Proof of dual-topology architecture of Fluc $F^-$ channels with monobody blockers

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Fluc-type $F^-$ channels—used by microorganisms for resisting fluoride toxicity—are unusual in their quaternary architecture: they are thought to associate as dimers with the two subunits in antiparallel transmembrane orientation. Here, we subject this unusual structural feature to a direct test. Single purified Fluc channels recorded in planar lipid bilayers are constitutively open, with rare, short-lived closings. Using combinatorial libraries, we generated synthetic binding proteins, 'monobodies,' that specifically bind to Fluc homologues with nanomolar affinity. Reversible binding of monobodies to two different Fluc channel homologues is seen in single-channel recordings as long-lived nonconducting events that follow bimolecular kinetics. By applying monobodies sequentially to the two sides of the bilayer in a double-sided perfusion manoeuvre, we show that Fluc channels present monobody-binding epitopes to both sides of the membrane. The result establishes that Fluc subunits are arranged in dimeric antiparallel orientation.
on channels of the newly discovered Fluc family\(^1,2\) protect unicellular microorganisms against the toxicity of aqueous F\(^-\)/C\(_0\) anion, an environmentally ubiquitous inhibitor of key enzymes in energy production and nucleic acid synthesis\(^3\). These F\(^-\)/C\(_0\)-specific channels keep cytoplasmic F\(^-\)/C\(_0\) below inhibitory levels by undermining the weak-acid accumulation effect of hydrofluoric acid that would otherwise occur in acidic niches encountered by bacteria, yeasts and protozoa\(^4,5\). Fluc channels function as dimers of small polypeptides (\(\approx 130\) residues) containing four transmembrane segments each, and mutually reinforcing lines of indirect evidence suggest that the two subunits are arranged in an antiparallel transmembrane orientation\(^2\). Though unprecedented among ion channels, dual-topology dimeric architecture is known in the multidrug efflux pump EmrE\(^6–9\), and many membrane transport proteins adopt an analogous motif, the inverted structural repeat, within a single polypeptide chain\(^10\). We were therefore motivated to determine the quaternary architecture of Fluc channels unambiguously. The many years of controversy\(^11\) over parallel versus antiparallel assembly of EmrE highlight the difficulties of distinguishing these alternative architectures. Engineered binding proteins have proven powerful in mechanistic and structural investigations of membrane proteins\(^12,13\). By combining single-channel recording with specific Fluc channel blockers selected from combinatorial libraries by protein engineering technologies, we now unequivocally establish the antiparallel arrangement of Fluc channels functioning in phospholipid membranes.

### Results

#### Selection of Fluc-directed monobodies

In ongoing efforts to develop crystallization chaperones suitable for structure determination, we generated engineered binding proteins termed ‘monobodies’ for two bacterial Fluc homologues, named Ec2 and Bpe\(^2\). Monobodies are single-domain proteins of \(\approx 10\) kDa, derived from the tenth fibronectin type III domain of human fibronectin\(^14,15\). They are selected from two different combinatorial phage-display libraries (Fig. 1a), termed ‘loop’ and ‘side,’ in which 16–26 positions are diversified using highly tailored amino acid compositions followed by gene shuffling

**Figure 1** | Selection of Fluc-directed monobodies. (a) Outline of monobody selection strategy. (b) Structure of monobody scaffold, showing residues (spheres) varied in combinatorial side and loop libraries. (c) Sequences of monobodies (variable regions in red) selected against Fluc homologues Ec2 and Bpe from side and loop libraries as indicated by ‘S’ or ‘L’ designators in monobody labels. All eight monobodies bind to their targets with submicromolar dissociation constants, and all except Mb(Bpe_S8) block Fluc channels in electrical recording assays. Upper sequence of each library indicates tailored variation as follows: ‘X’ denotes a mixture of 30% Tyr, 15% Ser, 10% Gly, 5% Phe, 5% Trp and 2.5% each of all the other amino acids except for Cys; ‘B’, a mixture of Gly, Ser and Tyr; ‘J’, a mixture of Ser and Tyr; ‘O’, a mixture of Asn, Asp, His, Ile, Leu, Phe, Tyr and Val; ‘U’, a mixture of His, Leu, Phe and Tyr; ‘Z’, a mixture of Ala, Glu, Lys and Thr, as previously reported\(^16\).
and further selection in the yeast-display format\textsuperscript{16}. These water-soluble, stably folded, cysteine-free, β-sheet proteins bind specifically to their targets with submicromolar dissociation constants. Figure 1 shows sequences of the monobodies selected against each homologue, as well as the locations of the loop and side residue variations on the protein surface.

**Monobodies block Fluc channels.** A small molecular size, short loops between transmembrane segments and stubby hydrophilic termini conspire to limit the amount of surface that Fluc channels can expose to aqueous solution. This limitation anticipates that in some cases a monobody’s footprint on the channel might lie close to the pore entrance or might even cover it. We were not entirely surprised, therefore, to find that seven of the eight monobodies used for crystallization trials also inhibit F\textsuperscript{−} current through the Fluc homologues against which they were selected. This effect is illustrated for both homologues under study here, with two different monobodies for each, in single-channel recordings in planar phospholipid bilayers (Fig. 2). Whereas Fluc channels are nearly always open under our recording conditions\textsuperscript{2}, the monobodies at submicromolar concentrations produce stochastic nonconducting ‘block’ events in the seconds-to-minutes range, kinetic details varying with the particular monobody tested. These recordings also attest to the specificity of the monobodies, since Bpe-directed monobodies at high concentration exert no effect on Ec2, and vice versa. To our knowledge, these monobodies are the highest-affinity specific inhibitors of any known anion channel.

We chose one of the channel–monobody pairs, Bpe/Mb(Bpe_L3), for a quantitative examination of the blocking process. Single-channel recordings (Fig. 3a) in the presence of this monobody (henceforth abbreviated ‘L3’) show that both blocked and open intervals are exponentially distributed (Fig. 3b), with time constants \( \tau_B \) and \( \tau_O \), respectively. Open times shorten and block times remain constant as monobody concentration increases (Fig. 3c), in quantitative agreement with expectations of a bimolecular blocking scheme with rate constants of blocker association \( k_{on} \) and dissociation \( k_{off} \):

\[
\text{Open channel} + \text{Mb} \underset{k_{off}}{\overset{k_{on}}{\longrightarrow}} \text{Blocked channel}
\]

\[1/\tau_O = k_{on} [\text{Mb}] \quad (1a)\]

\[1/\tau_B = k_{off} \quad (1b)\]

Kinetic parameters and corresponding equilibrium dissociation constant are reported in the legend of Fig. 3. Channel block may reflect physical pore occlusion by the monobody, but evidence for this is weakly suggestive at best; the results do not rule out an allosteric inhibition mechanism.

**Sidedness of monobody block.** We consistently observe single-channel block regardless of the side of the bilayer to which monobody is added. This finding would be perplexing if the channel were constructed with its two subunits in parallel transmembrane assembly, since in the liposomes used for fusion into planar bilayers, roughly half of the Fluc amino termini are exposed to the liposome interior and half to the outside solution\textsuperscript{2}. In contrast, the observation is in natural harmony with antiparallel assembly, wherein the monobody–binding surface on the dimeric channel—its epitope—would be exposed twice, once to each side of the membrane (Fig. 4a).

To definitively ascertain the sidedness of epitope exposure, we performed two-sided perfusion experiments on single Bpe channels (Fig. 4b). Channel block was recorded in the presence of monobody L3 added to one side of the membrane (upper trace). The blocker was then washed away by extensive perfusion,
Figure 4 | Two-sided exposure of monobody epitope. (a) Cartoon contrasting the presentation of monobody-binding epitopes (red surface) in channels with parallel or antiparallel architecture. (b) Sequential recordings of the same single Bpe channel with 100 nM monobody L3 initially on the trans side at 200 mV (upper trace), after washing trans monobody away (middle trace), and, after switching holding voltage to −200 mV, with 100 nM monobody L3 subsequently added to the cis side (lower trace). Dissociation constants estimated from limited records such as these were: 70 and 110 nM for cis– and trans-addition, respectively. (c) Analogous experiment with single Ec2 channel in the presence of 25 nM Mb(Ec2_S9) on the trans side (upper trace). After extensive trans-side perfusion to remove the monobody, the monobody was added to the cis chamber at 10-fold higher concentration (lower trace).

Discussion. These double-sided channel-blocking experiments demonstrate that homodimeric Fluc channels present their monobody-binding epitopes to both sides of the membrane simultaneously. This fact is inconsistent with the parallel-subunit assembly by which nearly all ion channel proteins are constructed, but it is expected of antiparallel architecture. Indeed, the result is actually demanded for a symmetric antiparallel homodimer, wherein the subunits adopt identical conformations. However, our results do not rule out an asymmetric antiparallel homodimer, wherein the subunits adopt identical conformations. Nevertheless, our results do not rule out an asymmetric antiparallel homodimer, as in EmrF6,8,9, a point that will require extensive future analysis of two-sided channel block. Skeptics might object that these experiments fail to prove parallel architecture because the epitope surfaces exposed to the two sides of the bilayer could arise from different channel sequences. We consider such a possibility plainly untenable, given the extreme rarity of obtaining Fluc-binding monobodies—on the order of 10 blockers selected from 10 billion variants—and from their high affinity and specificity of block, as indicated by the lack of monobody cross-reactivity between our two Fluc homologues.

Several basic questions regarding details of monobody action remain unaddressed here. For instance, we have no information on whether the two epitopes on opposite sides of the Fluc channel can be simultaneously occupied by monobodies. Likewise, the mechanism of monobody block—physical pore-plugging or allosteric pore-closure—is unknown. While these intriguing biophysical issues will require detailed future examination, they have no bearing on our inferences about Fluc’s unusual quaternary architecture; the mechanistic minitiae of monobody block stand entirely apart from the design of and structural conclusions from the experiments presented here.

Methods. Biochemical procedures and single-channel recording. We used two bacterial Fluc channel homologues, nicknamed Ec2 and Bpe (sequences 35% identical, 68% similar), whose molecular characteristics, expression, purification and reconstitution into liposomes and planar lipid bilayers were recently described in detail2.
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Author contributions

All authors contributed to experimental design, carried out experiments and shared in writing the manuscript.

Additional information

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