Research Article

Molecular and Immunogenic Properties of Apyrase SP01B and D7-Related SP04 Recombinant Salivary Proteins of *Phlebotomus perniciosus* from Madrid, Spain

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Sand fly salivary proteins are on the spotlight to become vaccine candidates against leishmaniasis and to markers of exposure to sand fly bites due to the host immune responses they elicit. Working with the whole salivary homogenate entails serious drawbacks such as the need for maintaining sand fly colonies and the laborious task of glands dissection. In order to overcome these difficulties, producing recombinant proteins of different vectors has become a major task. In this study, a cDNA library was constructed with the salivary glands of *Phlebotomus perniciosus* from Madrid, Spain, the most widespread vector of *Leishmania infantum* in the Mediterranean basin. Analysis of the cDNA sequences showed several polymorphisms among the previously described salivary transcripts. The apyrase SP01B and the D7-related protein SP04 were successfully cloned, expressed in *Escherichia coli*, and purified. Besides, recombinant proteins were recognized by sera of hamsters and mice previously immunized with saliva through the exposure to uninfected sand fly bites. These results suggest that these two recombinant proteins conserved their immunogenic properties after expression in a prokaryote system. Therefore, this work contributes to expand the knowledge of *P. perniciosus* saliva that would be eventually used for the development of tools for vector control programs.

1. Introduction

Leishmaniasis is still one of the most important vectorborne diseases in terms of incidence as two million people per year are affected worldwide [1]. The causative agent of the aforementioned disease is Leishmania spp., a flagellate parasite which is transmitted by infected phlebotomine sand flies (Diptera: Psychodidae) during blood feeding. Arthropod saliva is actively involved in the transmission of pathogens to its host as it contains a complex cocktail of antihaemostatic and immunomodulatory molecules that are inoculated into the host skin during blood feeding of both infected and noninfected sand flies [2]. Concretely, sand fly salivary components are known to play an important role in the establishment of Leishmania spp. infection [3]. In the last years, research on sand fly salivary proteins has greatly increased, suggesting that salivary proteins could be successfully assayed both as anti-Leishmania vaccine candidates and as markers of exposure to sand flies [4, 5]. As hosts are bitten, they develop both humoral and cellular responses against sand fly saliva [6]. Moreover, a positive correlation has been observed between the number of bites and antibody levels [4, 7, 8]. Therefore, host exposure to sand flies can be measured by evaluating humoral responses against salivary antigens. This methodology is being applied by mainly using salivary gland extracts [4, 9–12]. Recombinant salivary proteins have already been produced for sand fly species such as *Lutzomyia longipalpis* and *Phlebotomus papatasi* [4, 8, 10, 11, 13, 14]. Some of these proteins have been already proved as good markers of exposure [11, 14]. However, since salivary proteins display high specificity, it is necessary to produce immunogenic salivary proteins for other sand fly species.

In the western Mediterranean basin, *Leishmania infantum* is mainly transmitted by *Phlebotomus perniciosus* in an anthropozoonotic cycle where dogs have been traditionally considered the main reservoir [1]. However, other potential reservoirs such as hares (*Lepus granatensis*) have been recently involved in *L. infantum* transmission at a human leishmaniasis outbreak in Madrid [15, 16]. As a part of a management plan to control the disease in this environment, measuring exposure of reservoirs to the main vector involved, *P. perniciosus*, through the detection of anti-saliva antibodies, would be useful to evaluate whether actions taken to reduce leishmaniasis have been effective as previously done in India and Nepal [9].

In previous studies, our group described immunogenic salivary proteins of *P. perniciosus* and *P. argentipes*. These proteins were identified through the combination of twodimensional electrophoresis and Western blot with sera of mice and hamsters experimentally exposed to uninfected sand flies [17, 18]. Therefore, in this study, our aim was to obtain some of the *P. perniciosus* salivary immunogenic molecules as recombinant proteins, through a cDNA library from salivary glands, from *P. perniciosus* and subsequent studies of the immunogenicity of the purified salivary proteins. Moreover, several polymorphisms between transcripts of the *P. perniciosus* cDNA from Madrid were compared to the previously annotated ones that belonged to specimens from Italy [19].

2. Material and Methods

2.1. Sand Flies and Salivary Glands Collection. P. perniciosus sand flies were maintained at 27°C and 17:7 light-darkness photoperiod at the Medical Entomology Unit of the Instituto de Salud Carlos III (ISCIII), Madrid, Spain. This colony was established in 1987 from sand flies captured at a leishmaniasis endemic area of Madrid [20]. Salivary glands from recently emerged up to 1-day-old sand flies were dissected and stored in RNA*later* (Invitrogen, San Diego, CA).

2.2. Salivary Gland cDNA Library Construction. A cDNA library was constructed with mRNA isolated from 165 salivary glands using the Micro-FastTrack mRNA isolation kit (Invitrogen, San Diego, CA). After isolation, mRNA was reverse transcribted to cDNA and subsequently amplified by PCR following the instructions of the SMART cDNA library construction kit (Clontech). cDNAs were then fractionated by column chromatography before cloning. Directional cloning into λ TriplEx2 vector (Clontech) was achieved through *Sf*IAB flanking sites incorporated by PCR during the cDNA amplification. The pool of cDNAs cloned into λ TriplEx2 vector was packaged into phage particles following the manufacturer's instructions (Gigapack III Gold Packaging Extract, Agilent). The resulting cDNA library was amplified following general molecular biology protocols [21].

2.3. cDNA Library Screening, Sequencing, and Bioinformatics. After plating the library by infecting log phase XL1-blue cells (Clontech) in the presence of β -D-thiogalactoside (IPTG) and 5-bromo-4-chloro-indoly1- β -D-galactopyranoside (X-Gal), white plaques were randomly picked from the agar plates and analyzed by PCR using λ TriplEx2 primers (forward: 5'-TCCGAGATCTGGACGAGC-3' and reverse: 5'-TAATACGACTCACTATAGGGC-3'). Recombinant phages which presented the greatest insert sizes were converted into pTriplEx2 plasmid through Cre recombinasemediated site-specific recombination. Plasmid DNA was then isolated and sequenced using the primer 5' λ TriplEx LD: 5'-CTCGGGAAGCGCGCCATTGTGTTGGT-3' and the BigDye Terminator v3.1 Cycle Sequencing kit (PE Biosystems, Foster City, CA) in an ABI PRISM 3730XL DNA Analyzer (Applied Biosystems). Both strains of clones of interest were subsequently sequenced with reverse primer $3'\lambda$ TriplEx LD: 5'-ATACGACTCACTATAGGGCGAATTGGCC-3'. DNA electropherograms were manually inspected and corrected using Chromas program (McCarthy, Queensland, Australia). Sequence identities were determined by BLAST (http://blast.ncbi.nlm.nih.gov/). DNASTAR software (Lasergene, Madison, WI) was used for nucleotidic alignments (SeqMan program), and protein features were assigned by Protean tools. Amino acid sequences were aligned with ClustalW (http://www.ch.embnet.org/software/ClustalW.html) and refined using Boxshade server (http://www.ch.embnet .org/software/BOX_form.html). Glycosylation sites were predicted using NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/ services/NetNGlyc/) and NetOGlyc 3.1 Server (http://www .cbs.dtu.dk/services/NetOGlyc/) for N- and O-glycosylation sites, respectively [22, 23]. Phosphorylation sites prediction was done using NetPhos 2.0 Server (http://www.cbs.dtu .dk/services/NetPhos/) [24].

2.4. Cloning of Salivary Gland cDNAs. Salivary coding sequences of the apyrase SP01B and the D7-related protein SP04 were amplified by PCR from the corresponding pTriplEx2 plasmids using specific primers. cDNAs were cloned into PCR4-TOPO plasmid and transformed into TOP10 competent cells (Invitrogen, San Diego, CA). Restriction sites were incorporated by PCR from the recombinant PCR4-TOPO vector. SalI (forward and reverse) was used as a restriction site (represented as bold letters): SP01B: 5'-GTC-GACATGATATTGTTGAAATTG-3' and 5'-GTCGACTTA-CTTAATTCCTTTGGG-3' and SP04: 5'-GAGAGAGTCG-ACATGAATACCTTATTG-3' and 5'-GAGAGAGTCGAC-CTAATAATTTGTTAATG-3'. The primers were manually designed according to the sequences obtained. All primers were synthesized at the Genomic Unit of ISCIII. The highfidelity polymerase Pfu Turbo Hotstart (Stratagene, La Jolla, CA) was used in order to avoid point mutations. Coding sequences were excised from the plasmid by enzymatic digestion and ligated into pqE31 vector (Qiagen, Hilden, Germany). The recombinant expression vectors pqE31 were transformed into competent M15 (Qiagen, Hilden, Germany). All construction steps were verified by sequencing.

2.5. Expression and Purification of Recombinant Salivary Proteins. Different expression and purification conditions were tested in order to optimize the processes for these specific proteins. Cultures of M15 cells that contained the pqE31 recombinant plasmid for SP01B and SP04 were grown in Luria-Bertani medium containing ampicillin ($100 \mu g/mL$). Protein expression was induced by the addition of IPTG to a final concentration of 1 mM, and cultures were grown at 37°C for 3 hours. Bacterial cells were collected by centrifugation and lysed in 6 M guanidine hydrochloride, 100 mM NaH₂PO₄, 10 mM Tris-HCl, and pH 8. Cell debris was then separated by centrifugation, and the resultant supernatant was submitted to affinity chromatography by using Ni-NTA Superflow resin in prepacked 5 mL columns. Proteins were purified under denaturing conditions following the manufacturer's instructions (Qiagen, Hilden, Germany). Protein refolding was done by removing urea through dialysis against PBS (SnakeSkin Dialysis Tubing 10 kDa MWCO, Thermo Scientific, Goettingen, Germany). Proteins were then concentrated using the stirred ultrafiltration cell with 10 kDa MWCO membranes (Millipore, Bedford, MA) and quantified by gel in comparison with BSA standards.

2.6. Western Blotting. Western blots were performed following standard protocols. Briefly, $1 \mu g$ of recombinant protein was separated by SDS-PAGE and electroblotted onto a PVDF membrane which was incubated overnight in blocking buffer (3% BSA, Sigma, St. Louis, CA; 2% ECL Blocking reagent, Amersham, Piscataway, NJ). After washing, membranes were incubated for 2 hours with pooled sera (1:25) of either hamsters that were immunized against P. perniciosus saliva by the bite of uninfected sand flies through the exposure to 100 sand flies on a weekly schedule over 10 weeks [17] or mice exposed 13 times to 150 P. perniciosus (unpublished). Goat anti-hamster IgG and goat anti-mouse IgG peroxidaseconjugated antibodies (1:3500, Southern Biotech, Birmingham, AL and 1:500, AbD Serotec, resp.) were used, and immunogenic proteins were visualized by CN/DAB reagent following the manufacturer's instructions (Thermo Scientific, Goettingen, Germany). Experiments with sera of immunized and nonimmunized animals were carried out in parallel. For His-tag detection, mouse anti-RGS-His antibodies (1:3000, Qiagen, Hilden, Germany) were used in combination with the goat anti-mouse IgG peroxidase-conjugated antibodies (AbD Serotec).

3. Results and Discussion

3.1. cDNA Library Analysis. The expression library showed a titer of 3.6×10^6 and 17.6% of nonrecombinant clones. The amplified library displayed a larger number of independent clones (1.2×10^{12}) and a low number of nonrecombinant clones (1.92%). Therefore, this amplified library was used for subsequent analysis and cloning processes. 201 white plaques were randomly picked from IPTG/X-Gal plates and analyzed by PCR. 36 out of 201 plaques showing the greatest insert size were selected, converted into pTriplEx2, and further sequenced. Among the 25 complete transcripts, they were identified mostly as apyrases (SP01, SP01B), D7-related proteins (SP04, SP04B), yellow proteins (SP03B), ParSP25-like proteins (SP08), lufaxin-like proteins (SP06), and PpSP15-like proteins (SP02, SP09, and SP11). Complete sequences were annotated at EMBL nucleotide database, and NCBI accession numbers are shown in Table 1.

Overall, high degree of conservancy was found among the salivary transcripts we sequenced, and their best matches available in nonredundant databases were obtained from a cDNA library from *P. perniciosus* from Italy [19]. This finding is in contrast to the high level of divergence found for the salivary protein maxadilan of *L. longipalpis* from distinct locations [25].

In the case of maxadilan, the high level of polymorphisms elicits variant-specific antibodies with little cross-reactivity, and it has been suggested that sand flies may have evolved diversity in maxadilan as a strategy to evade the host immune response against this essential vasodilator peptide that facilitates blood feeding of L. longipalpis. Balancing selection might be maintaining many maxadilan alleles with equivalent vasodilatory potencies [25, 26]. On the other hand, our results match the description of a high degree of conservancy among salivary transcripts in two geographically distant Phlebotomus duboscqi sand fly populations in Africa, and it was suggested that sand flies belonging to the genus Phlebotomus show greater degree of conservancy than Lutzomyia spp. [27]. Following this trend, in previous experiments of our group, we did not find qualitative differences in the salivary protein profile among three colonies of P. perniciosus collected from different areas of Spain and reared under identical laboratory conditions [17]. Moreover, a recent comparison between transcripts from cDNA libraries constructed with P. papatasi strains from Israel and Tunisia showed a high level of conservancy [28]. Indeed, the high degree of conservancy found between the salivary transcripts of P. perniciosus from Spain and Italy may be a reflection of little evolutionary pressure from the host immune response on the analyzed salivary proteins in contrast with previous observations of maxadilan. Moreover, studies on the evolution of apyrase from several Phlebotomus species show high degree of conservancy, and the geographical pattern of genetic variation was consistent with neutral demographic processes mainly regional isolation and isolation by distance resulting from the changes that occurred during the late Pleistocene [29]. In the case of secretary salivary proteins, it is possible that the presence of multiple copies reflects an adaptation to increase the production of these important proteins for sand flies [30]. Salivary peptides often occur within and between gene families, for which there is evidence for overlapping functions [19]. Redundancy is a common property among salivary proteins of sand flies and other arthropods [17, 31], and it has been suggested that it might play a role in ensuring blood-feeding success [32].

Therefore, several polymorphisms were found when comparing the sequenced salivary transcripts in this work with their corresponding annotated transcripts. Although these polymorphisms are expected among populations, we aimed to *in silico* study in detail whether these polymorphisms could lead to changes in phosphorylation and glycosylation processes, structure, immunogenic properties, and functionality through bioinformatics-predictive programs. In order to confirm these single nucleotide polymorphisms (SNPs) both 5' and 3' strands were sequenced and aligned. Most of the SNPs were located among the 5' and 3' untranslated regions (UTRs) which are known for the high variability [33].

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The number of nucleotidic polymorphisms fits the values of nonidentical nucleotides (column 5) minus the gaps (column 4). ^b Protein antigenic regions were predicted by *in silico* bioinformatics (Jameson-Wolf index, Protean program, DNAstar, Lasergene). ^cN-, and O-glycosylation sites prediction performed with NetNGlyc 1.0 Server and NetOGlyc 3.1 Server.

^d Phosphorylation sites prediction performed with NetPhos 2.0 Server. ^e Alpha helix and beta sheet locations were predicted by Garnier-Robson algorithm (Protean program, DNASTAR, Lasergene).

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m f} {
m Two}$ deletions in the nucleotidic sequence of SP02 (HE985075) lead to a switch of the reading frame of the gene.

On the other hand, several SNPs were found on the translated region, and some of them resulted in amino acid changes. Substitutions in coding regions may influence protein structure, and therefore electrostatic forces and ligand affinity may involve changes in protein function [34]. Prediction of locations of alpha and beta regions, as well as the antigenic index and phosphorylation sites for the sequenced proteins, confirmed the presence of several changes from their best matches in databases as shown in Table 1. Polymorphisms found in SP01B and SP04 resulted in changes of the antigenic index from positive to negative values and vice versa when compared to the Italian strain (located at T¹⁶⁶ for SP01B and R⁵¹ for SP04). In the case of phosphorylation residues, most of the analyzed transcripts presented changes regarding to the P. perniciosus Italian strain. For instance, in the apyrase sequence SP01B, the amino acids T¹⁶³ and Y¹⁴⁸ were predicted to be phosphorylated in the Italian strain but not in the Spanish one (Figure 1). Although point mutations can change the ability of the protein to bind carbohydrates, the SNPs were not located at the N- and O-glycosylation sites as in silico predicted through bioinformatic programs (Table 1).

A description of the salivary proteins from *P. perniciosus* Spanish strain is listed below.

3.1.1. Apyrases. SP01 and SP01B belong to the protein family of *Cimex* apyrases which are typically found in sand flies and bed bugs. These enzymes hydrolyze nucleotide di- and triphosphates to orthophosphates and mononucleotides and act as potent antihaemostatic factors [13]. Transcripts coding for SP01 and SP01B in our library are conserved with the respective genes from *P. perniciosus* from Italy (Figure 1).

3.1.2. Odorant-Binding-Related Proteins. In sand flies two salivary protein families are included among the odorant-binding protein superfamily; the D7-related proteins and the PpSP15-like proteins [35, 36].

The SP04 and SP04B are classified as D7-related proteins. D7-related proteins are widely distributed in the saliva of haematophagous Diptera. One of the first cloned proteins from the saliva of *Aedes aegypti* was arbitrarily called D7 [37], and since then similar proteins have been identified in the saliva of other Nematocera such as mosquitoes, black flies, *Culicoides*, and sand flies [36]. Further studies showed that the odorant-binding-protein superfamily had a multigene organization and due to gene duplication processes produced two distinct forms of proteins called long and short forms [38]. Sand fly D7-related proteins are related to the long form [39].

In mosquitoes, D7 proteins are able to bind biogenic amines and leukotrienes, in addition to various components of the coagulation cascade, thus interfering with the haemostatic and host immune responses [40]. The role they play in sand flies has not yet been clarified however, their great representation at both transcriptomic and proteomic levels [17–19, 28, 41] and the conservation of the cysteinyl leukotrienes-binding motifs [42] suggest an involvement in counteracting the coagulation cascade [41]. Concretely,

the binding site for thromboxane A2 (TXA2) analogs in A. stephensi D7 protein (GenBank ID: 315583502) lies in a hydrophobic pocket in the N-terminal domain which accommodates a large portion of the fatty acid chain. Tyrosine at position 52 in A. stephensi D7 protein is known to stabilize the TXA2 analogs through hydrogen bonds [42]. In A. aegypti D7 protein (GenBank ID: PDB:3DXL_A), a mutation of this position from tyrosine to phenylalanine leads to a failure to bind TXA2 analogs [43]. In sand flies, most of the D7-related proteins conserved a residue of tyrosine in this position (Y⁶⁵); however, both SP04 from *P. perniciosus* Spanish and Italian strains contain a phenylalanine in that position (Figure 2). Moreover, in the D7-related protein SP04 from the P. perniciosus Spanish strain (GenBank ID: CCK73754), one of the cysteinyl leukotriene-binding motifs located at R⁵¹ showed a polymorphism (Figure 2). However, without experimental confirmation of the functionality of D7-related proteins in sand flies, these changes in the binding motif remain to be elucidated. The D7-related protein SP04B from the Spanish strain (GenBank ID: HE980443) showed two deletions between T^{546} and A^{547} that lead to a switch in the reading frame of the gene and an earlier stop codon (⁵⁴⁶TAG⁵⁴⁸, data not shown). The predicted protein should appear in the polyacrylamide gels at a theoretical molecular weight (Mw) of 20.7 kDa, however, it had not been found at that Mw [17, 19]. In order to check whether all SP04B clones present in this library included this mutation, we amplified the SP04B gene with primers flanking the CDS, the cDNA library as a template, and further sequenced the extracted DNA. We found the complete sequence for SP04B without that mutation, confirming that in our library both clones are present.

SP02, SP09, and SP11 belong to the PpSP15-like family which have been found only in sand flies and represent the most abundant family among sand fly salivary proteins [19, 41, 44]. PpSP15-like proteins were described as highly polymorphic and derived from a multicopy gene family [19, 30]. The sequences of these proteins show a high degree of variability in addition to certain conserved cysteine residues [28]. In P. papatasi, large numbers of variants of the PpSP15 gene were observed, and differences between alleles were small and resulted in only few amino acid substitutions [30]. Therefore, polymorphisms within transcripts are expected, as shown in other species. Some transcripts, such as SP02, were found to be highly representative within the cDNA library (Figure 3). One of the sequenced clones of SP02 (Gen-Bank ID: HE985075) presented a single nucleotide deletion between A³⁴⁰ and T³⁴¹ that leads to a switch in the reading frame of the gene. As a consequence, the putative-translated protein (GenBank ID: CCM43815) would differ in sequence and length since it contains an earlier stop codon (⁴¹⁷TGA⁴¹⁹, Figure 3). Although these changes do not seem to affect its immunogenicity, as the polymorphisms were found within an area of low antigenicity according to the Jameson-Wolf index (data not shown), the function of this protein family remains unknown. Interestingly, immunization with P. papatasi SP15 protected mice from cutaneous leishmaniasis caused by Leishmania major [45].

Dub_ABI20151	22	DNYK <mark>SIVKLCEL</mark> YKVADKYSFSMK-DEHH
Pap_AF261768	22	DNYKSIVKVGBLIEVG-DKYSVKMK-KEDH
Ser_ADJ54110	22	APQCGKSFNFAILADLDKKSISKTDANNFKSIVKLGELTQVGTKYDIVMKNKEDR
Ara_ACS93496	21	QKSFT <mark>SIVKYGEL</mark> KDNGERYTLTMK-SENL
Ari_AAX56357	21	QKSFTSIVRYGELKDNGBRYTLSIK-SENL
PerIta_ABB00907	22	QKSTSIVKYGELKDNGBRYTLSLK-SENL
PerSpa_CCK33660	22	QKSTSIVKYGELKDNGERYTLSLK-SENL
Tob_ADJ54077	22	QKSFTSIVKYGELKHNGERYTLSLK-SENL
Arg_ABA12135	22	GRQFTSIVKYGELRDNGENYDLTMK-SQNL
Lon_AF131933	22	
Int_AFP99246	18	
Aya_BAM69107	22	RT NSI KIDELRHNTRTG YNFVIS-RVKK
Hum_Q8WVQ1	69	PPTHNAHNWRLGQAP-ANWYNDTYPLSPPQRTPAG-IRYRIAVIADIDTESRAQEENTOFSYLKKGYLTLSDSGDKVAVEWDKDHG-
Dro_CAL26008	77	-SYTDARSMDNGGPV-VOAYNATYPLTSPMVLRG VINYRIAMIAD DTSSKVSKGDGSST MRSY KKGYLTYTMARSEIOISWDDGAPI
Cim_AAD09177	30	FMLVQSYELGHASGETNANSKYPLTTPVEENLK-VRFKIGVISDDDKNAVSKDESNTWVSTYLTGTLEWEKSTDKITVQWDKGNEK
		$\mathbf{v} + \mathbf{v} + $
Dub_ABI20151	77	EVFTKYAYKGRGAELSEFLYYKWKLYTFDDKSGIVFKLKNNADLYPWYLLANGDOQVDOFKAEWATTKGDKYVGSTGISSDS-TGKL
Pap_AF261768	77	EIFTKYAYK <mark>GGGAELSEFLIYKWKLYTFDDKSGIVFRLKTNADLIPWVTLANGNGDQTDGFKAEWATTK</mark> GDKMYVGSIGISFTDK-TGKL
Ser_ADJ54110	78	EIFTKYAYR <mark>GRGAELSEF</mark> LRFNRKLYSFDDKSGIVFQLKDNADLVPWVVLANGDGNQKDGFKAEWATAKDGK <u>YVG</u> SIGLS, TDK-SGIP
Ara_ACS93496	75	HYFTRYAYK <mark>GRGAELSELLYFN</mark> NK LYSIDDKTGIIFE VKHGD LIPWVILSNGDG NQKD <mark>GFKAEWATVKGDKLIVGSTG</mark> /P*FNDKHQIL
Ari_AAX56357	75	HYFTRYAYNGRGAELSELLYFNNKLYTIDDKIGIIFEVKHGDLIPWVILSNGDGNQKNGFKAEWATVKGDKLIVGSIG PNFEEKTQSL
PerIta_ABB00907	76	HYFTRYSYNGRGAELSELLYFNDKLYTIGDKIGIVFEVVHGDLIPWVILSNGPGNQKDGFKAEWATVKGDKLYVXSIGMIELDKRTGTI
PerSpa_CCK33660	76	HYFTRYSYNGRGAELSELLYFNDKLYTIGDKTGIVFEVVHGODLIPWVILSNGPGNQKDGFKAEWATVKGDKLWYKGSTGMTOLDKRTCTI
Tob_ADJ54077	76	HYFTRYAYNGRG AELSELLYFNDXLYTIGDKIGIVFEVKHGODLIPWVILANGPGNQKDGFKAEWATVKDDKLYVGSIG / TFLDKRTGNI
Arg_ABA12135	76	HYFTRFAYNGRGAELSELLNFNSKLFTVDDKTGIVFEVKYGONLIPWVILANGNSNKQEGMKAEWATRKGDKMYVGSIGLMMYNEKTKET
Lon_AF131933	78	PVSURVEFKERGAELSEIVVENNKLYTVDDKSEITERITKDEKEFPWVILADADEORPDEKEEWATIKDDTIVVESIGVLKFTS
Int AFP99246	75	PVTOKYGENGEG ELSEVVVVNN LYTEDDK GTTER TKD ELHPWV LANGDGNRPDGEKAEWAT IKSNT VVGSTGVI KDKNGKPS
Ava BAM69107	79	PVSTRECYKGRGAELSET, TENKOLYTEDDK GTVER TKD KU PWVVI ANGDGKOPDGFKAEWAT VKNDK YVGSTG T KDEKGNAN
Hum O8WVO1	154	VLESH JAFK GROMELS D. VENGKLYS VDDFT GVVYQ FE-GSKAVPWVT SDGDGTVEKGEKAEWLAVKDEBL YVGGLCKE TTTTGDVV
Dro CAL 26008	166	VIESAFALKGEMELSELVTENGELUTEDDETG TYE V-NDKE PWVTLLDGDCHSAKGEKAEWATVKEGTLYVGSMCKE TTSAGDEE
Cim A A D09177	116	KYKSKYSYC CROMELSEL V TENONULTEDDETCI VYL K-DDK V VDWWLADCDCKNSKCEKSEWATEKACN VVCSSCKE-TTKECTLE
Cim <u>-</u> ritibo)1//	110	
Dub_ABI20151	166	
Dub_ABI20151 Pap_AF261768	166 166	* NSNSIM KEIDQDGKULSLNMKQYMDKKSSVMK PNGFIWHEAVNNSKLNQMUL PRKCELP DTNTEETIGCNKIIIASENEQI NSNSIM KEIDQDGKUSLDMKEQYDKIK-SAMK PNGFIWHEAVNMSKLNQMVF PRKCERP DTKTEETIGCNKIIIASENEDI
Dub_ABI20151 Pap_AF261768 Ser_ADJ54110	166 166 167	* NSNSLWIKETDQDGKULSLNMKQYYDK K-SVMK PNGFIWHEAVNWSKLKNQWIL PRKCSELP DTNTEETIGCNKTIIASENFQI NSNSLWIKETDQDGKUQSLDWKEQYDKIK-SAMK PNGFIWHEAVNWSKLKNQWIF PRKCSERP DTKTEETIGCNKTIIASENFEI NTSSLWIKETDKEGRUQNKNWKYYEAVK-KAMN PNGFVWHEAVNWSPIKKQWIF PRKCSEPD DTKTEEDIGCNEILLADAVKT NTSSLWIKETDKEGRUQNKNWKYYEAVK-KAMN PNGFVWHEAVNWSPIKKQWIF PRKCSEPD DTKTEEDIGCNEILLADAVKT
Dub_ABI20151 Pap_AF261768 Ser_ADJ54110 Ara_ACS93496	166 166 167 164	* NSNSLWIKEIDQDGKVLSLNMKQYVDK/K-SVMKIPNGFIWHEAVNWSKLKNQWVLPRKCSELPDTNTEETIGCNKIIIASENFQI NSNSLWIKEIDQDGKVQSLDWKEQYDK/K-SAMKIPNGFIWHEAVNWSKLKNQWVFIPRKCSERPDTKTEETIGCNKIIIASENFEI NTSSLWIKEIDKEGRVQNKNWEKYYEAVK-KAMNIPNGFVWHEAVNWSPIKKQWVFIPRKCSDLPVNTETEENIGCNEIIIADAVFKT DSNALWIKEISTEGEVININMKSQYSKVK-NAMGIPSSVGFVWHEAVNWSPRKNLWVFMPRKCITEYTALVEERTGCNQIIIANEDFSQ
Dub_ABI20151 Pap_AF261768 Ser_ADJ54110 Ara_ACS93496 Ari_AAX56357	166 166 167 164 164	* NSNS W KEIDQDGKULSLNWKQYWDKK-SVWK PNGFIWHEAVNWSKLKNQWVL PRKCELPEDTNTEETIGGNKIIIASENEQI NSNS W KEIDQDGKUGSLDWKEQYDKIK-SAMKIPNGFIWHEAVNWSKLKNQWVFIPRKCERPEDTKTEETIGGNKIIIASENEEI NTSS W KEIDKEGRUONKWEKYYEAVK-KAMNIPNGFIWHEAVNWSPIKKQWVFIPRKCEDLPINTETEDNIGGNEIIADAVFKT DSNA W KEISTEGEUTNINWKSQYSKVK-NAMGIPSSVGFIWHEAVNWSPRKNLWVFIPRKCITEYITALVEBRIGGNQIIIANEDFIQ NTYSLWIKEISKEGEVTNINWKSQYSKVK-NAMGIPSSVGFIWHEAVNWSPRKNLWVFIPRKCITEYITALVEBRIGGNQIIIANEDFIQ
Dub_ABI20151 Pap_AF261768 Ser_ADJ54110 Ara_ACS93496 Ari_AAX56357 PerIta_ABB00907	166 167 164 164 165	* NSNSI WIKETDQDGKVLSLNWKQYWDKIK-SVMK PNGFIWHEAVNWSKLKNQWVL PRKCEEPPDTNTEETIGCNKIIIASENFQI NSNSI WIKETDQDGKVQSLDWKEQYDKIK-SAMKIPNGFIWHEAVNWSKLKNQWVF PRKCEEPPDTKTEETIGCNKIIIASENEEI NTSSI WIKETDKEGRVQNKNWEKYWEAVK-KAMN PNGFIWHEAVNWSPIKQWVF PRKCEDPDTKTEETIGCNKIIIASENEEI DSNAIWIKEISKEGEVTNINWKSQYSKVK-NAMGIPSSVGFVWHEAVNWSPRKNLWVFNPRKCITEY TALVEBRTGCNQIIIANEDFIQ NTYSI WIKEISKEGEVTNINWKSQYSKVK-NAMGIPSSVGFVWHEAVNWSPRKNLWVFNPRKCITEYTISQVEEKTGCNQIIIANEDFIQ SKNALWVKVIDHNGEVTSINWENQYKKVK-DAMGISS-GFVWHEAVNWSPRKNLWVFNPRKCITEYTISQVEEKTGCNQIIIANEDFIQ
Dub_ABI20151 Pap_AF261768 Ser_ADJ54110 Ara_ACS93496 Ari_AAX56357 Perlta_ABB00907 Perlsa_ABB00907	166 167 164 164 165 165	* NSNSLWLKEIDQDGKULSLNWKQYMCKK-SVMK PNGFIWHEAVNWSKLNQWIL PRKCSELP DTNTEETIGCNKIIIASENEQI NSNSLWLKEIDQDGKUQSLDWKEQYDKLK-SAMK PNGFIWHEAVNWSKLNQWFIPRKCSERP DTKTEETIGCNKIIIASENEI NTSSLWLKEIDKEGRUNKWEKYMEAVK-KAMNIPNGFIWHEAVNWSPIKQWVFIPRKCSELP DTKTEETIGCNKIIIASENEI NTSSLWLKEIDKEGRUNKWEKYMEAVK-KAMNIPNGFIWHEAVNWSPIKQWVFIPRKCSELP DTKTEETIGCNKIIIASENEI NTSSLWLKEISTEGEVTNINKSQYSKV-NAMG PSSVGFVWHEAVNWSPR NLWVFIPRKCTEY TALVEETGCNQII ANEDFOQ SKNALWKVIDHNGEVTSINWENQYKKV-DAMG SSGFVWHEAVNWSPR NLWVFIPRKCSRQP SAQIEEHTGCNQII ANEMFND STNALWVKVIDHNGEVTSINWENQYKKV-VAMG SSGFVWHEAVNWSPR NLWVFIPRKCSRQP SAQIEEHTGCNQII ANEMFND
Dub_ABI20151 Pap_AF261768 Ser_ADJ54110 Ara_ACS93496 Ari_AAX56357 PerIta_ABB0907 PerSpa_CCK33660 Tob_ADJ54077	166 167 164 164 165 165	* NSNSIM KEIDQDGKVLSLNNKQYYDK K-SVMK PNGFIWHEAVNNSKL NQW L PRKC ELP DINTEBIIGCNKIIIASENFQI NSNSIM KEIDQDGKVQSLDMKEQVDKI -SAMK PNGFIWHEAVNNSKL NQW FIPRKC ELP DINTEBIIGCNKIIIASENFQI NTSSIM KEIDKEGVQNKNMKSVYEAV-AKMN PNGFIWHEAVNNSPI KQW FIPRKC IDLP NTETEDNIGCNEIIADAVEKT DSNA WIKEISTEGEVININKSQYSK/-NAMG PSSVGFIWHEAVNNSPI KQW FIPRKC TEY TALVERTGCNQII ANEDFQ NTYSIM KEISKEGEVININKSQYSK/-NAMG PSSVGFIWHEAVNNSPR NLW FIPRKC TEY TALVERTGCNQII ANEDFQ SKNA WIKVIDHNGEVISINMENQYKK/-NAMG SSGFIWHEAVNNSPR NLW FIPRKC RQP SAQIEHTGCNQII ANEDFQ SKNA WIKVIDHNGEVISINMENQYKK/-DAMG SSGFIWHEAVNNSPR NLW FIPRKC RQP SAQIEHTGCNQII ANEDFDQ SKNA WIKVIDHNGEVISINMENQYKK/-DAMG SSGFIWHEAVNNSPR NLW FIPRKC RQP SAQIEHTGCNQII ANEDFDQ SKNA WIKVIDHNGEVISINMENQYKK/-DAMG SSGFIWHEAVNNSPR NLW FIPRKC RQP SAQIEHTGCNQII ANENFDD
Dub_ABI20151 Pap_AF261768 Ser_ADJ54110 Ara_ACS93496 Ari_AAX56357 PerIta_ABB00907 PerSpa_CCK33660 Tob_ADJ54077 Arg_ABA12135	166 167 164 164 165 165 165 165	* NSNSI WIKETDQDGKULSLNWKQYWDKIK-SVWK PNGFIWHEAVNWSKLKNQWUL PRKCELPEDTNTEETIGGNKIIIASENEQI NSNSI WIKETDQDGKUGSLDWKQYDKIK-SAMKIPNGFIWHEAVNWSKLKNQWUFIPRKCERPEDTKTEETIGGNKIIIASENEEI NTSSI WIKETDKEGRUNNWEKYYEAVK-KAMNIPNGFIWHEAVNWSPIKQWUFIPRKCEDLPINTETEENIGGNIIIANEDBSQ NTYSIWIKETSTEGEUTNINWKSQYSKVK-NAMGIPSSVGFIWHEAVNWSPIKNLWUFIPRKCITEY TALVERTGCNQIIIANEDBSQ NTYSIWIKETSKEGEUTNINWKSQYSKVK-NAMGIPSSVGFIWHEAVNWSPIKNLWUFIPRKCITEY TALVERTGCNQIIIANEDBTQ SKNALWIKUTDHNGEUTSINDENQYKKVK-DAMGISSGFIWHEAVNWSPIKNLWUFIPRKCITEY TALVERTGCNQIIIANEDBTQ SKNALWIKUTDHNGEUTSINDENQYKKVK-DAMGISSGFIWHEAVNWSPIKNLWUFIPRKCITEY SQUEEKTGCNQIIIANEDBTQ SKNALWIKUTDHNGEUTSINDENQYKKVK-DAMGISSGFIWHEAVNWSPIKNLWUFIPRKCIRQP SAQIEDHTGCNQIIIANEDBTD SKNALWIKUTDHNGEUTSINDENQYKKVK-DAMGISSGFIWHEAVNWSPIKNLWUFIPRKCINQA SAQIEDHTGCNQIIIANEDBTD SKNALWIKUTDHNGEUTSINDENQYKKVK-DAMGISSGFIWHEAVNWSPIKNLWUFIPRKCINQA SAQIEDHTGCNXIIIANEDBTD SKNALWIKETSKNGEUKSIDHHKQYEAVK-KALGITNGFIWHEAVTWSSHIKLWUFIPRKCINQA SAQIEDTTGCNXIIIANEDBTD
Dub_ABI20151 Pap_AF261768 Ser_ADJ54110 Ara_ACS93496 Ari_AAX56357 PerIta_ABB00907 PerSpa_CCK33660 Tob_ADJ54077 Arg_ABA12135 Lon_AF131933	166 167 164 164 165 165 165 165	* NSNSI MIKETDQDGKVLSLNMKQYMDKIK-SVMK PNGFIWHEAVNWSKLINQWIL PRKCEEP DTNTEETIGCNKIIIASENFQI NSNSI WIKETDQDGKVQSLDWKEQYDKIK-SAMKIPNGFIWHEAVNWSKLINQWF PRKCEEP DTKTEETIGCNKIIIASENFEI NTSSI WIKETDKEGRVQNKNEKYMEAVK-KAMMIPNGFIWHEAVNWSPIKQWF PRKCEEP DTKTEETIGCNKIIIASENFEI NTSSI WIKETSKEGEVTNINWSQYSKV-NAMG PSSVGFVWHEAVNWSPR NLWF PRKCITEY TALVEBRTGCNQII ANEDFSQ NTYSI WIKETSKEGEVTNINWSQYSKV-NAMG PSSVGFVWHEAVNWSPR NLWF PRKCITEY TSQVEEKTGCNQII ANEDFSQ SKNALWIKVIDHNGEVTSINWENQYKKV-DAMG SSGFIWHEAVNWSPR NLWF PRKCITEY TSQVEEKTGCNQII ANEDFSQ SKNALWIKVIDHNGEVTSINWENQYKKV-DAMG SSGFIWHEAVNWSPR NLWF PRKCITEY TSQVEEKTGCNQII ANEDFSD SKNALWIKVIDHNGEVTSINWENQYKKV-VAMG SSGFIWHEAVNWSPR NLWF PRKCINQA SAQIEDTGCNKII ANEDFSD SKNALWIKVIDHNGEVTSINWENQYKKV-VAMG SSGFIWHEAVNWSPR NLWFFIRGENGENGENGENTGCNKII ANEDFSD SKNALWIKVIDHNGEVTSINWENQYKKV-VAMG SSGFIWHEAVNWSPR NLWFFIRGENGENGENGENGENTGCNKII ANEDFSD SKNALWIKVIDHNGEVTSINWENQYKKV-VAMG SSGFIWHEAVNWSPR NLWFFIRGENGENGENGENGENGENGENGENGENGENGENGENGENG
Dub_ABI20151 Pap_AF261768 Ser_ADJ54110 Ara_ACS93496 Ari_AAX56357 PerIta_ABB00907 PerSpa_CCK33660 Tob_ADJ54077 Arg_ABA12135 Lon_AF131933 Int_AFP99246	166 167 164 165 165 165 165 163 165	* NSNSIW KEIDQDGKVISIN KQYYDK K-SVMK PNGTWHEAVNWSKL NQWVL PRKC ELP DTNTEETIGCNKIIIASENDOI NSNSIW KEIDQDGKVQSLDWKQVDKK -SVMK PNGTWHEAVNWSKL NQWVL PRKC ERP DTKTEETIGCNKIIIASENDOI NSNSIW KEIDKEGRVONKNEKYYEAV-KAMN PNGTWHEAVNWSPI KLWVF PRKC TEY TALVEERTGCNQIIIADAVEKT DSNALWIKEISTEGEVTNINKSQYSKV -NAMG PSSVGFVWHEAVNWSPI NLWVF PRKC TEY TALVEERTGCNQIIIADAVEKT SKNALWIKVIDHNGEVTSINKENQYKKV -DAMG SSGFVWHEAVNWSPR NLWVF PRKC TEY TSQVEEKTGCNQII ANEDFOD SKNALWIKVIDHNGEVTSINKENQYKKV -DAMG SSGFVWHEAVNWSPR NLWVF PRKC TEY TSQVEEKTGCNQII ANEDFOD SKNALWIKVIDHNGEVTSINKENQYKKV -DAMG SSGFVWHEAVNWSPR NLWVF PRKC RAP SAQIEHTGCNQII ANEDFOD SKNALWIKVIDHNGEVTSINKENQYKKV -DAMG SSGFVWHEAVNWSPR NLWVF PRKC RAP SAQIEHTGCNQII ANEDFOD SKNALWIKVIDHNGEVTSINKENQYKKV -DAMG SSGFVWHEAVNWSPR NLWVF PRKC RAP SAQIEDTGCNQII ANEDFOD SKNALWIKVIDHNGEVTSINKENQYKKV -DAMG SSGFVWHEAVNWSPR NLWVF PRKC RAP SAQIEDTGCNQII ANEDFOD SKNALWIKKITKDGVVTSHDVKKVK -DAMG SSGFVWHEAVNWSPR NLWVF PRKC RAP SAQIEDTGCNQII ANEDFOD SKNALWIKEIDKNGEVISINKENQYKKV -NAMG SSGFVWHEAVNWSPR NLWVF PRKC RAP SAQIEDTGCNXII ANEDFOD SKNALWIKEIDKNGEVISINKENQYKKV -NAMG SSGFVWHEAVNWSPR NLWVF PRKC AKKVSAQIEDTGCNKII ANEDFOD SKNALWIKEISKOEVISINKENQYKKV -NAMG SNGFVWHEAVNWSPR NLWVF PRKC AKVSAGIEDTGCNKII ANEDFOD PG-SQWIKKISKOEVISHKVFNON -NLWYFNON -GFVWHEAVTWSPF KQWVF PRKC KPJSQENEETGCNKII ANEDFON SKNALWIKEISKOEVISHKVFNON -NLWYFNON - NLWYFNON - NLWYF
Dub_ABI20151 Pap_AF261768 Ser_ADJ54110 Ara_ACS93496 Ari_AAX56357 Perfba_CK33660 Tob_ADJ54077 Arg_ABA12135 Lon_AF131933 Int_AFP99246 Aya_BAM69107	166 167 164 165 165 165 165 163 165 163	* NSNSIM KEIDQDGKULSLNMKQYWDKK-SVMK PNGFUHEAVNNSKLNQWUL PRKCELPDTNTEETIGCNKIIIASENFQI NSNSIM KEIDQDGKUSLDUKEQVDKIK-SVMK PNGFUHEAVNNSKLNQWUL PRKCELPDTNTEETIGCNKIIIASENFQI NTSSIM KEIDQDGKUSLDUKEQVDKIK-SVMK PNGFUHEAVNNSPIKQWUF PRKCELDPUNTETENIGCNEIIADAVFKT DSNA MKEISTEGEVININKSQVSKU-NAMG PSSVGFUHEAVNNSPIKQUF PRKCITEY TALVEERTGCNQIIAANEDFSQ NTYSIM KEISKEGEVININKSQVSKU-NAMG PSSVGFUHEAVNNSPIKQUF PRKCTEY TALVEERTGCNQIIAANEDFSQ NTYSIM KEISKEGEVININKSQVSKU-NAMG PSSVGFUHEAVNNSPIKQUF PRKCTEY TALVEERTGCNQIIAANEDFSQ NTYSIM KEISKEGEVININKSQVSKU-NAMG PSSVGFUHEAVNNSPIKNLWF PRKCTEY TALVEERTGCNQIIAANEDFTQ SKNA MKVIDHNGEVTSINMENQYKKU-DAMG SSGFUHEAVNNSPIKNLWF PRKCRQP SAQIEBTTGCNQIIAANEDFTQ SKNA MKVIDHNGEVTSINMENQYKKU-DAMG SSGFUHEAVNNSPIKNLWF PRKCRQP SAQIEBTTGCNQIIAANEDFTD SKNA MKEIDKNGEVISINMENQYKKU-DAMG SSGFUHEAVNNSPIKNLWF PRKCRQP SAQIEBTTGCNXII ANEDFTD SKNA MKEIDKNGEVISINMENQYKKU-CAMG SSGFUHEAVNNSPIKNLWF PRKCRQP SAQIEBTTGCNKII ANEDFTD SKNA MKEIDKNGEVISIDHKQYEAU-KAG TNGFUHEAVNNSPIKNLWF PRKC KAPFSQELBETTGCNKII ANEDFTD NSDS WKEISKNGEVKSIDHKQYEAU-KAG TNGFUHEAVTNSPFKUF PRKCRAFFSCHAEKSRQIEBTTGCNKII ANEDFTK SIMKKITKDGVTSEDTSANFKL-KAN PNGFUHEAVTNSPFKUF PRKCRAFFSCHAEKSRQIEBTTGCNKII ANEDFTK SIMKKITKDGSVTSEDJSANFKL-KAN PNGFUHEAVTNSPFKUF PRKCRAFFSCHAEKSRGENKII ANEDFTK SIMKKITKDGSVTSEDJSANFKL-KAN PNGFUHEAVTNSPFKUF PRKCRAFFSCHALTTSSEASGCNLIIAANEDFTK SIMKKITKDGSVTSEDJSANFKL-KAN PNGFUHEAVTNSPFKUF PRKCRAFFSCHALTTSSEASGCNLII ANEDFTK SIMKKITKDGSVTSEDJSANFKL-KAN PNGFUHEAVTNSPFKUF PRKCRAFFSCHALTTSSEASGCNLIIAANENFKN SIMKKITKDGSVTSEDJSANFKL-KAN PNGFUHEAVTNSPFKUF PRKCRAFFSKCHAAFSCHAFT
Dub_ABI20151 Pap_AF261768 Ser_ADJ54110 Ara_ACS93496 Ari_AAX56357 PerIta_ABB00907 PerSpa_CCK33660 Tob_ADJ54077 Arg_ABA12135 Lon_AF131933 Int_AFP99246 Aya_BAM69107 Hum_08WVQ1	166 167 164 165 165 165 165 165 163 165 169 243	* NSNSI WIKETDQDGKULSLNWKQYWDKIK-SVMK PNGFIWHEAVNWSKLKNQWIL PRKCEEP DTNTEETIGCNKIIIASENEQI NSNSI WIKETDQDGKUGSLDWKQWDKIK-SVMK PNGFIWHEAVNWSKLKNQWIF PRKCEEP DTKTEETIGCNKIIIASENEEI NTSSI WIKETDKEGRUNNWKYYEAV-KAMN PNGFIWHEAVNWSPIKQWIF PRKCEEP DTKTEETIGCNKIIIASENEEI DSNALWIKETSTEGEVTNINWKSQYSKV-NAMG PSSVGFIWHEAVNWSPRINLWIF PRKCITEY TALVEERTGCNQIIIANEDFIQ NTYSLWIKETSKEGEVTNINWKSQYSKV-NAMG PSSVGFIWHEAVNWSPRINLWIF PRKCITEY TALVEERTGCNQIIIANEDFIQ SKNALWIKUTDHNGEVTSINDENQYKKV-DAMG SSGFIWHEAVNWSPRINLWIF PRKCITEY TALVEERTGCNQIIIANEDFIQ SKNALWIKVTDHNGEVTSINDENQYKKV-DAMG SSGFIWHEAVNWSPRINLWIF PRKCITEY TALVEERTGCNQIIIANEDFID SKNALWIKVTDHNGEVTSINDENQYKKV-DAMG SSGFIWHEAVNWSPRINLWIF PRKCIRQF SAQIEDHTGCNQIIIANEDFID SKNALWIKVTDHNGEVTSINDENQYKKV-DAMG SSGFIWHEAVNWSPRINLWIF PRKCIRQF SAQIEDHTGCNQIIIANEDFID SKNALWIKVTDHNGEVTSINDENQYKKV-DAMG SSGFIWHEAVNWSPRINLWIF PRKCIRQF SAQIEDHTGCNQIIIANEDFID SKNALWIKVTDHNGEVTSINDENQYKKV-AAMG SSGFIWHEAVNWSPRINLWIF PRKCIRQF SAQIEDHTGCNXIIANEDFID SKNALWIKVTDHNGEVTSINDENQYKKV-AAMG SSGFIWHEAVNWSPRINLWIF PRKCIRQF SAQIEDTGCNKIIIANEDFID SKNALWIKVTDHNGEVTSINDENQYKKV-AAMG SSGFIWHEAVNWSPRINLWIF PRKCIRQF SAQIEDTGCNKIIIANEDFID SKNALWIKVTDHNGEVTSINDENQYKKV-AAMG SSGFIWHEAVNWSPRINLWIF PRKCIRAF SAQIEDTGCNKIIIANEDFID SKNALWIKVTDHNGEVTSINDENQYKKV-AAMG SSGFIWHEAVNWSPRINLWIF PRKCIRAF SAQIEDTGCNKIIIANEDFID SKNALWIKVTDHNGEVTSINDENQYKKV-AAMG SSGFIWHEAVNWSPRINLWIF PRKCIRAF SAQIEDTGCNKIIIANEDFID SKNALWIKKITKDGVTSHDWITKKIL-KALNIPNGFIWHEAVNWSPRINLWIF PRKCIRAF SAQIEDTGCNKIIIANEDFIND PG-SQWIKKITKDGVTSHDWITKKIL-KALNIPNGFIWHEAVNWSPIKULWIF PRKCIRAF SAQIEDTGCNKIIIANEDFIND SKNALWIKKITKDGVTSHDWITKKIL-KALNIPNGFIWHEAVNWSPIKULWIF PRKCIRAF SAQIEDTGCNKIIIANEDFIND PG-SQWIKKITKDGVTSHDWITKKIL-KALNIPNGFIWHEAVIWSPIKEWIFIPRKCHIATIFSENSACONLIIIANEDFIND PG-SQWIKKITKDGVTSHDWITKKIL-KALNIPNGFIWHEAVIWSPIKEWIFIPRKCHIATIFSENSACONLIIANEDFIND SKNALWIKKITKDGVTSHDWITKKIL-AALNIPNGFIWHEAVIWSPIKENFFIPRCHIATIFSENSACONLIIANEDFIND SKNALWIKEITKDGVTSHDWITKAL-AANNAKARIPPO-GFIWHEAVIWSPIKAUFIPRKOKIFIPRKCH
Dub_ABI20151 Pap_AF261768 Ser_ADJ54110 Ara_ACS93496 Ari_AAX56357 PerIta_ABB00907 PerSpa_CCK33660 Tob_ADJ54077 Arg_ABA12135 Lon_AF131933 Int_AFP99246 Aya_BAM69107 Hum_Q8WVQ1 Dro_CA126008	166 167 164 165 165 165 165 165 163 165 169 243 255	* * NSNSI MIKETDQDGKVLSLNMKQYMCK -SVMK PNGFIWHEAVNWSKL NQW L PRKC ELP DTNTEETIGCNKIIIASENFQI NSNSI WIKETDQDGKVQSLDWKQYDKIK-SAMKI PNGFIWHEAVNWSKL NQW F PRKC ELP DTNTEETIGCNKIIIASENFEI NTSSI WIKETDKEGRVQNKNWEKYMEAV -KAMM PNGFIWHEAVNWSPR NLW F PRKC TEY TALVEBRTGCNQII ANEDFSQ NTYSI WIKETSKEGEVTNINWSQYSKV-NAMG PSSVGFVWHEAVNWSPR NLW F PRKC TEY TALVEBRTGCNQII ANEDFSQ NTYSI WIKETSKEGEVTNINWSQYSKV-NAMG PSSVGFVWHEAVNWSPR NLW F PRKC TEY TALVEBRTGCNQII ANEDFSQ SKNAL WIKVIDHNGEVTSINWENQYKKV-DAMG SSGFIWHEAVNWSPR NLW F PRKC TEY TSQVEEKTGCNQII ANEDFSQ SKNAL WIKVIDHNGEVTSINWENQYKKV-DAMG SSGFIWHEAVNWSPR NLW F PRKC TEY TSQVEEKTGCNQII ANEDFSQ SKNAL WIKVIDHNGEVTSINWENQYKKV-DAMG SSGFIWHEAVNWSPR NLW F PRKC NQA SAQIEBHTGCNQII ANEDFSD SKNAL WIKVIDHNGEVTSINWENQYKKV-DAMG SSGFIWHEAVNWSPR NLW F PRKC RAP SAQIEBTGCNQII ANEDFSD SKNAL WIKVIDHNGEVTSINWENQYKKV-VAMG SSGFIWHEAVNWSPR NLW F PRKC RAP SAQIEBTGCNQII ANEDFSD SKNAL WIKVIDHNGEVTSINWENQYKKV-VAMG SSGFIWHEAVNWSPR NLW F PRKC RAP SAQIEBTGCNXII ANEDFSD SKNAL WIKVIDHNGEVTSINWENQYKKV-VAMG SSGFIWHEAVNWSPR NLW F PRKC RAP SAQIEBTGCNXII ANEDFSD SKNAL WIKEISNGEVISINWENQYKKV-VAMG SSGFIWHEAVNWSPR NLW F PRKC RAP SAQIEBTGCNXII ANEDFSD SKNAL WIKEISNGEVISINWENQYKKV-AMG SSGFIWHEAVNWSPR NLW F PRKC RAP SAQIEBTGCNXII ANEDFSD SKNAL WIKEISNGEVISINWENQYKKVAMG SSGFIWHEAVNWSPR NLW F PRKC RAP SAQIEBTGCNXII ANEDFSD SKNAL WIKEISNGEVISINWENQYKKVAMG SSGFIWHEAVNWSPR NLW F PRKC RAP SAQIEBTGCNXII ANEDFSD SKNAL WIKEISNGEVISINWENQYKKVAMG SSGFIWHEAVNWSPR NLW F PRKC RAP SQUEBTGCNXII ANEDFSD SKNAL WIKEISNGEVISINWENQYKKIKIAANK PNGFIWHEAVTWSPF KQW F PRKC ALF SQUEBTGCNKII ANEDFSD SKNAL WIKEISNGEVISINWENCHAR PROFERATION SSH KLW F PRKC ALF SAQIEBTGCNKII ANEDFSD SKNAL WIKEISNGEVISINWENCHAR PROFERATION SSH KLW F PRKC ALF SAQIEBTGCNKII ANENFN TQ-SIWIKEISNGEVISINWENGAL AFAN PNGFIWHEAVINGSI KEW F PRKC ALF SKDGCALEBTGCNKII ANENFN NGNF STRUKEISNGEVISINGANGAR AFANGAR PNGFIWHEAVINGSI KEW F PRKC BAR SAQIEBTGCNKII ANENFN NENFERMIKV SYMAA R-AAAG QPP-CTIHESGTWEETRWFFIPRA QER SK
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Dub_ABI20151 Pap_AF261768 Ser_ADJ54110 Ara_ACS93496 Ari_AAX56357 PerIsa_ABB00907 PerSpa_CCK33660 Tob_ADJ54077 Arg_ABA12135 Lon_AF131933 Int_AFP99246 Aya_BAM69107 Hum_Q8WVQ1 Dro_CAL26008 Cim_AAD09177 Dub_ABI20151 Pap_AF261768	166 167 164 165 165 165 165 165 163 165 169 243 255 205	* * * * * * * * * * * * * * * * * * *
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FIGURE 1: Multiple sequence alignment of apyrases from sand flies and other related sequences: L. longipalpis (Lon), Lutzomyia intermedia (Int), Lutzomyia ayacuchensis (Aya), Phlebotomus arabicus (Ara), Phlebotomus ariasi (Ari), P. perniciosus Italian strain (PerIta), P. perniciosus Spanish strain (PerSpa), Phlebotomus tobbi (Tob), Phlebotomus argentipes (Arg), P. duboscqi (Dub), P. papatasi (Pap), Phlebotomus sergenti (Ser), Homo sapiens (Hum), Drosophila melanogaster (Dro), and Cimex lectularius (Cim). Accession numbers are indicated in the sequence name. Sequences without a signal peptide were aligned with ClustalW and refined using Boxshade server, and the percentage of the identities or similarities that must agree for shading was set at 80%. Black background shading represents identical amino acids, and grey shading designates similar amino acids while white shading indicates no similarity. (*) and (Ø) indicate changes in the prediction of the antigenic index and secondary structure, respectively, between P. perniciosus Spanish and Italian strains as performed by Protean (DNASTAR, Lasergene). (1) signs above amino acids indicate changes in phosphorylation sites as predicted by NetPhos 2.0 Server, and the amino acid affected by the prediction on the phosphorylation site is encircled. Binding sites of nucleotides and Ca²⁺ are represented by (**v**) and (+), respectively, as predicted for the human apyrase [52].

		▲	*	♠ ○ ♠	
Ser_ADJ54113	20	- LQY PRNADQTLWAFRTCQRRE	SDSSLLQNWFQ KL	-PNNEATHCYVQCSWIHLGI	YNKKDGSIKVDKVKQQFTSR-GIE PGD
Pap_AAL11048	20	-WKYPRNADQTLWAFRTCQRRE	SDNNILKKWYTWEL	-PNDEKTHCYVKCVWIHLGI	YSKNTKSLRVNKIEKQFTSR-GVAIPSD
Dub_ABI20190	20	- WRFPRNGDQTYWAFNTCQRQT	TDIESVKLWDQWLL	PNNAATHCYIKCVFIHLG	YNEQEKAINIDAVKKQFKSR-GLE PKD
Ara_ACS93520	20	- WQYPRNADQTLWAFRACOREG	KNPALVRKWMNWEL	-PDDTETHCYVKCVWTYLCS	YNEDRKSIKIDTVKKQYKAR-GVT PNG
Arg_ABA12141	20	- WOYPRNADOTLWAFRTCOREV	SDQALLPKIYKWEL	-PDNADTHCYVKCVWMNLCS	YDFNSKSINVDTIATQYETR-GLTVPAD
PerIta_ABA43051	20	- WOYPRNADOTLWAWRSCOKEHI	-GDDQALLKKMLK EI	-PDDKVTHCFIKCTWIHLCM	YDEKTKTIRVDKVKQOFEGR-KLPVPAE
PerSpa_CCK73754	20	- WOYPRNADOTLWAWRSCOKEHI	-GDDOAL KKRLK E	PDDKVTHCFIKCTWIHLCM	YDEETKTIRVDKVKOOFEGR-K PVPAE
Tob_ADI54092	20	- WOYPRNADOTLWAWRSCHKEHS	-GGDOAL OKWMK EI	-PDDKVTHCLVKCIWIHLCM	YDAKTKTIRVDKVKOOFEDR-K PVPSE
Ari_AAX55660	20	-WKYPRNADOTLWAWRSCOKGN	YDPELVKKWMAFEI	PDDEVTHCY IKCYWTHLC	YDETSOTIRADRVKOOFKAR-G SVPAE
Ava_BAM69176	20	- WOYPRNADOTLWAFKTCOREA	KDOKFLEKWKKWEL	-PSSDETYCYVKCVWKNLCS	YNDADNSIKI A ACOFRSR-G NYPAS
Lon_AAL16051	20	- WODVRNADOTLWAYRSCOKNP	EDKDHVPOWRKFEL	PDDEKTHCYVKCVWTRLGA	YNENENVFKIDV TKOFNER-G EVPAG
Int_AFP99251	19	- WODVRNADOSLWAYKSCMRDP	KDVDLVPKWRKFIF	-PNDERTOCYVKCLWTRLGA	YNODTKSFNTOR AKOFASO-G SVPDE
Ans_315583502	1	OPWKALDA OALYVYKRCYEDHLPS	GSDRKTY TLWNA R E-	-PNDAIDHCYAKCVLTGDO	YDPOENAFKSDR PVO OAY-KTITOSK
Aed PDB 3DXL A	1	MGPFDPEMUET TRCMEDNLED	GANRLPM AKWKE INE	PVDSPATICE GKCVLVRTCL	YDPVAQKEDASV QEORKAYPS GEKSK
			·····		
					A A
Ser ADI54113	102	DSISEPIDESEKA	DKIIGFFRNNVONITA	YCTIPESDKWAEH-POV	PKGTK SOFCNAE-REKGNK-DOKTA
Pap AAL11048	102	KSMECETDCSCKATV	DKTISEENNNVADIBTA	YGTIEESNKWYAON-PDVK	PKGTKISKECKAKNBENGES-NCKHA
Dub ABI20190	102	KSI SCRTDCSCKALY	EKTTPEFKNNFONIBTA	YGTR ESDKW AKH-POVK	PKRTRASEECTAE-KEKGETKNCRRA
Ara AC\$93520	102	SK GCPTDCSCTC V	KK IDEFISOKTNI.OM	YGTTEASNEWYSKN-PNVK	PKGTK SDECKAENBEGGKEGTCKHA
Arg ABA12141	102	ENI KCATDCSCFA M	KKTIDEFLKEKDNLOHA	YGTIPESNKW AOH-PDVK	PKRTRISEFCTGKEGGKEGSCKHA
PerIta ABA43051	102	SK EGPTDCDCFK V	RATIDAOMKNYETA	YGTYDGSDAW AEH-DETE	PKNTK SEFCKGREGGKEGTCKHA
PerSpa CCK73754	104	SK ECPTDCDCEK V	RK KARLDAOMKNY TA	YGTYDGSDAW AEH-D TU	PKKTK SEFCKGREGGKEGTCKHA
Tob ADI54092	104	SOL CCPTCCSCEK VI	KKTKAFLDTOMANYRTA	YGTY CSDEW AKH-PETK	
Avi A AVEE660	104	SH ECSTCCSCVT V	KKTRAFLETOMPNYRTA	YGTV ESDKW ANN-PETK	PKRTKISDECKGREAGTEGTCKHA
AII_AAA33000	102		OKTIKEFI FOKANTOHA		PKCVKISOFCKCKFKSCCKHV
Lop AAL16051	102		DKSMKBEKSHEMDE NA	VATY CODEW SKN-P VK	
Int AED00251	102			VATN SEEKNENAH-D TK	
Ame 215592502	101	OKEVTEVOKALAANAKSOSOVD VI			OKCESEHAVOEKRAUKONKOSEUKINORR
Alls_313363302	89	W = A V A N = -A V K O P S NND C A AVE	KAVDDVHKAHKDTS NI	HONK I KCI FKI CK I	
ACCID DD DALA	89				
		▲		▲	A A
Ser_ADI54113	178		DEDYKPUYE KI	EIPCISNDK A-ECKEAS	GOR CE KVSDA VDERSNAAGLKAAVK
Pap AAL11048	179	CSAYYYRLV	DEDEEPUHERLI	EIKGESNED D-ECTKOTS	SGGGGCORSDA VDCLKNKKSAALEAALO
Dub ABI20190	179	CSLYYYRFV	DEDYQPHYIS KI	DIAGITDKO N-DCRDKAR	REKKGCKVGDA VRCLRLINKOGLIATVE
Ara ACS93520	180	CSMYYYRLVI	DEDN VUPERKI	NIQCIPGPK E-ECRRIAS	SKSCCKVSDA VSCLNKINSOGFIAADK
Arg ABA12141	178	CSMYYYRLV	DEDN VIIPE KI	KMNGTPEEAFD-GCBKDAS	SKETGCKVCDV YDC NALNSDGFQAVIK
PerIta ABA43051	180	CSMYYYTTI VI	DEDN VIIPE KI	PGISESD K-QCBDAAS	SKKSGCOVADT VDCLNKINPTGLKTALN
PerSpa CCK73754	180	CSMYYYRLVI	DEDN VIIPERKI	PGISESD K-QCRDAAS	SKKSCCOVADT YDCLNKINPTGLKTAIN
Tob ADI54092	182	CSMYYFRLV	DEDN VUPERKI	PGISESD K-ECEDVAS	SKKTGCKVADE VECLHNVNBKVIK
Ari AAX55660	178	CSMYYYRLV	DEDN VIIPE KI	PGILDSQ E-QCBDQAS	SETCCKVCDT VNCLNRINPEGLKKAIN
Ava BAM69176	175	CSMYYFRIV	DEDNI VVPERKI	PCYPEPK Q-ECRNKAF	ATTGCKVADV YECKRDFPTYLSMILO
Lon_AAL16051	176	CSMYYYRLI	DEDN VIIPESNI	PDYPEDK E-ECRNEAR	SANECK-SSV YOCLENADKSALDASIN
Int_AFP99251	175	CSFYYFRLI	DEDN IMPRSDI	PCYPKSA E-ECRNOVA	STNCCK-SSE YECLSKADKPALDSALE
Ans 315583502	178	YKLTGSPELKDAIDCIFRGLRYM	DTGKVDEIV	RDFNLINKSE EPEVRSVLA	SCKCSEAYDYYVCLWNSRLKOHFKNAFD
Aed PDB 3DXL A	176	ROYTVLDDALFKEHTDCVMKGIRY	TKONOLDVEEVKRONKL	NKDTKALEEVLNDC SKEP	SNAKEKSWHYYKCIV ESSVKDDFKEAFD
			• • •	· · •	
Ser_ADJ54113	245	ILDDQSAKY			
Pap_AAL11048	246	ILDDQSARTY			
Dub_ABI20190	246	RLDIESWKY			
Ara_ACS93520	247	KLDEESSRSY			
Arg_ABA12141	245	RWDDE DSTY			
PerIta_ABA43051	245	TLDEQSLTNY			
PerSpa_CCK73754	245	TLDEQSLTNY			
Tob_ADJ54092	243	ML			
Ari_AAX55660	243	TLDEQSLTLY			
Aya_BAM69176	240	NYDNESEYY			
Lon_AAL16051	240	ILDEFSGRY			
Int_AFP99251	237	QLDNWSERY			
Ans_315583502	260	FHELRSADYAYLLRGKVYENPEKVK	EEMKKLNTTVHF-		
Aed_PDB_3DXL_A	266	YR VRSQIYAFNLPKNQAYSKPAVQ	SQVMEIDGKQCPQ		

•

FIGURE 2: Multiple sequence alignment of D7-related proteins from sand flies and other related sequences: *L. longipalpis* (Lon), *L. intermedia* (Int), *L. ayacuchensis* (Aya), *P. arabicus* (Ara), *P. ariasi* (Ari), *P. perniciosus* Italian strain (PerIta), *P. perniciosus* Spanish strain (PerSpa), *P. tobbi* (Tob), *P. argentipes* (Arg), *P. duboscqi* (Dub), *P. papatasi* (Pap), *P. sergenti* (Ser), *A. stephensi* (Ans), and *A. aegypti* (Aed). Accession numbers are indicated in the sequence name. Sequences without a signal peptide were aligned with ClustalW and refined using Boxshade server, and the percentage of the identities or similarities that must agree for shading was set at 80%. Black background shading represents identical amino acids, and grey shading designates similar amino acids, while white shading indicates no similarity. (*) indicates changes in the prediction of the antigenic index between *P. perniciosus* Spanish and Italian strains as performed by Protean (DNASTAR, Lasergene). (**4**) denotes conserved cysteine residues. The cysteinyl leukotriene-binding motif is indicated by (□) [42], and the mutation of one of the amino acids that integrate this motif is designated by (**v**). Tyrosine in position 52 in *A. stephensi* stabilizes TXA2 analogs and is highlighted with (•).

3.1.3. Yellow Proteins. The yellow proteins are found in the saliva of insects, and they were named after observations in *Drosophila*, where mutation of a given gene gave a yellow phenotype, indicating that they are involved in melanization processes and pigmentation [46]. In sand flies, yellow proteins are widely represented at both transcriptomic and proteomic levels, showing molecular weights around 41–45 kDa and a wide range of isoelectric points [17, 19, 35, 41, 44]. Recently, it has been demonstrated that yellow proteins from *L. longipalpis* are able to bind and therefore inhibit the effects of several biogenic amines such as serotonin, norepinephrine, epinephrine, and histamine. Therefore,

Ari_AAX56359	21	D PEWKCERDFKKIDQNCFRPCT AINBVDN-K RUARKN EN KKF IDNNT KPEVNDDEKH LDCWNTI-KSIEAS	SSRT
Ara_ACS93524	21	ERPEKKCERIFKTEDUNCVRPOVYAIMHEVDN-KYRIERKNIEIYKKFIDYKAVKPEVNGEKHILDEWNSM-KSTEAS	STKSE
PerSpa_CCM43814	21	EXPEYKCR: DFKTEDKNCFLSCT: KNYHFIDN-K: RUE: KN EN KKFITUYKT KPNVSEKDLEKH LDCWDKFQKSPEAS	3TRPE
PerSpa_CCM43817	21	EXPEYKCR DFKTEDKNCFLSCT KNCHEIDN-K RIERKN EN YKKFITIYKT KPNVSEKDIEKHILDCWDKFQKSPEAS	STRPE
PerSpa_CCM43816	21	EXPEYKCR DFKTEDKNCFLSCT KNYHFIDN-K MIERKN EN KKFITDYKT KPNVSEKVLEKH LDCWDEFQKSPEAS	3TRPE
PerIta_ABA43048	21	EXPEYKCR.DFKTEDKNCFLSCT.KNYHFIDN-K.RIE.KN EN KKFITDYKA KPNVSDNDLEKH LDCWDKFQKSPEAS	3TRPE
PerSpa_CCM43815	21	EKPEYKCR DFKTEDKNCFLSCT KNYHEIDN-K RIERKN EN KKF TDYKT KPNVSEKD EKH LDCWDKFQKSPEAS	STRP E
Aya_BAM69146	22	EPPERKCIR ELARTDEVCILHCSY SYYCFTDE-NYRTTKKH ET RDV ISYNAVPGNEK-NK FDH KAGADAANATKPKS	3HN-D
Int_AFP99230	21	E+PEKKCIQELGKTQSSCILHCEYNHYGFTDE-NYRITKKHKKKRKSYPLSDK-SKLFGH RACGDRANAKKPKS	3TE-D
Lon_AAD32197	21	EHPEEKCIR ELARTDENCILHCIYSYYCFVDK-NERIAKKHYQKEKKILVTEDAVPKKEK-KKILEH EAGADSANADQPQI	ſKD−E
Arg_ABA12143	21	LDPHQKCT SDQNISRDCVLHCE KYYGFADD-Q NLN AHRNVLRNV LKYHV TNDQVEK - DEH EKCAKEANPKAQS-	-AEKD
Tob_ADJ54085	21	ERPSRKCRRELMEFEDECVIHCEVKYYRFIDDSRQITPVQRKN INV KKYGAFGMDQDESQIDKK MKGAHEVNKKTPVF	ESESD
Pap_AAL11047	21	ENPSKKCEEKFKNDASKMACIPHCK QYYCEVAM-DNNIA PE RT SNV IKYNVVDKSLK-AD RKI HECAKKVKKQAREI	DSHWL
Ser_ADJ54117	21	ETPENKCIAKHRANNLKETCIPQCKYEYYGEVGE-DYNTTYQH RTFSNTIKYNA DVSKK-HELRKL QKCEKRVKNQARNI	OSHWL
Dub_ABI20148	21	FGEHPEAYCIKKHQNEDFDCLVHCKFKHYIFTDD-QYNTRDYH RNLADFLIKYNVVAAKKR-GEVEKHLRSCVESSRKKAGG	J
Ari_AAX56359	104	KCEQVNNFER CVIDKN-ILNYPVYFNALKKINKNTNV	
Ara_ACS93524	104	KCERVNNFER CVIDKN-ILNYPVYFNALKKINKNTNV	
PerSpa_CCM43814	107	KCEKVNNFER CVIDKN-IFDYPIYFNALKKINYITKV	
PerSpa_CCM43817	107	KCEKVNNFERCVIDKN-IFDYPIYFNALKKINYITKV	
PerSpa_CCM43816	107	KCEKVNNFER CVIDKN-IFDYPIYFNALKKINYITKV	
PerIta_ABA43048	107	KCEKVNNEER CVIDKN-IFDYPIYFNALKKINYITKV	
PerSpa_CCM43815	107	NVKKSTIKDU TRISLIUFTSIL	
Aya_BAM69146	105	CYKIIHYYR CVVDGK-ILSWNSYAAAIIKYDKTKNV	
Int_AFP99230	105	CQKINDYHR CIVDEK-FLTFNRYYLAVNKYDKTINV	
Lon_AAD32197	105	CTKINKYYR CVVDGK-ILPWNSYADAIIKFDKTLNV	
Arg_ABA12143	105	^K CRRVLKYSL CVVNDSKLFTYNTYMKALIRFGHTFNL	
Tob_ADJ54085	108	KCKKINQYYICAVLDHSIFQYSAYAKAITEFEKTINV	
Pap_AAL11047	108	NCRTTIN YR CII TDK-RIGPQR DRAIQEYDKTIN	
Ser_ADJ54117	108	NCRTTIE YYR CIVADP-MINYRKEDKAI IEYDKTINV	
Dub_ABI20148	104	NCESIFKYYT CITDER-IFINKYDDA KLYDKTFTYVTRS	

FIGURE 3: Multiple sequence alignment of PpSP15-like proteins from sand flies: *L. longipalpis* (Lon), *L. intermedia* (Int), *L. ayacuchensis* (Aya), *P. arabicus* (Ara), *P. ariasi* (Ari), *P. perniciosus* Italian strain (PerIta), *P. perniciosus* Spanish strain (PerSpa), *P. tobbi* (Tob), *P. argentipes* (Arg), *P. duboscqi* (Dub), *P. papatasi* (Pap), and *P. sergenti* (Ser). Accession numbers are indicated in the sequence name. Sequences without a signal peptide were aligned with ClustalW and refined using Boxshade server, and the percentage of the identities or similarities that must agree for shading was set at 80%. Black background shading represents identical amino acids, and grey shading designates similar amino acids, while white shading indicates no similarity. (Ø) indicates changes in the prediction of the secondary structure between *P. perniciosus* Spanish and Italian strains as performed by Protean (DNASTAR, Lasergene). (\downarrow) signs above amino acids indicate changes in phosphorylation sites as predicted by NetPhos 2.0 Server, and the amino acid affected by the prediction on the phosphorylation site is encircled. (**A**) denotes conserved cysteine residues.

they act as vasodilators and as inhibitors of platelet activation, itch, and pain [47]. Yellow proteins are conserved among sand fly species as shown in Figure 4.

3.1.4. Par25-Like Proteins. This protein family displays a molecular weight around 25 kDa and an isoelectric point of 4.4–5 due to the great proportion of acidic residues (Figure 5). There are conserved regions rich in certain amino acid residues, and this protein family has been identified in *Adlerius (P. arabicus)*, where it represents the second most abundant protein family in the salivary glands cDNA, and *Larroussius (P. ariasi, P. perniciosus,* and *P. tobbi)* [19, 41, 44, 48]. Although its function is still unknown, several members of the sand fly ParSP25-like family stand to be highly immunogenic. Concretely, *P. perniciosus* SP08 is highly recognized by the sera of mice, hamsters, and dogs exposed to the bites of this vector [7, 17]. Moreover, a plasmid encoding *P. ariasi* SP25 strongly elicits both humoral and delayed-type hypersensitivity responses in mice [48].

3.1.5. Lufaxin-Like Proteins. SP06 from *P. perniciosus* Spanish strain (GenBank ID: CCK18305), a component of the 33 kDa family protein, shares 44% sequence identity with lufaxin from *L. longipalpis* (GenBank ID: AAS05319, Figure 6) whose

antithrombotic and anti-inflammatory effects have been recently described [49]. These alkaline proteins of molecular weight around 32–36 kDa seem to be specific for sand flies, and they have been identified in species of both genera *Phlebotomus* and *Lutzomyia* [19, 28, 35, 41, 49–51].

The transcript coding for a hypothetical protein P119 (GenBank ID: HE985078) was found among the sequenced cDNAs. This predicted protein shares homology with several hypothetical proteins of different insects, being the best match from *A. aegypti* (GenBank ID: XM_001663068). However, since it lacks signal peptide, it will not probably be secreted into the salivary gland lumen and would possibly be found in the cells of the gland walls.

3.2. Cloning, Expression, and Purification of Salivary Proteins. cDNA sequences of the salivary proteins SP01B and SP04 were successfully cloned in frame into expression vectors as confirmed by sequencing. Different expression conditions of the apyrase SP01B (37.3 kDa) and the D7-related protein SP04 (28.9 kDa) were assayed, and the best results were obtained when we induced cultures with 1 mM IPTG during 3 hours at 37°C. Batch purification of both 6xHis-tagged proteins was achieved by His-tag affinity chromatography using Super flow Ni-NTA resin under denaturing conditions. Adequate

		♠ Ø↓
PerIta_ABA43050	19	HVERENAMK ISYEG
PerSpa_CCK33661	19	HVEREYAWKNISYEGPUDAL NIDNIPT GVHDAINKKI I I AV PR SPQIPLT (DE
Tob_ADJ54080	19	HVEREYANK ISYEGFANDAAIKKI FAVPR FPQ PFT TE
Ari_AAX44093	19	HVEREYANK IFEG
Ara_ACS93501	19	HVEREYANK ITYEGRANK ITYEG
Arg_ABA12136	19	HVEREYAWRNVTFEGVNPSSYNVLHSPTGFAYDAETQKLFVAVPRRYPQVPHTLTE
Aya_BAM69185	19	YVETGYSWSNTTEGDTKDYKPRNNPTAFAHDPEGYKLFISTPR*LPQVPYTAE
Int_AFP99235	19	YVEIGYSMS. IFFEG
Ser_ADJ54123	19	DVERA AMK ITTEN
Pap_AGE83095	19	DVERFYAMRN TITED
Dub_ABI20172	19	DVGRLYENSKIDIVGFILL STATES OF STATES
Lon_AAS05318	19	ADTOGYKWKO LYNN
Ang XP_312785_2	29	EFEKVEENKISYTNLPDRSKGSTDNET QAYNNEM ATHHKN-RLFIT IPR RPGILAT NV
Dro_XP_002097461	24	DPMIEVFKWKQLDFYNGKDGYMDLWSRLCIPDPHVYNSRKCLGSSNSGASSTGSFIQYNNVPQGVTHFRRRLFVTVPRRQEGIPSTL
PerIta_ABA43050	77	
PerSpa_CCK33661	77	
Tob ADI54080	77	
Ari AAX44093	77	TAKHP
Ara ACS93501	//	
Arg ABA12136	77	DISKHP
Ava BAM69185	//	RKKHP
Int AEDQ0235	77	NTVMHPGYPVERAPKI SKFTGUSSKDLVSVYQPVID CRRLWIVDIGAVEYSGDDAGKYKTQKPAVI VYDLKKDHYPD IRY
Ser ADI5/123	77	DTVKHPQP1DRAPELDK+SGRSSKDFVSAYQPVIDECRRLWIDVGQVEYSGDDSQKYSKQKPALIVIDDNKSNYPE/RV
Dep ACE82005	77	DITKYNR-SEVRSPPLSKFNSQSKEKFTS, YQPVIDDCRRLWIDVGKVDYHKKD-NEYPTKNPE I APDINQPGNPEVRY
Pap_AGE85095	77	DTRNYNP-SEIRSPPFSKFNSQSGREFTS YQPVIDDCRRLWY DVGQVDYRKHG-NEYPTKNPE A DDNQEGNPEVRY
Dub_ABI20172	77	STRSYNS-AERDOPPIDKESGKSKKPLTSVYQPVIDDCRRLWVLDVGIVEVEAER-KTYPTKNPALVAFDLTKPNYPBIRY
Lon_AA505518	77	DAKNSLGVKGKHSPLINKESGHKTGKELTS YQPVID CRRLW VVDIGSVEYRSRGAKDYPSHRPALVAVDIKQPNYPD VRY
Ang_XP_312/85_2	93	DMTKVSRGDRSPPFQAYPSYSINELQPQEPDLHKLISVYRTRVDACERIAWFVDTGM_YPGNRMQVQRPQWIIDLKRDQLVRY
Dro_AP_00209/461	133	YIDLAMDG-WNQSBNGRAMPNFALNQYNASEQNWVSVYRTSVDACGRWFVDTGMLEFPNNRQQIRRPSIWUIDLANDRLI
		A .
PerIta_ABA43050	152	
PerSpa_CCK33661	152	PSK AGSHTIPEGGEAVDYTNPKEGCGKTVYTTNEEDNTL VYDDEK DSKISHGSEKP -HD STJSHDG-KOYK BVG EGV
Tob_ADJ54080	152	PSKLAGPNPTGEGGEAVDYTNPKEGCGKOFTYTTNEDDNTLUVYDGEK DSKTSHGSEKPE-HDSTLSHNG-EQYKYBVG EGT
Ari_AAX44093	152	
Ara_ACS93501	152	DRK SC-NDI GEODT INVINDTECCK
Arg_ABA12136	154	TAK 30 WE SHORT DWINE LEGGE THE TIME NIL WING CODER DESTINGTED TO THE REAL AND THE
Ava_BAM69185	159	
Int_AFP99235	150	CDN AS AF IS GOLAV DVININGUOIE
Ser_ADI54123	157	SDN AI-SPIIFGG AVDVINNAGDSVI VIING NSLV DVAI NSKFIDASED-KE IFSHFINAGSDK KVG FGI
Pap AGE83095	157	TGD AQ IPLGFQGFAVDVINPCGINIDE-I VIINFI NIL VIDFAN DARFKDDSFKP DAR HING-KEHP IIG FG
Dub ABI20172	157	EGD AR-SPLGFGGFAVDVINPNGNCARSDE-T YITNFI NAL VIDAN NAKFNDDSFXP PGA FFNHRG-EQIS IAG FGI
Lon AAS05318	157	IGNAAR IPLG GGFAVDVVVPR-RCGRNDERT VYTANEV INSL VYDRRRSDAVLRDDSFRP - GV ITTHNG-KEHRLEIG FG
Dro XP 002097461	160	FPIR VE-PPTYEGEFAVDVAMPRGDCSE
Ang XP 312785 2	1/8	PAS VREGVEMAS IMOVEATIOCDAA AVIPOLVANA HVYGLRENDMSFNHSSPAH PIRAALNVAC URFE DDCVESI
1115-11-012/00-2	193	KRFE PQSIVEIGRGLASITVDVDARRCADAFAMIPDLMRRLHVY LRSDRIW SB EHSYFNFDPLSDDLNIGG-QTFRMDDO
		▲
Perlta_ABA43050	236	LEDR DPECN-RPAYYI ACSSTK FESTIKI LIKEKCAKFDPVN CNRCPHT AVALVYDPKTKVIFFABSD ROVSCWNT-KPLN
PerSpa_CCK33661	236	LGDRDPEGN-RPAYYI AGSSTKLFESTKILKEKGAKFDPVNLGNRGPHTEAVALVYDPKTKVIFFAESDSROVSCWNT-KPLNI
Tob_ADJ54080	236	LGDRDPEGN-RPAYYIAGSSTKLFESTKILKOKGAKFDPVNLGNRGPHSEAVALAYDPKTKVIFFAESDSRQISCWNI-KPLNI
Ari_AAX44093	235	LGDRDPEGN-RPAYYLCCSSTKLFESTEALKKKCAKFDPVRLCDRCRHT ALALVYDPKTKVLFFAESDSROLSCWNT-KPLNI
Ara_ACS93501	235	LGDRDPEGN-RPAYYLGGSSTKLFESTEYLKKKGAKFDPVRLGDRGPKTBALALAYDPKTKVIFFAEADTROVSCWNT-KPLNI
Arg_ABA12136	238	LGDR AT GN-RMAYYL ACSSTKLYKSUGALKKKCARFDPIR LGDRCPYT HAT TLVYDPKUKVIFFAPSI TROVSCWNT-TPLDS
Aya_BAM69185	242	LGDRDKOGN-RPAYYIAGSSTKVYSNTKELKTKGGSLNPTLHGDRGPHTDAVALAYDPGHKVIFFAESDIROVSCWHV-TELKI
Int_AFP99235	243	LEDR NAGN-RPAYYLAGSSTK VSNTKOLKTKGTTLTPKLHGDRGKHO ALALAYDSVHKVLEFABSD ROVSCHHV-MELKI
Ser_ADJ54123	242	LGDRDKNGH-BSAVYLAGSSTKLYNSTASLKEKDTHLKPTLLGERGFKTHALALAYDPKTKVTFFVESNSRQVSCWNT-MELKI
Pap_AGE83095	244	I GORNKOCH-REAVY A CSSTK VYSNTASI KEKRASI KETI I CERCEKTEATAT AVDEKTKVTEEVESDSBC VSCWNT-KEUTI
Dub ABI20172	243	I GORNKEGN-REPAYY A CSSTK, VRDTKU LKKKCSKI, VRKI, CDRCVKT FA TAT AVDRETKVI FFARDSRC (SCWIT-KELK)
Lon AAS05318	243	LORDSECN-REDAVY A CSATK VSNTKEL KOKCKI NEFL CNRCKYMDATATATAVDEKTKVTEGABANTKOVSCWNTKMDIEN
Ang YP 312785 2	245	
Dro VD 002007461	200	ESATI C VCPA S-RUVEHD ASTNET VSKVI KOCNAARSDHCDEDI CTRCPSTOSTMHWDRTCVIEFAFUKSCVCWX
D10_AF_002097401	275	
n		
Perita_ABA43050	318	KNTDVILASAKFIYGSDISVDSESQLWFISTCHPPIPNLKLTFDKPHIRLRVDTAKAIR-RTRGEVKPRKP
PerSpa_CCK33661	318	KNTDVI ASAKFIYGSDISVDSESQLWFISTGHPPIPNLKLSFDKPHIRLRVDIAKAIH-RTRGEVKPRKP
Tob_ADJ54080	318	KNIDVIYASSKFIYGSDISVDSESQLWFISNGQPPIDNLKLTFDKPHIRLRWDIAKAIR-RTKGEVKPPKP
Ari_AAX44093	317	KNTDVIYASSKFIFGIDIQIDSDSQLWFLSNGQPPIDNLKLTFDKPHIRLRUDIKNSIR-RTRGEVKPIKKP
Ara_ACS93501	317	KNTDVIYASAKFIYGIDISIDSESQLWFISNGHPPVENLKLSFDKPHIRLRWDIEKSIR-RTRGEVKPIKKP
Arg_ABA12136	320	NHTDVIYSDARFIECIDISVDSNSTLWVMANCHPPVDDPDIVNNEFYKPQIRLYVDIRKSIR-RTRCDVNGNKP
Aya_BAM69185	324	ENTDVIYSYARFIC CDISVDKKGTLWFNSNGYPP KDAEKLKFYDRKTRLRWNTYNV P-YSKGNPDYKGPQGIPV
Int_AFP99235	325	ENTRY SYARE FOR DISENVINE SNGYPP NDAEKLKFONKKIRLENAMERVVK-YAKONPNYKAPPSIPV
Ser_ADJ54123	324	ENTGVIYSNAYFVEG DIMVD DGILWFYANGHPP DEPKLEFHKROIRLEVPIHRA R-LOPCEMKKNKKV
Pap_AGE83095	326	KNVGVIYTNAYFVEGIDIMVDADSTLWFYSNAHPPTELPKLDFDKROIIRLYWPTHRAIR-NLPGEVRKPK
Dub_ABI20172	325	ENVGVIYSSAKINFÄTDMMVDSKGFLWFMSNGQPPFD-EKMKYEDPHIRLKUKIKKAIKGEKRG0G
Lon_AAS05318	326	KNTDVY TSSREVEC DTSVD-KGGLWEMSNGFPP RKSEKFK-YDFPRYR MDTOEAIAGTAGDMNA
Ang_XP_312785_2	350	NHAVWHLDNREL YP DTSD DGVLWVL NNLPVWIYGRLNESDYNFR RODPAVAL GTKODNVA
Dro XP_002097461	364	SKPFSTENHGSVYSN SEM YPSDLTIDEEGYIWV SNSMPIFVYSKLDVOKYNFRIW QATSLAKR-GTVCE

FIGURE 4: Multiple sequence alignment of yellow proteins from sand flies and other related sequences: *L. longipalpis* (Lon), *L. intermedia* (Int), *L. ayacuchensis* (Aya), *P. arabicus* (Ara), *P. ariasi* (Ari), *P. perniciosus* Italian strain (PerIta), *P. perniciosus* Spanish strain (PerSpa), *P. tobbi* (Tob), *P. argentipes* (Arg), *P. duboscqi* (Dub), *P. papatasi* (Pap), *P. sergenti* (Ser), *Anopheles gambiae* (Ang), and *D. melanogaster* (Dro). Accession numbers are indicated in the sequence name. Sequences without a signal peptide were aligned with ClustalW and refined using Boxshade server, and the percentage of the identities or similarities that must agree for shading was set at 80%. Black background shading represents identical amino acids, and grey shading designates similar amino acids, while white shading indicates no similarity. (Ø) indicates changes in the prediction of the secondary structure between *P. perniciosus* Spanish and Italian strains as performed by Protean (DNASTAR, Lasergene). (\downarrow) signs above amino acids indicate changes in phosphorylation sites as predicted by NetPhos 2.0 Server, and the amino acid affected by the prediciton on the phosphorylation site is encircled. (**A**) denotes conserved amino acids contained in the ligand-binding pocket.

Dub_ACS93514	22	DRGVDGH K-TLDDHDYGDLAEYD-BDHKKUID-SDIB SGG TRG EDDDDEYTRDHYGHGTN GV Q RRGQSSSCON (SR
Ara_ACS93513	22	DRGVDGH K-TLDDHDYGDLAEYD-BDHHKVID-BSDIELSGGTTGHEDDDDDYTRDHHYCHGSNDGVDQRRGQSSSGGNAGSR
Ari_AAX55664	22	DRGVDGH RTQDDHDYSELAEYDD DDPHQEVIDG DE PHD SGG RLS HED DDDDRHYCHRGE RENSRGRN GSR
PerIta_ABA43056	26	YRANGDYGYSYBNH VVNG-DEDHEKTNSKFDDDD-YLFSHGY AYDD DD DRQGYSRGCGG GDS
PerSpa_CCK33662	26	YRANGDYGYSYBNHHVVNG-DEBHBKHNSKFDDDD-YLLSHCYAYDD DD DRQGYSRGCCGGGGGGDS
Tob_ADJ54100	22	DRGVDGHNRDHE DYDYTHMDDYDG BN H VTNG-DE HE TRG AKS EDDDDD-YLFSHGYDGYDD ED H RQSYSTGGGGTGDG
		øø
Dub_ACS93514	108	SRUNQEHSYDPYSGQRAPSI SESDEYEHSGDY-NSQSQQYSSBPSSHLUDQYLHLIQLQGVPSDLAQYAETYLQHAKN
Ara_ACS93513	108	SRCNQEHSYDPYSGQRAPS SESDEYEHSGDY-NSQSQQYSSSPSSHLVDQYLHLIQLQQVPSDLAQYABTYLQHAKN
Ari_AAX55664	100	NRGSEEQSYDPYSHERAPTYSESSEYDHSGDYDNSNYQQHSSTPSSYSNIDHYLHLIQLHSVPSDLAQYADSYLQHSKN
PerIta_ABA43056	99	SRDPGFYRRGSQEQSYDPHSGQTAPGYSESSEYEHSGDYDNSQNQQYSSIPSNANVNLDDQYLHLIQLHSIPSDLVQYAESYLTHAKN
PerSpa_CCK33662	99	SRDPGFYRRGSQEQSYDTHSGQTAPGYSESSEYEHSGDYDNSHNQQYSSTPSNANVNLIDQYLHLIQLHSIPSDLVQYAESYLTHAKN
Tob_ADJ54100	109	GSDPGFQRGGGQDQSYGPHSGQRAPG SESNEYEHSGDYDNSHYQQYSSTPSTANQLDYYLNQIQLHSVPSDLAQYAESYLKSAKD
		Ø
Dub_ACS93514	185	TISTYGYQAKDFEKIRPCLESVVKYFNLLNIDLANEYNRCKRKCFLDRLNSYTTAISQYTVTTSACINSRMH
Ara_ACS93513	185	TIRTYGYQAKDFEKIRPCLESVVKYFNLLNMDLANEYNRCKRKCFLDRLNSYTTAISQYTVTTSACINSRMH
Ari_AAX55664	179	SIR <mark>yyashakdfekirpclesvv</mark> kySnlln <mark>d</mark> dlak <mark>eyi</mark> rc <mark>orkcylerlnsytsaisqytvttn</mark> acinnrlh
PerIta_ABA43056	187	SIRYYAVHAKDFERIRPCLESVIKYFNMLNDDLAREYVRCOROCYLDRLNSYTTAISQYTVTTNACINNRLN
PerSpa_CCK33662	187	SIRYYAVHAKDFENIRPCFESVTKYFNMLNDDLAREYVRCOROCYLDRLNSYTTAISQYTVTTNACINNRLN
Tob_ADJ54100	195	SIRYYAAHAKDFEKIRPCLEAVMKYFNILNDDLAKEYVRCOROCFLDRLISYTSAISQHTVTTNNCINORMY

FIGURE 5: Multiple sequence alignment of Par25-like proteins from sand flies: *P. arabicus* (Ara), *P. ariasi* (Ari), *P. perniciosus* Italian strain (PerIta), and *P. perniciosus* Spanish strain (PerSpa), *P. tobbi* (Tob), *P. duboscqi* (Dub). Accession numbers are indicated in the sequence name. Sequences without signal peptide were aligned with ClustalW and refined using Boxshade server, and the percentage of the identities or similarities that must agree for shading was set at 80%. Black background shading represents identical amino acids, and grey shading designates similar amino acids, while white shading indicates no similarity. (Ø) indicates changes in the prediction of the secondary structure between *P. perniciosus* Spanish and Italian strains as performed by Protean (DNASTAR, Lasergene). (\downarrow) signs above amino acids indicate changes in phosphorylation sites as predicted by NetPhos 2.0 Server, and the amino acid affected by the prediciton on the phosphorylation site is encircled.

		Ø
PerSpa_CCK18305	23	IKVIREDDRDDYLLGKPDNTDEELLAVSTEDEIKNTCANPK-MKCTNNATHPVLDESDPKKRGISSIHVESTPDGPVNLEEKNKEOSKSS
PerIta_ABA43054	23	IKVIRFDDRDEYLLGKPDNTDEELLYSTFDFIKNTCANPK-MKCTNNATHFVLDFSDPKKRCISSIHVFSTPDGPVNLEFENKPRSKSS
Lon_AA\$05319	24	DGDEYFTCKYKEKDETDFTASYGLKEDPCQIVLGYKCSNNQTHEVDNEKTNKKSCISATKTSYPKINQNSDLTKN-
PerSpa_CCK18305	112	IYCQVGGIGQSYCLLVFKKKERREDALVDIRGLKTCSLKERYTSGDPKKTDAYGMAYKFDKNDNWSIKREGVKQWKRSGNEIFYRKN
PerIta_ABA43054	112	IYCQVGGIGQSYCLLVFKKKERREDALVDIRGLKTCSLKERYTSGDPKKTDAYGMAYKFDKNDNWSIKREGVKQWKRSGNEIFYRKN
Lon_AAS05319	102	YCQTGGIGTDNCKLVFKKKKRQIAAN IYGPAKKCSFKRYIGDELHVDSYG PYQFDQEHGMNERYN FKDTRFSTEVFYHKN
PerSpa_CCK18305	194	GLMNHQIRYLSKFDKYTVTREMVVKHRAKKFTMDFSNYGQYRISFLDVYWFQESVKHKPKLPYIYYNGECLPSNKTCQL FDADEPITY
PerIta_ABA43054	194	GLMNHQIRYLSKFDKYTVTREMVVKHRAKKFTMDFSNYGQYRISFLDVYWFQESVKHKPKLPYIYYNGECLPSNKTQLVFDADEPITY
Lon_AAS05319	185	<u>GLFNTQITYLAEEDSTSEARE TAKDIKKKFSTILPNEEYKRISFLDVYWFQETMRKKPKYPYIHYNGECSNENKTCELVFDTDELMTY</u>
PerSpa_CCK18305	276	AFVKVFSNPDHNEPRLRHADLGRG
PerIta_ABA43054	276	AFVKVFSNPDHNEPRLRHADLGRG
Lon 1 1 505210	267	

FIGURE 6: Alignment of SP06 from *P. perniciosus* Italian strain (PerIta), *P. perniciosus* Spanish strain (PerSpa), and lufaxin from *L. longipalpis* (Lon). Accession numbers are indicated in the sequence name. Sequences without a signal peptide were aligned with ClustalW and refined using Boxshade server, and the percentage of the identities or similarities that must agree for shading was set at 80%. Black background shading represents identical amino acids, and grey shading designates similar amino acids, while white shading indicates no similarity. (\emptyset) indicates changes in the prediction of the secondary structure between *P. perniciosus* Spanish and Italian strains as performed by Protean (DNASTAR, Lasergene). (\downarrow) signs above amino acids indicate changes in phosphorylation sites as predicted by NetPhos 2.0 Server, and the amino acid affected by the prediciton on the phosphorylation site is encircled.

expression pattern was attained for these salivary proteins as the corresponding Coomassie blue-stained bands matched the theoretical Mw, and Western blot revealed the presence of His-tagged protein (Figure 7 and Figure 8(b), resp.).

The apyrase SP01B and the D7-related protein SP04 were chosen for expression and purification since they had previously described as antigens for mice, hamsters, and dogs [7, 17], and they are highly represented in the saliva as seen in *P. perniciosus* salivary proteome [17, 19]. Apyrases have been pointed as good vaccine candidates since they display putative MHC epitopes, and a recombinant apyrase of *P. ariasi* produced protective cellular DTH responses in mice

[27, 29, 48]. Besides, apyrases are conserved proteins among sand flies and therefore good candidates for a wider vaccine [29].

3.3. Immunogenicity of Recombinant SP01B and SP04. Both recombinant proteins SP01B and SP04 were recognized by the sera of hamsters and mice exposed to sand fly bites in Western blot (Figure 8(a)).

Therefore, immunogenicity of the recombinant proteins was conserved for at least these animals after expression in a prokaryote system. Thus, we hypothesize that part of their



FIGURE 7: Coomassie blue-stained SDS-PAGE gel illustrating the expression and purification of the recombinant salivary proteins SP01B and SP04. Precision Plus Dual Xtra Standard (BioRad) was used as a marker (M). Lanes 1 and 2 show the bacterial extract before and after protein-expression induction by the addition of IPTG. Lane 3 shows the purified recombinant proteins.



FIGURE 8: (a) Recognition of *P. perniciosus* recombinant salivary proteins SP01B and SP04 by Western blot using pooled sera of control and immunized mice and hamsters (marked as - and +, resp.). (b) Western blot with His antibodies confirmed the presence of the recombinant His-tagged proteins SP01B and SP04.

immunogenicity is due either to lineal epitopes or conformational ones after an appropriate refolding. Posttranslational modifications that may occur in nature might contribute to their immunogenicity but do not seem to be essential for the recognition of mice and hamster sera as the recombinant proteins lack them. Previously, we had found that SP01B was recognized by the sera of both hamsters and mice while SP04 was highlighted only by hamster sera [17]. However, in the current experiments, both recombinant proteins were recognized by hamster and mice sera. In the case of SP04, the discrepancy observed with previous results may be due to the differences in the schedule followed to immunize the mice (100 flies weekly over 10 weeks versus 13 times exposure to 150 flies). Moreover, the anti-saliva IgG levels from *P. perniciosus*

mice immunized 13 times to 150 times were much higher than those sera from the other set of mice (data not shown).

4. Conclusions

Several polymorphisms were found between transcripts from the cDNA library of salivary glands of *P. perniciosus* and the cDNA library previously annotated [19], and further studies should be done in order to determine the biological meaning of all these polymorphisms. On the other hand, high level of conservancy was found between salivary transcripts of *P. perniciosus* from Spain and Italy thus, little antigenic variation is expected suggesting that recombinant salivary proteins could be used in different geographic areas where this sand fly species is present. Moreover, we successfully cloned, expressed, and purified SP01B and SP04 salivary proteins of P. perniciosus. Further characterization of these recombinant proteins will give additional information about their function, especially for SP04, as the functionality of the D7-related proteins has not been experimentally confirmed in sand flies. In addition, we preliminary tested the immunogenicity of these proteins with hyperimmune sera of mice and hamsters experimentally exposed to sand fly bites. Yet, these proteins should be further tested with sera of other reservoirs such as dogs, cats, hares, rabbits, and also with human sera in order to assess if they preserve immunogenicity for these species or not. In addition, further characterization of cellular immune responses of these recombinant proteins should be carried out to determine whether they could be selected as vaccine candidates against leishmaniasis.

Furthermore, to ensure that the best epidemiological markers are selected, it should be necessary to evaluate several recombinant proteins with sera of different hosts to select the most widely recognized proteins and test them alone or in combinations to cover a wide range of host immune responses. In this sense, additional works are in progress to obtain other recombinant proteins.

Conflict of Interests

The authors declare no conflict of interests.

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