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Report

Membrane Thickness Cue for Cold Sensing in a Bacterium

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Summary

Thermosensors are ubiquitous integral membrane proteins found in all kinds of life. They are involved in many physiological roles, including membrane remodeling, chemotaxis, touch, and pain [1–3], but, the mechanism by which their transmembrane (TM) domains transmit temperature signals is largely unknown. The histidine kinase DesK from *Bacillus subtilis* is the paradigmatic example of a membrane-bound thermosensor suited to remodel membrane fluidity when the temperature drops below ~30°C [1, 4] providing, thus, a tractable system for investigating the mechanism of TM-mediated input-output control of thermal adaptation. Here we show that the multimembrane-spanning domain from DesK can be simplified into a chimerical single-membrane-spanning minimal sensor (MS) that fully retains, in vivo and in vitro, the sensing properties of the parental system. The MS N terminus contains three hydrophilic amino acids near the lipid-water interface creating an instability hot spot. Mutational analysis of this boundary-sensitive beacon revealed that membrane thickness controls the signaling state of the sensor by dictating the hydration level of the metastable hydrophilic spot. Guided by these results we biochemically demonstrated that the MS signal transmission activity is sensitive to bilayer thickness. Membrane thickness could be a general cue for sensing temperature in many organisms.

Results and Discussion

Temperature sensing is essential for the survival of living cells. A striking but mysterious example of temperature-sensing mechanism occurs in *Bacillus subtilis* during cold adaptation when the membrane-associated histidine kinase DesK is activated by changes in membrane fluidity (Figure S1, available online). How DesK discriminates the surrounding lipid environment to promote membrane remodeling upon a drop in ambient temperature is unknown.

In vivo and in vitro experiments have demonstrated that DesK acts as a kinase at cold temperatures, autophosphorylating a conserved histidine within the kinase domain [1, 4, 5]. The phosphoryl group is then transferred to the receiver aspartate in the DNA-binding response regulator DesR. Phosphorylated DesR activates the transcription of the *des* gene coding for the acyl lipid desaturase Δ5-Des [6, 7]. Unsaturated fatty acids, products of Δ5-Des activity, promote a more fluid membrane that appears to switch DesK from a kinase to a phosphatase state [1]. Consequently, the concentration of DesR-phosphate declines and transcription of the gene *des* is terminated [1, 6]. The crystal structure for the entire cytoplasmic portion of DesK has provided a molecular framework to interpret the basic mechanistic principles by which the sensor protein alternates between output autokinase and phosphatase activities [4]. Nevertheless, given the critical role that lipid-protein interaction plays in prokaryotic function a fundamental question arises: which are the transmembrane (TM) helix rearrangements underlying the catalytic transitions along the signaling pathway? In this work we address this issue by reengineering the TM domain of DesK into a minimal sensor (MS) that is functionally equivalent, yet 5 times smaller. We found that the N-terminal domain of MS is able to measure the contraction and expansion of the bilayer to adjust the signaling state of the catalytic cytoplasmic domain of DesK. This novel finding extends the conceptual framework that ruler-like mechanisms control processes as diverse as the very long chain fatty acids (VLCFAs) length [8], the bacteriophage lambda tail length [9], and the needle length of type III secretion machines [10].

Role of the N-Terminal Bilayer-Spanning Segment in DesK Sensing

Membrane-topology bioinformatics analysis predicts that DesK has four well-defined TM-spanning helices and an unusual N terminus amphipathic stretch (residues 8–30), classified as a possible TM helix (Figures S2A and S2B). The substituted-cysteine accessibility method (SCAM) [11] approach based on the controlled membrane permeability of sulfhydril reagents was used to directly determine the topology of DesK (Figure 1A and Figure S2C). This experimental analysis confirmed the existence of the four TM segments predicted by the topology programs, and revealed that the amphipathic stretch (residues 8–30) constitutes an additional membrane-spanning domain (hereafter named TM1). Thus, DesK has five TM-spanning segments with the N terminus located on the external side of the plasma membrane (Figure 1B). To analyze the role of the TM helices in cold sensing, the gene regions encoding for each of the TM segments were removed. Then, we engineered *desK*⁻ cells to produce the DesK deletion variants in response to an inducer (Figure 1C). To monitor the signaling states of the truncated versions in vivo, we assayed the levels of β-galactosidase activities coded by a *lacZ* reporter gene fused to the desaturase promoter, which is activated only when there is flux of phosphate from DesK to DesR (Figure S1). Because western-blot analysis showed that all the DesK deletion mutants were located in the membrane fraction (Figure S2D), we proceeded to examine their ability to respond to cooling. Deletions of the TM1 (Δ25) or TM1 plus TM2 (Δ56) result in a kinase-on state (Figure 1C), whereas subsequent deletions (Δ83 and Δ119) produce a kinase-off state but maintain

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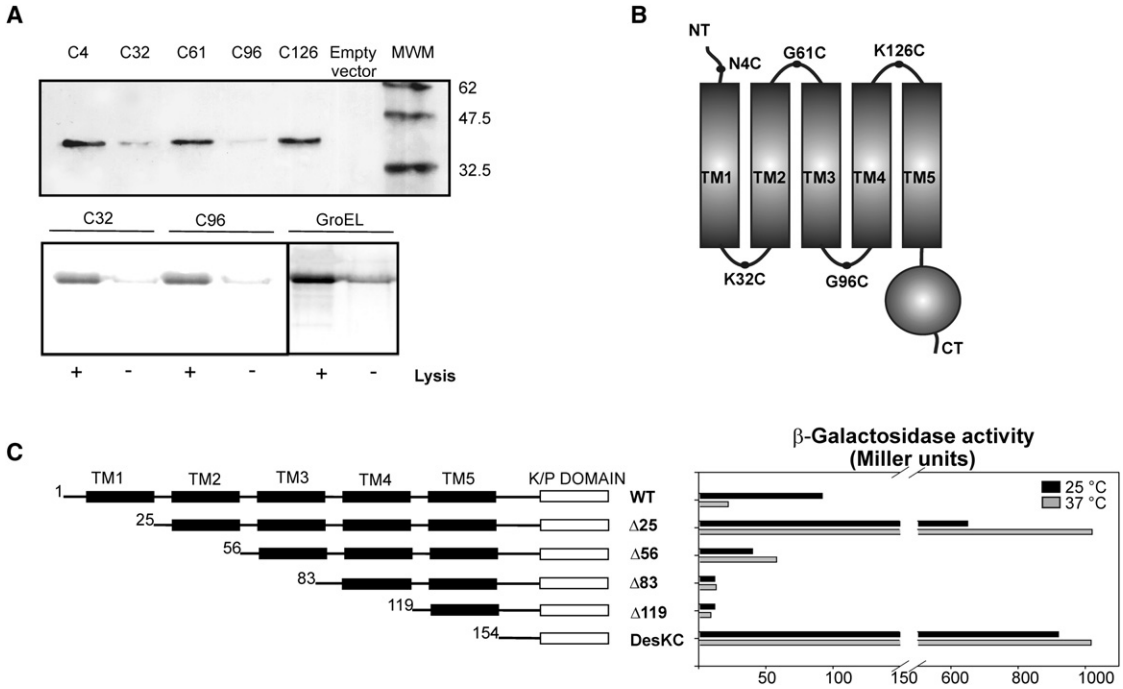

Figure 1. Membrane Topology of DesK and Deletion Analysis

(A) Determination of the topology of DesK. Five amino acids that according to hydropathy analysis are located in the loop regions (Figure 1B and Figure S2B) were replaced by cysteine in five different constructs (N4C, K32C, G61C, G96C, K126C). Accessibility of Cys residues to the membrane impermeant biotinylation reagent 3-(N-maleimidylpropionyl) biocytin (MPB) was determined by the SCAM method. C4, C61, and C126 were strongly labeled by MPB in intact cells (upper panel). SCAM analysis of intact (−) or lysed (+) cells revealed that C32 and C96 were detected mainly in lysed cells, similarly to cytoplasmic GroEL (lower panel).

(B) Topology model of DesK. Rectangles define the TMs oriented with the cytoplasm below the figure. N terminus (NT) and C terminus (CT) are indicated. The locations and names of amino acids substituted by cysteine and used for SCAM analysis are indicated.

(C) Sequential deletions of DesK hydrophobic segments. The rectangular black boxes represent predicted TM segments and the white box the cytoplasmic catalytic domain of DesK. *B. subtilis desK*$^{-}$ cells harboring each of the DesK deletion mutants were grown at 37°C to an OD of 0.3 at 525 nm and then divided into two samples. One sample was transferred to 25°C and the other kept at 37°C. Aliquots were taken every hour and β-galactosidase activities were determined. The values are representative of three independent experiments and correspond to 4 hr after the shift. See also Figure S2.

phosphatase activities (Figure S2E). Surprisingly, the deletion of the first TM (Δ25) promotes *des* transcription levels 7- to 20-fold higher than wild-type DesK at 25°C or 37°C, respectively, behaving similarly to DesKC (DesK lacking the complete TM region, residues 154–370, Figure S2B), which is completely deregulated in vivo (Figure 1C and [5]). This result likely reflects elevated autokinase activity of the Δ25 DesK variant and hence an elevated DesR phosphorylation in this strain. Taken together, these results imply that the TM1 domain plays a central role in modulating DesK kinase activity.

The Input-Output Properties of DesK Can Be Captured in a Single TM Segment

To unravel the mechanism of DesK thermosensing, we engineered simplified models by designing and testing fusions of DesKC, to individual TM1 and TM5 of the sensor as shown in Figure 2A. The goal was to determine whether any of these constructs was able to modulate the in vivo activity of DesKC in response to thermal signals. We focused on TM1 and TM5 segments because TM1 is necessary to turn off kinase activity under normal growth conditions (Figure 1C), whereas TM5 is directly connected to the catalytic core via a two-helix coiled coil, which ultimately controls the signaling state of DesK [4]. We also wondered whether cold-induced motions of the regulatory TM1 could be directly propagated to TM5 to control DesK activity. Thus, we constructed a chimera, named TM1/5, where the first 17 residues of the N-terminal domain of TM1 are peptidically linked to the last 14 residues of the C-terminal domain of TM5. To complete the sensor, we fused the chimera to DesKC (Figure 2A). Strikingly, whereas the TM1-DesKC and TM5-DesKC protein fusions were unable to adjust the signaling state of DesKC, a thermal regulation very similar to that promoted by wild-type DesK was only observed with the chimera TM1/5-DesKC (Figure 2B). Indeed, TM1/5 maintained levels of phosphatase activity similar to wild-type DesK (Figure S2E). These experiments combined with the finding that TM1/5 is indeed associated to the plasma membrane (Figure 2C), demonstrates that the 31-residue long single-membrane-spanning TM1/5 segment, is functionally highly similar to the 151-residue long five-membrane-spanning N-terminal domain of DesK: the minimalization did not compromise the sensor activity in any appreciable way. Because both the sensing and transmission properties of the wild-type sensor domain were captured into the single hybrid TM1/5, we called this TM segment "minimal sensor" (MS). To directly validate this finding, we studied the influence of temperature on the catalytic activity of MS-DesKC by reconstituting it in a pure lipid system. To this end, MS-DesKC was expressed in a cell-free system and cotranslationally integrated into liposomes of *Escherichia coli* phospholipids. These lipids undergo a reversible change of state from a fluid (disordered) to a nonfluid (ordered) array of fatty acyl chains when the temperature is

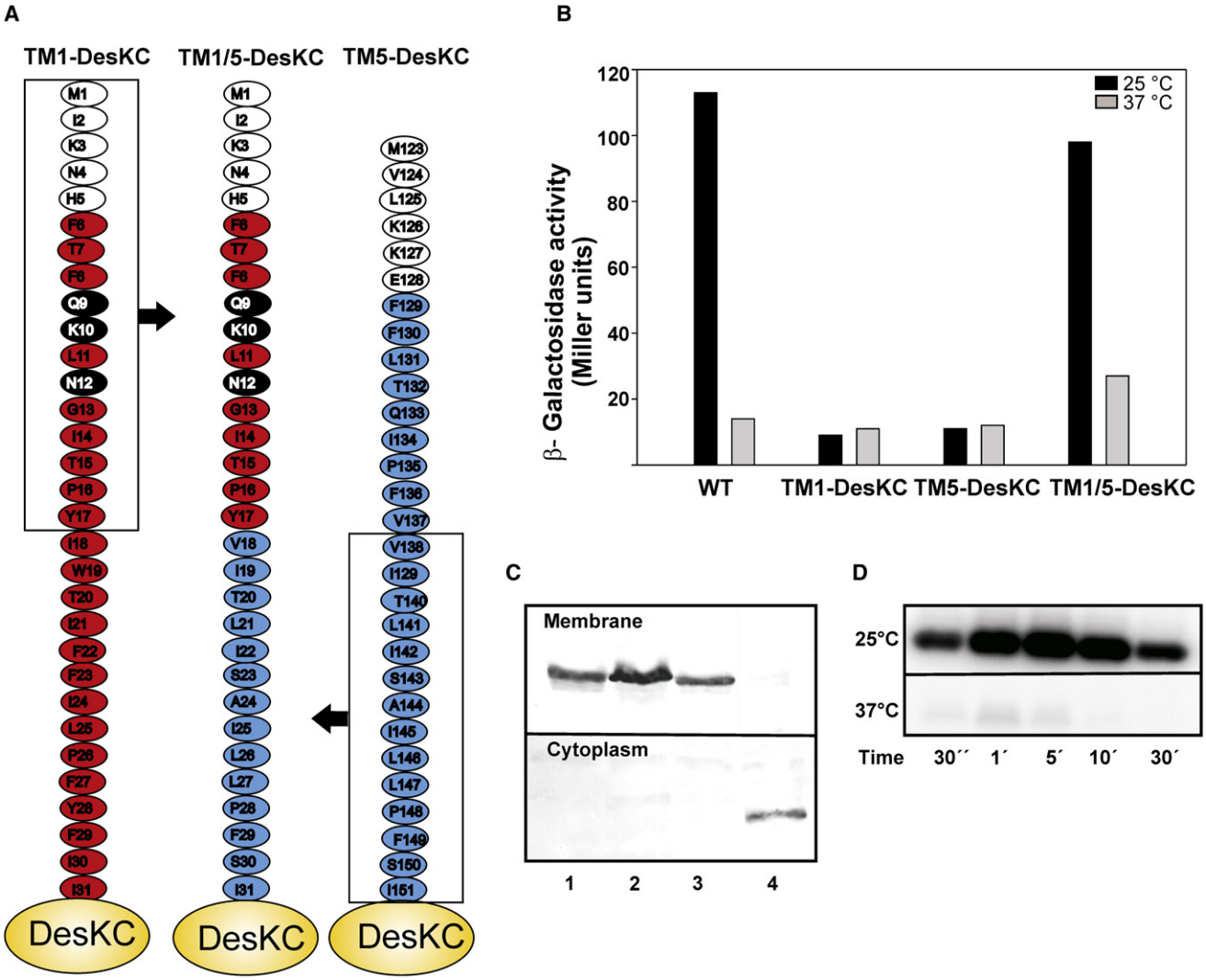

Figure 2. Engineering the TM Segments of DesK

(A) DesK fusion constructs. Fusion proteins were obtained by linking TM1 (amino acids 1–31), TM5 (amino acids 123–151), and TM1/5 (amino acids 1–17 of TM1 and 138–151 of TM5) to DesKC. Note that amino acid numbering was modified in TM1/5. Extracellular amino acids are depicted in white, hydrophobic TM amino acids are highlighted in red (TM1) or blue (TM5), and hydrophilic amino acids of the SB motif in black.

(B) *B. subtilis* CM21 (*desK*$^{-}$) was transformed with plasmid pHPKS directing the expression of either full-length DesK or the fusion constructs and β-galactosidase activities were determined as in Figure 1C.

(C) Western blot of fusion proteins. The membrane and cytosolic fractions from CM21 cells transformed with plasmids expressing the constructs TM1-DesKC (lane 1), TM5-DesKC (lane 2) or TM1/5-DesKC (lane 3), or cytosolic DesKC (lane 4) were subjected to SDS-PAGE, transferred to a nitrocellulose membrane, and analyzed by Western Blot using an anti-DesKC antibody.

(D) Autokinase activity of the TM1/5-DesKC (MS-DesKC) integrated in pure *E. coli* liposomes at 25°C and 37°C.

decreased from 37°C to 25°C [12]. As shown in Figure 2D, the autokinase activity of the MS-DesKC was significantly upregulated (~50 fold) when the temperature was decreased from 37°C to 25°C, demonstrating that the MS domain plays a role equivalent to the full-length TM domain of DesK, with no other proteins involved in signaling propagation. More importantly, the lipid properties are sensed by MS, because the cytoplasmic domain alone (DesKC) is unresponsive to the cold signal [4].

Mutational Evidence for Bilayer Thickness Control of MS Signaling

How can MS sense cold stimulus? The N terminus of MS has an unusual cluster of hydrophilic amino acids (Q9, K10, and N12) near the lipid/water interface (Figure 2A). These polarities, especially the charge-bearing K10, constitute a built-in instability when fully buried in the lipid phase due to the thermodynamic cost associated with dehydrating polar groups [13]. This suggests a hydration/stabilization switch turned on by a decrease in membrane width. This "sunken-buoy" (SB) motif would be implicated in temperature sensing so that an unstable state with deprivation of hydration of the polar cluster (low-T membrane expansion [14, 15]) is associated with the kinase activity, whereas stabilization through hydration (high-T membrane narrowing [14, 15]) would promote phosphatase activity. Thus, it is possible that the interfacial positioning of the SB-motif be involved in signaling-state regulation. To test this idea the SB-motif was replaced by hydrophobic amino acids (Q9L, K10A, N12A). Indeed, the

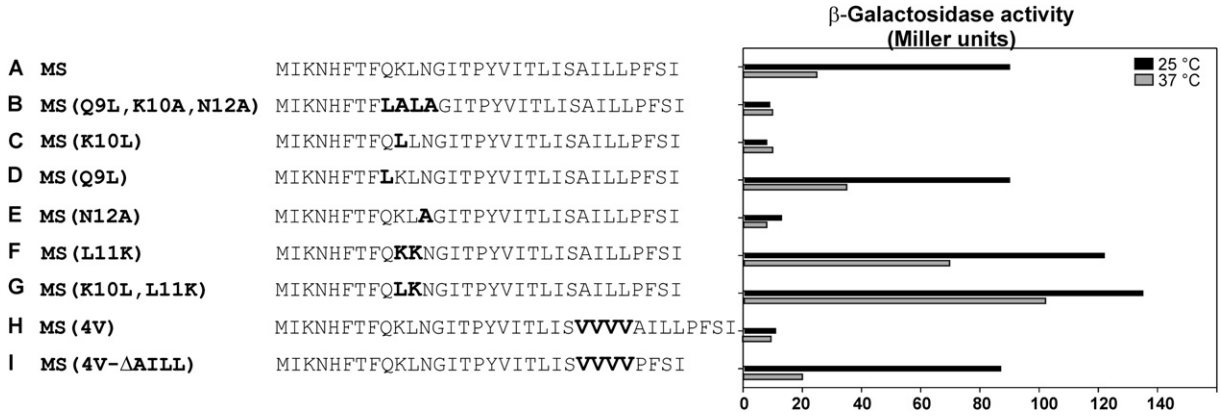

Figure 3. The Interfacial Position of the SB Hydrophilic Motif Determines the In Vivo Activity of MS-DesKC

The effect of point mutations or insertions in the SB motif of MS-DesKC was analyzed by measuring β-galactosidase activities in strain CM21, as described in Figure 1C.

$MS_{(Q9L,K10A,N12A)}$ triple mutant was unable to stimulate desaturase transcription upon a temperature downshift (Figures 3A and 3B). To assess the functional importance of individual residues within the SB-motif, we replaced them for A or L. K10 and N12 appear to be major contributors to MS cold sensing, whereas the replacement Q9L was not sufficient to affect the ability of MS to detect cooling (Figures 3C–3E).

Charged residues found in TM segments can move their polar groups toward the interface by snorkeling [16, 17], adopting configurations that orient their polar atoms to partially escape from the hydrophobic membrane core toward the interface. Because the largest snorkeling distances are achieved by lysine and because it is the only charged residue of the SB-motif, we expected that any factor purposely introduced to enhance its dehydration would promote the kinase state of the sensor. Thus, we assessed the effect of shifting K10 one position downwards into the lipid phase or adding an extra K to the MS segment. We expected that such modifications should be sufficient to promote a kinase-dominant state in MS-DesKC. Accordingly, we added an extra K at position 11, constructing the system $MS_{(L11K)}$-DesKC or transferred K10 to position 11, giving $MS_{(K10L,\ L11K)}$-DesKC (Figures 3F and 3G). In each case, the response regulator activation of the mutants was significantly higher than that of the MS-DesKC (40% and 50% at 25°C, and 280% and 400% at 37°C, respectively). These data confirm the functional role of the SB-motif and reveal that we can redirect the flow of thermal information in the MS-DesKC by altering the interplay of the SB-motif with the lipid/water interface.

These experiments support the hypothesis that at low temperatures, when the membrane becomes thicker due to an increase in lipid ordering [15], the SB-motif is trapped inside the anhydrous environment, a destabilization that promotes kinase activity. Conversely, at higher temperatures the lipids become more disorganized and the membrane becomes narrower, allowing the SB-motif to reach the aqueous environment, and stabilizing the kinase-repressed state of DesK. Thus, the SB-motif appears to respond to a bilayer thickness cue rather than to a *B. subtilis* specific compositional feature of the membrane. If so, an increase in the length of the MS nonpolar region should enhance the chance that the SB-motif becomes water exposed, regardless of temperature-promoted variations in membrane thickness. Insertion of four valine residues between S23 and A24 of the MS-DesKC ($MS_{(V4)}$-DesKC) was indeed found to stabilize the protein in a kinase-off state (Figure 3H). To corroborate that this effect was solely dependent on the MS length, we removed four hydrophobic residues (A24, I25, I26, L27) located just below the 4V cluster, and the ability to mediate temperature perception and signal transduction for the resulting variant $MS_{(4V\text{-}\Delta AIIL)}$ was effectively restored (Figure 3I).

Effect of Bilayer Thickness on the Autokinase Activity of MS-DesKC

To provide a solid biochemical ground to our mutational analysis, we studied the influence of membrane thickness on the autokinase activity of MS-DesKC reconstituted into a series of phosphatidylcholines (PCs) containing monounsaturated fatty acyl chains of different chain length: dimirystoleoil (14:1-PC), dipalmitoleoyl (16:1-PC), and dieicosenoyl (20:1-PC). Phase transition temperatures for all these lipids are <25°C [18], so that all the lipids are in the liquid crystalline phase at the assay temperature. Consistent with the notion that the SB motif is a thickness-measuring device, we found that the autokinase activity of MS-DesKC was markedly dependent of the fatty acyl chain length with the highest activity in PC-containing acyl chains of 20 carbons and lower activities in bilayers with shorter fatty acyl chains (Figure 4). These data, combined with the mutagenesis analysis shown in Figure 3, reveal a bilayer thickness ruler-like mechanism, in which the distance between the SB motif and the lipid-water interface regulates the activation/deactivation of MS-DesK.

Might bilayer thickness cues represent a strategy by which cold sensors process thermal information? Some sensitive ion channels of the transient receptor potential (TRP) family that have been implicated in cold sensing contain a lysine-arginine rich box (TRP box), close to the polar/apolar membrane interphase [19]. Perhaps, motions of amphipathic α helices in response to membrane thickness could be involved in the signal transduction mechanism used by cold sensors to maintain thermal homeostasis.

Experimental Procedures

Topology Determination of DesK via SCAM

SCAM was performed following the methods described by Zhu and Casey [11] and Bogdanov et al. [20]. Briefly, *E. coli* BL21 cells expressing each cysteine substitution mutant (N4C, K32C, G61C, G96C, and K126C,

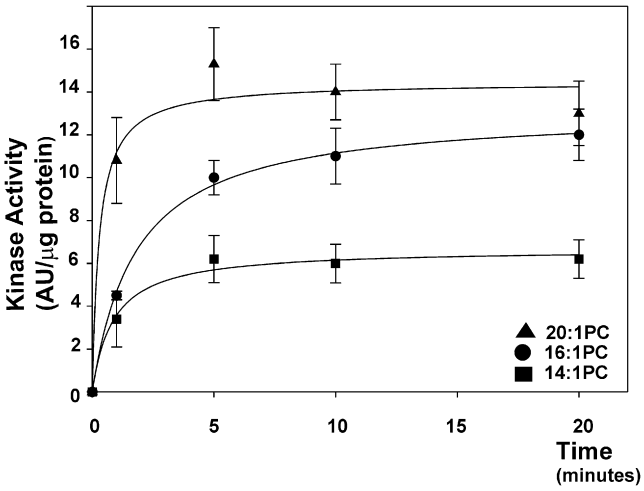

Figure 4. Effects of Phosphatidylcholine Fatty Acid Chain Length on MS-DesKC Autophosphorylation

MS-DesKC was reconstituted with phosphatidylcholines of the given chain length and autophosphorylation of the proteoliposomes was measured at 25°C at the indicated times. Quantification of the phosphoproteins was performed by densitometry and plotted as AU/μg protein versus time. The values are the mean of three independent experiments, and error bars represent the standard deviation of the values obtained.

Figure 2B), or bearing the empty plasmid were grown at 37°C in Luria-Bertani medium to a OD = 0.4, and then were induced with 0.5 mM isopropyl-beta-thio galactopyranoside (IPTG) at 30°C for 3 hr. Cells were centrifuged, washed in phosphate-buffered saline (PBS) pH 7 and incubated 30 min with conjugation buffer (CB) (PBS 1×, ethylenediaminetetraacetic acid disodium salt [EDTA] 1 mM) and 10 mM dithiothreitol (DTT). After washing twice with CB, cells were resuspended in CB and incubated for 30 min at 30°C with 0.5 mg ml^{-1} of the membrane-impermeant biotinylation reagent (+) biotinyl-3-maleimidolpropionamidyl-3,-6 dioxaoctanediamine (MPB, Pierce). Cells were washed with buffer CB and lysed by addition of 100 μl of PBS 1×, EDTA 5 mM, Triton X-100 1%, phenylmethylsulfonyl fluoride (PMSF) 1 mM. The biotynilated His-tagged DesK proteins were isolated with UltraLink immobilized neutravidine according to the manufacturer instructions (Pierce), analyzed by SDS-PAGE and western blot with anti-His antibodies (Qiagen). To test eventual labeling of internal Cys residues in intact cells, we also controlled the biotinylation of cytoplasmic proteins by checking GroEL biotinylation, which was detected by blotting with anti-GroEL polyclonal antibodies [21].

Proteoliposomes Obtainment and Purification

To obtain large multilamellar vesicles, we hydrated 20 mg of lipids (*E. coli* polar lipids, 1,2-dimyristoleoyl-*sn*-glycero-3-phosphocholine [14:1-PC], 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphocholine [16:1-PC], and 1,2-dieicosenoyl-*sn*-glycero-3-phosphocholine [20:1-PC], Avanti Polar Lipids, USA) in 1 ml of hydration buffer (20 mM Tris-HCl [pH 8], 250 mM sucrose and 100 mM K_2SO_4), and incubated the suspension at 37°C in a vortex mixer for 3 hr [22]. For liposome generation, the suspension was then extruded >20 times through a 400 nm filter via a hand-held extrusion device obtained from Avanti.

Plasmid pvxMS was incubated in Roche RTS 100 or 500 HY Kit reactions (Roche, Penzberg, Germany) in the presence of 4 mg ml^{-1} of the indicated liposomes, in accordance with the manufacturer's instructions. After the reaction was completed, the mixture was placed on the bottom of a step sucrose gradient (1.6, 1.2, and 0.2 M sucrose) and centrifuged at 30,000 rpm in a SW40 rotor for 18 hr at 4°C. After centrifugation, proteoliposomes were washed with 30 mM Tris-HCl pH 8, resuspended in hydration buffer, used for protein concentration determination, and analyzed by SDS-PAGE followed by western blot and biochemical characterization.

In Vitro Phosphorylation

For the autokinase assay, proteoliposomes containing 3 μg 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 dithiothreitol, 20% v/v glycerol, 50 mM KCl, 1 mM $MgCl_2$, 25 μM ATP, and 0.25 μCi/μl [γ-^{32}P] ATP); different time points aliquots were taken and subjected to SDS-PAGE on 12% polyacrylamide gels [5]. Radioactive gels were transferred to nitrocellulose filters and the radioactivity of phosphorylated proteins was visualized with a Typhoon 9200 PhosphorImager screen (STORM840, GE Healthcare) and quantified with ImageQuant software (version 5.2). The values obtained were expressed as arbitrary units (AU). 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.

Supplemental Information

Supplemental Information includes two figures, one table, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2010.06.074.

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