The Journal of Experimental Biology 210, 733-740
Published by The Company of Biologists 2007
doi:10.1242/jeb.001289

Identification and characterization of a salivary adenosine deaminase from the sand fly *Phlebotomus duboscqi*, the vector of *Leishmania major* in sub-Saharan Africa

Hirotomo Kato[1,2,*], Ryan C. Jochim[1,3,*], Phillip G. Lawyer[4] and Jesus G. Valenzuela[1,†]

[1]*Vector Molecular Biology Unit, Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 12735 Twinbrook Parkway, Room 2E-22C, Rockville, MD 20852, USA,* [2]*Department of Veterinary Hygiene, Faculty of Agriculture, Yamaguchi University, Japan,* [3]*Preventive Medicine and Biometrics, Emerging Infectious Diseases, Uniformed Services University of the Health Sciences, Bethesda, MD 20814 and* [4]*Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, NIH, Rockville, MD 20852, USA*

*These authors contributed equally to this work
†Author for correspondence (e-mail: jvalenzuela@niaid.nih.gov)

Accepted 18 December 2006

Summary

Two transcripts coding for an adenosine deaminase (ADA) were identified by sequencing a *Phlebotomus duboscqi* salivary gland cDNA library. Adenosine deaminase was previously reported in the saliva of the sand fly *Lutzomyia longipalpis* but it was not present in the saliva of the sand flies *Phlebotomus papatasi*, *P. argentipes*, *P. perniciosus* and *P. ariasi*, suggesting that this enzyme is only present in the saliva of sand flies from the genus *Lutzomyia*. In the present work, we tested the hypothesis that the salivary gland transcript coding for ADA in *Phlebotomus duboscqi*, a sister species of *Phlebotomus papatasi*, produces an active salivary ADA.

Salivary gland homogenates of *P. duboscqi* converted adenosine to inosine, suggesting the presence of ADA activity in the saliva of this species of sand fly; furthermore, this enzymatic activity was significantly reduced when using either salivary glands of recently blood-fed sand flies or punctured salivary glands, suggesting that this enzyme is secreted in the saliva of this insect. This enzymatic activity was absent from the saliva of *P. papatasi*. In contrast to other *Phlebotomus* sand flies, we did not find AMP or adenosine in *P. duboscqi* salivary glands as measured by HPLC-photodiode array. To confirm that the transcript coding for ADA was responsible for the activity observed in the saliva of this sand fly, we cloned this transcript into a prokaryotic expression vector and produced a soluble and active recombinant protein of approximately 60 kDa that was able to convert adenosine to inosine. Extracts of bacteria transformed with control plasmids did not show this activity. These results suggest that *P. duboscqi* transcripts coding for ADA are responsible for the activity detected in the salivary glands of this sand fly and that *P. duboscqi* acquired this activity independently from other *Phlebotomus* sand flies. This is another example of a gene recruitment event in salivary genes of blood-feeding arthropods that may be relevant for blood feeding and, because of the role of ADA in immunity, it may also play a role in parasite transmission.

Key words: sand fly, saliva, ADA, adenosine deaminase, insect saliva, *Phlebotomus*.

Introduction

In their saliva, blood-feeding arthropods have potent pharmacologically active components that help them counteract the hemostatic and inflammatory system of the vertebrate host each time they attempt to take a blood meal (Ribeiro, 1987). Vasodilators, anticoagulants and inhibitors of platelet aggregation are part of this salivary mixture (Ribeiro and Francischetti, 2003). Recently, with the technological advances in DNA and protein sequencing, novel and unexpected molecules with potential biological activities have been isolated from the saliva of blood-feeding arthropods. Such molecules include hyalorunidase, nucleotidases, novel apyrases, amine-binding proteins, tissue-factor pathway inhibitors and others (Ribeiro and Francischetti, 2003). Another such protein is adenosine deaminase (ADA), which was identified from transcripts of a salivary gland cDNA library of the New World sand fly *Lutzomyia longipalpis* (Charlab et al., 2000) and the mosquitoes *Culex quinquefasciatus* and *Aedes aegypti* (Ribeiro et al., 2001). This protein or the transcript coding for this protein have also been

identified in other organisms including bacteria, fruit flies, mice and humans (Charlab et al., 2001).

Adenosine deaminase (E.C. 3.5.4.4) catalyses the conversion of adenosine and 2′-deoxyadenosine to inosine and 2′-deoxyinosine, respectively (Cristalli et al., 2001). This enzyme is evolutionarily conserved and has a beta alpha, 8 barrel structure and zinc ion in the catalytic site (Wilson et al., 1991). Adenosine deaminase deficiency in mice results in the impairment of T and B cell function (Resta et al., 1997) due to the accumulation of adenosine resulting in severe combined immunodeficiency (SCID).

The role of ADAs in insects, particularly in the saliva of blood-feeding insects, was proposed to be in the hydrolysis of adenosine, a molecule involved in pain perception (Charlab et al., 2001). The activity of this enzyme in blood-feeding insects was demonstrated in the saliva of *L. longipalpis* (Charlab et al., 2000), *C. quinquefasciatus* and *Ae. aegypti* (Ribeiro et al., 2001) and from the activity of the recombinant salivary ADA from *L. longipalpis* (Charlab et al., 2001).

Of interest, ADA enzymatic activity or the transcripts coding for this enzyme were not present in the salivary gland of the sand flies *P. argentipes*, *P. papatasi*, *P. ariasi* and *P. perniciosus*, which belong to the genus *Phlebotomus* (Anderson et al., 2006; Charlab et al., 2000). Instead, the saliva of *P. papatasi* and *P. argentipes* contains large amounts of adenosine and adenosine monophosphate (AMP) (Ribeiro and Modi, 2001). Therefore, it appeared that ADA activity was only present in the saliva of *Lutzomyia* sand flies and not in *Phlebotomus* sand flies. Recently, transcriptome analysis of the salivary glands of the sand fly *Phlebotomus duboscqi*, a sibling species of *P. papatasi*, resulted in the identification of a transcript with homologies to ADA (Kato et al., 2006). In the present work, we tested whether there is ADA activity in *P. duboscqi* and whether the identified transcript codes for this activity. Because *P. papatasi* does not have ADA activity, but has large amounts of adenosine and AMP in the saliva, we also tested for the presence of adenosine and AMP in the saliva of *P. duboscqi* sand flies.

Materials and methods

Sand flies and preparation of salivary gland homogenate (SGH)

Phlebotomus duboscqi Theodor, Mali strain, were reared using a mixture of fermented rabbit food and rabbit feces as larval food. Adult sand flies were offered a cotton swab containing 20% sucrose and were used for dissection of salivary glands at 5–7 days after emergence. Salivary glands were stored in groups of 10 pairs in 10 μl phosphate-buffered saline (PBS). Salivary glands were disrupted by ultrasonication in 1.5 ml conical tubes. Tubes were centrifuged at 10 000 ***g*** for 2 min and the resultant supernatant used for the studies.

Salivary gland cDNA library

The *P. duboscqi* salivary gland cDNA library was made as previously described (Kato et al., 2006). Briefly, mRNA was isolated from 55 salivary gland pairs using the Micro-FastTrack mRNA isolation kit (Invitrogen, San Diego, CA, USA). The PCR-based cDNA library was made following the instructions for the SMART cDNA library construction kit (BD-Clontech, Palo Alto, CA, USA) with some modifications (Kato et al., 2006). The *P. duboscqi* cDNA library was sequenced as previously described using an Applied Biosystems 3730xl DNA Analyzer (Foster City, CA, USA) and a CEQ 2000XL DNA sequencing instrument (Beckman Coulter, Fullerton, CA, USA) (Kato et al., 2006).

Phylogenetic analysis

Consensus protein sequences were compared to related sequences from sand flies as well as non-sand fly species obtained from GenBank. Sequences were aligned using ClustalX (Jeanmougin et al., 1998) and manually refined using BioEdit sequence editing software (http://www.mbio.ncsu.edu/BioEdit/page2.html). Phylogenetic analysis was conducted on protein alignments using Tree Puzzle version 5.2 (Schmidt et al., 2002). Tree Puzzle constructs phylogenetic trees by maximum likelihood, using quartet puzzling, automatically estimating internal branch node support (1000 replications). Derived trees were visualized using MEGA (Molecular Evolutionary Genetics Analysis) version 3.1 (http://www.megasoftware.net/) (Kumar et al., 2004).

Enzymatic assays

Measurement of activity was performed in quartz microcuvettes using 60 μl samples (Starna Cells, Atascadero, CA, USA). 20 μmol l^{-1} adenosine in PBS was added to the cuvette, followed by addition of the enzyme source. After mixing the solution by pipetting, the absorbance between 220 and 300 nm was monitored at 1.5 or 3.0 min intervals using a Lambda 18 spectrophotometer from Perkin Elmer (Norwalk, CT, USA).

Molecular sieving-high-performance liquid chromatography

Molecular sieving–high-performance liquid chromatography (MS-HPLC) was carried out using a Dionex Summit system and Chromeleon software (Dionex, Sunnyvale, CA, USA). For analysis, 20 μl of sample was applied to a Superdex Peptide PC 3.2/30 column (Amersham Biosciences, Piscataway, NJ, USA) using 10 mmol l^{-1} $NaPO_4$, 150 mmol l^{-1} NaCl, pH 6.5 as the mobile phase at a flow rate of 150 μl min^{-1} for 30 min of separation. Detection was performed using a photodiode array detector and a 3-D chromatogram generated using Chromeleon software. Adenosine and AMP standards (500 pmol) were applied separately. Single pairs of salivary glands from *P. papatasi* and *P. duboscqi* were sonicated in PBS, clarified by centrifugation and applied to HPLC.

Expression of P. duboscqi ADA

DNA fragments encoding mature *P. duboscqi* ADA protein were amplified and inserted into the cloning site of the pCRT7/NT-TOPO vector (Invitrogen). The primers used for PCR amplification of the mature ADA encoding fragments

were PDBL_P02_G04_VF (5′-GTTTTGGACATTTCGAA-CATTA-3′) and PDBL_P02_G09_VR (5′-TGGCTCCAA-ATGATTCAGACA-3′) for 2G4 (PduM73) and PDBL_P02_G09_VF (5′-CTTTGAAAATTAAACCGAAA-CGA-3′ and PDBL_P02_G09_VR for 2G9 (PduM74). *Escherichia coli* strain BL21(DE3)pLysS cells (Invitrogen) were transformed with the recombinant plasmid and grown in LB broth containing ampicillin (50 μg ml^{-1}). Production of recombinant protein was induced by addition of IPTG to a final concentration of 1 mmol l^{-1} and at 27°C for 3 h. The recombinant protein was purified from the supernatant of bacterial sonic lysate using a MagneHis Protein Purification System (Promega, Madison, WI, USA) and dialysed with Centricon Plus-20 (Millipore, Bedford, MA, USA) to remove imidazole from the elution buffer before further enzymatic analysis.

Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) and western blotting

The samples were treated with NuPAGE LDS sample buffer (Invitrogen) and analysed on NuPAGE 10% Bis-Tris gels (Invitrogen) with NuPAGE MES SDS running buffer (Invitrogen). To estimate the molecular mass of the samples, SeeBlue markers from Invitrogen (myosin, bovine serum albumin, glutamic dehydrogenase, alcohol dehydrogenase, carbonic anhydrase, myoglobin, lysozyme, aprotinin, and insulin, chain B) were used. After electrophoresis, the gels were stained with SimplyBlue™ SafeStain Coomassie® (Invitrogen) or SilverQuest™ Silver Staining (Invitrogen).

For the western blotting, the proteins in the gel were transferred to nitrocellulose membrane (Invitrogen) using NuPAGE transfer buffer (Invitrogen). After blocking with 5% milk in Tris-buffered saline containing 0.1% Tween-20, pH 8.0 (TBST), the membrane was incubated with alkaline phosphatase (AP)-conjugated anti-His_6/G antibody (Invitrogen) for 1 h at room temperature. After three washes with TBST, the blots were developed by addition of 5-bromo-4-chloro-3-indolyl-1-phosphate and nitro blue tetrazolium for visualization.

Results

By sequencing a *P. duboscqi* salivary gland cDNA library, we have identified two transcripts coding for a protein homologous to ADA, an enzyme that metabolizes adenosine to inosine (Kato et al., 2006). The first transcript (PduM73; NCBI accession number DQ835357) of 1846 bp codes for a secreted protein of 57.6 kDa with an isoelectric point of 5.5 (Fig. 1); the second transcript (PduM74) of 1810 bp codes for a protein of 57.2 kDa with an isoelectric point of 5.8 (data not shown). Multiple sequence comparison of *P. duboscqi* ADA with homologues from dipterans such as the sand fly *L. longipalpis* and the mosquitoes *Ae. aegypti*, *Ae. albopictus* and *Culex pipiens* and from mammals such as mice, rats and humans shows an overall low level of identity (Fig. 2); however, the amino acids forming part of the active site (His_{116}, His_{118}, Ala_{121}, Gly_{328}, His_{355}, Glu_{358}, Gly_{381}, Asp_{440}, Asp_{441}) are highly conserved (Fig. 2). The ADA from *P. duboscqi* and from other insects is larger than the ADA from mice, rats or humans; a large string of approximately 80 amino acids at the N-terminal region is not present in the mammalian ADA. Additionally, the signal peptide sequence is not present in the mammalian ADA (Fig. 2). Phylogenetic analysis of ADA from different organisms produced a tree with two distinct clades, one containing ADA from dipteran blood-feeders and the other clade containing other organisms including *Leishmania*, *Plasmodium*, *Entamoeba*, mice, rats and humans (Fig. 3). Within the dipteran blood-feeders clade, sand flies form a distinct group, separate from mosquitoes.

Salivary gland ADA activity

Because of the discovery of the ADA transcripts in the salivary gland cDNA library of *P. duboscqi*, we wanted to test whether the saliva of this sand fly had ADA activity. For this, SGH of *P. duboscqi* was incubated in the presence of adenosine and the reaction was followed spectrophotometrically by scanning from 220 nm to 300 nm every 3 min. The substrate adenosine absorbs at 265 nm, and the product of ADA activity, inosine, absorbs at 241 nm. The equivalent of 0.2 salivary

```
PduM73  MFPRLIVWLLAASAVHAVLDISNIKPKRDYENFLQKYAEYADDEVDRSVGSDITLSLKEKFVNQYLMDLKTEELKAGLKN
PduM74  MFPRLVFCLLAASAVHASL---KIKPKRSYDNFLEQYDNYATDEMDRSVGSDITLSHKEEIVNQYLMDLKTEELKAGLKN

PduM73  PSQFIPSNHFFSVLDRINSSEIFKIIRRMPKGAILHAHDTALCSTDYVVSITYRDHLWQCADPKTGALQFRFSKESPKNT
PduM74  PSQFIPSNHFFSVLNRINSSEIFKIIRRMPKGAILHAHDTALCSTDYVVSLTYRDDLWQCADPKTDALQFRFSKKSPENT

PduM73  DTCQWTPVSEERKNQGEEQYNSKLRSQLSLYNTDPINRSRDVDSIWNDFMGLFGVNFGLLTYAPVWKDYYKQFLKEMMED
PduM74  DTCQWTRVSEERKNQGEEQYNSKLRSQLSLYNTDPINRSRDVDSIWNDFMGLFGVNFGLLTYAPVWKDYYKQFLKEMMED

PduM73  GVQYLELRGTLPPLYDLDGKIYNEEQVVEIYYNVTEEFKKENSTFIGAKFIYAPVRFVNATGIKTLTTTVKQLHERFPDF
PduM74  GVQYLELRGTLPPLYDLDGKIYNEEEVVEIYHNVTEEFKKENSTFIGAKFIYAPVRFVNATGIKTLTTTVKQLHERFPNF

PduM73  LAGFDLVGQEDKGGPLIGFSRELLELPESINFFFHSGETNWNGMTDDNLIAAVTLGTKRIGHGYALFKHPRVLKQVKKDK
PduM74  LAGFDLVGQEDKGGPLIGFSRELLELPESINFFFHSGETNWNGMTDDNLIAAVTLGTKRIGHGYALFKHPRVLKQVRKDK

PduM73  IAIEVCPISNQVLRLVADMRNHPGSILLANKKYPMVISSDDPSFWEATPLSHDFYMAFMGLASYHQDLRMLKQLAINSLE
PduM74  IAIEVCPISNQVLRLVADMRNHPGSILLANKKYPMVISSDDPSFWEATPLSHDFYMAFMGLASYHQDLRMLKQLAINSLE

PduM73  YSSMTLEEKTNAMKLWEAEWEKFIKELETEVFSLLE
PduM74  YSSMASEEKTNAMKLWEAEWEKFIKELETKV-SLLE
```

Fig. 1. Amino acid alignment of the two adenosine deaminase (ADA) molecules derived from transcripts found in *P. duboscqi* salivary glands. Amino acids shaded black are identical and those shaded gray are similar. The secretory signal peptide is italicized.

Fig. 2. Clustal alignments of invertebrate putative salivary adenosine deaminase (ADA) of *P. duboscqi* (PduM73 and PduM74), *Lutzomyia longipalpis*, *Aedes aegypti*, *Aedes albopictus* and *Culex pipiens* and mammalian ADA (mouse, rat and human). Arrowheads indicate conserved amino acids located in the active site of the mammalian enzyme. Black shading indicates amino acid sequence identity, and gray regions indicate conserved amino acid substitutions.

gland pairs (0.2 μg) of *P. duboscqi* converted adenosine to inosine in 30 min (Fig. 4A); by contrast, the same amount of SGH of *P. papatasi* had no effect on adenosine (Fig. 4B). Differential spectrum shows, in better detail, the decrease of adenosine (265 nm) and, over time, the increase of inosine (241 nm) in the presence of *P. duboscqi* SGH (Fig. 4C), indicating the presence of ADA activity in the salivary gland of this sand fly. To test whether this activity is secreted in the saliva of *P. duboscqi*, we compared ADA activity from SGH of unfed sand flies (intact saliva), from SGH of recently blood-fed sand flies (loss of secreted protein by salivation during feeding) and from punctured salivary glands (loss of all or the majority of the salivary contents and therefore any enzymatic activity). Salivary glands from unfed sand flies had the highest ADA activity while preparations from the salivary glands of recently blood-fed sand flies had approximately 70% less activity (Fig. 5). Finally, ADA activity was not detected in the preparations of punctured salivary glands (Fig. 5). Additionally, the amino-terminal sequence of the native protein was detected in the secreted fraction of the SGH of this sand fly (Kato et al., 2006). These data suggest that the molecule responsible for this activity is secreted in the saliva of this sand fly.

Lack of adenosine and AMP in the saliva of P. duboscqi

It was previously shown that *P. papatasi* and *P. argentipes* do not have transcripts coding for the enzyme ADA in their salivary glands or the activity was not detected within their salivary glands (Anderson et al., 2006; Charlab et al., 2000); however, it was shown that these sand flies have large amounts of adenosine and AMP within their salivary glands (Ribeiro et al., 1999). Although counterintuitive, due to the presence of ADA activity, *P. duboscqi* salivary glands were tested for the presence of adenosine and AMP by subjecting *P. duboscqi* SGH to MS-HPLC, and the eluted products were detected by photodiode array detection. As expected, and as previously shown, analysis of *P. papatasi* SGH resulted in the presence of two major peaks with the same retention times as adenosine (18.5 min) and AMP (14.5 min), respectively (Fig. 6). By contrast, *P. duboscqi* SGH showed no peaks at the retention times of adenosine and AMP (Fig. 6). Only a peak at 5 min was observed, which is the secreted proteins from the salivary glands, as determined by the retention time and the absorption spectra at 280 nm (Fig. 6). These data suggest that, in contrast to *P. papatasi* and *P. argentipes*, *P. duboscqi* does not have AMP or adenosine in its salivary glands and that it contains the active salivary ADA.

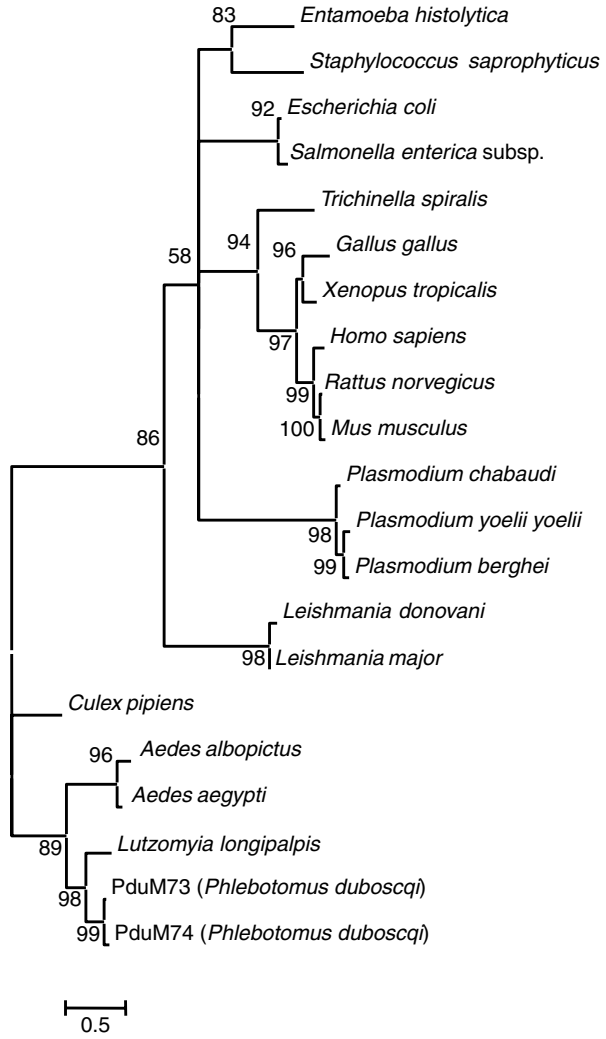

Fig. 3. Phylogenetic tree analysis of putative adenosine deaminase (ADA). Branch lengths are proportional to genetic distance calculated by the ClustalW program. The scale bar represents 0.5% divergence.

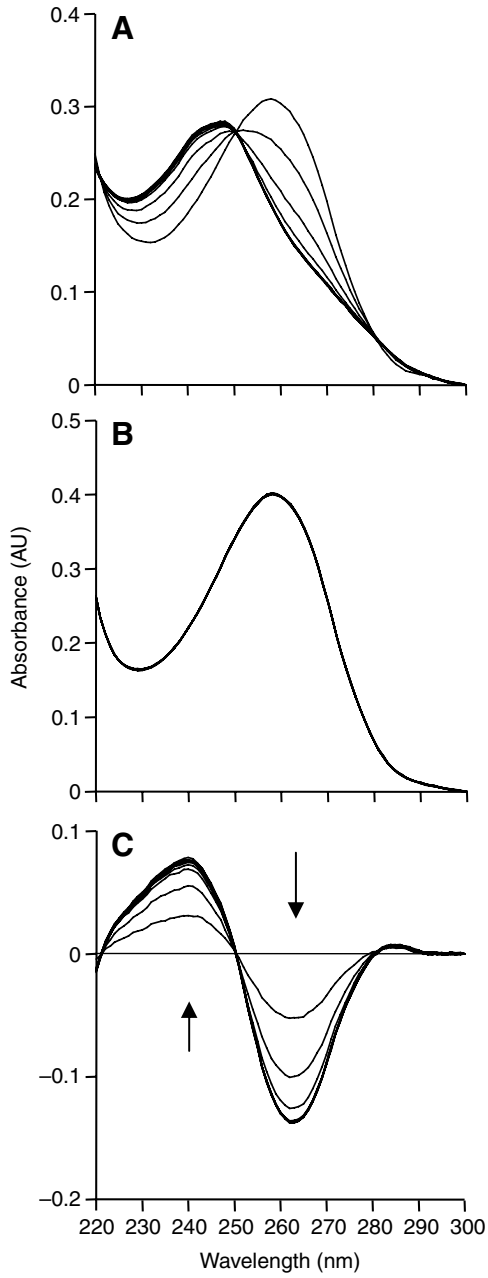

Fig. 4. ADA activity of salivary homogenates of *P. duboscqi*. A cuvette containing 20 μM adenosine in PBS was scanned at 3 min intervals for 30 min following addition of salivary homogenate equivalent to 0.2 pairs of salivary gland from *P. duboscqi* (A) and *P. papatasi* (B). (C) Differential spectra of the data in (A) obtained by subtracting each scan from the scan at time zero. The arrows indicate the direction of change of the spectrum over time.

Expression and activity of recombinant P. duboscqi salivary ADA

In order to determine if the ADA activity detected in *P. duboscqi* SGH was related to the transcript coding for this enzyme, we cloned the two transcripts coding for this protein into the PCRT7NT-TOPO bacterial expression vector. The soluble expressed proteins were purified from the supernatant of bacterial lysate by nickel magnetic beads, and an aliquot was subjected to western blot analysis and detected using anti-histidine antibody. This revealed a protein of approximately 60 kDa, which is the estimated molecular mass of the predicted ADA including the 4-kDa N-terminal addition that includes His_6G and Xpress™ peptide epitopes (Fig. 7A, lanes 1 and 2). No protein of this molecular mass was detected in the supernatant of bacteria expressing the empty vector (Fig. 7A, lane 3). Furthermore, a soluble expressed protein with the same migration pattern was detected by Coomassie blue staining (Fig. 7B, lanes 1 and 2), and this protein was not detected in samples from bacteria transformed with control plasmid (Fig. 7B, lane 3). The purified soluble recombinant proteins were then tested for the presence of ADA activity. Both sand fly recombinant proteins had a high level of ADA activity as detected spectrophotometrically by the conversion of adenosine to inosine (Fig. 8A,B). This activity was not detected in the supernatant of bacteria transformed with control plasmid

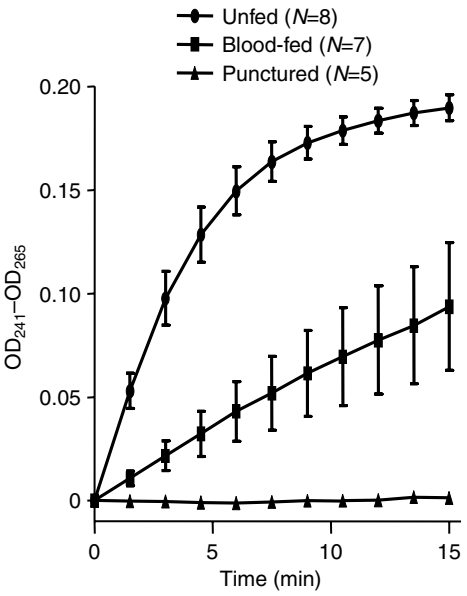

Fig. 5. Salivary adenosine deaminase (ADA) activity from salivary gland homogenate (SGH) of unfed and blood-fed sand flies and from punctured salivary glands. A cuvette containing 20 μmol l^{-1} adenosine in PBS was scanned at 1.5 min intervals for 15 min following addition of salivary homogenate equivalent to 0.2 pairs of salivary gland.

(Fig. 8C). These data suggest that the *P. duboscqi* transcript coding for an ADA is responsible for the ADA activity detected in the saliva of this sand fly.

Discussion

Phlebotomus duboscqi is a proven vector of *Leishmania major* in sub-Saharan Africa. It belongs to the subgenus *Phlebotomus*, together with the sand fly vector *P. papatasi*. Knowledge of the repertoire of salivary activities or molecules from the saliva of *P. duboscqi* is very limited. We recently sequenced a large number of transcripts from the salivary gland of *P. duboscqi* and identified a transcript coding for the enzyme ADA (Kato et al., 2006). This is the first report of ADA in a sand fly from the genus *Phlebotomus*, including data from transcriptome analysis from the salivary glands of *P. papatasi, P. ariasi, P. argentipes* and *P. perniciosus* sand flies (Anderson et al., 2006).

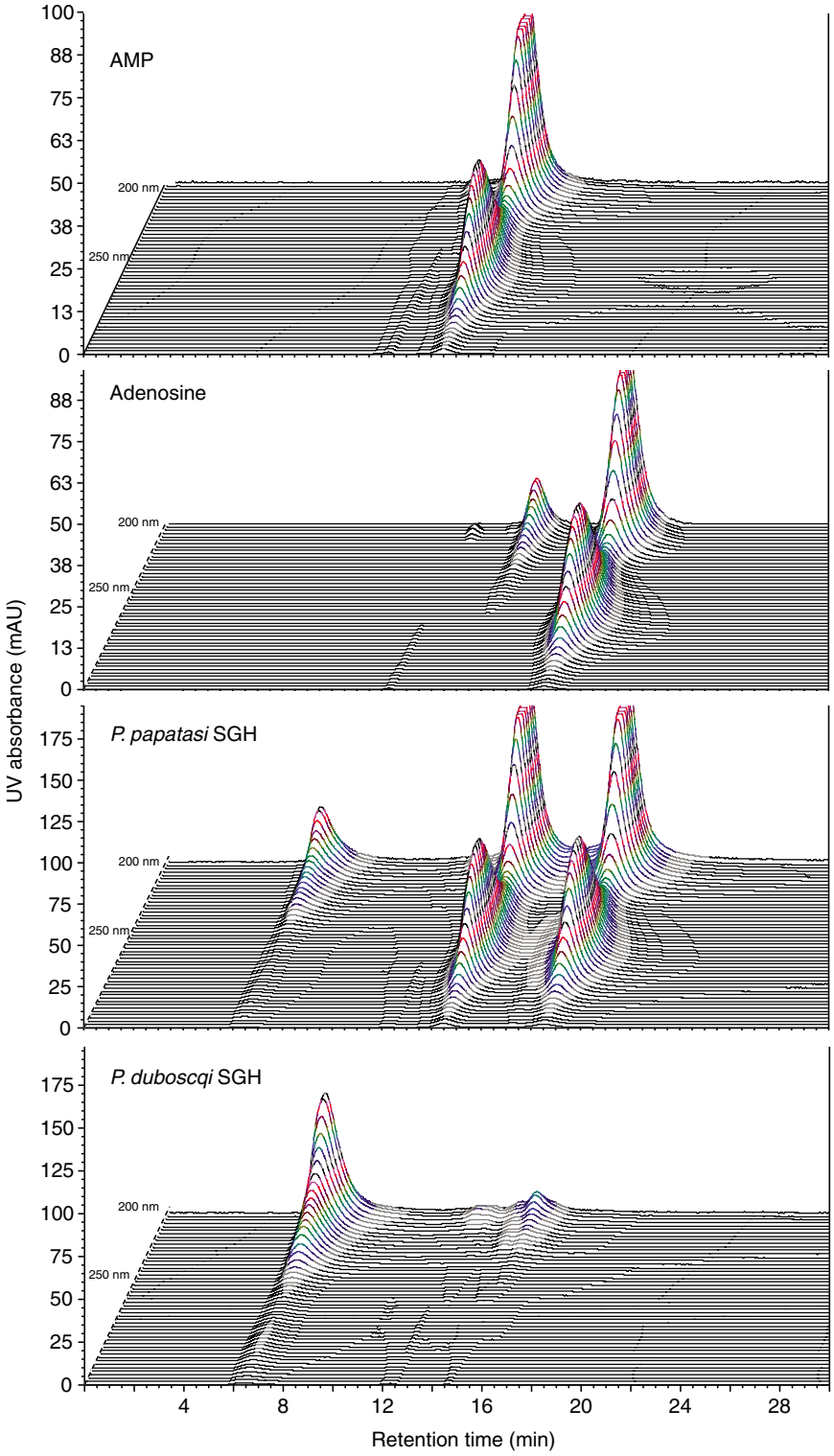

Fig. 6. Three-dimensional chromatographic display of photodiode array data obtained from MS-HPLC of *P. papatasi* and *P. duboscqi* salivary gland homogenate (SGH). The three-dimensional data show retention time on the *x*-axis, UV absorbance on the *y*-axis (in milli absorbance units) and the UV absorbance spectra on the *z*-axis (from 200 nm to 320 nm). Adenosine and AMP standards show retention times of 18.5 and 14.5 min, respectively.

In the present work, we have demonstrated the presence of ADA activity in the saliva of *P. duboscqi* and we also demonstrated that the soluble recombinant protein produced from the transcript coding for this enzyme exhibited ADA activity. Phylogenetic analysis placed *P. duboscqi* ADA

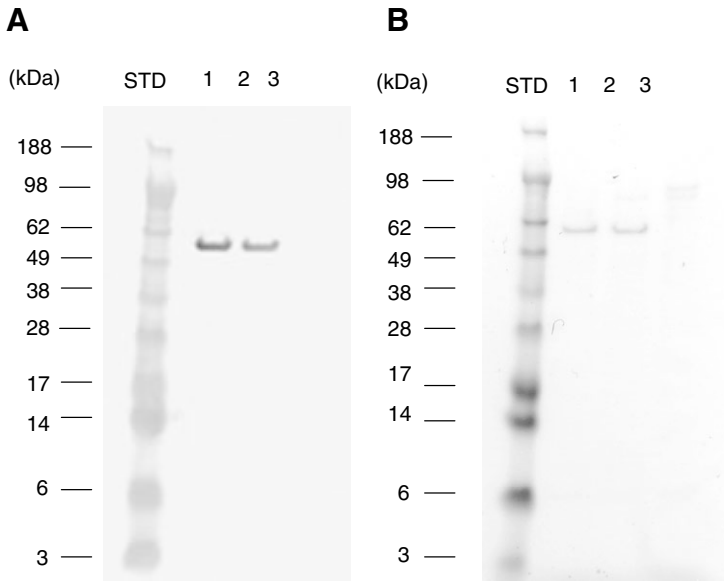

Fig. 7. Expression of recombinant *P. duboscqi* salivary adenosine deaminase (ADA). cDNAs encoding *P. duboscqi* salivary ADA (PduM73 and PduM74) were cloned into PCRT7NT-TOPO vector, and recombinant proteins were expressed in *E. coli*. The affinity column-purified proteins (lane 1, PduM73; lane2, PduM74; lane 3, empty plasmid vector) were analyzed by SDS-PAGE and then subjected to (A) western blotting and (B) Coomassie blue staining.

in the same clade with ADA from other blood-feeding arthropods. This group belongs to the ADGF/CECR1 family of proteins identified previously in *Sarcophaga peregrina*, *Lutzomyia longipalpis*, *Drosophila*, *Aplysia* and humans (Dolezelova et al., 2005). This sub-family of ADAs has an extended N-terminus region and is targeted for secretion. These data suggest that *P. duboscqi* acquired this activity independent of other *Phlebotomus* sand flies. The question remains as to what the role of this protein is in blood feeding. It was previously speculated that the activity may be related to the hydrolysis of adenosine, an important component in pain perception and in immunity (Charlab et al., 2001). What is puzzling is that other *Phlebotomus* sand flies do not have ADA in their salivary glands but they have large amounts of adenosine and AMP, very active vasodilators and platelet inhibitors. Neither adenosine nor AMP was present in the saliva of *P. duboscqi*, as demonstrated in here. *Lutzomyia longipalpis* also lacks adenosine and AMP in its saliva; however, it has maxadilan, a very potent vasodilator (Ribeiro et al., 1989). Maxadilan was not identified in the *P. duboscqi* cDNA library (Kato et al., 2006). Therefore, it appears that *P. duboscqi* may contain a novel vasodilator that will replace the lack of vasodilatory activities exerted by AMP and adenosine in other *Phlebotomus* sand flies. The fact that ADA is present only in *P. duboscqi* and not in other *Phlebotomus* sand flies examined to date emphasizes the ability of blood-feeding arthropods to acquired independent strategies to overcome or modulate the host hemostatic, inflammatory and immune system.

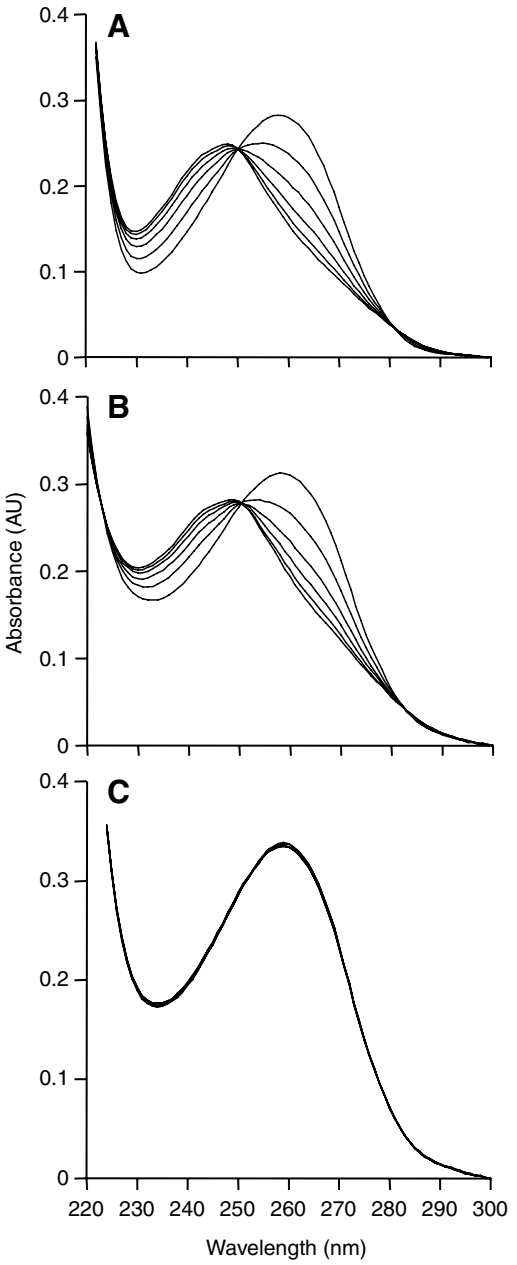

Fig. 8. Enzymatic activity of recombinant *P. duboscqi* salivary adenosine deaminase (ADA). A cuvette containing 20 μmol l^{-1} adenosine in PBS was scanned at 1.5 min intervals for 15 min following addition of recombinant ADA (A, PduM73; B, PduM74) or supernatant of bacteria transformed with control plasmid (C).

ADA has an important role in immunity as a result of the effects of adenosine, 2-deoxyadenosine and the hydrolytic product of these compounds (Cristalli et al., 2001). Further work will be necessary to determine the effect of this enzyme in parasite transmission. It may be possible that this enzyme changes the environment in the skin where the *Leishmania* parasite is deposited by the sand fly. Inosine is the primary metabolite of adenosine by ADA. Inosine has been shown to inhibit the production of proinflammatory cytokines including

TNF-α, IL-1, IL-12, MIP1-α and INFγ in stimulated macrophages and spleen cells (Hasko et al., 2000). Additionally, adenosine and inosine can alter cutaneous vasopermeability by activating A_3 receptors on mast cells (Tilley et al., 2000). Then it may be possible that inosine may favor a Th2 environment that will benefit parasite establishment in the skin of the mammalian host.

We would like to thank Dr J. M. C. Ribeiro for critical discussion on this work, Dr Fabiano Oliveira and Dr Jennifer Anderson for reviewing this manuscript, Dr Robert Gwadz for his continuous support and Nancy Shulman for editorial assistance. This project was funded by Grant 1Z01AI000932-02 to J.G.V. from the Division of Intramural Research, National Institutes of Allergy and Infectious Diseases, National Institutes of Health.

References

Anderson, J. M., Oliveira, F., Kamhawi, S., Mans, B. J., Reynoso, D., Seitz, A. E., Lawyer, P., Garfield, M., Pham, M. and Valenzuela, J. G. (2006). Comparative salivary gland transcriptomics of sandfly vectors of visceral leishmaniasis. *BMC Genomics* **7**, 52.

Charlab, R., Rowton, E. D. and Ribeiro, J. M. (2000). The salivary adenosine deaminase from the sand fly *Lutzomyia longipalpis*. *Exp. Parasitol.* **95**, 45-53.

Charlab, R., Valenzuela, J. G., Andersen, J. and Ribeiro, J. M. (2001). The invertebrate growth factor/CECR1 subfamily of adenosine deaminase proteins. *Gene* **267**, 13-22.

Cristalli, G., Costanzi, S., Lambertucci, C., Lupidi, G., Vittori, S., Volpini, R. and Camaioni, E. (2001). Adenosine deaminase: functional implications and different classes of inhibitors. *Med. Res. Rev.* **21**, 105-128.

Dolezelova, E., Zurovec, M., Dolezal, T., Simek, P. and Bryant, P. J. (2005). The emerging role of adenosine deaminases in insects. *Insect Biochem. Mol. Biol.* **35**, 381-389.

Hasko, G., Kuhel, D. G., Nemeth, Z. H., Mabley, J. G., Stachlewitz, R. F., Virag, L., Lohinai, Z., Southan, G. J., Salzman, A. L. and Szabo, C. (2000). Inosine inhibits inflammatory cytokine production by a posttranscriptional mechanism and protects against endotoxin-induced shock. *J. Immunol.* **164**, 1013-1019.

Jeanmougin, F., Thompson, J. D., Gouy, M., Higgins, D. G. and Gibson, T. J. (1998). Multiple sequence alignment with Clustal X. *Trends Biochem. Sci.* **23**, 403-405.

Kato, H., Anderson, J. M., Kamhawi, S., Oliveira, F., Lawyer, P. G., Pham, V. M., Sangare, C. S., Samake, S., Sissoko, I., Garfield, M. et al. (2006). High degree of conservancy among secreted salivary gland proteins from two geographically distant *Phlebotomus duboscqi* sandflies populations (Mali and Kenya). *BMC Genomics* **7**, 226.

Kumar, S., Tamura, K. and Nei, M. (2004). MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief. Bioinform.* **5**, 150-163.

Resta, R., Hooker, S. W., Laurent, A. B., Jamshedur Rahman, S. M., Franklin, M., Knudsen, T. B., Nadon, N. L. and Thompson, L. F. (1997). Insights into thymic purine metabolism and adenosine deaminase deficiency revealed by transgenic mice overexpressing ecto-5′-nucleotidase (CD73). *J. Clin. Invest.* **99**, 676-683.

Ribeiro, J. M. (1987). Role of saliva in blood-feeding by arthropods. *Annu. Rev. Entomol.* **32**, 463-478.

Ribeiro, J. M. and Francischetti, I. M. (2003). Role of arthropod saliva in blood feeding: sialome and post-sialome perspectives. *Annu. Rev. Entomol.* **48**, 73-88.

Ribeiro, J. M. and Modi, G. (2001). The salivary adenosine/AMP content of *Phlebotomus argentipes* Annandale and Brunetti, the main vector of human kala-azar. *J. Parasitol.* **87**, 915-917.

Ribeiro, J. M., Vachereau, A., Modi, G. B. and Tesh, R. B. (1989). A novel vasodilatory peptide from the salivary glands of the sand fly *Lutzomyia longipalpis*. *Science* **243**, 212-214.

Ribeiro, J. M., Katz, O., Pannell, L. K., Waitumbi, J. and Warburg, A. (1999). Salivary glands of the sand fly *Phlebotomus papatasi* contain pharmacologically active amounts of adenosine and 5′-AMP. *J. Exp. Biol.* **202**, 1551-1559.

Ribeiro, J. M., Charlab, R. and Valenzuela, J. G. (2001). The salivary adenosine deaminase activity of the mosquitoes *Culex quinquefasciatus* and *Aedes aegypti*. *J. Exp. Biol.* **204**, 2001-2010.

Schmidt, H. A., Strimmer, K., Vingron, M. and von Haeseler, A. (2002). TREE-PUZZLE: maximum likelihood phylogenetic analysis using quartets and parallel computing. *Bioinformatics* **18**, 502-504.

Tilley, S. L., Wagoner, V. A., Salvatore, C. A., Jacobson, M. A. and Koller, B. H. (2000). Adenosine and inosine increase cutaneous vasopermeability by activating A(3) receptors on mast cells. *J. Clin. Invest.* **105**, 361-367.

Wilson, D. K., Rudolph, F. B. and Quiocho, F. A. (1991). Atomic structure of adenosine deaminase complexed with a transition-state analog: understanding catalysis and immunodeficiency mutations. *Science* **252**, 1278-1284.