Characterization of Enzymes from *Legionella pneumophila* Involved in Reversible Adenylylation of Rab1 Protein*†§

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**Background:** Covalent modification of small GTPases by pathogens is an emerging field in current research.

**Results:** Quantitative analysis of effects, kinetics, and substrate specificities of adenylylation by DrrA and deadenylylation by SidD was performed.

**Conclusion:** Adenylylation and deadenylylation are means to tightly regulate Rab1 function by *Legionella* proteins.

**Significance:** This study increases our understanding of *Legionella pneumophila* subverting Rab protein function during infection.

After the pathogenic bacterium *Legionella pneumophila* is phagocytosed, it injects more than 250 different proteins into the cytoplasm of host cells to evade lysosomal digestion and to replicate inside the host cell. Among these secreted proteins is the protein DrrA/SidM, which has been shown to modify Rab1b, a main regulator of vesicular trafficking in eukaryotic cells, by transfer of adenosine monophosphate (AMP) to Tyr77. In addition, *Legionella* provides the protein SidD that hydrolytically reverses the covalent modification, suggesting a tight spatial and temporal control of Rab1 function by *Legionella* during infection. Small angle X-ray scattering experiments of DrrA allowed us to validate a tentative complex model built by combining available crystallographic data.

We have established the effects of adenylylation on Rab1 interactions and properties in a quantitative way. In addition, we have characterized the kinetics of DrrA-catalyzed adenylylation as well as SidD-catalyzed deadenylylation toward Rab1 and have determined the nucleotide specificities of both enzymes. This study enhances our knowledge of proteins subverting Rab1 function at the *Legionella*-containing vacuole.

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* The abbreviations used are: GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; LCV, Legionella-containing vacuole; SAXS, small-angle X-ray scattering; GDI, GDP dissociation inhibitor; ITC, isothermal titration calorimetry; P4M, phosphatidylinositol-4-phosphate binding domain; GppNHp, guanosine 5′-O-(3′-O-methyl)triphosphate; mantGDP, 2′-Deoxy-3′-O-(N-methylanthraniloyl) guanosine 5′-O-diphosphate; mantGTP, 2′-(or-3′)-O-(N-methylanthraniloyl) guanosine 5′-triphosphate, trisodium salt.

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‡ This article contains supplemental Figs. 1–4.

1 Experimental and theoretical scattering profiles, P(r) functions, SAXS envelopes, and atomic models reported in this paper have been deposited in the BIOISIS database (www.bioisis.net) under accession number BID 1DrrAP.

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the cell into a *Legionella*-containing vacuole (LCV), the bacterium injects more than 250 proteins into the host cell using a type IV secretion system termed DOT/ICM (defect in organelle trafficking/intracellular multiplication) (11, 12). Several of these proteins have been observed to modify Rab1 function, i.e., of a Rab protein involved in regulation of vesicular trafficking between the endoplasmic reticulum and the Golgi apparatus, thereby recruiting endoplasmic reticulum-derived vesicles to the LCV and establishing it as a replication vacuole (10). The first protein from *Legionella* reported to directly modify Rab1 function was DrrA (defect in Rab recruitment A), which was originally described as a protein containing GDP dissociation inhibitor (GDI) displacement factor and GEF activity (13, 14). Later it was demonstrated that it contains only GEF activity in a central domain containing residues 340–533, but that no active displacement (GDI displacement factor activity) occurs (15). The C-terminal domain of the protein was reported to bind phosphatidylinositol-4-phosphate with high affinity (16, 17), and the N-terminal domain was shown to be an adenylyltransferase catalyzing the covalent attachment of an AMP moiety to Tyr$^{77}$ in the switch II region of Rab1 (18). It was shown that the modifications has only small effects on DrrA-mediated nucleotide exchange, whereas catalysis by the GAPs LepB and TBC1D20 is strongly inhibited. Additionally, the human effector protein Mical-3 is not capable of binding to Rab1 in the modified state, whereas in contrast, the *Legionella* Rab super-effector LidA binds both Rab1 and adenylylated Rab1 (AMP-Rab1) with high affinity (18–20). The necessity of a protein catalyzing the demodification of Rab1 has been proposed after identification of the adenylyltransferase domain of DrrA (18). Confirming this hypothesis, recently a protein named SidD from *Legionella* was demonstrated to catalyze the hydrolysis of AMP-Rab1 (21, 22).

Here we report the characterization of the biochemical effects of DrrA-catalyzed modification of Rab1 on the interaction with different proteins in a quantitative manner. We determined the kinetic parameters of adenylation by DrrA and deadenylation by SidD and investigated the nucleotide preferences of these enzymes. Additionally, we present a structural model of full-length DrrA containing three distinct domains and corroborate the model by small angle x-ray scattering experiments.

**EXPERIMENTAL PROCEDURES**

**Protein Expression, Purification, and Modification—Mical-3, DrrA, and DrrA$_n$ (N451A/R453A/D480A/S483A), Rab1b$_{1-174}$ and Rab1b$_{3-174}$ were prepared, and Rab1 was adenylylated as described previously (15, 18, 23, 24).**

SidD$_{36-507}$ and TBC1D20$_{1-362}$ were cloned into a modified pET19 expression vector with N-terminal His$_6$ tag and tobacco etch virus cleavage site and expressed in *Escherichia coli* BL21 CodonPlus(DE3)-RIL (Stratagene) by induction with 0.2 mM isopropyl-1-thio-β-d-galactopyranoside and 0.5 mM isopropyl-1-thio-β-d-galactopyranoside overnight at 20 and 19 °C, respectively. Purification of SidD$_{36-507}$ was achieved by Ni$^{2+}$ affinity chromatography and final gel filtration in 20 mM Hepes pH 7.5, 100 mM NaCl, 2 mM dithioerythritol, 1 mM MgCl$_2$. Purification of TBC1D20$_{1-362}$ was achieved by Ni$^{2+}$ affinity chromatography, tobacco etch virus cleavage of the His$_6$ tag, and final gel filtration in 20 mM Hepes, pH 7.5, 100 mM NaCl, 2 mM dithioerythritol. LepP$_{325-618}$ was cloned into a modified pTriEx2 vector containing an N-terminal His$_6$ tag and PreScission cleavage site. Expression was achieved in *E. coli* BL21 CodonPlus(DE3)-RIL (Stratagene) by induction with 0.2 mM isopropyl-1-thio-β-d-galactopyranoside overnight at 20 °C and purified analogous to TBC1D20$_{1-362}$ with cleavage by PreScission protease and final gel filtration in 20 mM Hepes, pH 7.5, 150 mM NaCl, 2 mM dithioerythritol. Preparative nucleotide exchange on Rab1 and AMP-Rab1 was achieved as described previously (18).

**SAXS Measurements and Modeling—Small angle x-ray scattering (SAXS) data were collected at the European Molecular Biology Laboratory (EMBL) X33 beamline on the storage ring DORIS III (Deutsches Elektronen-Synchrotron (DESY)) (25).**

**Protein buffer consisted of 20 mM Hepes, pH 8.0, 50 mM NaCl, 1 mM MgCl$_2$, 2 mM dithioerythritol. Samples were measured at 20 °C in a minimum of three solve concentrations, ranging from 2 to 7.5 mg/ml. The data were recorded using a 1-M PILATUS detector (DECTRIS) at a sample-detector distance of 2.7 m and a wavelength of 1.5 Å, covering the range of momentum transfer $0.012 \, \text{Å}^{-1} < s < 0.6 \, \text{Å}^{-1}$ (here, $s = 4 \pi \sin(\theta)/\lambda$, where $\theta$ is the scattering angle). No measurable radiation damage was detected on comparison of four successive time frames with 30-s exposures. The data were processed with ATSAS package (26, 27) using standard procedures, averaged after normalization to the intensity of the transmitted beam, corrected for buffer contribution, and extrapolated to infinite dilution using the program PRIMUS (28).

The buffer-subtracted data were extrapolated to infinite dilution and further used for analysis and modeling. The radius of gyration ($R_g$) was determined from the Guinier approximation (29). Maximum complex dimensions $D_{\text{max}}$ and the interatomic distance distribution functions $P(r)$ were calculated using GNOM (30).

Ten ab initio models generated using DAMMIN (31) and GASBOR (32) were averaged with DAMAVER (33), which provides a value of normalized spatial discrepancy. Normalized spatial discrepancy values close to 1 indicate that the models are similar. The reference GASBOR model was aligned with the theoretical x-ray model using Supcomb13 (34). Evaluation of the theoretical scattering curves was done using x-ray-based model of full-length DrrA and fitting to the experimental scattering data was performed using CRYSOL (61, 62).

**Enzyme Kinetics of DrrA and SidD—Both adenylylation by DrrA and deadenylation by His$_6$ SidD$_{36-507}$ were measured using the change in tryptophan fluorescence as reported previously (35) in a FluoroMax-3 spectrophotometer (HORIBA Jobin Yvon) with excitation at 297 nm and emission at 340 nm at 25 °C (buffer: 20 mM Hepes, pH 7.5, 100 mM NaCl, and 5 mM MgCl$_2$). For all adenylation experiments, the quadruple mutant DrrA$_n$ (N451A/R453A/D480A/S483A) was used to exclude effects of the GEF activity of DrrA (15, 24), and all measurements of DrrA-catalyzed adenylation were performed in the presence of 2.5 units of yeast inorganic pyrophosphatase (New England Biolabs) to degrade pyrophosphate produced in the reaction.
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Data fitting of hyperbolic curves plotting initial velocities (v) divided by the enzyme concentration ([E]) against substrate concentrations ([S]) was performed using the following equation.

\[
\frac{v}{[E]} = \frac{[S]}{[S] + K_m} \quad (Eq. 1)
\]

Data fitting of sigmoidal curves plotting initial velocities (v) divided by the enzyme concentration ([E]) against substrate concentrations ([S]) was performed using the following equation.

\[
\frac{v}{[E]} = \frac{[S]^o}{[S]^o + s_0^3} \quad (Eq. 2)
\]

For determination of Michaelis-Menten parameters from single progress curves, we used KinTek Explorer (36, 37) and the following models for adenylylation by DrmA and deadenylylation by SidD (nucleotidylylated Rab1 is abbreviated NMP-Rab1).

Rab + DrmA \rightleftharpoons Rab·DrmA \rightarrow AMP-Rab + DrmA

REACTION 1

NMP-Rab + SidD \rightleftharpoons NMP-Rab·SidD \rightarrow Rab + NMP + SidD

REACTION 2

**GAP and GEF Assays and Determination of Intrinsic Nucleotide Exchange and GTP Hydrolysis Rates**—GAP assays for LepB and TBC1D20 using mantGTP-loaded Rab1 and AMP-Rab1 were done in a stopped flow apparatus (Applied Photophysics) with excitation at 365 nm and a 395-nm cut-off filter at 25 °C in 20 mM Hapes, pH 7.5, 50 mM NaCl, 5 mM MgCl2.

Nucleotide exchange was measured with mantGDP-loaded Rab1. Fast measurements in the presence of DrmA (residues 9–218) were performed using a stopped flow apparatus (Applied Photophysics) with excitation at 366 nm. Emission was detected using a 420-nm cut-off filter. Long term measurements without enzyme were performed using a FluoroMax-3 (HORIBA Jobin Yvon) with excitation at 360 nm and emission measurements at 440 nm with data collection at 20-s intervals. All measurements were performed in 25 mM Hapes, pH 8, 50 mM NaCl, 5 mM MgCl2, and 5 mM dithioerythritol at 25 °C.

For data analysis of hyperbolic curves plotting observed rate constants from single exponential fits of the progress curves (k_{obs}) against enzyme concentrations ([E]), the following equation was used.

\[
k_{obs} = \frac{[E] \times k_{cat}}{[E] + K_m} \quad (Eq. 3)
\]

Intrinsic GTP hydrolysis was measured using preparatively GTP-loaded Rab1 and AMP-Rab1 in 20 mM Hapes, pH 7.5, 50 mM NaCl, 2 mM dithioerythritol, 2 mM MgCl2 at 25 °C. The amount of bound GDP and GTP was quantified spectrometrically (A_{254 nm}) at different time points using HPLC (buffer, 50 mM potassium phosphate, pH 6.6, 10 mM tetrabutylammonium bromide, 8% (v/v) acetonitrile column, Prontosil 120-3-C18 column, AQ 3.0 μm, Bischof chromatography).

**Isothermal Titration Calorimetry (ITC) Measurements**—Interactions of Mical-31841–1990 and Rab1GppNHp or AMP-Rab1GppNHp were measured using an ITC200 microcalorimeter (MicroCal). Measurements were carried out in 20 mM Hapes, pH 7.5, 50 mM NaCl, 2 mM MgCl2 at 25 °C. 500 μM Mical-31841–1990 in the syringe was injected into the cell containing either 50 μM Rab1GppNHp or 50 μM AMP-Rab1GppNHp. Analysis of the data was performed using the Origin 7.0 Software provided by the manufacturer (MicroCal, LLC ITC).

**RESULTS**

**Biochemical Effects of Rab Adenylylation**—We have previously demonstrated that adenylylation of Rab1 in the switch II region has dramatic effects on interactions with GAPs and the human effector protein Mical, whereas the GEF activity of DrmA was only slightly inhibited (18). We have now characterized these effects further by determining equilibrium dissociation constants and enzyme parameters.

Independent K_m and k_{cat} values for GAP catalysis by LepB and TBC1D20 were determined for unmodified Rab1, whereas only catalytic efficiencies (k_{cat}/K_m) could be measured for AMP-Rab1 (Fig. 1, A and B, Table 1). The catalytic efficiencies of LepB and TBC1D20 toward AMP-Rab1 are decreased by factors of 1000 and 20, respectively.

Interestingly, although the overall catalytic efficiency (given by k_{cat}/K_m) of the DrmA GEF domain is only reduced by a factor of ∼3 by the covalent modification of Rab1, the K_m value is almost an order of magnitude higher for AMP-Rab1 than for Rab1 (Fig. 1C, Table 1), but this is partly compensated for by a higher k_{cat} value with modified AMP-Rab1.

In addition to the catalytic efficiencies of GAPs and GEFs, we also determined the affinity of the human effector protein Mical-3 (38) for Rab1 using ITC. For unmodified Rab1, a K_D value of 2.6 μM was observed, whereas no binding could be detected in the case of AMP-Rab1, indicating at least 2 orders of magnitude lower affinity (Fig. 1D, Table 1).

In contrast to the interactions of AMP-Rab1 with specific interaction partners, the intrinsic rates of GDP-GTP exchange and GTP hydrolysis were not significantly affected by the covalent modification with AMP (Fig. 1, E and F). Intrinsic GTP hydrolysis rate constants of 4.3 × 10^{-5} s^{-1} and 5.0 × 10^{-5} s^{-1} for Rab1 and AMP-Rab1, respectively, and an intrinsic mantGDP dissociation rate constant of 2.9 × 10^{-5} s^{-1} for both Rab1 and AMP-Rab1 were determined.

**A Structural Model of DrmA**—Since the protein DrmA was identified as a factor that can subvert Rab1 function in Legionella-infected cells, multiple crystal structures of the different domains of DrmA, but not the full-length protein, have been reported (15, 16, 18, 39, 40). The N-terminal adenylyltransferase domain (residues 1–339) is structurally not well characterized because only fragments containing residues 9–218 (Protein Data Bank (PDB) ID 3NKU) and 193–550 (PDB ID 3L0I) were successfully crystallized. Because further crystalliza-
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FIGURE 1. Adenylylation-induced effects on Rab1. All data for Rab1 are shown in blue curves, and data for AMP-Rab1 are shown in green curves. GAP-catalyzed GTP hydrolysis for Rab1 and AMP-Rab1 was measured for different concentrations of LepB325–618 and TBC1D201–362. A and B, resulting curves were fitted using a single exponential equation, and resulting $k_{\text{obs}}$ values were plotted against concentrations of LepB325–618 (A) and TBC1D201–362 (B) in the experiments. C, GEF-catalyzed nucleotide exchange using DrrA340–533 was measured similarly for AMP-Rab1. For comparison, previously published data of DrrA340–533-catalyzed nucleotide exchange on Rab1 are also shown (blue dotted curve (15)). Data fitting for A–C (see “Experimental Procedures”) led to the $k_{\text{cat}}/K_m$ and $k_{\text{cat}}/K_p$ values shown in Table 1. D, ITC measurements for interaction of Mical-31841–1990 with Rab1$_1$GppNHp (left, $n = 0.880 \pm 0.011, \Delta H = -7772 \pm 162 \text{cal/mol}, \Delta S = -0.536 \pm \text{cal/mol/K}$, and AMP-Rab1$_1$GppNHp (right). E and F, in contrast to interaction with different proteins, intrinsic GTP hydrolysis ($k_{\text{obs}} = 4.3 \times 10^{-5} \text{s}^{-1}$ for Rab1; $k_{\text{obs}} = 5.0 \times 10^{-5} \text{s}^{-1}$ for AMP-Rab1) (E) and intrinsic nucleotide exchange rates ($k_{\text{obs}} = 2.9 \times 10^{-5} \text{s}^{-1}$ for Rab1 and AMP-Rab1) (F) were not affected by the covalent modification.

### TABLE 1

| Enzyme parameters and equilibrium dissociation constants |
|--------------------------------------------------------|
| The table summarizes the determined enzyme parameters (GAPs LepB and TBC1D20, GEF DrrA) and equilibrium binding constants of the human effector protein Mical for Rab1 and AMP-Rab1, respectively. — indicates not detectable or applicable. |
| **Protein** | **Rab1** | **AMP-Rab1** | **Rab1** | **AMP-Rab1** | **Rab1** | **AMP-Rab1** | **A** | **B** |
|-------------|----------|--------------|----------|--------------|----------|--------------|-------|-------|
| LepB325–618 (GAP) | $32.4 \pm 3.7$ | — | $59.2 \pm 11.8$ | — | $5.5 \times 10^{-4}$ | $0.005$ | — | — |
| TBC1D201–362 (GAP) | $17.9 \pm 1.9$ | — | $27.2 \pm 5.2$ | — | $6.6$ | $0.32$ | — | — |
| DrrA340–533 (GEF) | $8.5 \pm 0.04$ | $17.9 \pm 0.1$ | $28.7 \pm 0.4$ | $214.0 \pm 2.4$ | $2.5$ | $0.85$ | — | — |
| Mical-31841–1990 | — | — | — | — | $2.6$ | $>260$ | — | — |

Adenylylation-induced effects on Rab1. GTP hydrolysis for Rab1 and AMP-Rab1 was measured for different concentrations of LepB325–618 and TBC1D201–362. A and B, resulting curves were fitted using a single exponential equation, and resulting $k_{\text{obs}}$ values were plotted against concentrations of LepB325–618 (A) and TBC1D201–362 (B) in the experiments. C, GEF-catalyzed nucleotide exchange using DrrA340–533 was measured similarly for AMP-Rab1. For comparison, previously published data of DrrA340–533-catalyzed nucleotide exchange on Rab1 are also shown (blue dotted curve (15)). Data fitting for A–C (see “Experimental Procedures”) led to the $k_{\text{cat}}/K_m$ and $k_{\text{cat}}/K_p$ values shown in Table 1. D, ITC measurements for interaction of Mical-31841–1990 with Rab1$_1$GppNHp (left, $n = 0.880 \pm 0.011, \Delta H = -7772 \pm 162 \text{cal/mol}, \Delta S = -0.536 \pm \text{cal/mol/K}$, and AMP-Rab1$_1$GppNHp (right). E and F, in contrast to interaction with different proteins, intrinsic GTP hydrolysis ($k_{\text{obs}} = 4.3 \times 10^{-5} \text{s}^{-1}$ for Rab1; $k_{\text{obs}} = 5.0 \times 10^{-5} \text{s}^{-1}$ for AMP-Rab1) (E) and intrinsic nucleotide exchange rates ($k_{\text{obs}} = 2.9 \times 10^{-5} \text{s}^{-1}$ for Rab1 and AMP-Rab1) (F) were not affected by the covalent modification.

The maximum particle dimensions ($D_{\text{max}}$) and the interatomic distance distribution function $P(r)$ were estimated using GNOM (30). The $P(r)$ function (Fig. 2C) showed a somewhat increased $D_{\text{max}}$ value ($15.5 \pm 0.5 \text{nm}$) as compared with the theoretical model ($D_{\text{max}} = 14.6 \text{nm}$), which can be attributed to the missing termini in the theoretical model (missing residues...
1–15 and 640–647) or a more extended conformation of the complex in solution. The theoretical pattern calculated from the x-ray-based model can be well fitted to the experimental data with a discrepancy $\chi^2$ value of $\chi^2$ (supplemental Fig. S1A).

Ab initio modeling using DAMMIN identified an excluded volume of $175 \text{ nm}^3$, which is in good agreement with the expected molecular mass of the complex ($\approx 95 \text{ kDa}$). Normalized spatial discrepancy values were below 0.7, indicating that independent reconstructions converged to a similar shape. Higher resolution ab initio models, consistent with the expected molecular mass, were built using GASBOR.

The reference GASBOR model was aligned to an x-ray-based model, showing a good agreement with the latter (Fig. 2B), hence suggesting that the x-ray-based model is a close representation of the complex in solution. However, one has to keep in mind that a membranous environment and additional, yet to be identified protein interactions might influence the conformation of DrrA.

**Kinetics of DrrA-catalyzed Adenylylation**—First measurements of adenylylation by DrrA using high concentrations of Rab1-GppNHp as substrate showed a fast initial rate of low amplitude followed by a linear phase in the progress curve (supplemental Fig. S2). This kinetic behavior was not easily explainable by simple competitive inhibition, but rather by allosteric inhibition by a reaction product affecting the turnover number ($k_{cat}$) of DrrA. Because the generated pyrophosphate was a possible cause of this product inhibition, we repeated the measurement in the presence of pyrophosphatase, which led to a more rapid overall time course that could be well fitted based on a simple Michaelis-Menten model without product inhibition. Therefore, all experiments for determination of Michaelis-Menten parameters were performed in the presence of pyrophosphatase. Interestingly, systematic analysis of the dependence of the initial rates on substrate concentrations showed sigmoidal kinetic behavior for Rab1-GDP, but not Rab1-GppNHp, using ATP as the cosubstrate. The sigmoidal fit resulted in a Hill parameter of 1.7, indicating possible dimer formation of Rab1-GDP at high concentrations as was shown for Rab9 and Rab27 (42, 43). Interestingly, the switch II region of the dimeric GDP-bound Rab9 was reported to adopt an active-like conformation, which might explain the increased reaction rates of adenylylation toward Rab1-GDP at higher concentrations (43). However, because the inactive GDP-bound state of Rab1 is not a physiologically relevant substrate, the sigmoidal kinetic behavior was not further investigated at this point.

Confirming the previously reported preference of DrrA-catalyzed adenylylation toward Rab1-GppNHp as compared with...
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**FIGURE 3. Kinetics of DrRA-catalyzed adenylylation.** A–C, values for \(v/[E]_0 \) (s\(^{-1}\)) were determined from initial slopes measured using constant concentrations of 15 nM DrRA, 2 mM ATP and varying concentrations of Rab1\(_{13–174}\)GppNHp (A), constant concentrations of 5 μM DrRA, 2 mM ATP and varying concentrations of Rab1\(_{13–174}\)/GDP (B), and constant concentrations of 15 nM DrRA, 10 μM Rab1\(_{13–174}\)GppNHp and varying concentrations of ATP (C). The data were fitted to obtain \(k_{cat}\) and \(K_m\) or \(S_{0.5}\) as described under “Experimental Procedures.”

**TABLE 2**

Kinetic parameters of DrRA catalyzed adenylylation

| Substrate               | \(k_{cat}\) (s\(^{-1}\)) | \(K_m\) or \(S_{0.5}\) (μM) | \(k_{cat}/K_m\) (10\(^{3}\) s\(^{-1}\) μM\(^{-1}\)) |
|-------------------------|--------------------------|----------------------------|----------------------------------|
| Rab1\(_{13–174}\)GppNHp | 53.4 ± 3.9               | 64.2 ± 12.7                | 8.3 ± 2.2                       |
| Rab1\(_{13–174}\)GDP    | 0.78 ± 0.01              | 362.9 ± 21.7               | 0.021 ± 0.001                   |
| ATP                     | (9.0 ± 0.4)              | 73.9 ± 10.2                | (1.2 ± 0.2)                     |

Rab1-GDP (18), \(k_{cat}\) values of 53.4 and 0.78 s\(^{-1}\) and \(K_m\) or \(S_{0.5}\) values of 64.2 and 362.9 μM were obtained for Rab1-GppNHp and Rab1-GDP, respectively (Fig. 3 and Table 2). Because of the relatively low \(K_m\) value for Rab1-GppNHp, calculation of independent \(k_{cat}\) and \(K_m\) values was also possible from a single progress curve, and the values obtained were in good agreement with the previously mentioned parameters (supplemental Fig. S3A). In summary, these findings indicate a 395-fold higher catalytic efficiency of DrRA toward the active state Rab1-GppNHp as compared with the inactive Rab1-GDP. Furthermore, a \(K_m\) value of 73.9 μM was measured for ATP (Fig. 3C and Table 2). Because determination of the catalytic parameters for ATP was performed at nonsaturating concentrations of Rab1-GppNHp, the corresponding \(k_{cat}\) and \(k_{cat}/K_m\) values are only apparent values and are shown in parentheses (Table 2).

**Kinetics of SidD-catalyzed Deadenylation**—Experiments using cell lysates from both *E. coli* and *L. pneumophila* showed a specific activity for removal of the AMP-modification in *Legionella*, but not in *E. coli* lysates (supplemental Fig. S4), confirming the presence of the previously identified deadenylase SidD (21, 22) and indicating the necessity of this specific enzyme for removal of the covalent modification. To obtain insights into the competition of adenylylation by DrRA and deadenylolation by SidD, we quantified the catalytic activity of recombinantly produced and purified SidD. Surprisingly, deadenylolation catalyzed by SidD was not significantly affected by the activation state of Rab1 (Fig. 4). \(K_m\) values of 2.6 and 3.9 μM and \(k_{cat}\) values of 13.1 and 17.8 s\(^{-1}\) for AMP-Rab1-GDP and AMP-Rab1-GppNHp, respectively, were determined (Table 3). Because of the low \(K_m\) values, the calculation of independent \(k_{cat}\) and \(K_m\) values was possible from single progress curves (supplemental Fig. S3B), indicating good agreement with the parameters obtained from Michaelis-Menten plots.

**Nucleotide Specificity of DrRA and SidD**—In addition to ATP, DrRA was also reported to be capable of using GTP as substrate for guanylylation of Rab1 (18). Hence, we systematically analyzed the substrate properties of various nucleotides. Besides ATP and GTP, DrRA could also utilize ADP and GDP, albeit at lower rates (Fig. 5A). Furthermore, pyrimidine nucleotides (CTP and UTP) were also tested. Interestingly, CTP showed similar substrate properties to ATP, whereas UTP showed only very low reaction rates (Fig. 5B), indicating an important role of the amino group at the C6 position of the purine ring and the C4 position of the pyrimidine ring, respectively, in recognition by DrRA. Determination of the initial velocities of the nucleotidylation reaction for each nucleotide indicated 60% activity for CTP, ~14% for ADP and GDP, and less than 10% for the remaining nucleotides tested as compared with ATP (Fig. 5C).

Because DrRA was capable of modifying Rab1 with various nucleotides, we consequently tested the reversibility using SidD in a fluorescence-based assay (Fig. 5E) and confirmed the results using electrospray mass ionization-MS at the end of each reaction (data not shown). For all nucleotide modifications catalyzed by DrRA, SidD catalyzed the demodification, albeit with slightly lower catalytic efficiencies for GMP, CMP, and UMP than for AMP (Fig. 5E and Table 4). The experiments show that ATP and AMP-Rab1 are the preferred substrates for DrRA and SidD, respectively, but other nucleotides might be used during infection as well.

**DISCUSSION**

During the past few years, posttranslational modifications and especially adenylylation of host cell proteins by pathogenic bacteria have become a topic of great interest (18, 44–50). Very recently, it has also been demonstrated for DrRA-catalyzed adenylylation that the modification can be reversed by the...
Characterization of adenylylating Enzymes from *L. pneumophila*

Here, we present a model giving deeper insight into the DrrA- and SidD-catalyzed events taking place at the LCV including knowledge on the kinetics of adenylylation by DrrA and deadenylation by SidD determined in this study and data on the time-dependent detection of different *Legionella* proteins at the LCV reported in literature. We speculate that the infection can most probably be divided into two phases regarding subversion of Rab1 function by *Legionella* (Fig. 6).

The first phase is referred to as the entrapment phase in Fig. 6. This phase is characterized by adenylylation of Rab1, and both DrrA and Rab1 are localized at the LCV (52). The adenylylation of Rab1 might be used as a tool to interfere with the control of Rab1 by the host cell, such as for example inactivation by GAPs. Furthermore, the covalent modification has been shown to inhibit binding of GDI, thereby not allowing extraction from the LCV membrane (24). During this entrapment phase, the supereffector LidA might act as a tethering factor for Rab1 with high affinity and is present at the LCV within 20 min after infection (20, 53). In contrast to LidA from *Legionella*, binding of the human effector protein Mical is not possible in the modified state. Further studies showing whether the covalent modification inhibits human effector proteins in general from binding to modified Rab1 would be of great interest. The absence of Rab1 effector proteins p115 and GM130 at the LCV during infection (54) indicates decreased binding affinities of these proteins to AMP-Rab1 as well.

The preference of DrrA-catalyzed adenylylation toward Rab1-GppNHp and the catalytic efficiency of the DrrA GEF domain of $2.2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ (15) suggest a consecutive order of DrrA function. After binding to the LCV via the P4M domain, DrrA recruits Rab1 by catalyzing nucleotide exchange, and only afterward, Rab1 can be covalently modified with AMP. This is further supported by our model of DrrA$_{fl}$, which we corroborated by small angle x-ray scattering as the localization of the active sites does not allow a simultaneous catalysis or even substrate channeling, but rather different events of Rab binding and release for nucleotide exchange and adenylylation by DrrA.

The second phase (referred to as the release phase in Fig. 6) starts approximately 6 h after internalization, when *Legionella* begins to replicate (53, 55, 56). Approximately at this time, DrrA loses its LCV localization (52), whereas both LepB and SidD can be detected at the LCV (21, 52). The delayed detection of SidD and LepB in these studies might indicate a generally lower expression level or a delayed transcription and secretion of these proteins as compared with DrrA. While binding of DrrA to the LCV membrane depends on the presence of phosphatidylinositol-4-phosphate, LepB most likely binds via putative transmembrane regions and might be enriched at the LCV during the course of infection even at low expression levels. As discussed later, a putative membrane binding domain has not been identified for SidD yet, and the distribution of SidD inside the host cell during infection is unknown.

Because LepB cannot catalyze GTP hydrolysis on AMP-Rab1, the order of events must be deadenylation of AMP-Rab1 by SidD followed by deactivation by LepB. Only at this point is GDI able to extract Rab1 from the LCV membrane so that Rab1 is removed from the LCV (24, 52).

Because adenylylation by ectopically expressed DrrA has been shown to have cytotoxic effects in cell culture experiments (14, 18), demodification and release of Rab1 from the LCV might be necessary to ensure survival of the host cell as long as *Legionella* replicates. We show that SidD has a high catalytic activity for both AMP-Rab1-GDP and AMP-Rab1-GppNHp. The high activity might indicate that SidD has evolved to ensure rapid and complete demodification of Rab1 at the LCV as well as other places inside the cell. Even in the presence of small remaining concentrations of DrrA, the high catalytic efficiency would ensure complete demodification. In this respect, it is of great interest to obtain more insight into the local concentrations of the enzymes DrrA and SidD and their substrates at the LCV during infection. The restriction to lateral diffusion for membrane-bound proteins, which effectively increases the...
local concentrations, might significantly influence the catalysis in an in vivo environment.

As an explanation of the ready acceptance of the GDP-bound form of AMP-Rab1 by SidD, AMP-Rab1/GDP, might adopt an active-like conformation of the switch II region as the crystal structure of AMP-Rab1b-GppNHp (PDB ID 3NKV) (18) showed a stacking interaction of the adenosine and Phe<sup>45</sup>, and this interaction might also occur in AMP-Rab1-GDP. This

| Nucleotide preference of SidD catalyzed deadenylylation | $k_{cat}/K_m$ values of deadenylylation by SidD were determined from single progress curves (Fig. 5E). |
|--------------------------------------------------------|--------------------------------------------------|
| AMP-Rab1                                               | 6.8                                              |
| GMP-Rab1                                               | 1.1                                              |
| CMP-Rab1                                               | 0.8                                              |
| UMP-Rab1                                               | 0.7                                              |

FIGURE 5. Nucleotide specificities of DrrA and SidD. A, Progress curves of adenylylation of 10 μM Rab<sub>1</sub><sub>1-174</sub>-GppNHp by 15 nM DrrA with 2 mM ATP (black curve), 2 mM GTP (red curve), 2 mM ADP (blue curve), or 2 mM GDP (green curve). The green star indicates the addition of 1.5 μM DrrA. B, progress curves of adenylylation of 10 μM Rab<sub>1</sub><sub>1-174</sub>-GppNHp by 15 nM DrrA with 2 mM ATP (black curve), 2 mM CTP (red curve), or 2 mM UTP (blue curve). The blue star indicates the addition of 75 nM DrrA. C, relative activities of nucleotidylation of Rab<sub>1</sub><sub>1-174</sub>-GppNHp were calculated using initial velocities of the progress curves from A and B. Error bars indicate S.D. D, the structural formulae of nucleobases are shown in the order of substrate specificity of DrrA for nucleoside triphosphates. E, progress curves of denucleotidylylation of 20 μM AMP-Rab<sub>1</sub><sub>1-174</sub>-GppNHp (black), 20 μM GMP-Rab<sub>1</sub><sub>1-174</sub>-GppNHp (red), 20 μM CMP-Rab<sub>1</sub><sub>1-174</sub>-GppNHp (green), or 20 μM UMP-Rab<sub>1</sub><sub>1-174</sub>-GppNHp (blue) by 4 nM SidD were fitted using KinTek Explorer to obtain catalytic efficiencies (Table 4). F, the structural formulae of nucleobases are shown in the order of substrate specificity of SidD for Rab1 modified with nucleoside monophosphates.
could therefore explain the low substrate specificity of SidD toward GDP or GTP/GppNHp-bound AMP-Rab1. Alternatively, SidD might solely recognize the amino acid sequence rather than any secondary structure of the switch II region of Rab1.

Although DrrA can use ATP, GTP, UTP, and CTP for nucleotidylation of Rab proteins, the determined catalytic parameters suggest a preference for ATP. This specificity is likely to be even more pronounced in vivo because the intracellular nucleotide concentrations are highest for ATP (ATP (3152 μM ± 1698 μM), CTP (278 μM ± 242 μM), GTP (468 μM ± 224 μM), and UTP (567 μM ± 460 μM) (57)). Therefore, these concentrations in conjunction with our biochemical nucleotidylation parameters suggest ~91% modification of Rab1 with AMP, less than 6% modification of Rab1 with CMP, and less than 3% modification of Rab1 with GMP, respectively, during infection. Modifications with UMP are most probably insignificant. This might therefore also explain the requirement that SidD is able to remove different covalently attached nucleotides. Supporting the preference of ATP for modification of Rab1, mass spectrometric analyses of cells infected with L. pneumophila detected AMP-Rab1, but no other nucleotide-modified Rab1 species (58).

Because SidD contains 507 amino acids, it is probably a multidomain protein. Identification and modeling of a protein phosphatase fold in SidD indicates N-terminal localization of the deadenylylation domain (59), raising the question of potential functions of the C terminus of SidD. Further characterization of the domain architecture as well as characterization of putative other domains will be of great interest. In this regard, the presence of a putative lipid binding domain for localization of SidD, as was shown for multiple other Legionella secreted proteins (12), and the intracellular localization of SidD during infection will give further insight into its purpose during infection at the LCV and possibly other places inside the cell.

After characterization of the antagonists AnkX and Lem3/lpg0696 for Rab1 modification (24, 35), this study gives further insight into the role of DrrA and SidD during infection. It is of great interest for future research to determine whether pathogens using Fic domain-containing proteins for adenyllylation of host cell GTPases (46, 60) have evolved similar antagonistic proteins to reverse the modification in a time-controlled manner during infection.

In this study, we have reported a model of the order of events taking place at the LCV based on the detailed characterization of DrrA and SidD and including known factors of Rab1 interaction. Looking at the amount of proteins secreted by Legionella with yet unknown function and the recent progress in identification of further Rab-interacting and -modifying proteins, identification of more factors for further fine tuning of Rab protein function and subversion of vesicular transport will be an interesting issue for future research.

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FIGURE 6. Timeline of Legionella infection. The timeline of Legionella infection is shown as a black bar with different proteins and approximate time points of their localization at the LCV reported in literature (human Rab1, red bar; Legionella DrrA, LepB, SidD, and LdA, blue bars). For further information, see under “Discussion” (figure partly adapted from Ref. 10). ER-derived, endoplasmic reticulum-derived.
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