Chapter 5: The Carboxyl Terminus of the *M. tuberculosis* Mechanosensitive Channel of Large Conductance (MscL)

5.1 Introduction

The intercellular regulation of eukaryotic ion channels is a well-established phenomenon and has been observed for sodium channels (Hanlon and Wallace, 2002; Trimmer, 1998; Volk et al., 2001), potassium channels (Hanlon and Wallace, 2002; Trimmer, 1998; Vanoye et al., 2002), calcium channels (Hanlon and Wallace, 2002; Sandoz et al., 2001; Trimmer, 1998), and P2X receptors (Denlinger et al., 2001). Regulation of these channels is achieved in two fashions: through interaction of terminal regions of the channel (typically the carboxyl terminus) with an intracellular β-subunit, or through direct carboxyl terminal modulation of the channel. Interestingly, the carboxyl terminal region of TREK-1, a mechano-gated mammalian two pore potassium channel, modulates channel response to bilayer tension (Maingret et al., 1999; Maingret et al., 2000). In particular, deletion of the TREK-1 carboxyl terminus greatly decreases the response of the channel to tension.

The cytoplasmic carboxyl terminal region of the mechanosensitve channel of large conductance (MscL) is generally considered to be unimportant for channel function. Carboxyl terminal deletion studies of the *E. coli* MscL homologue (Ec-MscL), the best studied MscL isoform, showed that extensive portions of this region could be removed without altering channel function (Blount et al., 1996; Hase et al., 1997). As well, extensive mutagenesis studies, including random mutagenesis, have uncovered no functionally interesting mutants in the Ec-MscL carboxyl terminal region (Blount et al., 1997; Blount et al., 1996; Ou et al., 1998). Recently developed gating models for Ec-

MscL and *M. tuberculosis* (Tb) MscL proposed no functional role for the carboxyl terminal region of either channel (Sukharev et al., 2001a; Sukharev et al., 2001b).

However, it is unclear that carboxyl terminal results are fully generalizable between Ec-MscL and Tb-MscL. Although the overall alignment between the channels is relatively high, an early sequence analylsis by our group indicated that their carboxyl terminal regions showed little homology (Maurer et al., 2000). As well, the Ec and Tb-MscL mutagenic profiles are not identical even in regions of high homology, such as the transmembrane domains (Maurer et al., 2000).

The carboxyl terminal region in the Tb-MscL high resolution crystal structure has two intriguing features (Figure 5.1) (Chang et al., 1998). First, the 33 terminal residues are missing, since insufficient electron density was collected to build chain traces for those amino acids. Second, the portion of the Tb-MscL carboxyl terminus that is visible in the crystal structure forms a highly charged pentameric helical bundle. Computational work has implied that if all ionizable residues are charged, the helical bundle would be electrostatically unstable (Elmore and Dougherty, 2001). As well, these computations predicted that charge neutralization might allow the formation of a helical bundle. TASP studies have shown that the formation of helical domains in the Tb-MscL carboxyl terminal region may be promoted by entropic restraints imposed by the overall assembly of the channel (Kochendoerfer et al., 2002). Nonetheless, the TASP studies do not highlight which portions of the region are involved in this helical structure. Thus, both

the structure and functional relevance of the Tb-MscL C-terminal region remain ambiguous.

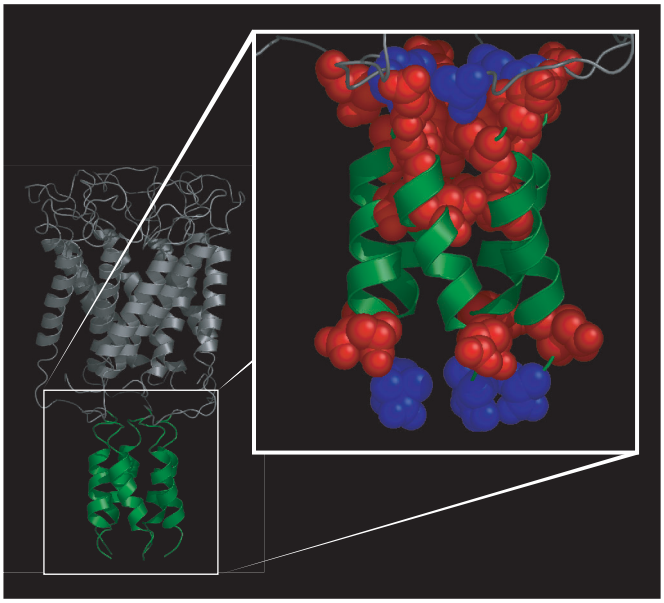

Figure 5.1: The carboxyl terminal region of the Tb-MscL crystal structure is highlighted, with all ionizable residues shown in spacefilling. Basic residues are depicted in blue and acidic residues are depicted in red.

Here we investigate the Tb-MscL carboxyl terminal region through a combination of site-directed mutagenesis, *in vivo* functional assays, and circular dichroism (CD) measurements. The utility of such *in vivo* assays in efficiently determining overall changes in MscL function upon mutagenesis has been well-established (Blount et al., 1997; Blount et al., 1996; Ou et al., 1998). In particular, mutations that reduce the tension threshold of channel gating show greatly decreased bacterial viability. These mutations commonly have been termed gain of function (GOF). Functional analysis of point mutations, in conjuction with CD measures of structural stability, establishes that

the Tb-MscL carboxyl terminal region plays an integral role in both channel structure and function.

5.2 Results

5.2.1 Growth Analysis

5.2.1.1 Deletion studies

Previous studies have shown that the carboxyl terminus of Ec-MscL can be deleted without affecting gating tension of the channel and with only minor effects on channel kinetics (Blount et al., 1996; Hase et al., 1997). However, the substantial sequence differences between MscL homologues in this region led us to investigate the carboxyl terminus of Tb-MscL (Maurer et al., 2000). Strikingly, deletion of the carboxyl terminus of Tb-MscL significantly affects channel function. Severe gain of function muations were observed upon deletion of the Tb-MscL carboxyl terminus region. The deletions and their pheotypical characterization are shown in Figure 5.2.

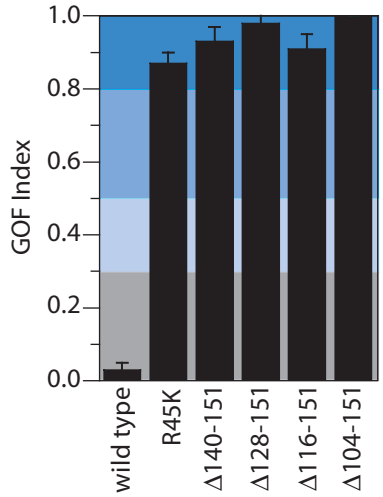

Figure 5.2: GOF index for carboxyl terminal deletion mutants of Tb-MscL.

Since the gross structural changes resulting from carboxyl terminus deletions induced hyperactivity, the effects of more subtle mutations were investigated. Initial studies focused on the negatively charged residues, since previous work had pointed to the significant charge concentration of this region (Elmore and Dougherty, 2001). Charge neutralization was undertaken by substitution of negatively charged amino acids with their neutral isosteric amide analogs; glutamic acid was replaced with glutamine and aspartic acid was replaced with asparagine. In most cases, these mutations resulted in channels that exhibited a GOF phenotype (Figure 5.3).

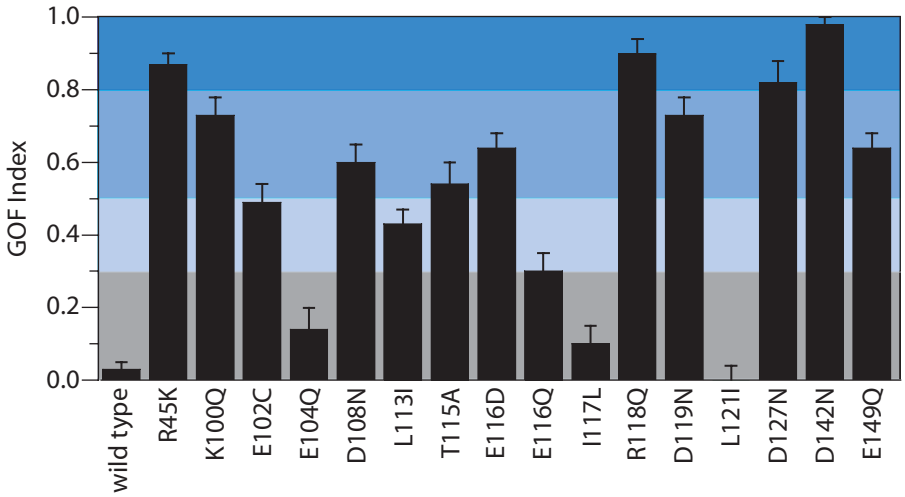

Figure 5.3: GOF index for single-site mutations in the carboxyl terminal region of Tb-MscL.

Following initial neutralization studies, mutation of some positive and neutral residues in this region was undertaken to explore the mutagenic landscape of the carboxyl terminus. Positive residues were mutated to glutamine, since this is the natural polar amino acid closest in volume. Mutations of leucine to isoleucine and isoleucine to leucine were performed as negative controls. In addition all residues of the almost universally

conserved TIERD domain that we previously identified were mutated (Maurer et al., 2000).

5.2.1.2 Ec-MscL Single-Site Mutations.

Analogous mutations to the negatively charged Tb-MscL single-site mutations were made in Ec-MscL as a control. Sites which were neutralized were Glu 107, Glu 108, Glu 118, Glu 119, Glu 124, Asp 127, and Glu 131. All of these muations showed phenotypically wild type behavior, as expected from the previous cleavage studies of the Ec-MscL carboxyl terminus.(Blount et al., 1996; Hase et al., 1997) The data are summarized in Figure 5.4.

5.2.2 Circular Dichroism Studies

Thermal denaturation was used to investigate if the Tb-MscL carboxyl terminus mutants caused structural perturbations in addition to inducing functional changes. Denaturation was monitored by the circular dichroism ellipticity at 220 nm. The denaturation curve for wild type Tb-MscL shows a single melting transition (T_m) around 60°C (Figure 5.5). A reduction of 35% in the CD signal is observed between 25°C to 95°C. Thus, significant helicity remains in the sample even after the observed transition, which most likely corresponds to the transmembrane domains. After melting, Tb-MscL regains the majority (>90%) of it original elipticity upon returning to 25°C.

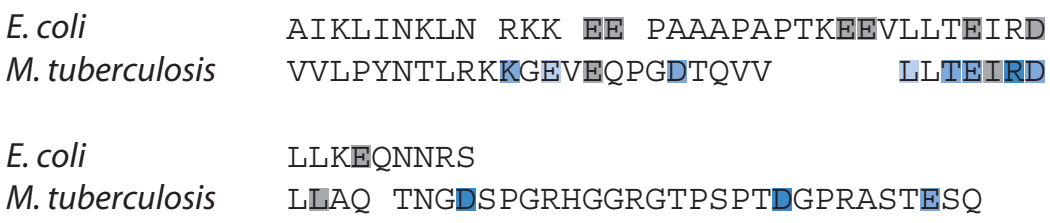

Figure 5.4: Sequence alignment of the carboxyl terminal regions of Tb-MscL and Ec-MscL. Sites at which mutation resulted in a GOF phenotype are shown in blue. The intensity of blue coloring corresponds to the GOF plots, with light blue signifying a slight GOF, medium blue indicating moderate GOF, and dark blue indicating major GOF. Sites at which mutations were phenotypically wild type channel are shown in gray.

The denaturation curves for the Tb-MscL carboxyl terminus mutants were qualitatively similar to that of wild type. However, changes in T_m arose for some mutants (Figure 5.5). In particular, major shifts in T_m to lower temperatures were noted for E116D and D142N. Conversely, the T_m shifted moderately to a higher temperature for D127N, and to slightly higher temperatures for E102C, E104Q, and D149N. Other carboxyl terminus single-point mutations showed no significant deviation in T_m from wild type Tb-MscL.

5.3 Discussion

5.3.1 Tb-MscL and Ec-MscL Are Different

Significant differences exist between the MscL homologues of *E. coli* and *M. tuberculosis*. The deletion profiles of the carboxyl terminal tail of these homologues are massively different. Additionally, we have shown that analogous single-point mutations in the carboxyl terminal region result in dramatically different phenotypic profiles for the two channels. Previously, similar results were obtained for mutations in the

transmembrane helices (Maurer et al., 2000). Recently, gating models have been proposed for both Tb-MscL and Ec-MscL by Sukharev and Guy (Sukharev et al., 2001a; Sukharev et al., 2001b.) These models do not reflect the observed differences in either the carboxyl termini or the transmembrane helices.

Additionally, these data clearly show that the Tb-MscL carboxyl terminal region plays an integral role in gating. Conversely, our controls in Ec-MscL confirm the previous observations that its carboxyl terminal region is not functionally relevant (Blount et al., 1996; Hase et al., 1997). The Sukharev/Guy model for Tb-MscL gating assigns no role to the carboxyl terminus and only includes residues 1-122. Clearly modifications of the current Tb-MscL gating model are needed to incorporate the entire carboxyl terminus and explain its functional role.

5.3.2 Structure-Function Studies of Tb-MscL

The combination of functional data with measures of structural stability for multiple mutations provides a unique opportunity to consider channel structure-function. In this study, some point mutations that exhibit an altered phenotype also exhibit changes in structural stability, however a correlation between altered phenotypic behavior and changes in structural stability is not always observed. This implies that altered phenotypic behavior does not result from the same structural phenomenon for all the mutations studied.

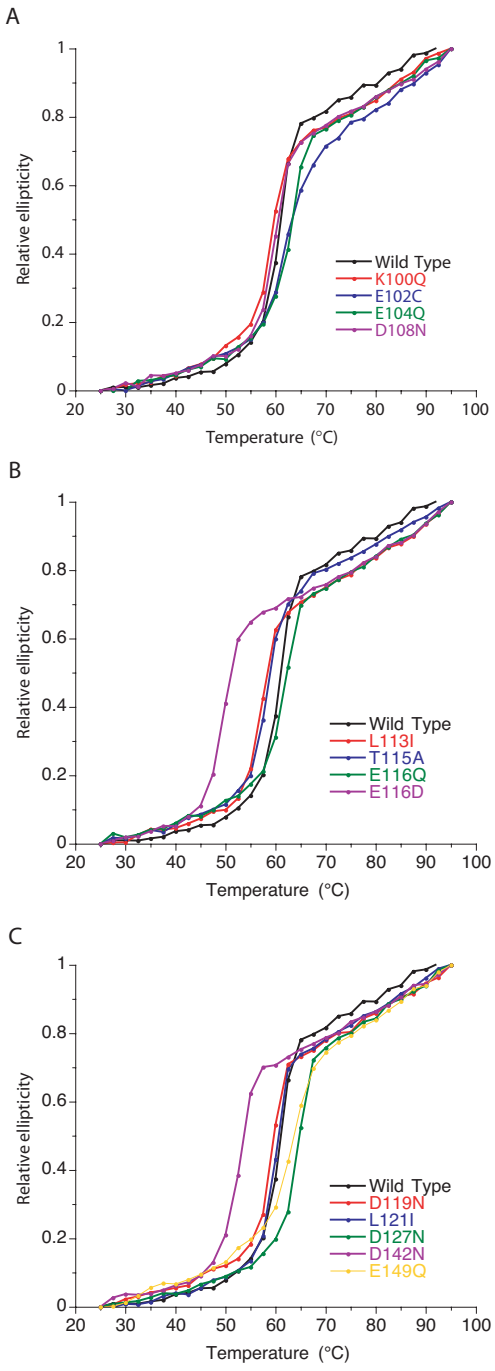

Figure 5.5: Circular dichroism thermal denaturation curves are shown for wild type Tb-MscL and a series of single-site carboxyl terminal mutations. A) Mutations in the upper portion of the crystal structure carboxyl terminal region. B) Mutations in the lower portion of the crystal structure carboxyl terminal region. C) Mutations to residues not in the crystal structure.

5.3.2.1 Upper portion of the crystal structure carboxyl terminal helix.

Only minor changes in structural stability were observed upon mutation of this region of the carboxyl terminus. However, significant functional differences are observed for mutations to this region. Most notably, Glu 104 and Asp 108 exhibit significant differences upon charge neutralization. This implies that these residues have different structural roles. However, the crystal structure proposes a similar enviroment for these residues, with both pointing toward the center of the carboxyl terminal bundle (Chang et al., 1998). Taken together, these data suggest that residues 104 and 108 may adopt slightly different conformations *in vivo* than those observed in the crystal structure.

Electrophysiological analysis of E104Q suggests that it has a lower gating tension threshold than wild type Tb-MscL. (Shapovalov, G., Bass, R., Rees, D. C., Lester, H. A., submitted.) However, one would not expect E104Q to exhibit a gain of function phenotype in our assay because its reduced gating tension is still similar to that observed for Ec-MscL. This confirms that mutations characterized as GOF in this study represent severely hyperactive MscL channels.

5.3.2.2 Lower portion of the crystal structure carboxyl terminal helix.

Mutations to all of the charged and polar residues in this region have significant functional consequences. However, changes in structural stability are only observed for E116D.

At position Glu 116 both structural and functional data were intriguing. Neutralization of Glu 116 by mutation to glutamine had no apparent effect on channel structure or

function. In striking contrast, shortening the carboxylic acid side chain by one methylene unit resulted in dramatic changes in both structural stability and channel function. Thus, residue 116 plays an important steric role in the carboxyl terminus.

Additionally, since the glutamic acid at position 116 can be mutated to the corresponding amide, this suggests that residue 116 either has a shifted pKa or that the negative charge is not important at this position. Position 108 may also exhibit a shifted pKa, since it is the only other residue in this region not to show a phenotypic effect upon charge neutralization.

Suprisingly, although no gross structural differences were observed, the apparently subtle L113I mutation shows a slight GOF phenotype. This may be explained by the lower helical propensity of isoleucine compared to leucine. As expected, the I117L mutation showed no effect.

5.3.2.3 Residues outside the crystal structure.

The structural and functional role of the Tb-MscL carboxyl terminal region is not limited to residues in the crystal structure. Mutation of residues missing from the Tb-MscL crystal structure produced channels with significant gain of function phenotypes. Specifically, the neutralization of any negatively charged residue in this region (Asp 119, Asp 127, Asp 142, or Glu 149) resulted in altered channel function. This was particularly remarkable for E149Q, which is only two residues from the end of the Tb-MscL protein.

Significant structural differences were also observed for mutations in this region. Most notably, D142N, which is only nine residues from the protein terminus, exhibited a massive shift in T_m to lower temperatures. Slight shifts to higher melting temperatures were observed for D127N and E149Q.

5.3.2.4 Speculation on the role of the Tb-MscL carboxyl terminal region.

Clearly, the Tb-MscL carboxyl terminal region plays an important role in gating. Our current data allow us to begin to create a crude working model of this region of the channel. Previous modeling has proposed a generally helical structure for the carboxyl terminus of many MscL homologues, including coiled coil regions for some species (Hamill and Martinac, 2001; Sukharev et al., 2001b). As well, TASP studies have implied that the assembled Tb-MscL carboxyl terminal region is highly helical (Kochendoerfer et al., 2002). This agrees with our observation that mutations to the carboxyl terminal region affect the thermal stability of a helical domain.

Mapping functional and structural stability data onto a helical wheel gives different results (Figure 5.6). Although the functional data show little discernable helical dependence, the structural stability data are consistent with a helical model of this region. In particular, all mutations that showed shifts in the thermal denaturation profile lie on one face of the helix.

However, we are not inclined to believe that the carboxyl terminus forms a long extended helix. This is because two different mutations separated by 25 residues both significantly

lower the melting temperature of the same transition. It seems unlikely that changing one residue on a long extended helical structure would cause such a notable change in stability, which we observe at two relatively distant sites. As well, it is less likely that helix stability would be severly compromised by single residue changes if the carboxyl terminal region primarily interacts with the membrane. Instead, it seems more reasonable that such mutations would be affecting interactions essential to the stability of a structural domain.

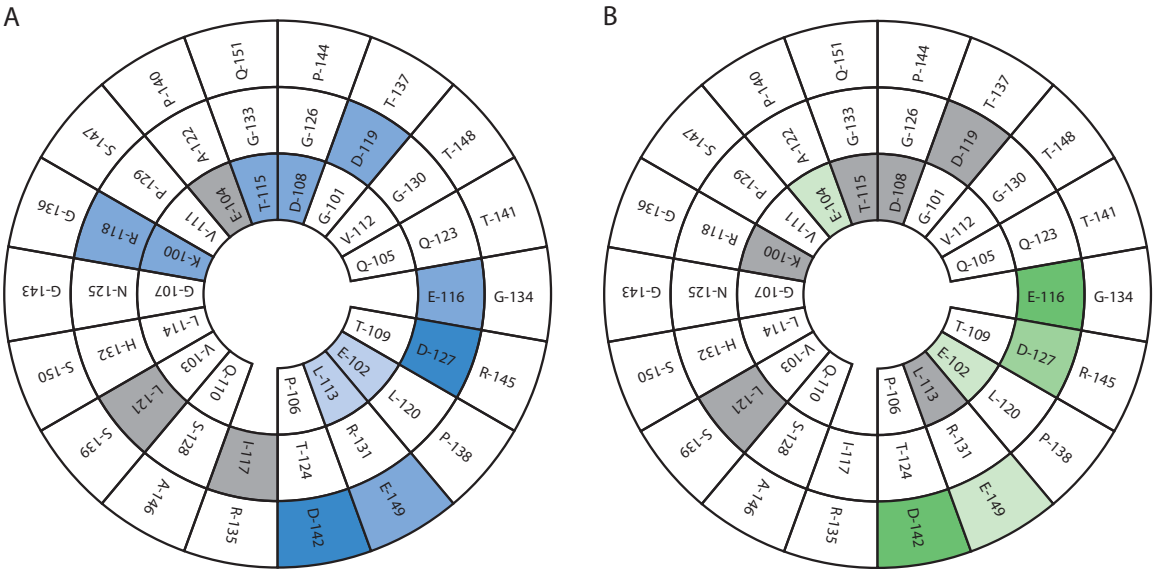

Figure 5.6: A) Helical wheel showing observed GOF phenotype for mutations in the carboxyl terminal region of Tb-MscL. Coloring corresponds to Figure 2. B) Helical wheel showing observed shifts in thermal stability for mutations in the carboxyl terminal region of Tb-MscL. Gray coloring indicates no shift, light green indicates a slight shift, medium green indicates a moderate shift, and dark green indicates a major shift.

Thus, we speculate that the carboxyl terminal region folds into a distinct subdomain such that the distal region interacts with the cytoplasmic helix visible in the crystal structure. A cartoon of such an interaction is shown in Figure 5.7. In this cartoon, the distal portion of

the carboxyl terminal region is depicted as an α-helix, based on the previously implied helicity of this region and the correspondence of our structural stability data to a helical model. However, it is quite possible that this portion of the structure adopts another well-defined conformation. In fact, secondary structure prediction programs, such as Jpred, only predict α-helicity for the upper portion of the carboxyl terminus.(Cuff et al., 1998) The high density of proline and glycine residues between sites 116 and 142 (32% of residues) would allow for the conformational arrangement needed for interactions between regions separated in sequence space. These residues may also imbue the region with the flexibility necessary for it to play an active role in the gating process. High-resolution electrophysiology on wild type and mutant channels may be extremely helpful in further refining the role this region plays during gating. For example, these techniques may be able to distinguish whether conformational changes in this region occur early or late in the gating process.

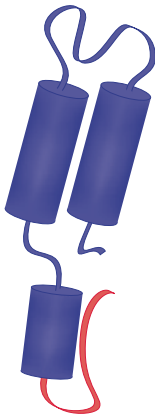

Figure 5.7: A cartoon depicted one possible conformation of a Tb-MscL single subunit. The residues in the crystal structure are shown in blue and the carboxyl terminus, which could not be resolved in the crystal structure, is shown in red.

5.3.2.5 Analogies to other ion-channel regulatory domains.

The carboxyl terminal domain of Tb-MscL plays a similar role to regulatory domains found in other eukaryotic ion channels and transporters. Similar carboxyl terminal regulation has been observed in TREK-1, a mammalian mechanosenstive ion-channel, despite low sequence homology between MscL and TREK-1 (Maingret et al., 1999; Maingret et al., 2000). This implies that perhaps carboxyl terminal regions commonly serve as regulatory domains in mechanosensitve channels.

Regulatory domains in bacterial ion channels provide useful models for understanding general aspects of eukaryotic ion-channel regulation. Intracellular regulation is common for eukaryotic ion-channels. For example, the Shaker B potassium channel exhibits amino terminus regulation, where cleavage of the regulatory domain results in a hyperactive channel (England et al., 1997; MacKinnon et al., 1993). Since bacterial channels can be easily modified and produced in very large quantities, their regulatory domains are amenable to a vast array of biochemical and biophysical techniques that are generally inaccessible to eukaryotic ion channels.

5.4 Conclusions

Here, we have further elucidated differences between homologues of the mechanosensitive channel of large conductance from *M. tuberculosis* and *E. coli*. In particular, the carboxyl terminus of Tb-MscL is an important regulatory domain, while this region has essentially no functional role in Ec-MscL. It is interesting to note that even the very end of the Tb-MscL carboxyl terminus appears critical for normal channel

regulation. This motif of intracellular regulation seems common between prokaryotic and eukaryotic ion channels.

Previously developed gating models for Tb-MscL and Ec-MscL assign no role to the carboxyl terminal domain of the channel (Sukharev et al., 2001a; Sukharev et al., 2001b). Our results support this assertion for Ec-MscL. However, these results clearly indicate that the carboxyl terminus plays a large role in Tb-MscL gating, which needs to be incorporated into future gating models.

The Tb-MscL carboxyl terminal region has provided a unique opportunity to obtain structural and functional data on a wide range of site-directed mutants of an ion channel. Currently, these sorts of studies are only feasible for bacterial ion channels, because unlike eukaryotic channels they can routinely be expressed on the scale required for many biochemical and biophysical studies.

5.5 Materials and Methods

5.5.1 *Plasmids and Strains.*

As previously described, Tb-MscL was encoded in the pET 19b vector; growth studies and protein expression were performed in MscL null *E. coli* BL21 (Chang et al., 1998; Maurer et al., 2000). Ec-MscL constructs were in the pB10b vector (Blount et al., 1996), and assays of Ec-MscL mutant function utilized the MJF465 *E. coli* strain, which is MscL, MscS, and KefA null (Levina et al., 1999). Both the pET 19b and PB10b vectors provide ampilicin resistance and IPTG control over protein expression. Site-directed

mutagenesis of both channels was performed using the QuikChange protocol (Stratagene, La Jolla, CA); all mutants were verified by sequencing and enzymatic digestion.

5.5.2 Bacterial Growth Studies.

Ec-MscL phenotypical characterization was carried out as previously described (Maurer and Dougherty, 2001). Bacterial growth studies for Tb-MscL were performed as previously described with slight modifications as noted below (Maurer et al., 2000). Frozen permastocks of Tb-MscL or Tb-MscL mutations were used to inoculate 2 mL cultures and the cultures were grown with shaking at 37˚C for 14 hours in LB media with 100 μg/mL ampicillin. The cultures were then diluted 1:10 in fresh LB media with ampicillin and grown for 1 h. Serial dilutions were performed to give dilutions of 1:10 through 1:1,000,000 in LB with ampicillin. The 1:1,000, 1:10,000, 1:100,000, and 1:1,000,000 dilutions were spotted (5 μL) onto 12-well LB agar plates with ampicillin and with or without 1 mM IPTG. The plates were grown for 20 h at 37˚C and scored as previously described. The reported GOF index was calculated by subtracting the ratio of the induced score to uninduced score from 1.

5.5.3 Protein Expression and Purification.

Frozen permastocks of Tb-MscL or Tb-MscL mutations were used to inoculate 25 mL of LB media with 100 μg/mL ampicillin. The cultures were grown overnight at 37˚C with shaking. LB cultures were used to inoculate 500 mL of TB media with ampicillin. The resulting TB cultures were grown to mid-log phase and then induced with 1 mM IPTG. After induction, the cultures were grown for an additional 2 hours at 37˚C or in some

cases for 3 hours at 30˚C. Bacteria were then pelleted, and resuspended in 50 mM Tris / 75 mM NaCl / 1% β-dodecyl maltoside (DDM) at pH 7.5 (10 mL) with Complete protease inhibitor cocktail (Roche, Indianapolis, IN). The suspensions were then probe sonicated (4 x 30 Sec.) and incubated with shaking for 1 hour at 37˚C. Following incubation, the suspensions were pelleted at 45,000 x g for 45 minutes and the resulting supernate was passed through a 0.2 μm filter. Difficulties were encountered when producing L117I and R118Q. These proteins showed extensive proteolysis of the carboxyl terminal tail that was not observed for other proteins.

All chromatography steps were carried out on a Vision Workstation (Applied Biosystems, Foster City, CA). The clarified supernate was subjected to two dimensional chromatography, using a POROS metal chelate affinity column (Applied Biosystems, Foster City, CA) charged with cobalt chloride in thebfirst dimension and a POROS HQ anion exchange column (Applied Biosystems, Foster City, CA) in the second dimension. Elution from the metal chelate column was achieved using an imidazole gradient (0.25 mM - 1 M at pH 7.5) with 0.05% DDM and elution from the HQ column was achieved using a sodium chloride gradient (0 mM – 1 M) in the presense of 10 mM Tris at pH 7.5 and 0.05% DDM. Buffer exchange and desalting were then performed using a HiPrep 26/10 desalting column (Amersham Pharmacia Biotech, Piscataway, NJ) charged with 1 mM phosphate buffer and 0.05% DDM at pH 7.2.

5.5.4 Circular Dichroism Thermal Denaturation.

All thermal denaturation was performed using an Aviv 62 DS circular dichroism spectrophotometer (Aviv Instruments, Lakewood, NJ). Purified Tb-MscL protein samples were diluted to approximately identical concentrations in 1 mM phosphate buffer (pH 7.2) for thermal denaturation. Circular dichroism scans (260-195 nm) showed equivalent helical propensities between samples. The denaturation consisted of heating from 25°C to 95°C, stepping every 2.5°C, and allowing the sample to equilibrate at each temperature for 5 min. After equilibration, the ellipticity of samples at 220 nm was measured, averaging the signal over 30 s.

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