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Modulation of Rab GTPase function by a protein phosphocholine transferase

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Abstract

The intracellular pathogen *Legionella pneumophila* modulates the activity of host GTPases to direct the transport and assembly of the membrane-bound compartment in which it resides[1–6]. *In vitro* studies have suggested that the *Legionella* protein DrrA post-translationally modifies the GTPase Rab1 by a process called AMPylation[7]. Here, mass spectrometry was used to investigate post-translational modifications to Rab1 that occur during infection of host cells by *Legionella*. Consistent with *in vitro* studies, DrrA-mediated AMPylation of a conserved tyrosine residue in the switch II region of Rab1 was detected during infection. In addition, a modification to an adjacent serine residue in Rab1 was discovered, which was independent of DrrA. The *Legionella* effector protein AnkX was required for this modification. Biochemical studies determined that AnkX directly mediates the covalent attachment of a phosphocholine moiety to Rab1. This phosphocholine transferase activity used CDP-choline as a substrate and required a conserved histidine residue located in the FIC domain of the AnkX protein. During infection, AnkX modified both Rab1 and Rab35, which explains how this protein modulates membrane transport through both the endocytic and exocytic pathways of the host cell. Thus, phosphocholination of Rab GTPases represents a mechanism by which bacterial FIC domain-containing proteins can alter host cell functions.

Legionella pneumophila is an intracellular pathogen that translocates proteins called effectors into the host cell cytosol using a type IV secretion system called Dot/Icm[8]. The *Legionella* protein DrrA (SidM) is an effector that targets the host GTPase Rab1[1–3, 5]. Initially identified as a Rab1-specific guanine nucleotide exchange factor (GEF), recent studies showed that the amino-terminal region of DrrA has structural similarity to glutamine synthetase adenylyl transferase (GS-ATase) and shares the catalytically important sequence

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Contributions:
SM, XL, KA performed research. XL conducted the mass spectrometry analysis, KA generated HEK293 FCγ3XFLAG-Rab1 stable cell line, JM conducted studies on the CBU_2078 protein, and SM conducted all other research. SM, XL, JM and CRR analyzed results and wrote manuscript.

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motif G-X_{11}-D-X-D, which enables DrrA to AMPylate the Tyr77 residue in the class II switch region of Rab1B[7]. To determine if the *in vitro* activity described for DrrA is biologically relevant we examined whether the endogenous DrrA protein mediates Rab1 AMPylation when delivered into host cells during *Legionella* infection.

Cells were infected with a strain of *Legionella* that has a functional Dot/Icm system that delivers effectors into host cells (WT) or an isogenic Δ*dotA* mutant that has a non-functional Dot/Icm system, and Rab1 protein was analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Fig. 1a). Two different modifications in the switch II region in Rab1 were detected after infection with WT *Legionella*. A fragment that corresponded to an AMPylated TITSSYYR peptide (*m/z* = 660.5) was detected. Unexpectedly, a form of this peptide with an unknown moiety of 183 Da (*m/z* = 578.5) was also detected (Fig. 1a). The Δ*dotA* mutant revealed that both modifications required the delivery of effector proteins into host cells during infection. Thus, Rab1 is modified during *Legionella* infection by AMPylation and by a second unknown post-translational mechanism.

Cells were infected with mutant strains of *Legionella* deficient in effectors that could be involved in AMPylation of Rab1. In addition to the Δ*drrA* mutant, a Δ*ankX* mutant of *Legionella* was examined. The AnkX protein contains a FIC domain, which for other bacterial effectors has been shown to have an enzymatic activity that promotes the AMPylation of small GTPases[9–13]. When microinjected into mammalian cells the AnkX protein disrupts membrane transport in the secretory pathway and interferes with the sorting of transferrin from early endosomes, consistent with AnkX being an effector that disrupts the activities of host membrane transport proteins, potentially by Rab AMPylation[14].

Rab1 AMPylation was not detected in the samples isolated from cells infected with the Δ*drrA* mutant, indicating that DrrA is the primary effector mediating Rab1 AMPylation *in vivo* (Fig. 1a). The unknown modification (*m/z* = 578.5) was detected after infection with the Δ*drrA* mutant, but was not detected after infection with the Δ*ankX* mutant. Thus, the unknown modification to Rab1 that occurs during infection requires AnkX. Defects in Rab1 modifications exhibited by these *Legionella* mutants were complemented upon the introduction of plasmids that restored DrrA and AnkX production (Supplementary Fig. 1a). MS/MS analysis revealed that the unknown 183 Da moiety was attached to Ser79 of Rab1A, adjacent to the Tyr80 residue AMPylated by DrrA (Fig. 1b). These residues correspond to Ser76 and Tyr77 in Rab1B.

Purified DrrA radiolabeled GST-Rab1 *in vitro* when ^{32}P-α-labeled ATP was used as a substrate, but no labeling was detected using ^{32}P-γ-labeled ATP, validating that DrrA mediates the attachment of AMP to Rab1 (Fig. 1c). The structurally distinct amino-terminal region of DrrA was sufficient for AMPylation[3, 15–17], and no AMPylation activity was detected for $DrrA_{340–533}$ or the $DrrA_{D110A, D112A}$ variant having the G-X_{11}-D-X-D adenylyl transferase domain inactivated. The effector AnkX was unable to efficiently AMPylate Rab1, the GTP-locked $Rab1_{Q70L}$ variant or the GDP-locked $Rab1_{S25N}$ variant, indicating that purified AnkX does not have robust Rab1 AMPylation activity (Fig. 1c).

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The nature of the unknown modification to Rab1 requiring AnkX was investigated further. Cells were transfected with a plasmid encoding either AnkX or the variant $AnkX_{H229A}$, which has the essential histidine residue in the FIC domain changed to alanine. Roughly 70% of the Rab1 isolated from cells producing AnkX had the 183 Da moiety attached, whereas, Rab1 isolated from cells producing the $AnkX_{H229A}$ protein was unmodified (Fig. 2a). Thus, AnkX is both necessary and sufficient to promote a novel post-translational modification to Rab1 by a process that requires a functional FIC domain.

For molecules <200 Da, the elemental composition can often be determined from a highly accurate mass measurement[18]. High-resolution MS measurements obtained for the modified Rab1 peptide isolated from cells producing AnkX revealed that the moiety attached to the Ser79 residue had an accurate mass of 183.0661 (Supplementary Fig. 1b). This moiety did not match any known post-translational modifications, but when a metabolite database (http://metlin.scripps.edu) was searched a near perfect match was made to the molecule phosphocholine, which has an exact mass of 183.0660. The protonated moiety attached to the Rab1 peptide was selected and dissociated by MS/MS/MS (MS^3) analysis. The fragments generated were of the sizes predicted for phosphocholine (Fig. 2b) and matched the MS/MS spectrum obtained following dissociation of a phosphocholine standard (Supplementary Fig. 2), suggesting Rab1 is phosphocholinated by AnkX.

If AnkX were functioning directly as a phosphocholine transferase, the host molecule most likely to be used as a substrate in this reaction would be CDP-choline, which is an intermediate used to synthesize phosphatidylcholine[19]. Indeed, phosphocholination of Ser79 on Rab1 was detected for *in vitro* reactions containing CDP-choline and AnkX, but not in reactions containing DrrA (Fig. 2c) or the $AnkX_{H229A}$ protein (Supplementary Fig. 3). Increasing the amount of AnkX in the *in vitro* reaction resulted in higher levels of phosphocholinated Rab1 being detected by anti-phosphocholine immunoblot analysis (Fig. 2d), validating that Rab1 is phosphocholinated by AnkX.

Phosphocholinated proteins in the size range of Rab GTPases were detected in lysates from cells producing AnkX and were not observed in lysates from cells producing $AnkX_{H229A}$ (Supplementary Fig. 4a). Phosphatidylinositol 4-phosphate and phosphatidic acid levels were not affected in cells producing AnkX, suggesting these is no indirect effect on phospholipid metabolism (Supplementary Fig. 4b). The intracellular pathogen *Coxiella burnetii* translocates a FIC-domain effector called CBU_2078 into host cells[20]. Although proteins reacting with the anti-phosphocholine antibody were found in the size range of small GTPases from cell lysates producing CBU_2078, there was no evidence of Golgi fragmentation or endosome enlargement in these cells (Supplementary Fig. 4a,c). Thus, defects in host membrane transport in AnkX-producing cells likely results from phosphocholination of a specific subset of Rab GTPases, although it can not be excluded that CDP-choline consumption might augment these effects.

The repertoire of Rab proteins that could be modified by *Legionella* effectors *in vivo* was investigated. Modifications to the Rab5 protein were not detected in cells producing either AnkX or DrrA (Supplementary Fig. 5). DrrA mediated the AMPylation of Rab6 on Tyr82, but phosphocholination of Rab6 mediated by AnkX was not detected (Fig. 3a and

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Supplementary Fig. 5). Rab35 is a Rab1 family member that regulates the sorting of cargo from early endosomes, and interfering with Rab35 function results in enlarged early endosomes[21]. Importantly, specific perturbations in Rab35 function result in a cellular phenotype that closely mirrors the defects in endosome morphology observed in cells microinjected with purified AnkX[14]. Phosphocholinated Rab35 was detected in samples isolated from cells producing AnkX, and AMPylated Rab35 was detected from cells producing DrrA (Fig. 3b). During infection, phosphocholination of Rab35 required AnkX and AMPylation of Rab35 required DrrA (Fig. 3c). Thus, AnkX has specificity for Rab1 family members.

To test whether previously described cellular disruptions mediated by AnkX required the FIC domain-dependent phosphocholine transferase activity (Fig. 4a), cellular phenotypes mediated by AnkX and the $AnkX_{H229A}$ mutant were compared. Disruption of the Golgi apparatus and a block in secretion of host alkaline phosphatase into the culture supernatant were observed in cells producing AnkX but not in cells producing $AnkX_{H229A}$ (Fig. 4b and Supplementary Fig. 7). Importantly, when the serine 79 residue in Rab1 was changed to alanine, the variant protein was no longer phosphocholinated by AnkX (Supplementary Fig. 8a), however, $Rab1_{S79A}$ interfered with secretion of alkaline phosphatase when produced in cells (Supplementary Fig. 8b). Thus, AnkX is targeting a residue in Rab1 that is critical for function. There was a significant increase in the number of cells containing enlarged early endosomes in cells producing AnkX compared to cells producing $AnkX_{H229A}$ (Fig. 4c and Supplementary Fig. 7), consistent with the function of Rab35 being perturbed by phosphocholination.

Because GEF proteins are essential for Rab activation, the effect of phosphocholination on the binding of Rab-specific GEFs was analyzed. The eukaryotic Connecdenn proteins are the only known GEFs for Rab35 and are required for Rab35 function *in vivo*[21]. A pronounced defect in binding of Connecdenn was observed for phosphocholinated Rab35 isolated from cells producing AnkX (Fig. 4d), which would explain why AnkX overproduction mimics the cellular phenotype observed when Connecdenn has been silenced in mammalian cells[21]. By contrast, there was no defect in the binding of phosphocholinated Rab1 with the GEF domain of DrrA (Fig. 4e), similar to what has been observed for DrrA interactions with AMPylated Rab1[7]. Thus, post-translational modifications mediated by the effectors AnkX and DrrA modulate the function of Rab GTPases during infection by tailoring the repertoire of proteins that interact with the modified GTPase.

The characterization of DrrA and AnkX provides an example of *Legionella* having structurally distinct proteins with different biochemical activities that modulate the function of host vesicle transport proteins similarly. This concept of functional redundancy has been postulated but not shown clearly. The differences observed in the *in vivo* specificities displayed by these two effectors, however, demonstrate that they are also likely to have roles in modulating Rab protein functions that do not overlap, which could explain why positive selection has led to the emergence of two different pathways to modify Rab protein function through post-translational modification.

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The reaction mediated by AnkX has similarities to the AMPylation reaction demonstrated for other FIC domain proteins. Both reactions use a nucleotide-based substrate as the donor molecule that mediates the post-translational modification process (Supplementary Fig. 9). Interestingly, in the AMPylation reaction, hydrolysis of the phosphoanhydrous bond results in protein modification by the 5'-ribonucleotide of the donor substrate, whereas, in the phosphocholination reaction, the 5'-ribonucleotide is presumably released and the phosphocholine group is transferred to the polypeptide chain.

There are several examples where post-translational modifications introduced by bacterial toxins or effectors, which were thought to be the exclusive domain of pathogens, were discovered to represent mechanisms used to regulate eukaryotic cell functions. Curiously, the inclusion of a phosphocholine moiety in a protein structure was suggested previously from studies examining peptides secreted by nematodes and from mammalian cells residing in the placenta [22, 23]. Thus, protein phosphocholination may also be used by eukaryotic organisms to modulate cellular functions.

Methods Summary

MS/MS analysis of Rab GTPases was conducted on immunoprecipitated proteins that were fractionated by SDS-PAGE, digested with trypsin in the gel, and extracted peptides were separated using nano-LC and electrosprayed directly onto a linear ion trap mass spectrometer (LTQ Velos, ThermoElectron, San Jose, CA) for MS and MS/MS analysis. All biochemical assays were conducted using purified proteins as described in the Methods section. The antibody TEPC-15 (Sigma) was used to detect phosphocholinated proteins by immunonoblot analysis.

Methods

Cell culture and transfection

COS7 and HEK293 cells were grown in DMEM (Dulbecco modified Eagle medium) from Gibco (Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum (FBS, Gibco). Cell lines were cultured at 37°C in 5% CO_2. For transfection, COS7 or HEK293 cells were added to 12-mm coverslips in 24 well plates at a density of 2.5×10^4 cells per well. Cells were transfected with 0.5 μg of each plasmid. Cells were fixed 18 hr after transfection in 4% PFA, permeabilized with 0.05% saponin and processed for immunofluorescence microscopy as described previously[14].

Fluorescence microscopy

Digital images were acquired with a Nikon TE300 microscope using a 100× 1.4 N.A objective lens and a Hamamatsu ORCA-ER camera controlled by IP Lab software.

Alkaline phosphatase secretion assay

Secretion assays were performed as described[24]. Briefly, HEK293 cells were co-transfected with a plasmid encoding secreted alkaline phosphatase and a plasmid encoding either GFP, GFP-AnkX or GFP-$AnkX_{H229A}$. After an 18 hr incubation period, cells were washed with

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PBS and fresh medium was added. Cells were incubated for 10 hr and then the alkaline phosphatase secretion index was determined by measuring the ratio of alkaline phosphatase protein secreted into the culture medium to the total amount of alkaline phosphatase protein in the assay well. The Tropix PhosphaLight System Kit (Applied BioSystems) was used to measure alkaline phosphatase activity and the Tecan Infinite M1000 plate reader with iControl Software was used to detect chemiluminescence. Data shown are the mean ± SD from three independent samples for each condition. Results were validated in two independent assays.

Protein purification and *in vitro* AMPylation

His-tagged and GST-tagged proteins were purified as described previously[1]. Purified GST-Rab1A (2 μg) was incubated with 0.4 μg of purified effector protein in buffer (20mM HEPES pH = 7.4, 100 mM NaCl, 1 mM $MgCl_2$ and 0.1 mM GTPγS) and incubated for 1 hr at 30°C in the presence of 2 μCi of ^{32}P-α-labeled ATP or ^{32}P-γ-labeled ATP (Perkin Elmer). Labeled proteins were identified by autoradiography following SDS-PAGE. *In vitro* AMPylation results shown are representative of three independent assays.

Bacterial strains and plasmids

The *Legionella* strains were grown on charcoal yeast extract plates as described previously[14]. The parental strain (WT) was *L. pneumophila* serogroup 1 strain Lp01, and the variant strains were all isogenic mutants described previously[1, 14, 25, 26], with the exception of the Δ*ankX*,Δ*drrA* double mutant, which was generated for this study using allelic exchange to introduce the Δ*ankX* mutation into the Δ*drrA* strain as described[14]. For all experiments, *Legionella* were isolated from charcoal yeast extract plates after growth for 2 days at 37°C. The plasmid pEFGPC2 (Clontech) was used for all GFP fusion constructs, the plasmid pJB1806 was used to produce DrrA and AnkX in *Legionella*, pQE30 (Qiagen) was used for all His-tagged constructs, and pGEX2TK (GE LifeSciences) was used for all GST-tagged constructs. The Rab plasmids were constructed using cDNA encoding human Rab1A, Rab5A, Rab6A, Rab35 and canine Rab1A.

Cell lines and *Legionella* infection

HEK293 FCγRII cells[27] were used to create the HEK293 FCγRII 3X-FLAG-Rab1A stable cell line. This cell line was used for assays examining Rab1 modifications during infection of host cells by *Legionella*. For each assay, cells grown to near confluency in two 10 cm dishes were infected with opsonized *Legionella pneumophila* strain LP01 (WT) or the isogenic mutants at an estimated multiplicity of 100 bacteria to 1 host cell. After incubation for 0.5 hr at 37°C the cells were lysed in buffer containing 20 mM HEPES pH = 7.4, 100 mM NaCl, 1 mM $MgCl_2$, 1% TritonX-100, 1 mM PMSF and protease inhibitor cocktail (Roche). Lysates were centrifuged at 13,000 rpm and the post-nuclear supernatant was then incubated with FLAG antibody-coated beads (Sigma) at 4°C for 1 hr, washed and the Rab1 protein was eluted using FLAG peptide. Samples were then processed for MS/MS analysis. This same approach was used to assay modifications to Rab35 after infection, except that the HEK293 FcγRII cell line was transfected with an expression plasmid producing 3X-FLAG-Rab35 and then infected 18 hr after transfection.

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Golgi and early endosome disruption assay

The disruption of Golgi and early endosomes was assessed in COS7 cells producing GFP-AnkX, GFP-AnkX$_{H229A}$, GFP-CBU_2078 or GFP alone. Cells were fixed with 4% PFA 18 hr after transfection and stained with mouse monoclonal antibodies specific for either GM130 or EEA1 (BD Transduction Laboratories) at a dilution of 1:200. Golgi and endosome morphology in GFP-positive cells was assessed by fluorescence microscopy. The Golgi was completely fragmented in nearly all the cells producing AnkX, whereas, no significant fragmentation was observed compared to the background of untransfected cells in cells producing GFP-AnkX$_{H229A}$, GFP-CBU_2078 or GFP alone. The early endosome disruption index represents the percentage of cells producing the indicated GFP protein that displayed an enlarged endosome phenotype as determined by EEA1 staining. The data represent the mean ± s.d., from three independent replicates in which 200 cells were counted for each protein. *P*-values were computed using Student's unpaired t-test. Data shown were validated in two independent experiments.

In vitro phosphocholination assay

GST-tagged Rab1A (5 μg) attached to glutathione agarose was incubated with thrombin to remove the tag. Rab1A was incubated with 1 μg of purified effector protein in buffer (20 mM HEPES pH = 7.4, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM ATP) and incubated for 1 hr at 30° C in the presence of 1 mM CDP-choline. Samples were boiled in SDS-loading buffer and the Rab1 protein was excised after SDS-PAGE and analyzed by LC-MS/MS analysis. For the immunoblot analysis, the amounts of AnkX in each reaction varied from 0 μg to 1.0 μg, and the amount of modified Rab1 in each reaction was compared by immunoblot analysis using an antibody specific for phosphocholine (TEPC-15, Sigma) to detect modified Rab1 and an antibody specific for Rab1A (Santa Cruz Biotechnologies) to detect total Rab1. Data shown were validated in three independent experiments.

Detection of host proteins modified by AnkX and CBU_2048 *in vivo*

HEK293 cells in 24-well dishes were transfected with plasmids encoding GFP-CBU_2078, GFP-AnkX or GFP-AnkX$_{H229A}$ and cultured for 18 hr. Cells were lysed in buffer containing 20 mM HEPES pH = 7.4, 100 mM NaCl, 1 mM $MgCl_2$, 1% TritonX-100, 1 mM PMSF and protease inhibitor cocktail. The lysates were centrifuged at 13,000 rpm and 50 μg of the supernatant was separated by SDS-PAGE for immunoblot analysis using the anti-phosphocholine specific antibody TEPC-15 (Sigma).

GEF binding assays

To measure Connecdenn binding to modified Rab35, a plasmid encoding 3X-FLAG-Rab35$_{S22N}$ was co-transfected into cells together with a plasmid encoding GFP-CBU_2078, GFP-AnkX or GFP-AnkX$_{H229A.}$ The GDP-locked allele of Rab35 was used because it demonstrates enhanced Connecdenn binding. Cellular lysates were prepared as described above, and post-nuclear supernatant was incubated with 4 μg of purified Connecdenn (Genbank accession number NP_659414). 3X-FLAG-Rab35$_{S22N}$ was precipitated using anti-FLAG agarose (Sigma) and eluted from the beads using the 3X-FLAG peptide (Sigma). The eluted proteins were resolved by SDS-PAGE gel and proteins were identified after the

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gel was stained with Coomassie brilliant blue dye. The locations of 3X-FLAG-Rab35$_{S22N}$ and Connecdenn were determined by running purified Connecdenn and 3X-FLAG-Rab35$_{S22N}$ in adjacent wells. To measure DrrA binding to modified Rab1, HEK293 cells that stably produce 3X-FLAG-Rab1 were transfected with GFP-CBU_2078, GFP-AnkX or GFP-AnkX$_{H229A}$. 500 μg of post-nuclear supernatant was incubated with 0.5 μg of purified His-tagged DrrA$_{340–533}$. 3X-FLAG-Rab1 was precipitated using anti-FLAG agarose beads (Sigma). Immunoblot analysis was used to compare the amount of DrrA (anti-His), the amount of total 3X-FLAG-Rab1 (anti-FLAG, Sigma) and the amount of phosphocholinated Rab1 (TEPC-15, Sigma) after 2% of the total amount of 3X-FLAG-Rab1 precipitated from each reaction was fractionated by SDS-PAGE. Data shown were validated in three independent experiments.

Mass spectrometry

3X-FLAG-Rab proteins were immunoprecipitated from cells using anti-FLAG agarose beads, and separated by SDS-PAGE. The band corresponding to the 3X-FLAG-Rab protein was excised from the gel and treated with DTT to reduce disulfide bonds and then alkylated with iodoacetamide (IAM). Trypsin digestion was allowed to occur overnight. Resulting peptides were extracted from gel matrix and then resuspended in aqueous buffer before final LC-MS/MS analysis. Nanoflow reverse-phase LC separation was carried out on a Proxeon EASY-nLC system (Thermo Scientific). The capillary column (75 μm × 150 mm, PICOFRIT, New Objective, Woburn, MA) was packed in-house. A methanol slurry containing 5 μm, 100 Å Magic C18AQ silica-based particles (Microm BioResources Inc., Auburn, CA) was forced to run through an empty capillary (with a frit in the end) using a pressurized device. The LC mobile phase was comprised of solvent A (97% H_2O, 3% acetonitrile (ACN), and 0.1% formic acid (FA)) and solvent B (100% ACN and 0.1% FA). The nano-LC separation was performed with the following gradient: B was increased from 7% to 35% in 40 min and then raised to 90% in 3 min and kept there for 10 min before going back to 100% A for column equilibration. At the moment when peptides were eluted from the capillary column, they were electrosprayed directly onto a linear ion trap mass spectrometer (LTQ Velos, ThermoElectron, San Jose, CA) for MS and MS/MS analysis. A data-dependent mode was enabled for peptide fragmentation with one full MS scan followed by collision induced dissociation (CID) of the ten most intense peptide ions. Dynamic exclusion was enabled to preclude repeated analyses of the same precursor ion. MS/MS scans were processed and searched using MASCOT (Matrix Science Ltd. London, UK). The resulting peptide and protein assignments were filtered to keep only those identifications with scores above extensive homology. High resolution MS/MS analysis was performed and the data was acquired by a high-resolution mass spectrometer (Orbitrap) by the Keck Proteomic Facility at Yale University. All LC-MS/MS data was validated by at least two independent experiments.

Supplementary Material

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References

1. Murata T, et al. The *Legionella pneumophila* effector protein DrrA is a Rab1 guanine nucleotide-exchange factor. Nat Cell Biol. 2006; 8:971–977. [PubMed: 16906144]
2. Ingmundson A, Delprato A, Lambright DG, Roy CR. *Legionella pneumophila* proteins that regulate Rab1 membrane cycling. Nature. 2007; 450:365–369. [PubMed: 17952054]
3. Schoebel S, Oesterlin LK, Blankenfeldt W, Goody RS, Itzen A. RabGDI displacement by DrrA from *Legionella* is a consequence of its guanine nucleotide exchange activity. Mol Cell. 2009; 36:1060–1072. [PubMed: 20064470]
4. Machner MP, Isberg RR. Targeting of host Rab GTPase function by the intravacuolar pathogen *Legionella pneumophila*. Dev Cell. 2006; 11:47–56. [PubMed: 16824952]
5. Machner MP, Isberg RR. A bifunctional bacterial protein links GDI displacement to Rab1 activation. Science. 2007; 318:974–977. [PubMed: 17947549]
6. Nagai H, Kagan JC, Zhu X, Kahn RA, Roy CR. A bacterial guanine nucleotide exchange factor activates ARF on *Legionella* phagosomes. Science. 2002; 295:679–682. [PubMed: 11809974]
7. Muller MP, et al. The *Legionella* effector protein DrrA AMPylates the membrane traffic regulator Rab1b. Science. 2010; 329:946–949. [PubMed: 20651120]
8. Ninio S, Roy CR. Effector proteins translocated by *Legionella pneumophila*: strength in numbers. Trends Microbiol. 2007; 15:372–380. [PubMed: 17632005]
9. Yarbrough ML, Orth K. AMPylation is a new post-translational modiFICation. Nat Chem Biol. 2009; 5:378–379. [PubMed: 19448605]
10. Yarbrough ML, et al. AMPylation of Rho GTPases by *Vibrio* VopS disrupts effector binding and downstream signaling. Science. 2009; 323:269–272. [PubMed: 19039103]
11. Kinch LN, Yarbrough ML, Orth K, Grishin NV. Fido, a novel AMPylation domain common to fic, doc, and AvrB. PLoS ONE. 2009; 4:e5818. [PubMed: 19503829]
12. Roy CR, Mukherjee S. Bacterial FIC Proteins AMP Up Infection. Sci Signal. 2009; 2:pe14. [PubMed: 19293428]
13. Worby CA, et al. The fic domain: regulation of cell signaling by adenylylation. Mol Cell. 2009; 34:93–103. [PubMed: 19362538]
14. Pan X, Luhrmann A, Satoh A, Laskowski-Arce MA, Roy CR. Ankyrin repeat proteins comprise a diverse family of bacterial type IV effectors. Science. 2008; 320:1651–1654. [PubMed: 18566289]
15. Brombacher E, et al. Rab1 guanine nucleotide exchange factor SidM is a major phosphatidylinositol 4-phosphate-binding effector protein of *Legionella pneumophila*. J Biol Chem. 2009; 284:4846–4856. [PubMed: 19095644]
16. Zhu Y, et al. Structural mechanism of host Rab1 activation by the bifunctional *Legionella* type IV effector SidM/DrrA. Proc Natl Acad Sci U S A. 2010; 107:4699–4704. [PubMed: 20176951]
17. Suh HY, et al. Structural insights into the dual nucleotide exchange and GDI displacement activity of SidM/DrrA. EMBO J. 2009; 29:496–504. [PubMed: 19942850]
18. Gross ML. Accurate masses for structure confirmation. J Am Soc Mass Spectrom. 1994; 5:57–57. [PubMed: 24222515]
19. Li Z, Vance DE. Phosphatidylcholine and choline homeostasis. J Lipid Res. 2008; 49:1187–1194. [PubMed: 18204095]
20. Chen C, et al. Large-scale identification and translocation of type IV secretion substrates by *Coxiella burnetii*. Proc Natl Acad Sci U S A. 2010; 107:21755–21760. [PubMed: 21098666]

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21. Allaire PD, et al. The Connecdenn DENN domain: a GEF for Rab35 mediating cargo-specific exit from early endosomes. Mol Cell. 2010; 37:370–382. [PubMed: 20159556]
22. Lovell TM, et al. Identification of a novel mammalian post-translational modification, phosphocholine, on placental secretory polypeptides. J Mol Endocrinol. 2007; 39:189–198. [PubMed: 17766644]
23. Grabitzki J, Ahrend M, Schachter H, Geyer R, Lochnit G. The PCome of *Caenorhabditis elegans* as a prototypic model system for parasitic nematodes: identification of phosphorylcholine-substituted proteins. Mol Biochem Parasitol. 2008; 161:101–111. [PubMed: 18652849]
24. Kagan JC, Stein MP, Pypaert M, Roy CR. *Legionella* subvert the functions of Rab1 and Sec22b to create a replicative organelle. J Exp Med. 2004; 199:1201–1211. [PubMed: 15117975]
25. Zuckman DM, Hung JB, Roy CR. Pore-forming activity is not sufficient for *Legionella pneumophila* phagosome trafficking and intracellular growth. Mol Microbiol. 1999; 32:990–1001. [PubMed: 10361301]
26. Berger KH, Merriam JJ, Isberg RR. Altered intracellular targeting properties associated with mutations in the *Legionella pneumophila dotA* gene. Mol Microbiol. 1994; 14:809–822. [PubMed: 7891566]
27. Arasaki K, Roy CR. *Legionella pneumophila* promotes functional interactions between plasma membrane syntaxins and Sec22b. Traffic. 2010; 11:587–600. [PubMed: 20163564]

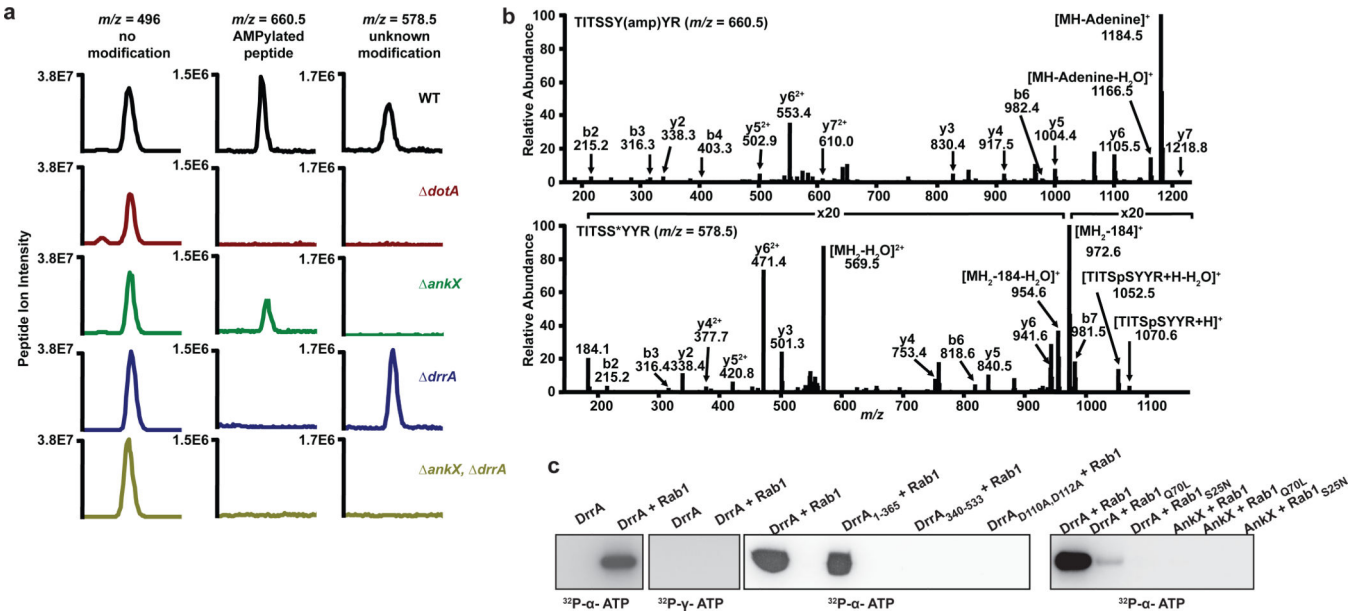

Figure 1. *Legionella* infection mediates two different post-translational modifications to Rab1

a, 3X-FLAG-Rab1 was isolated from HEK293 FcγRII cells after infection with the indicated *Legionella* strains. LC-MS/MS analysis produced the extracted ion chromatograms. The peak in each graph indicates the amount of Rab1 peptide TITSSYYR with no modifications (*m/z* = 496.0), peptide containing an AMP moiety (*m/z* = 660.5), and peptide with an unknown modification (*m/z* = 578.5). **b,** MS/MS spectra obtained for the AMPylated Rab1 peptide (top) and the Rab1 peptide with the unknown modification (bottom) showing mass to charge ratios of their fragments upon collision-induced dissociation. The AMPylated peptide (top spectrum) has a mass shift of 329 starting at y3 (y3 – y7), indicating a modification site at the first Tyr from the N-terminus of the peptide. The peptide with the unknown modification had a mass increase of 165 starting at y4 (y4 – y6), indicating the second Ser from the N-terminus was modified. **c,** Autoradiographs reveal AMPylated Rab1 from *in vitro* reactions containing the recombinant proteins indicated on the top of each panel and the radiolabeled nucleotides indicated below.

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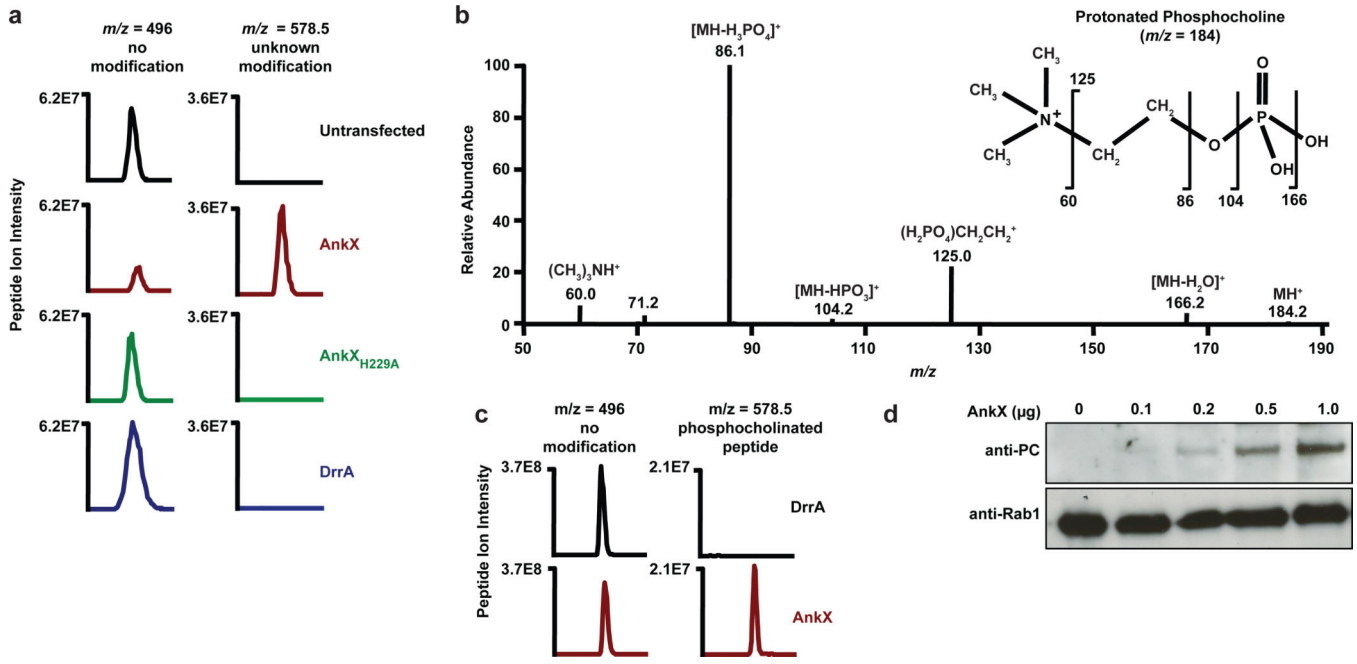

Figure 2. The *Legionella* effector AnkX functions as a Rab phosphocholine transferase

a, LC-MS/MS analysis of 3X-FLAG-Rab1 isolated from HEK293 cells that were either untransfected or transfected with a plasmid encoding GFP-tagged AnkX, AnkX$_{H229A}$ or DrrA. Extracted ion chromatograms indicate the amount of Rab1 peptide TITSSYYR with no modification (*m/z* = 496) and peptide with the unknown modification (*m/z* = 578.5). **b,** MS^3 analysis on the Rab1 peptide TITSSYYR with the unknown modification. The *m/z* = 184 peak corresponding to the protonated moiety attached to the Rab1 peptide was selected and subjected to further dissociation. Indicated are the fragments identified in the MS^3 spectrum that matched the fragments predicted upon dissociation of the protonated phosphocholine molecule. **c,** The peak in each graph indicates the amount of Rab1 peptide TITSSYYR with no modifications (*m/z* = 496) and phosphocholinated peptide (*m/z* = 578.5) after *in vitro* incubation of Rab1 with either DrrA or AnkX in the presence of CDP-choline. **d,** Immunoblots from *in vitro* reactions that contained Rab1 and the indicated amounts of AnkX. Blots were probed to detect phosphocholinated Rab1 (anti-PC) and total Rab1 (anti-Rab1) in each reaction.

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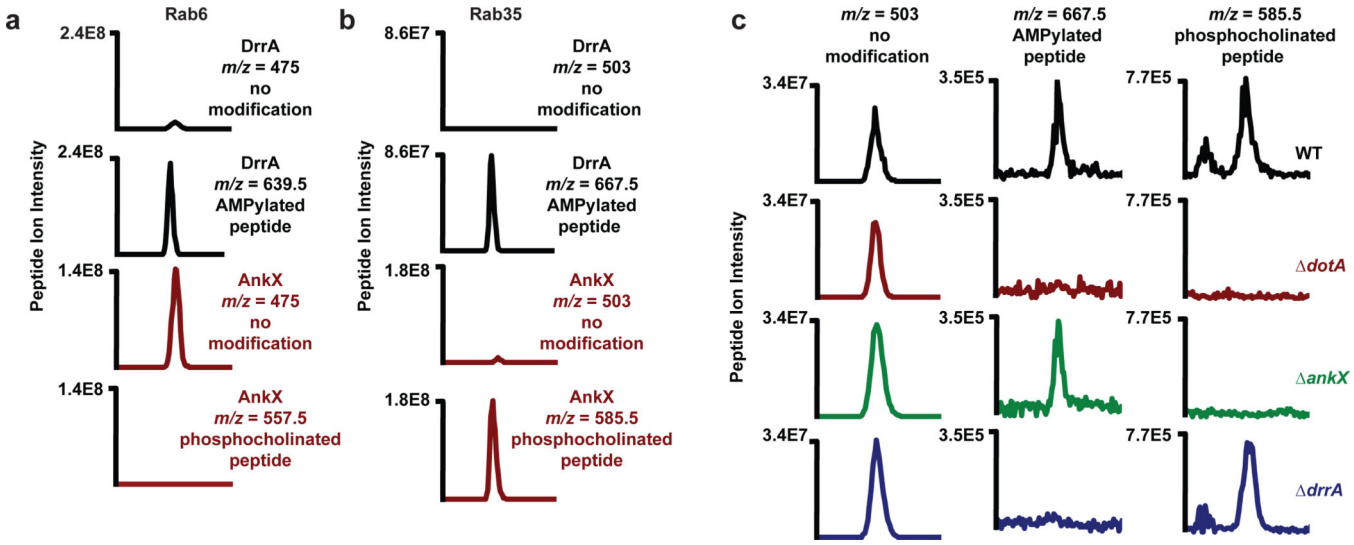

Figure 3. AnkX and DrrA have overlapping but non-identical Rab specificities

a, Extracted ion chromatograms of 3X-FLAG-Rab6 isolated from HEK293 cells producing either DrrA or AnkX. Shown are graphs for the unmodified Rab6 peptide SLIPSYIR (*m/z* = 475) and the AMPylated (*m/z* = 639.5) and phosphocholinated (*m/z* = 557.5) forms of this peptide. **b,** Extracted ion chromatograms of 3X-FLAG-Rab35 isolated from HEK293 cells producing either DrrA or AnkX. Shown are graphs for the unmodified Rab35 peptide TITSTYYR (*m/z* = 503.0), the AMPylated peptide (*m/z* = 667.5) and the phosphocholinated peptide (*m/z* = 585.5). **c,** 3X-FLAG-Rab35 was isolated from HEK293 FcγRII cells after infection with the indicated *Legionella* strains. The peak in each graph indicates the amount of Rab1 peptide TITSSYYR with no modifications (*m/z* = 503), peptide containing an AMP moiety (*m/z* = 667.5), and phosphocholinated peptide (*m/z* = 585.5).

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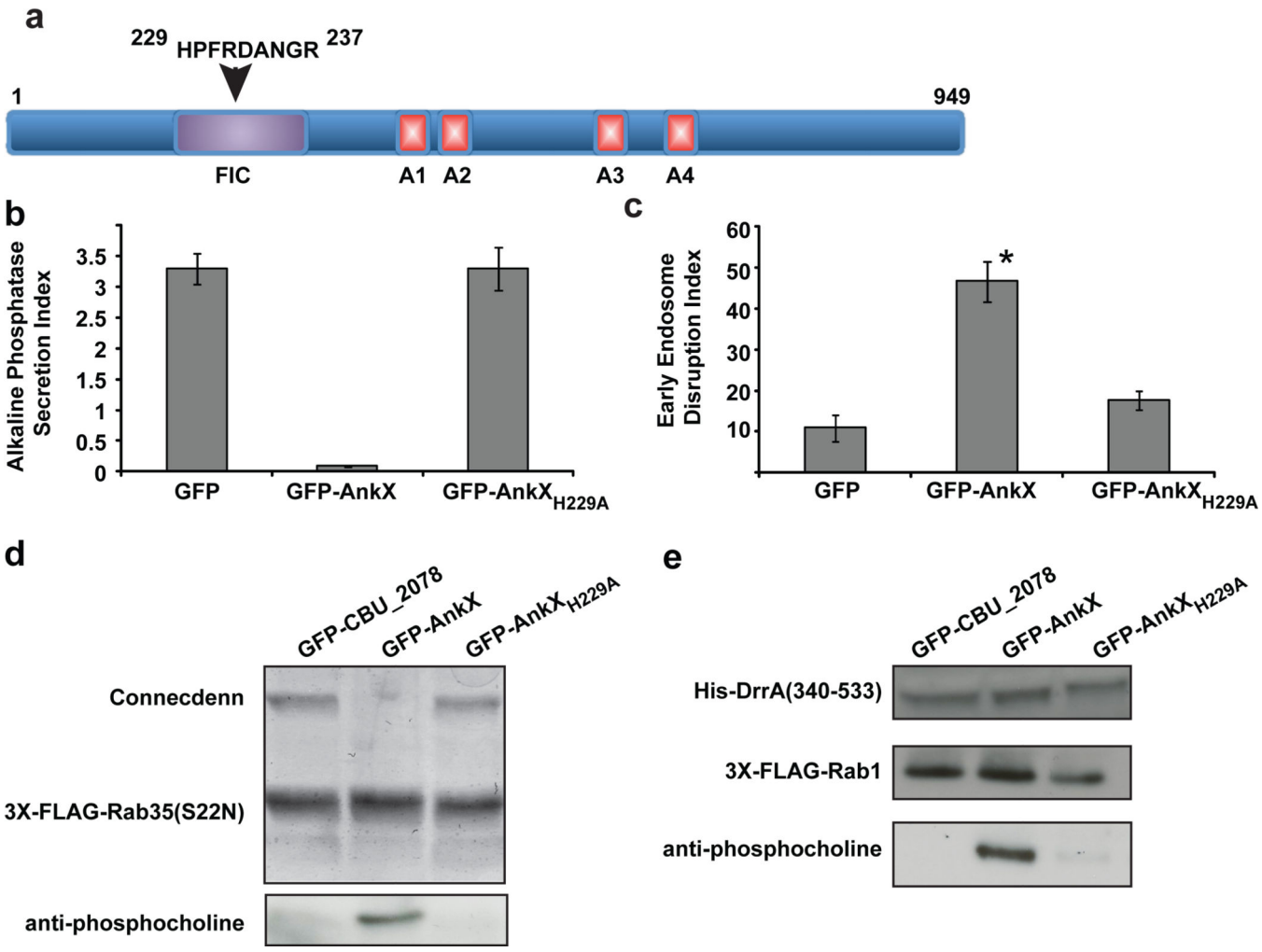

Figure 4. AnkX-mediated phosphocholination modulates the function of Rab1 and Rab35

a, Schematic representation of the AnkX protein showing the location of the FIC domain and the four predicted ankyrin repeat homology domains (A1-A4). The amino acid sequence in a conserved region of the FIC domain containing the essential His229 residue is shown. **b,** Secretion of alkaline phosphatase into the culture supernatant was measured for HEK293 cells producing either GFP, GFP-AnkX or GFP-AnkX$_{H229A}$ as indicated on the x-axis. Data are the mean ± standard deviation (s.d.) calculated from three independent sample wells. **c,** The disruption of early endosomes was assessed in COS7 cells producing either GFP-AnkX, GFP-AnkX$_{H229A}$ or GFP alone after staining for EEA1 (mean ± s.d., n = 200, *P<0.005 compared to the GFP alone control). **d,** Binding of recombinant Connecdenn to 3X-FLAG-Rab35$_{S22N}$ isolated from cells producing either GFP-CBU_2078 or GFP-AnkX or GFP-AnkX$_{H229A}$ was assessed by co-precipitation. The Coomassie-stained SDS-PAGE gel indicates the amount of Connecdenn and 3X-FLAG-Rab35$_{S22N}$ in each precipitate. The anti-phosphocholine immunoblot indicates that Rab35$_{S22N}$ isolated from cells producing GFP-AnkX was phosphocholinated. **e,** Binding of purified His-tagged DrrA$_{340–533}$ to 3X-FLAG-Rab1A isolated from cells producing either GFP-CBU_2078 or GFP-AnkX or GFP-AnkX$_{H229A}$ was assessed by co-precipitation. The immunoblots indicate the amounts of

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DrrA$_{340–533}$ and the levels of phosphocholinated 3X-FLAG-Rab1A present in each precipitate.