Reversible phosphocholination of Rab proteins by *Legionella pneumophila* effector proteins

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The *Legionella pneumophila* protein AnkX that is injected into infected cells by a Type IV secretion system transfers a phosphocholine group from CDP-choline to a serine in the Rab1 and Rab35 GTPase Switch II regions. We show here that the consequences of phosphocholination on the interaction of Rab1/Rab35 with various partner proteins are quite distinct. Activation of phosphocholinated Rabs by GTP/GDP exchange factors (GEFs) and binding to the GDP dissociation inhibitor (GDI) are strongly inhibited, whereas deactivation by GTPase activating proteins (GAPs) and interactions with Rab-effector proteins (such as LidA and MICAL-3) are only slightly inhibited. We show that the *Legionella* protein lpg0696 has the ability to remove the phosphocholine group from Rab1. We present a model in which the action of AnkX occurs as an alternative to GTP/GDP exchange, stabilizing phosphocholinated Rabs in membranes in the GDP form because of loss of GDI binding ability, preventing interactions with cellular GTPase effectors, which require the GTP-bound form. Generation of the GTP form of phosphocholinated Rab proteins cannot occur due to loss of interaction with cellular GEFs.

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Introduction

The facultative intracellular bacterium *Legionella pneumophila* injects several hundred proteins via a Type IV secretion system into the cytoplasm of cells it infects (Isberg *et al*, 2009). Many

of these proteins subvert or interfere with normal host cell vesicular transport processes, in particular via interactions with Rab and Arf family GTPases (Amor *et al*, 2005; Nagai *et al*, 2005; Machner and Isberg, 2006; Ingmundson *et al*, 2007; Isberg *et al*, 2009). In earlier work, secreted proteins that modified Rab or Arf activity by non-covalent, reversible interactions were described, but recently, two covalent modifications of the Switch II region of Rab1 catalysed by *Legionella* effectors have been identified. In one case, an AMP group is transferred to Tyr77 of the Switch II region of Rab1b (Tyr80 of Rab1a) and several other Rab proteins by the *Legionella* effector DrrA/SidM (Muller *et al*, 2010). The process of transfer of an AMP group to a protein amino acid side chain is referred to in the classic example of modification of glutamine synthetase in bacteria as adenylation (reviewed in Stadtman, 2001) but in recently discovered examples of AMP transfer by bacterial effector proteins also as AMPylation. This includes the first bacterial modification of this type to be discovered that operates on host cell GTPases, that of modification of Rho proteins by the FIC domain protein VopS from *Vibrio parahaemolyticus* (Yarbrough *et al*, 2009). Rho proteins are also adenylylated by another FIC domain protein, the secreted antigen IbpA from *Histophilus somni* (Worby *et al*, 2009). In the case of Rab protein modification by DrrA/SidM, it has been shown that the modification is reversed hydrolytically by the *Legionella* protein SidD (Neunuebel *et al*, 2011; Tan and Luo, 2011). In searching for evidence of adenylation of Rab1 in infected cells, another modification occurring on Ser79 of Rab1a was discovered (Mukherjee *et al*, 2011). This was shown to involve covalent attachment of phosphocholine (originating from CDP-choline) catalysed by the FIC domain containing protein AnkX. This modification appears to block early secretory events involving vesicular transport on microtubules after exit from the ER (Pan *et al*, 2008). The modification also occurs on Rab35, thereby inhibiting steps in the endocytic pathway and presumably helping to avoid early endosome maturation, an important mechanism for the establishment of *Legionella* containing vacuoles (LCVs; Pan *et al*, 2008).

Here, we describe the characterization of the phosphocholination reaction and the influence of the modification on interaction with Rab binding partners. In addition, we demonstrate that the *Legionella* effector protein lpg0696 has an enzymatic activity that can remove the phosphocholine group. We present a model for the action of AnkX, in which the key feature is generation of stably membrane-bound and GEF inert phosphocholinated Rab proteins in the GDP form, in contrast to the stably bound GTP form under normal physiological conditions.

Results

Phosphocholination of Rab1, Rab35 and peptides

As previously shown, Rab1 and Rab35 can be phosphocholinated by AnkX (Mukherjee *et al*, 2011). At the qualitative

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Figure 1 Phosphocholination of Rab1b monitored by ESI-MS and tryptophan fluorescence. **(A)** ESI-MS of a sample taken from the reaction mixture of the experiment with Rab1b:GDP described in **(C)** before addition of AnkX. wt stands for wild type. **(B)** ESI-MS of the Rab1b:GDP sample used for **(C)** taken ca. 400 s after the addition of 200 nM AnkX. **(C)** 10 μ M Rab1b:GDP (green symbols) was irradiated at 297 nm, and the fluorescence emission was measured at 340 nm as a function of time. The phosphocholination substrate CDP-choline was present at a concentration of 400 μ M, and the reaction was started at time zero by addition of 200 nM AnkX. The time dependence could be fitted by an exponential function with a rate constant of 0.020 s^{-1} . A similar experiment was repeated using 10 μ M Rab1b:GppNHp (red symbols). The fitted first order rate constant was 0.0075 s^{-1} . The fluorescence intensities have been normalized so that they begin at a relative fluorescence of 1 for each curve to ease the comparison. The actual fluorescence intensity is 30% lower for Rab:GppNHp than for Rab:GDP.

level, this could be confirmed by electrospray ionization-mass spectrometry (ESI-MS), with the resulting molecular ion peak having an additional mass of 165 Da in comparison with wild-type protein after incubation with AnkX and CDP-choline (Figure 1A and B; Supplementary Figure S1A and B). Interestingly, the peptide TITSSYYR could also be phosphocholinated, suggesting that recognition occurs at least partially at the level of the amino acid (aa) sequence alone (Supplementary Figure S1C). Incubation of Rab1b, AnkX and CDP or CTP led to slow phosphorylation of Rab1b in both cases (Supplementary Figure S1D and E), although pyrophosphorylation might be expected with CTP. It is possible that the phosphorylation seen with CTP arose from CDP impurities, and that CTP is not a substrate for AnkX.

Assays for phosphocholination and the kinetic characterization of AnkX

AnkX activity could be readily monitored in a continuous fashion using a coupled-enzyme assay in which CMP generated from CDP-choline by AnkX activity is converted to CDP by UMP/CMP kinase, followed by conversion of the ensuing CDP and ADP to their corresponding triphosphates and coupling to NADH oxidation as shown in Supplementary Scheme 1. The disappearance of NADH could be monitored sensitively by the loss of fluorescence intensity at 460 nm (Supplementary Figure S2). With the concentrations of AnkX, Rab1 and CDP-choline used, there was an initial exponential loss of absorbance with an amplitude corresponding to the concentration of Rab1, but this was followed by a linear decrease in fluorescence at lower rate that continued until the supply of NADH (50 μ M) was exhausted. Revealingly, if the

reaction was repeated using a concentration of CDP-choline equal to that of Rab1, after the initial exponential phase no further reaction occurred. The linear second phase could then be induced by addition of excess CDP-choline. This suggested that the linear phase is due to hydrolysis of CDP-choline by AnkX. This was confirmed by HPLC, showing that CMP was produced from CDP-choline by hydrolysis under the influence of AnkX.

The complication of CDP-choline hydrolysis by AnkX promoted a search for a further assay method. As shown in Figure 1C, addition of AnkX to a solution containing Rab1b:GDP and CDP-choline resulted in an exponential increase in tryptophan fluorescence with time. To confirm that the fluorescence change seen was due to the phosphocholination reaction, a sample taken after the fluorescence signal reached the plateau phase was subjected to ESI-MS (Figure 1B), showing complete conversion to phosphocholinated Rab1b (PC-Rab1b). Evaluation of the exponential time dependence gave an apparent k_{cat}/K_m value of $9.8 \times 10^4\text{ M}^{-1}\text{ s}^{-1}$, which will be somewhat lower than the genuine k_{cat}/K_m value, since the CDP-choline concentration used is not saturating. The phosphocholination reaction also occurred with the GppNHp bound form of Rab, but at about 40% of the rate with Rab:GDP (apparent $k_{\text{cat}}/K_m = 3.8 \times 10^4\text{ M}^{-1}\text{ s}^{-1}$; Figure 1C).

The tryptophan fluorescence signal was used for a more quantitative interpretation of the kinetics of phosphocholination by measuring initial rates of the reaction as a function of Rab1b and CDP-choline concentrations in a stopped-flow apparatus. In the first series of measurements, the CDP-choline concentration was held constant (1 mM) and the Rab1b concentration was varied (Figure 2A). The initial

Figure 2 Steady-state kinetics of phosphocholination of Rab1b. **(A)** Dependence of the initial rate of phosphocholination of Rab1b (v) on the Rab1b concentration at constant CDP concentration (1 mM). The AnkX concentration was 400 nM. The K_m value for Rab1b obtained from fitting a hyperbolic function to the data was $122 \pm 23 \mu\text{M}$. k_{cat} was calculated to be $27.4 \pm 2.3 \text{ s}^{-1}$. **(B)** Dependence of the initial rate of phosphocholination of Rab1b on the CDP-choline concentration at constant Rab1b concentration (1 μM) and 40 nM AnkX. **(C)** Double reciprocal plots of data of the type shown in **(B)** at three different Rab1b concentrations. A value of $254 \pm 76 \mu\text{M}$ for the K_m value of CDP-choline was calculated from these data.

rates plotted against the Rab1b concentration showed a hyperbolic dependence, and the K_m value for Rab1b was calculated to be $122 \pm 23 \mu\text{M}$, while k_{cat} was $27.4 \pm 2.3 \text{ s}^{-1}$. In a second type of experiment, the Rab1b concentration was held constant and the dependence of the initial rate on the CDP-choline concentration was measured. Series of this type were repeated for Rab concentrations of 1, 2 and 3 μM . It became clear from the first measurements that the low apparent K_m value for CDP-choline made these experiments technically challenging, since the lowest concentrations of CDP-choline that could be used and interpreted were near to or above the apparent K_m value. Despite this, the hyperbolic nature of the plot of rate against CDP-choline concentration was apparent, and the points could be fitted using a hyperbolic equation (Figure 2B). In principle, depending on the kinetic mechanism, different types of behaviour are to be expected when this experiment is repeated at different constant Rab1b concentrations. The two principal types of mechanism seen are those in which a ternary complex between the enzyme and both substrates is formed, and those in which binding of one substrate is followed by covalent transfer of a group of this substrate to the enzyme, followed by dissociation of a product, binding of the second substrate and transfer of the group to this molecule ('ping-pong' mechanism; see Supplementary Scheme S2). In the first type, there will be an increase of the apparent maximum reaction velocity (V_{max}) as the Rab concentration is increased, and either no dependence or an increase in the apparent K_m , depending on the exact mechanism, while in the second case (ping-pong) there is an exactly parallel increase in the apparent V_{max} and the apparent K_m values.

The results obtained with AnkX, Rab1b and CDP-choline agreed with the second scenario, since both the apparent K_m and the apparent V_{max} increased to the same extent on increasing the CDP-choline concentration. This is illustrated in the double reciprocal plots shown in Figure 2C, which led to approximately parallel lines, typical of ping-pong mechanisms. This then implies that AnkX is phosphocholinated in the first step, followed by release of CMP, binding of Rab1b and transfer of the phosphocholine group to Ser76 in the second covalent transfer step. A value of $254 \pm 76 \mu\text{M}$ for the K_m value for CDP-choline was calculated from the values of the intercepts and from the independently measured K_m value (122 μM) for Rab1b.

Support for the idea that the phosphocholination reaction involves a covalent intermediate arises from the observation that incubation of the FIC domain of AnkX (AnkX_{6–390}) with a large excess of CDP-choline yielded phosphocholinated AnkX_{6–390} that is detectable by ESI-MS (Supplementary Figure S3A and B). However, when only a small excess of CDP-choline over AnkX was used, no phosphocholinated product could be detected, in agreement with the notion that the intermediate is hydrolytically unstable and does not accumulate under these conditions. This would be expected if the autophosphocholination reaction occurs on histidine, in which case it could be the actual mechanistic intermediate for the overall reaction, but not if it occurs on serine, threonine or tyrosine, which would lead to a stable product.

The observation of a ping-pong mechanism for AnkX is in contrast to the direct transfer of AMP to a threonine residue in Rho proteins by VopS, which displays a direct transfer

sequential mechanism (Luong *et al*, 2010). While this mechanistic difference for the two FIC domain containing proteins is unexpected, it should be borne in mind that it is a different phosphate group of the nucleotide substrate that is attacked and transferred (α -phosphate for adenylation and β -phosphate for phosphocholination), so that mechanistic differences are not surprising.

A simple, rapid assay for monitoring several Rab1b interactions

After establishing the kinetic parameters of AnkX-mediated phosphocholination, we aimed at determining the consequences of this Rab modification on the interaction with several binding partners. The observation that the tryptophan fluorescence intensity of Rab1b:GppNHp was about 30% lower than that of Rab1b:GDP prompted us to use this signal to monitor processes in which the nucleotide state at the active site of the GTPase changed (GDP/GTP exchange, GTPase activity). Applying this initially to the nucleotide exchange reaction, addition of the *Legionella* GEF DrrA to Rab1b:GDP in the presence of excess GTP resulted in a reduction of tryptophan fluorescence, which was, however, biphasic. We speculated that in addition to GTP/GDP exchange, guanylation (Muller *et al*, 2010) of Rab1b also contributed to the fluorescence change. Repeating the experiment in the presence of ATP and GTP led to a markedly faster second phase than in the absence of ATP, in keeping with the fact that adenylation by DrrA is considerably faster than guanylation (Muller *et al*, 2010). Figure 3 demonstrates that this interpretation is correct, since use of the GEF domain of DrrA (DrrA_{340–533}) led to a monophasic decrease of fluorescence of ca. 30% in the presence of GTP or GTP and ATP. Subsequent addition of full-length DrrA containing GEF mutations that completely eliminate GEF activity (N451A, R453A, D480A and S483A) (Schoebel *et al*, 2009) but do not disturb adenylation properties led to a slow further decay of fluorescence in the case of GTP alone, or a rapid decay when ATP was also present. The larger decrease of fluorescence in the GTP-only experiment is probably due to a larger quenching effect of guanine than adenine on the tryptophan fluorescence after nucleotide transfer.

The experiments described in this and other sections show that significant changes in tryptophan fluorescence occur on phosphocholination, adenylation, nucleotide exchange and nucleotide hydrolysis. This signal was therefore used to investigate the interplay between these processes in subsequent experiments. In addition, some experiments were also performed using the fluorescent methylantraniloyl (mant) derivatives of GDP or GppNHp.

Effects of phosphocholination on Rab interactions

Rab proteins are involved in a large number of interactions with different classes of protein factors, including GEFs, Rab effectors (i.e., proteins interacting preferentially with the GTP-bound form of Rabs), GAPs, Rab escort protein (REP; binds to unprenylated Rabs and presents them to RabGGTase for geranylgeranylation) and GDP dissociation inhibitor (GDI; responsible for extraction of Rabs from membranes). In order to provide a basis for understanding the biological consequences of the modification, we examined the effects of phosphocholination on several of these interactions.

Figure 3 Monitoring successive GTP/GDP exchange and nucleotidylation of Rab1b by DrrA variants using tryptophan fluorescence. In the first part of the time course, exchange of 10 μ M Rab1b bound GDP by 100 μ M GTP was initiated by addition of 9.1 nM DrrA GEF domain (DrrA_{340–533}). After exchange was completed, nucleotidylation was initiated by addition of a further 100 μ M GTP (for guanylation; green curve) or 100 μ M ATP (for adenylation; red curve) together with 100 nM GEF-abrogated full-length DrrA.

Influence of phosphocholination on the GEF activity of DrrA and connecdenn 1

Using the tryptophan fluorescence assay, it was possible to first phosphocholinate Rab1b in the fluorescence cuvette and then add the Rab1-GEF domain of *Legionella* exchange factor DrrA GEF domain (DrrA_{340–533}) in the presence of excess GTP to observe the effect of phosphocholination on the nucleotide exchange reaction in the presence of connecdenn 1 (Figure 4A; note that on the timescale of these experiments, no significant exchange occurred in the absence of exchange factors). Comparison with the analogous experiment with unmodified Rab1b showed that the DrrA_{340–533} catalysed exchange reaction was partially inhibited. The k_{cat}/K_m values from individual time courses were $1.1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ for unmodified Rab1b and $2.4 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ for phosphocholinated Rab1b. A more detailed analysis of the influence of phosphocholination on the interaction with DrrA_{340–533} was made using 2'/3'-O-(N-methylantraniloyl)-dGDP (mantdGDP) as a fluorescent GDP analogue in the stopped-flow apparatus, making use of the reduction of mant-fluorescence of the nucleotide on dissociation from Rab1b and allowing measurements at excess DrrA_{340–533} over Rab1b. The results in Figure 4B show that there is an eight-fold reduction in the k_{cat}/K_m value on phosphocholination. This is slightly larger than the influence of adenylation on Tyr77 (reduction of a factor of 4 in GEF efficiency of DrrA; Muller *et al*, 2010). We can therefore conclude that phosphocholination has a relatively minor effect on the susceptibility of Rab1 to the GEF activity of DrrA, similarly to the situation with adenylation on Tyr77 (Muller *et al*, 2010).

The ready availability of connecdenn 1 (Allaire *et al*, 2006, 2010) as a mammalian GEF for Rab35 prompted us to test the effect of phosphocholination of Rab35 on its activity. Using unmodified Rab35:GDP, the k_{cat}/K_m value for the phosphocholination reaction was found to be $6.3 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ using tryptophan fluorescence, a factor of 3.8 slower than for Rab1b under identical conditions (Supplementary Figure S4). On addition of excess GTP and the GEF domain of connecdenn 1 (aa 1–403; connecdenn 1_{1–403}), no signal corresponding to GDP/GTP exchange could be observed, even at connecdenn 1_{1–403} concentrations that led to exchange within ca. 20 s

Figure 4 Influence of phosphocholination of Rab1b on the GTP/GDP reaction catalysed by DrrA. **(A)** 10 nM DrrA₃₄₀₋₅₃₃ was added at time zero to 7.5 μM Rab1b:GDP (green curve) or 7.5 μM PC-Rab1b:GDP (red curve). The GTP concentration was 100 μM. **(B)** Observed first order rate constants for the displacement of mantdGDP from its complex with Rab1b (green points) or PC-Rab1b (red points) as a function of DrrA₃₄₀₋₅₃₃ concentration. The slopes of the fitted lines give values for k_{cat}/K_m of $(1.8 \pm 0.04) \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ (Rab1b) and $(2.23 \pm 0.06) \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ (PC-Rab1b) for the exchange reaction. **(C)** Stopped-flow records of mantdGDP displacement from 100 nM Rab35:mantdGDP (green curve) or 100 nM PC-Rab35 (red curve) in the presence of 3 μM connectin 1₁₋₄₀₃. Rab35 wt stands for Rab35-CVIL (see Materials and methods).

using unmodified Rab1b. This was confirmed by the experiment of Figure 4C, which shows that mantdGDP displacement from Rab35:mantdGDP by connectin 1 in the presence of GDP could be easily observed in a stopped-flow experiment whereas no displacement from PC-Rab35:mantdGDP was detectable. This demonstrates that the GEF activity of connectin 1 is profoundly inhibited by phosphocholination of Rab35, in agreement with the lack of interaction between phosphocholinated Rab35 and connectin 1 showed by pull-down analysis (Mukherjee *et al*, 2011).

Influence of phosphocholination on the GAP activity of LepB and TBC1D20

The effect of phosphocholination on the interaction of Rab1b with GAPs was examined using both the tryptophan-based and mant-nucleotide fluorescence approaches. The large increase in tryptophan fluorescence occurring on GTP hydrolysis to GDP was utilized to follow this reaction on addition of the Rab1-GAP domain of *Legionella* LepB (LepB₃₁₇₋₆₁₈). As shown in Figure 5A, this reaction is significantly inhibited for phosphocholinated Rab1b (wt Rab1b: $k_{cat}/K_m = 7.5 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, PC-Rab1b $k_{cat}/K_m = 4.0 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$; factor of 19-fold). In the absence of GAPs, no significant hydrolysis occurred on the timescale of the experiments in Figure 5A and B. A further assessment of the inhibition was provided by following the increase in mantGTP fluorescence on hydrolysis to mantdGDP in the stopped-flow apparatus (Figure 5B). With the *Legionella* Rab1-GAP LepB, the k_{cat}/K_m was calculated to be $3.3 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ for wild-type Rab1b, and this was reduced to $2.2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ by phosphocholi-

nation (factor of 15). With the human GAP TBC1D20₁₋₃₆₂, there was a similar but smaller effect, with k_{cat}/K_m dropping from $5.7 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ for unmodified Rab1b to $1.5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ for phosphocholinated Rab1b (Figure 5C and D). This is similar to the situation with adenylylated Rab1b, where the inhibition of LepB activity was profound, but only moderate for TBC1D20₁₋₃₆₂ (Muller *et al*, 2010). It is of interest to note that the k_{cat}/K_m value obtained for the real substrate of LepB, namely Rab1b:GTP, was about a factor of 10 higher than for Rab1b:mantGTP. This is presumably due to a certain degree of disturbance of LepB binding to Rab by the mant group.

Influence of phosphocholination on adenylylation of Rab1b

Using the tryptophan fluorescence signal, the k_{cat}/K_m value for adenylylation of Rab1b:GppNHp by full-length DrrA (DrrA_{fl}) harbouring the N451A, R453A, D480A and S483A GEF-abrogating mutations (Schoebel *et al*, 2009) was found to be $3.0 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ (Figure 6). When the experiment was repeated using phosphocholinated Rab1b:GppNHp, a dramatic reduction of the adenylylation rate was observed. Considerably higher concentrations of DrrA were needed to observe the reaction, and the calculated k_{cat}/K_m value was $1.1 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$. Thus, adenylylation was strongly inhibited (by a factor of over 10^3) by prior phosphocholination.

Phosphocholination of AMP-Rab1b could not be detected using tryptophan fluorescence, but incubation of AMP-Rab1b AnkX and CDP-choline led to double modification, as shown by ESI-MS (Supplementary Figure S3C and D). This reaction

Figure 5 Influence of phosphocholination of Rab1b on the GAP activity of LepB and TBC1D1–362. (A) Increase of tryptophan fluorescence on addition of 7.5 nM LepB_{317–618} to 7.5 μ M Rab1b:GTP (green curve) or 7.5 μ M PC-Rab1b:GTP. (B) Observed first order rate constants for the hydrolysis of mantGTP in complex with Rab1b (green points) or PC-Rab1b (red points) as a function of LepB_{317–618} concentration. The k_{cat}/K_m values are $(3.3 \pm 0.1) \times 10^5 M^{-1} s^{-1}$ for Rab1b and $(2.2 \pm 0.05) \times 10^4 M^{-1} s^{-1}$ for PC-Rab1. (C) Increase of tryptophan fluorescence on addition of 10 nM TBC1D201–362 to 10 μ M Rab1b:GTP (green curve) or 10 μ M PC-Rab1b:GTP. (D) As in (B) but for TBC1D201–362. The k_{cat}/K_m values are $(5.7 \pm 0.14) \times 10^5 M^{-1} s^{-1}$ for Rab1b and $(1.5 \pm 0.44) \times 10^5 M^{-1} s^{-1}$ for PC-Rab1.

Figure 6 Influence of phosphocholination of Rab1b on adenylation by DrrA. The tryptophan intensity decreases exponentially with time after addition of DrrA_f (N451A, R453A, D480A and S483A) to 20 μ M Rab1b:GppNHp (green curve) or PC-Rab1b:GppNHp (red curve). The DrrA concentration was 10 nM for Rab1b and 1 μ M for PC-Rab1b.

appeared to be slower than phosphocholination of Rab1b, since the unmodified substrate was detectable under conditions that led to complete modification of Rab1b not harbouring the AMP residue.

Influence of phosphocholination of Rab1b on its interaction with Rab effectors

To examine the influence of the presence of a phosphocholine group on Rab1b on effector interactions, we generated Rab1b:mantGppNHp complexes for fluorescence stopped-

flow experiments. These experiments could be performed using changes in fluorescence intensity of the mant group upon interaction with effectors, or more reliably for the interaction with LidA, using the change in the fluorescence polarization signal. The observed pseudo first order rate constants for the association reaction between Rab1b:mantGppNHp or its phosphocholinated form and the mammalian effector MICAL-3 are plotted as a function of the MICAL-3 concentration (Figure 7A). For unmodified Rab1b, the rate constant for the association reaction was $2.9 \times 10^6 M^{-1} s^{-1}$, while the rate constant for dissociation was $20 s^{-1}$. After phosphocholination, these values were $9.5 \times 10^5 M^{-1} s^{-1}$ and $9.0 s^{-1}$, respectively. Thus, the association rate constant is reduced by a factor of ca. 3, but the concomitant decrease of the dissociation rate constant means that there was only a minor effect on the dissociation equilibrium constant (K_d) of the interaction ($K_d = 7.0 \mu M$ for unmodified Rab1b, $9.5 \mu M$ for phosphocholinated Rab1b). This is in contrast to the situation with AMP-Rab1b, where no interaction with MICAL-3 could be detected (Muller *et al*, 2010).

In the case of the Legionella effector protein LidA (Figure 7B), there was a slightly larger effect of phosphocholination on the association rate constant (wt Rab1b: $k_{on} = 4.6 \times 10^6 M^{-1} s^{-1}$, PC-Rab1: $k_{on} = 1.1 \times 10^6 M^{-1} s^{-1}$). In both cases, the dissociation rate constant was too slow to measure, as seen in general for Rab–protein–LidA interactions (Schoebel *et al*, 2011), so that a highly stable complex was formed in both cases, in agreement with the results obtained for AMP-Rab1b (Muller *et al*, 2010).

Figure 7 Influence of phosphocholination of Rab1b on its interaction with the effectors MICAL-3 and LidA. **(A)** Varying concentrations of MICAL-3₅₋₁₅₄ were mixed in a stopped-flow apparatus with Rab1b:mantGppNHp (green points) or PC-Rab1b:mantGppNHp (red points) and the interaction was monitored using the change in fluorescence polarization (excitation at 367 nm, emission via a 420-nm cutoff). The association rate constants were $(2.9 \pm 0.31) \cdot 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and $(9.5 \pm 2.3) \cdot 10^5 \text{ M}^{-1} \text{ s}^{-1}$ for the association with Rab1b:GppNHp and PC-Rab1b:GppNHp, respectively, while the corresponding dissociation rate constants were $20 \pm 1.7 \text{ s}^{-1}$ and $9.0 \pm 2.6 \text{ s}^{-1}$. **(B)** As in **(A)**, but using varying concentrations of LidA₂₀₁₋₅₃₃. The association rate constants obtained from the slopes of the fitted straight lines were $(4.6 \pm 0.2) \cdot 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and $(1.1 \pm 0.1) \cdot 10^6 \text{ M}^{-1} \text{ s}^{-1}$, respectively, for Rab1b:GppNHp and PC-Rab1b:GppNHp.

Influence of phosphocholination of Rab1b on its interaction with GDI

Geranylgeranylated Rab proteins are extracted from membranes by the generic solubilizing factor GDI. The interaction of Rab proteins with GDI can be conveniently examined using Rab proteins with a fluorescently labelled prenyl derivative at their C-terminus (Schoebel *et al*, 2009; Wu *et al*, 2010a,b). For this purpose, we used Rab1b:GDP-farnesyl-NBD, which shows a large increase in fluorescence on interaction with GDI. For this experiment, Rab1b:GDP-farnesyl-NBD was phosphocholinated in the fluorescence cuvette before addition of GDI. Interestingly and unexpectedly, phosphocholination catalysed by AnkX led to a significant drop in fluorescence (Figure 8A). Since the fluorescent prenyl derivative is remote from the position of phosphocholination, we interpret this result to indicate that the lipid group can interact with the globular part of Rab1, and that this interaction is affected by phosphocholination. As shown in Figure 8B, this change can be reversed by addition of the Legionella protein lpg0696, which we show in the next section to have the ability to remove the phosphocholine group from Rab1b. In Figure 8A, addition of GDI to PC-Rab1b:GDP-farnesyl-NBD caused only a slight increase in fluorescence, in contrast to the known very large increase in fluorescence in the absence of the phosphocholine group (Wu *et al*, 2010a), showing that phosphocholination inhibits the Rab1b–GDI interaction. Subsequent addition of lpg0696 protein resulted in a large, time-dependent increase in NBD fluorescence, showing that interaction with GDI could now take place as the phosphocholine group is removed from the Rab molecule. In Figure 8B, addition of GDI to Rab1b:GDP-farnesyl-NBD that had been first phosphocholinated and then dephosphocholinated led to a large rapid phase of fluorescence increase followed by a slower phase at a rate commensurate with the dephosphocholination reaction, suggesting that before addition of GDI, a dynamic equilibrium between phosphocholination and dephosphocholination had been established due to the simultaneous presence of AnkX and lpg0696 protein, and that complete binding to GDI only occurred after complete dephosphocholination.

Figure 8 Influence of phosphocholination of Rab1b on its interaction with GDI. **(A)** CDP-choline, AnkX, GDI and lpg0696 were added at the concentrations and times indicated to 50 nM Rab1b:GDP-farnesyl NBD. NBD stands for 7-nitrobenzol[1,2,5]oxodiazol. **(B)** The order of addition of GDI and lpg0696 was reversed in comparison with **(A)**, showing that influence of AnkX could be partially reversed by addition of lpg0696, and that GDI binding could now occur readily. The excitation and emission wavelengths were 479 and 525 nm, respectively.

Identification of lpg0696 as a dephosphocholinase

In recent publications, it has been reported that the adenylation of Rab1 by DrrA can be reversed by the protein SidD, the gene for which is situated adjacent to that of DrrA in the Legionella genome (Neunuebel *et al*, 2011; Tan and Luo,

Figure 9 Demonstration of dephosphocholinase activity of lpg0696. **(A)** Addition of 400 nM lpg0696 to a solution of 10 μM PC-Rab:GDP led to an exponential loss of fluorescence with an amplitude expected for removal of the phosphocholine group. **(B)** Steady-state kinetics of dephosphocholination of PC-Rab:GDP by lpg0696. The fitted parameters were $65.5 \pm 8.9 \mu\text{M}$ for K_m and $14.3 \pm 1.1 \text{ s}^{-1}$ for k_{cat} . **(C)** Model incorporating the consequences of phosphocholination of Rab proteins. The abbreviation mem stands for membrane. See the text for a discussion of this model.

2011). Encouraged by these findings, we examined the genes adjacent to AnkX. The AnkX gene (*lpg0695*) was situated next to the gene *proS* encoding for a prolyl-tRNA-synthetase and to *lpg0696*. A Blast search using the protein sequence of lpg0696 showed a relatively weak similarity to protein phosphatases, suggesting a possible role in the removal of the covalent modification dealt with here. We tested the protein product of gene *lpg0696* for dephosphocholinase activity using the change in tryptophan fluorescence. Addition of the protein to PC-Rab1b:GDP resulted in a time-dependent decrease in tryptophan fluorescence to the level expected for generation of unmodified Rab1b:GDP (Figure 9A). ESI-MS at the end of this phase showed that complete removal of the phosphocholine group had occurred (Supplementary Figure S5). These results suggest that lpg0696 is indeed the searched for dephosphocholinase.

Determination of the K_m value using a method analogous to that described for the phosphocholination reaction to approximate value of $65.5 \pm 8.9 \mu\text{M}$ for the K_m value and $14.3 \pm 1.1 \text{ s}^{-1}$ for k_{cat} (Figure 9B). While these individual values are not well determined due to the low degree of saturation at the highest PC-Rab1b concentration used, a reliable value of $2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ for k_{cat}/K_m can be calculated, meaning that it has a similar level of activity to AnkX.

Discussion

The results described lead to a characterization of the phosphocholination of Rab1b by AnkX and the dephosphocholination by lpg0696. Both the GDP and the GTP forms of Rab1b were modified, with a slight preference for the GDP form.

Rab35 was also a substrate for the modification, with a k_{cat}/K_m value that was ca. four-fold lower than for Rab1b. The phosphocholination reaction appears to occur via a ping-pong mechanism, that is, it probably involves a phosphocholinated enzyme intermediate. Although the phosphocholinated residue has not been identified, it is possible that it is the conserved histidine in the conserved HPFRDANGER sequence. A classical example for a ping-pong mechanism is that of nucleoside diphosphokinase (NDK), which catalyses the transfer of the terminal phosphate of a nucleoside triphosphate to a nucleoside diphosphate (Lascu and Gonin, 2000). In this case, the intermediate is a phosphohistidine species. Since this relatively unstable species can be generated even in the absence of a second (diphosphate) substrate, incubation of NDK with a nucleoside triphosphate alone leads to hydrolysis to the diphosphate and inorganic phosphate, since phosphorylated histidine residues are intrinsically unstable. This is exactly analogous to the situation with AnkX, which is able to hydrolyse CDP-choline, as described here.

Removal of the phosphocholine group appears to be catalysed by the product of the Legionella gene *lpg0696*, which has significant enzymatic activity ($k_{\text{cat}}/K_m = 2.2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$) towards phosphocholinated Rab1b in both the GDP and GTP forms *in vitro*. As further proof, during the review process of this manuscript, a study was published confirming that the product of the *lpg0696* gene of *Legionella pneumophila* can remove the phosphocholine group from phosphocholinated Rab1 (Tan *et al*, 2011).

The GEF activity of the Legionella protein DrrA was moderately reduced, whereas the GEF activity of the Rab35 GEF connectenn 1 was profoundly inhibited by Rab

phosphocholination. The strong inhibition of connectin 1 activity is probably connected with some of the defects in vesicular transport caused by AnkX (Pan *et al*, 2008), as discussed in more detail later.

Modification of Rab1b by phosphocholine weakened the GTPase activating effects of the *Legionella* GAP LepB and the mammalian GAP TBC1D20 on GTP hydrolysis by Rab1b moderately. The effect was more marked for LepB than for TBC1D20. This is similar to the situation with AMP-Rab1b, where the GTPase activation by LepB was strongly inhibited, whereas the TBC1D20 activation was only moderately affected (Muller *et al*, 2010).

Prior phosphocholination has a major effect on the rate of adenylation of Rab1b by DrrA/SidM, with the k_{cat}/K_m value being reduced by >3 orders of magnitude. Phosphocholination of AMP-Rab could only be observed by ESI-MS after incubation with high concentrations of AnkX, again indicating a much reduced rate of reaction. Doubly modified Rab1 species were also not detected in AnkX transfected cells (Mukherjee *et al*, 2011). It therefore seems unlikely that Rab1 bearing both modifications plays a significant biological role.

The interaction of Rab1b with Rab effectors was not significantly modified by phosphocholination. This is different to the situation with AMP-Rab, for which an interaction with MICAL-3 could not be detected (Muller *et al*, 2010). In contrast, stable complexes are formed between LidA and both PC-Rab and AMP-Rab, although it is possible that the affinity is reduced in comparison with the picomolar affinity seen for the Rab1b:LidA complex (Schoebel *et al*, 2011).

The Rab1b-GDI interaction was profoundly inhibited by phosphocholination. Although it has not yet been reported, it seems likely that adenylation has a similar effect when the interaction interface between Rab proteins and GDI is taken into consideration (Rak *et al*, 2003; Muller *et al*, 2010).

While it is not clear that all the effects described here are of biological significance, three of them are likely to be of major importance. The first of these is that the GDP-bound form of Rab1 is a good substrate (better than the GTP-bound form) for AnkX, which means that the phosphocholinated form of Rab1:GDP (or Rab35:GDP) could be produced on membranes. The second observation is that GDI cannot interact with PC-Rab1, meaning that PC-Rab1:GDP or PC-Rab35:GDP would be trapped on the membrane, since extraction by GDI would not be possible. Finally, PC-Rab:GDP trapped on membranes would not be able to exchange GDP for GTP under the influence of cellular GEFs, if we generalize the results obtained here and elsewhere (Mukherjee *et al*, 2011) on the inhibition of the interaction between Rab35 and connectin 1.

These results are incorporated into the model shown in Figure 9C. The starting off point in this model is the premise that Rabs with GDP bound are distributed between membranes and their cytosolic GDI-bound form, and that they only become stably attached to a distinct membrane after GEF activity (physiological branch of the scheme in Figure 9C) (Schoebel *et al*, 2009; Wu *et al*, 2010a). If Rabs are phosphocholinated before exchange takes place, then the non-physiological situation of a stably membrane-bound but inactive Rab will be induced, preventing interaction with cellular effectors. If this occurs on specific membranes/vesicles, it will lead to inhibition of vesicular transport, for instance at the level of transport on microtubules, as

reported (Pan *et al*, 2008). This effect would be made permanent if the native GEF activity is also inhibited, as shown for PC-Rab35 and connectin 1. Obviously, the localization properties of AnkX would be of significance in the envisaged scenario. Details of the localization of AnkX are not yet known, despite at least two publications addressing this point (Pan *et al*, 2008; Mukherjee *et al*, 2011). The available evidence suggests that localization to endomembrane structures after transfection of mammalian cells with AnkX, but early endosomes have been excluded as the site of localization.

In conclusion, as a working model, we suggest that phosphocholination of certain Rab proteins, including Rab1 and Rab35, occurs at specific membranes and leads to stable membrane attachment in a form (i.e., the GDP form) that cannot interact with cellular effectors. In contrast, interaction with the *Legionella* effector LidA would still be possible, since neither phosphocholination nor the presence of GDP rather than GTP bound to Rab inhibits the interaction with Rab1b (Schoebel *et al*, 2011). One of the features of *Legionella* infection is that vesicular material of ER origin is recruited by LCVs. Although the exact mechanism of this effect is not yet known, it is possible that the high-affinity interaction of LidA with Rab1 (and indeed other Rabs) is involved in recruitment of such vesicles/transport compartments.

Materials and methods

Protein preparation

Genes encoding for *Legionella pneumophila* AnkX and lpg0696 were obtained by PCR from *Legionella pneumophila* genomic DNA. The AnkX encoding genes were subcloned via *Sma*I and *Bam*HI restriction sites into a modified pET19 vector (pET19mod), whereas genes encoding for lpg0696 were subcloned via *Sall* and *Bam*HI restriction sites into pET19 vector. Both plasmids contain an N-terminal hexa-histidine (His_6) tag and a tobacco etch virus (TEV) protease cleavage sequence.

AnkX and lpg0696 were expressed in *Escherichia coli* BL21-CodonPlus (DE3)-RIL at 25°C overnight after induction with 0.2 mM IPTG (isopropyl- β -D-thiogalactopyranoside). From this point on, the procedures were identical for AnkX and the lpg0696 protein except that in the latter case 5% glycerol was added to all solutions and buffers. After cell lysis, the His_6 -tagged proteins were isolated from bacterial lysates by metal chelate affinity chromatography using a 5-ml Hi-Trap Ni^{2+} -NTA column (GE Healthcare) which was equilibrated with buffer A containing 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) pH 8.5, 500 mM LiCl, 2 mM β -mercaptoethanol. Proteins were eluted from the column using an imidazole gradient with 2–100% buffer B (buffer A + 500 mM imidazole). Fractions containing the protein of interest as judged by SDS-PAGE were pooled and dialysed overnight at 4°C in dialysis buffer (50 mM HEPES pH 8.5, 200 mM NaCl, 2 mM β -mercaptoethanol). Simultaneously, the His_6 tag was cleaved off by application of His_{10} -tagged TEV protease (0.06 mg per mg protein). Protein was separated from the His_6 tag and TEV protease by repeated Ni^{2+} -NTA affinity chromatography. Further purification was performed using gel filtration (Superdex 75 16/60; GE Healthcare) equilibrated with gel filtration buffer (20 mM HEPES pH 8.5, 200 mM NaCl, 2 mM dithioerythritol (DTE)).

The regions encoding for *Legionella pneumophila* LepB_{317–618} was obtained by PCR using *Legionella pneumophila* genomic DNA and inserted into a pOPIN vector by infusion cloning. The resulting construct was His_6 tagged at the N-terminus. Protein expression was in *E. coli* BL21-CodonPlus (DE3)-RIL at 20°C overnight after induction with 0.2 mM IPTG. Purification was achieved as for AnkX and lpg0696 with the exception that for affinity chromatography, 50 mM HEPES pH 8.0, 150 mM NaCl, 2 mM β -mercaptoethanol was used. Buffers used for dialysis contained 20 mM HEPES pH 7.5, 150 mM NaCl and 2 mM

β -mercaptoethanol, and for gel filtration 20 mM HEPES pH 7.5, 150 mM NaCl and 2 mM DTE.

The GEF domain of the human protein connectin 1 (connecdn 1_{1-403}) was expressed in Sf21 insect cells. Expression was performed with a cell concentration of 2×10^6 cells per ml for 3 days. After cell lysis, the His₆-tagged protein was isolated from the cell lysate using a 5-ml Hi-Trap Ni²⁺-NTA column (GE Healthcare) equilibrated with buffer A containing 50 mM HEPES pH 8.0, 150 mM NaCl and 2 mM β -mercaptoethanol. Proteins were eluted from the column with a 2–100% imidazole gradient (buffer A + 500 mM imidazole). Fractions containing the protein of interest, as judged by SDS-PAGE, were pooled and dialysed (50 mM HEPES pH 8.0, 100 mM NaCl, 2 mM β -mercaptoethanol). Simultaneously, His₆-PreScission Protease was applied to the protein to cleave off the His₆ tag. The protein was isolated from the tag and the protease by passing it over a Ni²⁺-NTA column again. Protein-containing fractions were pooled, concentrated and further purified on a gel filtration column (Superdex 75 16/60; GE Healthcare) equilibrated with 20 mM HEPES pH 7.5, 100 mM NaCl and 1 mM TCEP.

Recombinant UMP/CMP kinase was prepared as described by expression in *E. coli* (Segura-Pena *et al*, 2004); the expression plasmid was a gift of Dr Manfred Konrad, Max-Planck-Institute of Biophysical Chemistry, Göttingen. Rab1b, DrrA_{340–533} (Schoebel *et al*, 2009), LidA_{201–583}, TBC1D20_{1–362} and MICAL-3_{980–1130} (Muller *et al*, 2010) were expressed and purified as previously reported. Rab35-CVIL was prepared analogously to Rab1b-CVIL (Wu *et al*, 2010a).

The purity of the proteins used was monitored by SDS gel electrophoresis (see Supplementary Figure S6).

In-vitro phosphocholination of Rab proteins

Preparative phosphocholination was performed by incubating Rab proteins with AnkX in a 100:1 molar ratio in the presence of 1 mM CDP-choline (Enzo Life Sciences) for 2 h at RT. The completeness of the modification was monitored by Liquid Chromatography-Electrospray Ionization-Mass Spectrometry (LC-ESI-MS). The modified protein was purified using gel filtration (Superdex 75 16/60; GE Healthcare) in 20 mM HEPES pH 7.5, 100 mM NaCl, 2 mM DTE, 10 μ M GDP and 1 mM MgCl₂.

Nucleotide exchange on Rab1b and Rab35

Rab1b and PC-Rab1b were preparatively loaded with different mant 2'-(or-3')-O-(N-methylanthraniloyl)-nucleotides (mantdeoxyGDP, mantGppNHp, mantGTP). The protein was incubated for 2 h at RT in the presence of 10% glycerol, EDTA in five times molar excess over MgCl₂ and nucleotide in five times molar excess over protein. Buffer was exchanged afterwards three times by diluting the protein with the new buffer (20 mM HEPES pH 7.5, 100 mM NaCl, 1 μ M respective nucleotide and 2 mM DTE) and subsequent ultrafiltration, during which EDTA, glycerol and excess nucleotide were mainly removed. In some cases, GDP was displaced by a 10-fold excess of GTP in the absence of Mg²⁺ for 3–5 h in the cuvette used for the subsequent measurement, for example, monitoring of GAP activity using tryptophan fluorescence. The reaction was then started by addition of Mg²⁺ and GAP.

Similar procedures were followed for Rab35.

Kinetic investigations

Kinetic experiments were performed in 20 mM HEPES pH 7.5, 50 mM KCl, 1 mM MgCl₂ at 25°C using a stopped-flow apparatus

(Applied Photophysics) or a fluorescence spectrometer (Fluoromax-3, Horiba Jobin Yvon). For intrinsic fluorescence measurements, the excitation was at 297 nm in both types of apparatus. In the stopped-flow measurements, fluorescence intensity was monitored through a 320-nm cutoff filter. In the fluorescence spectrometer, an emission wavelength of 340 nm was used. For the steady-state kinetic measurements on Rab1b phosphocholination, one of the technical problems is conversion of the observed rate of the fluorescence change to a rate in concentration per unit time. A solution to this problem is to allow each phosphocholination reaction to proceed to completion, and then use the amplitude of the fluorescence change, which corresponds to the total Rab concentration in the experiment, to calculate the initial rate in units of μ M s⁻¹. Since each measurement would then last an inordinately long time using low enough concentrations of enzyme to allow comfortable measurement of the initial rate after manual mixing in the fluorescence cuvette, these reactions were performed in a stopped-flow apparatus, allowing the enzyme concentration to be chosen to allow measurement of a complete time course in 200–500 s. In practice, since the individual time courses of each experiment were well described by single exponential functions, the actual reaction rate was obtained by multiplying the observed first order rate constant by the concentration of Rab1b (for AnkX) or PC-Rab1b (for the lpg0696 protein). This is equivalent to determining the initial slope of the curve and converting it to concentration/time units by taking the total amplitude of the signal change to represent the total concentration of Rab1b used. Each point represents an average of three stopped-flow traces.

Experiments using the signal from the methylanthraniloyl group in the stopped-flow apparatus were carried out in 20 mM HEPES pH 7.5, 50 mM NaCl, 2 mM TCEP, 1 mM MgCl₂ at 25°C. The mant group was excited at 365 nm wavelength and emission was observed through a 420 nm cutoff filter. Association kinetics with Rab and LidA_{201–583} were monitored by the change in fluorescence polarization (excitation: 367 nm, 420-nm cutoff filter for emission).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: RSG, AI and MPM conceived and planned the project; PRG, KH and LKO performed the experiments; RSG and AI wrote the manuscript with input from the other authors.

Conflict of interest

The authors declare that they have no conflict of interest.

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