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Biochemical characterization of a unique cytokinin and nucleotide phosphoribohydrolase Lonely Guy protein from *Dictyostelium discoideum*

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A B S T R A C T

Lonely guy (LOG) proteins are phosphoribohydrolases (PRHs) that are key cytokinin (CK)-activating enzymes in plant and non-plant CK-producing organisms. During CK biosynthesis, LOGs catalyze the conversion of precursor CK-nucleotides (CK-NTs) to biologically active free base forms. LOG/PRH activity has been detected in bacteria, archaea, algae, and fungi. However, in these organisms, the LOG/PRH activity for CK-NTs and non-CK-NTs (e.g., adenine-NTs) has not been assessed simultaneously, which leaves limited knowledge about the substrate specificity of LOGs. Thus, we performed bioinformatic analyses and a biochemical characterization of a LOG ortholog from *Dictyostelium discoideum*, a soil-dwelling amoeba, which produces CKs during unicellular growth and multicellular development. We show that *Dd*Log exhibits LOG/PRH activity on two CK-NTs, N^6-isopentenyladenosine-5′-monophosphate (iPMP) and N^6-benzyladenosine-5′-monophosphate (BAMP), and on adenosine 5′-monophosphate (AMP) but not on 3′, 5′-cyclic adenosine-monophosphate (cAMP). Additionally, there were higher turnover rates for CK-NTs over AMP. Together, these findings confirm that *Dd*Log acts as a CK-activating enzyme; however, in contrast to plant LOGs, it maintains a wider specificity for other substrates (e.g., AMP) reflecting it has maintained its original, non-CK related role even after diversifying into a CK-activating enzyme.

1. Introduction

Cytokinins (CKs) comprise a major class of plant hormones that are best known for their roles in plant growth and development [1,2]. Naturally occurring CKs are adenine derivatives with either isoprenoid or aromatic side chains added to the N^6 position. The majority of what is known about CKs comes from plants. However, the repertoire of CKs continues to be expanded as there is an increasing number of non-plant and plant-associated organisms that produce CKs and contain conserved CK biosynthesis enzymes–e.g., isopentenyltransferase (IPT) and Lonely Guy (LOG) [3,4]. IPTs initiate CK biosynthesis through the addition of an isoprenoid side chain to the N^6 position of AMP/ADP/ATP to form a presumably inactive CK nucleotide [5,6]. The CK nucleotides can be converted into their biologically active free base forms via dephosphoribosylation in a one-step reaction catalyzed by the CK-activating LOG enzymes [7,8].

The first LOG enzyme was discovered in rice, *Oryza sativa*, and was named lonely guy after its loss-of-function phenotype which left the mutant with only one stamen and no pistil [7]. Functional characterization of LOGs in *Oryza sativa* and *Arabidopsis thaliana* revealed that these proteins have phosphoribohydrolase activity with CK nucleoside 5′-monophosphates, but not their di- or tri-phosphate derivatives, nor do they react with AMP [7,8]. As such, LOG proteins that are selective towards CK-NTs are referred to as classical LOGs. *LOG* genes belong to a superfamily (IPR031100) containing a highly conserved $PGG_xGT_{xx}E$ motif, which encode phosphoribohydrolases (PRHs) that vary in their substrate specificity. An initial LOG family protein possessing a $PGG_xGT_{xx}E$ motif was inaccurately annotated as a lysine decarboxylase (LDC), which has resulted in widespread mis-annotation of putative LOG proteins as LDCs (Pfam: PF03641) [9,10]. Recent studies in bacteria, archaea, algae, and fungi have shown that LOG orthologs, initially annotated as LDCs, all have LOG/PRH activity, not LDC activity [11–17]. The mis-annotation within databases is being corrected, and there are now two different classifications for the LOG protein family on InterPro: LOG proteins selective towards either purine/pyrimidine riboside 5′-monophosphates (NTs) (IPR031100) and LOG proteins selective towards cytokinin riboside 5′-monophosphates (CK-NTs) (IPR005269).

The soil-dwelling amoeba, *Dictyostelium discoideum*, is a well-established eukaryotic model organism for studying a wide range of cellular and developmental processes [18,19]. As a model organism, *D. discoideum* is unique in that researchers can study processes during single and multicellular life cycle stages. *D. discoideum* belongs to the

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Dictyostelia class within the Amoebozoa phylum, which evolved shortly after the line leading from plants to animals, but before fungi [20]. *D. discoideum* produces CKs across all life cycle stages and contains conserved genes for CK biosynthesis and metabolism that are largely understudied [21,22]. Intriguingly, it is among a select group of non-plant organisms that produce CKs [22,23]. Even more rare is its production of a novel CK, discadenine, that to date, has only been documented among the later diverged group 4 species of dictyostelids [22,24].

In this study, we employed the model organism, *D. discoideum*, to study CK metabolism and enhance our understanding of CK-mediated signaling beyond plants. First, we performed a BLASTp search and identified a putative LOG ortholog in *D. discoideum* (uncharacterized protein DDB0305758, hereafter referred to as *Dd*Log). Using multiple CK-NTs and non-CK-NTs as substrates, we show that *Dd*Log has LOG activity. In addition, we characterize the *in vitro* features of the enzyme.

2. Materials & methods

2.1. Bioinformatic analyses

Sequence alignments were performed using the BLASTp server on dictyBase. The predicted monomeric 3D structure was determined using Alphafold, and the proposed dimeric structure was generated using the SWISS-Model server and PDB 5.its.1.A as a template [25,26]. Both 3D structures were visualized using Mol* software (RCSB PDB) [27]. Multiple sequence alignment was conducted using MUSCLE software [28] in Lasergene MegAlignPro v. 17.4 (DNASTAR, Madison, Wisconsin, USA).

2.2. Cloning, expression, and purification of DdLog

The *Dd*Log gene sequence was codon optimized and synthesized at TWIST Biosciences with adapters for expression in *Escherichia coli* (San Francisco, California, USA; Supplementary Table 1A). Phusion high-fidelity polymerase (Fisher Scientific Company, Ottawa, Ontario, Canada) was used for all PCR amplifications. *Dd*Log was cloned into the *E. coli* expression vector, pOPINF, using the In-Fusion HD cloning kit (primers in Supplementary Table 1B; TakaraBio, San Jose, California, USA) [29]. 2xYT medium supplemented with ampicillin (100 μg/mL) and chloramphenicol (50 μg/mL) was used for expression. *E. coli* BL21-Codon Plus (DE3)-RIL (Fisher Scientific Company, Ottawa, Ontario, Canada) were grown to an OD_{600nm} of 0.6–0.8 and induced with IPTG (1 mM). The cells were subsequently grown for 4 h at room temperature prior to harvesting. Cells were lysed by sonication in binding buffer (50 mM Tris-HCl pH 8, 50 mM glycine, 500 mM NaCl, 5 % v/v glycerol, 20 mM imidazole) supplemented with EDTA-free protease inhibitor (Sigma Aldrich Canada, Oakville, Ontario, Canada), 50 μL DNase (New England Biolabs, Whitby, Ontario, Canada), and 0.2 mg/mL lysozyme (Sigma Aldrich Canada, Oakville, Ontario, Canada). Cellular debris was removed by centrifugation (5000 *g*, 50 min), and soluble proteins were purified with Ni-NTA agarose (Fisher Scientific Company, Ottawa, Ontario, Canada). Proteins were eluted stepwise using a 20–400 mM imidazole gradient in elution buffer (50 mM Tris-HCl pH 8, 50 mM glycine, 500 mM NaCl, 5 % v/v glycerol) and were buffer-exchanged into 38 mM Tris-HCl (pH 7.5). Protein concentrations were quantified using a Qubit 2.0 Fluorometer (Fisher Scientific Company, Ottawa, Ontario, Canada) and were separated by SDS-PAGE. The gel was stained with the Pierce™ Silver Stain kit (Fisher Scientific Company, Ottawa, Ontario, Canada) to assess purity and yield, and expression was further confirmed by western blotting (Supplementary Fig. 1).

2.3. Biochemical properties and phosphoribohydrolase assays

Optimal assay conditions including pH (3.0–9.0), temperature (5–60 °C), and incubation time (64-min period) were determined using 20 μM N^6-isopentenyladenosine-5′-monophosphate (iPMP). The incubation time was tested using three different enzyme concentrations (10 nM, 100 nM, and 500 nM) to select an optimal time within the linear range of product formation for the enzyme. *In vitro* assays contained 500 nM *Dd*Log and 20 μM substrate (N^6-isopentenyladenosine-5′-monophosphate, iPMP; N^6-benzyladenosine-5′-monophosphate, BAMP (both from Olchemim Ltd., Olomouc, Czech Republic); adenosine 5′-monophosphate, AMP; or 3′,5′-cyclic adenosine-monophosphate, cAMP (both from Sigma Aldrich Canada, Oakville, Ontario, Canada) in a total reaction volume of 40 μl (Tris-HCl buffer, pH 7.5, 38 mM). The reactions were screened in triplicate, incubated for 15 min at 25 °C, and quenched using two volumes of ice-cold methanol. The quenched reaction was centrifuged at 20,000 *g* for 10 min and transferred to a new microcentrifuge tube. Reactions containing no-enzyme or denatured enzyme were used as negative controls. The relative phosphoribohydrolase activity was calculated as follows: product peak area/(substrate peak area + product peak area) × 100.

2.4. Reaction product identification using HPLC-(ESI+)-HRMS/MS

Prior to processing, samples were spiked with 20 ng of isotopically labeled internal standards (IS) for compound identification or quantification through isotope dilution assay calculations (Supplementary Table 2). The reaction products were analyzed by high-performance liquid chromatography-positive electrospray ionization-high resolution tandem mass spectrometry (HPLC-(ESI+)-HRMS/MS) using a Dionex UltiMate 3000 HPLC (Thermo Scientific, San Jose, California, USA) coupled to a QExactive Orbitrap HRMS (Thermo Scientific, San Jose, California, USA). Reaction products were separated using either an Acclaim RSLC 120C18 column (AMP analytes; 2.2 μm, 3.0 × 100 mm; Fisher Scientific Company, Ottawa, Ontario, Canada) or a HALO C18 column (CK-NT analytes; 2.7 μm, 2.1 × 50 mm; Canadian Life Sciences, Peterborough, Ontario, Canada). For AMP, cAMP, and Ade detection, a 0.2 mL/min flow rate was used with a binary gradient of water (A) and acetonitrile (B), containing 0.08 % glacial acetic acid. The initial gradient (0 % B) was held for 1.25 min, linear gradient to 50 % B over 2.75 min, increased to 100 % B over 0.5 min and held at 100 % B for 2.5 min, before returning to initial conditions for 6 min of column re-equilibration (13.3 min total). For BAMP, BA, iPMP, separation and detection of products were assessed as previously described [30].

3. RESULTS and DISCUSSION

3.1. Identification of a Lonely Guy ortholog in Dictyostelium discoideum

BLASTp queries of the *D. discoideum* genome (http://dictybase.org) were conducted to identify putative LOG candidates based on amino acid similarity to previously characterized LOGs from bacteria, archaea, plants, and fungi. This analysis revealed a single putative LOG ortholog in *D. discoideum* with moderate sequence similarity (35–50 % identity) to other LOGs (Fig. 1A–D; Supplementary Table 3). The *D. discoideum* LOG (*Dd*Log) candidate is encoded by the uncharacterized gene, *DDB_G0281309*, which is comprised of two exons totalling 200 amino acids in length (Supplementary Table 1A). Bioinformatic analyses of *Dd*Log in Pfam revealed the presence of a lysine decarboxylase (LDC) domain owing to the highly conserved $PGG_xGT_{xx}E$ motif, while InterPro classifies it within the LOG family as a cytokinin riboside 5′-monophosphate phosphoribohydrolase LOG (InterPro ID: IRP005269). The LDC domain prediction is consistent with those found in other LOG proteins that were investigated and characterized as phosphoribohydrolases (PRHs) after showing no experimental LDC activity [9].

Structurally, the predicted *Dd*Log monomer exhibits a typical Rossmann α-β fold with seven parallel β-sheets and nine surrounding α-helices (Fig. 1A; Alphafold ID: AF-Q54UC5–F1) [25,26]. Using the SWISS homology modelling platform, *Dd*Log was searched against experimentally validated crystal structures to predict its *in vitro* conformation.

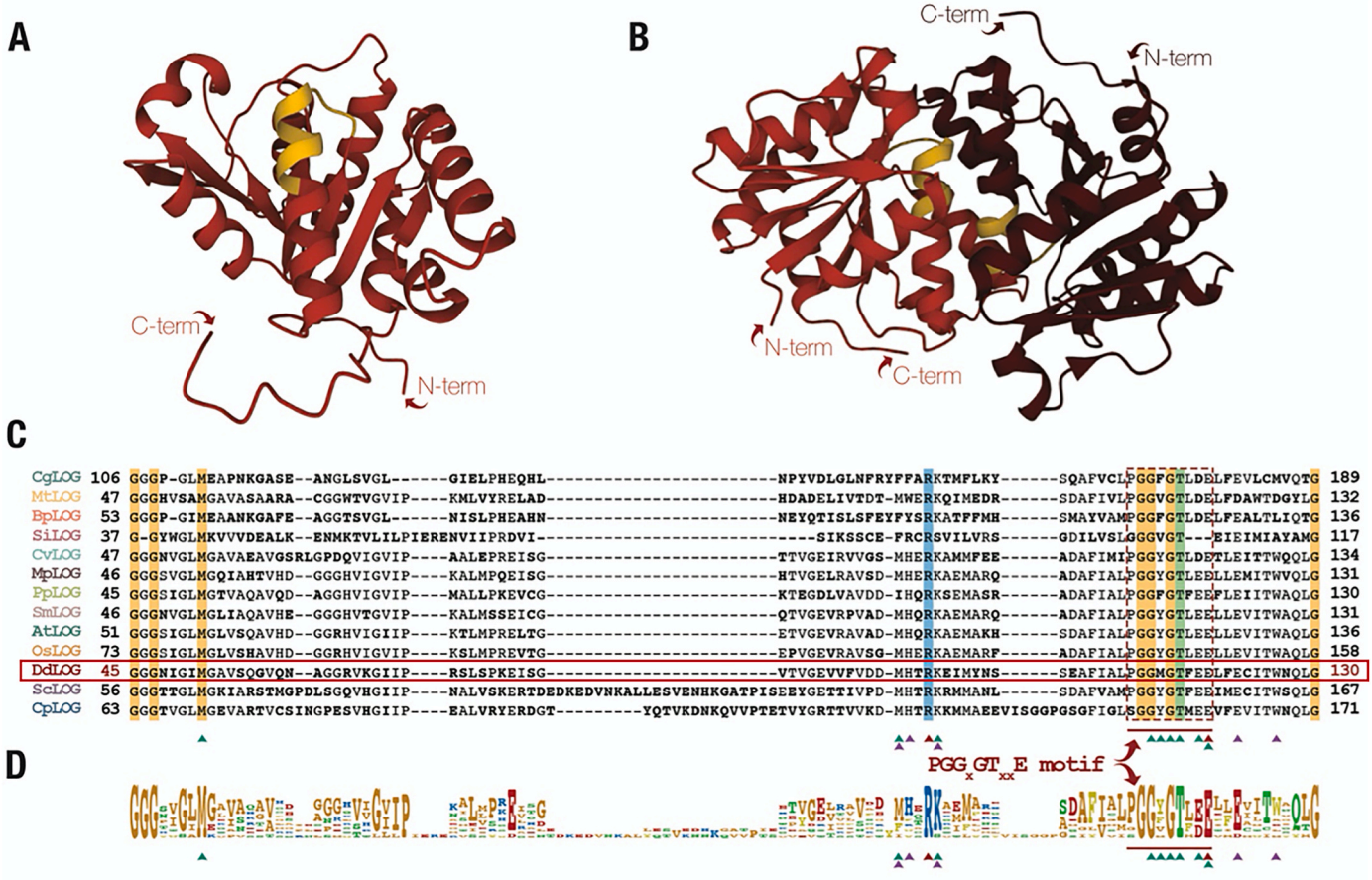

Fig. 1. Structural and conserved features of *Dd*Log. A) The predicted monomeric structure of *Dd*Log (Alphafold ID: AF-Q54UC5–F1) [25,26]. **B)** Proposed dimeric structure of *Dd*Log generated through SWISS-Model. The conserved $PGG_xGT_{xx}E$ motif is highlighted in yellow in **A** and **B,** and both structures were visualized using Mol* (RCSB PDB) [27]. **C)** MUSCLE alignments were performed using *D. discoideum* and 12 representative species with characterized or putative LOG orthologs (*Corynebacterium glutamicum*, *Mycobacterium tuberculosis*, *Bordetella pertussis*, *Sulfolobus islandicus*, *Chlorella variabilis*, *Marchantia polymorpha*, *Physcomitrium patens*, *Selaginella mollendorfii*, *Arabidopsis thaliana*, *Oryza sativa*, *Dictyostelium discoideum*, *Saccharomyces cerevisiae*, and *Claviceps purpurea*). The alignments were made and visualized with DNASTAR MegAlignPro and conserved functional residues across all 13 species are highlighted. The $PGG_xGT_{xx}E$ motif is indicated by the dashed red box and red line. The red, teal, and purple triangles represent residues involved in catalysis, AMP binding, and prenyl-binding respectively [12]. Dashes indicate gaps within the sequence alignment between orthologs. A full sequence alignment can be viewed in Supplementary Table 3. **D)** Sequence logo for the multiple alignment. The figure was generated with DNASTAR MegAlignPro, and colors are representative of amino acid chemistry.

This resulted in 485 different templates from various LOG and LDC-like proteins. The top 15 hits had at least 90 % coverage and 40 % sequence similarity to *Dd*Log. These top hits included characterized LOGs from *Corynebacterium glutamicum*, *Claviceps purpurea*, and *Mycobacterium tuberculosis*–all of which are classified as type-I LOGs, indicating they are dimeric *in vitro* (Fig. 1B) [12,31–33]. LOGs have been previously separated into two main clusters, each containing a sub-group, based on the phylogenetic analysis of over 120 different LOG-like proteins [31]. Analysis of the amino acid compositions indicate a structural basis for LOG proteins, with type-I proteins comprising dimeric structures and type-II proteins comprising hexameric structures. The subgroups are further categorized by differences in the amino acid composition at key residues involved in enzyme catalysis, substrate binding, and in the $PGG_xGT_{xx}E$ motif [31]. When comparing the sequence of *Dd*Log to the key residues of type-Ia/b and type-IIa/b LOGs, *Dd*Log is classified as a type-Ia LOG. This is consistent with the predicted homo-dimer oligo-state from the SWISS-MODEL homology report (Fig. 1B).

Multiple sequence alignment of *Dd*Log with putative or characterized LOG proteins from 12 other organisms showed numerous conserved amino acid residues, previously determined to be critical for PRH catalysis, AMP binding, and prenyl-binding (Fig. 1C–D) [12]. *Dd*Log shares the highest sequence identity with the classical plant LOG proteins, ranging from non-vascular bryophytes such as *Marchantia polymorpha* (50 %) and *Physcomitrium patens* (50 %), to vascular seed plants such as *Arabidopsis thaliana* (47 %) and *Oryza sativa* (48 %) (Supplementary Table 3). A consensus cladogram highlights the conserved domain architecture of the LOG protein family and suggests no major expansions of the protein either across species or throughout evolution (Supplementary Fig. 2; Supplementary Table 3). Interestingly, within the Uniprot database, the only proteins possessing at least a 50 % identify to the *Dd*Log protein sequence belonged to 5 different species of the Dictyostelia class, indicating that this gene is conserved across this class of organisms and is not unique to *D. discoideum* (Supplementary Table 4).

3.2. Biochemical properties of DdLog

His-tagged *Dd*Log was expressed in *E. coli* and purified with Ni-NTA agarose to assess its PRH and CK-activating activity. The purified, recombinant protein had the expected theoretical mass as determined through SDS-PAGE and Western blot (Supplementary Fig. 1). Optimal reaction conditions were determined for *Dd*Log using iPMP, and conversion from substrate to product was assessed using HPLC-(ESI+)-HRMS/MS. *Dd*Log had a temperature optimum of 25 °C (Fig. 2A). *Dd*Log showed the highest activity between pH 7.0 and 8.0 in Tris-HCl, with a pH optimum of 7.5 (Fig. 2B). The temperature optimum was slightly

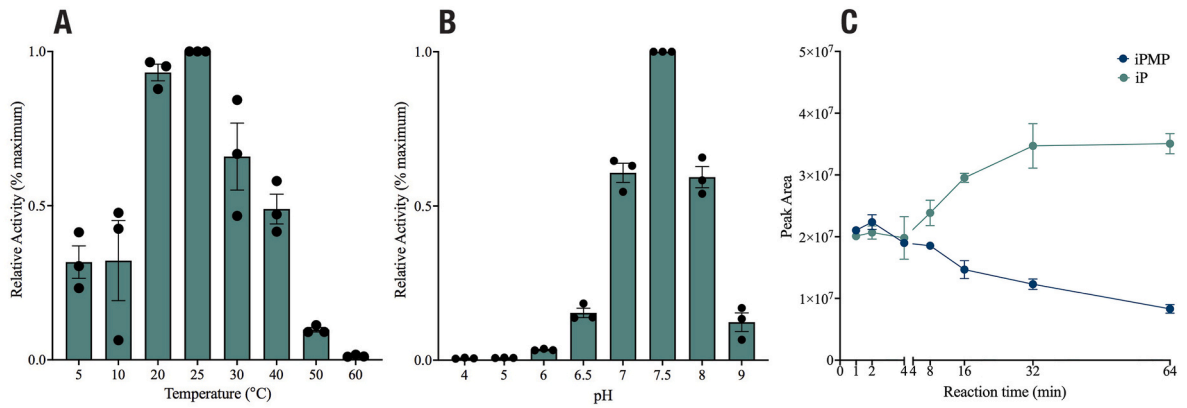

Fig. 2. *In vitro* characterization of purified and desalted recombinant *Dd*Log using iPMP as a substrate. **A)** Temperature optimum. **B)** pH optimum. **C)** iP formation over a 64-min incubation period using 500 nM *Dd*Log concentration. Values represent the mean product formation of iP from iPMP ± SEM (n = 3). A and B panels are shown as % maximum relative to the temperature and/or pH with the highest iP production. Panel C shows the peak area of product and substrate over a 64-min incubation period.

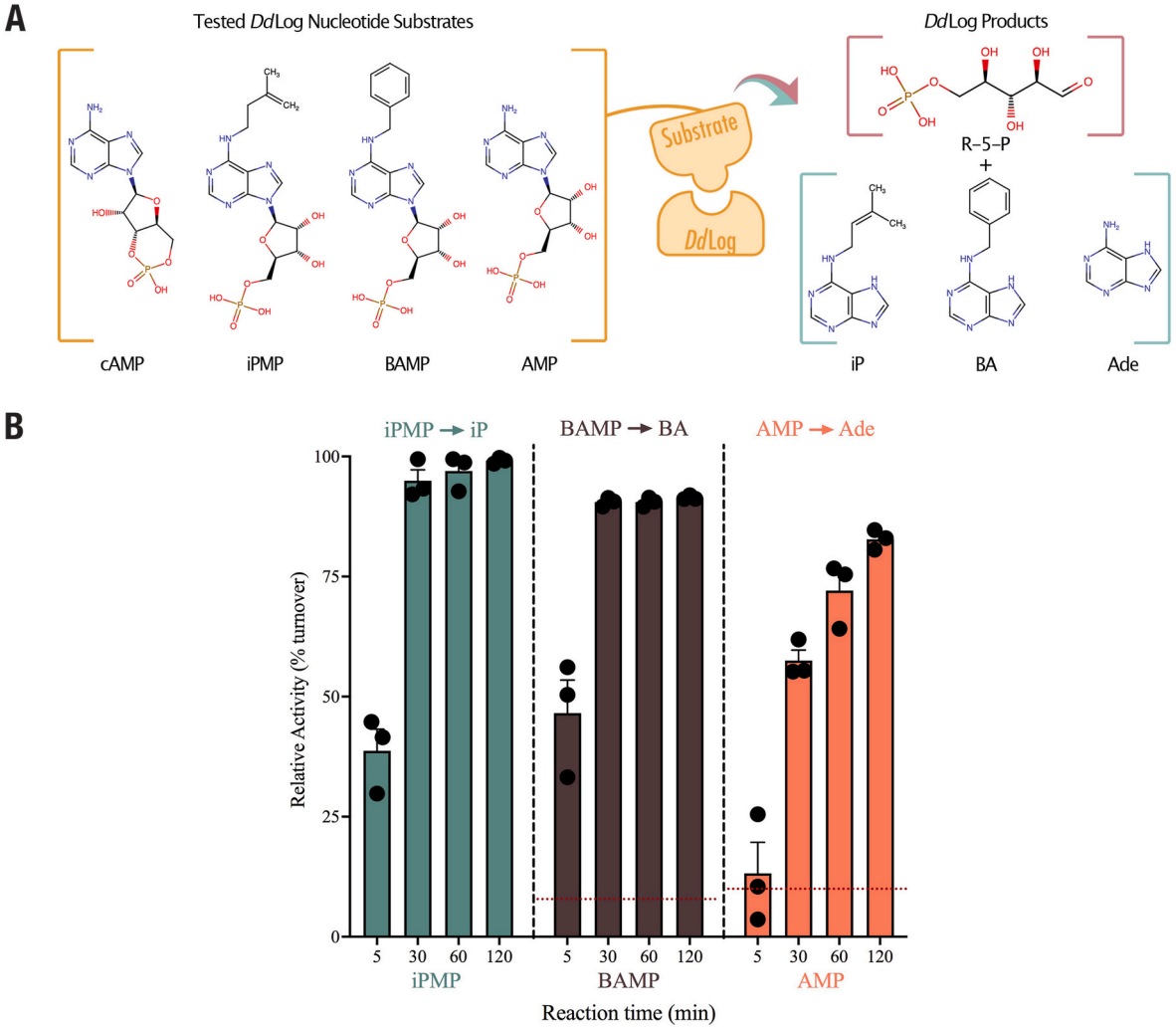

Fig. 3. Reaction scheme and relative phosphoribohydrolase activity of *Dd*Log. **A)** Reaction scheme of a phosphoribohydrolase (PRH) enzyme. Two cytokinin nucleotides (iPMP and BAMP) and two non-cytokinin nucleotides (AMP and cAMP) were assessed for their activity with *Dd*Log. Ribose-5-phosphate and the respective free base derivatives (BA, iP, or Ade) of the tested substrates are the breakdown products. **B)** Relative PRH activity. Values represent the mean % turnover ± SEM (n = 3). cAMP is not shown as it did not bind to *Dd*Log. The red dotted line marks residual substrate levels in denatured enzyme controls; there is no line for iPMP, which had less than 1 % turnover.

lower than observed in other CK-producing organisms, but the pH optimum was in line (see Supplementary Table 5 for comparison of experimental parameters). Under these conditions, product turnover was assessed to select an incubation time within the linear range of the enzyme (data not shown). A 500 nM enzyme concentration was selected as the optimal concentration for its linear range of product formation, and a 15-min incubation time for all subsequent assays was used based on the product formation plot (Fig. 2C).

3.3. *Dd*Log is a cytokinin-activating enzyme

Two CK-NTs, iPMP (isoprenoid CK produced in *D. discoideum*) and BAMP (aromatic CK-NT), and two non-CK-nucleotides, AMP and cAMP, were screened as potential substrates for *Dd*Log. These diverse substrate types enabled determination of whether the protein had LOG or PRH activity (Fig. 3A). A full scan HPLC-(ESI+)-HRMS/MS analysis detected the conversion of each substrate to their respective FB reaction products. Labeled internal standards enabled accurate compound identification of each free base product (Supplementary Table 2). No activity was found in the controls.

Of the tested substrates, *Dd*Log was able to hydrolyze iPMP, BAMP, and AMP, but not cAMP. These results support our hypothesis that *Dd*Log functions as a LOG protein that also retains the ability to hydrolyze non-CK-NTs (Fig. 3B). The turnover rates of the two CK-NT substrates, iPMP and BAMP, were higher than that of AMP at each of the four incubation times (Fig. 3B). Our previous CK-profiling study in *D. discoideum* indicates that iP-type CKs (iP, iPR, and iPMP) are the primary CK forms identified across the life cycle that allow for the production of discadenine in the fruiting body (see Ref. [23] for full details on concentrations). At 30 min, iPMP, had almost 95 % product turnover, BAMP had 90 %, while AMP had 57 %. In the few other known cases where both iPMP and AMP were used as substrates for determining PRH activity of LOGs, the affinity for iPMP was consistently higher than it was for AMP in *A. thaliana* (*At*LOG3 iPMP K_m: 14 μM; AMP: no binding), *O. sativa* (*Os*LOG iPMP K_m: 12 μM; AMP: no binding), *C. purpurea* (*Cp*LOG iPMP K_m: 4 μM; AMP: 28 μM), and *M. tuberculosis* (*Mt*LOG iPMP K_m: 6 μM; AMP: 73 μM) [7,8,11,34]. Unfortunately, in many recent studies that characterized LOGs in non-plant organisms, CK-NTs were not tested as substrates; thus, we have no such values to use as a comparison. Our relative PRH activity data, as measured by percent turnover for all three substrates, indicate that LOGs have a higher affinity towards CK-NTs than AMP (Fig. 3B). From the combined data, we conclude that *Dd*Log acts as a hybrid LOG enzyme, encompassing a broader substrate specificity than previously tested LOGs with activity for both CK-NTs and non-CK-NTs (e.g., AMP).

Insight into the nature of our hybrid *Dd*Log may come from the proposed dual function of LOG proteins in other organisms. A putative LOG ortholog was identified in *Saccharomyces cerevisiae* during a screen for genes that confer resistance to the mutagenic effects of certain base analogs [35,36]. The *S. cerevisiae* LOG was studied for its role in nucleotide sensing and metabolic detoxification of base analogs that could be misincorporated into RNA or DNA [36–39]. Carlsson et al. [36] showed that *S. cerevisiae* Log1 conferred resistance against two commonly used base analogs that have toxic effects on either DNA or RNA, 6-N-hydroxylaminopurine and 5-fluorouracil. Resistance of Log1 against 5-fluorouracil was partially dependent upon *HAM1*, a gene that encodes inosine triphosphate pyrophosphatase and lies upstream from *LOG1*. The authors concluded that Ham1 and Log1 likely serve as gatekeepers against non-canonical bases, thus protecting against the misincorporation of a broad range of toxic base analogs into nucleic acids. This combined evidence paired with the known role of LOG proteins as CK-activating enzymes prompted the proposal that LOGs initially had roles in nucleotide sensing and metabolic detoxification and that their CK activation roles were a more recent development [36].

STRING analysis of *Dd*Log against the *D. discoideum* proteome identified an ortholog to human and yeast inosine triphosphate pyrophosphatase (*D. discoideum* gene ID: DDB_G0286495; protein ID: DDB0238062–denoted ItpA) as the most highly predicted functional partner to *Dd*Log (database version 11.5, https://string-db.org/) [40]. It would be of interest to further assess the relationship between *Dd*Log and ItpA in nucleotide sensing and metabolic detoxification. Based on their specificity towards CK-NTs, it seems the LOGs in *A. thaliana* and *O. sativa* are CK-specific enzymes which have lost original functions of metabolic detoxification, whereas *D. discoideum* may have retained this original function whilst also expanding its substrate range for CK-NTs.

4. Conclusion

CKs play an essential role during the life cycle of *D. discoideum*, yet much of their biosynthesis and action mechanisms remain uncharacterized. Through biochemical and bioinformatic analyses, we revealed *Dd*Log is a key enzyme involved in CK activation in *D. discoideum*. We showed that *Dd*Log functions as a classical LOG protein, hydrolyzing BAMP and iPMP, but it also acts as a standard PRH with non-CK-NTs, such as AMP. Our structural analyses indicate that *Dd*Log adopts a homo-dimer oligostate *in vitro*, and sequence data supports a type-Ia classification. Together, these findings indicate that *Dd*Log is a CK-activating enzyme in *D. discoideum* which has also retained its original metabolic detoxification role that is yet to be explored.

CRediT authorship contribution statement

Megan M. Aoki: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Conceptualization. **Anna B. Kisiala:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis. **Scott C. Farrow:** Writing – review & editing, Methodology, Formal analysis. **Craig R. Brunetti:** Writing – review & editing, Supervision, Conceptualization. **Robert J. Huber:** Writing – review & editing, Supervision, Conceptualization. **R.J. Neil Emery:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2024.101756.

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