

# Phenylalanine-508 mediates a cytoplasmic–membrane domain contact in the CFTR 3D structure crucial to assembly and channel function

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**Deletion of phenylalanine-508 (Phe-508) from the N-terminal nucleotide-binding domain (NBD1) of the cystic fibrosis transmembrane conductance regulator (CFTR), a member of the ATP-binding cassette (ABC) transporter family, disrupts both its folding and function and causes most cystic fibrosis. Most mutant nascent chains do not pass quality control in the ER, and those that do remain thermally unstable, only partially functional, and are rapidly endocytosed and degraded. Although the lack of the Phe-508 peptide backbone diminishes the NBD1 folding yield, the absence of the aromatic side chain is primarily responsible for defective CFTR assembly and channel gating. However, the site of interdomain contact by the side chain is unknown as is the high-resolution 3D structure of the complete protein. Here we present a 3D structure of CFTR, constructed by molecular modeling and supported biochemically, in which Phe-508 mediates a tertiary interaction between the surface of NBD1 and a cytoplasmic loop (CL4) in the C-terminal membrane-spanning domain (MSD2). This crucial cytoplasmic membrane interface, which is dynamically involved in regulation of channel gating, explains the known sensitivity of CFTR assembly to many disease-associated mutations in CL4 as well as NBD1 and provides a sharply focused target for small molecules to treat CF. In addition to identifying a key intramolecular site to be repaired therapeutically, our findings advance understanding of CFTR structure and function and provide a platform for focused biochemical studies of other features of this unique ABC ion channel.**

ABC transporter | cystic fibrosis | domain interactions | modeling | protein misfolding

The cystic fibrosis transmembrane conductance regulator (CFTR) plays a fundamental role in metazoan epithelial ion transport, providing a rate-limiting step in the regulation of salt secretion and reabsorption (1). Secretory diarrhea results from persistent activation of CFTR by enterotoxins (2). When CFTR is absent or defective in humans, normal salt and water homeostasis in epithelial tissues cannot be maintained, resulting in the accumulation of macromolecular secretions and chronic infection and inflammation of the airways (3). At the biochemical and cell biological levels, CFTR is distinguished primarily by two characteristics. First, belonging to the human C subfamily of ATP-binding cassette (ABC) transporters, CFTR is unique as the only member of the entire family known to function as an ion channel (4). Progress toward understanding how fundamentally similar structures accomplish rapid bidirectional ion permeation in the case of an ion channel, and much slower vectorial active transport by transporters, is beginning to shed new light on the mechanism of each (5). The second feature of CFTR that is of fundamental importance is the major CF-causing mutation, deletion of Phe-508 ( $\Delta F508$ ), which results in misfolding and misassembly of the complex multidomain protein (6).

The in-frame deletion of the single Phe-508 codon present in  $\approx 90\%$  of CF patients prevents conformational maturation in the

endoplasmic reticulum (ER). Any mutant protein that reaches the cell surface remains thermally unstable, capable of only partial chloride channel activity, and is rapidly endocytosed and degraded (7). *In vitro* experiments with the isolated first nucleotide-binding domain (NBD1) reveal that the  $\Delta F508$  mutation decreases folding yield (8). However, other than the absence of the Phe-508 residue from its surface, the overall 3D structure of the domain is little altered (9–11). This observation led to the hypothesis, for which there is some support (12), that Phe-508 may mediate a critical interaction between NBD1 and a site elsewhere in the protein. As yet, the hypothesis has not been confirmed nor has the site been identified. There are indications that it is probably not in NBD2 (13, 14). However, gaining a fuller understanding of both the impact of the  $\Delta F508$  and the utilization of the ABC architecture to generate ion channel activity has been hampered by the lack of 3D structural information. Although the crystal structure for the isolated NBD1 is available (9), there is only a low resolution structure of the whole protein (15). High-resolution structures of several bacterial ABC transporters have recently been determined by x-ray crystallography (16–19). These structures provided important new insights into the mechanisms by which these proteins accomplish transmembrane translocation of their substrates (20). Although functioning as a channel rather than either an importer or an exporter, CFTR is a member of the exporter class of ABC proteins. Sequence and biochemical similarities between several of these exporters, and those for which atomic structures have been determined, Sav1866 (16) and MsbA (21), suggest that there are likely to be strong structural similarities as well.

## Results

**Molecular Modeling.** We constructed a 3D structure of CFTR by molecular modeling [for detailed methods, see [supporting information \(SI\) Text](#)]. CFTR consists of nucleotide-binding domains NBD1 and NBD2, membrane-spanning domains MSD1 and MSD2, and a regulatory region called the R domain (Fig. 1). There are existing 3D structures of NBD1 and also a homology model of NBD2 derived from structures of NBDs of other ABC transporters (22). To arrive at a structural model of the complete CFTR, we built models of the membrane-spanning domains from the known structure of the full-length ABC exporter, Sav1866 ([SI Fig. 5](#)). Full-length ABC proteins can be grouped into two classes according to the number and conformation of

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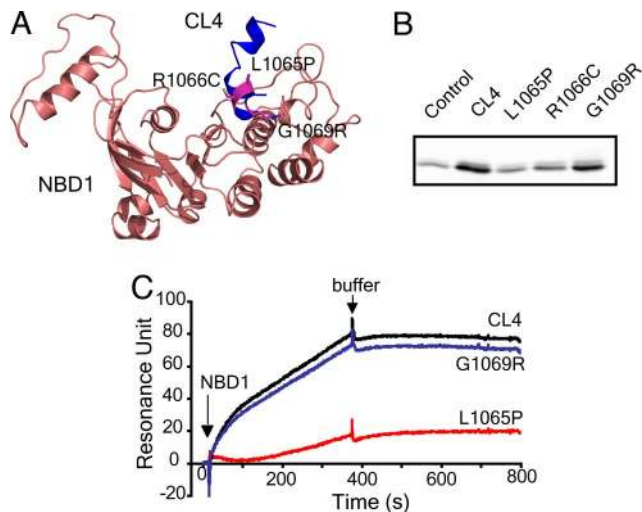
The authors declare no conflict of interest.

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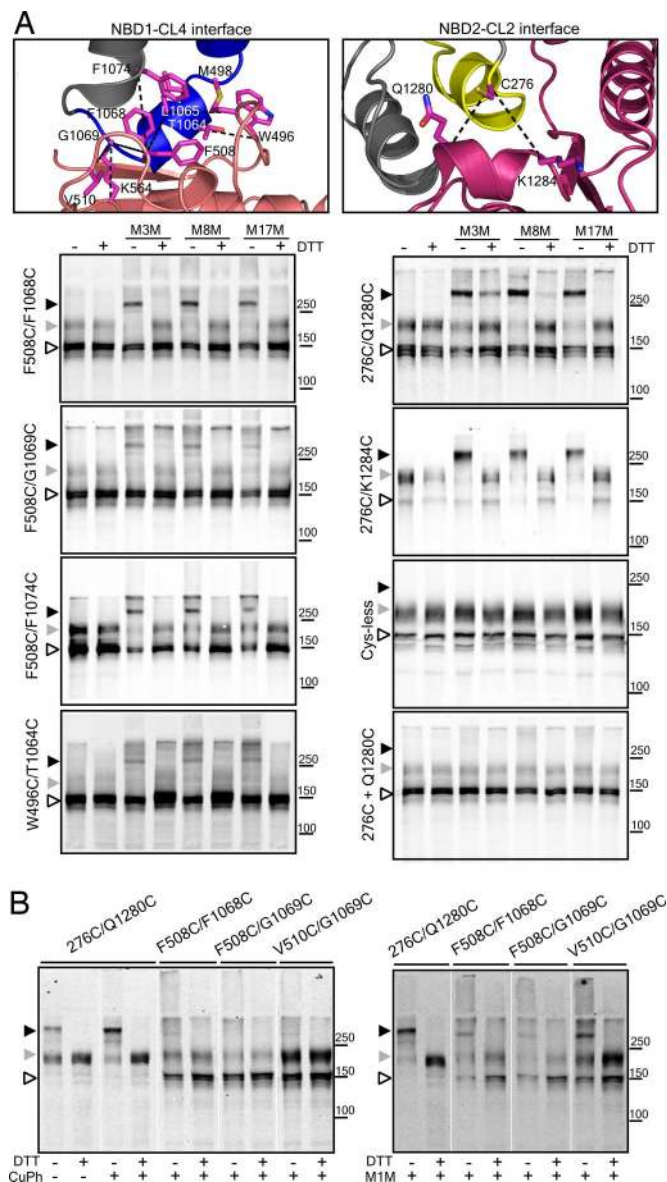


**Fig. 2.** CL4 peptide binding to NBD1. (A) Location of disease-associated mutations (L1065P, R1066C, and G1069R) at the NBD1/CL4 interface. (B) Disease-causing mutations in CL4 abolish or diminish the CL4 and NBD1 interaction. Biotinylated CL4 or its mutant peptides immobilized on NeutrAvidin beads were incubated with purified recombinant human NBD1 (see *SI Text*). Bound proteins were eluted with sample buffer and detected by Western blotting with CFTR antibody 660. NeutrAvidin beads without bound peptide were used as control. (C) CL4 binds to NBD1 as detected with surface plasmon resonance. Biotinylated peptides were immobilized on a BiAcore streptavidin sensor chip to 200 resonance units. NBD1 was injected, and the binding was detected by surface plasmon resonance and BiAcore 2000. The binding of NBD1 to the chip without peptide was subtracted from NBD1 binding to the peptides.

CL4 peptide was nearly completely ablated by a folding mutation (L1065P) but only slightly diminished by one influencing channel function (G1069R).

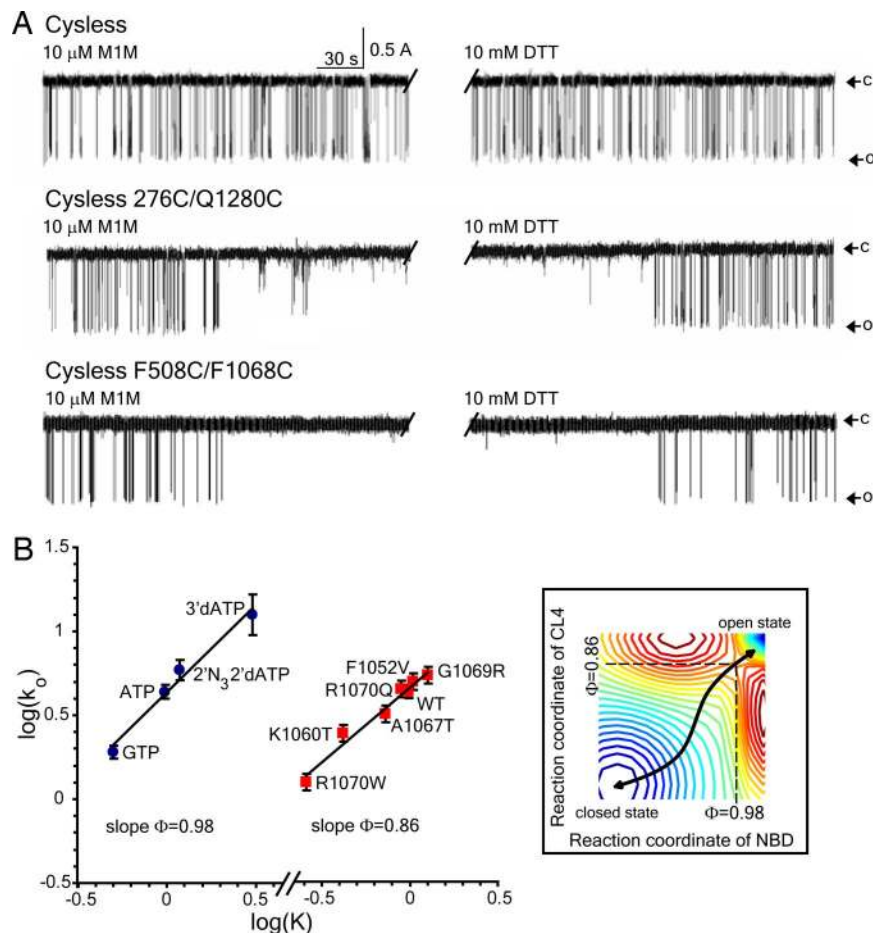
**Cysteine Cross-Linking.** As a second means of assessing the NBD1/CL4 interface and to locate specifically the contact sites between CL4 and NBD1, pairs of cysteine residues were introduced into a functional Cys-less CFTR construct (26) at positions in CL4 and NBD1 that are in close proximity according to our modeled structure (Fig. 3*A*, *Top Left*). As seen in the *Left* column of Fig. 3*A*, several such Cys pairs could be cross-linked by bifunctional methane-thiosulfonate (MTS) reagents. In control experiments where separate constructs containing individual rather than both members of these pairs were coexpressed, cross-linking did not occur (*SI Fig. 7*). Strikingly, Phe-508 apparently plays a central role in this interface because it can be cross-linked to cysteines introduced at many positions in CL4. These positions include Leu-1065, Phe-1068, Gly-1069, and Phe-1074 (Fig. 3*A* and *SI Fig. 8*). Cross-linking also occurs between cysteines substituted for another hydrophobic residue (Val-510) near Phe-508 on the NBD1 surface and G1069C in CL4 (*SI Fig. 8*). In addition to the Phe-508-containing NBD1 surface patch, residues in other regions of the domain also interact with CL4 residues as evidenced by cross-linking of Cys pairs involving amino acids closer to the Q loop (Gln-493), including W496C/T1064C and M498C/L1065C as well as nearer the Walker B motif (Asp-572) such as K564C/G1069C (Fig. 3*A* and *SI Fig. 8*). These data confirm the extensive NBD1/CL4 contact apparent in the structure.

Most of the specific Cys pair cross-links were mediated by MTS reagents with spacer arms ranging from 3.9 to 24.7 Å in length, which initially seemed somewhat surprising but must indicate considerable flexibility in the positioning of each member of the pair with respect to each other (Fig. 3*A* and *B*, *Right* and *SI Fig. 8*). Nevertheless, the proximity or relative orientation of the F508C/F1068C, F508C/G1069C, and V510C/G1069C



**Fig. 3.** Cross-linking of interfacial cysteine pairs. (A) Confirmation of the interaction, in cells, between NBD1 and CL4 (MSD2) and between NBD2 and CL2 (MSD1). Phe-508 participates in an apparent aromatic cluster with residues from CL4 (see also *SI Fig. 10*). CL4 also interacts with other regions in NBD1 as suggested by cross-linking of residues close to the Q loop (W496C/T1064C and M498C/L1065C) and a residue near the Walker B motif (K564C/G1069C). HEK293 cells transiently transfected with Cys-less CFTR containing Cys pairs were harvested and incubated in the presence of 200  $\mu$ M MTS cross-linkers of different spacer arm lengths for 15 min at room temperature. Samples with or without DTT were subjected to SDS/PAGE and Western blotting with mAb596. Cross-linked species migrate above 250 kDa. (Right, *Bottom two*) Controls showing the lack of effects of cross-linking of the Cys-less construct and individually expressed and mixed single cysteine constructs (labeled 276C + Q1280C) under the same conditions (see also *SI Fig. 8*). (B) (Right) Cross-linking by the shortest reagent 1,1-methanediyl bismethanethiosulfonate (M1M), in isolated membranes, indicates close contact and probable mobility of residues across both interfaces. (Left) There is greater propensity for disulfide bond formation at the CL2/NBD2 interface (276C/Q1280C) than at the CL4/NBD1 interface. Open arrowhead, immature core-glycosylated CFTR (band B); gray arrowhead, mature complex-glycosylated CFTR (band C); black arrowhead, cross-linked mature protein.

pairs permitted very little disulfide bond formation on oxidation catalyzed by copper phenanthroline, i.e., only a very small proportion of mature band was converted to cross-linked band



**Fig. 4.** Role of domain–domain interactions in CFTR channel gating. (A) Inhibition of CFTR channel gating by cross-linking. Single-channel recording after exposure to 10  $\mu$ M M1M from the *cis* side of the bilayer followed by 10 mM DTT. For Cys-less CFTR, the last 4 min from the total 20 min of M1M treatment is shown in the first half of the trace before the interruption. The last 4 min of the total 20 min of DTT treatment is shown in the second half. O, open state; c, closed state. (Top) Cys-less CFTR ( $n = 4$ ). (Middle) Cys-less CFTR with 276C/Q1280C ( $n = 3$ ). (Bottom) Cys-less CFTR with F508C/F1068C ( $n = 4$ ). (Middle and Bottom) Change in functional state in the middle of a 4-min portion of the total 20-min recording. No effect of MTS reagents was observed in constructs containing single cysteines that contribute to cross-linkable pairs (SI Fig. 11). (B) NBD1 and CL4 participate early in the gating cycle. Bronsted plots for wild-type CFTR gated by 2 mM nucleotide ligands shown by each experimental point (Left) and substitutions of CL4 residues (Right). Both graphs are linear with the slope  $\Phi$  values indicated. Points on both graphs are shown as mean values  $\pm$  SEM of at least four different experiments. (Inset) Hypothetical free energy landscape of CFTR gating where troughs (stable states) are colored blue and crests (unstable states) are red. At the transition state (saddle point), NBD1 has proceeded 98% toward the open state, and CL4 has proceeded 86%.

(Fig. 3B, Left). In contrast, in all cases where strong cross-linking occurred, only the mature form of CFTR but not the immature one was converted to the cross-linked species of slower mobility (Fig. 3 and SI Fig. 8). This finding provides strong evidence that residue associations occur only in the mature native form of the protein. There is indirect evidence that the Phe-508 side chain is involved in both domain assembly (11) and channel gating (26). Our present findings identify the site of the interdomain association and in so doing confirm that the so-called domain swapping described by Dawson and Locher in the Sav1866 exporter (16) and also observed in the P-glycoprotein multidrug exporter (27) and the MsbA lipid exporter (21) occurs in CFTR.

Unlike Sav1866, which is a homodimer containing two identical NBDs and MSDs, CFTR contains two distinct domains of each type. The structural model predicts that domain swapping occurs symmetrically between NBDs and MSDs in opposite halves of the molecule. Cysteine cross-linking confirms that domain swapping occurs between CL2 and NBD2 (Fig. 3). In fact, the Q1280C and K1284C substitutions in NBD2 of Cys-less CFTR containing the native Cys-276 residue in CL2 (illustrated in Fig. 3A, Top Right) enabled cross-linking by all MTS reagents

tested. Furthermore, disulfide bond formation between cysteines at positions 276 and 1280 occurred spontaneously because of oxidation during membrane isolation and was further promoted by copper phenanthroline (Fig. 3B, Left). This observation indicates close contact between the native Cys-276 residue in CL2 and Gln-1280 in NBD2. Thus, when NBD2 is synthesized to complete the CFTR structure, it integrates with CL2 in MSD1. However, although there are many disease-associated mutations in both NBD1 and CL4 that compromise assembly, few have been identified in either NBD2 or CL2 (www.genet.sickkids.on.ca/cftr). This finding now can be understood in view of the realization that incorporation of NBD2 is not required for the correct assembly of the other domains into a structure that satisfies ER quality control (13, 14).

**Role of Interfaces in Channel Gating.** It is likely that both of these interfaces mediate conformational signals elicited by ATP binding and hydrolysis to the channel gate. If this is the case, it is unclear whether the channel-activating stimuli, ATP binding and phosphorylation, should influence the formation of cross-links between cysteines on either side of an interface or whether their cross-linking

should influence channel gating. Treatment of cells with cAMP-elevating stimuli, which caused CFTR phosphorylation by PKA, did not alter cross-linking of cysteines between either CL4 and NBD1 or CL2 and NBD2 (SI Fig. 9). With membranes isolated from these cells, high concentrations of nucleotides [MgATP, adenosine 5'-[ $\beta,\gamma$ -imido]triphosphate (AMP-PNP), or ADP + vanadate] with or without phosphorylation by PKA also did not strongly influence formation of the cross-links. These results would imply that if conformational signals were being transmitted, structural elements on either side of the interface may move in concert. If so, channel gating might or might not be expected to be influenced by covalent cross-linking between cysteines on either side of the interface. In fact, we found that single-channel gating, which persists after the introduction of a Cys pair at each interface, was completely inhibited by cross-linking (Fig. 4A). In both cases, this inhibition was completely reversed on reduction with DTT. Hence, these points of contact are integral elements of the structure, and covalent coupling between residues on either side restricts channel activity. This restriction is unlikely to be caused by prevention of signal transmission *per se* but probably reflects the restriction of dynamics at the interfaces, which also is suggested by cross-linking by reagents of different lengths. The lack of effect of gating stimuli (PKA + ATP) on the interactions between the NBDs and the CLs (SI Fig. 9) also suggests that there may be coordinated movements of the two sides of the interfaces.

As an independent means of comparing the stages in the gating reaction at which NBD1 and CL4 participate, rate equilibrium-free energy relationship (REFER) analysis (28) was applied (Fig. 4B). The slopes ( $\Phi$  values) of the linear log/log plots relating the rate constants of single-channel opening and the equilibrium constants approach 1 ( $\Phi = 0.98$ ) for different channel-activating nucleotide ligands and only a slightly lesser value for multiple amino acid substitutions in CL4 ( $\Phi = 0.86$ ). These values indicate that ligand binding is essentially complete before the transition state for channel opening is reached, whereas CL4 has proceeded 86% of the way to the open state at the transition state. Thus, both parts of the structure appear to participate early in the gating cycle, consistent with a dynamic functional role of the NBD1/CL4 interface. Significantly, a  $\Phi$  value near 1 for NBD1 was also obtained by similar analysis of different chimeric constructs of the domain (29).

## Discussion

Thus, a 3D structure of CFTR derived by molecular modeling reveals important aspects of the interfaces between the NBDs and MSDs of CFTR. The most critical contact in conformational maturation during biosynthesis, which is precluded by the  $\Delta F508$  mutation, is between a site containing that residue on the surface of the N-terminal NBD and a cytoplasmic loop in the C-terminal MSD. This finding is consistent with and helps to explain previous observations of disruption of CFTR membrane domains by the  $\Delta F508$  mutation (13, 14). The mutation is known to act at two levels: the first to decrease the folding yield and transitions between intermediate states in the folding of NBD1 itself, and the second, to prevent an interaction of the NBD1 surface elsewhere in the protein. We have identified the site of this interaction, dependent

on the phenylalanine aromatic side chain. It appears to participate in an aromatic cluster with residues from CL4 (Fig. 3A and SI Fig. 10), which may contribute to the stability of this vital tertiary interaction as in other proteins (30, 31). The confirmed 3D structure of this interfacial site provides precise information on what has to be restored or mimicked to counteract the defect caused by the mutation. The corresponding crossing-over between NBD2 and MSD1 is less crucial to maturation of the protein because NBD2 is the last portion of the polypeptide to be translated and may be incorporated posttranslationally (12).

In addition to establishing the precise intramolecular contact formed by the Phe-508 side chain in the assembly of the N- and C-terminal portions of the protein, our findings provide some insight into the role of this interface in the regulation of the CFTR channel. The formation of covalent cross-links between cysteines on either side of this interface arrests channel gating, indicating a requirement for dynamic contact at these sites. This finding applies to both the Phe-508-containing NBD1/CL4 interface and the counterpart between NBD2 and CL2. These interactions between NBDs and MSDs in opposite halves of the molecule, which have been referred to as domain swapping (16) or intertwining (21) between subunits of homodimer ABC transporters, are probably also important in the transmission of regulatory signals. The fact that the ability of these interfaces to be cross-linked is not perturbed by the stimuli that activate channel gating is consistent with their action as connecting joints between other portions of the molecule that may undergo larger ranges of motion. This interpretation is supported by the REFER analysis of single-channel kinetics, which suggests that conformational movements in ligand-binding sites and cytoplasmic loops between transmembrane helices are tightly coupled.

## Methods

**Structural Modeling.** The Homology suite from INSIGHTII (Accelrys) was used to construct an atomic model of CFTR MSDs, The x-ray structure of NBD1 (9) and an existing homology model of NBD2 (22) were used. The R domain was approximated by an ensemble of conformations derived by *ab initio* folding.

**CL4-NBD1 Binding.** Biotinylated synthetic peptides corresponding to wild-type and mutant versions of a CL4 peptide were immobilized on NeutrAvidin beads and used to pull down purified human NBD1 protein kindly provided by P. J. Thomas (University of Texas Southwestern Medical Center, Dallas, TX). Binding also was assayed by surface plasmon resonance (BIAcore 2000).

**Cysteine Cross-Linking.** HEK293 or BHK cells or membranes expressing Cys-less CFTR containing specific Cys pairs were incubated with MTS reagents of different chain lengths and analyzed by SDS/PAGE with or without reduction with 30 mM DTT and Western blotting.

**Single-Channel Recording.** Measurements were made in planar lipid bilayers as described (26), and REFER analysis of gating kinetics was performed according to Mitra *et al.* (28).

Details of all of these procedures are in SI Text.

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