 1997; Brenner et al., 1998). Manual scrutiny of the prokaryotic sequences supports this conclusion. Several stretches of CIC sequence are invariant among the known eukaryotic family members, and these appear throughout the entire membrane-spanning region, ~400 residues, and only in this region. In these regions, the prokaryotic proteins display high sequence identity to their eukaryotic counterparts (Fig. 1). The roles that these conserved sequences play in channel function have not been experimentally established; however, certain of these, when mutated, lead to changes in both gating and permeation (Fahlke et al., 1997b,d; Ludewig et al., 1997), as though they either directly mediate these functions or are necessary for proper folding and assembly of CIC channels.

### Overexpression, Purification, and Reconstitution of EriC

We cloned one of the above CIC genes into an *E. coli* expression vector and added an NH<sub>2</sub>-terminal hexahistidine sequence for large-scale production and straightforward purification of EriC, a putative CIC-type channel product of the *yadQ* gene. After an overnight room-temperature induction of *E. coli* bearing the pEriC plasmid, a protein band of ~38 kD, absent in uninduced controls, was observed on SDS-PAGE of bacterial membrane fractions (Fig. 2). This protein was detergent-extracted from the bacterial membrane fraction and purified on a Ni-chelate column. The apparent molecular mass of ~38 kD is smaller than that calculated from the sequence (51 kD), but the integrity of the full-

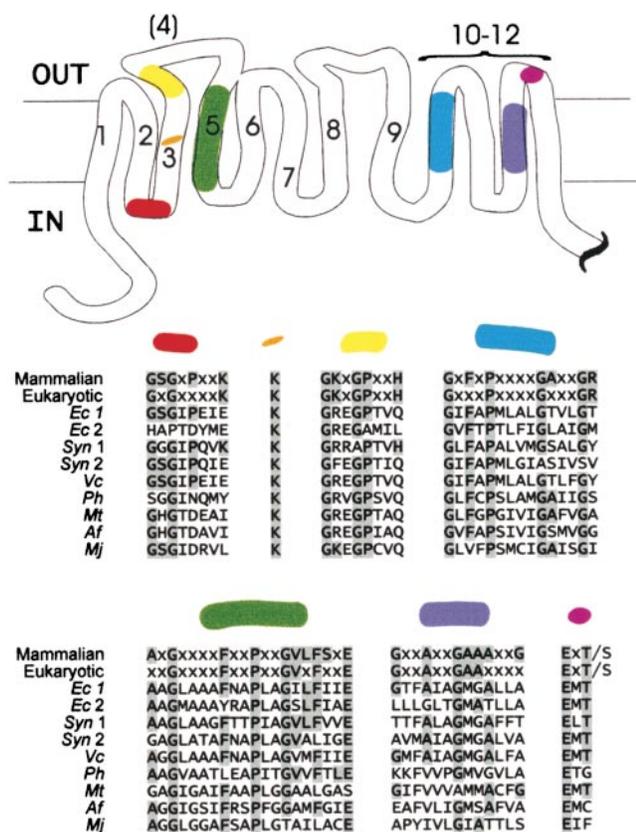


Figure 1. Conservation of prokaryotic CIC channels. Regions of protein that contain residues invariant among mammalian or all eukaryotic CIC channels are color-coded and mapped onto the CIC transmembrane topology model of Schmidt-Rose and Jentsch (1997). The topology of transmembrane stretches 5-7 is currently unresolved (Fahlke et al., 1997c; Jentsch et al., 1999). The COOH-terminal cytoplasmic domain, which contains no invariant regions, ranges from ~170 to 420 residues in the mammalian CICs and 6 to 475 residues in the prokaryotes (35 residues in EriC). Corresponding sequences of the prokaryotic CIC genes identified by BLAST searches are shown below the eukaryotic and mammalian consensus sequences (x, variant residue). Prokaryotic species and sequence references are as follows. *Ec*, *Escherichia coli* (Blattner et al., 1997); *Syn*, *Synechocystis* sp. strain PCC 6803 (Kaneko et al., 1996); *Vc*, *Vibrio cholerae* (TIGR, preliminary sequence data); *Ph*, *Pyrococcus horikoshii* (Kawarabayasi et al., 1998); *Mt*, *Mycobacterium tuberculosis* (Cole et al., 1998); *Af*, *Archaeoglobus fulgidus* (Klenk et al., 1997); *Mj*, *Methanococcus jannaschii* (Bult et al., 1996). EriC corresponds to *Ec 1*.

length protein is indicated by two observations. First, robust interaction with the metal-affinity column indicates that the NH<sub>2</sub>-terminal hexahistidine tag is present; second, an EriC construct carrying an eight-residue, COOH-terminal 1D4 epitope (Molday and MacKenzie, 1983), showed a similar apparent molecular mass, detected on immunoblots (data not shown). Typical yields of purified EriC are 0.5-1 mg/liter culture.

EriC is unambiguously a CIC-family protein at the level of primary sequence, but does it function as an ion channel? To assess this protein's ability to catalyze passive transmembrane flux of <sup>36</sup>Cl<sup>-</sup>, we reconstituted

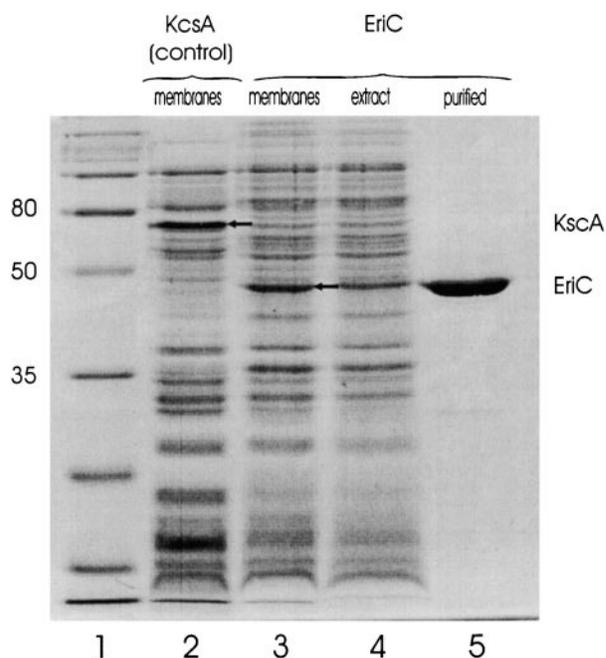
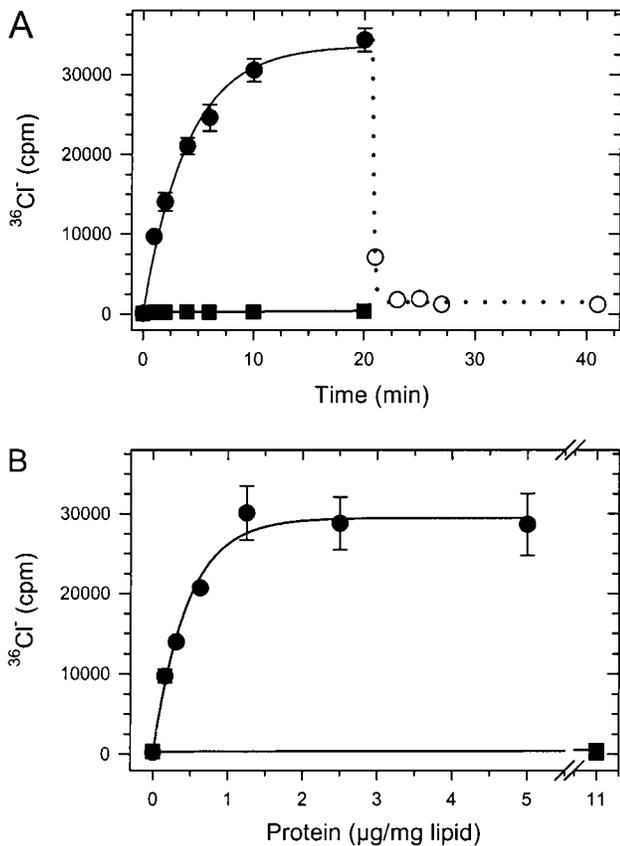


Figure 2. Overexpression and purification of EriC. Protein samples were run on a 13% polyacrylamide gel. (1) Molecular mass markers (broad range; Bio-Rad Laboratories), (2) control membranes (600 μg protein, estimated as 0.6 A<sub>280</sub>-ml) from *E. coli* induced to express KcsA, (3) membranes from *E. coli* expressing EriC (600 μg protein), (4) clarified membrane extract (equivalent to 600-μg membranes), (5) 9 μg purified EriC.

EriC into liposomes and performed a concentrative influx assay (Goldberg and Miller, 1991). Vesicles were loaded with a high concentration (450 mM) of Cl<sup>-</sup>, and <sup>36</sup>Cl<sup>-</sup> influx was measured at low (2.5 mM) external Cl<sup>-</sup>. The resulting Cl<sup>-</sup> gradient will polarize any Cl<sup>-</sup>-selective liposome membrane (positive potential inside), and <sup>36</sup>Cl<sup>-</sup> will accordingly accumulate inside the liposome. Liposomes that have failed to incorporate Cl<sup>-</sup> channels or have sprung nonselective leaks will not accumulate <sup>36</sup>Cl<sup>-</sup> in this assay. The results of Fig. 3 demonstrate that EriC is a Cl<sup>-</sup>-selective channel; tracer Cl<sup>-</sup> accumulates into EriC-containing vesicles with a time constant of ~4 min. Permeabilization of the liposomes to K<sup>+</sup> with valinomycin causes rapid loss of Cl<sup>-</sup>, a result demonstrating that the movement of <sup>36</sup>Cl<sup>-</sup> is conductive, not electroneutral. The final level of tracer rises in a saturating fashion with the amount of EriC reconstituted, as the population of channel-containing liposomes increases. Control protein-free vesicles or vesicles reconstituted with KcsA, a bacterial K<sup>+</sup> channel, fail to transport <sup>36</sup>Cl<sup>-</sup>, although the latter vesicles accumulate <sup>86</sup>Rb<sup>+</sup> under similar conditions (Heginbotham et al., 1998).

#### Absolute Estimation of Functional Activity

It is desirable to know if the observed flux activity is due to a major, minor, or minuscule fraction of the purified



**Figure 3.**  $^{36}\text{Cl}^-$  flux through reconstituted EriC. Concentrative uptake of  $^{36}\text{Cl}^-$  was followed as in materials and methods. (A) Time course of accumulation of  $^{36}\text{Cl}^-$  in vesicles reconstituted with 4.5  $\mu\text{g}$  EriC/mg lipid (●, mean  $\pm$  SEM,  $n = 4$ ) or without protein (■,  $n = 1$ ).  $^{36}\text{Cl}^-$  release was measured after addition of valinomycin at 21 min (○,  $n = 1$ ). (B) Protein concentration-dependent accumulation of  $^{36}\text{Cl}^-$  into vesicles reconstituted with EriC (○, ●) or KcsA (■). Uptake was measured at 20 min (mean  $\pm$  SEM,  $n = 3$ ).

EriC protein. However, the observed influx kinetics are inadequate to quantify activity in our preparations because the absolute rate of  $\text{Cl}^-$  uptake catalyzed by a single EriC channel is unknown. Instead, we employ an assay that does not require knowledge of absolute flux rates, but merely relies on the assumption that any liposome permeable to  $\text{Cl}^-$  will equalize internal and external concentrations of this ion at thermodynamic equilibrium. Vesicles are loaded to equilibrium with two tracers:  $^{36}\text{Cl}^-$ , which is permeant exclusively to EriC-containing liposomes, and  $^3\text{H}$ -glutamate, a trapped volume marker that is impermeant to all liposomes on the experimental time scale. The loaded vesicles are then diluted into tracer-free solution of identical ionic composition, and  $^{36}\text{Cl}^-$  is allowed to flow out (in exchange for unlabeled  $\text{Cl}^-$ ) until equilibrium is achieved. Any vesicle containing one or more functionally active EriC molecules releases  $^{36}\text{Cl}^-$  and retains impermeant glutamate; vesicles devoid of EriC channels will trap both  $^{36}\text{Cl}^-$  and

$^3\text{H}$ -glutamate. Glutamate is thus used as an internal standard to measure the total intravesicular volume to which the  $\text{Cl}^-$ -accessible volume can be compared.

As EriC is reconstituted at increasing protein/lipid mass ratio, the fraction of  $\text{Cl}^-$ -impermeable volume,  $f_0$ , falls exponentially from a protein-free value of unity (Fig. 4), as demanded by a Poisson distribution governing the random incorporation of  $N_E$  EriC channels into  $N_L$  liposomes (Goldberg and Miller, 1991). The absolute value of intravesicular volume,  $\theta$ , does not change systematically over this range of protein concentration (data not shown), which roughly spans the range of 0.2–4 channels per liposome. The protein concentration dependence of  $f_0$  quantitatively obeys the expectations of Eq. 3, where the crucial fit quantity, the fraction of active protein, is  $s = 1.9$ . Since we have based our analysis on a utopian model in which protein molecules distribute perfectly into uniform spherical vesicles, it is perhaps not surprising that we reach a physically impossible conclusion of 190% activity. As discussed below, several assumptions in this analysis are expected to lead to uncertainty in the estimation of EriC activity, so we can easily rationalize this absurd value. The important point of this calculation is that a major fraction of the purified EriC protein—perhaps 100%—is responsible for the functional activity detected in the assay.

#### *Ion Selectivity of EriC*

The concentrative uptake assay was used in two ways to gauge the ionic selectivity of EriC. In the first set of experiments, internal  $\text{Cl}^-$  was replaced with various test anions. Permeant anions support concentrative uptake, while impermeant anions do not. Of the anions tested (Fig. 5 A), only  $\text{Cl}^-$ ,  $\text{Br}^-$ , and  $\text{NO}_3^-$  are permeant by this criterion. In the second set of experiments, we added test ions to the external solution to see which of these would collapse the liposome membrane potential and thereby impede influx. In this assay,  $\text{SCN}^-$ , and to a lesser extent  $\text{I}^-$  and  $\text{F}^-$  score as permeant in addition to  $\text{Cl}^-$ ,  $\text{Br}^-$ , and  $\text{NO}_3^-$ . The discrepancy observed with  $\text{SCN}^-$  is not disconcerting;  $\text{SCN}^-$  both blocks and permeates some eukaryotic CICs (White and Miller, 1981; Fahlke et al., 1997b; Rychov et al., 1998) and, if acting in such a fashion here, would also inhibit influx. In other words, the flux assay in which the test anion is applied externally does not distinguish a permeant ion from a strong blocker. In any case, the two assays taken together demonstrate (a) that EriC-mediated fluxes are highly selective for anions over cations, and (b) that among anions the selectivity sequence is roughly similar to that found for CIC-0 and CIC-1 (Rychov et al., 1998; Jentsch et al., 1999):  $\text{Cl}^-$ ,  $\text{Br}^- > \text{NO}_3^- > \text{I}^-$ ,  $\text{F}^- \gg \text{H}_2\text{PO}_4^-$ , glutamate $^-$ . It is worth noting, however, that these selectivity assays are performed under ionic

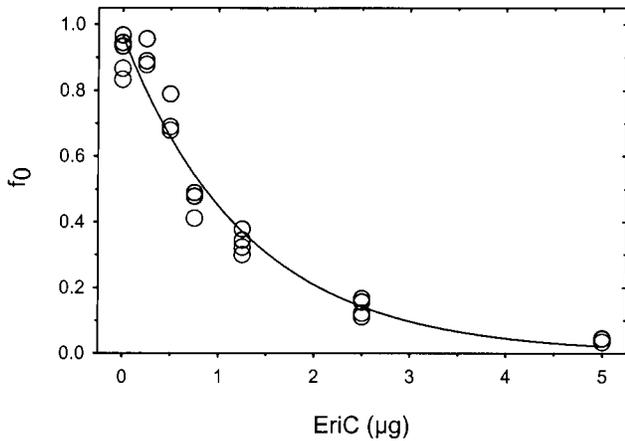


Figure 4. Quantification of EriC activity. The fraction of vesicles containing no active  $\text{Cl}^-$  channels,  $f_0$ , was determined for vesicles (3.8 mg lipid) reconstituted with the indicated amount of EriC. The data were fit to Eq. 3 as described in the text. Parameters of the fit were:  $M_E = 102,000$  g/mol;  $\theta = 5.2$   $\text{cm}^3/\text{g}$  ( $\pm 0.2$ ,  $n = 26$ );  $\sigma = 2.3 \times 10^6$   $\text{cm}^2/\text{g}$ . This latter parameter was calculated from molecular surface area, 61  $\text{\AA}^2$  (Rand and Parsegian, 1989), an estimated molecular weight of the phospholipid (750 g/mol), and a factor of 0.93 to account for liposome curvature.

conditions and with methods very different than in electrophysiological measurements, and that no single interanionic selectivity sequence applies to all eukaryotic CIC channels.

#### *EriC Is a Homodimer*

The most extensively studied eukaryotic CIC channels, CIC-0 and CIC-1, are both homodimers, a quaternary structure underlying their double-barreled behavior (Middleton et al., 1994b, 1996; Ludewig et al., 1996; but see Fahlke et al., 1997a). But is this dimeric characteristic merely an idiosyncrasy of these two CICs, which populate the same muscle-type subfamily, or is it a general property of the entire CIC family? Since EriC is evolutionarily distant from the eukaryotic CIC subfamilies, its quaternary structure would provide valuable insight into this question. We used three different techniques to assess the oligomeric state of EriC in detergent micelles: cross-linking, gel filtration, and velocity sedimentation.

Glutaraldehyde, a nonspecific cross-linking agent, has been used convincingly to report the oligomeric state of several membrane proteins (Canals, 1992; Craig, 1982a,b; Rabon et al., 1990). Reaction of glutaraldehyde with EriC gives a clean result (Fig. 6). Within 10 min of reaction, the 38-kD band is quantitatively converted into a band migrating at  $\sim 90$  kD; no conversion to higher mass species occurs over the next 60 min. The possibility of intermolecular cross-linking is largely eliminated by the control experiment showing no cross-linking of EriC dispersed under denaturing conditions in SDS. In addition, variation of the protein-detergent

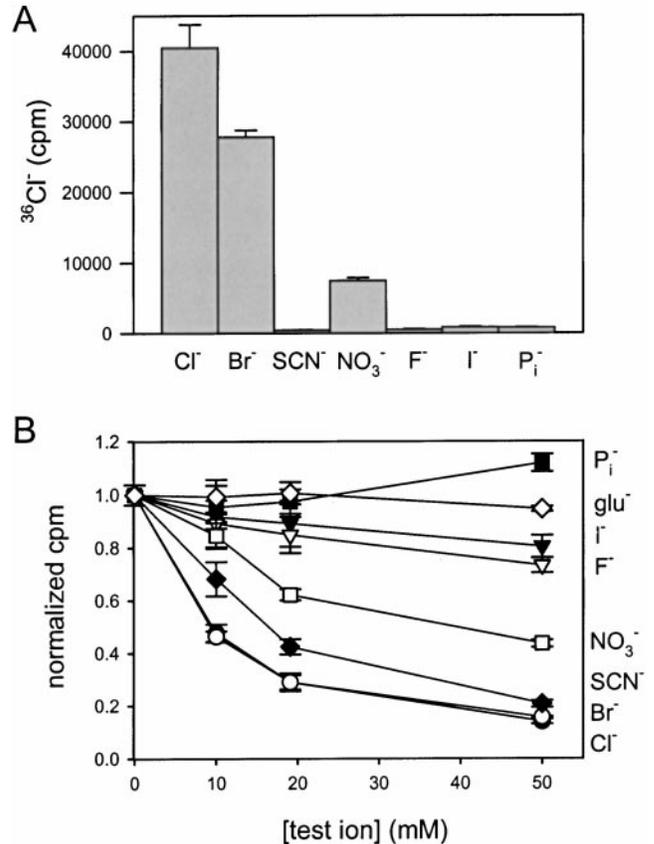


Figure 5. Ion selectivity of EriC. (A) Effect of internal ion on uptake of  $^{36}\text{Cl}^-$ . Vesicles were reconstituted with 1.9  $\mu\text{g}$  EriC/mg lipid in 450 mM KX, 20 mM Na-MES, pH 6.2. Uptake was measured at 13 min; data points represent mean  $\pm$  SEM of three or range of two measurements. (B) Effect of external ions on  $^{36}\text{Cl}^-$  uptake. Vesicles were reconstituted with 3.7 (10 mM test ion) or 3.0 (19 and 50 mM test ion)  $\mu\text{g}$  EriC/mg lipid. Uptake was measured at 13 min. Data (mean  $\pm$  SEM,  $n = 3$ ), are normalized to uptake with no test ion added.  $\text{glu}^-$ , glutamate.

ratio over two orders of magnitude failed to alter the cross-linking, a result that rules out artifactual dimerization resulting from multiple protein molecules sharing the same micelle (Fleming et al., 1997). Moreover, similar results were obtained with EriC reconstituted at very low density (0.09  $\mu\text{g}/\text{mg}$  lipid), where most liposomes have either one EriC channel or none. Cross-linking was robust to varying glutaraldehyde or protein concentrations; while the rates of cross-linking increased with these variables, the overall pattern, particularly the absence of high aggregates, was unchanged.

To complement the cross-linking experiments, we analyzed EriC by gel filtration chromatography and compared its migration to a reference membrane protein of known size, the  $\text{K}^+$  channel KcsA, a 74-kD homotetramer (Heginbotham et al., 1997). EriC runs slightly ahead of KcsA on a Superdex gel filtration column (Fig. 7). This result suggests that the EriC channel is substantially larger than its monomer molecular mass of 51 kD.

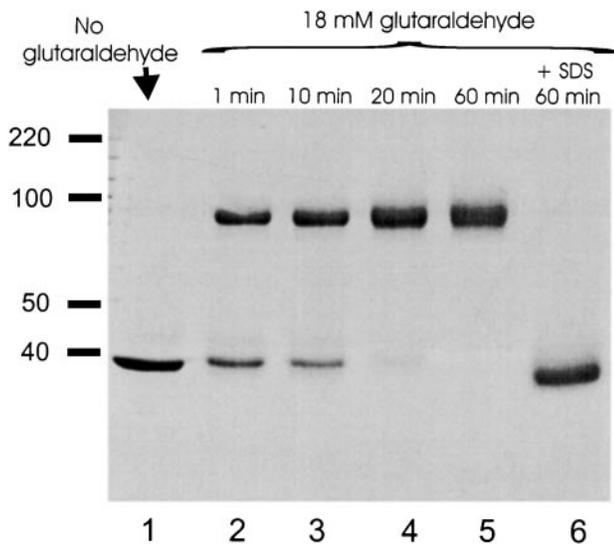


Figure 6. Glutaraldehyde cross-linking of EriC. EriC was treated with glutaraldehyde as in materials and methods, and the samples were run on a 10% polyacrylamide gel. (1) 6  $\mu$ g EriC, no glutaraldehyde, (2–5) 6  $\mu$ g EriC cross-linked for 1, 10, 20, or 60 min, respectively, (6) 6  $\mu$ g EriC incubated with 1% SDS before reaction with glutaraldehyde for 60 min.

Since these gel filtration results cannot distinguish dimers from higher-order oligomers, we also analyzed velocity sedimentation profiles of EriC and again used KcsA as a membrane protein size standard. Qualitatively, EriC sediments more rapidly than KcsA (Fig. 8). A fit of the sedimentation data to a Fujita function (Williams, 1972), which determines both the sedimentation and diffusion coefficients and hence molecular mass, yields 92 kD for KcsA and 126 kD for EriC. These estimates are both  $\sim$ 25% higher than the formula size of tetrameric KcsA and dimeric EriC, a result easily rationalized by bound detergent. A value of 126 kD for EriC would be difficult to reconcile with a trimer or higher oligomer.

We attempted to carry out equilibrium sedimentation experiments in neutral-density detergents, to eliminate rigorously the contribution of bound detergent to the measured mass of the protein (Reynolds and Tanford, 1976; Reynolds and McCaslin, 1985; Fleming et al., 1997). These experiments failed, however, since EriC was unstable in all neutral-density detergents tested, as indicated by rapid aggregation accompanied by high-order cross-linking (data not shown). Nevertheless, the three independent lines of evidence presented here argue powerfully that the functionally active form of EriC used for reconstitution is a homodimer.

#### DISCUSSION

At the current early stage of understanding the chemistry of integral membrane proteins, prokaryotes have served as the singular bearers of high-resolution struc-

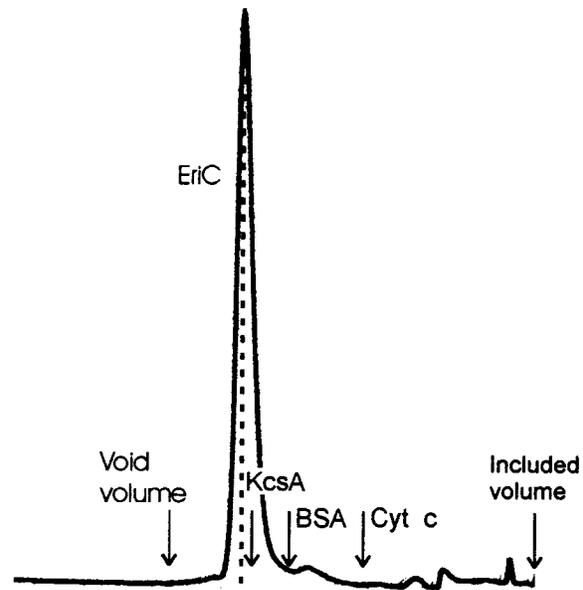


Figure 7. Superdex gel filtration. Purified EriC (230  $\mu$ g) was applied to a Superdex FPLC column, and elution was monitored by 280-nm absorbance. A dashed line is drawn under the EriC peak; arrows mark the peak positions of a void volume marker (blue dextran), KcsA (74 kD), serum albumin (67 kD), cytochrome c (13 kD), and an included volume marker (imidazole).

tural information about ion channels. Porins,  $K^+$  channels, and mechanosensitive channels from bacteria have been expressed at high levels in *E. coli* as a prelude to crystallization and structure determination (Weiss et al., 1991; Doyle et al., 1998; Chang et al., 1998). No eukaryotic ion channel has been successfully expressed in any heterologous systems at levels high enough even to contemplate crystallization. It is therefore encouraging that the CIC family of  $Cl^-$  channels is represented widely in prokaryotes.

We have overexpressed a prokaryotic CIC channel, EriC, the product of one of the two CIC genes in *E. coli*. The biological role of this channel is unknown, but the present results make it clear that it is in fact a CIC-type  $Cl^-$  channel. The purified protein promotes the passive, conductive flux of  $Cl^-$  and other anions across liposome membranes, and the ionic selectivity of this effect is reminiscent of vertebrate CIC channel electrophysiology. We attempted but failed to observe single-channel current fluctuations induced by EriC in planar lipid bilayers; this negative result is disappointing but not surprising in light of the extremely low conductances of many eukaryotic CIC channels (Jentsch et al., 1999). In the liposome flux assay, the time scale (seconds) of EriC-catalyzed  $^{36}Cl^-$  release is substantially lower than expected (milliseconds) for a channel of conventional unitary current (1 pA) open all the time. In the absence of direct electrophysiological information, we have no

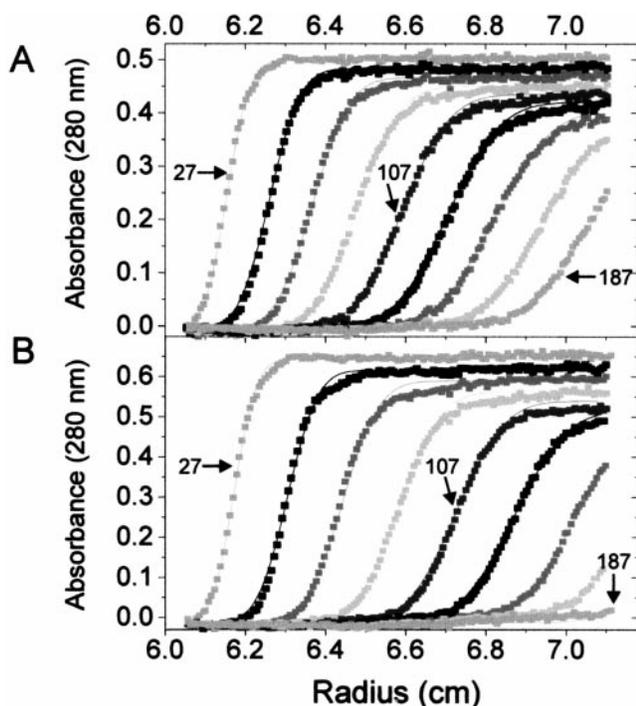


Figure 8. Velocity sedimentation of Eric.  $A_{280}$  in each sample cell is plotted as a function of radius from the axis of rotation. Each scan was made at a different time during the centrifugation run; the scans made at 27, 107, and 187 min are specifically labeled. Fits to the Fujita-MacCosham function yields values for the sedimentation coefficient,  $s$ , and diffusion coefficient,  $D$ , which in turn yield estimates of molar mass,  $M_w$  (A) KcsA:  $s = 6.73$  S,  $D = 6.55 \times 10^{-7}$  cm<sup>2</sup>/s,  $M_w = 92$  kD. (B) EriC:  $s = 8.43$  S,  $D = 6.47 \times 10^{-7}$  cm<sup>2</sup>/s,  $M_w = 126$  kD.

way of knowing whether the low fluxes reflect low channel current, low open probability, or both.

From a biochemical standpoint, perhaps the most important property to quantify for any new protein preparation, even more important than purity or yield, is functional competence. This measurement is difficult for uncharacterized ion channels reconstituted into liposomes, and our value implying 190% activity (Fig. 4) is certainly disconcerting. We have used a limiting Poisson method of “counting” the fraction of liposomes that carry no channels in a sample containing  $N_L$  liposomes and  $N_E$  EriC channels, a fraction  $s$  of which are functionally active. If the insertion of channels into a uniform population of liposomes is random, then this fraction,  $f_0$ , must obey a Poisson distribution (Eq. 4):

$$f_0 = \exp(-sN_E/N_L). \quad (4)$$

We estimate  $f_0$  directly from the fraction of  $\text{Cl}^-$ -impermeable liposomes, and  $N_E$  is known. The experimental samples consist of a known mass of lipid corresponding

to a fixed liposome surface area, and the total intravesicular volume is directly measured. If the liposomes were uniform spheres of known lipid surface density, this information would be sufficient to determine  $N_L$  and hence to derive a functional activity value  $s$ .

However, estimation of the number of liposomes is subject to several sources of error. First, the estimated value of activity varies with the cube of the bilayer surface area per mass of lipid,  $\sigma$ , in Eq. 3. The reconstituted liposomes used here are formed from a phosphatidylethanolamine-rich, undefined, complex mix of lipids. Molecular surface areas of phosphatidylethanolamines vary substantially (55–72 Å<sup>2</sup>) depending on hydrocarbon chain, lipid composition in mixed bilayers, and other factors (Rand and Parsegian, 1989); if used in Eq. 3 these would lead to an approximately twofold range of estimated activity values (average 190%, range 140–320%). Second, large unilamellar liposomes such as these are not expected to be spherical in general; ignoring this kind of nonideality always overestimates activity in the trapped- $\text{Cl}^-$  assay since any departure from a spherical shape increases the surface-to-volume ratio. For example, if the liposomes were on average approximated by a squat cylinder (height = radius), this effect alone would lower the activity estimated above into the range 80–180%. Unfortunately, we have no experimental data on liposome morphology, so we cannot rigorously quantify the expected errors in our estimate of  $s$ . Nevertheless, this uncertainty provides a plausible rationale for an activity value that is “impossibly” high. Another source of ambiguity arises from the assumption that the liposomes are uniform in size, which is certainly false (Mimms et al., 1981); however, calculations indicate that this effect is relatively minor, producing errors of <20% in either direction, depending on the size distribution of the liposome population. Together, the magnitude of these uncertainties dictates that we cannot estimate the value of  $s$  to better than two- to threefold accuracy. Nonetheless, the key inference of the experiments—that the flux activity we observe is mediated by a nontrivial fraction of the purified EriC—is preserved. The hydrodynamic properties of EriC further argue that a major, not a minor, fraction of the protein is active; in both gel filtration and analytical centrifugation experiments, purified EriC behaves as a properly folded, monodisperse macromolecule.

EriC behaves, as do the muscle-type channels CIC-0 and CIC-1 (Middleton et al., 1994a; Fahlke et al., 1997a), as a homodimer in both micellar and bilayer environments. None of the three methods used to assess quaternary structure is in itself rigorous. Nevertheless, a dimeric structure for EriC is strongly supported by the consistency of these three independent, complementary techniques. Gel filtration and velocity sedimentation behavior argue that EriC is larger than a

monomer; velocity sedimentation additionally indicates that it is smaller than a trimer; chemical cross-linking marks it as a dimer. These results underscore the structural homology between EriC and the eukaryotic CICs and thereby identify homodimeric architecture as a general hallmark of the CIC family rather than a peculiarity of the muscle-type subfamily.

CIC channels carry out many crucial biological functions, but conventional mutagenesis studies have provided only limited glimpses of CIC molecular architecture. Since EriC is functionally active as a Cl<sup>-</sup> channel and may be obtained in milligram quantities, this protein is an excellent candidate for future structural studies.

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