Contributions of the Different Extramembranous Domains of the Mechanosensitive Ion Channel MscL to Its Response to Membrane Tension

(Received for publication, September 14, 1999, and in revised form, October 20, 1999)

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MscL is a mechanosensitive channel that is gated by tension in the membrane bilayer alone. It is a homooligomer of a protein comprising two transmembrane segments connected by an external loop, with the NH₂ and COOH termini located in the cytoplasm. The contributions of the extramembranous domains of the channel to its activity were investigated by specific proteolysis during patch-clamp experiments. Limited proteolysis of the COOH terminus or the NH₂ terminus increased the mechanosensitivity of the channel without changing its conductance. Strikingly, after cleavage of the external loop of each monomer, the channel was still functional, and its mechanosensitivity was increased dramatically, indicating that the loop acts as a spring that resists the opening of the channel and promotes its closure when it is open. These results indicate that the integrity of most of the extramembranous domains is not essential for mechanosensitivity. They suggest that these domains counteract the movement of the transmembrane helices to which they are connected, thus setting the level of sensitivity of the channel to tension.

Mechanosensation and mechanotransduction, the processes by which mechanical force is detected and transduced into electrical and chemical signals by living cells, are at the basis of the physiology of osmoregulation, touch, hearing, proprioception, as well as detection of wind and gravity by plants. Since their discovery by patch-clamp experiments (1, 2), mechanosensitive ion channels (Msc)¹ have been hypothesized to play a major role in these processes. These channels gate in response to changes in membrane tension and are present in animal cells as well as in plant cells and bacteria (3—7). The molecular identification of these channels proved to be difficult, and their mechanism is not understood. The situation in this ion channel field has thus been in sharp contrast with that known for voltage-gated or ligand-gated channels for which a wealth of information is available today.

High conductance ion channels that are stretch-activated are present in Gram-negative and Gram-positive bacteria (8) and in Archaea (9). They have been proposed to catalyze the efflux of osmolytes and potassium upon osmotic down-shock (10—13). In Escherichia coli, patch-clamp experiments have revealed stretch-activated conductances ranging from 100 to 1,500 pico Siemens (in 0.1 M KCl). By order of increasing conductances, three families of Msc can be distinguished: MscM (M for mini), MscS (S for small), and MscL (L for large) (14).

Prokaryotic Msc can be solubilized in detergent and functionally reconstituted in giant liposomes amenable to patch-clamp recording. This property has allowed purification of MscL, the channel of the highest conductance, and cloning of its corresponding gene, mscL (15). Expression of the mscL gene was shown to be necessary and sufficient for the activity of this channel (15—17). The gene encodes a 15-kDa small protein of 136 residues. Cross-linking studies (17) and two-dimensional crystallization of MscL at low resolution (18) suggested that the functional channel is a homohexamer. Recently, three-dimensional crystals of the MscL homolog from Mycobacterium tuberculosis were obtained (19), allowing a determination by x-ray crystallography of its structure in the closed conformation to 3.5 Å resolution. In the crystal, the channel is organized as a homopentamer. Each subunit comprises two transmembrane segments connected by an external periplasmic loop, with the NH₂ and COOH termini located in the cytoplasm. The closed pore is lined by the five M1 helices (on the NH₂-terminal side) tilted at an oblique angle with respect to the membrane. The M2 helix returns to the cytoplasm along the outside of the closed pore. The COOH-terminal cytoplasmic domain consists of helices packed together to form a helical bundle. Because MscL is the first purified protein with unambiguous mechanosensitive activity, whose gene is available and whose structure is known, it becomes a model system to study the molecular mechanism of mechanosensation. It is a very simple system: an oligomer of a small protein which is able to change its conformation in response to a variation in membrane tension. However, despite this simplicity, its molecular mechanism is still unknown.

In this study, we have examined the contributions of the NH₂ and COOH termini and the external loop of the channel to its mechanosensitivity. This was achieved by proteolysis of these extramembranous domains (Fig. 1) during patch-clamp experiments performed on the native membrane or in liposomes in which the purified protein was reconstituted. We could demonstrate that limited proteolysis of either the COOH terminus or the NH₂ terminus increased the mechanosensitivity of the channel. Importantly, the channel remained functional after cleavage of the only external loop of each subunit, such that its mechanosensitivity was increased dramatically. The results
are interpreted within the framework of a molecular model for the gating mechanism of MscL.

EXPERIMENTAL PROCEDURES

Preparation of Giant E. coli Protoplasts—Giant E. coli protoplasts for which the plasma membrane is accessible to a patch electrode were prepared from E. coli lpp ompA cells (14). Cells were grown in 50 ml of LB, pH 7.2, supplemented with 30 mM MgSO4 to an OD660 of 0.12–0.15 before the addition of cephalexin (80 μg/ml). Giant round cells (5–7 μm in diameter) were harvested 3–4 h later. Cells resuspended in 10 mM Tris-HCl buffer, pH 7, 100 mM NaCl, 400 mM sucrose, were incubated at 37 °C for 30 min in the presence of lysozyme (400 μg/ml). 2 μl of this suspension was deposited in the patch-clamp chamber and covered with 50 μl of 10 mM Tris-HCl, pH 7, 1 mM EDTA, 100 mM KCl. 2 ml of 10 mM HEPES-KOH, pH 7.4, 100 mM KCl, 10 mM MgCl2, then was added to the patch-clamp chamber for recording.

Preparation of Giant Proteoliposomes—Recombinant MscL proteins and mutant (Δ110–136) proteins were purified in one step, using a glutathione S-transferase fusion system, as described previously (16). A few microliters of purified protein in 50 mM octyl β-glucoside was added to 1 ml of 10 mM HEPES-KOH, pH 7.4, 100 mM KCl, 25 mM octyl β-glucoside containing 1 mg of sonicated lipids (azolectin from soybean, type IV-S, Sigma) to achieve a lipid to protein ratio of 500–2,500. After a 20-min incubation, 160 mg wet weight of SM-2 Bio-Beads (Bio-Rad) was added to the suspension to remove the detergent (20). The suspension was agitation for 3 h, the Bio-Beads were discarded, and the suspension was centrifuged for 30 min at 90,000 rpm using a TL100 Beckman ultracentrifuge. The pellet was resuspended in 15 μl of 10 mM HEPES-KOH, pH 7.4. The proteoliposomes were then fused into giant proteoliposomes amenable to patch-clamp recording, using a cycle of dehydration-rehydration as described previously (21). Rehydration was performed in 10 mM HEPES-KOH, pH 7.4, 100 mM KCl. 2 μl of the giant proteoliposome suspension was deposited in the patch-clamp chamber and diluted with 2 ml of the bath solution for electrophysiological recording.

Electrical Recording—Single-channel activity was measured using the methods of Hamill et al. (22). Patch electrodes were pulled from Pyrex capillaries (Corning code 7740) and were not fire polished before use. Whether using cells or proteoliposomes, recordings were performed in the excised patch mode, and the internal face of the membrane patch could be superfused by solution coming from the outlet of a manifold connected to a series of five piped inlets, allowing easy change of solution. The flow rate of the solutions was 50–100 μl/min. Because a flow of solution can sometimes activate the channels by itself, the patch was always superfused with a control solution before application of protease. Trypsin (toxophenylalanyl chloromethyl ketone-treated), chymotrypsin (N′-tosyl-L-lysine chloromethyl ketone-treated), carboxypeptidase Y (from baker’s yeast), leucine aminopeptidase (from porcine kidney), and Pronase E from Streptomyces griseus were from Sigma. Control experiments were performed using bovine serum albumin in the bath or in the pipette. Negative pressure (suction) in the pipette was applied by syringe and monitored with piezo-electric pressure transducer (Bioblock scientific). Unitary currents were recorded using a Biologic RK-300 patch-clamp amplifier with a 10-gigaohms feedback resistance and stored on digital audio tape (Biologic DTR 1200 DAT recorder). Records were subsequently filtered at 1 kHz (~3 dB point) through a four-pole Bessel low pass filter, digitized off-line at a rate of 2 kHz, and analyzed on a personal computer, with a program developed by G. Sadoc (Gif sur Yvette). Data were plotted on a Hewlett-Packard laserjet printer, using Sigmaplot software (Jandel).

Open probability P_o multiplied by the unknown number N of channels in a given patch, versus the applied suction, were fitted with a Boltzmann distribution of the form

\[ N \cdot P_o = N \cdot P_{max} (1 + \exp(\rho (a - p)))^{-1} \]  

where \( P_{max} \) is the maximum probability of channel being open, \( p \) is the suction, \( P_{max} \) is the suction at which the open probability is 0.5, and \( a \) is the sensitivity to the applied suction.

For the native membrane as well as for proteoliposomes, the convention for the membrane potential is the same and assigns zero level to the pipette. The contents of all the pipettes, bath, and perfusion solutions are given in the figure legends.

RESULTS

Effects of Proteases on MscL Channels in the Native Membrane—The patch-clamp experiments were performed on giant E. coli protoplasts (Fig. 6B in Ref. 14). After excision, the inside-out patches were superfused with a control solution similar to the bath solution. Negative pressure (suction) was raised progressively until the activation of the MscL channels and was then kept constant. The control solution was then exchanged for a solution containing a protease. As shown in Fig. 2, the superfusion of the patch by trypsin (250 μg/ml) led

Fig. 1. Trypsin- and chymotrypsin-sensitive sites of the extramembranous parts of the MscL subunit. The topology of the E. coli MscL subunit in the membrane is based on PhoA fusion experiments (16) and the structure of the M. tuberculosis MscL (18). Trypsin-sensitive sites, lysines (K) and arginines (R) outside the transmembrane domain M1 and M2, are shown as filled circles. The aromatic phenylalanines (F) outside M1 which are potential chymotrypsin sites are shown as filled boxes.

![Image of proteoliposomes](image-url)
MscL reconstituted in liposomes. Patch-clamp experiments were performed on giant proteoliposomes. After excision, pressure was applied to the inside-out patch as indicated. The patch was superfused first with the bath solution then with the bath solution containing chymotrypsin or carboxypeptidase or aminopeptidase, as indicated. In all cases, application of the proteases increased the activity of the channels, which, however, remained mechanosensitive and closed upon release of pressure. Bath medium: 100 mM KCl, 10 mM HEPES-KOH, pH 7.4. Pipette medium: similar to bath medium with, in addition, 1 mM MgCl₂, 0.1 mM CaCl₂. The membrane potential was +10 mV. Panel A, effect of carboxypeptidase (500 µg/ml). The asterisk indicates a transient decrease in channel activity which was observed at the onset of carboxypeptidase superfusion. Panel B, effect of chymotrypsin (300 µg/ml). Panel C, effect of aminopeptidase (500 µg/ml).

Effects of Proteases Applied in the Bath on Purified MscL Reconstituted in Liposomes—The MscL proteins alone form the ion channel (15–17). However, it cannot be ruled out that in the plasma membrane it may interact with other proteins which could modulate its sensitivity. To examine whether the effects described above are caused by the action of the proteases acting on the channel itself or on other associated proteins, we turned to a reconstituted system. The purified MscL was reconstituted in liposomes which were fused, by dehydration-rehydration, into giant liposomes amenable to patch-clamp recording. A first concern in such studies is the orientation of the protein in the pure lipid bilayer compared with the native system. The MscL channel is weakly voltage-dependent. In the plasma membrane, at a given applied pressure, the channel tends to be more open at low positive potentials than at low negative potentials (14). A first hint that the proteins were reconstituted with the right side orientation came from the observation of a voltage dependence of the same polarity in liposomes as in the native membrane (not shown). This was confirmed further by the experiments described in this paper.

After excision from a giant liposome, superfusion of the patch by trypsin (250 µg/ml) resulted in an effect similar to that described above for patches performed on the plasma membrane (n = 13). Examination of the amino acid sequence and topology of the MscL monomer indicated that sites sensitive to trypsin are present in both amino and carboxyl termini (Fig. 1). However, hydrophobic residues, which are weaker potential targets for chymotrypsin, are present in the COOH terminus. As shown in Fig. 3A, superfusion of carboxypeptidase Y (500 µg/ml) first led to a transient decrease in channel activity (marked by an asterisk on the figure), which was systematically observed, followed by a rapid and significant increase in the channel activity (n = 12). There was no change in unit conductance. Importantly, independent of the length of time during which carboxypeptidase was superfused on the patch (up to 5 min), no loss of channel activity was ever observed. Similarly, as shown in Fig. 3B, superfusion of the patch by chymotrypsin (300 µg/ml) led to a rapid increase in the activity of the channels, at constant applied pressure, without a change in unit conductance (n = 11). The potential chymotrypsin cleavage sites (aromatic residues) are present in the NH₂ terminus (Fig. 1). However, hydrophobic residues, which are weaker potential targets for chymotrypsin, are present in the COOH terminus. We therefore tested the effect of aminopeptidase. As shown in Fig. 3C, superfusion of the patch by aminopeptidase (500 µg/ml) also led to an increase in the channel open probability at constant applied pressure (n = 6). Taken as a whole these experiments indicate that limited proteolysis of either the COOH or the NH₂ terminus of the channel increased its sensitivity to pressure. In all cases, the channels remained mechanosensitive and could be closed and reopened at will upon decrease or increase of applied pressure.

Previous studies led to the conclusion that most of the COOH-terminal extension plays no role in mechanosensitivity.
Indeed, a mutant (Δ110–136), in which the last 27 residues had been deleted, was still functional, whereas deletion of 6 additional residues suppressed channel activity totally (23, 24). The mechanosensitivity of the Δ110–136 mutant was reported to be similar to that of the wild type. We reconstituted the purified Δ110–136 mutant in giant liposomes. The threshold of activation was not modified, but the slope of the activation curve was enhanced considerably compared with the wild type. Superfusion of excised patches by carboxypeptidase led nevertheless to an increase in channel activity at constant applied pressure (n = 3, not shown). Taken together, these results indicate that deletion of residues of the COOH terminus before and after residue 110 modulates the mechanosensitivity of the channel.

We examined the effect of proteases on channel open probability versus applied pressure described by a Boltzmann distribution. In general, the pressure required for half-activation (where \( P_o = 0.5 \)) varied from patch to patch. The open probability was thus first determined before protease treatment, by

![Graph showing pressure dependence of the MscL channel reconstituted in liposomes before and after superfusion of the proteases in the bath.](image)

The pressure dependence of the MscL channel reconstituted in liposomes before and after superfusion of the proteases in the bath. Open probability \( P_o \) multiplied by the number \( N \) of channels in the patch versus the applied pressure, at fixed membrane potential (+10 mV) before (open circles) and after (full circles) the application of proteases in the bath to an excised inside-out patch is shown. Each point was obtained by integrating the current through open channels in a 15-s segment of recording and by dividing the integral by the time of recording multiplied by the unitary current. The data were fitted to a Boltzmann distribution of the form \( N P_o = N P_{o\max} (1 + \exp \left[ \frac{a}{p_{1/2} - p} \right])^{-1} \), where \( N \) is the unknown number of channels in the patch, \( P_o \) is the open probability, \( P_{o\max} \) is the maximum open probability, \( p \) is the pressure, \( p_{1/2} \) is the pressure at which the open probability is 0.5, and \( a \) is the sensitivity. In panels A, B, C, and F, after determination of open probabilities under control conditions, the indicated protease was applied on gating channels for 5–7 min. The patch was washed, and the new open probabilities were determined again for each value of applied pressure. In D and E, after determination of open probabilities under control conditions, the pressure was released to close the channels, and the indicated protease was applied to the patch for 5–7 min. The patch was then washed before determination of the new open probabilities. Ionic conditions were as in Fig. 3.

Panel A, trypsin (250 μg/ml) superfused on gating channels. \( p_{1/2} \) was 53 versus 44 mm Hg, and \( 1/a \) was 3.9 versus 1.8 mm Hg for the intact and proteolyzed channels, respectively. \( N P_{o\max} \) was 65. Panel B, chymotrypsin (300 μg/ml) superfused on gating channels. \( p_{1/2} \) was 62 versus 28 mm Hg, and \( 1/a \) was 5 versus 3.4 mm Hg for the intact and proteolyzed channels, respectively. \( N P_{o\max} \) was 30. Panel D, chymotrypsin (300 μg/ml) superfused on closed channels. \( p_{1/2} \) was 74.5 versus 42 mm Hg, and \( 1/a \) was 7 versus 3 mm Hg for the intact and proteolyzed channels, respectively. \( N P_{o\max} \) was 80. Panel E, carboxypeptidase (500 μg/ml) superfused on gating channels. \( p_{1/2} \) was 52 versus 36.4 mm Hg, and \( 1/a \) was 3.7 versus 1.3 mm Hg for the intact and proteolyzed channels, respectively. \( N P_{o\max} \) was 5. Panel F, chymotrypsin (300 μg/ml) was first superfused on gating channels followed by carboxypeptidase (500 μg/ml). \( p_{1/2} \) was 67 versus 11.3 mm Hg, and \( 1/a \) was 5.9 versus 1.02 mm Hg for the intact and proteolyzed channels, respectively. \( N P_{o\max} \) was 20.
application of pressure increased stepwise every 15 s. The protease was then superfused on the gating channels for 5–7 min, the patch was washed with control solution, and increased pressure was applied again for the open probability determination (Fig. 4, A, B, C, and F). In control conditions, a full curve could not always be obtained because of the fear of breaking the patch. Nevertheless, a clear shift of the curve to lower pressures was observed after treatment either by trypsin or chymotrypsin or carboxypeptidase, which remained mechanosensitive and could be closed upon release of pressure. Panel C, in contrast, the presence of carboxypeptidase (500 µg/ml) in the pipette did not produce any variation in channel activity. The membrane potential was +10 mV. The ionic conditions were as in Fig. 3.

Furthermore, we examined whether chymotrypsin or carboxypeptidase was effective when applied to closed channels. After application of increased pressure to the patch under control conditions, pressure was released to close the channels, and the protease was superfused for 5–7 min. The patch was then washed with the control solution and the open probability at different pressures was determined again. As shown in Fig. 4, D and E, both chymotrypsin and carboxypeptidase were able to induce a shift of the curve to lower pressure, indicating that they are active on closed channels as well.

**Effects of Proteases Present in the Pipette on Purified MscL Reconstituted in Liposomes**—Outside-out patches cannot be obtained with giant proteoliposomes. To examine the effects of a protease on the outside of a patch, the protease was added to the pipette solution. After seal formation and excision of the patch, pressure was applied rapidly until activation of the channels. The pressure was kept constant, and the channel activity was monitored. Increase in channel activity was observed when chymotrypsin (300 µg/ml) or trypsin (250 µg/ml) was present in the pipette, but an upward drift of the baseline was observed upon application of the pressure. Reduction of trypsin or chymotrypsin concentration to 50 and 60 µg/ml, respectively, completely suppressed the mechanical instability of the patches. Under these conditions, we observed either with trypsin (n = 33) (Fig. 5A) or chymotrypsin (n = 5) (Fig. 5B) an increase in channel activity at constant pressure, without a change in channel unit conductance. After 1 or 2 min, the channels became exquisitely sensitive to pressure, and application of pressure as low as 3–5 mm Hg was in general sufficient to activate the channels. In two cases, the channels were even observed to gate at apparent zero applied pressure. However, the channels remained mechanosensitive in that decrease (or increase) of pressure always resulted in a decrease (or increase) in open probability. We never observed, for instance, a permanently open level, within 20 min of continuous recording. A similar activation of the channels was also observed when Pronase (250 µg/ml), which has no specificity, was present in the pipette (n = 4). Importantly, when control experiments were performed with carboxypeptidase (500 µg/ml) in the pipette, no activation of the channels was observed (n = 9) (Fig. 5C, compare with Fig. 3A). We next examined how the presence of trypsin or chymotrypsin in the pipette affects the relationship between channel open probability and applied pressure. As shown in Fig. 6A, both proteases induce a dramatic shift of the activation curve reflected in a decrease pressure required for half-activation and a significant increase in the sensitivity of the channel to membrane tension. Moreover, the presence of trypsin or chymotrypsin or Pronase in the pipette drastically altered the channel open time. Fig. 6B, which displays successive segments of recordings obtained after seal formation, in the presence of trypsin in the pipette, shows how the channel open time increased progressively with time.

Trypsin or chymotrypsin in the pipette was effective on gating channels but not on closed channels. In a typical experiment in which trypsin was present in the pipette, after excision of the patch, pressure was increased progressively until, at 50 mm Hg, the channels started to gate with a fast kinetics. Pressure was then released immediately to close the channels. After 10 min, pressure was applied again. The channels started to gate at 46 mm Hg. The channel kinetics was similar to that of control experiments characterized by short openings. Pressure was maintained, and the channel activity increased rapidly. After 2 min, the threshold of activation dropped to 3 mm Hg, and the channel kinetics exhibited characteristic long open times (not shown). This type of experiment was repeated four times with trypsin and two times with chymotrypsin with a similar result. We also incubated the giant proteoliposomes for 20 min in a bath containing 50 µg/ml trypsin. The liposomes were then patched with pipettes that contained no trypsin. Under these conditions, the threshold of activation for the channels was always relatively high (between 25 and 60 mm Hg), as in control experiments. The long open times were not observed.

Finally we looked for the effects of proteases on both sides of
FIG. 6. Proteolysis of the external part of the channel alters its pressure dependence and drastically modifies its kinetics. Panel A, open probability $P_o$ versus the applied pressure, at fixed membrane potential (+10 mV), of the MscL channel after activation by trypsin (50 μg/ml) (circles) or chymotrypsin (60 μg/ml) (squares) present in the pipette. After seal formation, pressure was applied, and the activation of the channels was monitored for 10 min until a steady state was reached. The open probability at each applied pressure was then determined. Each point was obtained by integrating the current through open channels in a 15-s segment of recording and by dividing the integral by the time of recording multiplied by the unitary current and the total number of channels in the patch. The data were fitted to a Boltzmann distribution as defined in Fig. 5. After the action of trypsin, $p_{1/2}$ was 5 mm Hg, and $1/\alpha$ was 0.4 mm Hg. After the action of chymotrypsin, $p_{1/2}$ was 7.3 mm Hg, and $1/\alpha$ was 0.4 mm Hg. The broken line represents the Boltzmann distribution obtained by averaging the parameters of all of the control curves (in the absence of proteases) displayed in Fig. 5 ($p_{1/2} = 67$ mm Hg; $1/\alpha = 4.85$ mm Hg). Panel B, trypsin in the pipette altered the channel kinetics, increasing the open time. Successive segments of recordings were obtained at different times, as indicated, following seal formation and excision of the patch, with 50 μg/ml trypsin in the pipette. For each segment, only the amount of pressure necessary to activate one open channel level was applied.

the channel. The full effect of trypsin, present in the pipette, was observed. After that, chymotrypsin was perfused in the bath, followed by carboxypeptidase. A further slight enhancement in the activity of the channels was always observed after perfusion of the proteases in the bath. However, the channels always remained mechanosensitive; a permanently open level was never observed.

DISCUSSION

In this study we examined the effect of different proteases on the mechanosensitivity of MscL either in the native membrane or in a reconstituted system. Our results indicated that MscL had the same orientation in the plasma membrane as in giant liposomes. This conclusion is based on the following findings. First, trypsin or chymotrypsin, when present in the pipette, activated the reconstituted channels in a different manner than when perfused in the bath. Second, carboxypeptidase activated the reconstituted channels when it was perfused in the bath, but not when it was present in the pipette. Therefore, in patch-clamp experiments performed on the plasma membrane and on giant liposomes, the amino and carboxyl termini were accessible from the bath, whereas the periplasmic loop faced the pipette. Because, with the reconstituted system, all experiments always yielded identical results, we concluded that MscL was in all cases reconstituted with the native orientation in giant liposomes.

The experiments performed by applying proteases in the bath clearly indicate that limited proteolysis of either the NH$_2$ terminus or the COOH terminus resulted in an increased mechanosensitivity of the channels. This effect was characterized by both a reduction in $p_{1/2}$ and an increase in the slope of the curve in the Boltzmann distribution. These results modify somewhat the conclusion of previous studies, performed on mutated channels, that most of the COOH-terminal extension plays no role in mechanosensitivity (23, 24). The interest of the
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approach that has been used here is that it allows monitoring of the activity of channels in the same patch before and after application of proteases. The approach that uses mutated channels necessarily implies comparison of channel activities recorded in different patches, which may be more problematic. A limitation of the protease approach, however, is that the precise cleavage sites are unknown. Chymotrypsin cleavage sites are likely to be Phe-7 and Phe-10 (Fig. 1), indicating that the COOH-terminal extension plays a modulatory role, perhaps through interactions with other molecules.

Independently of its importance for the understanding of the channel mechanosensitivity, the finding that a limited proteolysis of the NH2 or COOH terminus of MscL increases its sensitivity to membrane tension might have a physiological relevance. In particular, it is remarkable that the numerous homologs of MscL which have now been found all have substantial cytoplasmic COOH-terminal extension. Several residues in this extension are conserved (25). This fact is difficult to understand, if, as suggested previously, this part of the protein plays no part in the channel function. A suggestion raised by our results is that the COOH-terminal extension plays a modulatory role, perhaps through interactions with other molecules.

The most striking result of this study is that the channel remains mechanosensitive after the external loop has been cleaved. As discussed above, the external loop, the only non-membranous part of the channel on the periplasmic side, is accessible from the pipette in the reconstituted system. Therefore the change in channel kinetics and mechanosensitivity observed when trypsin or chymotrypsin is present in the pipette indicates that the loop was cleaved at one or two of the trypsin- or chymotrypsin-sensitive sites present in this region of the molecule (Fig. 1). Indeed, it is extremely unlikely that cleavage might have occurred at sites present in the transmembrane segments because this is never observed for membrane proteins. Given the symmetry of the homo-oligomer, one may expect that after a steady state has been reached, all of the subunits of the channels have been cleaved. Importantly, the effect of trypsin or chymotrypsin was only observed when the channel was gating, indicating that its opening results in a conformational change of the loop which exposes its protease-sensitive sites. Reconstitution of functional membrane proteins from proteolytic fragments or from genetically cleaved fragments has been reported for membrane protein (for review, see Ref. 26). However, to our knowledge, this is the first time that the activity of a membrane protein has been monitored at the level of a single molecule after cleavage of all of the loops of the protein. That, after this treatment, the channel retained its main characteristic, mechanosensitivity, and that its conductance had not been altered, attest to the importance of the interactions between the transmembrane helices for both the stability and activity of the molecule. We are left with the remarkable conclusion that two unconnected segments plus a few amino acids on each side of the membrane are sufficient for the activity of a mechanosensitive channel. The integrity of the loop seemed not to be essential for mechanosensitivity itself. Therefore the external loop appears to act as a spring that resists opening of the channel and promotes its closure when it is open. Its stiffness sets the level of sensitivity of the channel to membrane tension.

Taken as a whole, the results presented here strongly suggest that all of the different extramembranous parts of the channel counteract the movement of the transmembrane helices triggered by a change in membrane tension. The COOH termini interacting with each other by forming a bundle (19) would resist the movement of the M2 helices. The cytoplasmic NH2 termini may also interact with each other and resist the movement of the M1 helices. The geometry of the external loop counteracts the movement of the two helices relative to each other. This leads us to propose that both M1 and M2 helices move when the channel opens. This could be possible if membrane tension itself, or thinning of the membrane upon stretch, modifies the orientation of each helix in the membrane and/or specifically modifies the interaction in the membrane between the two helices of an MscL subunit. The movement of these helices would be at the basis of mechanosensation for this molecule. It is not known how membrane tension or membrane thinning can affect transmembrane helices in membrane proteins, and it is therefore unclear what makes MscL helices specifically sensitive to membrane tension. The importance of the transmembrane helices for mechanosensation which is proposed here is consistent with a previous report indicating that several residues in both helices are highly conserved in MscL homologs (25). Moreover, a recent study has highlighted the importance of helix M1 for mechanosensitivity. Randomly mutagenized mscL genes were expressed in bacteria that were screened for gain-of-function mutants with impaired growth. The most severe mutations, which resulted in channels gating at anomalous low tension, were found on one facet of transmembrane helix M1 (27).

In the closed conformation, as revealed by x-ray diffraction, the pore formed by M1 helices, is a funnel whose radius varies from 18 Å on the periplasmic side to 2 Å on the cytoplasmic side, where it is occluded (19). Therefore, opening the channel requires a tilting of the M1 helices that are presumed to be pulled away from each other upon application of tension to the membrane. MscL, which has a high conductance and is able to catalyze the release of molecules such as thioredoxin (12), has a very large pore whose diameter was estimated to be around 40 Å by electrophysiological experiments (28). Given the mean distance between α-helices in membrane proteins, it is doubtful that only M1 helices are sufficient to make such a large pore,
and it is therefore probable that M2 helices also participate in the formation of the open pore (28). These considerations lead to the putative model of Fig. 7 which enables us to interpret the whole of the data presented here. An increase in membrane tension modifies the interactions between M1 and M2 helices or between these helices and the core of the membrane causing both helices to tilt, with the M2 helices intercalating between the M1 helices. These movements are opposed by the non-membranous domains, thus setting the level of mechanosensitivity of the channel. On each α-helix, the amino acids immediately adjacent to the membrane on the cytoplasmic side certainly play an important role because their deletion results in an inactive channel (23, 24). This could not be seen in our experiments, probably because these residues are too close to the membrane to be accessible to proteases, but it is of interest to note that trypsin or chymotrypsin, perfused in the bath, totally abolished the activity of another mechanosensitive channel, MscS, in patch-clamp experiments performed on the native membrane (29). It is possible that these cytoplasmic residues are important to keep the helices in an appropriate orientation relative to the plane of the membrane.

Are the results described here relevant for the understanding of the molecular mechanisms of mechanosensitive channels outside the bacterial domain? Although bacterial mechanosensitive channels have been shown to gate in response to bilayer tension alone, it is usually considered that the tethered model is more likely to describe the functioning of eukaryotic mechanosensitive channels. In this model, a direct connection is hypothesized between the gate of the channel and proteins located in the extracellular matrix and/or the cytoskeleton (30). A displacement of the channel relative to the extracellular matrix or the cytoskeleton will cause the channel to open or close. In the case of mechanotransduction in Caenorhabditis elegans, for instance, several non-membranous proteins are believed to interact with the mechanosensitive channel (31, 32). The MEC-4 and MEC-10 proteins, which belong to the DEG/ENaC superfamily, are likely to encode subunits of this mechanosensitive channel. These proteins have a similar predicted topology that includes two transmembrane domains, an extracellular loop, and internal COOH and NH2 termini (31, 32). It is clear, however, that modifications of the cytoskeleton might alter the mechanical properties of the membrane. Furthermore, the results presented here indicate that interactions with the end terminals and/or the loop of such proteins might greatly alter their mechanosensitivity, even if the channels are gated by membrane tension alone. Although no sequence similarity exists between MscL and the members of the DEG/ENaC superfamily, the overall structural similarity is striking. It is therefore possible that some of these channels share a common mechanism, for which the scheme outlined above could provide a working model.

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