Toward an understanding of the biochemical and pharmacological complexity of the saliva of a hematophagous sand fly *Lutzomyia longipalpis*

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Edited by Fotis C. Kafatos, European Molecular Biology Laboratory, Heidelberg, Germany, and approved October 26, 1999 (received for review July 23, 1999)

The saliva of blood-sucking arthropods contains powerful pharmacologically active substances and may be a vaccine target against some vector-borne diseases. Subtractive cloning combined with biochemical approaches was used to discover activities in the salivary glands of the hematophagous fly *Lutzomyia longipalpis*. Sequences of nine full-length cDNA clones were obtained, five of which are possibly associated with blood-meal acquisition, each having cDNA similarity to: (*i*) the bed bug *Cimex lectularius* apyrase, (*ii*) a 5′-nucleotidase/phosphodiesterase, (*iii*) a hyaluronidase, (*iv*) a protein containing a carbohydrate-recognition domain (CRD), and (*v*) a RGD-containing peptide with no significant matches to known proteins in the BLAST databases. Following these findings, we observed that the salivary apyrase activity of *L. longipalpis* is indeed similar to that of *Cimex* apyrase in its metal requirements. The predicted isoelectric point of the putative apyrase matches the value found for *Lutzomyia* salivary apyrase. A 5′-nucleotidase, as well as hyaluronidase activity, was found in the salivary glands, and the CRD-containing cDNA matches the N-terminal sequence of the HPLC-purified salivary anticlotting protein. A cDNA similar to α-amylase was discovered and salivary enzymatic activity demonstrated for the first time in a blood-sucking arthropod. Full-length clones were also found coding for three proteins of unknown function matching, respectively, the N-terminal sequence of an abundant salivary protein, having similarity to the CAP superfamily of proteins and the *Drosophila* yellow protein. Finally, two partial sequences are reported that match possible housekeeping genes. Subtractive cloning will considerably enhance efforts to unravel the salivary pharmacopeia of blood-sucking arthropods.

The blood-feeding mode evolved independently in at least six different groups of arthropods (1). These insects and ticks had to solve independently the problem of how to deal with their hosts' defense against blood loss. Fifty million years ago, when dinosaurs became extinct, and mammals became the main food source, a new problem was posed by blood platelets, which are much more efficient than bird or reptile (or dinosaur?) thrombocytes in preventing blood loss. Blood-feeding arthropods, even those in the same family, had to discover new pharmacological products to deal with this new feeding problem. Perhaps because of these evolutionary reasons, diverse pharmacological products are being discovered in the saliva of such arthropods, which comprise approximately 15,000 species in nearly 400 genera (1).

Many of the most devastating human diseases are transmitted by insect and tick vectors. Vector saliva is an important factor in the transmission of arboviruses by ticks (2) and mosquitoes (3) and in the transmission of *Leishmania* protozoa by sand flies (4). Indeed, mice preexposed to sand-fly saliva are at a much reduced risk of severe leishmaniasis (5), suggesting that characterization of vector salivary components can lead to the discovery of pharmacologically interesting molecules and, at the same time, to important vaccine targets.

The discovery of salivary gland-derived proteins or peptides from hematophagous arthropods normally follows the route: (*i*) identification of active components in total saliva or salivary gland homogenates, and (*ii*) obtaining peptide sequences by N-terminal degradation or sequencing of protein digest fragments, useful for (*iii*) the design of primers for PCR-based strategies to obtain a useful cDNA fragment, (*iv*) which is used to screen cDNA libraries to obtain the primary sequence of the protein or peptide of interest. In the present work, the sequence of discovery was reversed. We used a PCR-based cDNA subtractive cloning strategy, coupled with biochemical techniques, to find, to our knowledge, products in the salivary glands of the sand fly, *Lutzomyia longipalpis*. The approach was found heuristic, leading to discoveries of new salivary enzymatic activities in addition to speeding up discovery of full-length clones corresponding to previously purified proteins. The coupling of a molecular approach with biochemical and pharmacological testing of the activities can significantly increase the rate of discovery of interesting salivary compounds and will help in identifying novel vaccine targets.

Materials and Methods

Sand flies were reared in the Walter Reed Army Institute of Research as described previously (6). Salivary glands from adult female flies were obtained at 0–1 d after emergence for mRNA isolation or after 2 d for bioactivities.

Enzyme Assays. Hyaluronidase assays were done in 50 μl Hepes buffer 10 mM, pH 7.4/0.15 M NaCl (HS) containing 30 μg/ml hyaluronic acid and the indicated amounts of salivary gland extracts. Reactions were stopped by adding 50 μl of freshly prepared 2.5% cetylpyridinium chloride in 2% NaOH. Absorbance (turbidity) was read at 405 nm after exactly 5 min, with inclusion of a blank and standards of hyaluronic acid. Nucleotidase assays were done by measuring inorganic phosphate release (7). Isoelectric focusing gels (pH 3–9) were done in a Phast system from Pharmacia. Amylase assays used *p*-nitrophenyl α-D-glucosides (PG1 through PG8) obtained through Calbiochem in HS and the indicated amounts of salivary gland homogenates. Substrate and products were examined by RP-HPLC, as described (8).

HPLC. Size-exclusion HPLC used a TSK G2000SW column (7.5 × 600 mm) perfused at 1 ml/min with HS. RP-HPLC of salivary products was done with a Hamilton PRP3 column (2 × 25 mm) perfused at 0.2 ml/min with a gradient of 5% to 60% acetonitrile

This paper was submitted directly (Track II) to the PNAS office.

Abbreviation: pI, isoelectric point.

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Table 1. Full-length cDNA clones containing secretory leading sequences from *L. longipalpis* salivary glands

Category clone	Size, bp	ORF*	Signal† peptide	Mature‡ protein, Da	Predicted pI§	Sequence¶ similarity	Confirmation
Blood feeding							
LuloAPY	1,071	325	21/22	35,071	9.11	*Cimex* apyrase (49/38)	pI, activity
Lulo5NUC	1,839	572	25/26	60,628	6.94	Mouse 5′-nucleotidase (54/46)	5′-Nucleotidase activity
LuloHYA	1,249	376	16/17	42,289	7.98	*Polistes* hyaluronidase (46/38)	Hyaluronidase activity
LuloAC	621	160	19/20	16,732	8.40	*Rattus* selectin (45/37)	Anticlotting activity, Edman
LuloRGD	519	76	23/24	6,160	3.77	RGD motif (no matches)	ND
Sugar feeding							
LuloAMY	1,699	497	18/19	54,023	6.12	*Drosophila* amylase (67/60)	α-Amylase activity
Unknown function							
LuloAG5	905	271	19/20	28,886	8.85	*Drosophila* antigen-5 (38/33)	ND
LuloYELLOW	1,409	412	18/19	45,181	5.78	*Drosophila* yellow protein (36/26)	ND
LuloSL1	713	139	20/21	13,929	8.59	No significant matches	Edman, MS

*Number of amino acids of the translation products; †Signal peptide cleavage site predicted by the SIGNALP (12) program; ‡Predicted molecular mass (Da); §pI of the putative mature proteins; ¶amino acid sequence similarities and identity (in parentheses) always representing a continuous sequence length longer than 75% of the predicted protein sequence, by using GCG-BESTFIT to nearest BLAST match of nonredundant National Center for Biotechnology Informaiton database, both using BLOSUM-62 matrix. ND, not determined.

in 0.1% trifluoroacetic acid in 40 min. Anticlotting assays used 10-μl aliquots either immediately after size exclusion HPLC or after drying 20-μl aliquots from RP-HPLC fractions in the presence of 10 μg BSA and resuspending them in 50 μl HS. Anticlotting assays were done in a microtiter plate, as described (9).

mRNA Isolation and cDNA Library. mRNA was isolated from 123 pairs of salivary glands and 54 carcasses (whole body minus head, legs, and wings) of 0- to 1-d-old female *L. longipalpis* by using the Micro-FastTrack mRNA isolation kit (Invitrogen). A cDNA library was subsequently prepared from ≈100 ng of salivary gland mRNA by using the SMART PCR cDNA Library Construction Kit (CLONTECH). The obtained cDNAs were ligated to the Lambda Zap II vector (Stratagene). The unamplified library has a complexity of 2.34×10^6 recombinants.

Protein Analysis. Edman degradation and mass spectrometry measurements were done at the Harvard Microchemistry facility under the direction of William Lane.

Subtraction cDNA Library. The PCR-Select cDNA Subtraction Kit (CLONTECH) was used to generate a subtracted cDNA library enriched for salivary gland-specific sequences. Briefly, tester and driver cDNAs were prepared from salivary gland and carcass mRNA, respectively, by using the SMART PCR cDNA synthesis strategy (CLONTECH). Subtracted cDNAs were cloned into pCR-Script vector (Stratagene) and sequenced as described (10).

Salivary-Gland cDNA Library Screening and cDNA Clone Isolation. Subtracted cDNA inserts were used as probes to isolate the corresponding full-length clones from a *L. longipalpis* salivary gland cDNA library, as previously described (10). cDNAs were sequenced by using first the M13 forward and reverse primers and then custom primers constructed from the internal sequences of the individual full-length clones. cDNAs are indicated to be salivary-gland specific when they hybridize only to total salivary gland cDNA but not to carcass cDNA. Briefly, subtracted cDNA inserts were labeled by PCR with dUTP-digoxigenin (Genius System; Boehringer Mannheim) by using specific forward and reverse primers and hybridized against salivary gland or carcass total cDNA according to the dot-blot hybridization and detection protocol provided by the Genius System.

Sequence Analysis. Sequence similarity searches were performed by using the BLAST (11) program. Cleavage-site predictions of the mature proteins used the SIGNALP (12) program.

Results

Taking into consideration the pitfalls associated with attributing biochemical function on the basis of cDNA sequence information, the cDNAs obtained in this study (nine full-length clones and two partial clones) can be grouped into four categories: those most likely associated with blood feeding; those most likely associated with sugar feeding; those most likely secreted but with unknown function; and those most likely associated with housekeeping of the salivary glands. The proteins encoded by the full-length cDNA clones contain potential signal peptide sequences, suggesting they are secreted. The data corresponding to the full-length cDNAs and related protein products, e.g., size of the cDNA clones, number of amino acids, molecular weight, and isoelectric point (pI) of the predicted protein products, signal peptide cleavage sites, and percent similarity/identity to closest match on the nonredundant National Center for Biotechnology Information protein database, are summarized in Table 1.

cDNAs Most Likely Associated with Blood Feeding. All five clones below hybridized only to salivary gland cDNA and not to carcass cDNA (data not shown).

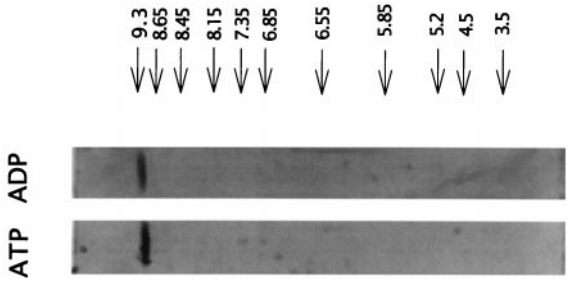

Fig. 1. Isoelectric focusing gel (negative image) of the salivary apyrase of *L. longipalpis*, indicating the basic nature of the enzyme. After the run, gels were exposed to 50 mM Tris·Cl, pH 8.3/0.1 M NaCl/20 mM $CaCl_2$/5 mM either ATP or ADP. Reaction was allowed to proceed for 30 min, allowing for calcium phosphate to precipitate, and was stopped by change to the above solution not containing nucleotide. Two pairs of homogenized salivary glands were used per lane.

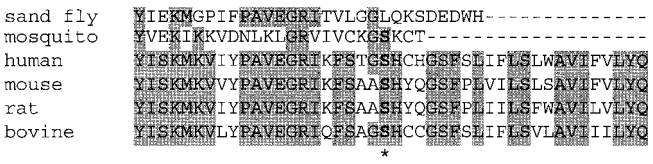

Fig. 2. C-terminal region of the 5′-nucleotidases from Lulo5NUC, *A. aegypti* apyrase (1703351), and human (112825), mouse (539794) and bovine (461441) 5′-nucleotidase. Note that the insect enzymes do not have the terminal hydrophobic stretch. Lulo5NUC also does not have the consensus serine (*), where the inositol phosphate anchor is predicted to be linked to the mammalian proteins.

LuloAPY (AF131933). This cDNA has high similarity to the salivary-gland apyrase cDNA of the bed bug *Cimex lectularius* (10). After removal of a potential signal peptide, the processed protein is predicted to have a pI of 9.1, in good agreement with the experimentally observed pI of 9.3 for an apyrase enzymatic activity in *L. longipalpis* salivary gland extracts (Fig. 1). Similarly to *Cimex* apyrase, which depends solely on Ca^{2+} (13), *L. longipalpis* apyrase was previously reported also to depend solely on Ca^{2+} (14).

Lulo5NUC (AF132510). The protein encoded by this cDNA shows strong similarities to 5′-nucleotidases and phosphodiesterases. Similarly to the salivary apyrase of the *A. aegypti* mosquito (15), it appears to be secreted because it shows an N-terminal potential signal peptide but lacks the hydrophobic C-terminal region, including the consensus serine (16) expected for membrane-anchored enzymes (Fig. 2). Following up on the discovery of Lulo5NUC, we investigated whether a secretory 5′-nucleotidase could be expressed in *L. longipalpis* salivary glands. Indeed, divalent metal-independent 5′-nucleotidase activity was found, in contrast to ADP and ATP hydrolysis, which are Ca^{2+} dependent (Fig. 3). After a blood meal, salivary 5′-nucleotidase decreased 92% from its original value ($P < 0.001$, Mann–Whitney test; $n = 10$) (data not shown), indicating its secretory nature.

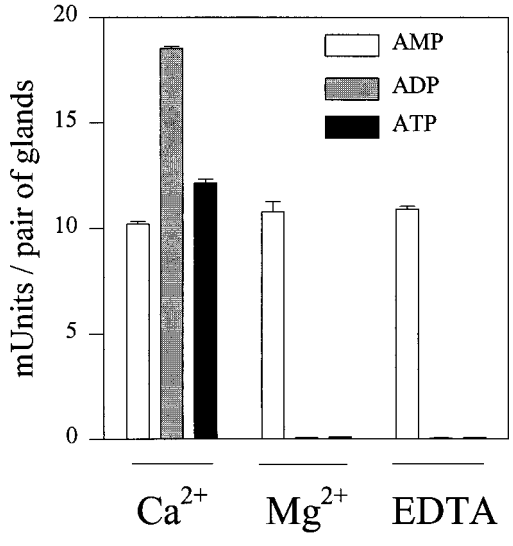

Fig. 3. Hydrolysis of AMP, ADP, and ATP by salivary gland homogenates of *L. longipalpis* and its dependence on divalent cations. Reaction medium was one pair of homogenized salivary glands/ml/50 mM Tris·Cl/0.1 M NaCl/2 mM nucleotide/2 mM divalent cation, or 0.2 mM EDTA and no divalent cation added. One milliunit = 1 nmol orthophosphate released/min at 37°C. Notice calcium dependence for ATP and ADP hydrolysis but not for AMP. Also note that Mg^{2+} does not substitute for Ca^{2+}.

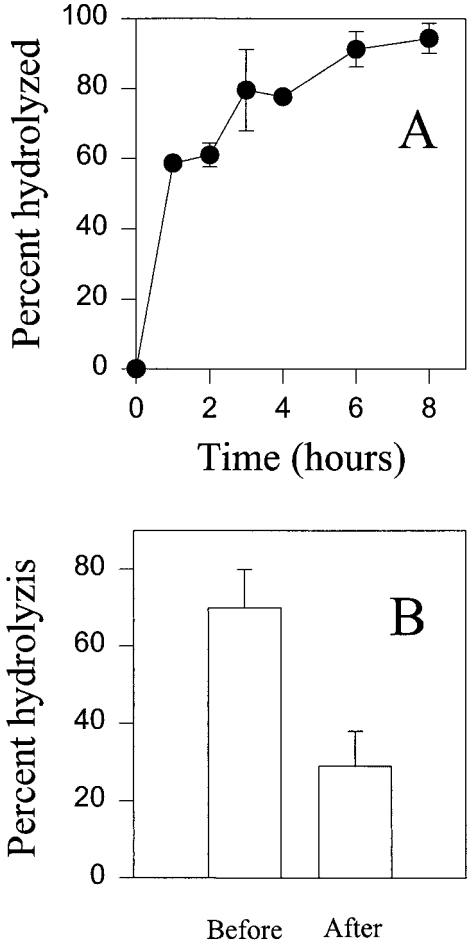

Fig. 4. (*A*) Hyaluronidase activity in *L. longipalpis* salivary glands. The graph indicates hydrolysis of hyaluronate after incubation with one pair of salivary homogenate per 50 μl reaction mixture. (*B*) Decrease of salivary hyaluronidase after a blood meal.

LuloHYAL (AF132515). This cDNA yielded high similarity to invertebrate (bee and vespid) and vertebrate hyaluronidases, indicating the possibility of a hyaluronidase enzyme in *L. longipalpis* salivary glands. This prediction was confirmed and the secretory nature of this activity indicated by its significant loss after a blood meal (Fig. 4).

LuloAC (AF131932). The protein encoded by this cDNA has a C-type (Ca^{2+}- dependent) lectin domain signature, also known as the carbohydrate-recognition domain, and shares similarities to different families of proteins presenting this domain, such as selectins and the macrophage-mannose receptor. Of interest, snake venom proteins active on the clotting cascade also have this signature (17, 18). While purifying the salivary anticlotting from *L. longipalpis*, we found that the predicted amino acid sequence of the LuloAC cDNA from L20 to K37 matched the result from Edman's degradation of the purified salivary anticlotting activity, as shown in Fig. 5. The first 19 amino acids of the deduced protein were thus considered to form the signal peptide. This is in agreement with the sequence analysis of the unprocessed protein, which identified the N-terminal part of the ORF as a potential signal peptide with a predicted cleavage site between residues 19 and 20 (Table 1).

LuloRGD (AF132516). This is a very abundant cDNA that appeared several times in our sequencing project. It has no significant matches in the GenBank database; however, it has an

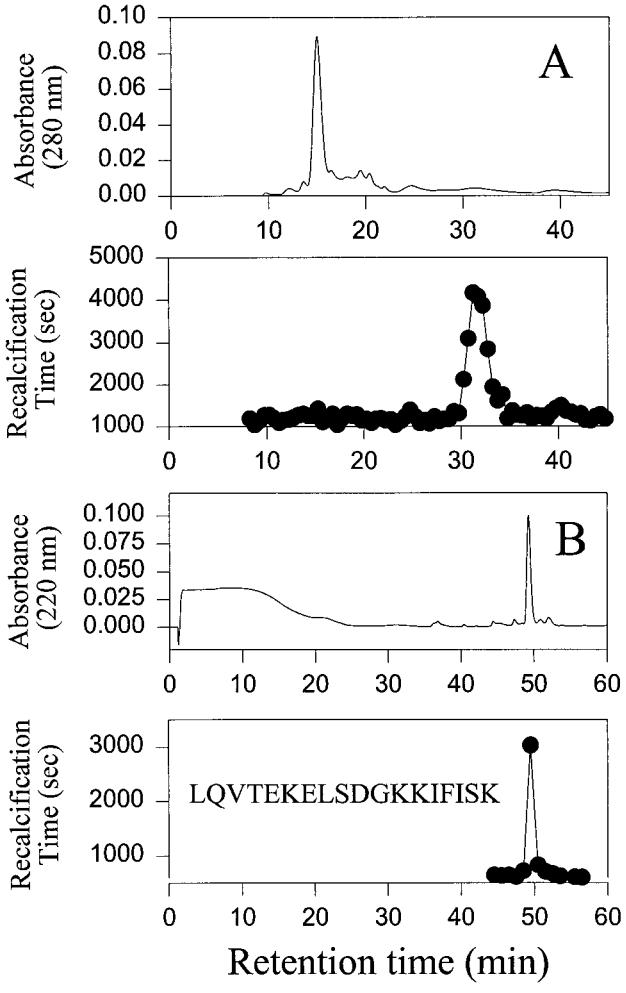

Fig. 5. (*A*) Molecular sieving chromatography of 500 pairs of *L. longipalpis* salivary glands on a TSK-2000SW column perfused with 10 mM Hepes, pH 7.0, and 0.15 M NaCl for 30 min, then a 10-min gradient to 1 M NaCl. Eluted fractions were tested for anticlotting activity on a human plasma recalcification time assay, shown in the symbols. (*B*) RP fractionation of the most active fractions of the size-exclusion chromatogram on a Hamilton PRP-3 column running a gradient from 10% to 60% acetonitrile in water plus 0.1% trifluoroacetic acid. Symbols indicate the plasma recalcification time with aliquots from the column after drying and reconstitution in 10 mM Hepes, pH 7.0/0.15 M NaCl. The sequence obtained by N-terminal Edman degradation is shown.

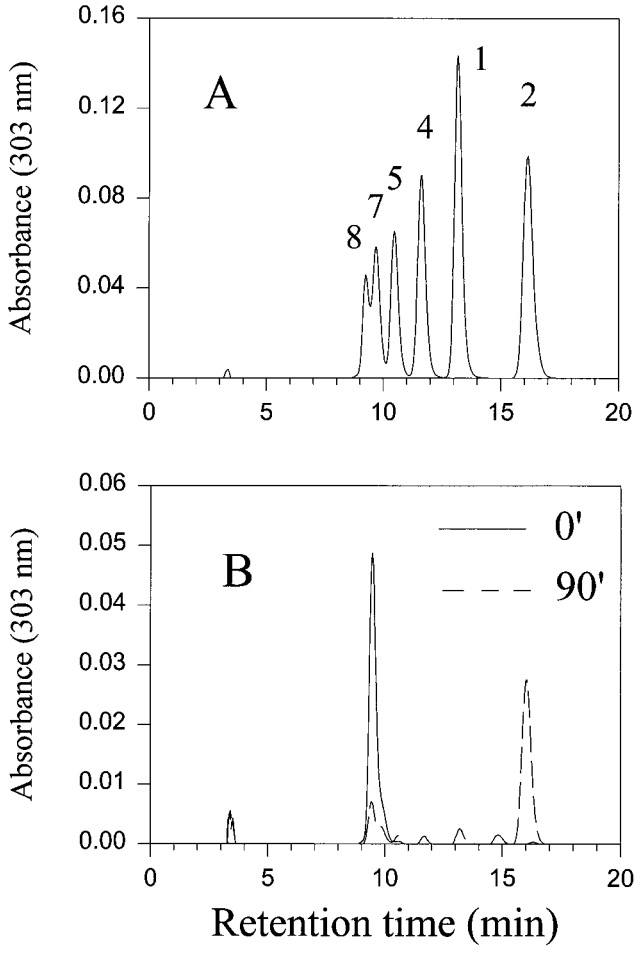

Fig. 6. (*A*) Standards of *p*-nitrophenyl glucosides on RP-HPLC. The numbers refer to the glucoside chain length. The absorbance of the *p*-nitrophenyl group at 303 nm is shown in the ordinate. (*B*) Result from incubating one pair of salivary gland homogenate with 0.1 mM *p*-nitrophenyloctaglucoside at 0- and 90-min incubation. Note conversion of the octaglucoside to the maltoside derivative.

RGD motif, which could be involved in inhibition of platelet aggregation (19).

cDNA Most Likely Associated with Sugar Feeding. The salivary gland-specific cDNA LuloAMY (AF132512) codes for a protein matching many invertebrate amylases, including the putative salivary amylase of the *A. aegypti* mosquito (20). Because no amylase activity has ever been detected in the salivary gland of hematophagous animals, we investigated whether this activity could be found in *L. longipalpis*. Indeed, salivary homogenates degrade *p*-nitrophenyl octaglucoside to the maltoside derivative (Fig. 6), demonstrating the presence of a true α-amylase and absence of α-glucosidase, which is common in mosquito salivary glands (21).

cDNAs Most Likely Associated with Secreted Proteins of Unknown Function. *LuloAG5 (AF132511).* The predicted product of this nonsalivary gland-specific cDNA shows significant sequence similarity to a wide group of mostly secreted proteins comprising the CAP family. Members of this family include vespid venom allergen antigen 5, fire ant and *Drosophila* antigen 5-related proteins, mammalian cysteine-rich secretory proteins, and the helothermine protein of Mexican beaded lizard venom (22, 23).

LuloYELLOW (AF132518). The protein coded by this salivary gland-specific cDNA shares significant sequence similarity to the *Drosophila* yellow protein (24) and with royal jelly proteins and bee milk protein (25).

LuloSL1 (AF132517). One abundant salivary protein from *L. longipalpis* was purified by molecular sieving and RP chromatography (not shown). N-terminal degradation yielded 30 residues of information, and matrix-assisted laser desorption/ionization mass spectrometry (MALDI) indicated a molecular mass of 13,920 Da. The entire amino acid sequence was represented in one of the clones of the subtraction library and, in agreement with MALDI data above, the predicted processed protein has a molecular mass of 13,929 Da. The N-terminal sequence of the native protein is found between the E21 and V50 of the deduced protein. Indeed, the deduced protein contains a hydrophobic stretch identified as a potential signal peptide with a predicted cleavage site between residues 20 and 21. This salivary gland-specific clone has no significant matches in the BLAST database (Table 1).

cDNAs Most Likely Associated with Housekeeping Functions. LuloNaKATPASE (AF132513) and LuloCYT (AF132514) are partial clones that respectively have similarities to the α chain of the sodium potassium ATPase and cytochrome *c* oxidase subunit I genes. They are probably related to housekeeping functions, and we made no effort to characterize them further.

Discussion

Formerly, only the cDNA coding for the vasodilator maxadilan in *L. longipalpis* was known (26). A calcium-dependent apyrase activity was also previously reported (14). In this paper, we report nine full clones and two partial cDNA clones from the salivary glands of this sand fly. cDNA sequence information allowed us to discover activities in sand fly salivary glands and to increase speed in identifying full clones from partial amino acid sequence data from purified proteins. Whether the activities fully correspond to the reported salivary gland-specific cDNAs or result from other gene products, or more than one gene product, remains to be determined. Final assignment of the cDNAs to salivary activities may need expression and/or purification of salivary proteins of interest, tryptic digestion, and MS/MS of the fragments and their comparison to the predicted peptide map by using the correspondent cDNA clone sequences.

Even without a precise gene assignment, however, the discovery of unique cDNAs from the salivary glands of *L. longipalpis* on the basis of similarity searches gave insights into salivary functions that can now be examined. Indeed, a hyaluronidase cDNA and enzymatic activity were found in *L. longipalpis* salivary glands. This is the first report of a hyaluronidase activity in a hematophagous insect salivary gland, although it was previously identified in tick saliva (27), leeches (28), and the worm *Ancylostoma* (29). *L. longipalpis* has very short mouth parts (30) and feeds from intradermal hemorrhagic pools. Salivary hyaluronidase may help diffusion of other pharmacological substances, such as the vasodilator maxadilan (26), which causes the skin erythema typically formed during the feeding of *L. longipalpis*. This activity may also help the spread of such pathogens as *Leishmania* and arboviruses. We have also found a cDNA matching *Cimex* Ca^{2+}-dependent apyrase, which may code for the Ca^{2+}-dependent apyrase of *Lutzomyia*. This cDNA predicts a protein with a pI of 9.1, in agreement with the observed pI for *Lutzomyia* apyrase. In addition, we found evidence for a secretory 5′-nucleotidase activity in a hematophagous arthropod. Although apyrase hydrolyses ATP and ADP to AMP—thus destroying two nucleotides with inflammatory (31) and hemostatic (32) activities—continuation of hydrolysis of AMP allows accumulation of adenosine, which is a potent vasodilator and antiplatelet substance (33, 34). Of interest, the Old World sand fly *Phlebotomus papatasi* has pharmacological amounts of both AMP and adenosine in its salivary glands (35) but no 5′-nucleotidase, underlining the diversity of solutions for the combination of salivary antihemostatic compounds even in two flies of the same family. Of note, the salivary anticlotting of *L. longipalpis* has similarity to members of the selectin family, which has both calcium-binding and carbohydrate-binding domains. This is also the first time that an anticoagulant molecule containing a carbohydrate-recognition domain-like structure is found other than in snake venom. Final proof of its anticlotting activity, as well as other possible activities such as inhibiting neutrophil aggregation, awaits production of recombinant protein for further studies, as is also the case with the RGD-containing peptide possibly associated with inhibition of platelet aggregation (36).

Sand flies, like mosquitoes, feed on sugar meals to obtain an energy source between blood meals. Unlike mosquitoes, which have abundant salivary α-glucosidase activity (21), sand flies do not have such activity (J.M.C.R., unpublished work). Having found a salivary gland-specific cDNA similar to those coding for amylases, we searched for and found salivary amylase activity. Although amylase cDNA is described in the salivary gland of mosquitoes (20), enzymatic salivary amylase activity from a hematophagous arthropod has never been reported. Of interest, sand flies are known to feed directly from plant leaves and stems and thus possibly ingest a diet rich in starch (37).

The cDNAs LuloAG5, LuloYELLOW, and LuloSL1 were found with no evidence for their function. Their translation products are presumably secreted because they have typical signal peptides. The protein encoded by LuloAG5 shares significant similarities to members of a widely distributed family of mostly secreted proteins named CAP family (23, 38), the highest values being with antigen 5 (or antigen 5-related) proteins of *Drosophila*, wasps, and fire ants. Interestingly, a cDNA fragment with similarity to the wasp and ant antigen 5 allergens has also recently been identified in the salivary glands of the malaria vector *Anopheles gambiae* (39). CAP proteins may be associated with the physiology of the secretory process itself, thus explaining its ubiquity and conserved motifs. Other biochemical functions of this large protein family may have been recruited for specific roles, such as in helothermine (40), a salivary toxin from a poisonous lizard. Most other functions of the CAP family remain speculative. LuloYELLOW shares sequence similarity to a gene found in *Drosophila* that causes a yellow mutant (24) when it is disrupted. Its function is thus associated with cuticle pigmentation. Because cuticle pigmentation involves catecholamine metabolism, it is possible that the LuloYELLOW secretion product may catabolize vasoconstrictor catecholamines. The putative translation product of LuloSL1 cDNA matched the N-terminal sequence of an abundant salivary-gland protein. It is also possible that SL1, as well as the putative *Lutzomyia* antigen 5 and yellow–related proteins, have some unknown antihemostatic activity, are "stabilizers" of the other secretory products, or serve to help lubricate the insect mouth parts.

Two cDNA fragments described here have high similarity to the α-subunit of the Na^+-K^+ATPase and to the mitochondrial cytochrome *c* oxidase subunit I. Their representation in a subtracted library may indicate increased expression of genes related to ion transport and cellular energy metabolism in glandular organs, as compared with the average cell in the organism. Because it has been proposed that the pace of evolution of salivary gland products of hematophagous insects may in fact be caused by host species diversity or host immunity (41), the two cDNAs coding for housekeeping genes (LuloCYT and LuloNaKATPASE), together with the additional cDNAs described in this paper, may be of utility in evaluating this hypothesis. Comparison of the sequence diversity of these cDNAs among members of the *Lutzomyia* genus in general and *L. longipalpis* species complex in particular may yield insights into the evolution of blood feeding.

Additional methods may be needed to grasp the full compositional complexity of the saliva of blood-sucking arthropods, particularly for nonsalivary-specific sequences that were subtracted out, and may code for important salivary proteins. Direct mass sequencing of a total cDNA library may yield additional information, as may our continuing attempts to obtain and characterize additional sequences from this subtracted library. Ultimately, the complex salivary antihemostatic potion unique to each blood-sucking arthropod will be known.

We thank Drs. David L. Sacks, Helge Zieler, and Louis H. Miller for critical reading of the manuscript, Ms. My Pham for skillful technical help, and Drs. Thomas Kindt and Robert Gwadz for their support and encouragement.

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