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Generation and Evaluation of a Large Mutational Library from the *Escherichia coli* Mechanosensitive Channel of Large Conductance, MscL

IMPLICATIONS FOR CHANNEL GATING AND EVOLUTIONARY DESIGN*[S]

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Random mutagenesis of the mechanosensitive channel of large conductance (MscL) from *Escherichia coli* coupled with a high-throughput functional screen has provided new insights into channel structure and function. Complementary interactions of conserved residues proposed in a computational model for gating have been evaluated, and important functional regions of the channel have been identified. Mutational analysis shows that the proposed S1 helix, despite having several highly conserved residues, can be heavily mutated without significantly altering channel function. The pattern of mutations that make MscL more difficult to gate suggests that MscL senses tension with residues located near the lipid headgroups of the bilayer. The range of phenotypical changes seen has implications for a proposed model for the evolutionary origin of mechanosensitive channels.

Since its cloning in 1994 by the Kung labs (1), the mechanosensitive channel of large conductance (MscL)[1] has developed into a prototype ion channel for understanding cellular mechanosensation (2–5). Much of what is known about the function of MscL has been gained through investigation of the *Escherichia coli* channel using electrophysiology and mutagenesis (3–5). Electrophysiological characterization of Ec-MscL in both bacterial spheroplasts and reconstituted lipid vesicles has demonstrated that MscL is opened by tension from the lipid bilayer (1), quantitated the tension required to open MscL (6), predicted the pore size of the open channel (7), and suggested that there are several discrete steps on the opening pathway (6). Mutagenesis studies also determined that the first transmembrane region of Ec-MscL lined the pore and established an occlusion of the channel in the vicinity of residue Gly-22 (8–11).

A breakthrough in the study of MscL came with the report from the Rees labs (12) of the high resolution crystal structure of MscL from *Mycobacterium tuberculosis* (Tb-MscL). This result confirmed many of the essential conclusions of the earlier mutagenesis studies, while also clarifying some confusion concerning the stoichiometry of the channel and providing a wealth of new insights into the molecular details of the structure.

The image of Tb-MscL produced by Rees and coworkers is undoubtedly of the closed state of the channel. Of the many challenges associated with the detailed study of channels and other membrane proteins, a major issue is the elucidation of the several states (open, closed, intermediate, desensitized) associated with such structures and the mechanisms of the transitions among them. For a type of bacterial K^+ channel images of the open and closed state are now available (13–15), but this is not the case for MscL or other channels. As such, other approaches to analyzing the open state of the channel have been employed.

Interesting results have been obtained from molecular dynamics simulations beginning from the closed state of MscL (16–18). At present, however, it is not possible to run such simulations long enough to see the transition from closed to open state. As a result, the *de novo* construction of molecular models for the open state and various intermediates on the opening pathway has been attempted.

In particular, a detailed, atomic-level gating model for Ec-MscL has been developed by Sukharev, Guy, and coworkers (SG) (19–21). Although the Rees crystal structure is of Tb-MscL, SG chose to model Ec-MscL so that use could be made of the much larger collection of experimental data that exist for this homologue. These data were used extensively in developing the computational model. In addition to emphasizing key residues identified from the mutagenesis studies, the SG model naturally identified a number of other key interactions that make important contributions in the open state and in a key intermediate state identified along the gating pathway. Recently, the Perozo lab has developed a structural model of the transmembrane domains based on spin labeling data that supports many features of the SG model (22).

To test specific features of the SG model, it would be useful to evaluate the many proposed structural contacts by site-directed mutagenesis. However, the protocols for evaluating MscL mutants, either through electrophysiology or growth studies, are time-consuming and tedious. Also, two distinct MscL mutant phenotypes have been identified using electrophysiology: gain of function mutations (GOF), which are mutations that open spontaneously or with less tension than required to open wild type MscL, and loss of function mutations (LOF), which are mutations that cannot be gated or require more tension than wild type MscL to gate. Previous mutagenic studies, however, have generally evaluated GOF mutations

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[S] The on-line version of this article (available at http://www.jbc.org) contains two supplementary tables.

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[1] The abbreviations used are: MscL, mechanosensitive channel of large conductance; Ec-MscL, *E. coli*-MscL; Tb-MscL, *M. tuberculosis*-MscL; SG model, model developed by Sukharev, Guy, and coworkers (19–21); GOF, gain of function; LOF, loss of function; HOEM, high osmolyte enhanced M9.

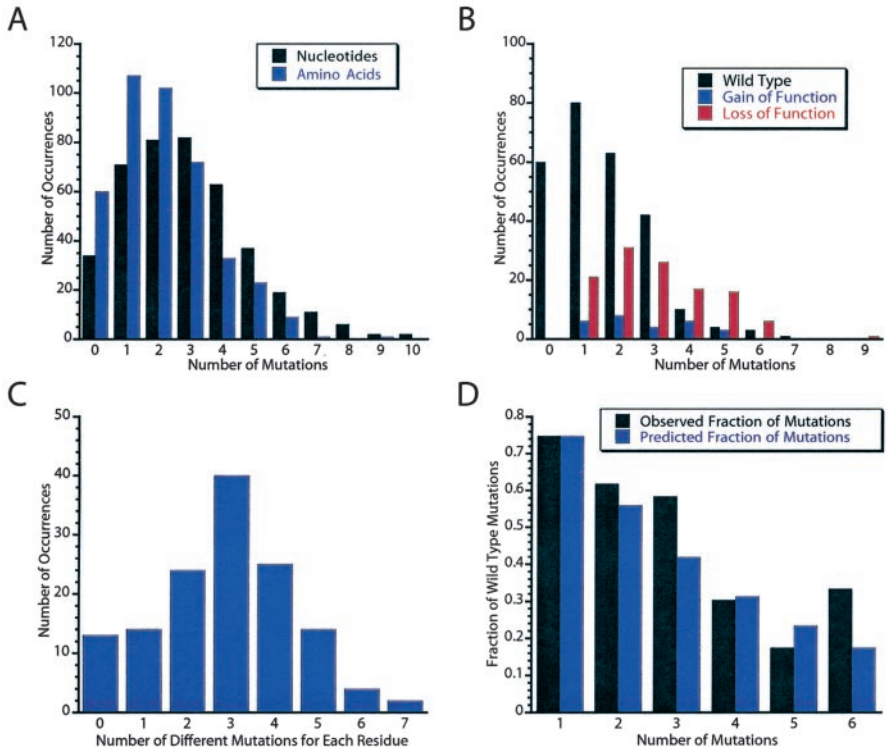

FIG. 1. *A*, distribution of nucleotide and amino acid mutations observed in the library of 408 sequenced Ec-MscL constructs. *B*, phenotypic distribution of sequenced Ec-MscL constructs. *C*, distribution of the number of amino acid mutations observed for a given residue in Ec-MscL. *D*, comparisons of the observed number of phenotypically wild type mutations with the predicted number of phenotypically wild type mutations.

only, because the LOF assay is especially tedious.

Recently, we have developed a high-throughput, fluorescence-based method for the phenotypical characterization of both LOF and GOF mutations in Ec-MscL (23). This approach allows for the rapid screening and identification of mutations that are phenotypically wild type, gain of function, or loss of function. We now report the results of an extensive random mutagenesis study of Ec-MscL, looking at mutations with both possible phenotypes. We have greatly expanded the number of MscL mutants with an altered phenotype, especially for the LOF phenotype. These mutagenesis results have been used to evaluate several key features of the SG gating model. In addition, our mutagenesis results implicate particular regions of MscL as being involved in tension sensing and may discriminate between two proposed evolutionary models of MscL.

EXPERIMENTAL PRODECURES

Plasmids and Strains—The Ec-MscL protein was encoded under the control of an isopropyl-1-thio-β-D-galactopyranoside-inducible promoter in pB10b, as previously described. All experiments were carried out in the MJF465 bacterial strain that is lacking the *MscL*, *MscS* (*YggB*), and *KefA* genes.

Random Mutagenesis—Random mutagenesis of Ec-MscL was performed using the Diversify PCR Random Mutagenesis kit (Clontech) under buffer condition six. A standard PCR reaction using TITANIUM *Taq* DNA polymerase was carried out in the presence of 640 μM $MnSO_4$ and 80 μM dGTP. Following PCR, the reaction products were subcloned into fresh pB10b vector using the *Bgl*II and *Xho*I restriction sites.

Phenotypical Characterization of MscL Mutations—Phenotypical characterization of Ec-MscL mutations was carried out as previously described with slight modification to allow growth in deep 96-well plates. Single colonies resulting from the subcloned random Ec-MscL mutations were grown in a 2-ml 96-well growth block using 200 μl of Luria Bertani medium supplemented with ampicillin (100 μg/ml) for 14 h at 37 °C in a shaking incubator (400 rpm). To prevent evaporation and allow gas exchange, the plate was covered with two sheets of 0.5 mil polyester. The Luria Bertani culture (1 μl) was then used to induce a 200-μl culture in HOEM medium supplemented with ampicillin (100 μg/ml) and isopropyl-1-thio-β-D-galactopyranoside (1 mM). The cultures were covered with polyester and grown for 7 h and 45 min at 37 °C in a shaking incubator (400 rpm). In a 96-well plate the HOEM cultures (10 μl) were diluted 20-fold by addition of solutions (190 μl) of various osmotic strengths containing propidium iodide and SYTO 9 (Molecular Probes). Each HOEM culture was subjected to eight different downshock solutions. The downshock solutions were prepared by mixing HOEM medium with water in the following ratios: 1:0, 4:1, 13:7, 1:1, 3:5, 1:3, 1:7, and 0:1. After mixing, the 96-well plate was incubated at 37 °C in a plate incubator for 90 min. The plates were then read using a Gemini XS plate reader (Molecular Devices) with an excitation wavelength of 485 nm and emission wavelengths of 530 and 630 nm. Phenotypes were determined from the resulting downshock curves as previously described. In previous work (23) we have shown that this screen faithfully reproduces the phenotypical variation seen in a series of G22X mutants, and so no rescreening using electrophysiology was performed.

DNA Sequencing—For DNA sequencing, the remaining Luria Bertani cultures were diluted with TB medium supplemented with ampicillin (100 μg/ml) and grown to saturation. Mini-prep DNA was obtained for sequencing using either a spin mini-prep kit (Qiagen), a 96-well mini-prep kit (Millipore), or a mini-prep robot (Laragen, Inc., Los Angeles, CA or ACGT, Inc., Northbrook, IL). Big dye terminated automated DNA sequencing (Applied Biosystems, Inc., Foster City, CA) was performed by either the Caltech Sequence Analysis Facility (Pasadena, CA), Laragen, Inc., or ACGT, Inc.

RESULTS

The Library—A library of random mutations was created using a standard sloppy PCR protocol, as described under "Experimental Procedures." Starting with this library, 408 Ec-MscL constructs gave reliable sequencing data and were phenotypically screened. The resulting constructs had a modal distribution centered at 2–3 nucleotide changes per construct and one amino acid change per construct. The distribution of mutations is shown in Fig. 1*A*. Of the 408 sequenced constructs, 348 contained at least one amino acid change in the MscL protein.

Of the constructs that contained at least one amino acid point mutation, 64% were phenotypically wild type, 29% were phenotypically loss of function, and 7% were phenotypically gain of function. Looking only at the constructs with a single amino acid change, 74% were wild type, 20% were loss of function, and 6% were gain of function. Of course, the wild type phenotype described here could be indistinguishable from very

weakly LOF or GOF phenotypes that might be detectable by detailed electrophysiological studies. The GOF and LOF phenotypes are unambiguous. These results are thus in striking contrast to previous work, which has primarily identified gain of function mutations.

As expected, increasing the number of mutations increased the probability of a channel showing an altered phenotype (GOF or LOF). Fig. 1*B* shows the number of occurrences of a particular phenotype as a function of the number of amino acid point mutations. The large number of phenotypically wild type mutations observed, coupled with the observation of phenotypically wild type constructs that contain multiple mutations (up to seven), suggests that MscL is relatively tolerant to mutation.

Ninety percent of the amino acids that make up Ec-MscL were mutated at least once in our sequenced random mutation library. A modal distribution was observed centered around three amino acid changes per Ec-MscL residue (Fig. 1*C*). Up to seven different amino acid changes were observed at a single Ec-MscL position. Assuming a single nucleotide change per codon in Ec-MscL, there are 843 possible non-silent point mutations. Forty-six percent (390) of all possible point mutations were observed in our sequenced random mutation library.

Evaluating Multiple Mutations—Single site mutants are, of course, straightforward to interpret, whether they give wild type, GOF, or LOF phenotypes. On the other hand, interpreting the results for structures with multiple mutations can be challenging. If we assume that the occurrence of compensating LOF and GOF mutations is rare, multiple mutations that are phenotypically wild type arise from a collection of phenotypically wild type mutations. To test this assumption we can use the number of single site, wild type mutations to predict the expected number of multisite wild type mutations. That is, if 75% of single site mutants are wild type, then we expect the fraction of double mutants that are wild type to be $(0.75)^2$. The predicted and actual fractions of wild type phenotypes for multiple mutants are shown in Fig. 1*D*, and the agreement is acceptable. We therefore assume that a mutation that occurs in any wild type construct is a "wild type" mutation and would produce a wild type phenotype if it occurred as a single mutant. This also means that such a mutation does not contribute to GOF or LOF phenotypes.

Constructs with multiple mutations and an altered phenotype are more difficult to interpret, because any single mutation could give rise to the observed phenotype. Pseudo-single site, altered phenotype mutation data can be generated, however, by subtracting the wild type data from the non-wild type data. In this process, if the same mutation is observed in both a wild type construct and an altered phenotype construct, the mutation is assumed not to give rise to the altered phenotype. For example, the double mutant V23A/K117T is observed to be gain of function, and the mutation K117T is observed in a wild type construct. From this we conclude that the mutation V23A gives rise to the observed gain of function phenotype. Additional pseudo-single site data can be obtained by assuming that mutations to Ec-MscL beyond residue 110 are wild type. It has been shown that deletion of residues 110–136 in Ec-MscL does not dramatically alter the gating of the channel (8, 24).

Similar to the analysis of wild type mutations, the number of LOF and GOF mutations can be predicted from the number of single site mutations, using the assumption that one mutation gives rise to a particular phenotype. The number of observed mutations is again similar to the predicted value (data not shown).

Based on this reasoning, the library contains 289 single site and pseudo-single site mutations: 237 wild type structures along with 7 GOF and 45 LOF mutants. These mutations are mapped onto the MscL sequence in Fig. 2. Fig. 3*A* gives a pictorial definition of the various domains of the receptor. A listing of all mutants prepared is given in the tables under supplemental information.

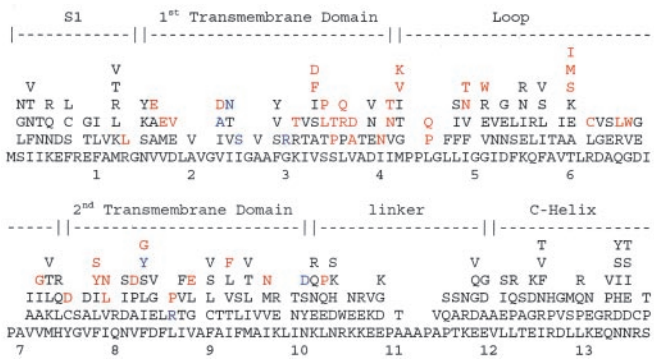

FIG. 2. **Observed single site and pseudo-single site mutations and their phenotypes are shown above the wild type Ec-MscL sequence.** Amino acid mutations that are phenotypically wild type are shown in *black*, mutations that are phenotypically loss of function are shown in *red*, and mutations that are phenotypically gain of function are shown in *blue*.

Interestingly, the phenotypically altered single site data suggest that TM2 is functionally significant. Two new gain of function mutations are observed in this region, F83Y and L86R, along with many loss of function mutations. With the exception of N100D, mutations in TM2 with altered phenotypes had not been observed in previous random screenings of Ec-MscL.

Previous work had identified roughly 30 gain of function mutations in Ec-MscL, and the 7 GOF constructs observed here add 5 new entries to that list. In contrast, previous work had identified only 10 loss of function mutants, and so the discovery of 45 new LOF mutants greatly expands the list and has significant implications for channel function and design, as discussed below. Additionally, the 237 single site wild type mutations or pseudo-single site wild type mutations provide additional information that was not previously available.

DISCUSSION

Using a standard sloppy PCR protocol and a recently developed high-throughput screen, we have been able to evaluate 408 Ec-MscL constructs, of which 348 contained at least one altered amino acid. The coverage of mutations is good and fairly uniform (Figs. 1*A* and 2). We feel the results have significant implications for several aspects of MscL research, including the details of the SG model and possible tension-sensing regions.

Previous work had identified roughly 30 gain of function mutations in Ec-MscL, and the 7 GOF constructs observed here add 5 new entries to that list. In contrast, previous work had identified only 10 loss of function mutants, all of which were variants at the Gly-22 site that also produces GOF mutants (11). As such, the discovery of 45 new LOF mutants (Fig. 2) greatly expands the list and has significant implications for channel function and design, as discussed below. No doubt the small number of previously observed LOF mutants reflects the greater challenge in performing conventional LOF assays. The fluorescence assay used here overcomes this limitation. Using this assay 29% of the constructs containing one or more mutations were LOF, and 20% of all constructs containing precisely one mutation were LOF (Fig. 1*B*). In sharp contrast, only 7% of all constructs containing at least one mutation were gain of function and only 6% of constructs with precisely one mutation were GOF (Fig. 1*B*).

Concerning global features of the channel, it is interesting

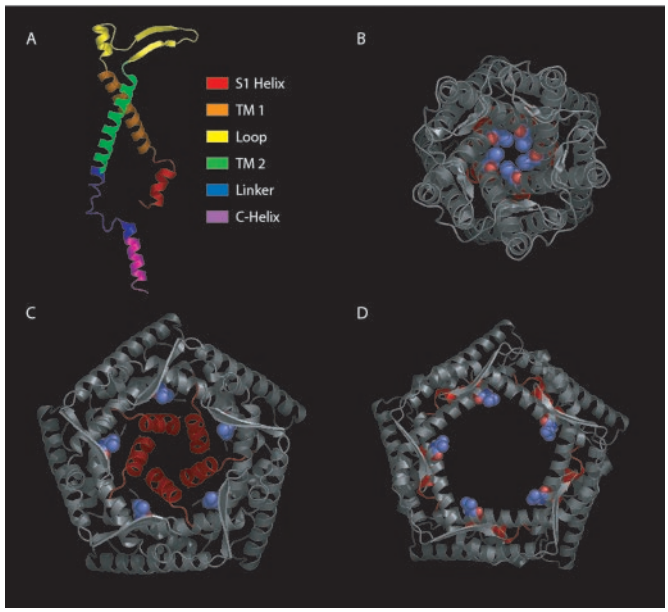

FIG. 3. *A*, a single chain of the SG closed state model highlighting the various regions of the protein. *B–D*, top views of the SG closed, intermediate, and open state models with the Val-23 plug shown in Corey-Pauling Koltun and the putative S1 helix shown in *red*.

that phenotypically altered single site data suggest that TM2 is functionally significant. Two new gain of function mutations are observed in this region, F83Y and L86R, along with many loss of function mutations. With the exception of N100D, mutations in TM2 with altered phenotypes had not been observed in previous random screenings of Ec-MscL.

Implications for the SG Model—The detailed model developed by SG is based on a number of reasonable assumptions and additional creative ideas to close some key gaps. The starting point for the SG model is a modified homology model of the Tb-MscL crystal structure of the closed state. A new helix (Fig. 3, *S1*) was added to the amino terminus of the protein, corresponding to a region that was not resolved in the Tb-MscL crystal structure. Also, because the loop region of MscL is not well conserved between the *E. coli* and the *M. tuberculosis* homologues, a *de novo* model for the Ec-MscL loop region, not based on the crystal structure of Tb-MscL, was developed. Additionally, the carboxyl terminus of the protein was adjusted to make room for the S1 region. The various regions of the model are shown in Fig. 3*A*. We note that SG have generously made the coordinates of their models freely available, which greatly facilitates efforts to develop experimental tests of the model.

A key feature of the SG model is the presence of an expanded, intermediate state on the gating pathway. In this state the occlusion of the pore seen in the crystal structure is broken, but an occlusion formed by the modeled S1 helices remains. This pre-expanded state was considered necessary to explain the electrophysiology of Ec-MscL. Fig. 3, *B–D*, shows a top view of the SG model for the closed, expanded intermediate, and open states, respectively. It could well be there are several states of intermediate conductance in the MscL channel, and it is not possible with a screen such as the one employed here to determine where along such a pathway a given mutation is exerting its influence. Nevertheless, it seems reasonable to assume that mutations of residues proposed to play a key structural role should have a functional consequence.

In the present work, we use specific point mutations to probe features of the SG model. It is difficult for a study of point mutations to provide compelling evidence in support of or opposed to the global structural changes inherent in the SG model, and we note that the global model for the first and second transmembrane domains has received some experimental support (1, 20–22). Still, evaluating particular amino acids is justified by the fact that the SG model was created with a strong reliance on the assumption that highly conserved residues are important and should be involved in complementary interactions in the closed, pre-expanded, and/or open states (19). Relying on sequence conservation is inherently challenging for MscL, because conservation across the family is not nearly as extensive as is typical for homologous proteins. Also, a detailed sequence analysis from our labs (25) produced two distinct MscL subtypes with Tb-MscL, the sequence for which a crystal structure exists, lying in a different family from Ec-MscL, the sequence modeled by SG.

As shown in Fig. 2 and the supplemental material, many conserved residues can be mutated and yet still produce a wild type phenotype. Attempts to correlate mutational tolerance with sequence conservation were made by applying the Rao physiochemical scoring matrix (26) to the observed mutational data and our sequence alignment, but no correlation was found. These results highlight the previously noted fact that sequence conservation among the MscL channels is not high, with some tendency for homologues to cluster into specific subtypes.

The supplemental material shows the proposed interacting partners for the conserved residues in each state based on the SG model and the mutations we have observed for these residues. It is clear from this table that many of the residues postulated to be involved in key interactions in the various states of the model can be mutated and yet still produce a wild type phenotype. This calls into question the degree to which a given interaction is essential for proper channel function. Furthermore, no direct correlation is observed between sequence conservation and mutational tolerance for a given residue. As can be seen in the supplemental material, mutations were observed for most of the conserved residues, with many producing a wild type channel. This is especially true for residues located in the amino and carboxyl termini of the protein. In the non-terminal regions of MscL, the number of sites having mutations that give rise to an altered phenotype is not appreciably different between the conserved and non-conserved residues. Here, we focus on select residues that have been proposed by SG to play key roles in the gating mechanism.

The Role of S1 and the Five Conserved Phenylalanines—The SG model makes many predictions about key interactions involved in various states of MscL. SG observe five highly conserved phenylalanines in the MscL family and assign significant functional roles to all of them. The first two, Phe-7 and -10, are proposed to reside in the hypothetical S1 helix that is not observed in the Tb-MscL crystal structure. The third phenylalanine, Phe-29, resides in the first transmembrane domain, and the final two phenylalanines, Phe-85 and -93, reside in the second transmembrane domain. Our data suggest that none of these residues is critical to channel function.

The SG model postulates that in the closed state Phe-7 and -10 interact with each other to occlude the pore and serve as a secondary gate. We observe mutagenic tolerance at Phe-7 for serine, cysteine, and leucine and mutagenic tolerance at Phe-10 for leucine and isoleucine. Although the Phe-10 mutations could be considered conservative, those at Phe-7 are certainly not, arguing against a key Phe-7-Phe-10 interaction. Note that Sukharev *et al.*, (20) had previously shown, using electrophysiology, that the F7C mutant was functional in the absence of disulfide formation.

In the pre-expanded states of the SG model, it is postulated that Phe-10 from one subunit interacts with Phe-7, Ile-3, and

Ile-4 of the neighboring subunit. Mutations that have been observed to be phenotypically wild type at Ile-3 are phenylalanine, asparagine, threonine, and valine; at Ile-4, asparagine and threonine give wild type behavior. The significant mutagenic tolerance at these positions is supported by the observation of Blount *et al.* (8) that residues 2–4 of Ec-MscL can be deleted without significantly altering channel function. These results suggest that the blockage in the pre-expanded state of the channel, which is necessary to explain the electrophysiology of MscL, may not be because of the postulated S1 helices.

As with the closed and pre-expanded states, Phe-7 and -10 are proposed to exert significant influence over the open state. In the open state of the channel, Phe-7 is postulated to interact with Phe-93, and Phe-10 is postulated to dock between Phe-29 and -85. Again mutagenic tolerance is observed for Phe-29, -85, and -93. Mutations of Phe-29 to serine, valine, and tyrosine are tolerated, mutation of Phe-85 to leucine is tolerated, and mutations of Phe-93 to leucine and serine are tolerated. It seems unlikely that the proposed aromatic-aromatic interactions play critical roles in the function of MscL.

Although one could argue that mutational tolerance at any given site might not make a compelling case, the range of conserved residues that can, in fact, be mutated without phenotypical consequences suggests that the S1 helix developed for the SG model may not play a crucial role in MscL function. In fact, of 26 mutations made to S1, 25 produce a wild type phenotype. The lack of an important functional role for the S1 helix is in agreement with the intermediate proposed by Perozo *et al.* (22). However, the lack of an expanded intermediate in the Perozo model does not fit well with the electrophysiology data (6).

An Important Aspartic Acid—All conformations of the SG model are proposed to be stabilized by a salt bridge between Arg-13 and Asp-18, and our results support this conclusion. Interestingly, D18E is observed to be phenotypically wild type, but the non-conservative mutations D18V and R13L are observed to be phenotypically loss of function (Fig. 2). The R13L mutant is the only mutation in S1 that is not wild type.

Unimportant Salt Bridges—Two other salt bridges were specifically noted by SG as important for stabilization of the open state: Arg-8–Asp-127 and Glu-9–Arg-126. Clearly these salt bridges are not necessary for gating, because Glu-9 can be mutated to threonine and glycine, Arg-126 can be mutated to proline and histidine, and Asp-127 can be mutated to valine and glycine, with all mutants giving a wild type phenotype. The lack of importance of these salt bridges is further highlighted by the fact that Ec-MscL truncated at position 110 is phenotypically wild type (8, 24).

The Tension Sensor—In an effort to gain some insight into which residues are most crucial to channel function, we have mapped onto the proposed closed state structure of Ec-MscL the distribution of single site and pseudo-single site loss of function mutations (Fig. 4). We assume that LOF mutants are more likely associated with key functional regions of the protein than GOF mutants, and the discovery of a large number of new LOF mutants is thus a key feature of this work. LOF mutations are clearly concentrated in the loop region and in the regions of the protein near the headgroups of the lipid bilayer. The carboxyl-terminal domain is completely devoid of LOF mutations, and residues in the proposed S1 helix and the middle regions of the transmembrane domains are, for the most part, mutagenically tolerant.

The lipid headgroups provide a mechanism for specific protein-lipid interactions. Molecular dynamics simulations of Tb-MscL have established strong interactions between the lipid headgroups and the MscL channel (18, 27). The same simulations have shown that the number of hydrogen bonds between the protein and the lipid varies as a function of the lipid headgroup.

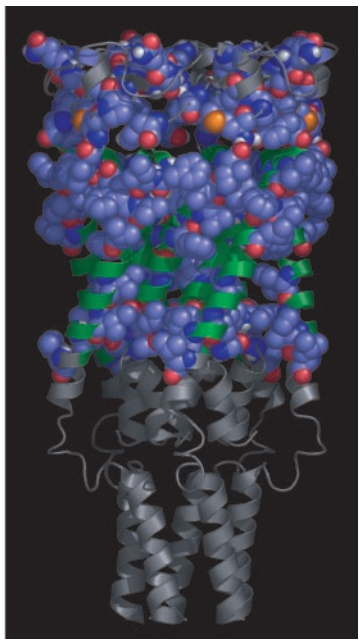

FIG. 4. **Loss of function mutations shown as Corey-Pauling-Koltun on the SG closed state structure.** TM1 and TM2 backbones are highlighted in *green*.

Several lines of experimental evidence also point to a key role for lipid-protein interactions in gating mechanisms. Gating tensions for Ec-MscL in native *E. coli* membranes, which are predominately phosphatidylethanolamine, are measurably higher than gating tensions for Ec-MscL reconstituted into phosphatidylcholine (3), and the hydrogen bonding potentials of these two lipids are substantially different (18, 27). Martinac and Hamill (28) have shown that small changes in lipid chain length can greatly affect the mechanosensitive gating of gramicidin A. Furthermore, Martinac and coworkers (29, 30) have shown that reconstitution of Ec-MscL into vesicles made up of lipids with progressively shorter chain lengths results in a channel that is progressively easier to open. Additionally, Martinac and Hamill (28, 31) have argued that changes in bilayer thickness due to tension, which they estimate to be less than 1.5 Å, are important for the gating of mechanosensitive channels.

Further, although less direct, support for key lipid-protein interactions comes from the observation by Jones *et al.* (32) that Ec-MscL gates upon heat shock. One aspect of thermal adaptation for *E. coli* is a change in membrane composition, with shorter lipids being more common at higher temperatures (33, 34). In addition, significant differences in gating tensions exist between Ec-MscL and Tb-MscL, and the native lipid bilayers of *M. tuberculosis* and *E. coli* are different (33–37). Taken together, these experimental observations are consistent with our observation that LOF mutants are clustered near the lipid headgroups, where the interactions with the protein are the strongest.

Evidence for important protein-lipid interactions in gating has significant implications for efforts to model the gating transition. In particular, the very detailed model proposed by SG was based on calculations that did not include any representation of the bilayer. Despite the greatly increased computational cost of adding explicit membranes and water to such calculations, it may be necessary in order to explain fine details of the gating mechanism.

The Loop—A significant number of the single site and pseudo-single site loss of function mutations observed for Ec-MscL are located in the loop region (Fig. 4). Previous work by us and others has implicated this region as being important in MscL gating (8, 25, 38, 39). For Tb-MscL it has been shown that modification of a charged hydrogen bond in the loop results in mutations that are phenotypically gain of function (25). Martinac and coworkers (38) have shown that proteolytic cleavage of the loop results in gain of function behavior for Ec-MscL. Conversely, Blount *et al.* (8) have shown that deletion of a single residue, Gln-56, from the Ec-MscL loop results in a loss of function phenotype. This has led to the hypothesis that the loop serves as a spring connecting the first and second transmembrane domains. The observed loss of function mutations in this region further support this hypothesis.

Evolutionary Arguments about Mechanosensitive Channels—Morris and coworkers (40, 41) have proposed two hypotheses to account for the evolution of mechanosensitive ion channels. The first hypothesis is that mechanosensitive channels have evolved with either specialized mechanogating regions, a global structure that renders them susceptible to bilayer tension, or both. The second, less conventional, hypothesis is that mechanosensitive channels respond to bilayer tension because it has been evolutionarily impossible, undesirable, and/or unnecessary to eradicate intrinsic protein characteristics that result in mechanosensitivity. That is, mechanosensitivity is a natural feature of all membrane channels. Those channels that are not mechanosensitive or have high tension thresholds like MscL must have acquired special structural features to *diminish* the innate mechanosensitivity of ion channels. Morris and coworkers have argued that the second hypothesis is more compelling than the first, citing evidence that mechanosensitivity is harder to design out of a protein than to design into a protein. Their evidence for this is the mechanosensitivity of Shaker-IR and the "ample selection of MscL mutants that produce a channel more mechanosusceptible than the wild type" (*i.e.* GOF mutants) (40).

We assert that true mechanosensitive channels conform to the first hypothesis and that MscL is one of these channels. Our mutational data clearly show that a mutation is much more likely to create a *loss* of function phenotype than a gain of function. This implies that it is more difficult to design mechanosensitivity into MscL than it is to design mechanosensitivity out of MscL. As noted above, the reason that previous random mutagenesis studies of MscL did not uncover many phenotypically loss of function channels is that the screening assay used was incapable of distinguishing loss of function mutations from wild type. The hypothesis that mechanosensitivity is difficult to design into "true" mechanosensitive channels is further supported by random mutagenesis studies on MscS performed by Blount and coworkers (42). These studies show that it is extremely difficult to identify mutations that make MscS easier to gate. Additionally, our single site and pseudo-single site loss of function data point to a specialized mechanogating region as required by the first hypothesis. Although mechanosensitivity may exist in non-mechanosensitive channels, it seems clear that true mechanosensitive channels have been designed to open under very specific conditions.

CONCLUSIONS

Although the general mechanism of gating proposed by SG may or may not describe the opening pathway for Ec-MscL, it seems likely that some details of the model need further refinement. Many of the highly conserved residues, which are assigned important roles in the model, can undergo dramatic mutations while preserving a phenotypically wild type channel. In particular, the putative S1 helix bundle was assigned the important role of forming a second gate in the closed and intermediate states, but that conclusion is not supported by the data presented here.

The clustering of loss of function mutations along the lipid headgroups at the top and bottom of the membrane may indicate that this region is responsible for tension sensing in MscL. Additionally, the identification of many loss of function mutations in the loop region provides further evidence that the loop serves as a transduction domain connecting the first and second transmembrane domains. In general, phenotypically altered mutations occur in the first transmembrane domain, loop, and second transmembrane domains. This argues for the importance of these regions in the gating of MscL. The putative S1 helix, the linker, and the carboxyl-terminal helix are relatively tolerant to mutation.

The mechanosusceptibility of Ec-MscL seems to be the evolutionary purpose of Ec-MscL and not just an evolutionary byproduct. MscL has a specific gating tension that can be increased or decreased by mutation. Additionally for MscL, our data show that it is easier to design mechanosensitivity out of the channel than to design mechanosensitivity into the channel.

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