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Molecular Dissection of the Large Mechanosensitive Ion Channel (MscL) of *E. coli*: Mutants with Altered Channel Gating and Pressure Sensitivity

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Abstract. In the search for the essential functional domains of the large mechanosensitive ion channel (MscL) of *E. coli,* we have cloned several mutants of the *mscL* gene into a glutathione S-transferase fusion protein expression system. The resulting mutated MscL proteins had either amino acid additions, substitutions or deletions in the amphipathic N-terminal region, and/or deletions in the amphipathic central or hydrophilic C-terminal regions. Proteolytic digestion of the isolated fusion proteins by thrombin yielded virtually pure recombinant MscL proteins that were reconstituted into artificial liposomes and examined for function by the patch-clamp technique. The addition of amino acid residues to the N-terminus of the MscL did not affect channel activity, whereas N-terminal deletions or changes to the N-terminal amino acid sequence were poorly tolerated and resulted in channels exhibiting altered pressure sensitivity and gating. Deletion of 27 amino acids from the C-terminus resulted in MscL protein that formed channels similar to the wild-type, while deletion of 33 C-terminal amino acids extinguished channel activity. Similarly, deletion of the internal amphipathic region of the MscL abolished activity. In accordance with a recently proposed spatial model of the MscL, our results suggest that (i) the N-terminal portion participates in the channel activation by pressure, and (ii) the essential channel functions are associated with both, the putative central amphipathic α-helical portion of the protein and the six C-terminal residues RKKEEP forming a charge cluster following the putative M2 membrane spanning α-helix.

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Introduction

Since their discovery in embryonic chick skeletal muscle (Guharay & Sachs, 1984) and frog muscle (Brehm, Kullberg & Moody-Corbett, 1984), mechanosensitive (MS) ion channels have been documented electrophysiologically in various cell types, including microbes (Sachs, 1988; Morris, 1990; Martinac, 1993; Sackin, 1995), with increasing evidence suggesting important physiological roles for these channels (Medina & Bregestovski, 1988; Erxleben, 1989; Zhou et al., 1991; Berrier et al., 1992; Lewis, Ross & Cahalan, 1993; Naruse & Sokabe, 1993; Oliet & Bourque, 1993; Franco-Obregon & Lansman, 1994). Microbes have been used as model systems to advance the understanding of basic biological principles and in the case of MS channels, *Escherichia coli* was instrumental in the sequencing and cloning of the first mechanosensitive ion channel gene, *mscL,* encoding the 17 kD protein monomer underlying the activity of the large conductance bacterial MS ion channel (MscL) (Sukharev et al., 1993; Sukharev et al., 1994*a,b*; Hamill and McBride, 1994). Since its discovery, several genes homologous to *mscL* have been found in other Gram-negative (Parra-Lopez et al., 1994; Fleischmann et al., 1995; Sukharev et al., 1997) and Gram-positive bacteria (Matsushita, Jung & Okabe, 1995). The amino acid sequence of the MscL is unique in that it has no homology with known voltage- or ligand-gated ion channels. Hydropathy plot analysis has revealed a highly hydrophobic protein for which a tentative multimeric (homohexamer) structural model has been recently proposed (Sukharev et al., 1996; Blount et al., 1996*a*). The model predicts two α-helical hydrophobic membrane spanning domains, M1 and M2, and two α-helical amphipathic domains, the

N-terminal S1 and the central S2-S3 domains (Fig. 1*A*). In the present study, we have generated recombinant *mscL* mutants with various alterations to the N-terminus and deletions in the internal or C-terminal regions of the MscL, and examined the recombinant mutant proteins in patch-clamp experiments.

Materials and Methods

Molecular Cloning

The *mscL* wild-type gene and the two C-terminal deletion mutants Δ110 and Δ104, generated by PCR (kindly provided by P. Blount, University of Wisconsin) were subcloned into the plasmid vector pGEM11Zf(+) (Promega) as a *Xho*I DNA restriction fragment in the desired orientation (Häse, Le Dain & Martinac, 1995). The internal deletion mutant was generated by digesting the plasmid carrying the wild-type *mscL* gene with *Eco*RV followed by religation. The wild-type and deletion mutant genes were then excised from the vector as *Bam*HI-*Eco*RI DNA restriction fragments and ligated into the *Bam*HI-*Eco*RI cut plasmid vector pGEX-2T (Pharmacia, LKB Biotechnology, Uppsala, Sweden). For generation of the N-terminal deletion mutants NBE, NBE-ΔEV, and NBE-Δ110, the pGEX-2T vector was cut with *Bam*HI and the overhanging DNA ends were then filled in with the Klenow enzyme followed by digestion with *Eco*RI. The wild-type, internal, and C-terminal deletion mutant *mscL* genes were excised from the pGEM11Zf(+) vector as *Nru*I-*Eco*RI DNA restriction fragments and ligated into the pGEX-2T vector. For generation of the mutant with 20 additional amino acids (H6), the pGEM11Zf(+) construct carrying the wild-type *mscL* gene was first cut with *Hind*III, treated with Mung Bean nuclease to remove the overhangs, and then digested with *Eco*RI. For the mutant with the N-terminal amino acid substitutions (P6) the wild-type *mscL* gene was first cloned into a *Sma*I-*Eco*RI cut pUC18 as a *Nru*I-*Eco*RI fragment and then excised as a *Pst*I (Mung Bean nuclease-treated)-*Eco*RI fragment. Both *mscL* carrying DNA fragments were then cloned into the pGEX-2T vector cut *Bam*HI(filled in)-*Eco*RI. All constructs were subjected to DNA sequence analysis using [^{35}S]dATP labeling with the Sequenase DNA Sequencing Kit (USB, Amersham Life Science) and pGEX-specific primers (Pharmacia) according to the manufacturer's instructions.

Protein Reconstitution

Recombinant MscL proteins were reconstituted in artificial liposomes using a method similar to that described previously (Häse et al., 1995). Azolectin liposomes composed of soya-bean phosphatidylcholine (Sigma) and 10% cholesterol were prepared according to the modified dehydration-rehydration method (Delcour et al., 1989) which is nowadays a standard reconstitution method to study MscL channel activity (Sukharev et al., 1993; Sukharev et al., 1994*a,b*; Häse et al., 1995). Total purified proteins were mixed with the liposomes at the desired protein:lipid ratio, which were (i) WT: 1:6000, (ii) NBE: 1:1000, 1:200 or 1:100, (iii) ΔEV: 1:1000 or 1:200, (iv) H6 1:2000, (v) P6 1:1000, (vi) Δ110: 1:4000, (vii) Δ104: 1:2000, and (viii) NBE-Δ110: 1:1000 or 1:500, (ix) NBE-ΔEV: 1:200. Protein concentrations were determined using the D_C protein assay (Bio-Rad), and protein samples were analyzed by 12% and 15% SDS-PAGE (Fig. 1*C*).

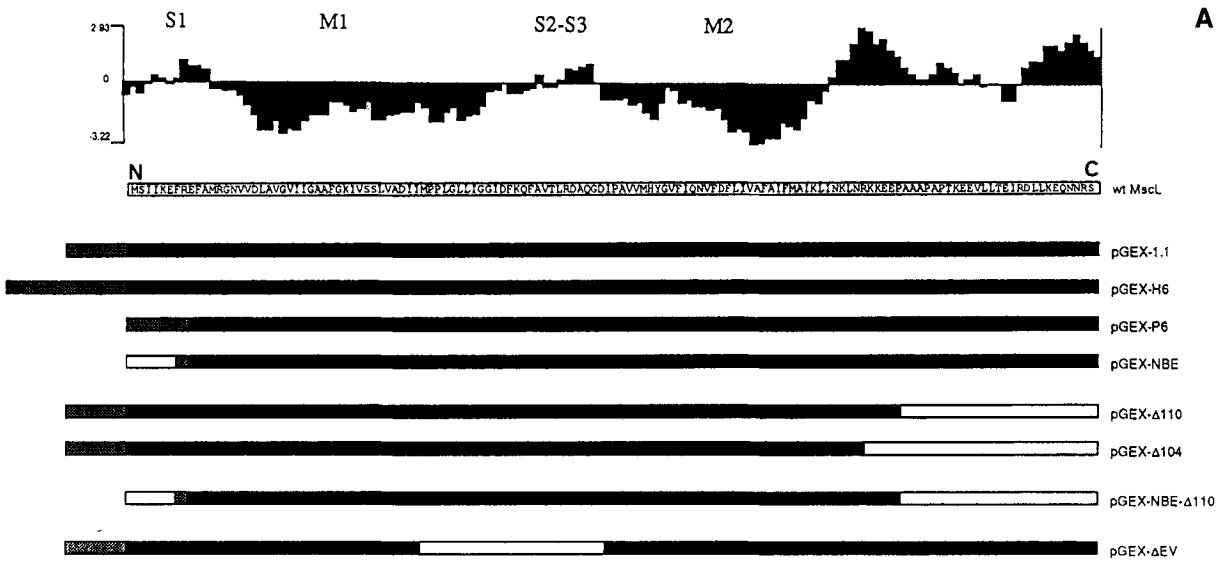
Electrophysiology

The standard patch-clamp technique (Hamill et al., 1981) was used to record single-channel currents of reconstituted MscL proteins in excised patches of unilamellar blisters arising spontaneously from multilamellar liposomes, as reported previously (Häse et al., 1995). The bath and pipette recording solution had the following standard composition: 200 mM KCl, 40 mM $MgCl_2$, 5 mM HEPES, pH 7.2 adjusted with KOH (Sukharev et al., 1994*a*; Häse et al., 1995). Channel activation was achieved by applying suction to the pipette by mouth or syringe attached to the port used to apply pressure during seal formation. Pipettes were manufactured using a Flaming/Brown micropipette puller (P-87, Sutter Instrument, Novato, CA). All pipettes were standardized with respect to shape and size, and had bubble numbers in 100% ethanol of 3.5–4.0 corresponding to resistances in recording solution of 17–26 MΩ. Single-channel currents were digitized at 5 kHz using WinTida (Heka Electronics, Heidelberg, Germany) or pClamp6 (Axopatch, Axon Instruments, Foster City, CA) acquisition software, filtered at 1 kHz and analyzed off-line with commercial software (pClamp6) or programs written in this laboratory.

Current and pressure were digitized simultaneously, with a piezoelectric transducer (differential type, ±5 psi, Omega Engineering, Stamford, CT) providing the pressure signal. Activation pressure was calculated as the pressure at which the first full channel opening was observed. Conductance measurements were made from *I-V* plots of single-channel amplitude and voltage, or 3.2-sec voltage ramps over the range −60 to +60 mV. The channel conductance was determined from the slope of the current resulting from this ramp, following correction for the leakage current.

Results

We have previously used a common method for expressing recombinant proteins in *E. coli* by generating a plasmid encoding a glutathione S-transferase (GST-MscL) fusion protein (Häse et al., 1995). The N-terminus of the recombinant wild-type MscL protein obtained upon thrombin cleavage of the corresponding fusion protein, contains nine additional amino acids which are not present in the native purified wild-type 136 amino acid MscL monomer (Sukharev et al., 1994*a*). In addition to the wild-type gene, several *mscL* mutants were cloned into the plasmid vector pGEX-2T (*see* Materials and Methods) to generate various GST-MscL fusion proteins (Fig. 1). The N-terminal deletion mutant (pGEX-NBE) resulted from cloning the *mscL* gene into the GST-MscL expression system using the unique *Nru*I restriction site, resulting in loss of the first eight amino acid residues of the native MscL and the addition of two novel residues following thrombin cleavage (Fig. 1*B*). Further N-terminal mutants were generated using other restriction sites present in the multiple cloning region of the plasmid vectors, resulting in either the addition of 20 novel amino acids (pGEX-H6) or the substitution of the first eight amino acids of the native MscL protein by nine novel residues (pGEX-P6) (Fig. 1*B*). The internal 28 amino acid deletion of MscL (pGEX-ΔEV) was generated by removing the internal *Eco*RV DNA restriction fragment of the *mscL* gene. The C-terminal deletion mutants were generated by PCR and kindly provided by P. Blount (University of Wisconsin, Madison). Double mutants of the *mscL* gene with both N-terminal and internal or C-



pGEX-1.1, pGEX-Δ110, pGEX-Δ104, pGEX-ΔEV **B**

Thrombin

Leu	Val	Pro	Arg	Gly	Ser	Leu	Glu	His	Arg	Glu	Asn	Asn	Met ...
CTG	GTT	CCG	CGT	GGA	TCC	CTC	GAG	CAT	AGG	GAG	AAT	AAC	ATG...
				Bam HI		*Xho* I							

pGEX-NBE, pGEX-NBE-ΔEV, pGEX-NBE-Δ110

Thrombin

Leu	Val	Pro	Arg	Gly	Ser	Glu ...
CTG	GTT	CCG	CGT	GGA	TCC	GAA ...
				Bam HI-*Nru* I		

pGEX-P6

Thrombin

Leu	Val	Pro	Arg	Gly	Ser	Val	Asp	Ser	Arg	Gly	Ser	Pro	Glu ...
CTG	GTT	CCG	CGT	GGA	TCG	GTC	GAC	TCT	AGA	GGA	TCC	CCC	GAA ...
				Bam HI-*Pst* I		*Sal* I		*Xba* I		*Bam* HI	*Sma* I-*Nru* I		

pGEX-H6

Thrombin

Leu	Val	Pro	Arg	Gly	Ser	Met	His	Ala	Ala	Ala	Ser	Arg	Gly	Pro	Gly	Ser	Leu	Glu	His	Arg	Glu	Asn	Asn	Met ...
CTG	GTT	CCG	CGT	GGA	TCT	ATG	CAT	GCG	GCC	GCA	TCT	AGA	GGG	CCC	GGA	TCC	CTC	GAG	CAT	AGG	GAG	AAT	AAC	ATG ...
				Bam HI-*Hind* III		*Nsi* I		*Not* I			*Xba* I		*Apa* I		*Bam* HI		*Xho* I							

Fig. 1. (*A*) The amino acid sequence of the *mscL* ORF and the corresponding Kyte-Doolittle hydropathy plot with the two predicted α-helical transmembrane domains (M1, M2) and α-helical amphipathic regions (S1, S2–S3) are shown. The terminal ends of the protein are labeled N and C respectively. The boxes below the plot indicate the amino acid changes in the recombinant MscL protein with empty boxes representing deleted regions and hatched boxes showing added amino acid residues (*B*) The DNA nucleotide triplets and corresponding amino acids at the junction between the GST and MscL portions of the GST-MscL fusion protein are shown for the recombinant mutants. The thrombin cleavage site is indicated by the arrows. The *mscL* ATG start codon and corresponding methionine residue are shown in bold face. Note that the N-terminus of the recombinant wild-type MscL is nine amino acids longer in comparison to the native wild-type MscL. (*C*) *E. coli* cells expressing the various purified MscL preparations were analyzed on 15% SDS-PAGE and silver stained. For unknown reasons, purified wild-type MscL monomers migrate as double bands on the gel, as reported previously (Sukharev et al., 1994*a,b*). Also, note trace amounts of "background" proteins visible in lanes 3–8, the amount of which varied between preparations. The arrow on the right indicates the position of MscL protein bands and bars on the left indicate the positions of molecular weight standards.

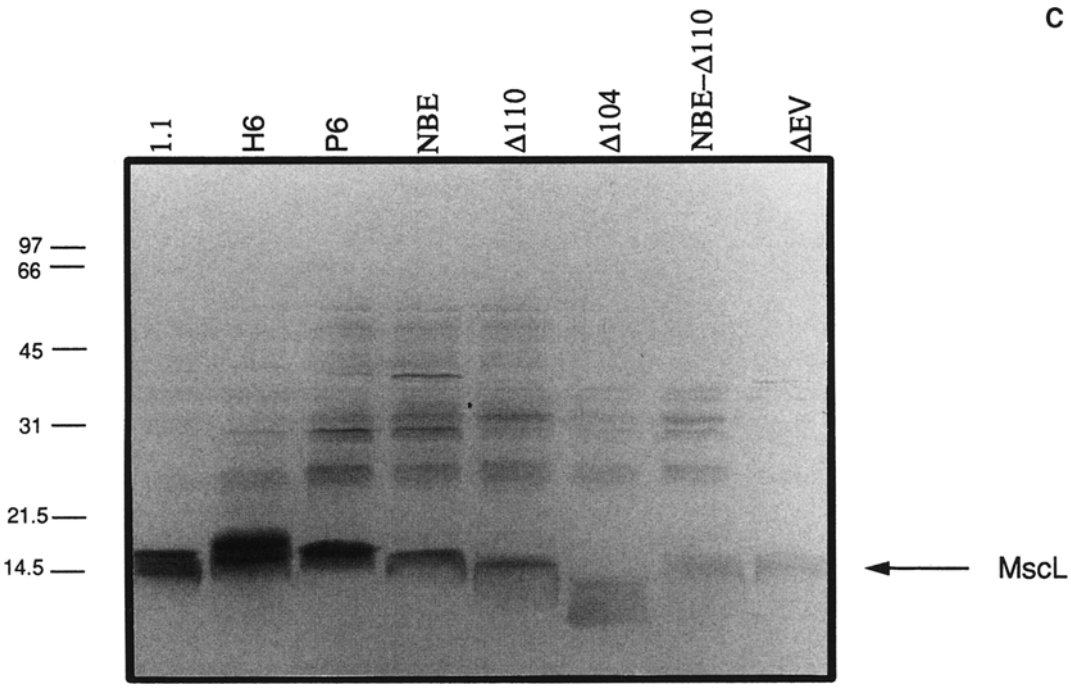


Fig. 1. Continued.

terminal deletions were generated from the single ΔEV mutant generated in our lab or the Δ110 construct provided by P. Blount and the techniques used to generate the N-terminal mutants (Materials and Methods, Fig. 1*A*). After expression, purification and thrombin digestion of the wild-type and mutant GST-MscL fusion proteins, the proteins were examined using SDS-PAGE (Fig. 1*C*). The molecular weight of the particular MscL protein bands corresponded to that expected from their DNA sequences. In addition, all *mscL* gene constructs were confirmed by DNA sequencing analysis (Materials and Methods).

The trace amounts of contaminating proteins visible in the gel varied between different preparations used for the reconstitution experiments. However, none of these proteins interfered with the channel activity of MscL, since the activity of the recombinant wild-type MscL (Häse et al., 1995) is similar to that of the reconstituted native wild-type MscL (Sukharev et al., 1994*a*). Furthermore, the amounts of purified MscL varied between different mutants in all of our preparations, with the tendency to be reduced as the hydrophobicity of the mutant MscL proteins increased (compare lanes 1 and 2 for WT and H6 mutant with lanes 6 and 7 for Δ104 and NBE-Δ110 mutants, Fig. 1*C*).

For functional analysis the MscL proteins were reconstituted into artificial liposomes, and their activities examined by the standard patch-clamp technique (Hamill et al., 1981). Application of negative pressure to the patch resulted in activation of MS channels after a threshold activation pressure is crossed; with relaxation of the pressure resulting in closure of the channels (Martinac et al., 1987; Häse et al., 1995). When the patch is held at a pressure above threshold, the MS channels gate between the open and closed state with an open probability dependent upon the applied pressure. In general, the steady-state activity of MS channels in *E. coli* can be well described by a Boltzmann distribution (Martinac et al., 1987). As we reported previously for the recombinant wild-type MscL (Häse et al., 1995), continuous application of pressure in many patches resulted in an increase in channel activity with time, suggesting that channels were not in an equilibrium state with the applied mechanical force. We also found this to be the case with the mutant channels examined in the present study. Consequently, we have not attempted fitting the channel activation by pressure to a Boltzmann distribution. However, as an example of the dependence of channel activity upon the applied pressure, Fig. 2 illustrates the gating of the recombinant MscL's of the H6 and Δ110 mutants at several pressures. A further complication in describing the pressure-dependence of the channels in the present study occurs with the N-terminal deletion and substitution mutants, since the pressures required for channel activation were within the range of lytic tensions sufficient to break the liposome patches (compare activation pressures in Table 1 with patch breakage usually occurring at 170 ± 3 mm Hg (n = 86 patches).

Figure 3 shows recordings of channel activity characteristic of the reconstituted recombinant wild-type and

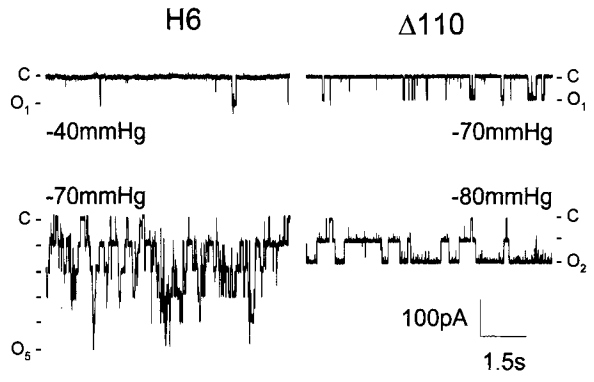


Fig. 2. Current traces of recombinant H6 and Δ110 mutant channels recorded at two different negative pressures demonstrating the preservation of the pressure-dependence of the MscL channels in these mutants. Negative pressures were as indicated. The results for each mutant were obtained from the same patch. Data are 8-sec recordings obtained at the pipette voltage of −20 mV. C and O indicate the closed and open states of the channels respectively.

several mutant MscL proteins at pipette voltages of opposite polarity and negative pressures in the pressure range required for threshold activation of the particular mutant channels. Whereas the recombinant wild-type channels exhibited steady long openings with rare excursions to subconducting levels at pipette voltages of either polarity (Fig. 3*A*), gating of the Δ110 deletion mutant channel lacking 27 residues in the C-terminal region (Δ110–136), exhibited frequent transition to subconducting levels of variable size (asterisks, Fig. 3*A*). Analysis of 7 patches (at ±20 mV) containing the Δ110 mutant channel, revealed that subconducting levels <85% of the full conductance and duration >10 msec, occurred at a frequency of 0.58 ± 0.16 events per second as opposed to a frequency of 0.07 ± 0.04 events per second for the wild-type channel at the same pipette voltages (analysis of 5 patches).

Two deletions to the C-terminal portion of the MscL protein were examined. While the Δ110 C-terminal deletion mutant formed functional channels, the Δ104 mutant lacking 33 residues (Δ104–136) failed to exhibit active channels (Table 2). Similarly, the ΔEV mutant with a 28 amino acid internal deletion (Δ41–68) that includes the central amphipathic S2-S3 region (Fig. 1*A*), also failed to exhibit channel activity (Table 2).

The channels formed by MscL proteins of the N-terminal mutants H6 (addition of 20 amino acids), or P6 (first eight residues substituted by nine novel amino acids resulting in a less charged N-terminus compared to that of the wild type), did not show marked differences in gating and conductance compared with the wild-type channels (Fig. 3, Fig. 4, Table 1). In contrast, gating of the N-terminal deletion mutant NBE (Δ1–8 plus two novel amino acids) was characterized by frequent brief, openings at pipette voltages of both polarities (Fig. 3*B*) as we reported previously (Häse et al., 1996). Similar changes to gating kinetics were observed with the NBE-Δ110 double mutant (Fig. 3).

The conductance of the reconstituted MscL channels was calculated from the current amplitude of the single-channel openings and the applied pipette voltage, or from voltage-ramp experiments (Fig. 4) (Table 1). As reported previously (Häse et al., 1995), the average conductance of the recombinant wild-type channel in the voltage range −60 to +60 mV is approximately 3.2 nS (Table 1), with a slight rectification observed at positive pipette voltages. Interestingly, in all the MscL mutants examined in the present study, the conductance was the least affected feature. The full conductance of the NBE mutant channels were comparable to that of the wild type channel, as estimated by measurements of current in response to voltage ramps and steps (Table 1). The MscL of the double mutant, NBE-Δ110, exhibited an increased number of events of amplitudes smaller than the wild-type full conductance (Fig. 3, expanded trace). Given the very low frequency of observing active channels for the NBE-Δ110 mutant even at protein:lipid ratios much higher than those required for other active channel mutants (Table 1), obtaining sufficient data for complete analysis was difficult. However, there is a conductance observed for this mutant that approximates that of the wild-type (Fig. 3*B*).

In contrast to conductance, pressure sensitivity varied markedly between several of the mutant channels. Substantially larger negative pressures (≈140 mm Hg) than those sufficient to activate the recombinant wild-type channel (≈70 mm Hg), were required to observe and sustain channel activity of the P6, NBE or NBE-Δ110 mutant channels (Table 1). Furthermore, unlike the recombinant wild-type MscL that could be activated in approximately 95% of the patches, the NBE, P6 and Δ110 mutant channels could only be activated in approximately 60% of all patches examined at comparable or higher protein:lipid ratios (*see* Materials and Methods), while NBE-Δ110 was much less active (Table 1). Unlike the deletion or substitution mutants, the N-terminal H6 mutant, with an additional twenty amino acids, formed channels that were activated in approximately 90% of the patches examined and negative pressures of ≈70 mm Hg similar to the response of the wild-type channel (Table 1). No channel activity was observed in liposome patches containing MscL proteins of either Δ104, ΔEV, or the NBE-ΔEV mutant. Based on probabilities calculated for not observing any channel activity in a series of consecutive experiments (Table 2), we concluded that these mutants do not form functional channels. Presumably, the particular mutations are those most likely to affect regions of the protein essential for channel function or may not allow correct (normal) protein folding.

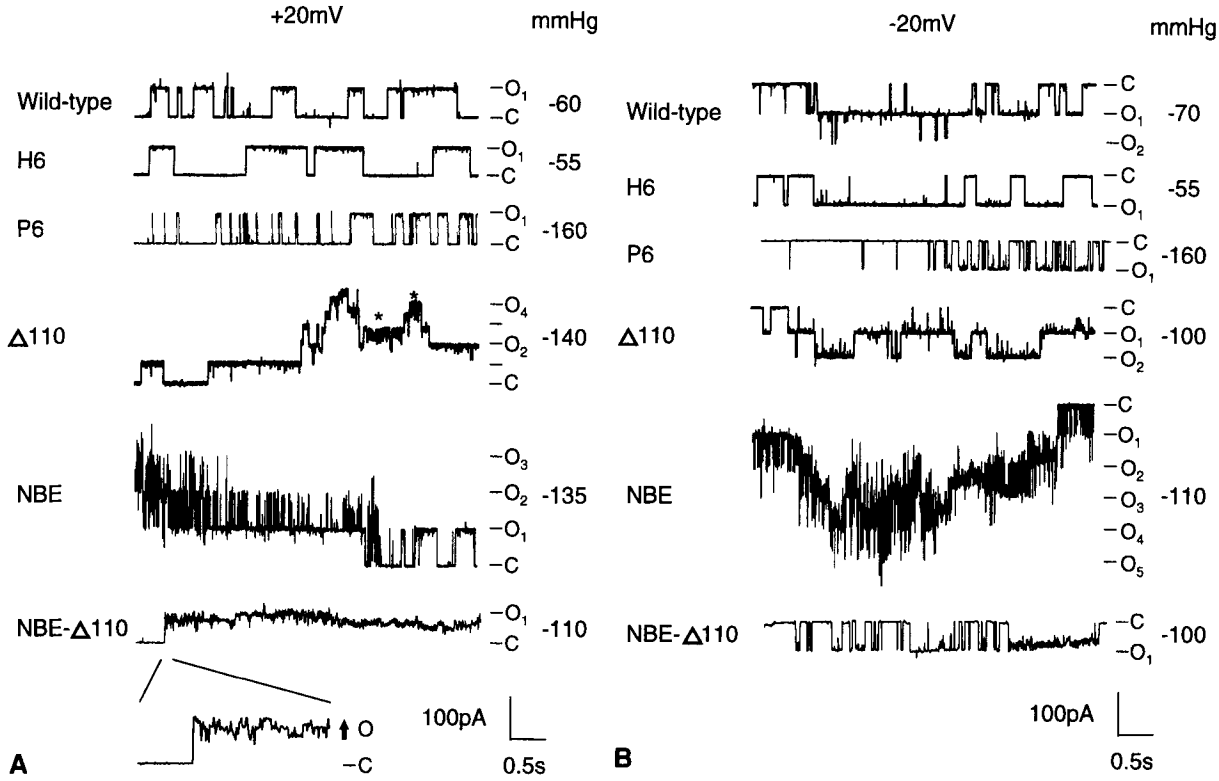


Fig. 3. Current traces of recombinant wild-type and various mutant channels recorded in liposome patches. Data are 5-sec recordings at pipette potentials of (*A*) +20 mV, and (*B*) −20 mV. Recordings of all mutant channels were obtained from the same patch at both pipette voltages, except for the NBE-Δ110 double mutant, where each recording was from a separate patch. Negative pressures were as indicated. C and O designate the closed and open states of the channels respectively. Subconducting levels are indicated by an asterisk. Note expanded view of NBE-Δ110 recording showing distinct channel transitions.

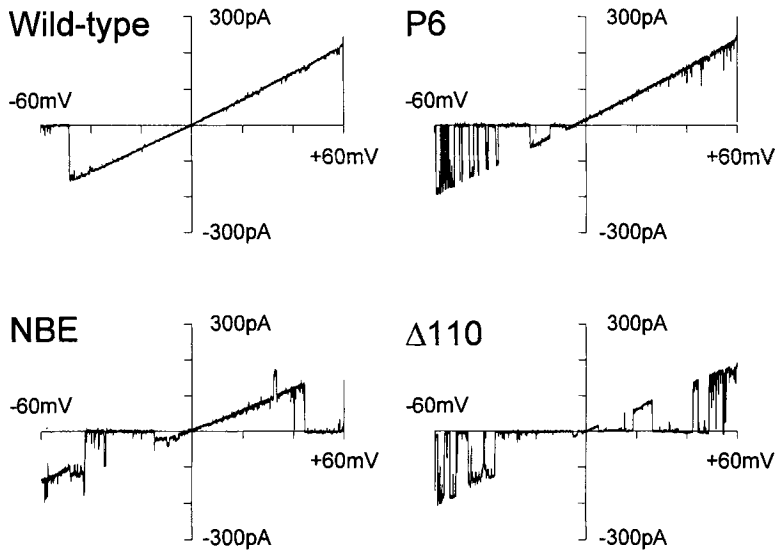


Fig. 4. Current-voltage plots for single channel currents obtained from voltage ramp protocols over the range −60 to +60 mV for the wild-type, P6, NBE, and Δ110 mutant channels. The voltage was linearly changed over a 3.2-sec time course. Plots are shown following subtraction of the leakage current. Note the subconductance levels in the NBE and Δ110 traces.

Discussion

We report here the results of a systematic exploration to determine functional motifs within the MscL protein responsible for the mechanosensitive channel gating and kinetics. Two deletions of a large portion of the C-terminal region of the protein were examined. Similar to an independent study in which the C-terminal deletion

Table 1. Summary statistics of active recombinant MscL proteins

	Conductance		Negative activation pressure (mm Hg)	Negative pressure Range (mm Hg)	Percent Inactive
	Positive (nS)	Negative (nS)			
WT	3.1 ± 0.1 (10)	3.2 ± 0.1 (10)	74 ± 9 (8)	40–115	6% (17)
H6	3.0 ± 0.1 (6)	3.1 ± 0.1 (8)	70 ± 5 (13)	45–115	7% (14)
P6	3.2 ± 0.1 (11)	3.1 ± 0.1 (11)	138 ± 6 (7)	110–160	15% (20)
Δ110	3.0 ± 0.1 (4)	3.3 ± 0.1 (6)	70 ± 7 (13)	30–100	40% (25)
NBE	3.0 ± 0.2 (7)	3.2 ± 0.2 (8)	144 ± 4 (22)	110–180	26% (39)
NBEΔ110			120 ± 5 (9)	100–140	81% (80)

Data are mean ± SE from *n* estimates (in parentheses). Conductances were measured at either positive or negative pipette voltages. *Negative activation pressure* is the pressure at which the first full channel opening is observed, and is presented as an average across patches. *Negative pressure range* indicates the pressures over which channels were observed. *Percent inactive* is the number of patches examined at pressures greater than the mean activation pressure (*Negative activation pressure* column) for which no channel activity was observed ("blank patches") presented as a percentage of the total number of patches examined at the pressures greater than the mean activation pressure (in parentheses).

Table 2. Summary statistics of nonactive recombinant MscL proteins

	Protein : Lipid	Number of blank patches	Negative average pressure	Negative pressure range	% Activity maximum	Prob. <%
			(mm Hg)	(mm Hg)		
Δ104	1:2000	16	169 ± 6	80–220	44%	0.83
ΔEV	1:200	30	160 ± 6	130–230	22%	0.90
NBE-ΔEV	1:1000; 1:200	40	178 ± 4	150–250	13%	0.93

All patches examined for these mutants exhibited no pressure-dependent channel activity. Data are mean ± SE from the number of patches indicated. *Negative average pressure* is the mean pressure at which these mutants were tested. *Negative pressure range* is the range of pressures over which these mutants were tested. *Percentage activity maximum* is the maximum activity that can be expected for a particular mutant given its number of consecutive blank patches (at a significance level of $P > 0.95$). *Prob.* <5% represent the likelihood that the channel activity for each particular mutant was less than 5%, given the number of consecutive blank patches examined. Note that the average pressures are greater compared with the average pressures for activation in Table 1, and furthermore, the protein to lipid ratios were higher for these mutants compared with those of wild-type or H6 (*see* Material and Methods).

mutants of MscL were examined in giant spheroplasts (Blount et al., 1996*b*), deletion of 27 terminal amino acids (Δ110) still gave a pressure-activated functional channel (Fig. 2), while deletion of further 6 amino acids (Δ104) abolished channel activity (Table 2). In addition, we found the Δ110 mutant to exhibit a significantly increased tendency to adopt subconducting levels. This approximately ten times greater incidence of transitions to such subconducting levels in the Δ110 mutant may indicate instability of the open configuration of the mutant channel protein, suggesting that the C-terminal region may contribute to stabilization of functional conformations of the MscL channel. The Δ110 and Δ104 data together suggest that the six residues (104–109, RKKEEP) that form a charge cluster following the supposed M2 membrane spanning α-helix (Fig. 1*B*), are crucial for channel function. The NBE-Δ110 double mutant data provides further support to the view that the C-terminal portion may be important, but not necessarily exclusive, in stabilization of the open conducting configuration of the channel homomultimer, since the NBE mutant alone also showed a change in gating characteristics.

Deletion of the central amphipathic region (ΔEV) also provided a nonfunctional protein. We can conclude that this region is at the least, essential for formation of functional MscL channels. However, it is also conceivable that all the mutants with large deletions may not form functional channels because of improper folding of the MscL into secondary and tertiary protein structure, and therefore may not insert correctly into the lipid bilayer.

Our data demonstrate that a few amino acid deletions, as well as complete substitution of the amphipathic N-terminal domain of the MscL resulted in marked alteration in channel activation by pressure. Large additions of ten and twenty amino acids to the N-terminus of

the MscL (≈7–15% of the length of the monomer) did not affect channel gating and pressure sensitivity, while short deletions or substitutions altered these parameters. This study has provided evidence that the level of pressure activation can be affected by deletions and changes in the overall charge of the N-terminal region by reducing the number of charged amino acid residues from five to two (WT *vs.* P6, Fig. 1*A* and *B*, Table 1). This result suggests that the N-terminus may represent a "mechanosensitive structural element" required for the activation of the channel homomultimer by pressure. Indeed, an independent study has shown that chymotrypsin, which may react with the phenylalanine associated proteolytic sites present at the N-terminus of the MscL, had a marked effect on the channel sensitivity *in situ* in giant spheroplasts as well as with the purified MscL protein reconstituted in liposomes, by increasing channel activity at a constant pressure (A. Ghazi, *personal communication*). Our results are to some extent comparable with the results of a similar study in which very small (Δ2–4), but not larger (Δ2–12) N-terminal deletions were tolerated for the MscL channel function (Blount et al., 1996*b*). However, results of both studies suggest that the N-terminus may be important in protein folding.

Deletion of amino acids in the hydrophilic C-terminal domain did not significantly affect channel activity, until this deletion included a charged cluster of six additional amino acids, and thereby abolished channel activity. Furthermore, a deletion of 28 amino acids in the central M1 hydrophobic and S2-S3 amphipathic region extinguished channel activity. In addition to the result that the channel remains functional after approximately 25% of its amino acid sequence is deleted in the "minimal channel" of the NBE-Δ110 double mutant, the data suggest that the N- and C-terminal portions, which are most likely located outside the membrane bilayer (Sukharev et al., 1996), are not essential channel components but probably have regulatory functions. In summary, it appears that the N-terminal portion of the channel is required for normal channel responsiveness to pressure and probably plays a role in channel gating, while regions encompassing the putative membrane spanning α-helices M1 and M2 as well as the amphipathic domain S2-S3 (Fig. 1*A*), are essential channel components.

The MscL is at present the only mechanosensitive ion channel available for structure and function studies aiming at understanding the molecular basis of mechanosensation. Together with the recent work reported from the laboratory of Kung and coworkers (Blount et al., 1996*a,b*) this study helps provide some insights into the structural domains essential for MscL channel function and pressure activation. Future experiments will focus on the interplay of these domains within the protein monomer in providing the characteristic conductance, gating and pressure dependence of the large mechanosensitive ion channel.

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