Comparing and Contrasting Escherichia coli and Mycobacterium tuberculosis Mechanosensitive Channels (MscL)

NEW GAIN OF FUNCTION MUTATIONS IN THE LOOP REGION*

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Sequence analysis of 35 putative MscL homologues was used to develop an optimal alignment for Escherichia coli and Mycobacterium tuberculosis MscL and to place these homologues into sequence subfamilies. By using this alignment, previously identified E. coli MscL mutants that displayed severe and very severe gain of function phenotypes were mapped onto the M. tuberculosis MscL sequence. Not all of the resulting M. tuberculosis mutants displayed a gain of function phenotype; for instance, normal phenotypes were noted for mutations at Ala\(^{20}\), the analogue of the highly sensitive Gly\(^{22}\) for instance, normal phenotypes were noted for mutations at Ala\(^{20}\). Cross-linkable residues were substituted for the residues involved in the hydrogen bond, and cross-linking studies indicated that these sites are spatially close under physiological conditions. In general, mutation at these positions results in a gain of function phenotype, which provides strong evidence for the importance of the loop region in MscL channel function. No analogue to this interesting interaction could be found in E. coli MscL by sequence alignment. Taken together, these results indicate that caution should be exercised in using the M. tuberculosis MscL crystal structure to analyze previous functional studies of E. coli MscL.

The recent crystal structures of two bacterial ion channels, the KcsA potassium channel and the mechanosensitive channel MscL, provide unique opportunities to study ion channel structure-function relationships (1, 2). Concerning the MscL system, several different models for channel opening have been proposed by considering E. coli gain of function (GOF) mutations in light of the M. tuberculosis crystal structure (1, 3, 8). To evaluate critically these efforts, it is essential to assess the underlying assumption of the portability of Eco-MscL functional data to the Tb-MscL structure. Although the function of the E. coli channel has been extensively probed by site-directed and random mutagenesis, analogous studies of the M. tuberculosis channel have not been reported (9–15). Preliminary data have shown that wild type E. coli and M. tuberculosis MscL are similar electrophysiologically. Both channels exhibit a large single channel conductance, approximately 3.5 nS, and gate with similar tensions in reconstituted liposomes (17). However, the Tb-MscL channel exhibits twice the gating tension of Eco-MscL in E. coli spheroplasts (17). This difference may result from protein structural differences, a difference in interactions with lipids, or both.

Sequence alignment is essential to map previously studied E. coli GOF mutations onto the M. tuberculosis MscL sequence. In this work we report an optimal sequence alignment of 35 MscL homologues and an analysis of regions of conservation and variability. Consistent with previous studies, we find greater conservation in the transmembrane regions than in the loop or intracellular regions. Interestingly, the various channels clearly fall into subfamilies based on sequence similarity, with Eco-MscL and Tb-MscL in different subfamilies.

By using the optimal alignment, we have prepared Tb-MscL analogues of several critical Eco-MscL GOF mutations (Fig. 1A). Perhaps surprisingly, we find that several well established Eco-MscL GOF mutants do not translate to the Tb-MscL system. We also directly evaluate a predicted intersubunit hydrogen bond in the Tb-MscL crystal structure (Fig. 1B). Cross-linking studies establish that these residues are indeed close in the reconstituted channel and firmly establish the pentameric nature of the channel. Mutations of this pair generally lead to GOF mutants, suggesting an important functional role for this specific region of the channel. Interestingly, no analogous interaction is apparent in the Eco-MscL alignment. Our results indicate that the functional studies performed on the Eco-MscL channel may not map directly onto the Tb-MscL crystal structure.

MATERIALS AND METHODS

Sequence Analysis—Multiple sequence alignments were obtained using alignment of multiple sequences (AMPS) (20, 21), and consensus group analysis was performed using multiple EM for motif elicitation (MEME) (22, 23). The alignment was broken into regions, extracellular

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† The abbreviations used are: Eco-MscL, large mechanosensitive ion channel from E. coli; Tb-MscL, large mechanosensitive ion channel from M. tuberculosis; GOF, gain of function; MEME, multiple EM for motif elicitation; AMPS, alignment of multiple sequences; PAGE, polyacrylamide gel electrophoresis; NHS, N-hydroxysuccinimide; DDM, N-dodecyl β-maltoside; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; DCC, N,N'-dicyclohexylcarbodiimide.

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Fig. 1. M. tuberculosis MscL crystal structure. A, severe and very severe GOF mutations from Eco-MscL are mapped onto one subunit of the Tb-MscL crystal structure. B, the Arg^{45}-Gln^{55} hydrogen bond. The box shows a close-up of the hydrogen bond between the yellow and purple subunits. Figures were generated with MOLSCRIPT and Raster3D (18, 19).

**RESULTS**

**Sequence Analysis—**Although clearly related, the mechanosensitive channels from various organisms show moderate to low sequence identities. For example, the sequence identity of Tb-MscL compared with Eco-MscL is 37%, whereas the sequence identity of Bordatella bronchiseptica MscL compared with Mycobacterium leprae MscL is 15%. Therefore, development of an optimal alignment is not straightforward. For this reason, we have augmented sequence alignment approaches with MEME analysis, looking for patterns of conservation across the series. Fig. 2 shows an AMPS multiple sequence alignment and MEME group analysis of 35 putative MscL sequences. The MEME group analysis was used to make slight adjustments to the AMPS multiple sequence alignment using the indicated areas of conservation within the sequences. This alignment was further analyzed to determine which regions of MscL were most divergent using AMPS pairwise alignment of the full sequences and also of selected regions such as the first and second transmembrane domains, the extracellular loop, and the carboxyl terminus. Regional divisions were made by applying the previous definitions from the Tb-MscL crystal structure to the multiple sequence alignment (1). These alignments indicate general overall similarity for all regions of the protein; however, the loop region clearly shows the most variability. Contour plots showing scores for the AMPS pairwise alignments of the first transmembrane domain, the extracellular loop, and the carboxyl terminus are shown in Fig. 3.

**Mutational Mapping—**With an optimal alignment in hand (Fig. 2), we were able to map some of the very severe and severe mutations from Eco-MscL (11, 14) onto Tb-MscL. The most extensively probed type of mutation has been the so-called gain of function (GOF) mutation. This is observed in growth studies...
of *E. coli* expressing the mutant channel. It is assumed that a mutation that increases channel opening probability will, in effect, put a hole in the cell membrane, which is deleterious to growth. The screen thus identifies channels that have a higher open probability at ambient pressure, which is considered a gain of function (9, 11, 14).

Fig. IA shows the positions of these mutational sensitive sites mapped onto the Tb-MscL structure. In all cases the
alignment we obtain for these residues is the same as others have reported previously (1, 3, 5, 6). Site-directed mutagenesis of Tb-MscL at these positions was performed, converting the wild type amino acid to a residue shown in E. coli to give a GOF phenotype. The resulting mutations were analyzed using plate growth studies and scored using the system described under “Materials and Methods.” Typical plate growth results are shown in Fig. 4, and all results are gathered in Table I. A GOF phenotype was observed in L17Y, V21A, N44D, and N95D. Unexpectedly, normal growth was observed for A20E, A20R, and T28R, even though the aligned positions, especially Ala20, were shown to be very sensitive to mutation in Eco-MscL (11, 12, 14). That mutants displaying normal growth were indeed expressing a MscL channel was verified by SDS-PAGE analysis and Western blotting, which showed levels of protein expression within the variation seen for wild type Tb-MscL.

**Tb-MscL Loop Intersubunit Hydrogen Bond—** Examination of the Tb-MscL structure revealed an intersubunit hydrogen bond, Arg45-Gln51, located in the loop region of the channel (Fig. 1B). Suspecting that such a specific intersubunit contact may be important to function, we mutated this interaction to R45K/Q51E and R45C/Q51C to determine the proximity of these residues under physiological conditions by cross-linking analysis. The R45K/Q51E mutation was overexpressed and purified from E. coli. Cross-linking studies were performed in DDM micelles using EDC or DCC, with or without NHS activators. A typical SDS-PAGE Western blot of cross-linking products is shown in Fig. 5. Cross-linking is always seen, and in
some cases it is quite efficient. After treatment with 10 mM EDC and 10 mM sulfo-NHS, the majority of the observed cross-linked product is tetrameric or pentameric, establishing the high efficiency of this rationally designed cross-linking system. Cross-linking of the R45C/Q51C mutant produced similar results to the standard cross-linking of the salt-bridge mutant, but in no instance was highly efficient formation of tetramer and pentamer seen.

Since cross-linking studies confirmed the close proximity of Arg^{45} and Gln^{51} under physiological conditions, growth studies were used to assess the effects of mutations at these positions on channel function. The results of growth studies for some single and double mutants at these positions are summarized in Table II. All mutations at these positions, with the exception of R45K/Q51K, show a GOF phenotype.

### DISCUSSION

**Sequence Analysis**—The MEME sequence analysis has provided insight into the overall similarity of the MscL homologues. Not surprisingly, the homologues are most similar in the transmembrane regions and most divergent in the loop and carboxyl-terminal regions. The strong similarities in the transmembrane domains are highlighted by the fully conserved groups, II and VIII, and the highly conserved group III. Additionally, members of the MscL family that lack group III in the first transmembrane region tend to have a similar conserved group IV in this region.

The carboxyl-terminal and loop region are much less conserved. Despite the appearance of three consensus groups in the loop region, V, VI, and VII, these groups are clearly not universal. The carboxyl terminus is more highly conserved than the loop region, but it is clearly not as well conserved as the transmembrane helices. The carboxyl terminus contains two very highly conserved groups, IX and XIII, and the less conserved group XI. Mycobacteria do not contain group IX, but an analogous charged region is evident (group X). Previously it has been shown that a large portion of the carboxyl terminus in Eco-MscL can be deleted without affecting protein function (15). This is consistent with the lack of sequence conservation in this region.

To examine further the similarities and differences among MscL homologues, a pairwise alignment of the various regions was employed (Fig. 3). The pairwise alignments showed the same general trends observed with MEME analysis. In general, all regions of the MscL sequence are conserved; however, the loop region has pairs with sequences with poor alignment. To some extent the sequence pairs within the loop region can be used to group the homologues into subfamilies. The largest and most obvious subfamily includes *E. coli* MscL and other sequences containing MEME group VI. Another distinctive subfamily includes the Mycobacteria. Thus, by this analysis Eco-MscL and Th-MscL are in different subfamilies.

**Mutational Mapping**—Previous mutational analysis of Eco-MscL has focused mainly on the highly conserved transmembrane regions, with only a few randomly obtained mutations in the loop (9, 11, 12, 14, 15). For the transmembrane regions, one would expect the sequence homology mapping of the previously obtained GOF *E. coli* mutants onto Tb-MscL to give mutants with a GOF phenotype, due to the high sequence homology in these regions. Note that all alignments, the one reported here and those published previously, agree as to which residues in Tb-MscL correspond to previously studied GOF sites in Eco-MscL (1, 3, 5, 6).

For the majority of mutations studied (L17Y, V21A, N44D, and N95D), the GOF phenotype seen in Eco-MscL is also seen in Tb-MscL (Table I and Fig. 4). Surprisingly, however, mutations at Ala^{20} and Thr^{28} do not yield a GOF phenotype. The

| Mutant | Number of plates | Average score after 20 h | Average score after 40 h |
|--------|------------------|------------------------|------------------------|
|        | Uninduced | Induced | Uninduced | Induced | Uninduced | Induced |
| Wild type | 51      | 52       | 3.38     | 2.64     | 3.29      | 2.75     |
| L17Y    | 11      | 11       | 3.10     | 0.70     | 3.19      | 0.70     |
| A20E    | 13      | 13       | 3.38     | 3.47     | 3.38      | 3.47     |
| A20R    | 13      | 13       | 3.32     | 3.52     | 3.32      | 3.52     |
| V21A    | 11      | 11       | 2.85     | 1.10     | 2.85      | 1.10     |
| T28R    | 13      | 13       | 3.79     | 3.47     | 3.79      | 3.47     |
| N44D    | 11      | 11       | 3.27     | 0.57     | 3.27      | 0.57     |
| N95D    | 11      | 11       | 3.12     | 0.20     | 3.20      | 0.60     |

FIG. 4. Representative plate growth for mutations mapped from *E. coli* MscL to *M. tuberculosis* MscL. The left panel shows the uninduced control, and the right panel shows growth in the presence of isopropyl-1-thio-β-D-galactopyranoside (IPTG). In both panels samples were plated (left to right) from high concentration to low concentration. A GOF phenotype is observed for L17Y, V21A, N44D, and N95D. No difference from wild type growth is seen for A20E, A20R, and T28R.

FIG. 5. Cross-linking of the R45K/Q51E mutant of *M. tuberculosis* MscL. Purified R45K/Q51E *M. tuberculosis* MscL and wild type protein were cross-linked for 2 h at 4°C using EDC, DCC, EDC with sulfo-NHS, and DCC with NHS. The reactions were quenched with β-mercaptoethanol, run on a 4–15% SDS-polyacrylamide gel, and visualized by Western blotting with His antibody.
production of Tb-MscL protein for these mutants was confirmed by SDS-PAGE analysis. The lack of GOF phenotype for the A20E and A20R mutants is particularly surprising in light of recent work (14), which shows that all charged residues at this site give a very severe GOF phenotype in Eco-MscL. In fact, only Ala and Gly at these sites produce Eco-MscL with normal function (12).

**Tb-MscL Loop Intersubunit Hydrogen Bond—**Previous studies of mutations at Gly46 in Eco-MscL showed the GOF phenotype (11). We have seen similar behavior at the aligned Asn44 site in Tb-MscL. On examining the Tbs-MscL crystal structure, we observed an inter-subunit hydrogen bond involving the adjacent Arg45 site, with Gln51 serving as the partner (Fig. 1B). This significant intersubunit interaction suggested an interesting starting point to explore loop function.

Initially, the intersubunit hydrogen bond in the crystal structure was mutated to cross-linkable residues to examine whether these residues are in close proximity under more physiological conditions. The subtle mutation of R45K to Gln51 converts the hydrogen bond to a salt bridge. This should still be a favorable intersubunit contact, but the mutant is now susceptible to cross-linking by peptide bond-forming reagents. After overexpression and purification, the protein was exposed to a variety of cross-linking reagents and activators for 2 h at 4 °C. All reagents showed at least a weak pentameric band in the mutant with slight background cross-linking in wild type (Fig. 5). The background cross-linking is most likely due to cross-linking in the carboxyl terminus of the protein, which contains a number of glutamates, aspartates, and lysines.

The most interesting cross-linking results were seen with EDC and Sulfo-NHS. This combination gives mainly pentamer and tetramer for cross-linked products. The strong pentameric band in this designed system provides the best evidence to date that Tb-MscL is pentameric under physiological conditions. Under some conditions, a weak but is too weak to be seen as the intensity progressively falls off overexpression and purification, the protein was exposed to a variety of cross-linking reagents and activators for 2 h at 4 °C. All reagents showed at least a weak pentameric band in the mutant with slight background cross-linking in wild type (Fig. 5). The background cross-linking is most likely due to cross-linking in the carboxyl terminus of the protein, which contains a number of glutamates, aspartates, and lysines.

The most interesting cross-linking results were seen with EDC and Sulfo-NHS. This combination gives mainly pentamer and tetramer for cross-linked products. The strong pentameric band in this designed system provides the best evidence to date that Tb-MscL is pentameric under physiological conditions. Other cross-linking studies typically show progressively weaker band intensities on going from monomer to dimer to trimer, etc., analogous to our results with just EDC and other non-optimal conditions (Fig. 5) (24–26). Such observations allow open the possibility that a hexamer band is present and tetramer bands are seen, but the hexamer band is visible. This provides compelling evidence that no significant fraction of Tb-MscL is present in hexameric (or higher oligomerization) states when reconstituted in DDM micelles.

After confirming that the residues Arg45 and Gln51 are within interaction distance, we performed growth studies on both single and double mutants to examine channel function. All single mutants (R45K, Q51E, R45C, and Q51C) and all double mutants (R45K/Q51E, R45E/Q51E, and R45C/Q51C) except R45K/Q51K displayed a GOF phenotype (Table II). The lack of a GOF phenotype for the R45K/Q51K mutant is surprising and merits further study. Nevertheless, this region appears to be quite mutationally sensitive. Note that the R45K mutation is subtle, suggesting that the loop plays a central role in channel gating. Very recently it has been shown that proteolytic cleavage of the loop significantly alters channel gating (16), supporting our view of a critical functional role for this region.

By using the alignment of Fig. 2, there is no obvious Eco-MscL analogue to the Arg45-Gln51 hydrogen bond seen in Tb-MscL. Technically, the alignment is Leu47/Asp53 (Eco-MscL numbering), which is not a favorable interaction. There is no cationic or hydrogen bond donating residue near Leu47 that could pair with Asp53. However, residues on either side of Asp53 are hydrophobic, suggesting that perhaps the ion pair of Tb-MscL is replaced by a hydrophobic contact such as Leu7/Ile12 or Leu7/Thr14 in Eco-MscL. It would be interesting to investigate this possibility.

These studies suggest that although *M. tuberculosis* and *E. coli* MscL are similar, there are important differences. Thus, caution should be exercised when employing the Tb-MscL crystal structure to explain functional results for Eco-MscL. Most strikingly, mutations at Ala20 in Tb-MscL do not exhibit a GOF phenotype, despite the extreme sensitivity of the aligned Gly22 in *E. coli*. Additionally, the loop region of Tb-MscL appears highly sensitive to mutations, suggesting that the loop region in general and the Arg45-Gln51 intersubunit hydrogen bond in particular, merit further investigation.

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