Adenosine diphosphate (ADP) ribosylation is a reversible posttranslational modification involved in the regulation of numerous cellular processes. Prototype ADP ribosyltransferases (ARTs) from many pathogenic bacteria are known to function as toxins, while other bacterial ARTs have just recently emerged. Recent studies have shown that bacteria also possess enzymes that function as poly-ADP ribose (ADPr) glycohydrolases (PARGs), which reverse poly-ADP ribosylation. However, how bacteria manipulate host target proteins by coordinated reactions of ARTs and ADPr hydrolases (ARHs) remains elusive. The intracellular bacterial pathogen *Legionella pneumophila*, the causative agent of Legionnaires’ disease, transports a large array of effector proteins via the Dot/Icm type IV secretion system to host cells. The effector proteins, which mostly function as enzymes, modulate host cellular processes for the bacteria’s benefit. In this study, we identified a pair of *L. pneumophila* effector proteins, Lpg0080 and Lpg0081, which function as an ART and an ARH, respectively. The two proteins were shown to coordinate their enzymatic activities to conjugate ADPr to, and remove it from, a key arginine residue. The crystal structures of Lpg0081 and the Lpg0081:ADPr complex indicated that Lpg0081 is a macroD-type ARH with a noncanonical macrodomain, whose folding topology is strikingly distinct from that of the canonical macrodomain of which is ubiquitously found in eukaryotic PARGs and ARHs. Our results illustrate that *L. pneumophila* has acquired an effector pair that can coordinately manipulate mitochondrial activity via reversible chemical modification of ANTs.

**Significance**

Mitochondria are organelles of the central metabolism that produce ATP and play fundamental roles in eukaryotic cell function and thereby become targets for pathogenic bacteria to manipulate. We found that the intracellular bacterial pathogen, *Legionella pneumophila*, targets mitochondrial ADP/ATP translocases (ANTs), the function of which is linked to the mitochondrial ATP synthesis. This is achieved by a pair of effector proteins, Lpg0080 and Lpg0081, which have opposing enzymatic activities as an ADP ribosyltransferase (ART) and an ADP ribosylhydrolase (ARH), respectively, coordinately regulating the chemical modification of ANTs upon infection. Our structural analyses indicate that Lpg0081 is an ARH with a noncanonical macrodomain, whose folding topology is distinct from that of the canonical macrodomain of known eukaryotic, archaeal, and bacterial proteins.
adenosine triphosphate (ATP) translocases (ANTs) and interferes which ADP ribosylates an arginine residue in mitochondrial ADP/}

L. pneumophila is the causative agent of Legionnaires’ disease, a severe form of pneumonia (20). Using the Dot/Icm type IV secretion system (T4SS), this bacterium transports more than 300 effector proteins into host cells (21, 22). The extraordinary number of the L. pneumophila effector proteins has allowed systematic analyses of the functional relationships among them, leading to the identification of “metaeffectors” (23–26). A metaeffector designates an effector protein that can regulate the function of another effector protein (24). In addition to metaeffectors, bacterial effectors include enzymes that can reverse a chemical reaction catalyzed by other effectors. For instance, the de-AMPylation activity of SidD reverses DrtA (SidM)-mediated AMPylation of the host small GTPase Rab1 (27, 28). The unique mechanisms of deubiquitination found in L. pneumophila effector proteins are also examples of such effector functions; the noncanonical ubiquitination mediated by the SidE family proteins is reversed by the opposing catalytic activity of MvaC (29, 30), and ubiquitination mediated by the transglutaminase activity of Mvca (31). Regarding the emerging evidence of the reversible enzymatic modification of host proteins, we anticipated that L. pneumophila might have effector pairs that could modify host proteins with ADPPr in a reversible manner.

In this study, we identified an L. pneumophila ART, Lpg0080, which ADP ribosylates an arginine residue in mitochondrial ADP/adenosine triphosphate (ATP) translocases (ANTs) and interferes with mitochondrial respiration. We further identified another effector, Lpg0081, which can counteract the activity of Lpg0080 against ANTs. Structural and biochemical analyses revealed that Lpg0081 exhibits a unique ARH activity to remove mono-ADPPr moiety from the modified ANT, thereby reversing the Lpg0080-mediated down-regulation of mitochondrial activity.

Results

Lpg0080 Is a Putative ART. We conducted in silico analysis to identify L. pneumophila effector proteins that possess ART activities. An HHsearch (32) conducted against a PDB70 database revealed that three L. pneumophila proteins, namely, Lpg0080 (Ceg3), Lpg0181 (Lart1), and Lpg2523 (Lem26), are putative ARTs, as they can be aligned with bacterial toxins (Fig. 1A). Based on these alignments, we constructed a model structure of the plausible ART domain of Lpg0080, showing that the catalytically essential residues in the cholera toxin (CT) A1 subunit, CTA1, are in spatially equivalent positions in the model structure (Fig. 1B). The bacterial ARTs, CT, heat-labile enterotoxin (LT), and pertussis toxin (PT), are classified into the subclass of ARTs on the basis of the R-S-E triad motif or its variant in the catalytic domain (Fig. 1A). Lpg0080, Lpg0181, and Lpg2523 also have this triad and characteristically possess additional glutamic acid (thus a R-S-EXE motif), which determines the enzymatic specificity for arginine (33). We therefore assumed that Lpg0080, Lpg0181, and Lpg2523, are arginine-specific ARTs. In fact, Lpg0181 was recently reported to target a conserved arginine residue of NAD+-dependent glutamate dehydrogenase (GDH) enzymes that are present in fungi and protists (34).

To examine the ART activities of Lpg0080, Lpg0181, and Lpg2523 along with their potential targets in eukaryotic cells, we transiently expressed the GFP-tagged proteins in HEK293T-FcγRII cells and isolated ADP-ribosylated proteins from cell

![Fig. 1.](https://doi.org/10.1073/pnas.2122872119)

**A** Sequence alignment of Lpg0080 (Ceg3) with catalytic domains of bacterial ADP ribosetransferases (CT: cholera toxin from V. cholerae; LT: Heat labile enterotoxin from E. coli; PT: Pertussis toxin from B. pertussis; Lpg0181[Lart1] and Lpg2523[Lem26] from L. pneumophila). Red letters are expected catalytic residues. **B** Crystal structure of cholera toxin A1 subunit (CTA1) (PDB entry: 1XTC) and the model structure of Lpg0080 (Top). The model structure was built with the Phyre2 server program. The ribbon diagram was constructed with PyMOL software. The side chains of the expected catalytic residues are represented as green sticks. The regions represented in the structures are shown as orange- and blue-colored boxes for CTA1 and Lpg0080, respectively (Bottom). **C** The putative ARTs from L. pneumophila were expressed in HEK293T-FcγRII cells and ADP ribosylated proteins were isolated from the cell lysates using Af1521-conjugated beads. The samples were separated by SDS-PAGE, and the ∼30-kDa band was analyzed by LC-MS/MS.
lysates utilizing Sepharose beads conjugated with the Archaeoglobus fulgidus macrodomain protein AF1521 (35) (Fig. 1C). In cells producing Lpg0080, some ADP-ribosylated proteins were detected. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis revealed that the most prominent band (∼30 kDa) contained peptide fragments the sequences of which matched those of adenine nucleotide translocase 1 (ANT1)/SLC25A4, ANT2/SLC25A5, and ANT3/SLC25A6, which function as mitochondrial ADP/ATP carriers (36). We chose ANT2 as a model substrate, which produced the highest identification score, for further analyses.

Lpg0080 Can Modulate Mitochondrial ATP Synthesis by ADP Ribosylation of ANTs. By conducting a B. pertussis adenylate cyclase (CyaA) fusion assay, we confirmed that Lpg0080 is an effector protein that can be delivered to host cells via the Dot/Icm T4SS (SI Appendix, Fig. S1). When GFP-tagged Lpg0080 were ectopically expressed in human cells, a Lpg0080-mediated ADP ribosylation of ∼30 kDa proteins was detected from the Af1521-interacting proteins by probing with the ADPr-binding reagent (panADPr) (SI Appendix, Fig. S2). The coexpression of FLAG-ANT2 and GFP-Lpg0080 or its mutant GFP-Lpg0080(EE/DD) evidently showed that ADP ribosylation of ANT2 by Lpg0080 depends on the intact R-S-EXE motif, which is essential for catalytic activity (33) (Fig. 2A). The subcellular fractionation showed that ANT2 was present and modified by Lpg0080 exclusively in mitochondria (Fig. 2B). The transiently expressed Lpg0080 was not exclusively but significantly detected in the mitochondrial fraction, suggesting that the catalytic activity of Lpg0080 can be executed in mitochondria.

Next, we investigated the ANT residues modified by Lpg0080 using mass spectrometry of trypsin-digested ADP-ribosylated ANT2, but no ADP-ribosylated peptides were identified. In this analysis, the overall protein sequence coverage was over 80%, but several arginine residues were uncovered, including Arg140, Arg235, Arg236, and Arg237. We reasoned that the ADP-ribosylated ANT2 fragment could have escaped the mass spectrometry detection. The crystal structure of ANT in complex with the inhibitor, carboxyatractyloside (Protein Data Bank [PDB] entry: 1OKC) revealed that six transmembrane helices of ANT form a barrel with a cavity that is accessible from the matrix side of mitochondria (37). The conserved hexapeptide (RRRMMM signature), which is characteristic of mitochondrial carriers, is located at the edge of H5 facing the matrix (Fig. 2C) and is crucial for the nucleotide transport activity (37). As the RRRMMM signature contains uncovered Arg235, Arg236, and Arg237, we focused on these arginine residues and another uncovered arginine, Arg140. Among the individual mutation of these residues, only R236K failed to be ADP ribosylated by Lpg0080, showing that Arg236 is the...
target residue (Fig. 2D). We further confirmed that not only the arginine residue in ANT2, but also the corresponding ones in ANT1 and ANT3 are ADP ribosylated by Lpg0080 (SI Appendix, Fig. S3). These results clearly demonstrate that Lpg0080 functions as an arginine-specific ART and targets Arg236 in ANTs. Consistently, direct evidence of Lpg0080-mediated mono-ADP ribosylation of Arg236 in ANTs was provided using mass spectrometry in a study published during the revision process of our manuscript (38).

We expected that the chemical modification of the functionally important residue of ANTs would negatively impact on their ADP/ATP transport activity, which is coupled to mitochondrial ATP synthesis. To address this possibility, we measured the ATP levels in HEK293T cells transiently expressing HA-tagged Lpg0080 or its catalytically inactive mutant, Lpg0080(EE/DD) (Fig. 2E). To prevent glycolysis-mediated generation of ATP, 6 h prior to the measurement, we exchanged the medium for a serum- and glucose-free one supplemented with galactose. In this condition, adding an F$_F$F$ATP$ synthase inhibitor oligomycin almost entirely eliminated cellular ATP, showing that mitochondria-producing ATP can be exclusively measured in this analysis. The ATP level significantly declined in the presence of HA-Lpg0080 relative to the vector control but not in the catalytic mutant. This result is consistent with the expectation that the catalytic activity of Lpg0080 can affect mitochondrial ADP/ATP transport by ANTs.

**Lpg0081 Can Reverse the Lpg0080-Mediated Regulation of Mitochondrial ATP Synthesis by Demodification of ANTs.** Lpg0081, which is the product of the gene adjacent to lpg0080 (Fig. 3A) and is a T4SS-substrate effector protein (SI Appendix, Fig. S1) (39), was reported to have the ability to antagonize the defect in yeast growth conferred by the ectopic expression of Lpg0080 in a systematic survey of the Legionella metaeffectors (26). This finding led us to examine the functional relationship between Lpg0080 and Lpg0081. Upon infection of HEK293T-FcγRII cells with the wild-type *L. pneumophila* strain (Lp01), ADP-ribosylated proteins corresponding to the molecular weight of ANTs were not detected in AF1521 pulled-down fractions from cell lysates (Fig. 3B, Left). By contrast, the ADP-ribosylated proteins of ~30 kDa were detected in AF1521 pulled-down fractions from cell lysates infected with the Δlpg0081 strain but not with the Δlpg0080Δlpg0081 strain (Fig. 3B, Left). We further confirmed that complementation with a plasmid encoding the wild-type Lpg0080 but not Lpg0080(EE/DD) recovered the ATP ribosylation of ANTs upon infection with the Δlpg0080Δlpg0081 strain (Fig. 3B, Right). These results suggest that Lpg0080-mediated ADP ribosylation of ANTs is normally suppressed by the presence of Lpg0081 in the infection condition.

We considered that the induction/suppression of ADP ribosylation of ANTs might be temporarily controlled in the infected cells. We therefore examined the kinetics of the ADP ribosylation of ANTs in bone marrow–derived macrophages (BMDMs) infected with the wild-type, the Δlpg0081, and the Δlpg0080Δlpg0081 strains (Fig. 3C). The ADP ribosylation of ANTs was readily detectable at 4 h and 7 h after infection with the Δlpg0081 strain, while it was not fully detected in the cells infected with the wild-type or the Δlpg0080Δlpg0081 strain (Fig. 3C, Top). The level of ANTs in the cells was unchanged over time (Fig. 3C, Bottom). This result suggests that the modification is normally suppressed to an undetectable level if Lpg0081 is present in cells. The immunoprecipitation analysis using HEK293T-FcγRII cells transiently expressing 3xFLAG-ANT2 defined ANT2 as the target of bacterially delivered Lpg0080 (Fig. 3D). The enhanced level of the target enabled us to detect its ADP ribosylation in the cells infected with the wild-type strain albeit at the lower levels than that with the Δlpg0081 strain (Fig. 3D). The similar kinetics of ADP ribosylation of ANT2 between the cells infected with the wild-type and with the Δlpg0081 strains suggests that the activity of Lpg0080 is induced at the later stages of infection regardless of the presence of Lpg0081, and that Lpg0081 down-regulates the basal level of Lpg0080-mediated ADP ribosylation of ANTs.

To examine the direct involvement of Lpg0081 in the Lpg0080-mediated ADP ribosylation of ANTs, GFP-Lpg0080 and GFP-Lpg0081 were coexpressed with 3xFLAG-ANT2 in the cells, and the level of ADP ribosylation of ANT2 was analyzed (Fig. 3E). When both GFP-Lpg0080 and GFP-Lpg0081 were expressed, the level of ADP ribosylation of ANT2 was significantly lower than in the absence of GFP-Lpg0081 (Fig. 3E, Bottom). The levels of neither GFP-Lpg0080 nor those of 3xFLAG-ANT2 were altered by the presence of GFP-Lpg0081, excluding the possibility that Lpg0081 can affect stability of Lpg0080 or ANTs, which could lead to the apparent reduction of the level of ADP ribosylated ANTs (Fig. 3E, Top and Middle).

We expected that the presence of Lpg0081 in the cells can recover the ATP level that declined by the activity of Lpg0080 (Fig. 2D). We therefore conducted cotransfection of HEK293T cells with plasmids expressing HA-Lpg0080 and HA-Lpg0081 (Fig. 3F). The cellular ATP level reduced by the expression of HA-Lpg0080 was restored by the coexpression of HA-Lpg0081 but not of its derivative mutant, HA-Lpg0081(E387A), which was revealed to be defective in function (see below). Together, these results provide the evidence that Lpg0081 can antagonize the catalytic activity of Lpg0080 and that it plausibly reverses the Lpg0080-mediated down-regulation of mitochondrial nucleotide transport.

It is possible that Lpg0081 can alter the enzymatic activity of Lpg0080 by functionally interacting with Lpg0080. Alternatively, Lpg0081 may act on the same target, ANTs, and reverse the Lpg0080-mediated chemical modification by its potential enzymatic activity. To address the latter possibility, we conducted an in vitro experiment utilizing recombinant Lpg0081 and 3xFLAG-ANT2 isolated from cells expressing Lpg0080 (Fig. 3G). The products of the in vitro reaction with various concentrations of purified Lpg0081 were probed with the pan-ADPr reagent. The level of ADP-ribosylated ANT2 decreased in a Lpg0081 dose–dependent manner (Fig. 3G, Top), while the levels of ANT2 in the samples were not affected (Fig. 3G, Bottom). These results demonstrate that preexisting ADP$\gamma$S bound to ANT2 can be removed conceivably by enzymatic activity of Lpg0081. We further conducted another in vitro experiment utilizing mitochondria isolated from cells expressing Lpg0080 (Fig. 3H). Treatment with recombinant Lpg0081, but not with a mutant Lpg0081, containing alanine substitution of key active-site residues (F283A/E387A) (see below) reduced the level of ADP-ribosylated ANTs existing in mitochondria. This result suggests that Lpg0081 can access and execute its enzymatic activity to ADP$\gamma$S, which is bound to ANTs localized in mitochondria. Human recombinant ARH1 and PARG did not remove ADP$\gamma$S from ANTs, indicating that the activity of Lpg0081 against ADP-ribosylated ANTs is specific (Fig. 3H).

**Lpg0080 Can Impair Mitochondrial Respiration and Lpg0081 Can Reverse the Effect.** To elucidate the biological roles of Lpg0080 and Lpg0081, we investigated whether these proteins influence mitochondrial activity by measuring oxygen consumption rate (OCR) using an extracellular flux analyzer (Fig. 4).
Consistently with the previous observations (53, 58), infection with *L. pneumophila* led to a reduction of cellular oxygen consumption in a T4SS-dependent manner (Fig. 4A and B). We therefore assessed whether the function of Lpg0080 is involved in the modulation of mitochondrial respiration. Infection of HEK293T-FcγRII cells with the Δlpg0080 strain showed slightly but significantly higher basal respiration than with the wild-type (Lp01), revealing that lack of Lpg0080 resulted in attenuation of the infection-induced impairment of oxygen consumption (Fig. 4A and B). However, we did not observe significant effects on the impairment by infection with the Δlpg0081 strain.

We further investigated whether transfection with the Lpg0080-expressing plasmid can affect oxygen consumption of the cells (Fig. 4E and F). Expression of Lpg0080 prominently reduced...
Lpg0080 impairs mitochondrial respiration and Lpg0081 can antagonize the effect. (A and C) Oxygen consumption rate (OCR) profiles of HEK293T-FcγRII cells infected for 4 h with the indicated *L. pneumophila* strains. Oligomycin (final 1.5 μM), carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP, final 2 μM) and rotenone + antimycin A (final 0.5 μM) were sequentially added to each well according to manufacturer's instruction (arrows). OCR (100%) is defined as OCR of Lp01 infected cells just prior to oligomycin addition. Bars designate SE of mean (SEM) of triplicates. (B and D) Bar plot of OCR responsible to basal mitochondrial respiration of cells calculated from the data of panels A and C, respectively. Statistical analysis (t test) was conducted from the triplicated experiments. (E) OCR profiles of HEK293T-FcγRII cells transfected with indicated plasmids for 24 h. Oligomycin, FCCP and rotenone + antimycin A were sequentially added as in panels A and C. OCR (100%) is defined as OCR of vector-transfected cells just prior to oligomycin addition. Bars designate s.e.m. of triplicates. (F) Bar plot of OCR responsible to basal mitochondrial respiration of cells calculated from the data in E. Statistical analysis (t test) was conducted from the triplicated experiments.

Fig. 4. Lpg0080 impairs mitochondrial respiration and Lpg0081 can antagonize the effect. (A and C) Oxygen consumption rate (OCR) profiles of HEK293T-FcγRII cells infected for 4 h with the indicated *L. pneumophila* strains. Oligomycin (final 1.5 μM), carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP, final 2 μM) and rotenone + antimycin A (final 0.5 μM) were sequentially added to each well according to manufacturer's instruction (arrows). OCR (100%) is defined as OCR of Lp01 infected cells just prior to oligomycin addition. Bars designate SE of mean (SEM) of triplicates. (B and D) Bar plot of OCR responsible to basal mitochondrial respiration of cells calculated from the data of panels A and C, respectively. Statistical analysis (t test) was conducted from the triplicated experiments. (E) OCR profiles of HEK293T-FcγRII cells transfected with indicated plasmids for 24 h. Oligomycin, FCCP and rotenone + antimycin A were sequentially added as in panels A and C. OCR (100%) is defined as OCR of vector-transfected cells just prior to oligomycin addition. Bars designate s.e.m. of triplicates. (F) Bar plot of OCR responsible to basal mitochondrial respiration of cells calculated from the data in E. Statistical analysis (t test) was conducted from the triplicated experiments.

Crystal Structure of Lpg0081. Remote homology detection with HHpred (40) indicated that Lpg0081 (composed of 443 residues) exhibits sequence homology with PARG from *D. radiodurans* over 84 residues, which corresponds to a subregion of a macrodomain. This observation is consistent with the observed activity of Lpg0081 in the regulation of ANT modification by ADPr, but it did not indicate whether Lpg0081 is an enzyme or an ADPr-binding protein. To obtain further information, we determined the crystal structure of full-length Lpg0081 (SI Appendix, Table S1). The asymmetric units of the crystal contained two Lpg0081 molecules, whose structures were virtually identical to each other. The structure is a mixed α/β/α-fold with a quite irregular central β-sheet, composed of nine β-strands and stacked by α-helices and loops on both sides (Fig. 5A). A structural similarity search performed on the Dali server (41) revealed that Lpg0081 is most similar to a *L. pneumophila* effector protein MavL (Lpg2526) (PDB entry: 6OMI) (42), with a Z score of 24.4. Other hits with lower but significant Z scores (8.9 < Z > 4.8) included PARG from *T. curvata* (PDB entry: 3SII) (14), an ADPr-binding domain of human ARDT8/PARP14 (PDB entry: 3VFQ) (43) and macroD1 (PDB entry: 6LH4), a macroD-type ARH (44). The structural alignment of Lpg0081 to these proteins showed that Lpg0081 has a macrodomain (Fig. 5B). Surprisingly, the folding topology of the macrodomain in Lpg0081 is different from that in the other three proteins. The canonical macrodomain is composed of six β-strands and five α-helices, and the connectivity between the secondary structural elements is β1–β2–α1–α2–β3–β4–α3–β5–α4–β6–α5. By contrast, this connectivity corresponds to β5–β6–α4–α5 and β1–β2–α1–β3–α2–β4–α3 in Lpg0081 (both in macrodomain numbering), revealing that Lpg0081 contains an atypical macrodomain (Fig. 5B). This topological difference does not allow a normal sequence alignment. Instead, we performed sequence alignment in a piecewise manner: β5–β6–α4–α5 and β1–β2–α1–β3–α2–β4–α3 in the Lpg0081 macrodomain were aligned to β1–β2–α1–α2 and β3–β4–α3–β5–α4–β6–α5 in the canonical macrodomain, respectively (Fig. 5B). In addition to the topological differences, Lpg0081 contains an extra ~70-residue segment between β2 and α1 (macrodomain numbering), which can be considered as a domain insertion relative to the canonical macrodomains (Fig. 5B).

Using the structure-based multiple sequence alignment (Fig. 5C), we examined whether or not the catalytically important residues in PARGs or macroD-type ARHs are conserved in Lpg0081. The PARG signature sequence (GGG–X6–GEE) contains two consecutive glutamate residues that are found in both mammalian and bacterial PARGs and were identified as cellular OCR, while coexpression of Lpg0080 and Lpg0081 completely recovered it. These results support our hypothesis that ANT modification mediated by the ART activity of Lpg0080 impairs mitochondrial respiration, and Lpg0081 can antagonize the effect.
Fig. 5. Crystal structure of Lpg0081 reveals a noncanonical macrodomain. (A) Ribbon representation and folding topology. The macrodomain is highlighted in magenta, and the insertion domain in the macrodomain is in gold. The secondary structural elements are labeled in their order of appearance from the N terminus. (B) Topologies of canonical macrodomains and the macrodomain in Lpg0081. The diagram below shows that the first part of the canonical macrodomains (β1, β2, α1, α2) corresponds to the last part of the Lpg0081 macrodomain (β5, β6, α4, α5). The secondary structural assignment and topology diagrams were extracted using the PDBSUM server (http://www.ebi.ac.uk/thornton-srv/databases/pdbsum) (70). The canonical macrodomains are Tc PARG: T. curtiva PARG, Hs ARTD8 m2: macrodomain 2 in human ARTD8, Hs MacroD1: human MacroD1. (C) Structure-based multiple sequence alignment. The macrodomain in Lpg0081 was aligned separately with PARGs and with MacroD-type ARHs. The abbreviations are Tc: T. curtiva, Dr: D. radiodurans, Tt: T. thermophila, Hs: Homo sapiens, Oi: Oceanobacillus iheyensis, Tb: T. brucei, S2: SARS-CoV-2. The conserved sequence motifs are indicated by the rectangular boxes and labeled. The arrow indicates Y134 of Lpg0081, which interacts with bound ADPr. The secondary structural elements of the macrodomain in Lpg0081 are shown at the top of the alignment.
crucial residues for the catalytic activity (14, 45). The first Glu binds and orients the distal ribose (referred to as ribose 00 ), and the second Glu is known as the catalytic residue activating water molecules in the mechanism of hydrolysis (14). The mutation of either of these glutamate residues rendered T. curvata PARG inactive (14). A sequence (and a structural) comparison shows that Lpg0081 does not have this PARG signature sequence, including the consecutive Glu-Glu sequence (Fig. 5 C). Lacking this critical Glu-Glu motif, Lpg0081 is unlikely to be a PARG.

Most macroD-type ARHs, including human macroD1 and macroD2, generally have four conserved signature sequences: NAAN, Gly-Gly-Gly, DxxxH, and G[V/I/A][F/Y]G motifs (17, 46) (Fig. 5 Q). The first three motifs play an important role in positioning ribose 00 at an active site cleft by forming hydrogen bonds with the hydroxyl groups of the ribose ring via their side chains or backbone amides (46). The Asp and His residues of the DxxxH motif interact with each other to form a dyad, which has been proposed to serve as an electrophile interacting with the 2'-OH group of the ribose 00 ring (46, 47). The aromatic residue of the G[V/I/A][F/Y]G motif also plays a key role in positioning the ribose 00 ring (44). It is generally agreed that precise positioning of ribose 00 in a tight coordination network surrounding it is crucial for the catalytic reaction (18, 44, 46–48), where the hydrolytic water molecule is activated by the

Fig. 6. Crystal structure of Lpg0081:ADPr and mutagenetic analyses of Lpg0081. (A) ADPr binding to Lpg0081. (Left) 2Fo_Fc electron densities (contoured at 1.2 σ) are shown for ADPr and the putative catalytic water molecule (Wat). (Middle and Right) Comparison of ADPr binding between Lpg0081 and human MacroD2 (PDB entry: 4IQY). The key ADPr-interacting residues are shown as sticks and labeled. The dotted lines indicate the hydrogen bonds. The Ca traces of the conserved sequence motifs (NAAN, GGG, DxxH, G[V/I/F][Y/F]G) in MacroD2 are shown in green. The Y134, shown in cyan, does not have a corresponding residue in MacroD2. (B) Overall structure of the Lpg0081:ADPr complex in surface rendering, showing that C1' of ribose 00 is not buried. (C) Mutational effects on the ADP ribosylation of ANT2. The residues shown in (A) were individually substituted with alanine. The L. pneumophila Δlpg0081 strain was complemented with a vector expressing the wild-type or mutant Lpg0081. For controls, both the Δlpg0081 and Δlpg0080Δlpg0081 strains were complemented with an empty vector. HEK293T-FcRII cells were infected with the transformed L. pneumophila Δlpg0081 strains for 7 h at a MOI of 10. Bacterial expression of the Myc-tagged Lpg0081 derivatives were detected with anti-Myc antibody (Left, Top). The cell lysate was probed with panADPr reagent (left, middle) and the same blot was reprobed with anti-ANT2 antibody (Left, Bottom). The Δlpg0080Δlpg0081 strain harboring the empty vector is a negative control lacking the ADP ribosylation of ANT2. Representative data from the individually triplicated experiments are shown. Statistical analysis was conducted from the three individual experiments (Right). The data are represented as relative intensities of ADP ribosylated ANT2 against ANT2, which were normalized to the value of Δlpg0081 with the vector (gray bar).
pyrophosphate group of the bound substrate rather than by protein residue(s) (44, 48). The multiple sequence alignment shows that Lpg0081 apparently contains none of the four conserved sequence motifs (Fig. 5C), obscuring whether Lpg0081 is a macroD-type ARH.

**Lpg0081 Is a Noncanonical MacroD-Type ARH.** To clarify this issue, we determined the crystal structure of Lpg0081 bound to ADPr at a resolution of 2.7 Å (SI Appendix, Table S1). Lpg0081 undergoes considerable conformational changes to bind ADPr through extensive hydrophilic and hydrophobic interactions. The structure of the complex indicates that the conserved (or mostly conserved) interactions seen between ADPr and macroD-type ARHs are also observed for Lpg0081. Surprisingly, the single residues of Lpg0081 play the same roles of the NAAN and DxxH motifs of the canonical macroD-type ARHs. While the NAAN motif forms a loop and is juxtaposed to ribose, with the second Asn hydrogen bonds with the 2'-OH (and the 3'-OH) of the ribose ring (44, 49), Asn379 in Lpg0081 is not a part of such a loop, but it also forms hydrogen bonds with the 2'-OH and 3'-OH of ribose (Fig. 6A). Similarly, while the Asp-His dyad in the DxxH motif interacts with the 2'-OH of ribose via the Asp residue, Glu387 alone interacts with the 2'-OH of ribose in Lpg0081 (Fig. 6A). Likewise, Phe283 of Lpg0081 is closely juxtaposed to the C4'-C5' bond of ribose, similarly to the way that the Phe or Tyr residue of the G[V/I/A][F/Y]G motif does in other macroD-type ARHs (Fig. 6A). The other part of bound ADPr has extensive interactions with Lpg0081 or with water molecules, as has been observed in other macroD-type ARHs. Strikingly, a water molecule coordinated by the beta phosphate, Glu387 -NH, Pro370 C = O and the ribose' O is found in the Lpg0081:ADPr structure, which corresponds to a water molecule with the same hydrogen bonds at the same spatial position in the structure of human macroD2 (PDB entry: 4IQY) (18). It was proposed that the water molecule at this position attacked C1' of ribose via the course of the hydrolytic reaction (44), convincingly indicating that Lpg0081 is a macroD-type ARH with a noncanonical macrodomain. In support, the C1' atom of ribose is exposed to the outside of the protein (Fig. 6B), which is a requirement for binding to ADPr covalently linked to amino acid residue of the substrate protein via this atom. Another unique feature of ADPr binding is the contact interaction of Tyr134 with the adenine ring of the bound ADPr, as if it positioned this moiety at the active site. The loop containing Tyr134 is entirely missing in the canonical macroD-type ARHs (Fig. 6A), which is a requirement for binding to ADPr. The catalytic activity of Lpg0081, as described below.

To clarify the importance of these ADPr-interacting residues, we individually substituted them for alanine and conducted an infection experiment (Fig. 6C). The Δ_lpg0081 strain complemented by the plasmid-derived wild-type Lpg0081 reduced the level of ADP ribosylation of ANT s, while the strain complemented with each of the mutants, including Lpg0081 (Y134A), significantly lost the ability to reduce the level except Lpg0081(F324A) whose mutated residue positions far from bound ADPr (Fig. 6A). This result indicates that the residues positioned close to ADPr and to the putative water molecule are crucial for the ARH activity.

**Discussion**

Intracellular bacteria, including *L. pneumophila*, have evolved mechanisms to target cellular organelles for the establishment of their replicative niches. Early studies using electron microscopy showed that *L. pneumophila* associates with mitochondria during the process of the *Legionella*-containing vacuole formation (50, 51). Recent studies suggest that *L. pneumophila* can regulate mitochondrial functions using effector proteins in various ways. *Legionella*-encoded mitochondrial carrier protein LncP was shown to be targeted to the mitochondrial inner membrane of macrophages (52). Substrate transport analyses by reconstituting LncP into liposomes revealed its catalytic activity as an ATP transporter (52), although LncP lacks the RRRM domain signature. MitF can modulate mitochondrial dynamics via the recruitment of the host fusion protein DNM1L (53). LegS2, a sphingosine-1 phosphate lyase, regulates sphingolipid metabolism in mitochondria (54, 55). It has been shown that LegS2 has a role in maintaining mitochondrial morphology, because infection with a *L. pneumophila* ΔlegS2 strain-induced elongation of mitochondria, which is reminiscent of mitochondrial fusion (56), linked to an increase in cellular ATP during starvation (57). Interestingly, it has been proposed that LegS2 is partially involved in *L. pneumophila*-induced reversing of the F$_{1}$F$_{0}$-ATPase activity by conserving the mitochondrial membrane potential (58).

In this study, we identified two *L. pneumophila* effectors that can regulate mitochondrial function mediated by their enzymatic activities as an ART and an ARH. Lpg0081 ARH is a plausible partner for Lpg0080 ART in modulating the ADP ribosylation of ANTs in a reversible manner. We demonstrated that the chemical modification of transporters by Lpg0080 can impair mitochondrial respiration, and demodification mediated by Lpg0081 can suppress the impairment. Homologs of Lpg0080 and Lpg0081 are found in many but not all *Legionella* species (SI Appendix, Table S2), suggesting that *Legionella* have broadly acquired the mechanism of reversible ADP ribosylation by paired effectors via interaction with various host organisms.

In the HHpred and the Dali searches, Lpg0081 is most homologous to MavL. Just recently, the crystal structure of the apo form of MavL and ensuing analyses have been reported (42), showing that this protein interacts with the mammalian ubiquitin-conjugating enzyme UBE2Q1 and suggesting that it is a reader or eraser of mono-ADP ribosylated substrates. Remarkably, a structural alignment of Lpg0081 and MavL reveals that the folding topology of the macrodomain is the same between the two proteins, although the insertion domain in Lpg0081 is absent in MavL (SI Appendix, Fig. S4). Moreover, the key ADPr-interacting residues of Lpg0081 are identically or similarly conserved in MavL except for Asp351, and these residues occupy spatially similar positions (SI Appendix, Fig. S4). These observations suggest that MavL might also be a hydrolytic enzyme that cleaves an as-yet-unknown mono-ADP ribosylated substrate. The gene encoding MavL, lpg2526, locates in the vicinity of lpg2523, suggesting a possibility that Lpg2523/Lem26 and MavL (plausible ART and ARH, respectively) coordinately function to reversibly modify an unknown substrate(s), similar to Lpg0080 and Lpg0081. It is likely that Lpg0081 and MavL have divergently evolved from a common ancestor to recognize specific ADP-ribosylated substrate proteins, and these two noncanonical macrodomains and classic macrodomains represent an example of convergent evolution to acquire the same hydrolytic activity.

In the normal infection condition, ADP ribosylation of ANTs was readily detected only in the absence of Lpg0081. This suggests that Lpg0081 plays a basal role in suppressing the Lpg0080-induced down-regulation of mitochondrial activity, as mitochondrial damage might be a negative impact on bacterial
survival. We, however, observe that neither the Δlpg0081 nor the Δlpg0080Δlpg0081 strain has an apparent effect on the growth of L. pneumophila in BMDMs, even in the presence of 2-deoxyglucose (2-DG), an inhibitor of glycolysis (SI Appendix, Fig. S5). The 2-DG treatment consistently reduced growth of the wild-type L. pneumophila with the previous observation showing a requirement of host cellular glycolysis in optimal intracellular growth (53). The regulation of mitochondrial activity might be beneficial for L. pneumophila in certain specific circumstances.

The infection of host cells with intracellular bacteria, including L. pneumophila, can cause metabolic reprogramming, shifting to glycolysis-mediated energy synthesis from mitochondrial respiration (59). In the case of L. pneumophila infection, this shift is biphasic and includes both T4SS-independent and -dependent processes (53). Lpg0080 and Lpg0081 may coordinately modulate the mitochondrial function in specific cellular conditions. L. pneumophila may have a number of unidentified effector proteins that can target mitochondria or even ANTs. Investigating how these effectors can function in coordination and temporarily regulate mitochondrial activity together with Lpg0080 and Lpg0081 for bacterial benefit may be a goal for future work.

Materials and Methods

Bacterial Strains and Culture. The L. pneumophila and Escherichia coli strains used in this study are listed in SI Appendix, Table S3. Deletion strains were constructed by allelic exchange, as described previously (60). The luciferase-expressing strains were constructed as described previously (61). The L. pneumophila strains were grown at 37 °C in liquid N(2-acetamido)-2-aminothanesulfonic acid (ACES [7365-82-4; Sigma])-buffered yeast extract (AYE) media was harvested from a 2-d heavy patch grown on CYE plates with or without Japan SLC) as described previously (62). Transfection was performed using Lipofectamine 2000 (Invitrogen) as described in Fig. S6. The level of ATP in cells was supplemented with 5 mM galactose (Sigma) or 5.6 mM glucose (Nacalai), as well as 15 strokes of a 26-gauge needle. Equivalent amounts of postnuclear supernatant (PNS) and cytoplasmic and mitochondrial fractions were analyzed with immunoblotting.

Mitochondria Isolation. Mitochondria were isolated from HEK293T-FcYRII cells using Mitochondria Isolation Kit for Culture Cells (Thermo Fisher) with a detergent-free homogenization method (option B in the manufacturer's instructions). Instead of using a dounce homogenizer, cells were ruptured by 15 strokes of a 26-gauge needle. Equivalent amounts of postnuclear supernatant (PNS) and cytoplasmic and mitochondrial fractions were analyzed with immunoblotting.

Cellular ATP Measurement. HEK293T cells were transfected for 15 h, and the media were replaced with prewarmed glucose-depleted medium (WAKO) supplemented with 5 mM galactose (Sigma) or 5.6 mM glucose (Nacalai), as well as with 15 mM Hepes and 1 mM sodium pyruvate. The level of ATP in cells was measured using the Mitochondrial ToxGlo Assay kit (Promega).

Extracellular Flux Analysis. HEK293T-FcYRII cells were transfected or infected as described in SI Appendix. OCRs were measured using the extracellular flux analyzer Xfp (Agilent) and Seahorse Xfp Cell Mito Stress Test kit (Agilent).

CyAA Translocation Assay. The translocation of CyAA-fused proteins to cells was monitored as described previously (69) with minor modifications.

Intracellular Growth Assay. A luminescent L. pneumophila growth assay was conducted as described previously (63).

Data Quantification and Statistical Analysis. Student's t test was carried out with data from at least three independent experiments. Please see SI Appendix for a more detailed description of the materials and methods. All animal experiments were performed according to institutional guidelines. Animal experiments to prepare mouse BMDMs were approved by the animal care and use committee of Gifu University, Japan (Approval No. 30-19).

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