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ARTICLE

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The role of the periplasmic loop residue glutamine 65 for MscL mechanosensitivity

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Abstract The periplasmic loop of MscL, the mechanosensitive channel of large conductance, acts as a spring resisting the opening of the channel. Recently, a high-throughput functional screening of a range of MscL structural mutants indicated that the substitution of residue glutamine (Q) 65 with arginine (R) or leucine (L) leads to a wild-type (WT)-like and a loss-of-function (LOF) phenotype, respectively (Maurer and Dougherty *J. Biol. Chem.* 278(23):21076–21082, 2003). We used electron paramagnetic resonance (EPR) spectroscopy, single-channel recording and in vivo experiments to investigate further the effect of R and L mutation of Q65 on the gating mechanism of MscL. Structural analysis of Q65R and Q65L was carried out by coupling the site-directed spin labeling (SDSL) with EPR spectroscopy. A SDSL cysteine mutant of the isoleucine 24 residue (I24C-SL) in the first transmembrane domain, TM1, of MscL served as a reporter residue in EPR experiments. This was due to its strong spin–spin interaction with the neighboring I24C-SL residues in the MscL channel pentamer (Perozo et al.*Nature* 418:942–948, 2002). The effects of bilayer incorporation of lysophosphatidylcholine on the MscL mutants were also investigated. Functional analysis was carried out using patch-clamp recordings from these mutants and WT MscL reconstituted into artificial liposomes. Although our data are largely in agreement with the high-throughput mutational analysis of Maurer and Dougherty, this study shows that Q65R and Q65L form functional channels and that these mutations lead to partial gain-of-function (GOF) and LOF mutation, respectively. Overall, our study confirms and advances the notion that the periplasmic loop plays a role in setting the channel mechanosensitivity.

Keywords Mechanosensitive channel · Electron paramagnetic resonance · Lysophosphatidylcholine · Patch-clamp · Osmoregulation

Abbreviations ANOVA: Analysis of variance · D/R: Dehydration/rehydration · EPR: Electron paramagnetic resonance · GOF: Gain of function · GST: Glutathione S-transferase · Hepes: *N*-(2-Hydroxyethyl)piperazine-*N*′-ethanesulfonic acid · ID: Inner diameter · IPTG: Isopropyl-*β*-D-thiogalactoside · LB: Luria–Bertani · LOF: Loss of function · LPC: Lysophosphatidylcholine · MS: Mechanosensitive · MscK: Potassium-regulated mechanosensitive channel · MscL: Mechanosensitive channel of large conductance · MscS: Mechanosensitive channel of small conductance · OD: Outer diameter · PBS: Phosphate-buffered saline · SD: Standard deviation · SDSL: Site-directed spin labeling · TM1: First transmembrane domain · TM2: Second transmembrane domain · TPX: Specially designed polymethylpentene · WT: Wild type

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Introduction

Mechanosensitive (MS) channels are gated in response to mechanical stress exerted onto the cellular membrane. Bacterial MS channels were shown to directly respond to membrane tension developed solely in the bilayer membrane without the involvement of cytoskeletal

elements (Martinac et al. 1990; Markin and Martinac 1991; Hamill and Martinac 2001). Also, the addition of amphipathic compounds into different leaflets of the lipid bilayer were found to cause activation of bacterial MS channels without the application of negative pressure (Martinac 2004).

The structural model of Eco-MscL, where MscL is the MS channel of large conductance, in the closed state (Fig. 1) showed that the S1 helix and the linker region (residues 1–14) are connected to the first transmembrane domain, TM1, helix (residues 15–45). The TM1 is linked to the second transmembrane domain, TM2, helix (residues 76–100) by a periplasmic loop, the S2 (residues 46–75) and is followed by a C-terminal cytoplasmic helix, S3 (residues 101–110) (Sukharev et al. 2001; Gullingsrud and Schulten 2003). In the determination of the functional domains of the MscL, recombinant DNA techniques and site-directed mutagenesis have been employed to dissect the molecular basis of the gating mechanism of the MscL (Blount et al. 1996, 1997; Häse et al. 1997; Ou et al. 1998; Yoshimura et al. 1999). These studies revealed the importance of the highly conserved TM1 in channel-gating (Yoshimura et al. 1999; Ou et al. 1998) and the contributions of the extramembranous domains of the MscL, acting as a spring to resist the opening of the closed channel (Ajouz et al. 2000; Maurer et al. 2000; Park et al. 2004).

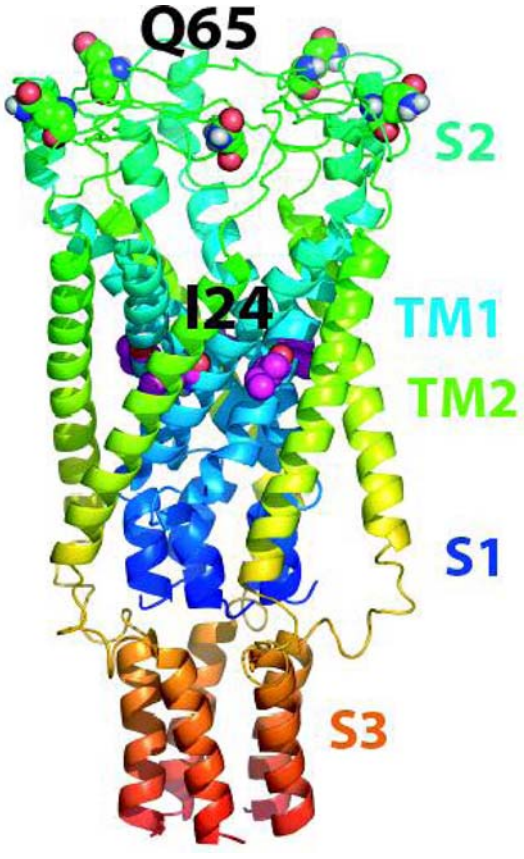

Fig. 1 The structure of the mechanosensitive channel of large conductance (*MscL*). *Balls* represent residues glutamine 65 (*Q65*) in the periplasmic loop and isoleucine 24 (*I24*) on the first transmembrane domain (*TM1*) helices.

Several studies have suggested that mutagenesis of the MscL alters the gating mechanism of the channel and leads to two distinct phenotypes (Blount et al. 1996, 1997; Ou et al. 1998; Yoshimura et al. 1999, 2001; Levin and Blount 2004). Gain-of-function (GOF) mutations increase the probability of spontaneous MscL gating and require lower membrane tension than the wild-type (WT) channel to open. Loss-of-function (LOF) mutations decrease the probability of spontaneous MscL gating and require higher membrane tension to gate the channel compared with the WT. Growth experiments were employed to differentiate between the two basic mutational phenotypes: GOF and LOF (Blount et al. 1997; Moe et al. 2000; Ou et al.1998; Yoshimura et al. 1999; Levina et al. 1999). Overall, GOF phenotypes were found to impair the growth of bacterial cells, whereas LOF phenotypes did not impair the growth (Blount et al. 1997; Moe et al. 2000; Maurer et al. 2000; Ou et al. 1998; Yoshimura et al. 1999; Levina et al. 1999). The results of growth experiments correlated well with single-channel recordings, which showed an increase in pressure sensitivity of the MscL GOF phenotypes. A decrease in pressure sensitivity or complete lack of channel activation in the LOF phenotype was also observed. The results indicated that in the presence of the inducing agent isopropyl-β-D-thiogalactoside (IPTG), GOF mutants could be identified by their poor growth rate or the total inhibition of the growth state (Blount et al. 1997; Ou et al. 1998; Yoshimura et al. 1999; Moe et al. 2000; Maurer et al. 2000). The LOF mutants were phenotypically characterized by an increase in bacterial death upon exposure to hypo-osmotic shock (Levina et al. 1999). Recently, a high throughput screening for mutagenic analysis of *E. coli* MscL (Maurer and Dougherty 2003) suggested that the mutagenesis of glutamine residue Q65 into hydrophobic leucine (L) led to a LOF mutation and the substitution with basic amino acid residue, arginine (R) at this position caused a WT mutation.

In this study, we examined in detail the role of glutamine residue Q65 at the tip of the periplasmic loop for MscL mechanosensitivity using a functional patch-clamp and structural spectroscopic analysis of mutated MscL proteins. Structural analysis of Q65 MscL mutants was carried out using cysteine mutagenesis and site-directed spin labeling (SDSL) coupled with electron paramagnetic resonance (EPR). This is an alternative strategy to X-ray crystallography, for example, in obtaining structural information on proteins since the mobility of the spin label is influenced by the local structures at the target residue, which can be determined by EPR spectroscopy (Biswas et al. 2001). We employed this method to monitor the effect of mutation on Q65 into R and L in the periplasmic loop of the MscL by monitoring the spectroscopy changes at the residue I24 that had been mutated into cysteine I24C on the TM1 and was chosen to be the reference residue because of its strong spin–spin interaction with neighboring I24C in the MscL pentamer

(Perozo et al. 2002a). In addition, the effects of lysophophatidylcholine (LPC) on the gating mechanism of MscL Q65 mutants were investigated as EPR experiments conducted by Perozo et al. (2002b) have illustrated that bilayer incorporation of LPC strongly favors the transition to the open state . Functional patch-clamp studies were carried out with MscL WT and mutant proteins reconstituted in liposomes. In vivo experiments under hypo-osmotic conditions were also conducted to discriminate osmotic lysis phenotype by applying osmotic downshock to the WT and mutant bacterial cells.

Methods

Production, expression and purification of glutamine 65 mutants of MscL

The mutation of glutamine 65 to L or R was performed by oligonucleotide mismatch site-directed mutagenesis using the Transformer Mutagenesis kit (BD Biosciences, Australia) and confirmed by DNA sequencing. The plasmid pGEX1.1 (Häse et al. 1995) containing WT MscL as a gene fusion with glutathione S-transferase (GST) was used as a template for mutagenesis. The primers CGC GAT GCG CTG GGG GAT ATC and CGC GAT GCG CGG GGG GAT AT for the mutation Q65L and Q65R, respectively, and the selection primer CGA GGT CGA CAA GCT TAT CGT GAC TG, which changes from the unique *Eco*RI site in pGEX1.1 to *Hind*III, were obtained from Sigma Genosis (Australia). The plasmids containing the mutants MscLQ65L and MscLQ65R were introduced into the triple KO ($MscL^{-}$, $MscS^{-}$, $MscK^{-}$, where MscS is the MS channel of small conductance and MscK is a potassium-regulated MS channel) MJF465 *E. coli* strains.

The purification of the GST-MscL mutant proteins (GST-Q65R and GST-Q65L) was as previously described (Häse et al. 1995). Briefly, after protein expression had been induced by 0.5 mM IPTG, membranes were solubilized in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.2, adjusted with NaOH) with 1.5% octylglucoside at room temperature overnight. The suspension was collected and 1 mL of glutathione-Sepharose 4B beads was added to the supernatant and the mixture was rocked at room temperature overnight. The beads were washed in PBS containing 1.5% octylglucoside and thrombin (1 unit/μg of protein) was added to the final wash. Mutant channel proteins were eluted off the column after 16 h and the final protein concentration was determined following established methods (Lowry et al. 1951; Bio-Rad Laboratories, USA).

Generation of cysteine mutants

Cysteine mutants I24C, Q65R/I24C and Q65L/I24C were generated by oligonucleotide mismatch site-directed mutagenesis using the Transformer Kit (Clontech Laboratories , USA), following the manufacturer's method. The mutagenic primers GGT GGG TGT CTG TAT CGG TGC GG, CGC TAC GCG ATG CGC GGG GGG ATA TCC CTG C and GCT ACG CGA TGC GCT GGG GGA TAT CCC TG were used for the mutation of I24C, Q65R/I24C and Q65L/I24C. *E. coli* strain M15 (pREP4:: Kan) (Qiagen, USA) was used as a host. The mutations were confirmed by dideoxy DNA sequencing.

The purification of the cysteine mutant was carried out as previously described (Blount et al. 1996). *E. coli* M15 strains harboring the mutated MscL were cultured and protein expression of 6 times His-tagged recombinant MscL mutant protein was induced with 0.5 mM IPTG. The membranes were solubilized in phosphate buffer (50 mM NaH_2PO_4, 300 mM NaCl, 1 mM imidazole, 1% Triton X, pH 8.0) with 0.1 mM tris(2-carboxyethyl)phosphine reducing agent (0.1 mM). All buffers were prebubbled with N_2 gas prior to use to minimize the formation of the disulfide bonds of the cysteine mutants. After the soluble protein fraction had been collected, Ni-nitrilotriacetic acid agarose resin was added to the supernatant and proteins were eluted off the column on the next day then desalted through a PD-10 desalting column (Amersham Pharmacia Biotech, NJ, USA). The protein was concentrated to approximately 2 mL using a Millipore stirred ultrafiltration unit (Amicon) through a regenerated cellulose filter and the concentration was determined as described earlier.

Protein reconstitution and electrophysiological recordings

Azolection lipids were prepared as described previously (Delcour et al. 1989; Sukharev et al. 1993). Lipids were dissolved in chloroform and dried under nitrogen to produce a fine lipid layer, resuspended in dehydration/rehydration (D/R) buffer [200 mM KCL, 5 mM *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid, Hepes, pH 7.2 with KOH] then bath-sonicated for 5 min. Purified MscL was added at the required protein-to-lipid ratio and incubated at room temperature for 60 min. Detergent was removed with the addition of Bio-Beads and incubation at room temperature for further 3 h. The proteoliposomes were collected by ultracentrifugation and resuspended in 40 μL D/R buffer. Aliquots of proteoliposomes were spotted onto glass slides and dehydrated for 4–6 h (vacuum dessicator, 4°C) followed by overnight rehydration under humid conditions. The improved patch-clamp technique (Hamill et al. 1981) was used to record single-channel currents of MscL from isolated membrane patches. An aliquot of the rehydrated proteoliposomes (4 μL) was placed in the ~800 μL patch-clamp chamber containing recording solution (200 mM KCl, 40 mM $MgCl_2$, 5 mM Hepes, pH 7.2 with KOH). Single-channel currents were

recorded using borosilicate glass pipettes, having a resistance of 4–6 MΩ. A gigaohm seal formed immediately between the pipette tip and the liposome by applying brief suction. After the formation of the seal, the pipette tip was passed through the solution–air interface to rupture the outer face of the closed liposome vesicle. The patch current signal was amplified and filtered (1 kHz) with an Axopatch 1D amplifier (Axon Instruments, Union City, CA, USA) and digitized at 5 kHz with Digidata 1200B using pClamp 8 acquisition software. Pressure applied via suction to the pipette was monitored with a piezoelectric pressure transducer (Omega Engineering, Stamford, CT, USA).

Spin labeling and EPR spectroscopy

Purified cysteine proteins were spin-labeled twice for 1 h with methanethiosulfonate spin label (Toronto Research) at 10:1 (label-to-channel) molar ratio. Labeling was carried out in detergent solution at room temperature and the proteins were stored at 4°C once they hade been spin-labeled. The excess, unreacted label was removed by dialysis in desalting buffer at 4°C. Proteins were reconstituted at a 1:500 channel-to-lipid molar ratio. After the detergent (Triton X) had been removed using Bio-Beads, the proteoliposomes were collected by ultracentrifugation at 100,000*g* (Beckman TL-100).

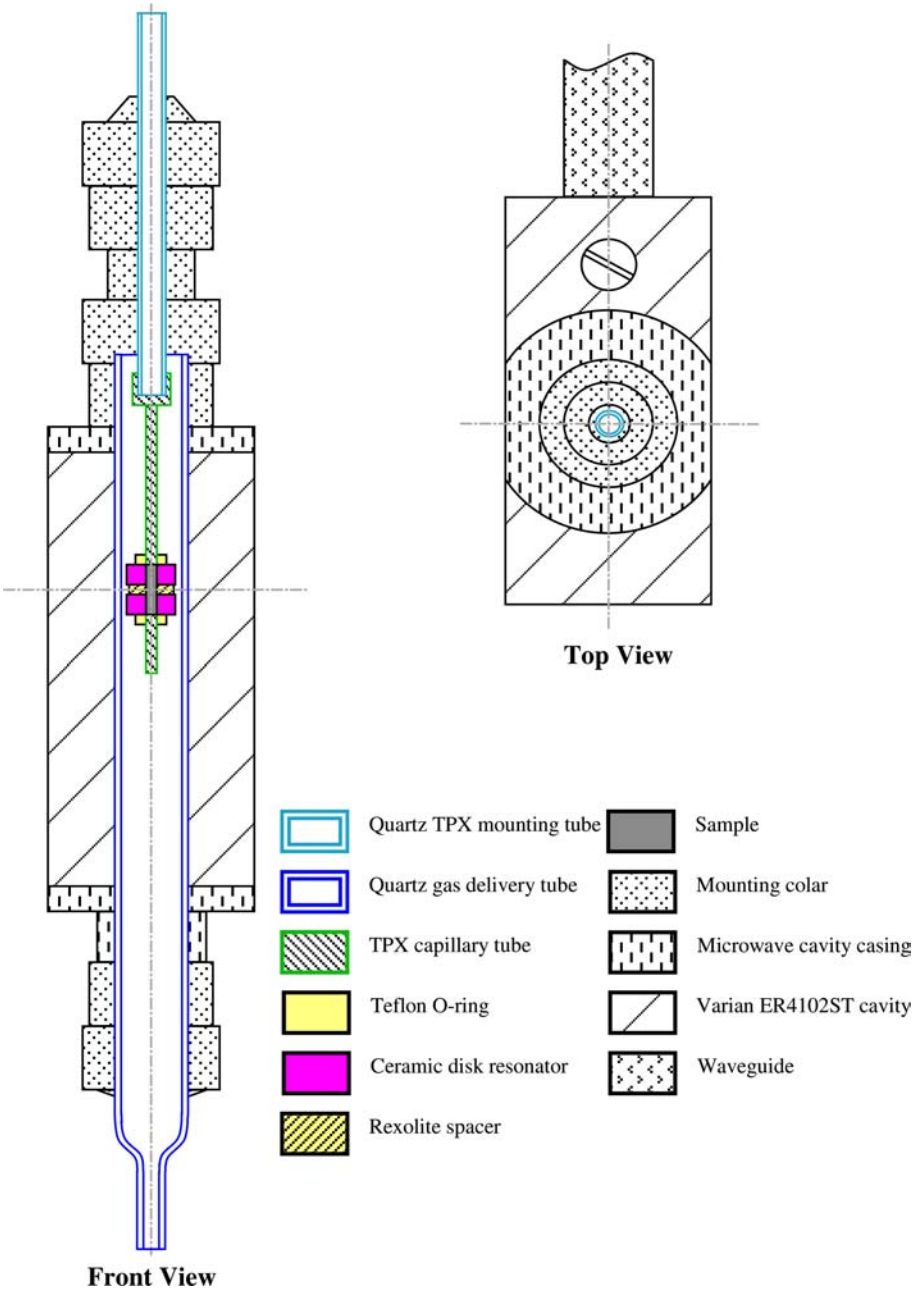

Fig. 2 Schematic representation of a pair of ceramic disk resonators in a Varian ER4102ST microwave cavity. Shown at 1:1 scale

Table 1 Electrophysiological properties of reference I24C and cysteine spin label mutants

MscL mutants	Percentage of patches exhibiting current (%)	Opening threshold pressure (mmHg)	Conductance (nS)
I24C	90.48 ($n=63$)	122.46 ± 34.50 ($n=57$)	3.19 ± 0.26 ($n=43$)
Q65R/I24C	78.38 ($n=74$)	138.10 ± 36.00 ($n=58$)	3.14 ± 0.36 ($n=36$)
Q65L/I24C	17.54* ($n=57$)	152.00 ± 63.74** ($n=10$)	3.00 ± 0.21 ($n=7$)

Shown are the mean ± the standard deviation (*SD*) of *n* patches.
MscL is mechanosensitive channel of large conductance
*$p=0.001$, **$p=0.023$ from I24C and Q65R/I24C as determined by analysis of variance (*ANOVA*)

Table 2 Properties of MscL glutathione S-transferase (*GST*)-Q65X mutants (mean ± SD)

MscL mutants	Percentage of patches exhibiting current (%)	Opening threshold pressure (mmHg)	G (nS)	$P_{1/2}$ (mmHg)	$1/\alpha$ (mmHg)	ΔG_0 (kT)
Wild type	100.00 ($n=37$)	73.24 ± 22.86 ($n=37$)	3.18 ± 0.31 ($n=37$)	98.26 ± 7.75 ($n=9$)	5.14 ± 1.83 ($n=9$)	21.08 ± 6.78 ($n=9$)
GST-Q65R	100.00 ($n=36$)	65.56 ± 17.96 ($n=36$)	3.22 ± 0.26 ($n=33$)	78.03 ± 16.95 ($n=10$)	4.35 ± 0.77 ($n=10$)	18.51 ± 5.63 ($n=10$)
GST-Q65L	100.00 ($n=48$)	86.60 ± 23.79* ($n=48$)	3.19 ± 0.23 ($n=42$)	92.86 ± 24.97 ($n=13$)	2.78 ± 0.98** ($n=13$)	35.50 ± 10.78*** ($n=13$)

Shown are the results from ANOVA.
G is the conductance of a single MscL channel, $P_{1/2}$ is the negative pressure at which the channel is opened 50% of the time, $1/\alpha$ is the channel sensitivity to negative pressure and ΔG_0 is the difference in free energy between the closed and open conformations of the channel.
*$p=0.0005$, **$p=0.0003$, ***$p=0.0001$ for channel properties compared with two other groups.

EPR spectra of spin-labeled MscL proteins were collected on an ESP 300E spectrometer (Bruker) coupled to an ER4102ST microwave cavity (Bruker). The system employed a pair of ceramic disk resonators, model DRT071E020C031A (Murata Manufacturing Co., Kyoto, Japan). The disks were reported to have a resonant frequency in the range of 9.18–9.98 GHz and a minimum quality factor, Q, of 20,000 at a microwave frequency of 10 GHz (RESOMICS 2000). Each disk had an outer diameter (OD) of 7.07 ± 0.05 mm, an inner diameter (ID) of 2.0 ± 0.1 mm, and a thickness of 3.14 ± 0.05 mm. The dielectric resonators were mounted on specially designed polymethylpentene (TPX) (Boedeker Plastics) capillary tubes, 1.60 ± 0.03-mm OD, 0.95 ± 0.03-mm ID. These tubes were permeable to both oxygen and nitrogen (Altenbach et al. 1994). The resonators were separated by a Rexolite (Boedeker Plastics) spacer, 1.56 ± 0.03-mm thick. Rexolite was selected for its low-dielectric loss and high stability properties, making it well suited to microwave applications (Boedeker Plastics; Jaworski et al. 1997; San Diego Plastics). The spacer was shown experimentally to yield maximum instrument sensitivity. The resonators and the spacer were secured to the TPX capillary tubes using a Teflon O-ring at either end. Sample volumes of approximately 8 μL were then loaded into the capillary tubes, such that the sample length was surrounded by the resonator assembly. The combined apparatus was mounted in the center of the microwave cavity via a 4.02 ± 0.03-mm OD quartz tube, inserted into the ends of the TPX capillary tubes. A 10.56 ± 0.03-mm OD, 8.76 ± 0.03-mm ID quartz gas delivery tube was inserted into the cavity so that it surrounded the TPX–ceramic disk assembly. This tube lowered the resonant frequency of the system and allowed the introduction of oxygen and nitrogen gas into the sample. Figure 2 shows a schematic representation of the apparatus. The combined system had a resonant frequency of 9.88–9.91 GHz and a Q of 1,400 in the presence of an aqueous sample. The resonant frequency could be adjusted with variations to the gap width between the two ceramic disks. This, however, lead to a decrease in the signal-to-noise ratio.

LPC incorporation in the bilayer

Single stocks of reconstituted, spin-labeled MscL protein were divided into six identical aliquots and LPC was added at the desired lipid-to-lipid molar ratio. Sample volumes were held constant. All the spectra were collected under the same conditions as described before.

In vivo assays: downshock experiments

Fresh overnight cultures of MscL mutants were diluted 1:100 into Luria–Bertani (LB) medium with appropriate antibiotics and grown to an optical density of 0.5–0.6 at 600 nm. The cultures were then combined with an equal volume of prewarmed LB medium plus antibiotics and 2 mM IPTG and grown for 1 h. Osmotic downshock

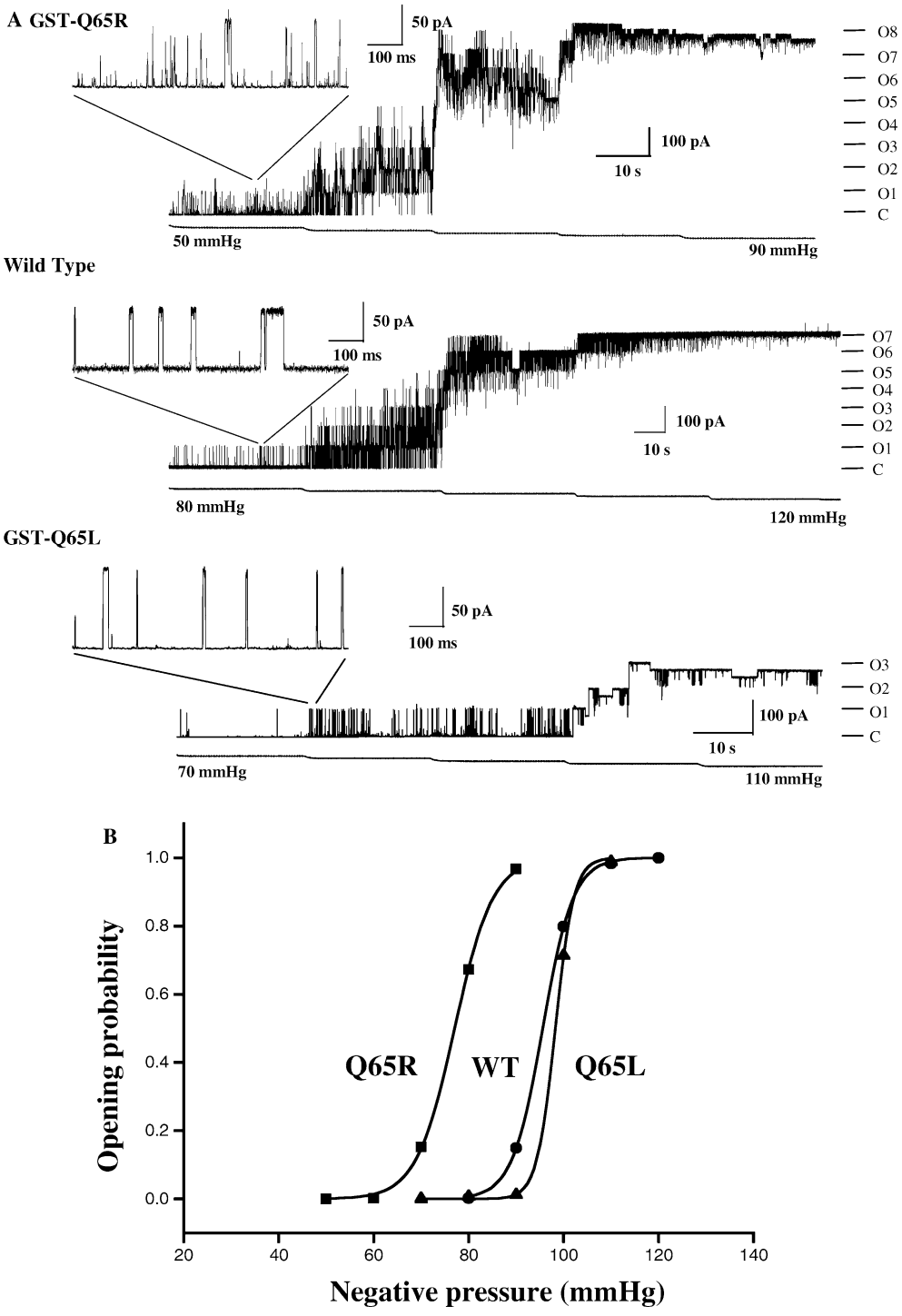

Fig. 3 Activation of MscL wild-type (*WT*) and glutathione S-transferase (*GST*)-Q65 mutants by negative pressure. **A** Current traces. Channel activities of MscL WT and MscL GST-Q65X were recorded at a pipette potential of +30 mV. *C* and O_n indicate closed and open states of *n* channels. The *lower trace* is the pressure trace and each pressure step is 10 mmHg. **B** The Boltzmann distribution curve for MscL WT and MscL Q65X. The negative pressures at which the open probability equals 50%, $P_{1/2}$, for GST-Q65R, WT and GST-Q65L are 77.22, 95.59 and 98.24 mV, respectively. The $1/\alpha$ values, where α describes the sensitivity to negative pressure of the channel, are 4.48, 3.21 and 1.94 mmHg and the free-energy ΔG_0 values are 17.14, 29.75 and 50.64 *kT*, respectively

was applied by 1,000-fold dilutions in sterile double-distilled water, whereas controls were either diluted into LB medium or LB plus 500 mM NaCl (LB500) followed by 20-min incubation at room temperature. Serial dilutions were then carried out for all samples and the samples were spotted onto LB plates with antibiotics. Viability following the osmotic shock was assessed by viable colonies plate count.

Results

Electrophysiological analysis of mutant channels

Cysteine mutation at position I24 caused an increase in the channel opening threshold pressure compared with WT MscL (Tables 1, 2). Q65X/I24C enhanced further

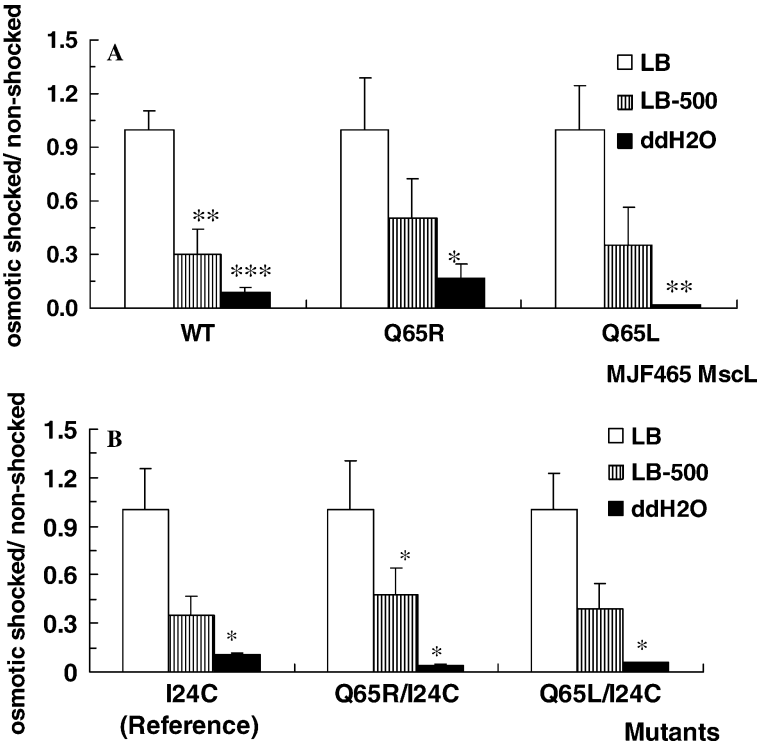

Fig. 4 In vivo studies of bacteria expressing MscL WT and mutants. **A** Ratio of osmotic-shocked to nonshocked for MJF465 MscL WT and Q65 mutants. *Error bars* show the standard errors of six experiments. Shown are the results from the Student *t* test, where $*p \leq 0.05$, $**p \leq 0.01$ or $***p < 0.001$ for cells grown in Luria–Bertani (*LB*) medium (nonshocked) compared with the same strain grown in LB500 or survival of cells upon osmotic downshock (ddH_2O). **B** Ratio of osmotic shocked to nonshocked for the reference I24C mutants Q65R/I24C and Q65L/I24C. Shown are the ratios ± the standard errors of four independent experiments; $*p < 0.05$ from the cell growth for the same strain in LB medium (nonshocked) as determined by the Student *t* test

this increase twofold (Table 1). In addition, the percentage of patches exhibiting current for the Q65L/I24C channel was very low compared with that of other mutants (Table 1). On the other hand, the effect of a single mutation at the Q65 locus was less, since channel activities could be recorded for both mutants (Fig. 3a). The opening threshold pressure for Q65R was similar to that of the WT, whereas Q65L required higher pressure for channel opening. The estimated free energy, ΔG_0, for Q65L was higher than that of MscL WT or Q65R (Table 2).

In vivo assays

This assay was designed to test the ability of expressed mutant MscL channels in rescuing *E. coli* cells from a severe hypo-osmotic shock. We expressed both MscL Q65 mutants in *E. coli* strain MJF465 (Δ*mscL* Δ*yggB* Δ*KefA*), which has a KO background for MscL, MscS, and MscK and MscL Q65/I24C mutants in *E. coli* strain M15. The results are expressed as the ratio of osmotic-shocked to nonshocked. Q65R showed better growth at higher osmolarity (LB500) and upon osmotic downshock (ddH_2O) compared with the WT strain. Mutant Q65L was less able to rescue bacterial cells from the severe hypo-osmotic shock (ddH_2O) (Fig. 4a). In vivo studies of I24C and Q65L/I24C showed similar results (Fig. 4b). However, Q65R with a cysteine replacement at the isoleucine I24 site increased the bacterial death upon osmotic downshock (ddH_2O), indicating that Q65R/I24C mutant cells were less viable upon downshock compared with both I24C and Q65L/I24C (Fig. 4b).

EPR spectroscopy studies

The introduction of the SDSL mutagenesis to a protein is essential for EPR studies. As described in the "Introduction", cysteine, which replaced residue isoleucine I24, which exhibits strong spin–spin interaction with its neighboring I24C residues in the MscL homopentamer (Perozo et al. 2002a), was chosen as the reference residue in EPR studies to report the changes in MscL mechanosensitivity caused by mutations at the glutamine Q65 site (Fig. 1). The structural changes were monitored by variations in the EPR spectra lineshape, which correspond to probe dynamics (H_0). The bilayer incorporation of LPC into the MscL was shown to cause massive EPR spectra changes owing to the changes in the transbilayer pressure profile (Perozo et al. 2002b). Narrowing of EPR spectra suggested that the addition of LPC strongly favored the transition to the open state of the MscL (Perozo et al. 2002b). Hence, the effects of increasing concentrations of LPC on the spin-labeled samples were examined. Additions of LPC to the liposome suspension showed an increase in the mobility of the spin label, as characterized by the sharpening of the peak in the EPR spectra (Fig. 5a). Dose-response curves to LPC were also obtained for the reference residue I24C and for mutants Q65R/I24C and Q65L/I24C by monitoring changes in their mobility parameter (ΔH_0) in the presence of LPC (Fig. 5b).

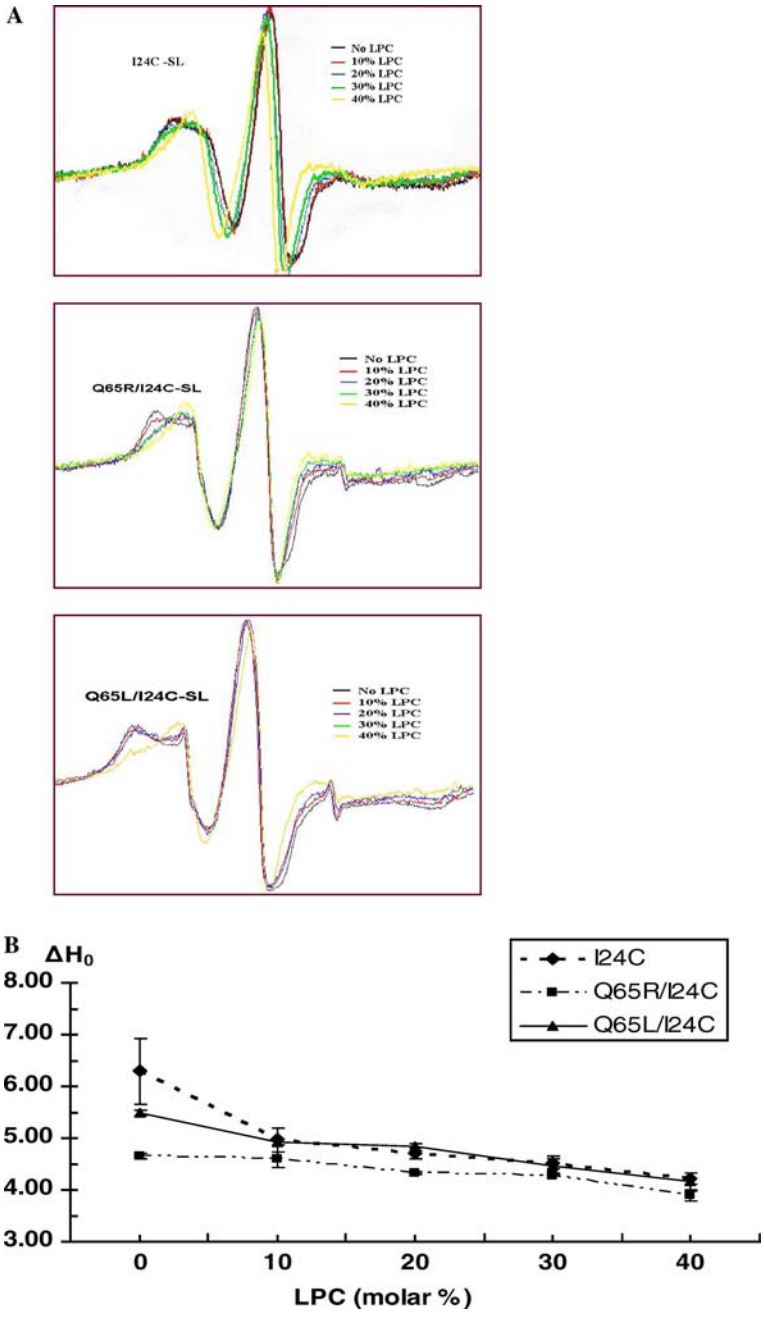

Fig. 5 **A** Room temperature continuous wave electron paramagnetic resonance spectra for the reference residue I24C and mutants Q65R/I24C and Q65L/I24C. Changes in spectra lineshape at increasing concentrations (mol%) of lysophosphatidylcholine (*LPC*). **B** Dose response curves for each mutant as changes in probe mobility versus concentration of LPC. *Error bars* show standard errors of three independent experiments

Discussion

Overall, our results are in accordance with the study conducted by Maurer and Dougherty showing that mutation of glutamine 65 into R and L leads to WT and LOF mutation. The single-channel recordings in our study showed that all mutant channels were fully functional (Fig. 3). In general, all I24C mutant channels required more pressure to open compared with WT MscL and Q65X single mutants (Table 1). Q65R mutation on average increased the pressure sensitivity of the channel but Q65L was less sensitive to membrane tension and required more pressure to open the channel. These results are in accordance with in vivo experiments, which showed that WT MscL and Q65R could survive better upon osmotic downshock compared with Q65L (Fig. 4a). In vivo studies of Q65R/I24C gave somewhat confusing results. In these experiments Q65R/I24C appeared to be more a LOF mutant than Q65L/I24C. However, LOF assays on GOF mutants, which have misfunctional channels, are difficult to interpret because the cells are already stressed (Fig. 4b). Thus, these findings seem to indicate that Q65L and Q65R are partial LOF and partial GOF mutants.

In the EPR studies, the effect of the mutation of the Q65 residue in the periplamic loop on MscL mechanosensitivity was examined by monitoring the mobility of

the reference residue I24C in the TM1 helix (Fig. 1). The substitution of the acidic Q65 residue with a basic R increased the mobility of the spin label, indicating free tumbling of the spin label at the I24C site. Hydrophobic substitution of Q65 into L showed no statistical difference to I24C (Table 3). Thus, the EPR experiments seem consistent with in vivo experiments, suggesting that Q65R/I24C channel is easier to open owing to its partially open conformation. Mutants I24C and Q65L/I24C appear to be in the closed conformation and harder to open.

Incorporation of LPC into the bilayer reconstituted with the reference residue I24C resulted in increased mobility of the spin label (Table 3). The narrowing of the spectra generated by LPC indicates a conformation towards the open state of the MscL. These results are consistent with the previous study (Perozo et al. 2002b), which demonstrated that addition of LPC strongly favored the transition of MscL to the open state.

The LPC-induced conformational changes of the channel could also be observed for Q65L/I24C, which showed statistical difference in probe mobility between no LPC and with increasing concentration of LPC ($p < 0.05$) (Table 3). On the other hand, Q65R/I24C showed statistical difference only after 40 mol% of LPC had been added (Table 3). The results suggest that Q65L/I24C is in the closed conformation, which correlates well with the channel requiring higher membrane tension to open compared with the WT channel. In contrast, the Q65R/I24C channel seems to exist in an intermediate conformation state on the way towards the open state, such that higher concentrations of LPC were required to observe a change in mobility of the spin label.

In summary, our observations obtained from single-channel recordings, in vivo studies and EPR experiments indicate that mutagenesis of the Q65 in the periplasmic loop of the MscL to basic R and hydrophobic L leads to partial GOF and partial LOF phenotypes, respectively. This result is in agreement with a study conducted by Li et al. (2004), who showed that substitutions at this position by tyrosine (Q65Y) or proline (Q65P) acted as a ''general'' suppressor of other GOF mutants (G26S and V23A). Together with the results of the study by Li et al. (2004) and the study by Maurer and Dougherty (2003) showing that mutagenesis of Q65 to a more hydrophobic residue leads to a LOF mutant whereas hydrophilic substitution results in a GOF mutation, our findings support the idea that hydrophilicity at the Q65 site is important for the proper function of the MscL. Future studies will be directed to further detailed characterizations of other critical regions in the periplasmic loop of MscL using the same functional and structural approach.

Table 3 Changes in probe mobility (ΔH_0) at increasing concentration of lysophosphatidylcholine (*LPC*) (mol%)

LPC	I24C	Q65R/I24C****	Q65L/I24C
0	6.287 ± 0.639	4.642 ± 0.060	5.475 ± 0.053
10	4.963 ± 0.221*	4.587 ± 0.153	4.919 ± 0.043**
20	4.712 ± 0.107*	4.324 ± 0.032	4.825 ± 0.060**
30	4.527 ± 0.125*	4.282 ± 0.013	4.458 ± 0.126***
40	4.213 ± 0.111*	3.883 ± 0.105***	4.158 ± 0.175***

Shown are the means ± the standard errors of three independent experiment and the results from the paired test.
*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ for the changes in probe mobility of the mutant without LPC compared with the same mutant with different concentrations of LPC. ****$p = 0.0465$ from I24C and Q65L/I24C as determined by one-way ANOVA

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