The *Rickettsia prowazekii* ExoU Homologue Possesses Phospholipase A₁ (PLA₁), PLA₂, and Lyso-PLA₂ Activities and Can Function in the Absence of Any Eukaryotic Cofactors *In Vitro*[▽]

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Here we have characterized the *Rickettsia prowazekii* RP534 protein, a homologue of the *Pseudomonas aeruginosa* ExoU phospholipase A (PLA) secreted cytotoxin. Our studies showed that purified recombinant RP534 PLA possessed the predicted PLA₂ and lyso-PLA₂ activities based on what has been published for *P. aeruginosa* ExoU. RP534 also displayed PLA₁ activity under the conditions tested, whereas ExoU did not. In addition, recombinant RP534 displayed a basal PLA activity that could hydrolyze phosphatidylcholine in the absence of any eukaryotic cofactors. Interestingly, the addition of bovine liver superoxide dismutase 1 (SOD1), a known activator of *P. aeruginosa* ExoU, resulted in an increased rate of RP534-catalyzed phospholipid hydrolysis, indicating that mechanisms of activation of the ExoU family of PLAs may be evolutionarily conserved. The mechanism of SOD1-dependent stimulation of RP534 was further examined using active site mutants and a fluorogenic phospholipid substrate whose hydrolysis by RP534 over a short time course is measureable only in the presence of SOD1. These studies suggest a mechanism by which SOD1 stimulates RP534 activity once it has bound to the substrate. We also show that antibody raised against RP534 was useful for immunoprecipitating active RP534 from *R. prowazekii* lysed cell extracts, thus verifying that this protein is expressed and active in rickettsiae isolated from embryonated hen egg yolk sacs.

Rickettsia prowazekii is the etiological agent of epidemic typhus fever in humans and is a select agent. This reemerging pathogen is associated with louse infestations within the human population that occur as the result of social conditions that precipitate a breakdown in hygiene and sanitation (5, 25, 33). *R. prowazekii* is an obligate intracellular parasitic bacterium that attaches to host cells, actively stimulates its own entry, escapes from the phagosome, grows to large numbers in the cytoplasm, and eventually lyses the host cell (for reviews, see references 4, 20, 45, 47, and 49). It is well established that phospholipase A (PLA) activity plays an integral role in *R. prowazekii* pathogen/host interactions. Attachment of rickettsiae to host cells immediately activates a rickettsial PLA thought to be involved in pathogen entry (39, 46, 51, 54, 56). Furthermore, *R. prowazekii*-derived PLA activity is thought to be responsible for the continuous but noncytolytic release of host cell-free fatty acid to the culture medium during infection (51–53). In addition to parasitizing eukaryotic cells, *R. prowazekii* is able to lyse mammalian red blood cells (RBCs) in a PLA-dependent fashion, although the biological significance of RBC lysis remains unclear (13, 30–32, 40, 48, 50, 55, 57). Despite the prominent role of PLA in *R. prowazekii* pathogenesis, the biochemical properties of the culprit PLA(s) remain poorly characterized.

The *R. prowazekii* genome encodes two putative PLA proteins, RP534 and RP602 (6), which are annotated as members

of the patatin superfamily of phospholipase/acyl hydrolase proteins. Patatin is a major storage glycoprotein in potatoes and possesses potent PLA/acyl hydrolase activity that becomes activated to serve as a defense mechanism against plant parasites (7, 21, 34, 41). Patatin-like PLAs are most highly homologous to eukaryotic group IV cytosolic PLA₂α and group VI calcium-independent PLA₂s, which are distinguished by a conserved serine-aspartic acid catalytic dyad within the active site (for reviews, see references 19 and 36). The amino-terminal half of the RP534 protein shows 27% sequence identity and 43% similarity to that of the ExoU PLA cytotoxin of *Pseudomonas aeruginosa*, including perfect conservation of residues within the active site (36). ExoU is one of four major exotoxins of *P. aeruginosa* secreted by the type three secretion system (17) and has been linked to strains that cause severe respiratory disease (1, 15, 38). A defining characteristic of *P. aeruginosa* ExoU (PaExoU) is its reported dependence on a eukaryotic cell cytosolic cofactor(s) to elicit PLA activity (14, 27, 28, 36, 37, 44). Sato and coworkers have identified the eukaryotic copper/zinc superoxide dismutase (SOD1) as a specific activator of ExoU activity *in vitro* (35). However, it remains unknown whether SOD1 is the only *P. aeruginosa* ExoU activator. Based on homology to *P. aeruginosa* ExoU, the goal of the present study was to characterize the *R. prowazekii* RP534 protein to determine if it is a *bona fide* PLA and whether it shares the SOD1-dependent activation properties of ExoU.

MATERIALS AND METHODS

Cell growth and chemicals. *Escherichia coli* was routinely grown in Luria-Bertani (LB Lennox) broth (Difco) supplemented with 100 μg/ml of ampicillin for plasmid maintenance (LBAp) at 37°C with aeration (200 rpm). Oligonucleotide primers were purchased from Integrated DNA Technologies. Chemicals

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were purchased from Sigma, molecular biology enzymes were purchased from New England BioLabs, fluorogenic phospholipids (PED6 and PED-A1) were purchased from Invitrogen, 1- α -1-palmitoyl-2-[1- 14 C]arachidonyl-phosphatidylcholine, ([14 C]PC-AA) (48 mCi/mmol, used at 0.05 μ Ci μ L $^{-1}$) and [1- 14 C]oleic acid ([14 C]OA, 58.2 mCi/mmol, used at 0.1 μ Ci μ L $^{-1}$) were purchased from Perkin Elmer, and 1-3-phosphatidylcholine, 1-palmitoyl-2-[1- 14 C]oleoyl ([14 C]PC-OA) (56 mCi/mmol, used at 0.025 μ Ci μ L $^{-1}$) was from GE Healthcare. All other phospholipids were purchased from Avanti Polar Lipids.

Expression plasmid construction and mutagenesis. Molecular cloning techniques were previously described (18). The *R. prowazekii* RP534 gene was cloned into the pET16b expression vector (Novagen) to incorporate an amino-terminal decahistidine (N-His₁₀) tag (plasmid designated pET16b-N-His₁₀-RP534). Site-directed mutagenesis was performed on pET16b-N-His₁₀-RP534 using Stratagene's QuikChange system to change a serine residue at position 100 to an alanine (S100A). A second, separate mutant was constructed by changing an aspartic acid residue at position 246 to an alanine (D264A). A pET28-*exoU* plasmid (a kind gift from Partho Ghosh, University of California, San Diego) encoding *P. aeruginosa* ExoU (PaExoU) was used for subcloning of *exoU* to generate pET16b-N-His₁₀-PaExoU.

All plasmids used in this study were verified by DNA sequencing using the ABI BigDye Terminator system (v.1), and reactions were resolved by the Iowa State DNA Facility on a fee-for-services basis. Sequence alignments were performed using the CodonCode Aligner software program (CodonCode Corp) or NCBI BLAST (2, 3).

Recombinant protein purification. The pET16b-N-His₁₀-RP534, pET16b-N-His₁₀-RP534(S100A), pET16b-N-His₁₀-RP534(D264A), and pET16b-N-His₁₀-PaExoU expression vectors were independently introduced into the C41[DE3] expression strain of *E. coli* (24) by electroporation. Strains were regenerated routinely, since we have previously observed that expression vectors encoding *R. prowazekii* proteins are not stably maintained in expression-proficient *E. coli* strains. Transformants were not used for more than 1 week, but liquid cultures that had been induced for protein expression were routinely stored for weeks as cell pellets at -80°C prior to protein purification. Standard bacterial growth and protein purification conditions were as described previously (18), with the following modifications. Cell lysates were loaded onto a nickel-nitrilotriacetic acid (Ni-NTA) resin using a Bio-Rad Profinia protein purification system and sequentially washed with 0.5 M NaCl, 20 mM Tris-HCl, 10 mM imidazole, and 10 mM β -mercaptoethanol (β ME), pH 8.0, with 0.5 M NaCl, 20 mM Tris-HCl, 30 mM imidazole, and 10 mM β ME, pH 8.0, and with 0.5 M NaCl, 20 mM Tris-HCl, 300 mM imidazole, and 10 mM β ME, pH 8.0. Purified protein was exchanged into 50 mM morpholinopropanesulfonic acid (MOPS), 50 mM NaCl, and 5 mM β ME, pH 7.4, and stored at 4°C . Note that for purification of N-His₁₀-PaExoU, β ME was omitted from all buffers. Purified protein concentrations were determined by spectrophotometry at 280 nm using an extinction coefficient of $\sim 35,730 \text{ M}^{-1} \text{ cm}^{-1}$. The purified RP534 proteins were stable for several weeks under these conditions. Purified proteins were analyzed by SDS-PAGE (10% Tris-glycine) and staining, and identities were verified by tandem mass spectrometry at the USA MCI Proteomics and Mass Spectrometry Laboratory on a fee-for-service basis.

Purified N-His₁₀-RP534 protein was used as an antigen to raise polyclonal rabbit antiserum (Sigma Genosys). Anti-N-His₁₀-RP534 antibody was affinity purified using Pierce's Aminolink Plus immobilization kit as per the manufacturer's directions.

In vitro phospholipase activity assays. The activities of purified recombinant PLA proteins were assayed using a radiolabeled phospholipid substrate incorporated into large unilamellar vesicles (LUVs) as described in reference 37. The stock LUVs were a 2.5 μ M equimolar mixture of phosphatidylcholine (PC) and phosphatidylserine (PS) (to give 5 μ M total phospholipid) containing either 16.8 μ M [14 C]PC-OA or 38.2 μ M [14 C]PC-AA and were diluted 1:20 into the PLA assays. Purified PLA₂ from *Naja mossambica mossambica* (Sigma) was used as a positive control. All samples were extracted using the acid-modified method of Bligh and Dyer (11, 22), resolved by thin-layer chromatography on silica gel G plates using the solvent system described in reference 16, and visualized by phosphorimaging (Cyclone; PerkinElmer). The PLA activity assay conditions are described in the legend to Fig. 1.

The activities of the recombinant PLA proteins were also assayed using fluorogenic phospholipid substrates (Invitrogen). The PED6 [N-((6-(2,4-dinitrophenyl)amino)hexanoyl)-2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt] substrate has a fluorescent moiety conjugated at the *sn*-2 position and contains a dinitrophenyl group conjugated to the polar head group to provide intramolecular quenching. Thus, when the activity of a PLA₂ hydrolyzes the *sn*-2 fatty acyl chain, the quenching is released and fluorescent signal is

measured. The PED-A1 [(N-((6-(2,4-dinitrophenyl)amino)hexanoyl)-1-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-2-hexyl-*sn*-glycero-3-phosphoethanolamine)] substrate differs in that the fluorescent moiety is conjugated at the *sn*-1 position and the *sn*-2 fatty acyl group is nonhydrolyzable and is used to measure the activity of PLA₁ enzymes. Substrates were dissolved into 50 mM MOPS-50 mM NaCl, pH 7.4, and assay conditions are described in the legends to Fig. 3, 4, and 5. Samples were removed from reactions at appropriate times and assayed for fluorescence on a NanoDrop 3300 Micro-Volume fluorospectrometer (Thermo Scientific) using 470-nm excitation/511 emission. The activity of the purified N-His₁₀-RP534 protein was also tested in the presence of the PLA inhibitors methyl arachidonyl fluorophosphate (MAFP) (concentrations are given in Fig. 3C) or arachidonyl trifluoromethyl ketone (AACOCF₃) at 10 μ M. MAFP was purchased as a methylacetate solution that was evaporated prior to dissolution of the MAFP directly into assay buffer. AACOCF₃ was dissolved in ethanol and added directly to the PLA assays. Ethanol alone was included as a vehicle control.

Immunoprecipitation of active RP534 from lysed cell extracts of purified *R. prowazekii*. All experiments described in this article were performed using the Madrid E strain of *R. prowazekii* (hen egg yolk sac passage 282), purified as previously described (18). For immunoprecipitation (IP) of the RP534 protein from *R. prowazekii*, purified rickettsiae were washed and suspended in 4 ml of buffer A (135 mM NaCl, 2.7 mM KCl, 5.4 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 5 mM β ME, and Roche's EDTA-free complete protease inhibitor cocktail [PIC] [at the manufacturer's suggested concentration], pH 7.4), lysed by ballistic shearing, and cleared of debris (18). *R. prowazekii* lysed cell extracts (~ 5 mg total protein, measured using the Bio-Rad DC protein assay) were incubated with 10 μ L protein A Sepharose beads (Pierce) for 10 min at 4°C with agitation followed by incubation with 24 μ g of affinity-purified anti-RP534 antibody and 10 μ L protein A Sepharose beads for 18 h at 4°C with agitation. The IP samples were washed six times in 0.5 ml of buffer A (no PIC), eluted in 0.2 ml of glycine buffer (0.2 M, pH 2), and neutralized by the addition of 20 μ L 1 M Tris-HCl (pH 9.0). Neutralized samples were dried on an Amicon YM-10 ultrafiltration membrane, solubilized in 60 μ L Laemmli loading dye (Bio-Rad), and resolved by SDS-PAGE (10% Tris-glycine). Gels were stained with Bio-Rad Biosafe Coomassie, and proteins were excised and analyzed by tandem mass spectrometry. Analysis of the RP534 protein by immunoblotting was performed using the protocol described in reference 8. Anti-RP534 rabbit antiserum was used at a 1:500 dilution, peroxidase-conjugated anti-rabbit secondary antibody (Sigma) was used at a 1:2,000 dilution, and blots were developed using enhanced chemiluminescence (horseradish peroxidase [HRP] ECL from GE Healthcare), and visualized using a Bio-Rad ChemiDoc XRS gel documentation system.

For isolation and assay of RP534 PLA activity from purified *R. prowazekii* lysed cell extracts (~ 2 mg total protein), the IP protocol described above was modified as follows. The protein A preclearing step was performed for 45 min, the affinity-purified anti-RP534 antibody and 10 μ L protein A step was performed for 2 h, beads were washed in 0.2 ml of buffer A (no PIC), and protein was eluted in a final volume of 0.25 ml with elution buffer containing 5 mM β ME. The IP flowthrough, washes, and eluate were tested for PLA activity using fluorogenic substrates as described above and in the legend to Fig. 5.

RESULTS

In vitro PLA₂ activity of purified recombinant RP534 protein. Considering that the *R. prowazekii* RP534 open reading frame is a homologue of the *P. aeruginosa* ExoU PLA₂ cytoxin, we predicted that RP534 would also require the addition of a eukaryotic cofactor(s) to observe PLA activity. Thus, the known activator of *P. aeruginosa* ExoU, bovine liver SOD1 (35), was specifically tested for activation of RP534 PLA activity *in vitro*. To this end, a recombinant, N-decahistidine-tagged RP534 protein expressed in *E. coli* was purified (Fig. 1A, lane 1) and assayed for PLA activity using LUVs containing [14 C]phosphatidylcholine (PC) as a substrate. PC was labeled at the *sn*-2 position with either [14 C]oleic acid (PC-OA) or [14 C]arachidonic acid (PC-AA). In these assays, a PLA₂ enzyme will produce 14 C-labeled free fatty acid, a PLA₁ enzyme will produce [14 C]lysophosphatidylcholine (LPC), and a deacylating enzyme will produce short 14 C-labeled fatty acyl chains. RP534 was able to hydrolyze PC-containing LUVs with

FIG. 1. *In vitro* assay of *R. prowazekii* RP534 PLA activity using radiolabeled phospholipid substrates. (A) Recombinant N-His₁₀-RP534, N-His₁₀-RP534(S100A), and N-His₁₀-RP534(D264A) were purified by immobilized-metal affinity chromatography (IMAC), resolved by SDS-PAGE, and Coomassie stained. The molecular mass markers shown to the left of the gels are in kDa. The expected size of the recombinant proteins is ~70 kDa. (B) Purified recombinant N-His₁₀-RP534, N-His₁₀-RP534(S100A), and N-His₁₀-RP534(D264A) were assayed for PLA activity using [¹⁴C]-LUVs as substrates; the products were extracted, resolved by thin-layer chromatography, and visualized by phosphorimaging. Recombinant PLA proteins (0.01 $\mu\text{g } \mu\text{l}^{-1}$) were incubated with or without bovine liver SOD1 (0.07 $\mu\text{g } \mu\text{l}^{-1}$) with 0.25 μM PC/PS LUVs ([¹⁴C]PC-OA-labeled) in 20 mM MOPS-5 mM β ME (pH 7.4) and incubated for 16 h at room temperature. Lane 8 shows a positive-control reaction using purified PLA₂ (0.07 mU μl^{-1}) from snake venom (*Naja mossaibica mossaibica*). Lanes 9 and 10 show the resolution of radiolabeled phospholipid and free fatty acid standards, respectively. Abbreviations to the right of the image are as follows: FFA, free fatty acid; PC, phosphatidylcholine; LPC, lysophosphatidylcholine. Each lane is labeled at the bottom to denote the proteins added to the assay and labeled at the top to show the amount of FFA released, expressed as a percentage of the total counts in the entire lane by densitometry. (C) The N-His₁₀-RP534 PLA₂ activity was measured kinetically by expressing the amount of FFA released as a percentage of the total counts in the entire lane by densitometry. N-His₁₀-RP534 (0.01 $\mu\text{g } \mu\text{l}^{-1}$) was incubated with 0.25 μM PC/PS LUVs ([¹⁴C]PC-OA labeled) (open squares), 0.25 μM PC/PS LUVs ([¹⁴C]PC-AA labeled) and bovine liver SOD1 (0.07 $\mu\text{g } \mu\text{l}^{-1}$) (filled diamonds), 0.25 μM PC/PS LUVs ([¹⁴C]PC-AA labeled) (open triangles), or 0.25 μM PC/PS LUVs ([¹⁴C]PC-OA labeled) (filled circles). Error bars represent standard errors of the average for 2 experiments for [¹⁴C]PC-AA-labeled LUVs and 3 experiments for [¹⁴C]PC-OA-labeled LUVs. (D) The stoichiometry of bovine liver SOD1-mediated activation of N-His₁₀-RP534 (at 0.01 $\mu\text{g } \mu\text{l}^{-1}$) was determined using 0.25 μM PC/PS LUVs ([¹⁴C]PC-OA labeled) with SOD1 added at 1:1, 5:1, 10:1, and 20:1 molar ratios. Reaction mixtures were incubated for 90 min (during the linear part of the reaction) and extracted, and FFA release was determined as above. Error bars represent standard errors of the averages from 3 experiments.

[^{14}C]*sn*-2 free fatty acid as the major reaction end product, and unexpectedly, this PLA₂ activity was observed in the absence of any eukaryotic cofactors (Fig. 1B, lane 2, and C, open squares and triangles). In the absence of eukaryotic cofactors, RP534 PLA₂ activity hydrolyzed ~80% of the total [^{14}C]-labeled free fatty acid over the 30-h time course when PC-OA was presented as a substrate (Fig. 1C, open squares) and ~35% of the total when PC-AA was the substrate (Fig. 1C, open triangles). We observed a dramatic increase in the reaction rate with both the PC-OA- and PC-AA-containing LUV substrates when RP534 PLA₂ activity was assayed in the presence of bovine liver SOD1 (Fig. 1C, compare filled diamonds and circles to open squares and triangles, respectively). Control reactions where the LUV substrates were incubated with SOD1 alone produced negligible amounts of free fatty acid (Fig. 1B, lane 1). Figure 1D shows that SOD1-mediated activation of RP534 PLA₂ activity was saturated at an ~5:1 molar ratio (SOD1/RP534). Intriguingly, we tested SOD1 from other sources, including bacteria, human erythrocyte, and bovine erythrocyte, but none was able to stimulate RP534 activity (data not shown). Our data, along with previous results for *P. aeruginosa* ExoU (35), suggest that it is not the dismutase enzymatic activity of SOD1 that is affecting RP534 activity *in vitro*.

We also purified and assayed two other recombinant N-His₁₀-RP534 proteins, each harboring a single alanine substitution at putative active site residue S100 or D264 (Fig. 1A, lanes 2 and 3). These sites were selected based on alignment with *P. aeruginosa* ExoU and other patatin homologues (36). Figure 1B shows that in either the absence or presence of SOD1, the RP534(S100A) (lanes 3 and 6) and RP534(D264A) (lanes 4 and 7) mutants displayed substantially diminished PLA₂ activities.

***In vitro* PLA₁ and lyso-PLA₂ activity of purified recombinant RP534 protein.** It is known that *P. aeruginosa* ExoU possesses PLA₂ and lyso-PLA activities (42). Our thin-layer chromatographic analysis of the PLA reaction products suggested that RP534 possesses PLA₁ and lyso-PLA₂ activities in addition to the predominant PLA₂ activity. Recall that the [^{14}C]PC substrates are labeled in the *sn*-2 position, and thus the activity of a PLA₁ will produce [^{14}C]LPC and the unlabeled *sn*-1 free fatty acyl group. Subsequent hydrolysis of the [^{14}C]LPC by a lyso-PLA₂ will release [^{14}C]free fatty acid. RP534 PLA reactions run in the absence of SOD1 showed a marked initial increase in the production of [^{14}C]LPC, which is indicative of PLA₁ activity. The amount of [^{14}C]LPC then decreased with time, which is indicative of lyso-PLA₂ activity (Fig. 2, open squares). Addition of SOD1 to the reaction appeared to ablate the initial increase in [^{14}C]LPC levels, but any existing [^{14}C]LPC was hydrolyzed by the lyso-PLA₂ activity (Fig. 2, filled circles). The drop in PLA₁ activity may have been due to the rapid depletion of substrate by the SOD1-stimulated PLA₂ activity. Alternatively, the addition of SOD1 may downregulate the PLA₁ activity. We are currently examining these interesting possibilities. While the overall trends shown in Fig. 2 are strongly suggestive that RP534 possesses PLA₁ and lyso-PLA₂ activities, we acknowledge that these data did not reach statistical significance when [^{14}C]LPC levels were compared to their starting time point, likely due in part to the observed variability between the three experiments.

FIG. 2. The N-His₁₀-RP534 PLA₁ and lyso-PLA₂ activities were measured by expressing the amount of LPC released as a percentage of the total counts in the entire lane by densitometry. N-His₁₀-RP534 (0.01 $\mu\text{g } \mu\text{l}^{-1}$) was incubated with 0.25 μM PC/PS LUVs ([^{14}C]PC-OA labeled) (open squares) or 0.25 μM PC/PS LUVs ([^{14}C]PC-OA labeled) and bovine liver SOD1 (0.07 $\mu\text{g } \mu\text{l}^{-1}$) (filled circles). The large panel shows the average, with data for each individual experiment shown to the right.

***In vitro* PLA₁ and PLA₂ activities of recombinant RP534 and PaExoU proteins.** Next, we used highly sensitive fluorogenic phospholipid substrates to examine the *in vitro* PLA₁ and PLA₂ activities of recombinant RP534 and *P. aeruginosa* ExoU (PaExoU; also purified as a recombinant protein expressed in *E. coli*; gel picture not shown). The PED-A1 substrate measures PLA₁ activity, and the PED6 substrate has been used previously to measure the PLA₂ activity of PaExoU (9, 35). Figure 3A shows that RP534-mediated hydrolysis of both the PED-A1 and PED6 substrates was dependent on the presence of SOD1 over the time course tested (compare open and filled symbols). These PED-A1 data lend further support to the conclusions drawn from Fig. 2 that RP534 possesses PLA₁ activity *in vitro*. The results presented in Fig. 3B did the following: (i) confirmed that PaExoU-mediated hydrolysis of the PED6 substrate occurred upon the addition of SOD1 (open and filled squares) and (ii) showed that PaExoU was unable to hydrolyze the PED-A1 substrate over the time course tested (open and filled circles). In fact, there was a modest quenching of the PED-A1 fluorescent signal in the presence of PaExoU and SOD1. PaExoU began to show a very modest amount of PED-A1 hydrolysis in the presence of SOD1 after 25 min of incubation, but this observation was not pursued further. As a control, we verified that incubation of either substrate with SOD1 alone showed no PLA activity (data not shown).

As further evidence that RP534 is a *bona fide* PLA enzyme, we tested its ability to hydrolyze the PED6 substrate in the presence of two known inhibitors of PLA₂ activity. Figure 3C shows inhibition of RP534 PLA₂ activity by nanomolar

FIG. 3. *In vitro* assay of *R. prowazekii* RP534 and *P. aeruginosa* ExoU PLA activities using fluorogenic phospholipid substrates. The N-His₁₀-RP534 (A) or N-His₁₀-PaExoU (B) PLA activities were measured using PED-A1 (PLA₁ activity) and PED6 (PLA₂ activity) as substrates. N-His₁₀-RP534 was used at a concentration of 0.02 μg μl⁻¹, and N-His₁₀-PaExoU was used at a concentration of 0.02 μg μl⁻¹ (these protein amounts are equivalent molar amounts). PLAs were incubated in 50 mM MOPS–50 mM NaCl (pH 7.4) with 29.7 μM

amounts of the potent, irreversible inhibitor MAFP (Fig. 3C). The reversible inhibitor AACOCF₃ inhibited recombinant RP534 activity at 10 μM (data not shown).

Mechanism of SOD1-stimulated RP534 PLA activity. The fact that RP534-mediated hydrolysis of the fluorogenic phospholipid substrates was entirely SOD1 dependent over a short time course furnished us with a sensitive assay to examine the mechanism of SOD1-mediated activation. The RP534 interaction with the PED6 substrate was tested using the RP534(D264A) mutant as a potential inhibitor of wild-type RP534 PLA₂ activity. We first verified that the RP534(S100A) and RP534(D264A) mutants were unable to hydrolyze the PED6 substrate in the presence or absence of SOD1 (data not shown). For the inhibition experiments, the PED6 substrate was preincubated with increasing amounts of the RP534(D264A) mutant protein for 20 min, followed by the addition of wild-type RP534 and SOD1 to the reaction mixture. Figure 4A shows that the addition of increasing amounts of the RP534(D264A) protein to the reaction mixture resulted in a concomitant reduction in the rate of SOD1-dependent PED6 hydrolysis by wild-type RP534. These data raised the interesting question of whether the RP534(D264A) mutant was sequestering the substrate or specifically forming an inactive complex with the wild-type RP534 protein. Figure 4B shows that preincubation of the PED6 substrate with the RP534(D264A) mutant protein also inhibited the activity of the snake venom PLA₂ protein, indicating that the RP534(D264A) mutant protein may be sequestering the PED6 substrate. As a control, we tested a non-PLA rickettsial protein previously characterized by our laboratory (18) to show that the observed RP534(D264A)-mediated inhibition of RP534 PLA₂ activity was specific (Fig. 4C). Together these data suggest a mechanism in which RP534 binds to its substrate and the rate of hydrolysis is stimulated upon the addition of SOD1.

The RP534 PLA is assayable in lysed cell extracts of *R. prowazekii*. Recombinant N-His₁₀-RP534 protein was used to prepare rabbit polyclonal antiserum to probe whether RP534 is expressed and active in purified *R. prowazekii*. Figure 5A shows that the RP534 protein was isolated from rickettsial lysed cell extracts by immunoprecipitation, and the identity of the protein was verified by mass spectrometry (MS). These data con-

PED6 (open squares), 29.7 μM PED6 and bovine liver SOD1 (0.2 μg μl⁻¹) (filled squares), 29.7 μM PED-A1 (open circles), or 29.7 μM PED-A1 and bovine liver SOD1 (0.2 μg μl⁻¹) (filled circles). The N-His₁₀-RP534 reaction mixtures contained 5 mM βME, and the N-His₁₀-PaExoU reaction mixtures contained no βME. Each data point is adjusted for background based on reactions incubated with substrate alone along the corresponding time course. Error bars represent standard errors of the averages from 3 experiments. (C) The effect of titrated amounts of the PLA₂ inhibitor MAFP on N-His₁₀-RP534 activity was tested using the PED6 substrate at a final concentration of 29.7 μM. N-His₁₀-RP534 (0.03 μg μl⁻¹) was incubated with MAFP (concentration range given in the figure) in the complete assay buffer with βME described for panel A for 10 min prior to the addition of SOD1 (0.2 μg μl⁻¹) to start the reaction. RP534 activity is expressed as the linear rate determined from a 5-min time course (average for 3 experiments is shown). The asterisk denotes samples in which the inhibition compared to results for the control was statistically significant by a one-tailed *t* test (*P* < 0.05).

FIG. 4. (A) The effect of titrated amounts of the N-His₁₀-RP534(D264A) mutant protein as a potential inhibitor of wild-type N-His₁₀-RP534 PLA activity was determined using the PED6 substrate at a final concentration of 2.97 μ M. Increasing concentrations of N-His₁₀-RP534(D264A) (0 μ g μ l⁻¹ [diamonds], 0.01 μ g μ l⁻¹ [squares], 0.02 μ g μ l⁻¹ [triangles], and 0.04 μ g μ l⁻¹ [circles]) were preincubated for 25 min with PED6 prior to the addition of wild-type N-His₁₀-RP534 (at 0.01 μ g μ l⁻¹) and SOD1 (0.2 μ g μ l⁻¹) to start the

assay. Error bars represent standard errors of the averages from 2 experiments. The asterisk denotes that all samples showed statistically significant inhibition compared to the uninhibited control by a one-tailed t test ($P < 0.05$). (B) The effect of titrated amounts of the N-His₁₀-RP534(D264A) mutant protein as a potential inhibitor of 0.07 mU μ l⁻¹ purified snake venom (*Naja mossaibica mossaibica*) PLA₂ activity was determined as described for panel A except that SOD1 was omitted from the assay. The asterisk denotes that all samples showed statistically significant inhibition compared to the uninhibited control by a one-tailed t test ($P < 0.05$). (C) The effect of titrated amounts of the N-His₆-GpsA rickettsial protein as a control for nonspecific inhibition of wild-type N-His₁₀-RP534 PLA activity was determined using the PED6 substrate at a final concentration of 2.97 μ M. Increasing concentrations of N-His₆-GpsA [0 μ g μ l⁻¹ (filled squares), 0.005 μ g μ l⁻¹ (open circles), 0.01 μ g μ l⁻¹ (open triangles), and 0.02 μ g μ l⁻¹ (open diamonds)] were preincubated for 25 min with PED6 prior to the addition of wild-type N-His₁₀-RP534 (at 0.01 μ g μ l⁻¹) and SOD1 (0.2 μ g μ l⁻¹) to start the assay. These N-His₆-GpsA protein concentrations were chosen to recapitulate the molar ratios of wild type to mutant RP534 protein used for panel A. Error bars represent standard errors of the averages from 3 experiments. Note that none of the lowest N-His₆-GpsA protein concentration data points showed statistically significant inhibition, only 50% of the middle N-His₆-GpsA protein concentration data points showed statistically significant inhibition, and all of the highest N-His₆-GpsA protein concentration data points showed statistically significant inhibition ($P < 0.05$ by a one-tailed t test).

firm two previous MS-based proteomic studies that detected RP534 in purified *R. prowazekii* (12, 43). The crude *R. prowazekii* lysed cell extracts displayed both SOD1-dependent (Fig. 5B, squares) and SOD1-independent (Fig. 5B, diamonds) PLA₂ activities using the PED6 substrate. The observation of SOD1-independent PLA₂ activity in *R. prowazekii* extracts using a fluorescence-based assay was not unexpected in light of a previous report (26). The SOD1-dependent increase in PLA₂ activity in the crude extracts (Fig. 5B) was statistically significant compared to results for the unstimulated samples, thus suggesting that the observed increase is due to activation of RP534. The large experimental error observed was likely due to the unavoidable variability inherent in preparations of *R. prowazekii* isolated from embryonated hen egg yolk sacs.

The RP534 protein was immunoaffinity purified from the *R. prowazekii* extracts using the anti-RP534 antibody and displayed the expected SOD1-dependent PLA₂ activity when assayed using the PED6 substrate (Fig. 5C). Interestingly, shorter incubation times between the anti-RP534 antibody and the *R. prowazekii* lysed cell extracts yielded protein with higher specific PLA₂ activity, whereas overnight incubations resulted in total inactivation of SOD1-stimulated PLA₂ activity (data not shown). Together, these data suggest that *R. prowazekii* synthesizes the RP534 protein and that in lysed cell extracts the protein becomes inactive with time. This observation is extremely interesting considering that purified recombinant N-His₁₀-RP534 purified from *E. coli* does not lose activity over weeks of storage at 4°C. We are further studying this phenomenon.

DISCUSSION

The present study has shown that purified, recombinant RP534 possesses PLA₁, PLA₂, and lyso-PLA₂ activities and

FIG. 5. Immunoaffinity purification of the wild-type RP534 PLA protein from *R. prowazekii* lysed cell extracts. (A) Affinity-purified

that bovine liver SOD1 can stimulate RP534 activity *in vitro*. The evidence demonstrating that RP534 is a *bona fide* PLA protein includes the following: (i) the observation that the recombinant RP534 PLA activities were measurable in the absence of any eukaryotic cofactors (Fig. 1B and C), (ii) the deleterious effect of mutating putative active site residues S100 and D264 on RP534 PLA activity (Fig. 1B), and (iii) the inhibition of RP534 activity by the known PLA₂ inhibitors MAFP and AACOCF₃ (Fig. 3C and data not shown). The *R. prowazekii* ExoU homologue also displayed some distinguishing characteristics compared to what has been published for *P. aeruginosa* ExoU by several independent groups (14, 27, 28, 36, 37, 42, 44). First, our direct comparison of the two enzymes using fluorogenic phospholipid substrates demonstrated that RP534 possessed a distinguishing PLA₁ activity. Second, published studies have shown that measurement of PaExoU activity *in vitro* requires the addition of eukaryotic cofactors. Conversely, we were readily able to measure RP534 PLA activity in the absence of any eukaryotic cofactor(s). Perhaps a recombinant PaExoU protein does not remain stable and active over the extended 30-h time course that was required to observe the near-complete hydrolysis of PC-OA by RP534 in the absence of a eukaryotic cofactor(s). We observed that purified recombinant PaExoU lost activity after storage at 4°C over a shorter period of time than was the case for RP534, although this was not pursued further (data not shown). Interestingly, despite these differences, both PLAs show conservation of their activation by a common eukaryotic cofactor, indicating that mechanisms of activation of the ExoU family of PLAs may be evolutionarily conserved. In addition, the RP534 protein was shown to be expressed in *R. prowazekii* purified from embryonated hen egg yolk sacs and when purified by immunoprecipitation displayed the expected SOD1-stimulated PLA₂ activity when assayed with the sensitive PED6 fluorogenic substrate (Fig. 5). The fact that the RP534 PLA reaction rates were slow compared to those for the snake venom PLA₂ controls seems to fit with the obligate intracytoplasmic lifestyle of *R. prowazekii*, which is typified by slow growth and filling of the host cell cytosol with pathogens prior to lysis and release.

anti-RP534 antibody was used for immunoprecipitation of RP534 from *R. prowazekii* lysed cell extracts. The elution fraction was resolved by SDS-PAGE, and protein was visualized by Coomassie staining (left panel) and immunoblotting with anti-RP534 antibody (right panel). The identity of RP534 was verified by mass spectrometry. MWM is the molecular size marker in kDa. (B) Total *R. prowazekii* lysed cell extracts were assayed for SOD1-activated PLA activity (indicative of RP534) using the PED6 substrate and assay buffer described in the legend to Fig. 3A. PLA assays contained 0.02 mg of rickettsial lysate alone (open diamonds) or 0.02 mg of rickettsial lysate with bovine liver SOD1 (0.2 μ g μ l⁻¹, filled squares). Error bars represent standard errors of the averages from 5 experiments. The asterisks denote samples in which the increase in SOD1-mediated stimulation of PED6 hydrolysis was statistically significant by a one-tailed *t* test ($P < 0.05$). (C) The immunoaffinity-purified RP534 protein was assayed for SOD1-activated PLA activity using the PED6 substrate and assay buffer described in the legend to Fig. 3A. Representative results from a single rickettsial preparation are shown using optimal IP conditions. Assays contained 20 μ l of the eluted RP534 protein alone (open diamonds) or 20 μ l of the eluted RP534 protein with bovine liver SOD1 (0.2 μ g μ l⁻¹, filled squares).

The RP534 PLA demonstrated notable differences in activity depending upon the substrate used in the assay. In the absence of SOD1, the RP534 PLA showed a higher rate of [14 C]PC-OA hydrolysis than with [14 C]PC-AA when these substrates were presented as PC/PS-mixed LUVs (Fig. 1C). Although the addition of SOD1 to the reaction resulted in similar rates of [14 C]PC-OA and [14 C]PC-AA hydrolysis, it was apparent that more of the [14 C]PC-OA substrate was consumed (Fig. 1C). Furthermore, the PED6 substrate, which is composed of a modified ethanolamine head group and a fluorophore in the *sn*-2 position, was hydrolyzed by RP534 only in the presence of SOD1 over a short time course (Fig. 3A). Together these data suggest that the fatty acyl group in the *sn*-2 position and the nature of the polar head group may influence RP534 substrate recognition prior to its activation by SOD1 or any other unknown eukaryotic cofactor(s).

The identification of ExoU homologues in the obligate intracellular pathogenic rickettsiae and the free-living, opportunistic pseudomonad raises interesting questions regarding the ancestral acquisition and evolution of ExoU function in these phylogenetically unrelated organisms. It is postulated that the *P. aeruginosa* *exoU* gene was acquired via horizontal transfer, as indicated by its location in the genome and G/C content different from that of the core genome (23). Conversely, the *R. prowazekii* RP534 gene displays a high A/T content that is a hallmark of the *R. prowazekii* genome (6), indicating that it was likely acquired by an early ancestor. The homology between the *P. aeruginosa* ExoU and *R. prowazekii* RP534 PLAs is limited to the N terminus, which contains the putative PLA catalytic domains. Conversely, their C termini are large domains constituting half of the ~70-kDa protein sequence but share no identifiable homology. Divergence of their C-terminal domains may have been influenced by the different mechanisms by which these pathogens secrete the protein into the host cell milieu. *P. aeruginosa* secretes ExoU via the type three secretion system, which is not present in any *Rickettsia* species genome sequenced to date. The RP534 homologue of *Rickettsia typhi* has been shown to be released to the host cell cytosol (29), but the underlying mechanism(s) of release remains unknown. Other exciting possibilities inherent in the divergent C-terminal sequences of the RP534 and ExoU PLAs include possible explanations for our observed differences in PLA₁ activity and SOD1-mediated activation, possible differences in subcellular targeting/trafficking within the host cell, or differences in their ultimate effect on host cell physiology. Resolution of these questions may provide critical insight into our understanding of rickettsial obligate intracytoplasmic parasitism.

While the pathogenic role of the *R. prowazekii* ExoU homologue remains unknown, the ExoU homologue of *R. typhi* (RT0522) has recently been characterized and shown to be present in the host cytosol during infection (29). Similar to *P. aeruginosa* ExoU, secretion of the rickettsial ExoU PLA homologues would facilitate a perhaps necessary encounter with a eukaryotic activator, such as SOD1, to mediate the desired effects on the host cell. Interestingly, purified recombinant *R. typhi* RT0522 PLA activity was reported to be dependent upon the presence of eukaryotic cell extracts to stimulate activity *in vitro* and displayed weaker activity than purified *P. aeruginosa* ExoU when activity was assayed using a fluorogenic phospho-

lipid substrate (29). In our assay system using fluorogenic substrates, the purified *R. prowazekii* RP534 PLA had considerably greater PLA activity than purified recombinant *P. aeruginosa* ExoU (Fig. 3A and B) and that reported for *R. typhi* RT0522 (29). Considering that the *R. prowazekii* and *R. typhi* ExoU homologues share >90% identity, it will be important to resolve these apparent discrepancies by assaying *R. typhi* ExoU activity using the radiolabeled PC substrates employed in our study. Any similarities and/or differences might reflect differences in the virulence of these two rickettsial pathogens.

Analyses of all sequenced rickettsial genomes reveal an intriguing difference with respect to the status of ExoU homologues. Functional ExoU homologues are present in the typhus group rickettsiae, *R. prowazekii* (RP534 from this study) and *R. typhi* (RT0522 from reference 29), and rickettsial ExoU homologues also appear to be intact in the distant relatives *Rickettsia massiliae* and *Rickettsia bellii* (84% and 69% identity, respectively, with perfect conservation of putative active site residues, but their biochemical properties are unknown). Intriguingly, rickettsial ExoU homologues in all other sequenced rickettsial genomes, including the spotted fever group (e.g., *Rickettsia rickettsii* and *Rickettsia conorii*), are markedly degenerated/fragmented and may no longer be functional. These genomic analyses may shed light on previous studies implicating PLA₂ as critical to the interaction of both typhus group and spotted fever group rickettsiae with eukaryotic host cells (39, 46, 51, 54, 56). Homologues of RP602, the other rickettsial patatin-like PLA, have been identified in all sequenced rickettsial genomes to date. Interestingly, *Rickettsia felis* possesses two additional plasmid-borne gene copies (10). Thus, it is tempting to speculate that RP602 is a major player in rickettsia-host interactions and that the ExoU homologues have been maintained to play either a redundant or an evolved specialized role in the typhus group rickettsiae and possibly in *R. massiliae* and *R. bellii*. Alternatively, rickettsial ExoU homologues may be entirely dispensable and will eventually be lost from the core genome. In either case, one must be cautious in making such assertions until the biological role of RP602 homologues is fully established and the existing ExoU gene fragments from the spotted fever group rickettsiae are examined for any remaining activity or function. Our current focus is on investigating the role of RP534 PLA activity in *R. prowazekii* growth and pathogenesis.

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