The Rickettsia prowazekii Invasion Gene Homolog (invA) Encodes a Nudix Hydrolase Active on Adenosine (5’)-pentaphospho-(5’)-adenosine*

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The genomic sequence of Rickettsia prowazekii, the obligate intracellular bacterium responsible for epidemic typhus, reveals an uncharacterized invasion gene homolog (invA). The deduced protein of 18,752 Da contains a Nudix signature, the specific motif found in the Nudix hydrolase family. To characterize the function of InvA, the gene was cloned and overexpressed in Escherichia coli. The expressed protein was purified to near homogeneity and subsequently tested for its enzymatic activity against a series of nucleoside diphosphate derivatives. The purified InvA exhibits hydrolytic activity toward dinucleoside oligophosphates (Np₂N; n ≥ 5), a group of cellular signaling molecules. At optimal pH 8.5, the enzyme actively degrades adenosine (5’)-pentaphospho-(5’)-adenosine into ATP and ADP with a $K_m$ of 0.1 mM and $k_{cat}$ of 1.9 s⁻¹. Guanosine (5’)-pentaphospho-(5’)-guanosine and adenosine-(5’)-hexaphospho (5’)-adenosine are also substrates. Similar to other Nudix hydrolases, InvA requires a divalent metal cation, Mg²⁺ or Zn²⁺, for optimal activity. These data suggest that the rickettsial invasion protein likely plays a role in controlling the concentration of stress-induced dinucleoside oligophosphates following bacterial invasion. *Molecular & Cellular Proteomics* 1:179–185, 2002.

Rickettsia prowazekii is the etiologic agent of epidemic typhus and Brill-Zinsser disease. These illnesses are louse-borne rickettsioses that are reemerging worldwide (1, 2). The organism is an obligate intracellular Gram-negative bacterium growing only within the eukaryotic host cell cytoplasm (3). Humans are exposed to R. prowazekii through direct contact with contaminated body louse feces. The bacterium begins its life cycle in the human host by invading the epithelial cells via the process of induced phagocytosis (3, 4). Then, it rapidly escapes from the phagosome into the host cytoplasm where it replicates and eventually causes the invaded cell to burst (3, 4). Destruction of host cells is the basis of rickettsial pathogenesis (3). Although systemic approaches have revealed substantial information about the biology of rickettsial host cell invasion, the molecular basis underlying the invasive mechanism remains undefined.

Recently, the completion of the R. prowazekii genome project revealed the presence of the invasion gene homolog, invA (5). The invA open reading frame encodes a polypeptide of 161 amino acids with a predicted molecular mass of 18,752 daltons, containing a conserved motif called the Nudix box (Nucleoside diphosphanes linked to some other moiety, X) (6). Nudix boxes are present in the Nudix hydrolase family, a group of diverse enzymes that catalyze the hydrolysis of nucleoside diphosphate derivatives (6). Alignment analysis of the deduced amino acid sequence of R. prowazekii InvA demonstrated 37–44% identity to the putative invasion proteins of other invasive bacteria including Bartonella bacilliformis IalA, Neisseria meningitidis putative Ap₅A¹ pyrophosphatase, Helicobacter pylori InvA, Escherichia coli YgdP, Salmonella typhimurium putative invasion protein, Haemophilus influenzae InvA, and Pseudomonas aeruginosa invasion protein homolog (Fig. 1). Among these homologous genes, only B. bacilliformis IalA and E. coli K1 ygdP have been documented to be associated with host cell invasion (7, 8). Furthermore, purified IalA and YgdP were shown to be members of the dinucleoside oligophosphate pyrophosphatase subfamily of the Nudix hydrolases (9–11). Their specific substrates, dinucleoside oligophosphates, are considered to be a class of signaling molecules involved in cell stress responses, cell growth, and cell differentiation (12, 13). It has been proposed that these enzymes might play a role in enhancing the intracellular survival of the invading bacteria by regulating the stress-induced dinucleoside oligophosphate levels during host cell invasion (6, 9–13). In this paper, we have identified homologous genes in several Rickettsia species. Among these, the R. prowazekii invA was

¹ The abbreviations used are: Ap₅A, adenosine (5’)-tetraphospho-(5’)-adenosine; Ap₆A, adenosine (5’)-pentaphospho-(5’)-adenosine; HPLC, high performance liquid chromatography.
expressed, and the purified protein was characterized to be a member of the dinucleoside pentaphosphate pyrophosphatase subfamily of Nudix hydrolases.

**MATERIALS AND METHODS**

The R. prowazekii (Madrid E), Rickettsia typhi (Wilmington), Rickettsia rickettsii (Sheila Smith), Rickettsia akari (Kaplan), Rickettsia
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canada (CA410), and Rickettsia rhipicephali (CA871) were propagated in our laboratory (University of Maryland, Baltimore, MD). Primers utilized in this study were synthesized at the Biopolymer core facility, University of Maryland, Baltimore. The genomic DNA isolation kit was from Promega (Madison, WI). Enzymes used in standard cloning procedures were from Stratagene (La Jolla, CA) and Invitrogen. Calf intestinal alkaline phosphatase and inorganic pyrophosphatase were from Stratagene (La Jolla, CA). PCR 2.1 TA TOPO cloning vector and E. coli TOP10 competent cells were purchased from Invitrogen. The prokaryotic expression system, pET-24a(+) and E. coli DH5x and E. coli HMS174(DE3) hosts were obtained from Novagen (Madison, WI).

**Cloning of Rickettsia and Genomic DNA Isolation**—Rickettsiae were propagated in African green monkey kidney (Vero) cells (ATCC C1088) as described previously (14). 90–95% infected cells were harvested, and rickettsiae were purified by Renografin density gradient centrifugation (15). Genomic DNAs were isolated using a Wizard® DNA isolation kit and were quantified spectrophotometrically.

**Cloning of Rickettsial invA Homolog**—To investigate whether members of typhus group and spotted fever group rickettsiae, both human pathogens and non-pathogens, contain the invA gene homolog, primers based on the R. prowazekii invA sequence were used in PCR reactions. 0.1 μg of genomic DNA of typhus group, R. typhi and spotted fever group rickettsiae, R. rickettsii, R. akari, R. canadensis, and R. rhipicephali were subjected to PCR. The amplified fragments were cloned into the TA TOPO cloning vector, PCR 2.1, and recombinant vectors were transformed into competent cells for propagation. Plasmid DNA was purified and sequenced. Alignments were compared through blastn searches from GenBank®.

**Construction of the Expression Vector**—Full-length R. prowazekii invA (GenBank® accession number AJ235271) was amplified from 0.1 μg of R. prowazekii genomic DNA utilizing a forward primer incorporating an NdeI site (NdeI-invA; nucleotide 12769–12736; 5′ GGC CGC GGC ATA TGA GGA ATT CTT CTA ACA AAT) and a reverse primer incorporating a BarnHI site (BarnHI-invA; nucleotide 12284–12320; 5′ GGC CGC GGA TCC TTA CTG AAT TTA TGA TTC AAA). The amplicon was subcloned into PCR 2.1 TA TOPO cloning vector. The recombinant plasmid was transformed into E. coli TOP10 for propagation and sequenced to ensure the fidelity of the amplified gene. R. prowazekii invA was then excised from the TA cloning vector, gel-purified, and directionally cloned into the NdeI/BamHI sites of the pET-24a(+) expression vector. The resulting construct, pETinvA, was introduced into E. coli DH5x for further propagation, and the in-frame insertion of invA was confirmed by sequencing.

**Expression and Purification of R. prowazekii invA**—To obtain a high quantity of native InvA, it was necessary to use an enhancing expression protocol optimizing the production of soluble protein (9). In this procedure, the expression host, E. coli HMS174(DE3), was co-transformed with pETinvA and pGroESL expressing the chaperones GroEL and GroES. The transformed clones were recovered by double antibiotic selection (kanamycin and chloramphenicol resistance acquired from pETinvA and pGroESL, respectively). To express the recombinant InvA, two liters of prewarmed LB medium containing 30 μg/ml each of kanamycin and chloramphenicol was inoculated with 20 ml of an overnight culture of E. coli HMS174(DE3) carrying pETinvA and pGroESL. The culture was incubated at 37 °C on an A600 of 0.3 and then transferred to 18 °C. At an A600 of 0.8, the cells were induced with 0.5 mm isopropyl-β-D-thiogalactopyranoside and incubated at 18 °C overnight. The induced cells were harvested by centrifugation, washed with isotonic saline, and frozen at −80 °C. To extract the protein, the frozen cells (4 g) were resuspended in 2.5 volumes of TED buffer (50 mM Tris·Cl, pH 7.5, 0.1 mM EDTA, 0.1 mM dithiothreitol) and ruptured by three repetitive freeze-thaw cycles. The cell debris was removed by centrifugation to obtain the crude extract (Fraction I). A solution of 10% streptomycin sulfate was slowly added to Fraction I to a final concentration of 1.5%. The precipitated nucleic acids were removed by centrifugation, and the supernatant, Fraction II, was adjusted to 55% saturation with solid ammonium sulfate. The precipitated protein was dissolved in a minimal volume of TED buffer (Fraction III), applied to a 2.5 × 60-cm Sephadex G-100 gel filtration column, and eluted with 100 mM sodium chloride in TED buffer. Fractions were assayed for enzymatic activity. The active fractions were pooled, concentrated by pressure filtration in a Centricron Plus-20 microconcentrator, and adjusted to 20% glycerol (Fraction IV) before storage at −80 °C. Each step of the purification process was monitored by SDS-PAGE followed by Coomassie Blue staining. Protein concentration was measured according to Bradford (16).

**Enzyme Assays and Product Identification**—Enzymatic activity of purified InvA was assayed as described previously (9). Briefly, this procedure measures the conversion of a phosphatase-resistant substrate to a phosphatase-sensitive product by determining the liberated Pi. Potential substrates (1 mM) were incubated for 15 min at 37°C with InvA and calf intestinal alkaline phosphatase (4 units) in 50 μl of a standard reaction mixture containing 50 mM Tris·HCl, pH 8.5, and 5 mM MgCl2. The reaction was terminated by adding 250 μl of 4 mM EDTA, and the released Pi was measured according to the colorimetric procedure of Ames and Dubin (17). One unit of InvA catalyzes the hydrolysis of 1 μmol of substrate per min under these conditions.

HPLC was used to identify the reaction products as described previously (9). An assay mixture (250 μl) containing 50 μM Tris·HCl, pH 8.5, 5 mM MgCl2, 250 nmol Ap5A, and 330 ng of InvA protein was incubated up to 20 min at 37°C. Samples collected at different time points were analyzed on a reverse phase column, and the peaks were identified by comparison to standards.

**RESULTS**

**Cloning of the Rickettsial invA**

Using the polymerase chain reaction, full-length R. prowazekii invA (486 bp) was cloned, and its nucleotide sequence was identical to the published sequence. To determine whether invA homologs are present in other rickettsiae, PCR utilizing R. prowazekii invA gene-specific primers were performed on the genomic DNA of human pathogenic rickettsiae; R. typhi, R. rickettsii, R. akari, and the non-pathogenic Rickettsia species, R. canadensis and R. rhipicephali. Approximately 500 bp of PCR products were amplified (Fig. 2). The fragments were cloned and sequenced. Alignment analysis demonstrated that the nucleic acid sequences of the invA homologs in other rickettsiae were 96–98% identical to the published R. prowazekii invA.

**Expression and Purification of the InvA Protein**

Directional cloning of the full-length R. prowazekii invA into pET-24a(+) at the NdeI and BamHI sites allowed for the expression of unmodified protein under the control of the strong T7 promoter. When cells were grown and induced at 37°C, a large amount of InvA was produced; however, only a
limited amount was present in the soluble fraction of the cell extract (Fig. 3). Similar yield was observed in InvA expression at 18 or 37 °C in the presence of host chaperones (data not shown). The combination protocol, lowering the expression temperature and using a host overproducing chaperones, resulted in a substantial increase in the soluble, native form of R. prowazekii InvA (Fig. 3). SDS-PAGE analysis of the soluble fraction of the crude cell extract revealed novel protein bands at 58, 19, and 10 kDa representing GroEL, InvA, and GroES, respectively (Fig. 3). Purification of InvA was aided by the observation that most of the enzyme was released from the cell by a freeze-thaw cycle. Using the protocol as described under “Materials and Methods,” InvA was purified to near homogeneity as shown in Fig. 3.

Properties of the InvA Protein

Substrates—Because alignment analysis of the primary sequence demonstrated high homology to the Nudix hydrolases, we tested a number of substrates shown previously to be hydrolyzed by members of the family. Little or no activity was seen with NADH, GDP-mannose or ADP-ribose (Table I). However, as with IalA and YgdP, the InvA protein catalyzes the hydrolysis of members of the dinucleoside oligophosphate family. As summarized in Table I, InvA preferentially degrades adenosine Ap5A. It has no activity on dinucleoside oligophosphates (n ≤ 4), whereas Bartonella IalA prefers Ap3A. The relative activity of InvA on Ap2A and Gp3G decreases significantly with decreasing phosphate chain length. This trend is also observed with IalA and YgdP.

Reaction Products—To identify the products from the hydrolysis of the preferred substrate, Ap2A, a standard reaction was scaled up omitting alkaline phosphatase. Aliquots were collected after incubation for 0, 5, 10, 15, and 20 min and subsequently analyzed by HPLC. There was a decrease in the Ap2A during the incubation and concomitant formation of ATP and ADP (Fig. 4). No fraction of adenosine tetraphosphate was observed throughout the course of the reaction. The stoichiometry of the hydrolytic reaction may be described by Equation 1,

$$\text{Ap}_2\text{A} + \text{H}_2\text{O} \rightarrow \text{ATP} + \text{ADP} \quad \text{(Eq. 1)}$$

Kinetic Properties—Kinetic parameters of Ap2A hydrolysis by InvA, along with those of YgdP, for comparison, are summarized in Table II. The maximum velocity of InvA is 3.8 units/mg leading to the catalytic constant, $k_{\text{cat}}$, of 1.9 s$^{-1}$ compared with 1.0 s$^{-1}$ for YgdP.

Other Properties of the Enzyme—InvA elutes from the gel filtration column as a 19-kDa protein, behaving as a monomer.
in solution. Like most of the other characterized Nudix hydrolases, it prefers an alkaline pH, optimally at 8.5, and a divalent cation, Mg$^{2+}$ or Zn$^{2+}$, at 5 mM to be fully functional. Mn$^{2+}$ did not support the hydrolytic activity of InvA whereas it is required for IalA and YgdP. A comparison of the properties of InvA and the other two characterized invasion proteins is presented in Table III.

**DISCUSSION**

One aspect of our research is to describe the molecular basis underlying rickettsial host cell invasion. We have initially focused on the functional characterization of the *R. prowazekii* invA and have shown that this gene is present and conserved among the pathogenic and non-pathogenic *Rickettsia*, members of the typhus and spotted fever groups. We hypothesize that this particular gene might be important for host cell invasion and/or establishing and maintaining the obligate intracellular parasitism shared by rickettsiae. To characterize its function, *R. prowazekii* InvA was expressed, purified, and evaluated for its enzymatic properties. Initial attempts to express *R. prowazekii* InvA as a histidine-tagged protein for convenient protein purification was unsuccessful because of an extremely low expression yield.\(^2\) Expression of InvA as a fusion protein with the 40-kDa maltose-binding protein resulted in a high quantity of soluble product; however, the purified maltose-binding protein-InvA fusion protein exhibited a specific enzyme activity 200-fold lower than that of the native protein.\(^3\) Similar observations have been reported previously for vaccinia virus deoxyuridine triphosphatase, in that the native protein had a specific activity 36-fold higher than that of the glutathione S-transferase fusion protein.\(^18\) Utilizing the pET system, in combination with the expression-enhancing protocol described here, allowed for the expression of high quality, stable, native InvA, which was suitable for biochemical characterization. Our data indicate that *R. prowazekii* invA encodes a dinucleoside pentaphosphate pyrophosphatase, identifying it as a member of the subfamily of the Nudix hydrolases. This finding is consistent with previous reports in which the invasion-associated genes, *Bartonella* ialA and *E. coli* K1 ygdP, were also shown to encode members of this subfamily.\(^9-11\)

Considering its enzymatic activity, how might InvA be involved in host cell invasion or enable its intracytoplasmic existence? The dinucleoside oligophosphates are byproducts of the aminoacyl-tRNA synthetase reaction, found in prokaryotic and eukaryotic cells in small amounts.\(^12, 19\) However, during cellular stresses such as heat shock or oxidative stress, the concentration of dinucleoside oligophosphates in-

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Table II

| Parameters | R. prowazekii InvA | E. coli YgdP$^a$ |
|------------|-------------------|-----------------|
| $V_{\text{max}}$ (units$^b$/mg) | 3.8 | 3.3 |
| $K_{\text{cat}}$ (s$^{-1}$) | 1.9 | 1.0 |
| $K_m$ (mM) | 0.1 | 0.36 |
| $K_{\text{cat}}/K_m$ (s$^{-1}$ M$^{-1}$) | $1.9 \times 10^4$ | $2.7 \times 10^3$ |

$^a$ These data are taken from Bessman et al. (11).

$^b$ A unit of activity is 1 μmol of substrate hydrolyzed per min.
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### Comparison of the dinucleoside oligophosphate pyrophosphatases

| Amino acids | *R. prowazekii* InvA | *B. bacilliformis* IaIA | *E. coli* YgdP<br> |
|-------------|----------------------|-------------------------|-------------------|
| Molecular mass (kDa) | 161 | 170 | 176 |
| Quaternary structure | Monomer | Monomer | Monomer |
| Optimum pH | 8.5 | 9.0 | 8.5–9.0 |
| Metal requirement | Mg^{2+}, Zn^{2+} | Mg^{2+}, Zn^{2+}, Mn^{2+} | Mg^{2+}, Zn^{2+}, Mn^{2+} |
| Substrate preference | Ap_{A} | Ap_{A} | Ap_{A} |
| Hydrolysis products | ATP, ADP | ATP, AMP | ATP, ADP |

* These data are taken from Conyer and Bessman (9) and Bessman et al. (11).
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