

Legionella pneumophila regulates the small GTPase Rab1 activity by reversible phosphorylcholine

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Effectors delivered into host cells by the *Legionella pneumophila* Dot/Icm type IV transporter are essential for the biogenesis of the specialized vacuole that permits its intracellular growth. The biochemical function of most of these effectors is unknown, making it difficult to assign their roles in the establishment of successful infection. We found that several yeast genes involved in membrane trafficking, including the small GTPase Ypt1, strongly suppress the cytotoxicity of Lpg0695(AnkX), a protein known to interfere severely with host vesicle trafficking when overexpressed. Mass spectrometry analysis of Rab1 purified from a yeast strain inducibly expressing AnkX revealed that this small GTPase is modified posttranslationally at Ser₇₆ by a phosphorylcholine moiety. Using cytidine diphosphate-choline as the donor for phosphorylcholine, AnkX catalyzes the transfer of phosphorylcholine to Rab1 in a filamentation-induced by cAMP(Fic) domain-dependent manner. Further, we found that the activity of AnkX is regulated by the Dot/Icm substrate Lpg0696(Lem3), which functions as a dephosphorylcholinase to reverse AnkX-mediated modification on Rab1. Phosphorylcholine interfered with Rab1 activity by making it less accessible to the bacterial GTPase activation protein LepB; this interference can be alleviated fully by Lem3. Our results reveal reversible phosphorylcholine as a mechanism for balanced modulation of host cellular processes by a bacterial pathogen.

vesicle trafficking | posttranslational modification | type IV secretion

Intravacuolar bacterial pathogens have evolved various strategies to engage selectively with specific cellular compartments to acquire membrane materials to accommodate the expansion of their phagosome. After phagocytosis, *Legionella pneumophila*, the etiological agent of the potentially fatal Legionnaires' disease, initiates a unique trafficking route that bypasses the default phagosome maturation pathway. The *Legionella*-containing vacuole (LCV) sequentially engages in intimate interactions with several host organelles, including the endoplasmic reticulum (ER), mitochondria, and ribosomes (1). It now is well established that *L. pneumophila* actively converts its phagosomal membranes into membranes with characteristics of the ER. This model is supported by the observations that the LCV interacts intimately with the ER, and its membranes are enriched with several proteins such as binding immunoglobulin protein and calnexin specific for this organelle (2, 3). Furthermore, genetic and pharmaceutical interference with the formation of coat protein complex II vesicles blocks the maturation of LCVs (4, 5).

Successful conversion of the phagosome into a vacuole permissive of *L. pneumophila* replication is mediated by effectors translocated by its Dot/Icm type IV secretion system, which modulates various host cellular processes to coordinate the biogenesis of the LCV (6). More than 270 Dot/Icm substrates have been identified (7). However, with a few exceptions, the biochemical functions of most of these substrates are unknown, as are their contributions in the intracellular life cycle of *L. pneumophila* (6). Consistent with the notion that *L. pneumophila* actively intercepts membrane vesicles originating from the ER to remodel its phagosome (4), several bacterial proteins directly target key molecules critical for this phase of membrane transport. For

example, the entire activity cycle of the small GTPase Rab1, which regulates many important events in membrane transport, is hijacked by bacterial virulence factors. The multifunctional protein SidM/DrrA extracts from and/or competes with the GDP dissociation inhibitor (GDI) and subsequently activates it by its guanine nucleotide exchange factor (GEF) activity (8–10). As infection proceeds to 2 h, Rab1 is inactivated by LepB, a *L. pneumophila* GTPase activation protein (GAP), leading to its removal from the bacterial phagosome by a yet-unidentified GDI (11).

Two lines of evidence suggest that other Dot/Icm substrates are involved in the modulation of host membrane transport. First, deletion mutants of genes known to interfere with Rab1 activity did not cause defects in intracellular bacterial growth. Second, a number of Dot/Icm substrates have been shown to inhibit membrane trafficking in yeast (12). However, the biochemical functions of these proteins are unknown.

In a screen to identify *L. pneumophila* proteins capable of killing eukaryotic cells, we isolated several *L. pneumophila* genes that are toxic to yeast (13). Among the identified toxic proteins, Lpg0695 (AnkX) is a Dot/Icm substrate that contains multiple ankyrin-repeat homology domains (14). Furthermore, ectopically expressed AnkX strongly disrupts the secretion pathways of mammalian cells in a process that requires presence of the filamentation-induced by cAMP (Fic) domain in its N-terminal domain (14). With a core sequence of HPFx(D/E)GN(G/K)R, the Fic domains are critical for the adenylyl transferase activity (AMPylation) that stably modifies substrates by transferring an AMP moiety from ATP to the target proteins (15). However, such enzymatic activity has not yet been demonstrated, and the full spectrum of its cellular targets is unknown. By using yeast genetics as a tool, we identified a number of yeast genes capable of efficiently suppressing the yeast toxicity of AnkX. By mass spectrometry analysis of Rab1 purified from a yeast strain inducibly expressing AnkX, we found that AnkX is a phosphorylcholine (PC) transferase that targets the small GTPase Rab1 and possibly other proteins involved in the docking of vesicles in membrane trafficking to the *cis*-Golgi compartment. Further, we found that the activity of AnkX is regulated by Lem3, a Dot/Icm substrate that possesses a biochemical activity opposite that of AnkX to remove the PC moiety from Rab1. Our results suggest the spatial regulation of Dot/Icm substrate activity by other substrates.

Results

Several Genes Involved in Membrane Trafficking Between the ER and the Golgi Apparatus Suppress Yeast Toxicity of AnkX. To exploit yeast genetics in the dissection of Dot/Icm effector activity, we isolated a number of *L. pneumophila* genes exhibiting toxicity to

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this organism (13). To identify the potential cellular target of AnkX, we introduced a yeast genomic library into a yeast strain inducibly expressing AnkX; a number of clones capable of suppressing AnkX cytotoxicity were isolated. Sequencing revealed that each of these clones contained at least one gene involved in membrane trafficking, including the small GTPase Ypt1 (Rab1 for mammalian cells); Bet1p, Sec22p, and Bos1p, three SNARES involved in the ER to Golgi membrane transport (16, 17); and the tethering factor Uso1p (16). Most of the genes are essential for yeast viability, and many of them can suppress defects caused by *ypt1* mutation (18). When expressed independently, each of these genes exhibited suppressor phenotypes similar to the original clones without affecting the cellular levels of AnkX under inducing conditions (Fig. 1). To obtain a more complete list of suppressors, we constructed *sly1-20p*, a dominant mutant of the t-SNARE-associated protein Sly1, which suppresses *ypt1* deficiency (19), and found that this allele was able to suppress the toxicity of AnkX (Fig. 1*A*, strain vi). These results suggest that one or more of these proteins is the potential cellular target of AnkX.

AnkX is a PC Transferase That Modifies Rab1 at Ser₇₆. Although all characterized Fic domain proteins target small GTPases, and *ypt1* displayed the strongest suppressing activity (Fig. 1) (15), we were unable to detect AnkX-mediated AMPylation of Rab1 or other suppressors. To determine the potential effect of AnkX on Rab1, we purified GST-Rab1 from yeast strains inducibly expressing AnkX or its Fic domain mutant AnkX_{H229A} and subjected the protein to trypsin digestion and nanoflow liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. A mass shift of 165.1 ($m/z = 578.7$, $z = 2$) was observed in most (relative abundance >92%) of the tryptic fragment -T₇₂ITSSYYR₇₉- in GST-Rab1 coexpressed with wild-type AnkX (Fig. 2*A* and *B*). Such

increase was not observed in peptides from GST-Rab1 coexpressed with the AnkX_{H229A} mutant ($m/z = 496$, $z = 2$) (Fig. 2*A* and *B*). Further analysis revealed that this peptide contains a covalent modification of 165.1 D on Ser₇₆, the residue next to Tyr₇₇ (Fig. 2*C*), which was AMPylated by SidM/DrrA (20). This mass increase corresponds to the addition of a PC moiety, suggesting that AnkX functions as a PC transferase that potentially modifies Rab1 at Ser₇₆.

To examine directly whether AnkX functions as an enzyme to modify Rab1 with PC, we performed in vitro biochemical assays with cytidine diphosphorylcholine [methyl-¹⁴C] (¹⁴C-CDP-choline). Incubation of purified His₆-AnkX with GST-Rab1 and ¹⁴C-CDP-choline led to the production of ¹⁴C-phosphorylcholine-Rab1 (Fig. 3*A*, lane 2). Consistent with the observation that a mutation in the conserved histidine residue abolished its toxicity to eukaryotic cells (14), His₆-AnkX_{H229A} failed to catalyze the reaction, indicating the importance of the Fic domain in this enzymatic activity (Fig. 3*A*, lane 5). Further, His₆-AnkX did not label GST-Rab1_{S76A}, confirming that the PC modification is specific for Ser₇₆. The fact that ¹⁴C-choline-AnkX was detected in all samples containing wild-type protein indicated that AnkX modifies itself (Fig. 3*A*, lanes 1–3, and *B*, lane 1). Because detection of the ¹⁴C-choline incorporated into the target molecules requires at least 3-wk exposure of X-ray films to the gels, we established a Western blot-based detection with the PC-specific antibody TEPC 15 (21). This antibody recognizes both PC-Rab1 and PC-AnkX but not AMP-Rab1 (Fig. 3*B* and Fig. S1). Taken together, these results established that AnkX is a PC transferase that targets the Rab1 protein in a process that requires its Fic domain.

Phosphorylcholine Interferes with GTP Loading and LepB-Induced GTPase Activity of Rab1.

To determine the effects of phosphorylcholine (referred to as “PCylation” throughout the text) on the activity of Rab1, we first examined the loading of GTP to PCylated Rab1. Although at a low efficiency, incubation of ³⁵SyGTP with GDP-Rab1 led to significant loading of GTP (Fig. 4*A*). Inclusion of SidM, the GEF for Rab1, in the reactions significantly increased GTP incorporation (Fig. 4*A*). When SidM and Rab1 were used at a 1:40 ratio, similar amounts of ³⁵SyGTP associated with PCylated Rab1 were detected (Fig. 4*A*). However, PCylated Rab1 reproducibly received lower amounts of GTP when the molar ratio between these two proteins was increased to 100 (Fig. 4*A*). Thus, in these in vitro experiments, the ability of PCylated Rab1 to receive GTP was impaired, and this impairment could be compensated by higher amounts of SidM. Next, we examined the GTP hydrolysis activity of PCylated Rab1. The *Legionella* GAP protein LepB (11) induced efficient GTP hydrolysis by Rab1 or by Rab1 that had been incubated with AnkX_{H229A} and CDP-choline (Fig. 4*B*). As expected, AMPylation of Rab1 by SidM/DrrA blocked its GTPase activity even in the presence of LepB (Fig. 4*B*) (11). Although less severe than AMPylation, PCylated Rab1 blocked the GTPase activity induced by LepB (Fig. 4*B*). On the other hand, PCylation did not interfere detectably with the binding of Rab1 to SidM/DrrA or LidA (Fig. S2). Thus, PCylation interferes with the GTPase activity of Rab1, and this interference, along with other potential alterations in the property of Rab1, such as the accessibility to endogenous GEF or GAP, may account for the inhibition of eukaryotic secretory pathway by AnkX.

Lem3 Is a Dephosphorylcholinase That Modifies Rab1. The strong effects of AnkX on the host secretory process prompted us to hypothesize that other bacterial factors may be involved in regulating its activity. We first tested whether the PC moiety on Rab1 can be removed by potential *Legionella* enzymes by incubating PCylated Rab1 with total bacterial lysate. Mixing PC-Rab1 with total *Legionella* cell lysate did not cause protein

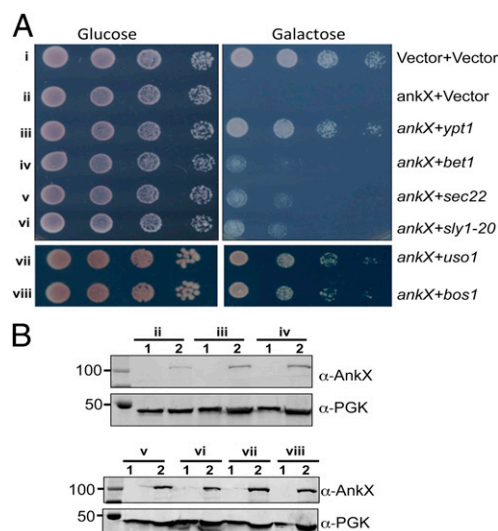


Fig. 1. Suppression of AnkX yeast toxicity by yeast genes involved in membrane trafficking. (*A*) Yeast expressing chromosomally encoded (obtained by integration) AnkX from the galactose-inducible promoter was transformed with plasmids expressing the indicated genes from the alcohol dehydrogenase promoter. Yeast cells diluted with water were spotted onto glucose or galactose medium. The growth of yeast cells was documented 3 d after inoculation. Strains harboring only the vectors (*Top row*) or only *ankX* and vector (*Second row*) were used as controls. (*B*) Expression of *ankX* in yeast strains harboring the suppressor genes. Yeast strains grown in medium supplemented with raffinose (1) were induced with galactose (2) for 7 h, and total proteins resolved by SDS/PAGE were probed by immunoblotting with an AnkX-specific antibody. The 3-phosphoglycerate kinase (PGK) was probed as a loading control. Note that AnkX is expressed in all strains in a galactose-dependent manner.

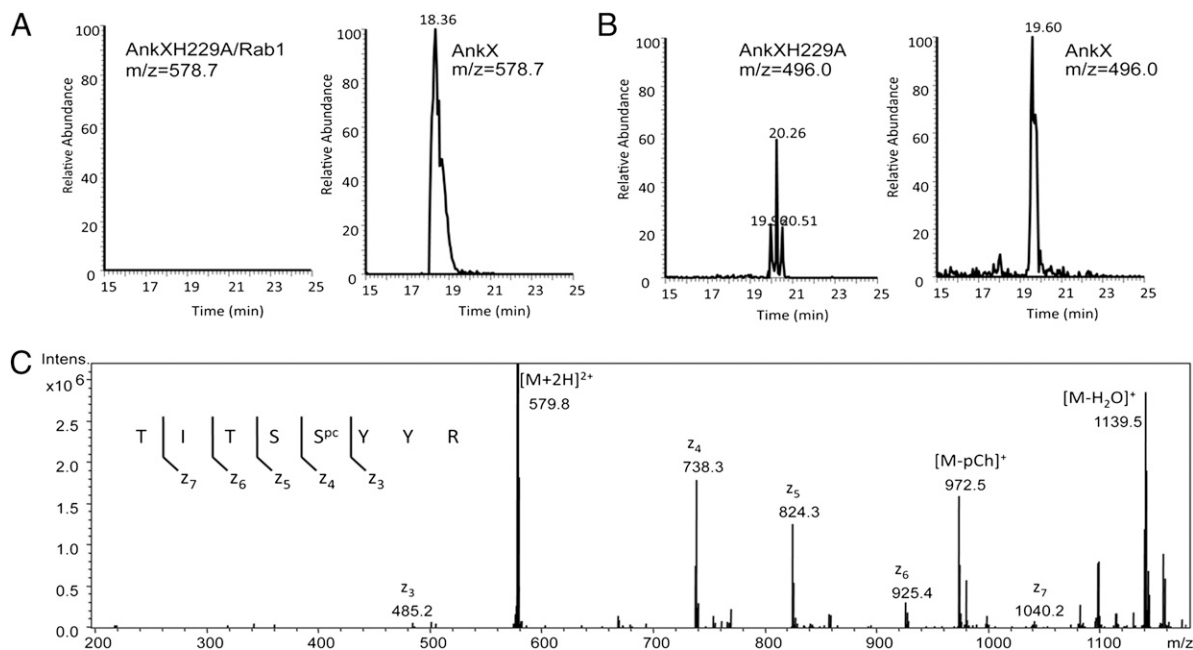


Fig. 2. Rab1 coexpressed with AnkX in yeast was modified on Ser₇₆ by phosphorylcholine. (A) Extracted ion chromatograms for the -T₇₂ITSSYYR₇₉- peptide ($m/z = 578.7$) in GST-Rab1 coexpressed with the AnkXH229A mutant (1.6% relative to all forms of the peptide; *Left*) or with AnkX (92.5% relative abundance; *Right*). (B) Extracted ion chromatograms for the -T₇₂ITSSYYR₇₉- peptide ($m/z = 496.0$) of GST-Rab1 coexpressed with the AnkXH229A mutant (96.3% relative abundance) or AnkX (6.8% relative abundance). (C) Electron-transfer dissociation MS/MS spectrum of modified -TITSSYYR- peptide. The mass shift for fragment ions z₄ through z₇ and lack of shift for z₃ shows that the phosphorylcholine occurred at Ser₇₆. Labeled sequence-specific z-ions indicate the site of modification.

degradation but led to an almost complete loss of the PCylation signals (Fig. 5A), suggesting the existence of enzymes capable of reversing the effect of AnkX on Rab1. Therefore, we initiated a screening to identify *L. pneumophila* genes capable of suppressing the yeast toxicity of AnkX by introducing a *L. pneumophila* genomic library (22) into the yeast strain inducibly expressing AnkX. Sequencing of 86 clones reproducibly exhibiting the suppression phenotype revealed that, although the size of inserts in these clones varied greatly, all clones contained *lpg0696*, also called “*lem3*,” which encodes a 570-amino acid Dot/Icm substrate (23). In *L. pneumophila* genome, *ankX* and *lem3* are separated by a 7-bp intergenic region and are transcribed in convergent orientations (24). Lem3 fully rescued the growth of the yeast strain harboring *ankX* on galactose medium (Fig. 5B, strains iii and iv). Such suppression is not caused by inhibition of AnkX expression in yeast, because AnkX in these yeast cells is readily detectable (Fig. 5C). In mammalian cells, Lem3 appeared to localize in the cytoplasm and did not cause any discernable change in cell morphology (Fig. S3). Lem3 was able to rescue the cell-rounding phenotype and the inhibition of secreted alkaline phosphatase (SEAP) secretion by AnkX (Fig. 5D–G). Lem3 was unable to suppress the yeast toxicity of SidM (Fig. S4) (22), suggesting its specificity toward the activity of AnkX. Like *ankX*, *lem3* is not essential for intracellular replication of *L. pneumophila* in bone marrow-derived macrophages or *Dictyostelium discoideum*, two commonly used host systems (Fig. S5). Together, these data suggest that Lem3 functions to antagonize the activity of AnkX.

The activity associated with total bacterial cell lysate in removing the PCylation signal from Rab1 suggested that Lem3 biochemically reverses the posttranslational modification on the target of AnkX. Because His₆-AnkX present in the samples may interfere with Lem3 activity, we first removed the PC transferase from the reactions by antibody neutralization and extensive washing (Fig. S6). When cleaned PCylated GST-Rab1 was incubated with His₆-Lem3 at a molar ratio of 5:1, about 80% of the

PCylated substrate was dePCylated in 10 min (Fig. 6A, lane 3). Further incubation for 30 min removed the PC moiety from Rab1 almost completely (Fig. 6B, lane 5). Similar kinetics of Lem3-mediated PC moiety loss from PC-GST-Rab1 were observed when the samples were analyzed by mass spectrometry, in which the

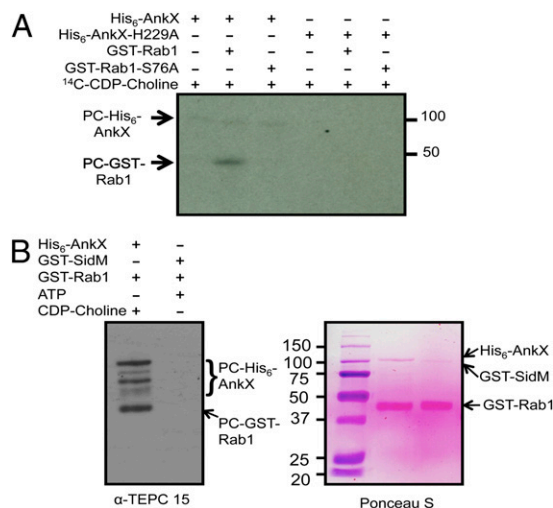


Fig. 3. AnkX is a PC transferase that modifies Rab1. (A) PCylation of Rab1 by AnkX requires the Fic domain and Ser₇₆ of the small GTPase. A series of reactions with the indicated components and ¹⁴C-CDP-choline was allowed to proceed for 30 min at 35 °C. After separation by SDS/PAGE, the incorporation of ¹⁴C-PC into the proteins was detected by autoradiography. The sizes of relevant protein markers (in kDa) are indicated. (B) The PC-specific antibody TEPC 15 can detect PCylated Rab1 and PCylated AnkX but not AMPylated Rab1. GST-Rab1 was incubated with His₆-AnkX and CDP-choline or with GST-SidM and ATP. After SDS/PAGE, proteins on the same membrane were detected by Ponceau S staining (*Right*) or by Western blot with the TEPC 15 antibody (*Left*).

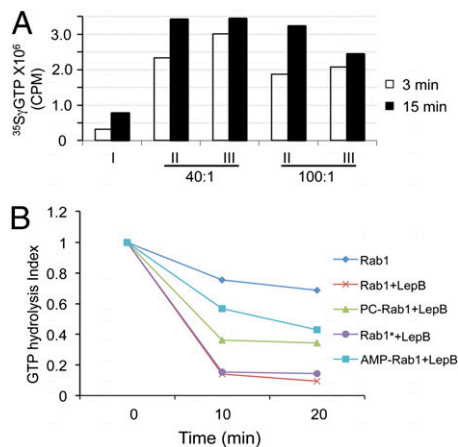


Fig. 4. PCylation of Rab1 affects its activity in GTP loading stimulated by SidM and hydrolysis induced by LepB. (A) GDP-loaded GST-Rab1 or GST-PC-Rab1 was incubated with ^{35}S GTP for the indicated time in reactions with different molar ratios of Rab1 and SidM. Radioactivity associated with the protein was determined by a scintillation counter. Reactions: I, Rab1 without SidM; II, Rab1 with SidM; III, PCylated Rab1 with SidM. (B) Differently modified GST-Rab1 was loaded with ^{32}P PyGTP, and GTPase activity was induced by adding LepB. Rab1* denotes the protein incubated with AnkXH229A and CDP-choline. The GTP hydrolysis index is the ratio of the radioactivity at the endpoint of the experiments and the radioactivity associated with Rab1 before LepB was added.

relative abundance of PCylated peptide -T₇₂ITSSYYR₇₉- dropped to about 20% after 10-min incubation with Lem3 and to less than

8% when incubation was extended to 30 min (Fig. 6B). In experiments to determine dose-dependent activity of Lem3, incubation of PC-GST-Rab1 with His₆-Lem3 at a similar molar ratio for 10 min led to an ~75% reduction of PCylated GST-Rab1 (Fig. 6C, lane 5). Importantly, the mass of the dePCylated peptide was identical to that of untreated Rab1 (Table S1), indicating that Lem3 catalyzed a simple hydrolytic reaction to remove the PC moiety from Rab1. Consistent with this observation, dePCylated GST-Rab1 can be remodified by AnkX and CDP-choline (Fig. S7). Further, Lem3 did not detectably affect LepB-induced GTP hydrolysis by Rab1, but treatment of PCylated Rab1 with Lem3 restored the accessibility of LepB to the small GTPase, leading to full GTP hydrolysis activity (Fig. S8). Taken together, these results establish that Lem3 is a dephosphorylcholinase that functions to regulate the activity of AnkX by reversing its posttranslational modification on Rab1.

Discussion

Posttranslational modification conferred by Fic domain proteins, particularly via AMPylation, has emerged as a recently recognized mechanism of signal transduction (15, 25). Our findings and the recent discoveries by Mukerjee and colleagues (26) have expanded our knowledge about the biochemical activity of the Fic domain, and our understanding of the biochemical capacity of proteins harboring this catalytic motif likely will continue to expand. For example, some enzymes may use CTP, GTP, or UTP as the substrate in the reaction (27). Our identification of Lem3 as a dephosphorylcholinase showed that, like other commonly used posttranslational modifications such as phosphorylation,

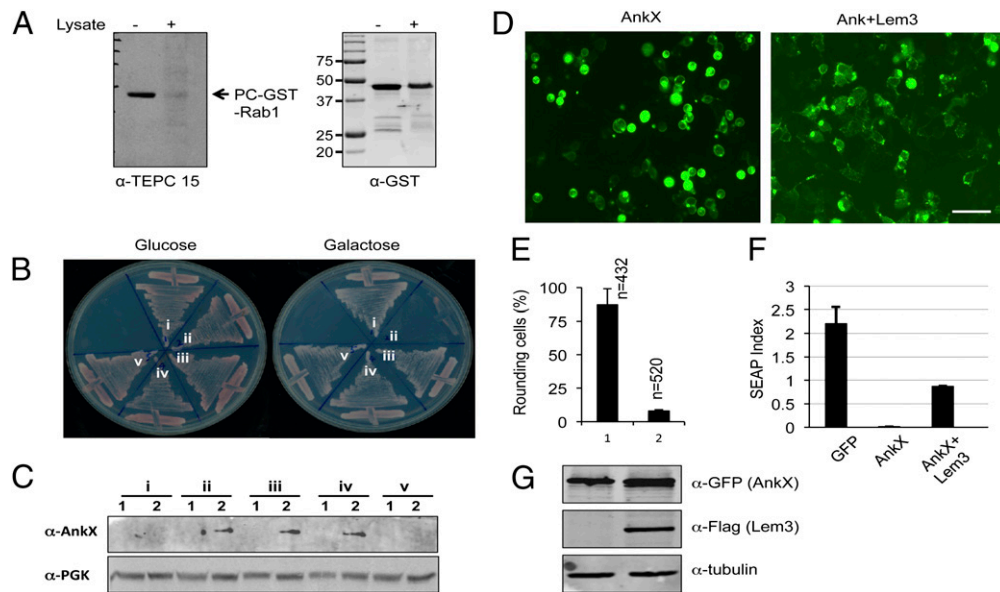


Fig. 5. Identification of a *L. pneumophila* gene capable of suppressing AnkX toxicity to eukaryotic cells. (A) Removal of PCylation signals from Rab1 by total-cell lysate of *L. pneumophila*. Soluble bacterial cell lysate was incubated with PCylated GST-Rab1, and proteins resolved by SDS/PAGE were detected for the levels of PCylation with the TEPC 15 antibody (Left) or with a GST-specific antibody (for GST-Rab1). The sizes of relevant protein markers (in kDa) are indicated. (B) The yeast strain W303(pGal::AnkX) was transformed with empty vector (ii), one of the original identified suppressor clones (iii), or plasmid harboring the *lem3* gene (iv). Yeast cells were streaked onto selective medium containing glucose (Left) or galactose (Right). Strains carrying the vector (i) or the *lem3* plasmid (v) were included as additional controls. Plates were incubated at 30 °C for 3 d before image acquisition. (C) Expression of *ankX* in yeast. SDS/PAGE-resolved lysates of indicated yeast cells uninoculated (1) or inoculated (2) with galactose were detected with an AnkX specific antibody. PGK was probed as a loading control (Lower). (D and E) Lem3 rescued the cell-rounding phenotype caused by AnkX in mammalian cells. (D) 293T cells were transfected with pEGFP::AnkX/pFlag (Left) or with pEGFP::AnkX/pFlag::Lem3 (1:1 molar ratio) (Right) for 24 h before image acquisition. (Scale bar, 50 μm.) (E) The same samples were used to quantitate the percentage of cells exhibiting the rounding phenotype. (F and G). Lem3 rescued the AnkX-mediated inhibition of SEAP secretion by mammalian cells. The indicated combinations of plasmids were used to cotransfect 293T cells with a plasmid that directs SEAP biosynthesis. Twenty-four hours after transfection, extracellular and intracellular SEAP levels were measured. (F) The SEAP index is the ratio between extracellular and intracellular SEAP activity. (G) The same samples were used to evaluate the expression of GFP-AnkX and Flag-Lem3. For quantitative assays, each experiment was performed in triplicate, and data from the samples were used to calculate SD. Similar results were obtained in at least three independent experiments.

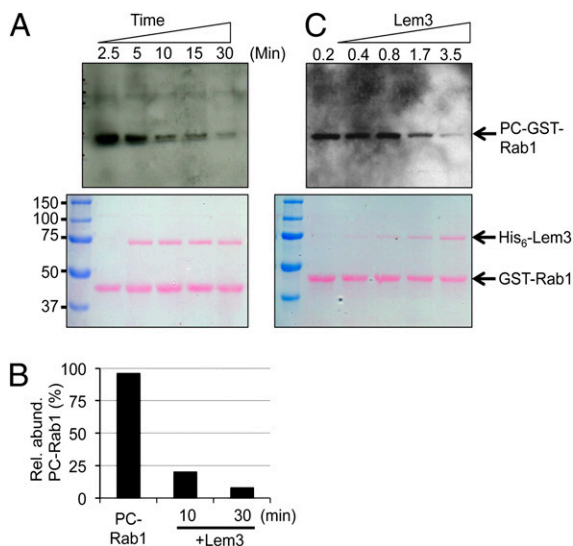


Fig. 6. Lem3 is a dephosphorylcholinase that removes the PC moiety from PCylated Rab1. (A and B) GST-Rab1 modified by AnkX and CDP-choline was incubated with His₆-Lem3 at a 5:1 molar ratio for the indicated time. (A) After SDS/PAGE, the levels of PCylated GST-Rab1 were analyzed by immunoblotting with the TEPC 15 antibody (Upper). Protein levels were detected by Ponceau 5 staining (Lower). (B) After SDS/PAGE, the levels of PCylated GST-Rab1 were analyzed by mass spectrometry of the GST-Rab1 protein bands. (C) Dose-dependent activity of Lem3. Ten micrograms of PCylated GST-Rab1 were incubated with the indicated amounts of His₆-Lem3 for 10 min. Proteins resolved by SDS/PAGE were detected first by Ponceau 5 staining (Lower), and the levels of PCylation were detected by Western blot (Upper). The sizes of relevant protein markers (in kDa) are indicated.

ubiquitination, and the more recently recognized AMPylation, PCylation is a reversible process regulated by specific enzymes.

AnkX has been shown to be necessary for efficient inhibition of the acquisition of lysosomal-associated membrane protein 1 by the LCV but not to be essential for intracellular growth of *L. pneumophila* under standard laboratory conditions (14). Similarly, Lem3 is dispensable for intracellular replication in two tested hosts. As suggested by a recent study, *L. pneumophila* appears to acquire clusters of Dot/Icm substrates for the expansion of its host range (28). These proteins may be required for maximal virulence under certain conditions or during the interactions of *L. pneumophila* with some hosts. The synteny of *ankX* and *lem3* provided another example of the regulation of the activity of the Dot/Icm substrate by closely linked genes. The ubiquitin ligase LubX targets the product of its neighboring gene *sidH* for proteasome degradation (29), presumably to prevent the latter from causing yet-unidentified detrimental effects in the development of LCVs. Similarly, SidD down-regulates the effects of SidM/DrrA by an enzymatic activity that is the opposite of AMPylase to allow efficient removal of Rab1 from the bacterial phagosome (22, 30). The close linkage of these gene pairs in the *L. pneumophila* genomes emphasizes the importance of properly regulating the activity of the proteins that directly target host processes. Many genes coding for Dot/Icm substrates form clusters, with some organized into operon-like structures (7). Investigation of the potential functional relationships among these closely linked genes could facilitate the study of their biochemical activities and their roles in *L. pneumophila* infection.

Although the functional relationship between AnkX and Lem3 is reminiscent of the relationship between SidM/DrrA and SidD, the role of Lem3 during *L. pneumophila* infection is not as clear as that of SidD. Furthermore, SidD appears to regulate the effects of SidM/DrrA temporally, but such regulation is not apparent for Lem3 on AnkX. Unlike many Dot/Icm substrates whose

expression is induced when the bacterium reaches postexponential phase, *ankX* and *lem3* appear to express constitutively throughout the entire bacterial growth cycle in broth (Fig. S9). Consistently, their protein levels in the cytosol of infected macrophages were constant during the first 8 h of infection (Fig. S10). Deletion of *lem3* did not cause detectable changes in infected cells, suggesting that the effect of AnkX during infection is subtle and probably highly specific for certain cellular compartments. Most of the yeast genes capable of suppressing AnkX toxicity are involved in the docking of ER-derived trafficking vesicles at the *cis*-Golgi compartment (16). Thus, it is tempting to speculate that Lem3 spatially regulates the activity of AnkX. It is possible that one of the functions of AnkX is to inhibit membrane-fusion events at the *cis*-Golgi compartment. Such cellular compartment-specific regulation can be achieved by specific targeting of the protein to its sites of action. The multiple ankyrin repeat in AnkX may be involved in localizing the protein to specific cellular compartments by interacting with proper resident proteins. Clearly, to substantiate this speculation, it is of paramount importance to determine the precise cellular localization of AnkX during *L. pneumophila* infection. Such inhibition could be beneficial to the pathogen because it might make more vesicles available for fusion with its phagosome, thus facilitating the membrane-remodeling process. On the other hand, Lem3 may localize at cellular sites such as the LCV, which are different from those at which AnkX localizes, to ensure that mislocalized AnkX does not “accidentally” prevent membrane fusion at the bacterial phagosome. Regardless of the mechanism, the activities of AnkX and Lem3 emphasize the importance of a balanced manipulation of host function during *L. pneumophila* infection and may explain the necessity for this pathogen to code for such a large effector repertoire.

Phosphorylcholation has been described in all domains of organisms, mostly on antigens of both prokaryotic and eukaryotic pathogens that play important roles in the modulation of the host’s immune system (31, 32). Because the modification leads to alternations in the activity of its target proteins, PCylation can be considered a form of signaling mechanism. This modification appears to play an important role in development and reproduction of *Caenorhabditis elegans* (33). Despite its wide use and the potentially important roles in many essential biological processes, few PC transferases that directly modify the backbone of target proteins have been identified, nor have enzymes that specifically reverse the modification (32). Further study of proteins with biochemical activities similar to those of AnkX and Lem3 likely will unravel novel cellular processes regulated by phosphorylcholation.

Materials and Methods

Bacterial, Yeast Strains and Plasmid Construction. All *L. pneumophila* strains used in this study were derivatives of the Philadelphia 1 strain Lp02 (34). Bacteria were grown and maintained with standard procedures (35). In-frame deletion mutants of *ankX* and *lem3* were constructed as described (36). In each case, the primers were designed so that the target gene was replaced by a 32-amino acid polypeptide consisting of the first and the last 15 residues and two amino acids encoded by recognition element of the restriction enzyme BamHI (36). For complementation experiments, the gene was expressed on the RSF1010-derived plasmid pJB908 (37). When necessary, antibiotics were used as described (35). For infection experiments, *L. pneumophila* strains were grown in AYE broth to the postexponential phase as judged by optical density of the cultures ($OD_{600} = 3.3\text{--}3.8$) as well as by an increase of bacterial motility. For expression in mammalian cells, genes were cloned into pEGFP-C1 (Clontech) or p4XFlag (Sigma). All constructs were verified by sequencing analysis. The sequences of primers used in this study are available upon request. Intracellular bacterial growth was performed with established methods (35). Other materials and methods used are listed in *SI Materials and Methods*.

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