The Gene, ialA, Associated with the Invasion of Human Erythrocytes by Bartonella bacilliformis, Designates a Nudix Hydrolase Active on Dinucleoside 5′-Polyphosphates*

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ialA, one of two genes associated with the invasion of human red blood cells by Bartonella bacilliformis, the causative agent of several diseases, has been cloned and expressed in Escherichia coli. The protein, IalA, contains an amino acid array characteristic of a family of enzymes, the Nudix hydrolases, active on a variety of nucleoside diphosphate derivatives. IalA has been purified, identified, and characterized as an enzyme catalyzing the hydrolysis of members of a class of signaling nucleotides, the dinucleoside polyphosphates, with its highest activity on adenosine 5′-tetraphospho-5′-adenosine (Ap4A), but also hydrolyzing Ap3A, Ap2A, GpG, and GpG. In each case, a pyrophosphate linkage is cleaved yielding a nucleoside triphosphate and the remaining nucleotide moiety.

Bartonella bacilliformis is the only bacterium known to invade human red blood cells, and it and other species of Bartonella are responsible for several maladies, including Carrión’s disease (Oroya fever, verruga peruana), cat scratch disease, trench fever, bacillary angiomatosis, and bacillary erythema. The disease is caused by Bartonella bacilliformis, which, when transformed into minimally invasive strains HB101 to test lethality and HMS174(DE3) for expression. The pIALa-transformed HMS174(DE3) cell line was then transformed with pGroESL.

Purification of IalA Protein—Eight liters of LB medium containing 100 µg/ml ampicillin and 64 µg/ml chloramphenicol were inoculated with 80 ml of an overnight culture of HMS174(DE3) cells containing pIALa and pGroESL. The culture was incubated at 37 °C to an A600 of 0.3, then transferred to 22 °C. At an A600 of 0.6, the cells were induced with 1 mM isopropyl-β-D-thiogalactopyranoside and incubated at 22 °C for an additional 24 h. The cells were harvested by centrifugation, washed by suspension in about 10 volumes of an isotonic saline solution, centrifuged again, and frozen at −80 °C. About 2 g of cells were obtained per liter of culture. Freezing the cells was essential for preparing the extract. The frozen cells were suspended in 5 volumes of buffer A (50 mM Tris-HCl, pH 7.5, 1 mM EDTA), and the supernatant, which contained the IalA protein, was collected after centrifugation (Fraction I).

A 10% streptomycin sulfate solution was slowly added to Fraction I to a final concentration of 1%, while the fraction was stirred on ice. After approximately 15 min, the supernatant was collected after centrifugation (Fraction II).

Fraction II was brought to 40% saturation by the slow addition of solid ammonium sulfate. After 15 min, the precipitate was collected by centrifugation and discarded, and additional ammonium sulfate was added to the supernatant to give a 60% saturated solution. This precipitate, containing the IalA protein, was collected and dissolved in a volume of buffer A representing a 15-fold concentration of the starting material (Fraction III).

Fraction III was loaded onto a 2.5 × 60-cm Sephadex G-100 column and eluted with buffer A containing 100 mM NaCl. The fractions containing purified IalA protein were combined, concentrated by pressure filtration in a Centricon 10 microconcentrator, and stored at −80 °C (Fraction IV).

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FIG. 1. Expression and purification of IalA. A 15% polyacrylamide gel containing 1% SDS, stained with Coomassie Blue, included the following: lanes 1 and 6, reference proteins of bovine albumin (66 kDa) egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), and soybean trypsin inhibitor (20 kDa); lane 2, 3 µl of a crude extract of E. coli pIL4Aa before induction with isopropyl-b-D-thiogalactopyranoside; lane 3, 3 µl of a crude extract of E. coli pET 11 b (no insert) 9 h after induction; lane 4, 3 µl of crude extract of E. coli pIL4Aa 9 h after induction; lane 5, enzyme Fraction IV, containing approximately the same number of enzyme units as present in lane 4.

Enzyme Assay—This assay measures the conversion of a phosphatase-insensitive substrate to a phosphatase-sensitive product.

The standard incubation mixture contained in 50 µl: 1 mM substrate, 50 mM Tris-HCl, pH 7.5, 1 mM ZnCl2, 3 units of calf alkaline intestinal phosphatase, 0.1–1.5 milliliters of enzyme. The reaction was terminated by the addition of 250 µl of 8 mM EDTA and analyzed for inorganic orthophosphate by the method of Ames and Dubin (7). A unit of enzyme hydrolyzes 1 µmol of substrate/min under these conditions.

RESULTS AND DISCUSSION

Expression and Purification of the IalA Protein—Cloning of the ialA gene, and transformation of the expression host, HMS174(DE3), with pIL4Aa produced a new, highly visible protein band of about 20 kDa in extracts of induced cultures when analyzed by polyacrylamide gel electrophoresis. However, all of the newly expressed protein was present in the insoluble fraction (inclusion body) of a low speed centrifugation, making it unsuitable for enzyme purification. Accordingly, we explored procedures for increasing the solubility of recalcitrant proteins, and found that the combination of growing the cells at a reduced temperature, and in the presence of chaperonins co-expressed from a second plasmid, produced significant quantities of soluble IalA protein. The partial purification of the IalA protein was facilitated by the ease with which it was extracted from the cells. Merely freezing and thawing the centrifuged packed cell mass causes the soluble IalA protein to leak out with the retention of most of the other proteins. This observation is reminiscent of another of the Nudix proteins we have studied, Orf17, a dATPase from E. coli, which we have cloned recently and are in the process of characterizing, is also a dinucleoside polyphosphate hydrolase.1 We therefore examined whether the IalA invasion protein also belonged to this family by assaying extracts of cells carrying the plasmid for diadenosine tetraphosphate hydrolase activity. Fig. 3 shows the appearance of the enzyme after induction. In the control culture containing the plasmid without the ialA insert, no commensurate activity was detected. After purification of the newly discovered enzyme, the substrate specificity was examined as shown in Table I. In both the diadenosine and diguanosine series, the tetraphosphate appears to be the preferred substrate, although more extensive kinetic measurements will be required to compare all the members of the series. As mentioned previously, none of the other compounds including nucleotide sugars, NADH, nucleoside triphosphates, or ADP-ribose, favored, by other members of the Nudix family, are significant substrates.

Products of the Reaction—In order to ascertain the pathway of hydrolysis of adenosine tetraphosphate, a scaled up standard reaction mixture (without alkaline phosphatase) was allowed to proceed until approximately 70% of the initial substrate was hydrolyzed. The products and remaining substrate were separated by high performance liquid chromatography as shown in Fig. 4. The disappearance of Ap4A2 was accompanied by the commensurate appearance of ATP and AMP. Neither inorganic orthophosphor nor pyrophosphate is produced during the course of the reaction, and the following equation may be written: Ap4A + H2O → ATP + AMP.

The products formed from the other substantial substrates were analyzed by paper electrophoresis according to Markham and Smith (14). Gp4G, as expected, yielded GTP and GMP. When the pentaphosphates were analyzed, the products found to be the triphosphate (ATP or GTP) and the respective diadiphosphate (ADP or GDP). With the hexaphosphate, Ap6A, ATP was the sole product. Thus, the feature common to all the reactions is the obligatory formation of a nucleoside triphosphate as one of the products, arguing that a nucleophilic attack by H2O is directed at the phosphorus atom in each of the substrates. This is in contrast to the MutT dGTPase and Orf17 dATPase, also members of the Nudix hydrolase family, which catalyze nucleophilic attacks on the β phosphorus as shown by NMR analysis of the products of dGTP or dATP hydrolyzed in H218O (8, 15). It would be of interest to do similar studies with the dinucleoside polyphosphate hydrolase since the amino acid motif of the nucleotide binding site and catalytic center is common to all members of the Nudix hydrolase family.

It is worth noting that the enzyme described here differs from the dinucleoside polyphosphate hydrolase recently reported by Cartwright and Mclellan (16). Their protein from Saccharomyces cerevisiae also contains the Nudix signature. However, it forms multiple products from Ap5A (ADP, Ap4, AMP, Ap3A) and Ap5A (ADP, ATP, AMP, Ap2A), and it also hydrolyzes ATP and Ap4A, which are not substrates for the enzyme described in this paper. On the other hand, the specificity of the Bartonella enzyme seems to parallel, most closely, the Ap4A

1 J. D. Walsh and M. J. Bessman, unpublished observation.

2 The abbreviations used are: Ap, adenosine 5'-tetraphospho-5'-adenosine. Other members of the family are abbreviated in an analogous manner.
pyrophosphatase purified and characterized from human placenta (17) and shown to have the Nudix motif (18).

Other Properties of the Enzyme—
The protein appears to be monomeric, eluting from a gel filtration column at a position corresponding to 20 kDa. It has a broad pH optimum between 7.5 and 9.0 and requires a divalent metal ion for activity. At pH 9.0, it has optimal and approximately equal activity in 10 mM Mg²⁺ or 1 mM Zn²⁺, and it is about 50% as active in 3 mM Mn²⁺.

The purified enzyme (Fraction IV) has a specific activity of 40

FIG. 3. Expression of diadenosine tetraphosphate pyrophosphatase in E. coli pIAlA. Cultures of E. coli pET (□) and E. coli pIAlA (●) were grown under standard conditions at 22 °C and monitored by A₆₀₀ measurements. At the indicated times after induction with isopropyl-β-D-thiogalactopyranoside, aliquots of both cultures were centrifuged, resuspended in buffer, sonicated, and Ap₄A pyrophosphatase activity was measured in the standard assay (E. coli pET (V), E. coli pIAlA (●)).

FIG. 4. Products of the diadenosine pyrophosphatase reaction.
A scaled up standard reaction mixture (omitting alkaline phosphatase) was allowed to proceed until approximately 70% of the initial substrate was hydrolyzed. The products were separated by high performance ligand chromatography on a C₁₈ reverse phase chromatography column (YMC, Inc.). A, zero time; B, after 60 min; C, standards.

### TABLE I

| Substrate | Substrate hydrolyzed (nmol) | Relative activity (%) |
|-----------|----------------------------|-----------------------|
| Ap₄A      | 12.3                       | (100)                 |
| Ap₃A      | 9.5                        | 77                    |
| Ap₂A      | 8.3                        | 67                    |
| Ap₁A      | <0.1                       | <1                    |
| Gp₄G      | 4.6                        | 37                    |
| Gp₂G      | <0.1                       | <1                    |
| ADP-glucose| <0.1                     | <1                    |
| ADP-mannose| <0.1                    | <1                    |
| ADP-ribose| <0.1                       | <1                    |
| GDP-glucose| <0.1                    | <1                    |
| GDP-mannose| <0.1                    | <1                    |
| ATP       | <0.1                       | <1                    |
| GTP       | <0.1                       | <1                    |
| Ap₄      | <0.1                       | <1                    |
| NADH      | <0.1                       | <1                    |
| NAD⁺      | <0.1                       | <1                    |

*Ap₄A, adenosine 5'-tetraphospho-5'-adenosine. Other dinucleoside polyphosphates are likewise abbreviated.

FIG. 2. A clustal sequence alignment (12) of IalA of Bartonella and three homologous proteins identified in a BLAST (6) search. The closest matches to the Bartonella protein are from Lupinus (lupin), E. coli, and Hordeum (barley). The accession numbers are listed in parentheses.
µmol/min/mg or a kcat of about 14/s.

What role does the hydrolysis of dinucleoside polyphosphates play in the invasion of red cells by B. bacilliformis? The dinucleoside polyphosphates themselves are an interesting group of cell signaling molecules broadly distributed in prokaryotes and eukaryotes and implicated in a wide variety of physiological responses, including stress or heat shock ("alarmones"), neurotransmission, platelet aggregation, cardiovascular regulation (for review, see Ref. 19), and in cell differentiation and apoptosis (20). With this plethora of diverse targets, it has been difficult to assign specific mechanisms to their modes of action. However, thought-provoking correlations are evident. We have seen that Suramin (number 89,268, The Merck Index), a powerful anti-helminthic, anti-parasitic drug is a potent inhibitor of the Ap4A pyrophosphatase, reducing its catalytic rate 50% at 10 µM concentration.3 Rotlan et al. have seen similar effects in extracts of rat brain (21). Suramin has also been shown to inhibit purinergic neurotransmission (22), ADP-induced platelet aggregation (23), and the non-adrenergic, non-cholinergic inhibitory action potential (24). This suggests that adenine nucleotides, implicated as signaling molecules in these processes, are also involved in cell invasion.

It is also noteworthy that B. bacilliformis is a member of the alpha proteobacteria and closely related phylogenetically to other intracellular parasites, including Rickettsia, Brucella, Rhizobium, and Agrobacterium. Several lines of evidence, including 16 S RNA sequencing (25), chaperonin homologies (26, 27), and their bioenergetics system (28), point to the Rickettsia as the most likely antecedents of mitochondria by invading eukaryotic cells. We have recently identified an open reading frame (GenBank™ accession number Z82300) in Rickettsia prowazekii, the causative agent of epidemic typhus, containing the Nudix box and highly homologous to the enzyme described in this paper. We plan to determine whether it also is a dinucleoside pyrophosphatase in order to assess how general the linkage is between invasiveness and homologues of IalA. In addition, our identification of the product of ialA as a dinucleoside pyrophosphatase should help in uncovering the role of the second gene associated with invasion, ialB.

3 G. B. Conyers and M. J. Bessman, unpublished observations.

Note Added in Proof—A recent report (Safrany, S. T., Caffrey, J. J., Yang, X., Bernbeneck, M. E., Moyer, M. B., Burkhardt, W. A., and Shears, S. B. (1998) EMBO J 17, 6599–6607) describes an enzyme purified from rat liver hydrolyzing diphosphoinositol polyphosphates. It contains the signature sequence of the Nudix hydrolases and is the first member of the family whose major substrate is not a nucleoside diphosphate derivative.

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