Correlating a Protein Structure with Function of a Bacterial Mechanosensitive Channel

MscL, a mechanosensitive channel found in many bacteria, protects cells from hypotonic shock by reducing intracellular pressure through release of cytoplasmic osmolytes. First isolated from Escherichia coli, this protein has served as a model for how a protein senses and responds to membrane tension. Recently the structure of a functionally uncharacterized MscL homologue from Mycobacterium tuberculosis was solved by x-ray diffraction to a resolution of 3.5 Å. Here we demonstrate that the protein forms a functional MscL-like mechanosensitive channel in E. coli membranes and azolitcin proteoliposomes. Furthermore, we show that M. tuberculosis MscL crystals, when re-solubilized and reconstituted, yield wild-type channel currents in patch clamp, demonstrating that the protein does not irreversibly change conformation upon crystallization. Finally, we apply functional clues acquired from the E. coli MscL to the M. tuberculosis channel and show a mechanistic correlation between these channels. However, the inability of the M. tuberculosis channel to gate at physiological membrane tensions, demonstrated by in vivo E. coli expression and in vitro reconstitution, suggests that the membrane environment or other additional factors influence the gating of this channel.

Mechanosensation, the process of detecting a mechanical stimulus, is integral to a vast array of sensory systems. These sensory systems encompass a span from hearing to proprioception to osmoregulation, yet are among the least understood at the molecular level. The advent of patch clamp analysis revolutionized the study of mechanosensitive systems. Electrophysiological evidence quickly implicated mechanosensitive (MS) channels as the primary transducing element in these sensory cascades (1, 2). Soon, MS channel activities had been detected in more than 30 cell types (3) including embryonic chick skeletal muscle cells and frog muscle, where they were first discovered (4, 5).

These systems have remained veiled because of the very low abundance of the channels or the tissues that bear them. In addition, the lack of tangible ligands for MS channels has hampered efforts to enrich a population by biochemical means. This veil began to lift when MS channel activities were detected in bacteria. Early studies in Escherichia coli giant spheroplasts (6) showed MS conductances, and soon similar activities were detected in giant protoplasts of Bacillus subtilis (7) and Streptococcus faecalis (8). At least three MS activities are now recognized in E. coli: MscL (mechanosensitive channel large conductance), MscS (Msc small), and MscM (Msc mini) (9). Several studies support a role for these bacterial MS channels in osmoregulation, serving as “emergency relief valves” in response to acute hypotonic shock (10–12).

Previously it was demonstrated that a 136-amino acid protein from E. coli formed a homo-multimeric membrane-bound complex that correlated with the large membrane conductance (13–15). This protein, MscL, became the first MS channel to be cloned and subjected to molecular dissection, presenting a simple, accessible system to assess how tension in a lipid membrane can effect the gating of an MS channel (16–24).

A great stride forward in our understanding of the MS phenomenon was made as the crystal structure of a putative MscL homologue from Mycobacterium tuberculosis (Tb-MscL) was solved to 3.5 Å (25). This structure agreed with earlier data in many important aspects (24). Most importantly, the homomultimer was composed of subunits bearing two transmembrane elements, with both termini residing within the cytoplasm (13).

Unfortunately, this homologue had not been shown to encode an MS channel activity. Hence, it was unclear how much of the knowledge gained about the functionally relevant residues and domains of the E. coli model system would be applicable to the structure derived from this putative orthologue.

Here we show by patch clamp analysis that the M. tuberculosis gene does indeed encode an MS channel activity. Furthermore, some analogous mutations in the M. tuberculosis channel lead to perturbation of channel gating as seen in the E. coli channel, strongly suggesting that these orthologues share a common molecular mechanism for detecting and responding to membrane tension. However, the insensitivity of the wild-type M. tuberculosis channel to membrane tension in the physiological range, when expressed in heterologous systems, suggests differences in environmental factors that normally contribute to the gating of this homologue.

EXPERIMENTAL PROCEDURES

Stocks and Cultures—All mscL genes initially were ligated into the multiple cloning site of the vector pBluescript II (Stratagene, La Jolla, CA) for sequence verification and amplification and subsequently moved to the expression vector pBl10 (26). The mscL-null E. coli strain PB104 (13) was used to host the intermediate cloning steps and the expression constructs. For whole-cell physiology experiments, E. coli strains Frag1 (27) and its derivative MJF455 (∆mscL::Ωm, ΔyggB) (12), were utilized. Cultures were grown at 37 °C in Lennox Broth (LB) with shaking at 250 rpm. For plasmid-bearing strains, ampicillin (100 µg/ml) was added, and expression was induced by addition of IPTG (1 mM) to mid-logarithmic phase cultures. Induced expression times were 1 h for in vivo experiments and 2 h for single channel analysis experiments.
Growth curves were generated from cultures of E. coli MJF455 expressing the MscL protein in trans. Cultures were inoculated from a single colony to 15 ml of LB plus ampicillin and IPTG and grown as above for the duration of the experiment.

### Gene Cloning and Site-directed Mutagenesis of Tb-mscL

The cloned wild-type M. tuberculosis mscL gene was a gift from D. Rees (25). Manipulations by polymerase chain reaction used Pfu DNA polymerase (Stratagene) under standard reaction conditions (28), and sequences were confirmed by analysis of both strands. The published sequence data were used to design oligonucleotide primers that encompassed the open reading frame and the native Shine-Delgarno sequence. Linkers XhoI(5') and XhoI(3') were added to facilitate the directional cloning of the fragment into pb10b. The oligonucleotide primers used were: 5' linker, AGA TCT AGA TCT GCA GAA AGC ACA TCG CAT GCT CAA; and 3' linker CTC GAG TCT CCG GAG GCT ATT GCG ATT CTG TGG.

The construction of the amino-terminal, deca-histidine-tagged protein and crystallization procedure has been described previously (25).

Site-directed mutagenesis of Tb-mscL was accomplished by polymerase chain reaction using Pfu DNA polymerase in a modified megaprimer protocol (29). The mutating primers used are: A20G, GCC GAT AGG, and 3' linker, AGA TCT AGA TCT GCA GAA AGG ACA TCG CAT GCT CAA. Purified amino-terminal histidine-tagged Tb-MscL protein was incorporated into synthetic azolectin membranes as described previously (30), with modifications (16, 30, 31). Excised, inside-out patches were examined at room temperature under symmetrical conditions using a buffer composed of 200 mM KCl, 90 mM MgCl2, 10 mM CaCl2, and 5 mM HEPES adjusted to pH 6.0. Records were gathered at −20 mV for channel pressure response experiments and +30 mV to −50 mV for determination of the current-voltage relationship and conductance. Data were acquired at a sampling rate of 20 kHz with a 5 kHz filtration using an AxoPatch 200B amplifier in conjunction with Axoscope software (Axon).

### RESULTS

#### Expression of M. tuberculosis mscL Yields an MS Channel Activity

As shown in Fig. 1A, the Tb-MscL structural gene yields a mechanosensitive channel activity when expressed in E. coli spheroplasts; no such channel activity was observed in the absence of expressed protein. The relatively short open time and large conductance of this channel were similar to several other MscL orthologues (26). However, the large degree of membrane tension required for gating distinguishes this channel from all of the other characterized MscL channels. Quantitatively, Tb-MscL requires about twice the tension of several other MscL orthologues to activate these channels in azolectin membranes (B and C) as compared with spheroplast membranes (A). D, the current-voltage relationship of the Tb-MscL channel was determined from data obtained from reconstituted purified protein (B).

![Fig. 1. The M. tuberculosis mscL gene product encodes a mechanosensitive channel activity as assayed by patch-clamp. A, shown are traces from wild-type Tb-MscL, expressed in E. coli spheroplasts, examined in excised patches at −20 mV. The closed state is defined as c; openings of successive individual channels are designated o1, o2, etc. The activation pressure threshold (mm of Hg) is indicated above the traces. B, a representative trace of enriched polyhistidine-tagged Tb-MscL reconstituted into azolectin liposomes using standard techniques and clamped at +20 mV pipette. C, Tb-MscL crystals were dissolved in a low salt buffer containing 0.05% dodecyl-β-D-maltoside and reconstituted into azolectin membranes and examined by patch clamp at +20 mV pipette. Note that lower pressures are required to activate these channels in azolectin membranes (B and C) as compared with spheroplast membranes (A). D, the current-voltage relationship of the Tb-MscL channel was determined from data obtained from reconstituted purified protein (B).](image)

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*P. C. Moe and P. Blount unpublished results.*
An in Vivo Assay of MS Channel Function Corroborates Electrophysiological Evidence—To test the functional nature of the Tb-MscL channel, an assay was designed to characterize the ability of expressed MS channels to rescue an acute hypotonic shock. This assay used the ability of expressed MS channels to rescue in vivo MscL channels. To test the functional nature of the Tb-MscL channel, it was demonstrated that the Tb-MscL channel is able to gate and effect a rescue. However, an alternative explanation would be that homologues from other species are inherently unable to suppress the lysis phenotype. We therefore performed this experiment using an MscL orthologue from B. subtilis (26) and supplemental data showing an alignment of the three MscL proteins used in this study. The MscL channel of B. subtilis (Bs-MscL), when assayed by patch clamp, was shown to have properties very similar to the E. coli MscL including the amount of tension required to gate the channel (26). When tested by this in vivo assay, the Bs-MscL channel was found to suppress lysis similar to Eco-MscL, demonstrating that an orthologue from a Gram-positive organism can substitute for the normal in vivo function of Eco-MscL (Table I). Hence, although some orthologues can functionally substitute as a conduit for solute efflux upon hypo-osmotic shock the Tb-MscL channel cannot, likely due to a significantly higher gating threshold.

Probing Tb-MscL Structure by Site-directed Mutation—Although the electrophysiological data suggested that the Tb-MscL is functionally related to the Eco-MscL, it was not clear if the two shared a deeper structural similarity. A common structure would imply a common mechanism; mutations that make the Tb-MscL channel more structurally similar to the Eco-MscL channel may also decrease its gating threshold to approach that of the E. coli channel. Furthermore, mutations that had been shown to affect the E. coli MscL (10, 16, 33) should yield analogous effects when applied to the Tb-MscL channel. Therefore, to mechanistically correlate the two channels, Eco-MscL and Tb-MscL, we generated several such site-directed mutations in Tb-MscL.

A previous random mutagenesis study identified several mutations within the Eco-MscL channel that, when expressed in E. coli, led to a slow or no growth gain-of-function phenotype and channels with lower gating thresholds in patch clamp (10, 16, 33). To determine if similar lesions would change the gating properties of the Tb-MscL, analogous mutations were generated at two of these sites. The first site chosen for substitution was Val-21, which correlates with the majority of MscS activity, suppressed this lysis (12). However, similar to observations made in E. coli spheroplasts, the reconstituted Tb-MscL channels still required more than twice the gating pressure seen for most other MscL orthologues when assayed by this technique. Although reconstituted Eco-MscL gates at less than 100 mm of Hg pipette pressure (13, 32), Tb-MscL typically requires ~200 mm Hg (Fig. 1).

To determine if the process used for crystallization of the Tb-MscL disrupted the native folding of the protein, crystals of the Tb-MscL were resolubilized, functionally reconstituted, and examined by patch clamp analysis. The electrophysiological behavior of these reconstituted channels was distinguishable from that of the purified polyhistidine-tagged Tb-MscL protein (Fig. 1C). These results indicate that the crystallization procedure does not irreversibly change the conformation of the Tb-MscL channel.

An Osmotic downshock survival and channel-gating threshold

| Strain   | Survivala (%) | Threshold (MscL/MscS)b (n) | Dwellc | τ2 | τ3 |
|----------|--------------|--------------------------|--------|----|----|
| Plasmid  | 0.07 ± 0.02*** | NA | NA | NA |
| Frag1    | 2.7 ± 0.3*** | 1.42 ± 0.03 | 8 | 28 |
| Eco-MscL | 2.8 ± 0.6*** | 1.42 ± 0.03 | 8 | 28 |
| Tb-MscL  | 0.3 ± 0.1 | 2.8 ± 0.35 | NA | NA |
| Be-MscL  | 3.2 ± 0.9*** | 1.39 ± 0.02 | 7 | 36 |
| A20G     | 0.08 ± 0.02*** | 2.68 ± 0.08 | 12 | ND |
| V21A     | 0.13 ± 0.03*** | 1.74 ± 0.05 | 9 | 55 |
| V21D     | 0.3 ± 0.2*** | 1.6 ± 0.03 | 8 | 7 |
| G24S     | 0.3 ± 0.1*** | 2.02 ± 0.02 | 12 | 3 |

a Shown, when significant, are the results from Student’s t test versus empty-plasmid control (#) and Student’s t test vs. wild-type Eco-MscL (*); significance levels are: * p < 0.05; ** p < 0.01; *** p < 0.001.

b Note that patch rupture/MscS = 2.73 ± 0.45 (S.D., n = 49).

b NA, not applicable; ND, not determined due to insufficient data.

d E. coli MscL electrophysiological data are from Blount et al. (16).

e Bs-MscL electrophysiological data are from (26).
analogous mutation (33), neither mutation led to a severe phenotype; the phenotype is primarily observed in high density cultures. In addition, as depicted in Fig. 4, when host viability (black bars) is compared with the channel sensitivity (gray bars), the Tb-MscL Val-21 mutants are also not competent for rescue of the host cell. However, the ability of these mutations to yield a more sensitive channel suggests that the Eco- and Tb-MscL share similar underlying molecular mechanisms for sensing and gating in response to membrane tension.

Another site chosen was glycine 24. A substitution of serine for glycine at the analogous site in *E. coli* MscL yielded a very severe GOF phenotype (33). Although, in the Tb-MscL channel, this mutation did reduce the gating threshold appreciably (Fig. 2, bottom; Table I), it did not perturb cell growth (Fig. 3). These results are corroborated by *in vitro* experiments that show that not only did expression of this mutant in *E. coli* not lead to a GOF phenotype but that this mutant was unable to rescue the MJF455 host strain from hypotonic shock (Fig. 4; Table I).

In a previous study, glycine 22 of the *E. coli* MscL was substituted with the full range of common amino acids (34).
That study found that placement of an alanine in this position led to a much higher gating threshold. Interestingly, the wild-type Tb-MscL contains an alanine at the corresponding position (Ala-20). Of the homologues that had been assayed previously for MS channel activity (26), only the MscL from Synechocystis contains an alanine rather than the highly conserved glycine at this site. The Synechocystis MscL is also the only other characterized homologue to have a gating threshold significantly higher than the rest of the orthologues (26). We therefore wanted to determine whether a single substitution of glycine for alanine at position 20 would reduce the gating threshold of the Tb-MscL channel. However, within the resolution of the assay, no substantial changes in channel sensitivity were observed (Table I). Furthermore, as seen in Fig. 4 and in agreement with the electrophysiological data, the A20G mutant was unable to rescue the host strain from hypotonic shock. Although the data suggest that the pressure sensitivity of the A20G mutant is not statistically different from that of the wild-type channel, the small population (n) of events observed for the wild-type channel reserves the possibility that the actual opening threshold is higher than that calculated. In fact, comparison of the wild-type Tb-MscL threshold with that of the A20G mutant shows that, at pressure levels at least 2.4 times the MscL threshold, Tb-MscL A20G activity was observed in 80% of successful patches as compared with only 15% for the wild-type channel. This would be possible if the events observed represent a sub-population of channels or channel openings that occurred at lower a membrane tension than the average. This is especially likely given that the gating threshold of these channels, particularly the wild-type, lies close to the average lytic limit of the membrane.

**DISCUSSION**

**Tb-MscL Encodes a MS Channel Activity Resembling the E. coli MscL**—Although the Eco-MscL channel has been studied for some time (6, 13, 15, 35, 36), only recently has the Tb-MscL channel emerged as a model system (25). Although the solved structure was consistent with models previously developed from biochemical, biophysical, and genetic clues (13, 18, 22), there was no evidence that this homologue actually encoded an MS channel activity. Such evidence was necessary before the Tb-MscL structure could be used to direct experiments aimed at the mechanism(s) of mechanosensitive channel gating. We therefore demonstrated functional channel activity for the Tb-MscL using a three-pronged approach. First, we patch-clamped *E. coli* spheroplasts expressing the native Tb-MscL in a heterologous expression system previously used for the study of other MscL orthologues (26). Second, we re-solubilized purified Tb-MscL protein into synthetic azolectin liposomes for patch clamp analysis. And finally, we re-solubilized Tb-MscL crystals and reconstituted the protein into azolectin liposomes for electrophysiological characterization. All three approaches unequivocally demonstrated that Tb-MscL is indeed a mechanosensitive channel. In addition, the latter observation demonstrates that the crystalization procedure does not irreversibly change the conformation of the Tb-MscL channel.

Although electrophysiological characterization of Tb-MscL in *E. coli* spheroplasts proved to be extremely difficult due to the very high pipette pressures required to gate this channel, channel events that were observed demonstrated that the channel is gated by membrane tension and has about the same conductance observed in other MscL orthologues (26). When reconstituted into liposomes, the inherent compliance of the azolectin bilayer allowed observation of extended channel records without patch rupture, nicely corroborating the sparse data gleaned from spheroplasts. Even in the liposome system, however, the gating pressures were ~2-fold higher than those required to gate other MscL orthologues that have been studied similarly (26).

**Live-cell Physiology Studies Implicate Environmental Factors in the Regulation of MS Channel Gating**—Recent works by several groups demonstrate that mechanosensitive channels play a role in osmoregulation by protecting the cell from acute hypotonic distress (11, 12, 24, 37). Here, we have exploited this vital cell system to develop an assay to canvass the ability of a given protein to protect the cell from hypotonic shock. This assay provides a rapid, efficient screen for MscL homologs and MS channels in general and permits the discrimination of apparent orthologues that may have broadly different properties.* in vivo.* Using a strain deficient in both of the predominant MS channels of the *E. coli* membrane, the majority of MscL, and totality of MscL activities, we have shown that expression of either *E. coli* or *B. subtilis* MscL will rescue these cells from acute hypotonic shock. Both of these channels have been shown previously to be very similar in both gating threshold and conductance (26). These results show that not only will re-expression of the native channel protect these cells but also that orthologues with similar properties, even from a Gram-positive organism, are competent to effect such a rescue.

In contrast, as is clearly apparent in Fig. 4, expression of the Tb-MscL channel is not sufficient to rescue these cells, presumably because the channel is not able to open before the cell envelope bursts. We know from electrophysiological evidence that the Tb-MscL channel is expressed and functional in the membrane of this strain; therefore, the observed phenotype is probably a direct consequence of the properties of the channel, *i.e.* its extremely high gating threshold in these membranes.

The observed differences in gating behavior of these MS channels might reflect not only the intrinsic properties of the channel protein but also peculiarities of the native environment of the channels. For instance, the Tb-MscL channel may have a much lower gating threshold when expressed in *M. tuberculosis* membranes. Such a shift could be due to the lipid environment or to diffusible modulators that interact with the channels. Indeed, the marked conservation in portions of the carboxyl-terminal region (26), which apparently plays no measurable role in MS channel activity *in vitro* (16, 17, 35), invites such a speculation.

**Mutagenesis Suggests That the Tb-MscL and the E. coli MscL Are Structurally and Mechanistically Similar**—An alignment and comparison of the Tb-MscL and Eco-MscL primary sequence shows that these proteins are remarkably similar and share 37% identity (see supplemental data for alignment), so it would be likely that the two share similar secondary and tertiary structures. This likelihood was functionally explored by generation of site-directed mutations at three different residues of the Tb-MscL protein as shown in Figs. 4 and 5.

The A20G mutation was invoked because alignment of 15 MscL orthologues showed nearly all bearing a glycine at the corresponding residue. A deviation, also an alanine, was in the MscL orthologues showed nearly all bearing a glycine at the corresponding residue. A deviation, also an alanine, was in the
gating threshold of the Tb-MscL channel was unsuccessful. The A20G channel still required high pipette pressures, like the wild-type Tb-MscL, to achieve the gating threshold and was unable to rescue cells from hypotonic shock. Hence, the high threshold for gating of the Tb-MscL channel cannot be attributed to this single difference between proteins.

The three other site-directed mutants presented here were patterned after lesions in the *E. coli* MscL channel that yielded the GOF phenotype. These mutant channels, when examined by patch clamp, were noted for their tendency to gate inappropriately or at lower than usual membrane tensions. All three mutants represent the most severe class recovered in the *E. coli* channel (33), but only the V21X substitutions in the Tb-MscL channel led to detectable, although mild, gain-of-function growth phenotypes (Fig. 3) when expressed in *E. coli*. Although these Tb-MscL mutants did not arrest growth, as observed with the Eco-MscL channel, the mature colonies of these mutants were conspicuously less opaque than their wild-type counterpart, and liquid cultures entered stationary phase at lower densities than wild-type or non-induced strains (Fig. 3). Why this phenotype is most pronounced at high density is, as yet, unexplained but could be due to changes in lipid or cell wall composition, sub-cellular localization, or other factors that are present (or absent) primarily under these conditions. In contrast to the V21X mutants, the G24S substitution increased the channel sensitivity (Fig. 2) but did not confer a detectable gain-of-function phenotype (Fig. 3). Although all of these mutants had gating thresholds approaching that of *E. coli*, none of these mutants could effect a rescue from hypotonic shock (Fig. 4).

When records from the V21X mutants were studied, it was noted that each mutant had a distinct behavior. Although both substitutions led to a marked 2-fold reduction in the gating threshold, a conservative substitution to alanine gave channels with wild-type conductance but extended open-dwell kinetics. In contrast, substitution by aspartate resulted in a noticeable perturbation of channel kinetics with an activity marked by many brief openings at a low probability of opening ($P_o$). This channel behavior appears to be a response to the introduction of a charged residue, because this substitution is relatively conservative in terms of size. Hydrophilicity, another distinction between these substitutions, has been implicated as a critical factor in the behavior of other mutants. A study that focused on the adjacent residue, glycine 22 in *E. coli*, noted a strong correlation between the hydrophilicity of the residue and the gating threshold of the mutant channel (34). In addition, a random mutagenesis study that screened for GOF mutants found that 14 of 18 mutations were between amino acids 13 and 30 of the Eco-MscL protein; all but one of these mutations were to more hydrophilic residues, and half were to charged amino acids (33).

**Mutational Analysis of Tb-MscL Advances a Model for Channel Gating**—The experimental observations suggest a model for MscL channel gating that postulates a hydrophobic “lock” as the major energy barrier between the closed and open conformations (20, 24, 25, 34). It is the transition of hydrophobic locking residues through an exposed, aqueous state that defines this barrier. A likely candidate for this aqueous environment is the vestibule of the opening channel. This model predicts that the Tb-MscL valine 21 participates in this lock. Consistent with this we find that Tb-MscL V21D, which adds a charge within this lock (24), has a decreased energy barrier (transition state), as demonstrated by the decreased open-dwell time; once the open state is attained, the channel can more easily return to the closed state, giving the activity a “flickery” nature. Note that with the V21A substitution, both valine and alanine are hydrophobic amino acids. Hence, the hydrophobic lock hypothesis alone cannot predict how the energy barrier will be changed. For instance, an analogous mutation in Eco-MscL leads to a flickery channel (33), whereas for Tb-MscL it does not. However, the observation that the Tb-MscL channel can become more sensitive to membrane tension without significantly shortening the open dwell-time suggests that the closed state of the V21A mutant can be destabilized without significantly changing the energy barrier between closed and open states. The apparent juxtaposition of these residues in the most constricted region of the closed channel (Fig. 5) implicates the perturbation of van der Waal’s interactions in the destabilization of the closed state. The locations of the three mutated residues within the tertiary structure of the Tb-MscL channel are depicted in Fig. 5. These residues are clustered at an apparent convergence of the TM1 helices. Although the Ala-20 and Gly-24 residues form a girdle surrounding the narrowest portion of the channel lumen, the Val-21 residues reach into the lumen, partially obstructing the pore. The relative positions of these residues are paralleled by the severity of the
phenotype rendered by their mutation. The most severe substitutions are, by far, at Val-21. The position of this residue and its contribution to the channel sensitivity suggests a crucial role in determining the gating transition energy barrier. Because similar effects are seen when the analogous site is mutated in the *E. coli* Mscl protein, it may be reasonable to designate this residue as the “gate latch,” controlling the earliest events of channel opening. Future experiments on this region of the molecule will be focused on understanding the cascade of events that culminate in the huge open-pore conformation of MscL.

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**Note Added in Proof**—An important work (Maurer, J. A., Elmore, D. E., Lester, H. A., and Dougherty, D. A. (2000) *J. Biol. Chem.* 275, 22238–22244) published since the submission of this manuscript, highlights additional gain-of-function mutants of the Tb-MscL Channel.

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