A lipid-mediated conformational switch modulates the thermosensing activity of DesK

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The thermosensor DesK is a multipass transmembrane histidine-kinase that allows the bacterium *Bacillus subtilis* to adjust the levels of unsaturated fatty acids required to optimize membrane lipid fluidity. The cytoplasmic catalytic domain of DesK behaves like a kinase at low temperature and like a phosphatase at high temperature. Temperature sensing involves a built-in instability caused by a group of hydrophilic residues located near the N terminus of the first transmembrane (TM) segment. These residues are buried in the lipid phase at low temperature and partially "buoy" to the aqueous phase at higher temperature with the thinning of the membrane, promoting the required conformational change. Nevertheless, the core question remains poorly understood: How is the information sensed by the transmembrane region converted into a rearrangement in the cytoplasmic catalytic domain to control DesK activity? Here, we identify a "linker region" (KSRKERERLEEK) that connects the TM sensor domain with the cytoplasmic catalytic domain involved in signal transmission. The linker adopts two conformational states in response to temperature-dependent membrane thickness changes: (*i*) random coiled and bound to the phospholipid head groups at the water-membrane interface, promoting the phosphatase state or (*ii*) unbound and forming a continuous helix spanning a region from the membrane to the cytoplasm, promoting the kinase state. Our results uphold the view that the linker is endowed with a helix/random coil conformational duality that enables it to behave like a transmission switch, with helix disruption decreasing the kinase/phosphatase activity ratio, as required to modulate the DesK output response.

signal transduction | membrane–protein interaction | two-component system | sensor biophysics

DesK is the histidine kinase of the DesKR two-component system. It is a membrane protein with five transmembrane segments and a cytoplasmic catalytic domain containing the dimerization and histidine phosphotransferase (DHp) domain and the ATP-binding (ABD) domain. DesK is a bifunctional enzyme such that when stimulated acts as a kinase and when not stimulated acts as a phosphatase for its cognate response regulator, DesR (1–3). Conditions that decrease the order of membrane lipids (cold-shock, growth in media devoid of branched-chain amino acids) activate the kinase conformation and result in phosphorylation of the response regulator DesR (4, 5). Once phosphorylated, DesR activates transcription of the target gene Δ5-*des*. The Δ5-desaturase enzyme inserts into the membrane and catalyzes introduction of double bonds into the lipids to restore membrane fluidity (6). On the contrary, conditions that increase membrane fluidity (growth at warm temperatures or in the presence of branched-chain amino acids) favor DesK phosphatase activity, removing the phosphoryl group from the response regulator to terminate Δ5-*des* transcription (4, 5, 7).

Remarkably, the multimembrane-spanning domain of DesK can be simplified into a chimerical single membrane-spanning segment, which results from linking the N terminus region of the first transmembrane segment with the C terminus of the fifth transmembrane segment. This chimerical segment connected to the cytoplasmic catalytic domain is still able to respond to changes in lipid fluidity like full-length DesK. It has been called minimal sensor-DesK (MS-DesK) (Fig. 1) (3) and can be used as a tool to decipher the mechanism of DesK thermosensing.

An important contribution that has highlighted DesK functioning is the finding that a region located at the N-terminal transmembrane segment (Lys10-Asn12), which has been called "the sunken buoy" (SB), is involved in the detection of changes in membrane thickness that occur as a consequence of temperature variations (3). It has been proposed that, at lower temperatures, the membrane is thicker due to a more ordered packing of lipids (8, 9) and forces the SB motif to be buried in the hydrophobic region of the lipid bilayer, favoring the kinase activity of DesK. At higher temperatures, the lipids are in a more disordered state, and the membrane becomes thinner. The motif may now "buoy" to the membrane interface, stabilizing the protein in a "kinase-off" state (3) (Fig.1). Another key contribution to understand DesK functioning is a crystallographic study of DesKC (DesK catalytic core), which has revealed that DesK dimers are stabilized through hydrophobic interactions between two helical hairpins that form a dimerization motif, DHp (10). The structural comparison of DesK in the phosphatase or kinase functional states suggested that interhelical rearrangements that change the twisting in the DHp domain could favor one or the other signaling state (10).

In this scenario, the emerging question is: How is the information sensed by the transmembrane region transmitted to

Significance

Environmental temperature variations affect most biological processes and reactions, and cells must adapt accordingly. One such example is the fine-tuned regulation of membrane fluidity and its impact on a large array of cell physiological processes. How do cells "sense" membrane fluidity? Here, we discovered that the bacterial histidine-kinase, DesK, transmits temperature information through lipid-mediated conformational changes of a region of the protein that links its membrane sensor domain with its catalytic domain and activates the expression of a desaturase, which allows membrane fluidity to be recovered at this lower temperature. Because many thermosensors and other types of sensors contain similar linker domains, the mechanism we describe here could prove a general theme.

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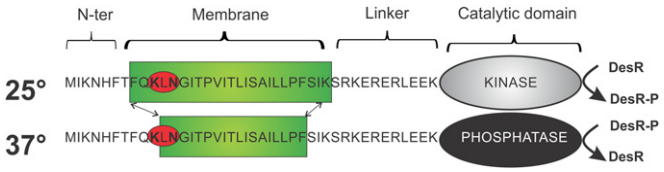

Fig. 1. Schematic representation of MS-DesK at 25 °C and 37 °C. The sequence of the transmembrane region and the connecting linker is indicated with a one-letter code. The SB motif is highlighted with a red circle, the catalytic cytoplasmic domain is represented with an oval, and the lipid bilayer is represented with a green rectangle. At 25 °C, the membrane is thicker, and the hydrophilic SB motif is buried in the hydrophobic core of the membrane. The catalytic domain has kinase activity (gray oval) and phosphorylates DesR. At 37 °C the membrane is thinner, and the SB floats at the water–lipid interface. The catalytic domain has phosphatase activity (black oval) and dephosphorylates DesR-P.

the cytoplasm and converted into a rearrangement in the catalytic domain that allows switching between kinase and phosphatase states? In this paper, we assessed the role of a linker region (KSRKERERLEEK) (Fig. 1) that connects the transmembrane sensing domain with the cytoplasmic catalytic domain. This region, although present in different constructs, was not solved in the crystal structure of the phosphatase state whereas it was nearly completely solved in the kinase state (10).

Here we used genetic, spectroscopic, and biochemical techniques to decipher the role of the linker in DesK signal transduction. We propose and validate the view that the linker is endowed with a helix/random coil conformational duality that enables its role as a transmission switch. Thus, the disruption of the helical structure between the transmembrane segment and the intracellular domain is mechanistically exploited to transmit temperature-dependent conformational changes from the transmembrane to the intracellular region.

Results

Charged Residues of the Linker Are Critical for DesK Signaling. The linker region connects the transmembrane region with the cytoplasmic domain of DesK and is located in close proximity to the inner leaflet of the lipid bilayer. We took advantage of the minimized version of DesK, MS-DesK, to introduce point mutations in the linker (KERERLEEK) and replaced three positively or three negatively charged residues with neutral ones, shown in bold: **A**E**A**E**A**LEEK (A3) and K**Q**R**Q**R**L**Q**E**K (Q3) (Fig. 2, *Lower*). To evaluate the capacity of these MS-DesK variants to sense membrane-thickness variations, they were expressed in a *desK*$^{-}$ strain, and the activity of the reporter gene β-galactosidase fused to the desaturase promoter (P*des-lacZ*) was measured in conditions of high or low lipid membrane fluidity (cells grown at 37 °C or grown at 37 °C and then cold-shocked to 25 °C, respectively). As shown in Fig. 2, MS-DesK stimulates transcription of the reporter when cells are shifted from 37 °C to 25 °C. Surprisingly, the negatively charged linker variant A3 shows a constitutive kinase activity regardless of membrane fluidity variations. On the contrary, the positively charged linker variant Q3 locks the sensor in a state unable to stimulate *des* transcription at 25 °C or 37 °C (Fig. 2) although this variant displays high phosphatase activity (Fig. S1).

The Linker Binds to Lipid Membranes. To analyze whether DesK linker variants interact with the phospholipid membrane and to characterize parameters that are required for such interaction, we designed the three corresponding peptides: WT (KSRKERERLEEK), A3 (KSRAEAEALEEK), and Q3 (KSRKQRQRLQEK) and prepared liposomes made of *Escherichia coli* lipids (mixture of anionic and zwitterionic phospholipids), which have a similar composition to that of *Bacillus subtilis* and are widely used for in vitro assays (3, 10) (*Materials and Methods*). Addition of the Q3 peptide to liposomes significantly increased turbidity, as measured by right-angle light scattering. Slow decrease of the scattering over time was due to visible flocculation observed after a few seconds. In contrast, no change in turbidity was observed after addition of A3 peptide, and, finally, a very moderate increase was observed for the WT peptide (Fig. S2*A*). A turbidity increase could be explained by liposome charge shielding due to the binding of positively charged peptides (WT and specially Q3) to the liposome surface, reducing electrostatic repulsion by liposomes and favoring flocculation.

At increasing salt concentrations, the WT and Q3 peptides lost their capacity to increase liposome turbidity, suggesting an essentially electrostatic interaction between the WT or Q3 peptides and liposomes (Fig. S2*B*). Because turbidity increase does not necessarily mean that the peptides quantitatively interact with lipids, liposome–peptide mixtures were ultracentrifuged and analyzed for the simultaneous presence of lipids and peptides by attenuated total reflection (ATR)-FTIR spectroscopy in terms of lipid and peptide content. Lipids were detected at 1,735 cm^{-1} assigned to the C = O signal of lipid ester bonds and the peptides around 1,650 cm^{-1} assigned to the C = O of the amide bond (11). The intensity of the band at 1,650 cm^{-1} recorded for the three peptides follows the propensity of the linker variants to interact with charged lipid polar head groups (Fig. 3*A*). This experiment was validated by dialyzing the peptide–liposome complexes previous to ATR-FTIR analysis (Fig. S2*C*). The basic amino acid content of the linker seems essential for the interaction with lipids because increasing positive charges (Q3 mutant peptide) increases binding, whereas decreasing them (A3 mutant peptide) has the opposite effect. To confirm the ionic interaction, we performed microdialysis peptide-release assays and ultracentrifugation of the peptide–liposome mixture in the presence of salts and analyzed the complexes by ATR-FTIR. These expermiments show that peptides are released from liposomes in the presence of salts (Fig. S2 *D* and *E*).

Does the Linker Change Its Conformation During DesK Activation? ATR-FTIR measurements were performed to determine the secondary structure of the peptides and to detect possible structural changes when incubated in the presence of liposomes. Table 1 shows a tentative secondary structure content prediction according to Goormaghtigh et al. (12). The secondary structure of the three peptides is mainly a mixture of helical and random structures.

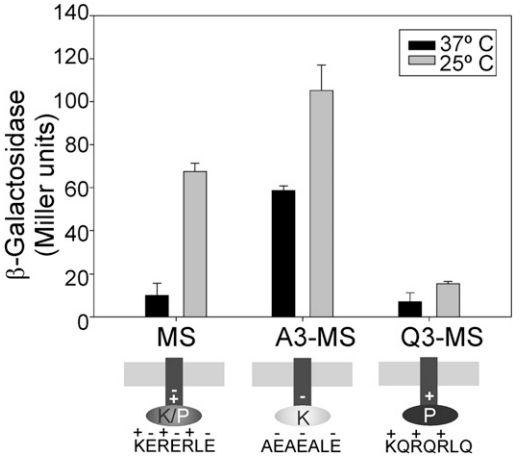

Fig. 2. Linker charges are critical to determine the signaling state of DesK. Cells expressing MS-DesK linker variants were grown at 37 °C or grown at 37 °C and transferred to 25 °C at an OD_{525} = 0.3. β-galactosidase activity was assayed every hour in independent triplicates. The results shown are expressed as the average of three independent experiments and correspond to 2 h after the cold shock.

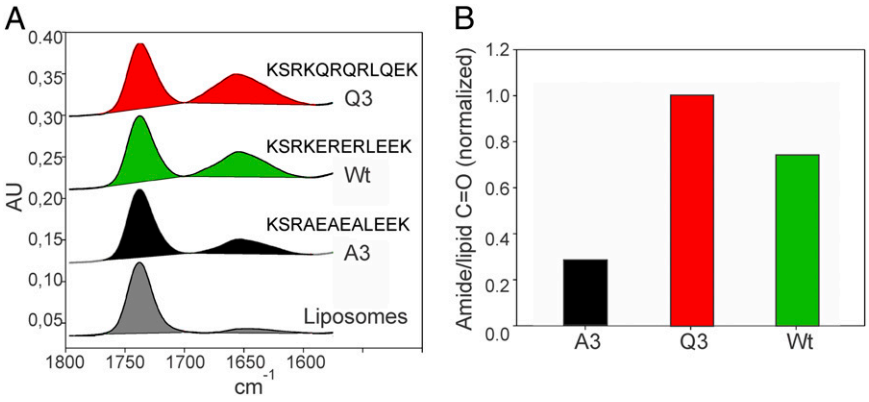

Fig. 3. Linker variants interact differentially with lipid membranes. (*A*) ATR-FTIR spectra of peptide–liposome complexes isolated by centrifugation. The 1,800–1,480 cm^{-1} region is displayed, with the absorption band of the C = 0 group of the lipid acyl chains (1,735 cm^{-1}) and the amide I band of the peptide (1,600–1,700 cm^{-1}); the absorbtion is expressed in arbitrary units (AU). (*B*) Quantification of bound peptide expressed as the ratio of the integrated amide I band of the peptide between 1,600 cm^{-1} and 1,700 cm^{-1} over the integrated C = O band at 1,735 cm^{-1} of the lipids shown in *A*. Results are corrected from the small-lipid contribution in the amide I region.

The A3 mutant peptide is more helical-structured than the other peptides (Table 1).

Nevertheless, the presence of liposomes stabilized the random-coil state of the WT even though a slight increase was also observed for the Q3 and A3 peptides, respectively (Table 1).

Analysis of the amide I and II bands in the 1,750–1,500 cm^{-1} region revealed a sharp maximum around 1,656 cm^{-1} in the amide I band and at 1,545 cm^{-1} in the amide II region characterizing an alpha-helical structure. Nevertheless, the hydrogen bonds are highly exchangeable because, when submitted to a D_2O-saturated vapor flux, the amide I peak shifted quickly and the amide II band, sensitive to the amide bond proton–deuterium exchange, showed its maximal exchange after 5 min. This observation suggests that the flexibility of the helix makes H-bonds accessible very quickly and might explain the propensity of the linker peptide to adopt a random structure and to interact with the lipids at the membrane interface (Fig. S3).

Membrane–Linker Electrostatic Interactions Play a Role in DesK Activity. MS-DesK was reconstituted in liposomes of commercial *E. coli* lipids.The autokinase activity was measured at 25 °C, a temperature that activates the kinase conformation, in the presence of γ^{32}-ATP at different salt concentrations. Consistent with the idea that electrostatic interactions affect the interaction of the linker with the membrane and modify the signaling state of the linker domain, we observed that the autophosphorylation of MS-DesKC was markedly dependent on salt concentration, with its highest activity at 400 mM KCl (Fig. 4*A*). The same effect was observed when NaCl was added to the reaction mixture (Fig. S4*A*).

At 37 °C, a temperature that activates the phosphatase conformation, the autokinase activity is negligible in the absence of salts (lane 1, Fig. 4*B*) but is recovered at increasing salt concentrations (lanes 2–4, Fig. 4*B*). A feasible interpretation is that salts disrupt the interaction of the linker with the lipid membrane favoring the kinase state. On the contrary, if the protein is not inserted into liposomes (the soluble truncated cytoplasmic catalytic domain DesKC or the detergent-treated MS-DesK), increasing salt concentrations do not raise autokinase activity (Fig. S4*B*).

Table 1. Secondary-structure prediction for DesK-linker variants

Peptide	Alpha	Beta	Turns	3–10 helix	Random	Increase in random
Wt	29.1	5.8	14.5	0	32.1	
Wt + liposomes	29.5	1.8	19.3	2	41.8	9.7
A3	41.3	1.5	13.5	0.1	28	
A3 + liposomes	42.6	1.6	15.8	1.5	32.4	4.3
Q3	17.1	5.8	20.5	2.4	45.2	
Q3 + liposomes	14.3	2.3	23.1	3	50.7	5.6

All numbers are expressed as percentages.

These findings support the hypothesis that protein–membrane electrostatic interactions are involved in signal transmission.

To test the idea that the electrostatic interaction in mainly due to the negatively charged membrane surface and positively charged residues of the linker, we reconstituted the MS-DesK in 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), which has a zwitterionic (neutral) polar headgroup, and tested the effect of salts in the kinase activity. As expected, in these neutral lipids, salts do not potentiate kinase activity (Fig. 4*C*).

To test our hypothesis that the kinase state of DesK is favored when the linker is detached from the membrane, liposomes made of *E. coli* lipids were incubated with the truncated cytoplasmic domain of DesK (DesKC). DesKC includes the linker region and in vivo shows constitutive kinase activity because it lacks the sensor transmembrane (TM) domain (2). Fig. 4*D* shows that the in vitro autokinase constitutive activity of truncated DesKC decreases when it is incubated with increasing amounts of liposomes containing anionic lipids. This experiment suggests that, in the presence of a lipid bilayer, a fraction of DesKC interacts via the linker with the negatively charged membrane surface, decreasing the kinase activity. This hypothesis is supported by the experiment showing that the activity of truncated DesKC is not inhibited by increasing the DMPC:protein ratio (Fig. 4*E*).

Can the Linker Conformation Reroute Transmission of the Signal? The critical region involved in membrane-thickness sensing is the sunken buoy (SB) motif located at the N terminus of the transmembrane segment of MS-DesK. Mutations that increase the hydrophilicity of this motif increase kinase activity whereas mutations that decrease the SB hydrophilicity abolish this activity (3). At this stage, we wondered which one, the SB motif or the linker, was dictating the final DesK-signaling state.

To analyze whether the linker is involved in signal transmission from the membrane to the cytoplasm and to determine whether the linker conformation contributes to define DesK signaling states, we combined point mutations in the transmembrane region that have been shown to lock DesK in the kinase or phosphatase state, respectively, with mutations in the linker. The inactive sensing mutant SB^- (Q9L,K10A,N12A), which shows a constitutive phosphatase state regardless of the temperature due to elimination of the sensor sunken buoy motif in the transmembrane region (3), was combined with the linker mutant A3 (constitutive kinase), giving rise to SB^-/A3 MS-DesK. On the other hand, the hyperactive mutant SB^+ (L11K), which shows constitutive kinase activity due to the presence of an extra charge in the sunken buoy (3), was combined with the linker mutant Q3 (constitutive phosphatase), giving rise to SB^+/Q3. Because the linker would be located downstream in the mechanism of signaling from the membrane to the cytoplasmic catalytic core, we expected that mutant SB^-/A3 would have a predominant kinase activity and SB^+/Q3 a predominant phosphatase activity,

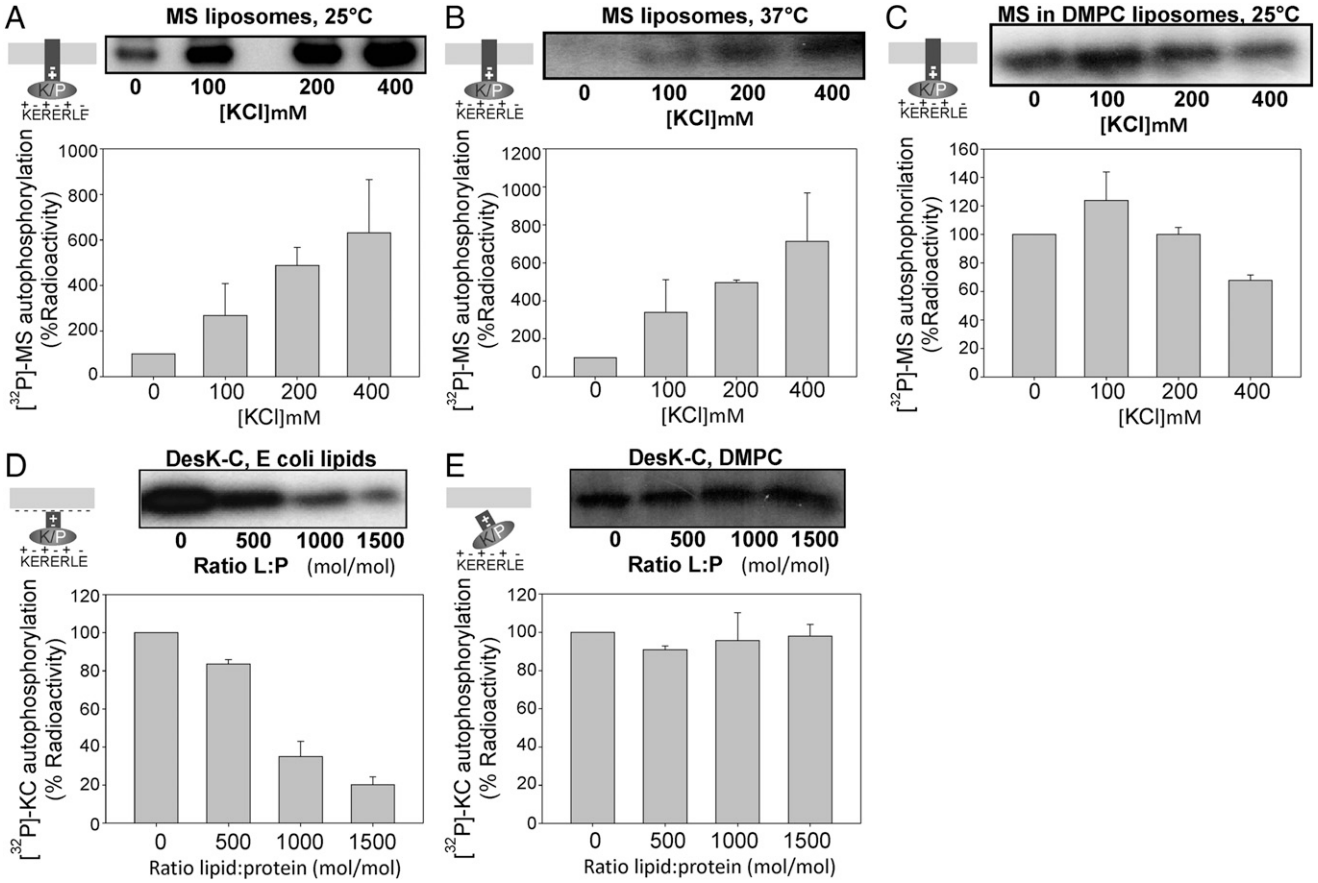

Fig. 4. Disruption of electrostatic interactions favor DesK autokinase activity. MS-DesK was reconstituted in liposomes of *E. coli* polar lipids (*A* and *B*) or DMPC lipids (*C*). Autokinase activity, shown in *Upper*, was assayed at 25 °C (*A* and *C*) or at 37 °C (*B*) in the presence of γ-32 ATP and increasing concentrations of KCl . The soluble truncated DesKC protein was incubated with increasing amounts of liposomes made of *E. coli* (*D*) or DMPC (*E*), and autokinase activity was measured at 25 °C. The total amounts of phosphorylated protein present in each well were determined by densitometry and plotted as the percentage of activity, considering 100% for the activity in the absence of salts (lane 1). The results correspond to the average of at least three independent experiments.

regardless of the signaling state dictated by the transmembrane region. These DesK variants were expressed in a *desK*$^{-}$ strain and tested for activity. We found that linker mutations that favor the kinase conformation can rescue SB^{-} transmembrane mutants locked in the phosphatase conformation (Fig. 5). Similarly, the behavior of the double mutant SB^{+}/ Q3 suggests that the Q3 linker mutations, which favor the phosphatase conformation, can rescue transmembrane mutants locked in the kinase conformation, decreasing kinase activity to wild-type levels (Fig. 5). Together, these results indicate that the linker is critical to transduce the signal and that charged residues play a crucial role in redirecting the signaling sensed by the transmembrane domain to the catalytic cytoplasmic domain.

Discussion

Rerouting Transmembrane Information. We identified a highly charged cluster of twelve residues (K32–K43) (Fig. 1) connecting the transmembrane domain with the cytoplasmic catalytic domain of

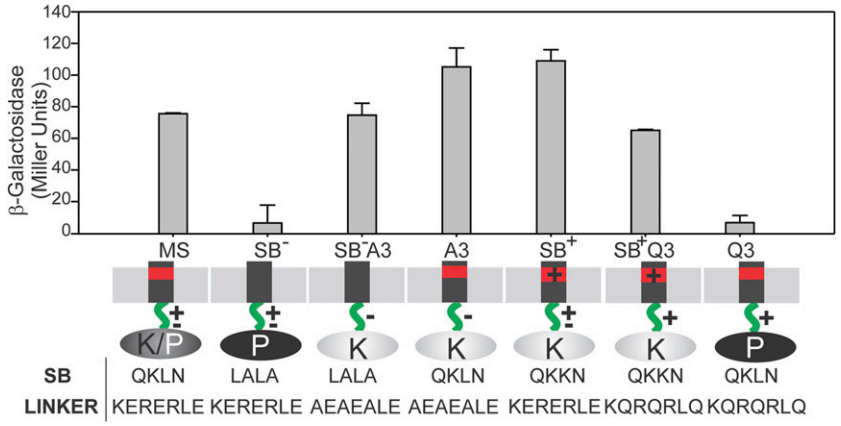

Fig. 5. Linker–membrane interaction dictates DesK signaling. *B. subtilis desK*$^{-}$ cells were complemented with either wild-type MS-DesK or MS-DesK variants with single mutations at the level of the membrane SB (SB^{-}, SB^{+}) or in the linker region (A3, Q3) or MS-DesK variants with combined mutations at both the transmembrane or linker region (SB^{-} A3/ SB^{+} Q3). β-Galactosidase–specific activities were determined every hour as described for Fig. 2. The results shown are the average of three independent experiments made 2 h after the cold shock.

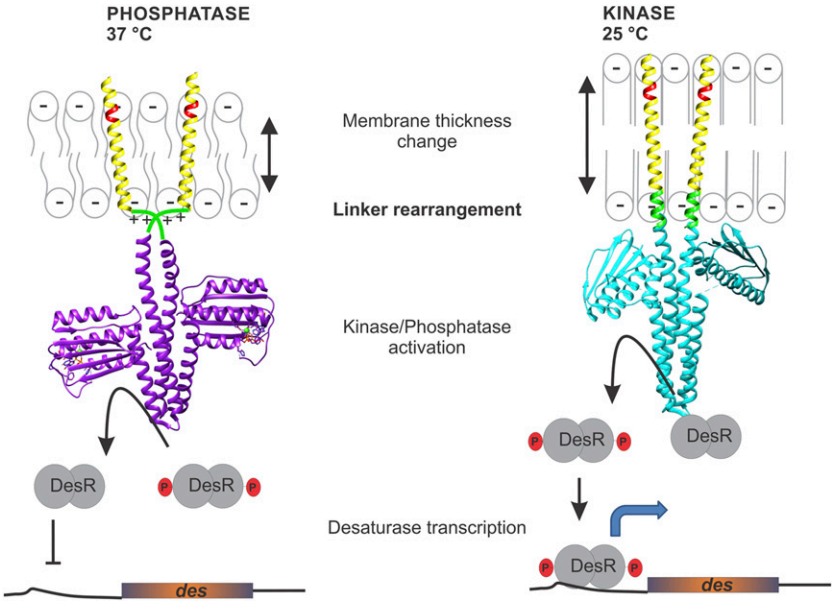

Fig. 6. Model for DesK activity upon temperature changes. The increase of the bilayer thickness prevents the interaction of the linker region (green) with the lipid bilayer and contributes to stabilize and favor the helical continuity between the transmembrane segment and the intracellular domain. Reversible interaction of the linker with the lipid bilayer surface would allow DesK to alternate between the kinase and phosphatase states.

MS-DesK. We propose that this motif, highly conserved in most species of the genus, is critical for signal transduction. We characterized two linker mutants, A3 and Q3, conferring, respectively, high and low kinase activity not only to MS-DesK but also to full-length DesK (Fig. S5). Structural infrared analysis suggests that the A3 linker has a higher tendency to form alpha-helix compared with the wild-type or Q3 linker variants. In contrast, the Q3 linker has a higher tendency to adopt a random-coil structure, and its higher conformational flexibility enables interactions between charged side-chain and the polar head groups of the membrane lipids. The role of the interaction with the lipids is supported by light scattering and infrared assays, which clearly demonstrate that the peptide Q3 binds stronger to liposomes than the peptide A3. The analysis of double mutants suggests that the linker is downstream from the stimulus perception in DesK signal transduction because mutations in the linker are dominant over constitutive mutations in the TM region. The transmission of the signal can thus be rerouted to one or the other state by modulating the linker–membrane interaction.

Dual Nature of the Linker–Membrane Interaction. We propose that the DesK linker has the ability to adopt a dual conformation: helix and random coil. The random-coil conformation is stabilized by electrostatic interaction with the lipid membrane interface whereas the helix is stabilized by the formation of intrahelical salt bridges between paired opposite charges. This conformational duality becomes the essence of a molecular transmission switch that will be at the same time sensitive to subtle conformational changes perceived by the sensing SB motif and able to transmit the signal.

A Working Model for Thermosensor DesK. We propose that, at high temperatures (thin membranes), the N-terminal SB motif is located at the water–membrane interface. The hydrophobic region of the TM is long enough to allow the linker to adopt the unstructured hydrophilic state (Fig. 6, *Left*), interacting with the charged lipids on the cytosolic side. Distribution of positively and negatively charged residues does not allow efficient clustering of positively charged residues on a helical structure that would favor a strong interaction with the negatively charged lipid, and a more random distribution is the way to optimize such an interaction.

The interaction of the linker with the lipid bilayer interface causes helix disruption, which favors the phosphatase conformation. This experimental observation is compatible with the crystallographic data reported for the cytosolic domain of the phosphatase-competent conformation (H188V), in which the linker region was not solved, a possible consequence of its high conformational flexibility (10).

At lower temperature, membrane thickness increases; the hydrophobic region of the TM is not long enough to match the hydrophobic core of the membrane and tends to bury the SB motif and its opposite counterpart, the N-terminal part of the KSRKERERLEEK linker motif, imposing a stress along the whole transmembrane region, which would force the linker to line up with the transmembrane part and adopt a more helical structure, protecting the otherwise exposed linker backbone from the hydrophobic membrane core (Fig. 6, *Right*). Burial of unpaired charged groups would be thermodynamically unfavorable; therefore, the charged residues are expected to increase their mutual coulombic interactions in the apolar environment of the membrane, making opposite charge pairs and triggering the formation of a continuous helix from the transmembrane region to the cytoplasmic part. The linker is thus removed from the membrane interface, adopting a helical structure. The helix is stabilized by a peculiar distribution of positive and negative residues that contribute to the formation of salt bridges in the helical register: n with $n + 3$ or n with $n + 4$ (n = the contour variable or residue number along the peptide chain). These salt bridges become stabilized and

Table 2. Cluster of charged residues at the junction between transmembrane and cytosolic domains in signaling proteins, channels, and transporters

Protein	Protein organization	Organism	Source
DesK	N-TM 5-KSRKERERLEEK—C	*Bacillus*	This work
KdpD	N-TM 4-EYLHRKSME—C	*E. coli*	(15)
OpuA	N-TM 7-EKEEENK—C	*L. lactis*	(16, 17)
TREK-1	N-TM 4-KKTKEE—C	Mammals	(18)
TREK-2	N-TM 4-KKTKEE—C	Mammals	(19)
MscL	N-TM 2-RKKEE—C	*E. coli*	(20)
Osm-9	N-TM 6-EERSESK—C	*C. elegans*	(21)
TRPA1	N-TM 6-DRFKKE—C	Mammals	(22)
TRPV4	N-TM 6-RLRRDR—C	Mammals	(23)

C, cytosolic domain; *C. elegans*, *Caenorhabditis elegans*; *L. lactis*, *Lactococcus lactis*; N, N-terminal domain; TM, transmembrane domain.

enhanced in the lipid phase because of the great decrease in charge shielding in the low dielectric medium (13).

This structural reorganization of the linker might constitute the basis of transmission and amplification of a signal sensed by the TM domain and transmitted to the catalytic domain and would result in modulation of the DHp interhelical twisting required to regulate DesK activity. Consequently, a continuous helix from the membrane to the cytoplasm would favor the kinase conformation. This idea is compatible with structural studies that show that, in the kinase conformation, the helical structure of the DHp extends further toward the membrane-proximal side of the protein (10). It is tempting to speculate that changes in tilting or rotation of the transmembrane segments resulting from a change in the membrane thickness could also play a role in the signaling process (14), stabilizing the linker in the bound or unbound conformation to finally determine the DesK signaling state. Interestingly, a cluster of positive and negative residues located at the water–lipid interface is also present in the tandem-pore K channel (TREK-1), the potassium sensor KdpD, the transient receptor potential proteins (TRPs), the OpuA transporter, and the mechanosensitive MscL channels (Table 2). For some of them, it has been suggested that the charged residues could interact with the negative surface of the membrane, modulating activity (15–18). We propose that highly charged residue clusters involved in signaling proteins and channels (Table 2) are able to adopt alternative states (unbound or bound to the membrane) in response to changes in mechanical properties of the lipid membrane, allowing this region to behave as a molecular switch for downstream protein activity.

Materials and Methods

Synthetic Peptides. Synthetic peptides were purchased from GL Biochem Ltd. Peptides corresponding to the linker sequence KSRKERERLEEK (WT) or KSRAEAEALEEK (A3 mutant) and KSRKQRQRLQEK (Q3 mutant) were all N-terminal–acetylated, C-terminal–amidated, and HPLC-purified. Their mass was confirmed by mass spectrometry. For FTIR experiments, samples were diluted in 10 mM HCl and lyophilized (three times) to remove traces of TFA.

Liposome–Peptide Complexes. Ten milligrams of *E. coli* polar lipid extract dissolved in chloroform (50 mg/mL) were dried under a nitrogen flux and then maintained overnight under vacuum in a lyophilisator. The dried film was rehydrated in a 10 mM Hepes-Na buffer at pH 7.3 and extensively vortexed to form liposomes. The resulting turbid suspension was transferred to a 1.5-mL polycarbonate tube and sonicated in a water bath sonicator (UCD-200 Bioruptor; Diagenode) to convert multilameller liposomes into unilamellar. Liposomes were mixed with peptides (10 mg/mL dissolved in water) in a 5:1 lipid/peptide ratio (wt/wt) in a final volume of 0.1 mL. After incubation at room temperature for 30 min, the liposomes were centrifuged at 120,000 rpm in a 42.2 Ti Beckman rotor at 20 °C for 1 h. When microdialysis was used, the peptide–liposome complexes were dialyzed by suspending a sample drop on a floating-disk filter with a 20-nm cutoff, which allows permeation of the free peptide, whereas the liposomes were retained on the filter. The sample recovered from the filter was used for ATR-FTIR measurements.

ATR-FTIR Measurement. Peptide–liposome complexes were spread on a 1-mm-square diamond ATR element (Goldengate Bridge Harrick Scientific) fitted to a Brucker IFS-55 FTIR spectrometer. The lipid samples (1 μL) were partially dried under nitrogen. Serial measurements were recorded at a 2 cm^{-1} resolution and averaged in the 4,000–900 cm^{-1} spectral region with a subtracted background. Further processing of the spectra (scaling, integration, and vapor subtraction) was made with in-house-made software (Kinetics) running under Matlab.

In Vitro Autophosphorylation Assay. These reactions were performed essentially as previously described (3). Briefly, for the autokinase assay, proteoliposomes containing 0.3 mg of MS-DesK protein were incubated at 25 °C or 37 °C in P buffer [50 mM Tris·HCl (pH 8), 200 mM NaCl, 1 mM DTT, 20% (vol/vol) glycerol, 50 mM KCl, 1 mM $MgCl_2$, 25 mM ATP, and 0.25 mCi/mL [γ-^{32}P]ATP]; at different time points, aliquots were taken and were run on SDS/PAGE 12%. The radioactivity of phosphorylated proteins in gels was visualized using a Typhoon 9200 PhosphorImager screen (STORM840; GE Healthcare) and quantified using ImageQuant software (version 5.2). The values obtained were expressed as the percentage of phosphorylated protein. Western blots were also performed to confirm the amount of protein loaded in each lane. All results shown are representative of at least three independent experiments.

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Supporting Information

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SI Materials and Methods

Plasmid and Strain Constructions. The *MS-DesK* gene and its variants were PCR-amplified from plasmid TM1/5-DesKC-pHPKS or from DesK-pHPKS for full-length DesK (1, 2). Site-directed mutagenesis was performed to introduce the mutations A3, Q3, SB+/Q3, and SB^{-}/A3 using different pairs of mutagenic oligonucleotides. The overlap-PCR products were cloned into the BamHI-XbaI sites of the expression vector pHPKS, a low copy number plasmid that places the coding regions under the control of the P*Xyl* promoter (2). The resulting plasmids were used to transform the *desK*−CM21 or AKP20 *Bacillus subtilis* cells (1, 3, 4). To induce the expression of DesK variants, 0.1% xylose was added to the growth medium. To overexpress and purify DesK variants for in vitro assays, the same amplicons were cloned into the NdeI-SalI sites of expression vector pET22 (Novagen), and the resulting plasmids were used to transform BL21 cells. All mutations were confirmed by DNA sequence analysis. Strains, full sequences, and detailed construction methods are available upon request.

Bacterial Strains and Growth Conditions. *B. subtilis* JH642 strains were grown in a rotary shaker (New Brunswick, model VS) operating at 250 rpm. Cells were either grown at 37 °C, or at 37 °C and then transferred to 25 °C at an $OD_{525} = 0.3$ in Spizizen salts supplemented with 0.1% glycerol, 50 μg/mL each tryptophan and phenylalanine, 0.05% casa amino acids, and trace elements (5, 6). β-galactosidase activity was assayed in independent triplicates (7). The results shown are the average of three independent experiments and correspond to 2 h after the cold shock.

Turbidity Measurements. Right-angle light scattering was measured in an SLM-8000C spectrofluorimeter with 400 nm as excitation and emission wavelengths. The sample was maintained under continuous stirring with a small magnetic bar in the measurement cuvette. Two microliters of peptide dissolved in water at 10 mg/mL were added at 50 and 100 s to a 0.1 mg/mL liposome suspension in 10 mM Hepes-Na buffer at pH 7.3 in a final volume of 1 mL. When required, KCl was added to the liposome suspension before peptide addition.

Protein Overexpression, Purification, and Reconstitution. The *Escherichia coli* BL21 strain was used as a host for plasmid pET 22b, which overexpressed MS-DesK and its variants. The overexpression and purification of this protein was performed following the Studier Method for Autoinduction of Protein Expression in the T7 System (8). Cells were resuspended in TNP buffer [50 mM Tris (pH 8), 200 mM NaCl, 1 mM phenylmethylsulfonyl fluoride) supplemented with 20 mM imidazole (TNPI-20) and a mixture of Triton X-100 and Brij 58, both at 0.5%. After treatment with lysozyme 1 mg/mL for 30 min, cells were disrupted by sonication and then centrifuged at 37,000 × *g* for 15 min. From the supernatant, MS-DesK in mixed micelles was purified by affinity chromatography using an Ni^{2+}-nitrilotriacetic acid agarose resin (Qiagen) at 4 °C for 1 h and dialyzed against 50 mM Tris (pH 8), 200 mM NaCl, 10% (vol/vol) glycerol, and 1 mM DTT.

Proteoliposome Preparation. Eight milligrams of lipids of *E. coli* polar lipids extract or 1,2-dimyristoyl-sn-glycero-3-phosphocholine lipids (Avanti Polar Lipids) were hydrated in 1 mL of hydration buffer [20 mM Tris·HCl (pH 8), 250 mM sucrose, and 100 mM K_2SO_4) and vortexed above the melting temperature. The resulting large multilamellar vesicle suspensions were disrupted by 10 freeze–thaw cycles. For liposome generation, lipid suspensions were extruded through a 200-nm pore size Whatman polycarbonate filter using a hand-held extrusion device (Avanti). A homogenous suspension of unilamellar liposomes was obtained. To insert the protein in liposomes, 0.24% (wt/wt) Triton X-100 was added to preformed liposomes and incubated for 20 min with continuous stirring. Then, purified MS-DesK was added at an 80/1 (wt/wt) lipid/protein ratio and incubated with continuous stirring during 1 h at 4 °C. The detergent was later removed by incubating the sample 8 h with SM2 Bio-beads (Biorad). This detergent-removal step was repeated three times, shifting the equilibrium toward the integration of the protein into the liposomes.

Proteoliposome-Purification Step. MS-DesK–containing proteoliposomes were separated from free liposomes and not-incorporated protein on a sucrose gradient by ultracentrifugation. The liposome suspension collected after the Bio-Beads treatment was placed on the bottom of a step sucrose gradient (1.6, 1.2, and 0.2 M sucrose) and centrifuged overnight at 30,000 rpm in an SW40 rotor at 4 °C. After centrifugation, the proteoliposome band floating on top of the gradient was harvested, washed with 30 mM Tris·HCl (pH 8.0) and ultracentrifuged in a Ti90 rotor at 45,000 rpm for 1 h at 4 °C. Proteoliposomes containing MS-DesK were resuspended in 200 μL of hydration buffer, glycerol 10%, and DTT 1 mM. The protein concentration was determined by Lowry, and the quality of the sample, as well as the efficiency of protein integration, was determined by SDS/PAGE followed by Western blot using a-His antibodies (Qiagen) and a-DesKC antibodies.

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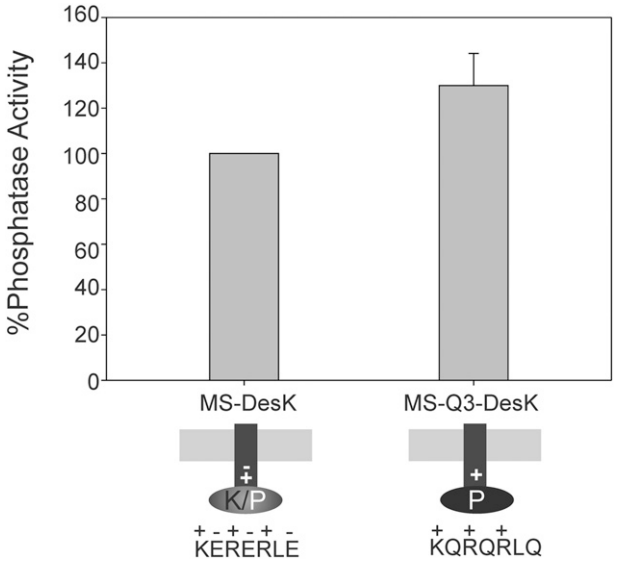

Fig. S1. MS-Q3-DesK conserves phosphatase activity. In vivo phosphatase assay was performed using strain AKP20, which contains a P*des-lacZ* construct and overexpresses phospho-DesR, in a desK−null background. This strain shows constitutive expression of the reporter gene β-galactosidase at 37 °C unless it is complemented with DesK variants exhibiting phosphatase activity (2, 3). AKP20 cells complemented with pHPKS plasmids carrying MS-DesK with the WT or Q3 linker variants were grown at 37 °C in the presence or absence of the inductor (xylose), and β-galactosidase activities were determined every hour. The difference in β-galactosidase activity in the absence or presence of the inductor reflects the phosphatase activity of each variant. A 100% difference corresponds to the MS-DesK with a WT linker.

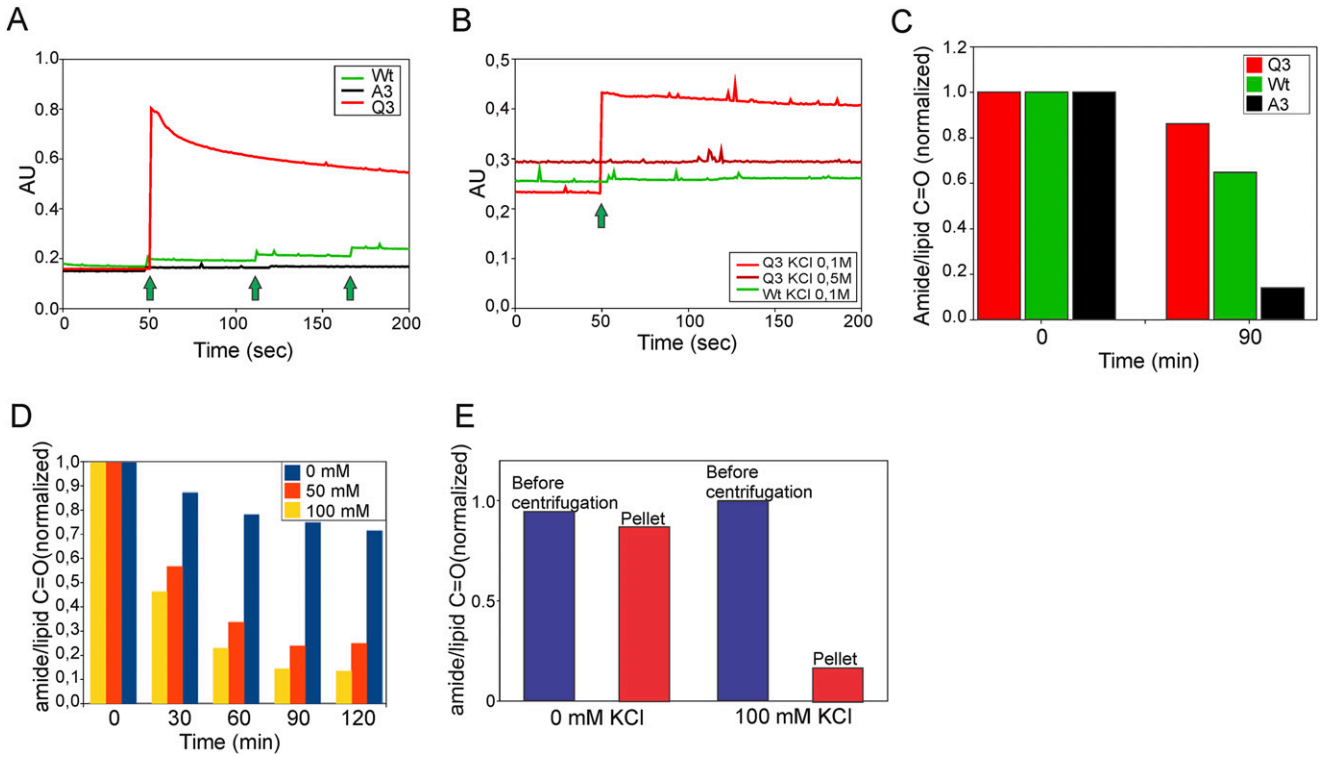

Fig. S2. Peptide–lipid interaction. Turbidity of liposomal suspensions upon sequential addition of peptides (arrows) measured by right-angle light scattering in the absence (*A*) or presence (*B*) of KCl. Attenuated total reflection (ATR)-FTIR analysis of peptide–liposome complexes subjected to different conditions: microdialysis treatment (*C*); increasing salt concentrations followed by microdialysis for the indicated time periods (*D*), or followed by ultracentrifugation (*E*). Quantification of bound peptide is expressed as the ratio of the integrated amide I band of the peptide between 1,600 cm^{-1} and 1,700 cm^{-1} over the integrated C = O band at 1,735cm^{-1} of the lipids.

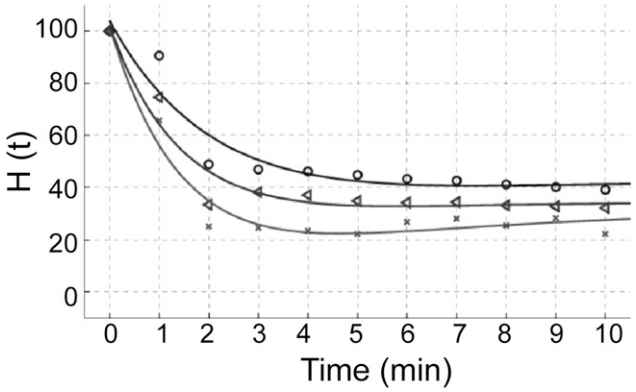

Fig. S3. Evolution of the percentage of exchange of the hydrogen of the peptidic amide group as a function of deuteration time. Peptides were mixed with *E. coli* polar lipid liposomes, and mixtures were flushed continuously with D2O-saturated nitrogen on the ATR element. Spectra were recorded every minute (32 scans averaged). The area of the amide II band (around 1,545 cm^{-1}), which is sensitive to the deuteration of the amide N-H group, was monitored over time and normalized on the lipid ester C = O group area (around 1,735 cm^{-1}). Circle, A3 peptide; triangle, WT peptide; cross, Q3 peptide.

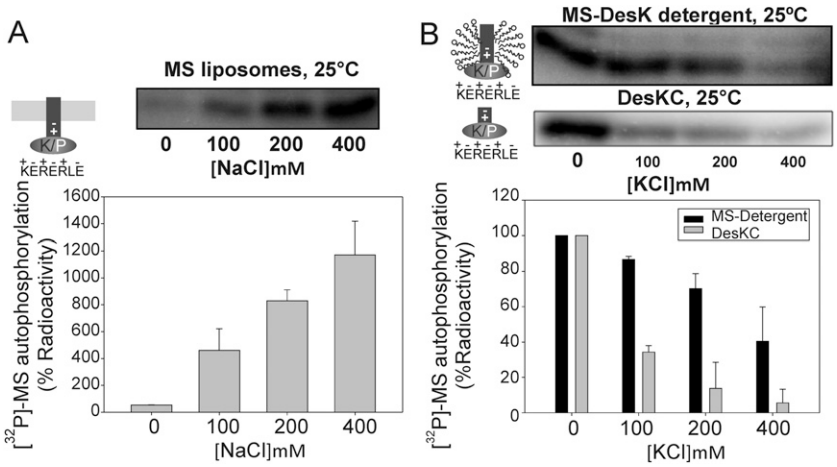

Fig. S4. Salt effect on autokinase activity of DesK variants. (*A*) The MS-DesK was reconstituted in liposomes of *E. coli* polar lipids, and autokinase activity (shown in *Upper*) was assayed at 25 °C in the presence of γ-32 ATP and increasing concentrations of NaCl. (*B*) The soluble truncated DesKC (gray bars) or the MS-DesK dissolved in Triton X-100 0.5%/Brij 0.5% (black bars) were incubated with increasing salt concentrations, and the autokinase activity was determined as in *A*. The total amounts of phosphorylated protein present in each well were determined by densitometry and plotted as the percentage of activity, considering 100% for the activity in the absence of salts (lane 1). The results correspond to the average of at least three independent experiments.

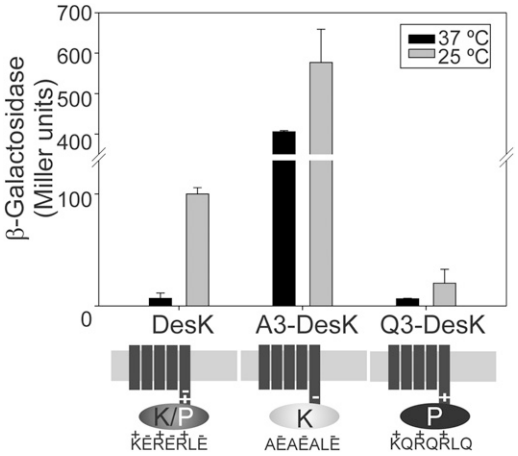

Fig. S5. CM21 cells expressing full-length DesK linker variants were grown at 37 °C or at 25 °C, and β-galactosidase–specific activities were determined every hour as described for Fig. 2. The results shown are the average of three independent experiments made 2 h after the cold shock.