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A topologically diverse family of fluoride channels

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Abstract

Dual-topology proteins are likely evolutionary antecedents to a common motif in membrane protein structures, the inverted repeat. A family of fluoride channels, the Flucs, which protect microorganisms, fungi, and plants against cytoplasmic fluoride accumulation, has representatives of all topologies along this evolutionary trajectory, including dual-topology homodimers, antiparallel heterodimers, and, in eukaryotes, fused two-domain proteins with an inverted repeat motif. Recent high-resolution crystal structures of dual-topology homodimers, coupled with extensive functional information about both the homodimers and two-domain Flucs, provide a case study of the co-evolution of fold and function.

Graphical abstract

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Introduction

The advent of the structural era in membrane protein biology revealed that many membrane transport proteins, built on common folds in classes as diverse as LeuT, CLC, MFS, aquaporin, NhaA, and GltPh, are comprised of two homologous domains, arranged antiparallel with respect to each other, in an arrangement known as an inverted repeat (discussed extensively elsewhere, for example, see[1–3].) This architecture is the vestige of a gene duplication and fusion so long ago that the structurally homologous domains no longer bear any discernable sequence identity. In almost all cases, that ancestral single domain gene has been lost to time. But dual-topology membrane proteins are rare examples in which that primitive architecture persists; they represent modern day, single-domain building blocks that assemble in the membrane with inverted architecture (Figure 1A).

The orientation of integral membrane proteins in bacteria can be predicted by the “positive inside rule,” originally articulated by von Heijne [4], which describes a nearly universal feature of membrane proteins – a large excess of arginines and lysines on the cytoplasmic loops. In 2006, von Heijne described a rare exception to the rule: a class of small, unusually hydrophobic proteins that he called dual-topology proteins [5]. Monomers of dual-topology proteins are inserted into cell membranes with no inward/outward-facing bias and can dimerize in an antiparallel orientation *in vivo* [6]. As the original controversy regarding this unusual pattern of membrane insertion has receded, dual-topology proteins have been recognized as likely evolutionary antecedents to inverted repeat [1,7,8].

Topological diversity in Fluc family fluoride channels

Using sequence analysis, a number of putative dual-topology protein families have been proposed [5,9,10], but only two have been characterized in great biochemical depth: the multidrug efflux pump EmrE[11,12], and a class of fluoride channels called Flucs (also known as crcB or FEX), which protect against toxic environmental fluoride ion (F^-) in weakly acidic conditions[13–15]. Flucs are ubiquitous in bacteria, and also found in archaea and free-living eukaryotes (where they are known as FEX proteins), including yeasts and protozoa, plants, and even simple marine animals such as sponges, sea anemones, and tunicates [16]. Fluoride export function has been established by genetic knockout strains of *Escherichia coli*, *Candida albicans*, *Saccharomyces cerevisiae*, and *Neurospora crassa*, which are all rendered hypersensitive to fluoride at environmental concentrations [14,16].

With this many biological test kitchens, the Flucs have evolved into a topologically diverse family that it is not constrained only to dual-topology homodimers. Modern Flucs sample each evolutionary way station: they are present in genomes as single genes that code for antiparallel homodimers [17] (Figure 1A), oppositely inserted pairs (~30% sequence identity) that transport fluoride as obligate heterodimers[15] (Figure 1B), and in eukaryotes, fused two-Fluc proteins linked by a TM helix that forces the conjoined subunits into an antiparallel orientation: an inverted repeat[16] (Figure 1C). This full assortment of topological states is rare, and unique among proteins with known function.

A phylogenetic tree constructed with ~500 representative bacterial and archaeal Fluc sequences shows that gene duplications leading to probable heterodimers have occurred at least four times in bacterial lineages: in Firmicutes, in actinomycetes, in cyanobacteria, and in a handful of alphaproteobacteria (Figure 2). A fifth possible duplication occurred among a clade of archaea nestled among the actinomycetes. Archaeal lineages spring from bacterial clades here and elsewhere in the Fluc phylogeny, perhaps indicating that these proteins were transmitted via lateral gene transfer.

All eukaryotic Flucs are two-domain inverted repeats, and the eukaryotic clade is the sole example of a fusion event. Topological inspection reveals a simple explanation for the paucity of fused two-domain Flucs: to retain antiparallel topology for a protein with an even number of helices, the fusion event must also introduce a membrane spanning helix. Fusion events are more common for antiparallel proteins with an odd number of helices, as evidenced by the number of independent duplication/fusion events in the DUF606 (domain of unknown function) family [18]. Sequence analysis suggests that the DUF606 family is topologically diverse, with dual topology, duplicated heterodimers, and fused representatives. The monomer possesses five transmembrane helices, and of the nine independent duplication events observed in bacteria, four have yielded fused two-domain proteins [18].

Conservation of biological role and function

In the Fluc family, these diverse topologies all serve a common biological purpose. Fluoride ion interferes with some of the most fundamental metabolic activities, and its export is therefore necessary for growth [19]. Isosteric with hydroxide, fluoride ion closely resembles the activated water molecule transition state that occurs during reactions catalyzed by essential metabolic enzymes, including enolase and pyrophosphatase [20,21]. Present at basal levels of 20–100 μM in typical marine and terrestrial environments[22], fluoride can enter and become trapped within cells via a weak acid accumulation pathway[13]. In the absence of a fluoride export system, cytoplasmic fluoride increases to levels as high as mM at a modestly acidic pH value of 5.5, a level well above the $\sim 100 \mu\text{M}$ inhibition constants for metabolic enzymes [13,16]. The weak acid accumulation phenomenon accounts for the counterintuitive notion of protection from an environmental toxin by an energetically passive channel. In addition, metabolizing cells tend to maintain a negative electric potential across the membrane, which provides an additional driving force for anion expulsion. (Though the most widespread, Fluc family channels are not the only fluoride export strategy that has evolved; some bacteria instead use fluoride selective proteins from the CLC family of anion channels and transporters, coupling fluoride export to proton import [23]).

Available evidence implies that the electrodiffusive (i.e. passive flow of fluoride down its electrochemical gradient) mechanism of Fluc channels is conserved across topologies. Single channel recordings of purified bacterial homodimers in planar lipid bilayers show 5–10 pS currents for the constitutively open single channels, equivalent to a throughput of $\sim 10^6$ ions/s [15]. Estimates of single molecule turnovers for fused *S. cerevisiae* proteins, inferred from patch clamp measurements of many-protein currents, yield a similar value of $\sim 10^6$ ions/s. This high turnover is consistent with a channel rather than a transporter mechanism

[24]. Heterodimeric representatives have not been as well characterized, but bulk liposome flux assays imply a high rate of transport of $>10^5$ ions/sec for the *Lactobacillus acidophilus* heterodimer [15].

The bacterial homodimers are ideally selective for fluoride over cations, and enormously ($>10^4$ -fold) selective for fluoride over other abundant cytoplasmic ions like chloride, a closely related halide that only differs in dehydrated radius by about half an Ångström (1.33 Å for fluoride versus 1.81 Å for chloride) [15]. This selectivity is both biologically necessary to avoid catastrophic membrane depolarization and unparalleled among ion channels for close ionic analogs. Such discrimination could probably not be achieved without dehydration of the fluoride ions in the pore [25]. Given the unusually high enthalpy of desolvation for fluoride ion ($\Delta H \sim 125$ kcal/mol, versus ~ 90 kcal/mol for chloride), selective recognition of fluoride ions is a chemically interesting puzzle in its own right, and the selectivity achieved by these proteins surpasses human attempts to develop host-guest compounds that selectively recognize fluoride over chloride [26]. The two-domain *S. cerevisiae* proteins also demonstrate fluoride /chloride selectivity at the limit of resolution of the patch-clamp assay (greater than 10-fold), although further measurements will be required to determine whether their selectivity matches their bacterial counterparts [24].

Symmetrical, antiparallel homodimer structure

Recent 2.1 Å and 2.6 Å crystal structures of homodimers from *Bordetella pertussis* (PDB: 5XB) and an *E. coli* virulence plasmid (PDB:5A43) revealed an elegant and unlikely architecture [27] (Figure 3). The previously unseen fold is striking for its perfect (non-crystallographic) two-fold symmetry about an axis parallel to the plane of the membrane, so that the channel presents identical faces to both the cytoplasmic and periplasmic sides of the membrane. The hourglass shaped dimer possesses two deep aqueous vestibules lined with conserved polar side chains (Figure 3A). Within each vestibule, a conserved arginine (Arg23) from the first transmembrane helix contributes to the overall electropositive environment. TM3 has a mid-membrane break of 5 residues, and the crossover of these breaks contributed by the two subunits defines the deepest reaches of the vestibules. The vestibules do not form a continuous aqueous pathway through the protein; instead a plug of protein ~ 10 Å thick separates the aqueous chambers (Figure 3B). At the center of this plug, at the heart of the protein, resides a deeply buried sodium ion (Na^+) located precisely on the two-fold symmetry axis (Figure 3A, 3C). Fulfilling the symmetry of the overall structure, the central sodium ion is present in an unusual tetrahedrally-coordinated conformation, where it is liganded by two symmetry-related backbone carbonyl oxygens from each subunit (Gly77 and Thr80), contributed by the mid-membrane breaks of TM3 (Figure 3D). Although sodium ions are typically coordinated by 5–6 ligands, coordination by four is not unprecedented [28,29]. The role of this central sodium ion has not been elucidated, but it certainly contributes to the general electropositivity of the protein core, and its location at the dimer interface suggests a possible role in homodimer affinity or specificity.

Two pore construction of homodimers

The two-fold structural symmetry of the channel begets functional redundancy: the cytoplasmic and periplasmic sides of the channel are identical, and it harbors two pores [27] extending antiparallel with respect to each other across the membrane (Figure 3B). The double-barreled structure is a surprise given the protein's unusually small size, just 128 residues (15 kDa) per subunit in the dimer. (Note that although the pores are related by 2-fold symmetry, and thus have opposite polarity, fluoride uses both conduits to diffuse passively down its gradient.) The pores are defined by four bound fluoride ions, arranged two-by-two, parallel to each other and perpendicular to the membrane plane (Figure 3B). The side chains that coordinate these ions include an absolutely conserved asparagine in TM2 (Asn 43) and a sequence of polar residues lining one face of TM4, spaced every fourth residue, called the TM4 polar track (Figure 4A). Although the absolute identity of the residues lining the TM4 polar track is not conserved among even close Fluc family members, the polar character is. Perhaps the most prominent feature of the fluoride ion coordination sphere is the "phenylalanine box," a symmetrical motif whose corners define the two pores of this double-barreled channel, with the electropositive edges of each phenylalanine coordinating a fluoride ion (Figure 4B). Such "anion-quadrupole" interactions have been observed in proteins before [30,31], notably predicted in the bestrophin family of anion channels [32]. Each pore is not confined entirely to an individual monomer, but is lined by side chains contributed from both. Mutation of these pore-lining residues decreases fluoride throughput, although a mutation has not yet been identified that relaxes discrimination against chloride [27,33].

Outside of these deeply buried crystallographic fluoride sites, the details of the permeation pathway are unclear. Although the observed fluoride binding sites are occluded from the vestibules (Figure 3A), it is tempting to imagine the vestibules' electropositive character drawing in and preliminarily desolvating fluoride ions. Mutation of the arginines and a conserved vestibule serine have deleterious effects on function in yeast proteins, supporting the notion that the vestibules are involved in ion transport [24]. Additional evidence that the vestibules provide part of the ion pathway comes from recent electrophysiological assays exploiting small, engineered proteins called "monobodies." These experiments showed that the soluble, toxin-like monobodies inhibit Fluc channels by physically blocking the pores, and that a negatively charged glutamate that acts as an electrostatic surrogate for fluoride is responsible for the pore occlusion [34,35]. Structures of Fluc monobody-complexes show that the loop on which this glutamate resides extends ~9 Å into the aqueous vestibules [27], providing compelling evidence that the vestibules contribute part of the permeation pathway.

Drift of two-pore construction after duplication

The double-barreled architecture raises the question: does Fluc need both pores? Fluoride flows through both pores in the homodimer, and mutagenic ablation of one cuts the channel's conductance neatly in half [33]. Analysis of sequences of fused eukaryotic FEX proteins and Fluc heterodimers suggests that both pores are not required: the residues that define one pore are well-conserved, whereas those that define the second have drifted substantially [27]. This pore degradation is particularly evident in the phenylalanine box

motif, for which each subunit contributes one phenylalanine per pore (Figure 4B, 4C). The fused eukaryotic proteins always retain the first Phe in the N-terminal domain, and the second Phe in the C-terminal domain, which combine to form one complete pore. The heterodimers also tend to conserve opposite phenylalanines in the two domains, although which pair is conserved differs among heterodimeric lineages (Figure 4C). Among pore-lining residues that are conserved in both domains of the *S. cerevisiae* FEX protein, including the TM2 asparagine, mutation of the one located in the degraded pore is less detrimental for fluoride resistance, confirming experimentally that the two homologous domains in FEX proteins have evolved substantial functional asymmetry[24]. It is not known whether the degraded pore retains the ability to bind anions, or still plays any functional role.

Future Directions

As a model of a dual-topology to inverted repeat evolutionary trajectory, key questions for the Fluc family include: Is the functioning homodimer truly symmetrical, as in the crystal structure, or does it adopt an asymmetric functional form, like fellow dual-topology homodimer EmrE[12]? How much of a constraint on function and folding is dual topology, and how are functions elaborated and improved upon once that limitation is lifted?

One could imagine obvious advantages of duplication. The homodimeric constraint enforces 2-fold symmetry and disallows much structurally extraneous evolutionary drift, even among functionally redundant features – exemplified in Flucs by the double-barreled construction. After duplication, some of the structural constraints relax, allowing more sampling in sequence space, a process that is archived in the degradation of the second pore in duplicated Flucs. Furthermore, the two subunits can adopt more specialized roles after duplication, once insertion bias develops and the subunits are fixed in their orientations, as in Figure 1B. This allows asymmetrical presentation to the periplasm and cytoplasm, and the development of side-specific features. There is clear value in developing asymmetry: perhaps the asymmetric protein displays a broader, electropositive vestibule to the inside only, funneling the dilute anionic toxin in the direction of export. Whether such imagined embellishments actually occur remains to be seen, and will require further structural and functional characterization of heterodimeric and inverted-repeat Flucs.

Further analysis of the central Na^+ is also likely to be revealing. In some respects, the binding site, located at breaks in TM helices, is reminiscent of Na^+ binding sites in Na^+ -coupled transporters. In other respects, such as the tetrahedral coordination sphere comprised by backbone carbonyls alone, the Na^+ binding site in the Flucs is singular. Understanding whether this binding site has broader implications requires determining a structural or functional rationale for the central Na^+ in the homodimers, determining whether the ion is conserved in heterodimers and inverted repeat proteins, and if so, whether asymmetric relaxation has provided additional ligands to an energetically favorable 5- or 6-coordinated form.

Other goals for the dual-topology protein field include identifying molecular functions for, and structurally characterizing other single domain building blocks, like the dual-topology

DUF606 family, which, like the Flucs, is particularly rich in topologies including inverted repeats [18], or the DedA family, which has a hydropathy profile intriguingly similar to LeuT proteins [9]. An analogous evolutionary case study with parallel single-domain and fused multi-domain counterparts is underway, with a number of recent crystal structures of two-domain SWEET and single-domain “semi-SWEET” sugar transporters, and the Pnu vitamin transporter, which is unrelated in sequence but similar in fold [8,36–40]. Like the Flucs, the fused SWEETS and single-domain semi-SWEETS are segregated in eukaryotic and prokaryotic genomes, respectively, suggesting some selective advantage for fusion in eukaryotes that is not as strong in prokaryotic membranes.

Via dual-topology Flucs and other modern day “building block” membrane proteins, we can glimpse folds that, for whatever reason, remained in evolutionary infancy. As the number of newly discovered membrane protein folds dwindles, and interest increases in engineering new functions onto existing folds, dual-topology proteins and their kin provide a unique opportunity to understand how nature’s fold prototypes changed in time.

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Highlights

- Flucs are highly selective electrodiffusive fluoride channels that protect microorganisms against cytoplasmic accumulation of toxic F^- ion.
- Fluc proteins are present in genomes as dual-topology homodimers, pairs of oppositely oriented heterodimers, and fused 2-domain constructs with inverted topologies.
- Recent crystal structures of dual-topology homodimers show a 2-fold symmetric structure with two antiparallel pores and an interfacial Na^+ ion at the heart of the dimer interface.
- Both pores function in the homodimers, but in heterodimers and 2-domain fused proteins, the second pore appears to have degraded.

Figure 1.

Schematic illustrating subunit topologies found in the Fluc family of fluoride channels. (+) symbols indicate arginine and lysine residues of representative bacterial Fluc homologues. A. Subunits of dual topology homodimers are inserted into the membrane in both inward- and outward-facing orientations, and can be identified by balanced (+) charge bias, as for this representative example from *Bordetella pertussis*. B. For Fluc heterodimers, subunit pairs are inserted into the membrane with preferred, fixed orientations that can be predicted by the “positive inside” rule [4], as in this representative heterodimer from *Lactobacillus acidophilus*. The termini of one subunit are always inward-facing (blue, positive charge bias on the termini and loop 2) and the termini of the other are always outward-facing (red, with positive charge bias on loops 1 and 3). C. Cartoon of a fused Fluc, with a transmembrane “inversion helix” (pink) enforcing the antiparallel domain architecture. Inverted repeat Flucs are found exclusively in eukaryotes, and have been experimentally determined to insert into the plasma membrane with the N-terminus facing the cytoplasm in *Saccharomyces cerevisiae*[24]. The factors that determine topology in eukaryotic membrane proteins are more complex than in bacteria [41]; thus, the charge distribution is not shown for this homologue.

Figure 2.

Unrooted phylogenetic tree constructed from ~500 representative Fluc proteins using maximum likelihood methods [42–44], with proposed duplication events indicated in color: Green: *Firmicutes* duplication; red: *Actinomycetes* duplication; blue: alphaproteobacteria duplication; violet: cyanobacteria duplication; yellow: archaeal duplication. Gene pairs were identified as likely heterodimers based on two criteria: the start of the second coding sequence falls within 100 nucleotides of the end of the first, and the two proteins have opposite loop charge biases, in accordance with the positive inside rule for membrane protein orientation, assessed by consensus prediction of membrane protein topology[45]. 92% of the pairs that met the first criterion also met the second. The remainder of pairs showed little positive charge bias, and thus prediction of the direction of insertion was uncertain. Amino acids likely to encode aqueous-exposed regions, inferred by structural alignment to the homodimeric Fluc structure, were trimmed from the alignment. This maneuver was essential to determine a phylogeny unbiased by the convergent evolution of positive charges on the inward-facing loops of heterodimers from divergent lineages. Nodes with >90% clade confidence according to approximate likelihood ratio test (aLRT [42]) are indicated by circles. Two structurally characterized homodimers, from *Bordetella pertussis* and a virulence plasmid isolated from *Escherichia coli*, are indicated by stars.

Figure 3.

Structure of dual-topology Fluc homodimer (PDB: 5FXB). A. Through membrane view of the channel. Volume of the aqueous vestibules are colored in cyan, fluoride ions in pink and violet, central Na^+ in blue, and conserved vestibule Arg23 and Phe box residues shown as sticks. Helices are labeled with arrows indicating polarity. The membrane boundaries are indicated by a dashed line. The non-crystallographic 2-fold symmetry axis is indicated by an arrow. Note that because of the channel's perfect 2-fold symmetry, the cytoplasmic and periplasmic sides of the channel are identical. B. View of channel in A rotated 90° . This view emphasizes the double-barrelled architecture; putative pores are highlighted by yellow lines. This view also shows that the aqueous vestibules do not connect; the span of protein separating the vestibules is referred to as the plug, and is indicated by dashed lines. C. Channel viewed perpendicular to the plane of the membrane. The two-fold symmetry axis is indicated by an arrow. Residues from the transmembrane helix 3 (TM3) break are shown as sticks. D. Detail from panel C showing coordination of the central Na^+ at the dimer interface.

Figure 4.

Pore construction in Fluc proteins. A. Fluoride coordination sphere in *Bordetella pertussis* Fluc homodimer (PDB: 5FXB). Two subunits are shown in green and gray, with the “central Na⁺” shown in blue. Fluoride ions are shown as pink and violet spheres, and coordinating residues Asn43, Phe box residues Phe82 and Phe85, and TM4 polar track residues Tyr104, Ser108, Ser112, and Thr116 shown as sticks. For clarity, only one of the two pores rendered as a cartoon with fluoride ions shown. B. Fluoride coordination by the phenylalanine box demarcates the two pores. Note that each subunit contributes a phenylalanine to the pore. Symmetry-related fluoride ions are colored identically. C. Alignments of Phe box region of Fluc homodimers, heterodimers, and fused 2-domain constructs. Conserved residues are bolded, and Phe residues contributing to the expected functional pore are highlighted red. For heterodimers, the inward- and outward-facing subunits are indicated, predicted based on positive charge bias. For fused two-domain proteins, the N-terminal (inward-facing) and C-terminal (outward-facing) domains are indicated.