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Ser/Thr-rich repetitive motifs as targets for phosphoglycan modifications in *Leishmania mexicana* secreted acid phosphatase

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The insect stage of the protozoan parasite *Leishmania mexicana* secretes a phosphomonoesterase in the form of a filamentous complex. The polypeptide subunits of this polymer are modified by phosphoglycans and/or oligomannosyl residues linked to phosphoserine. Based on peptide sequence data of a predominant 100 kDa protein of the filamentous complex, two tandemly arranged, single copy genes, *lmsap1* and *lmsap2*, were cloned and sequenced. *lmsap1* predicts a protein with features characteristic of acid phosphatases and a remarkable serine- and threonine-rich region of 32 amino acids close to the C-terminus. In the otherwise identical *lmsap2* product, this region is extended to 383 amino acids and is composed of short Ser/Thr-rich repeats. Deletion analysis demonstrates that *lmsap1* encodes the major 100 kDa protein of the complex while a minor 200 kDa component is derived from the *lmsap2* gene. Null mutants of either gene retain the ability to secrete acid phosphatase filaments, while a deletion of both genes results in *Leishmania* defective in enzyme formation. The Ser/Thr-rich domains are the targets for phosphoglycan modifications as shown by the expression of secreted fusion proteins composed of these C-terminal regions and the N-terminal domain of a lysosomal acid phosphatase.

Key words: acid phosphatase/genomic organization/ *Leishmania mexicana*/null mutants/phosphoglycan modification

Introduction

Protozoan parasites of the genus *Leishmania* are responsible for a spectrum of disease in tropical and subtropical regions, ranging from mild self-healing cutaneous lesions to lethal visceral forms (Alexander and Russell, 1992). *Leishmania* alternate between two developmental stages: the flagellated promastigotes, which live in the digestive tract of the sandfly vector, and the intracellular non-flagellated amastigotes, which reside in the phagolysosomes of mammalian macrophages. One remarkable property of all *Leishmania* species investigated is their ability to synthesize highly unusual glycoconjugates. The most thoroughly characterized is an abundant surface glycolipid of the promastigote stage termed lipophosphoglycan (LPG). LPG has a tetrapartite structure consisting of a lysoalkyl–*sn*–glycerophosphatidyl inositol membrane anchor, a phosphosaccharide core, phosphorylated oligosaccharide repeats and, mannose- and galactose-containing oligosaccharide caps at the non-reducing end (Turco and Descoteaux, 1992; McConville and Ferguson, 1993; Ilg *et al.*, 1994b). Moreover, *Leishmania* covalently modify polypeptides by phosphoglycans, as initially suggested by the reaction of monoclonal antibodies (mAbs) specific for oligosaccharide caps or phosphosaccharide repeats of LPG with secreted proteins (Jaffe *et al.*, 1990; Ilg *et al.*, 1991a, 1993) and by specific chemical deglycosylation (Bates *et al.*, 1990).

An example of a phosphoglycan-containing protein is the acid phosphatase (SAP) secreted by *L.mexicana* promastigotes. Interestingly, this enzyme is organized in long filaments composed of a central chain of globular particles and a surrounding glycocalyx (Ilg *et al.*, 1991b). It has been proposed that the filaments are assembled in the flagellar pocket, a secluded extracellular compartment at the base of the flagellum of the parasites (Stierhof *et al.*, 1994). The complex purified from the culture supernatant of promastigotes was suggested to be composed of at least three components: a 100 kDa glycoprotein, a 200 kDa component and additional non-covalently associated proteophosphoglycans (Ilg *et al.*, 1991b, 1993, 1994a). The SAP complex is modified by conventional N-linked glycans and by phosphoserine linked (oligo)mannosyl residues as well as capped phosphosaccharide repeats, representing a novel type of O-glycosylation (Ilg *et al.*, 1994a). It is shown here that the products of two related acid phosphatase genes contain clustered repetitive motifs that serve as targets for O-linked modification of phosphoserines by mannooligosaccharides and phosphoglycans, and that either gene product is able to polymerize to enzymatically active filaments.

Results

Cloning and sequencing of lmsap genes

Screening of a genomic library of *L.mexicana* DNA using oligonucleotides corresponding to the N-terminal and an internal sequence of the 100 kDa component of the purified SAP complex led to the isolation of two overlapping phage clones (Figure 1A) spanning a region of ~25.9 kb (Figure 1B). Mapping of the entire region revealed two *Pst*I fragments separated by 10.7 kb. Both fragments hybridized to the oligonucleotides, suggesting the presence of two tandemly arranged genes. Sequence analysis of the *Pst*I fragments (2 kb and 3.6 kb) indicated two open reading frames that were designated *lmsap1* and *lmsap2*, respectively (Figure 1B).

The sequence of *lmsap1* is shown in Figure 2. The

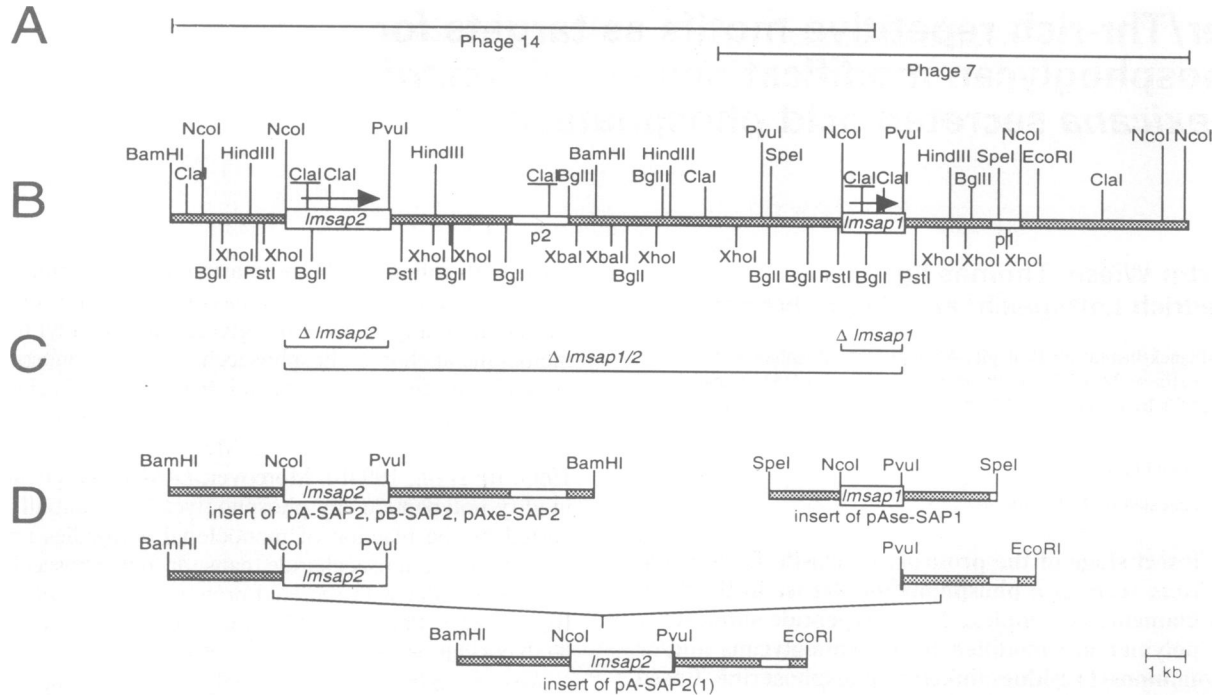

Fig. 1. Genomic organization of the SAP-locus and strategy for the targeted deletion of *lmsap* genes. **(A)** Two overlapping phage DNA fragments cloned from a genomic library of *Leishmania mexicana*. **(B)** Genomic organization of the secreted acid phosphatase genes *lmsap1* and *lmsap2* on an ~25.9 kb spanning DNA region. The open reading frames are marked by arrows indicating the direction of transcription. The sequences upstream and downstream of the two genes are identical at least to position −68 and +275 relative to the start and stop codons of the genes, respectively. Restriction enzyme cleavage sites are identical to at least 1.5 kb downstream of the genes but diverge thereafter. p1 and p2 indicate gene-specific probes used for Northern blot hybridizations (Figure 4). *Pst*I cleavage sites have not been mapped completely; underlined *Cla*I sites are dam-methylase sensitive in *dam*$^+$ bacteria. **(C)** Δ*lmsap1*, Δ*lmsap2* and Δ*lmsap1/2* indicate the regions replaced by *hyg* or *neo* genes in the targeted deletion of both alleles of either one or both *lmsap* genes. **(D)** Constructs used for replacement of *lmsap* open reading frames using *Nco*I and *Pvu*I restriction sites by either *hyg* or *neo* genes. For the Δ*lmsap1/2* deletion, the downstream region of *lmsap1* was fused to the protein-coding region of *lmsap2* via the common *Pvu*I restriction site as shown for pA-SAP2(1).

open reading frame predicts a protein of 537 amino acids. A typical signal peptide is followed by a sequence expected for the N-terminus of the 100 kDa protein in the SAP complex (calculated molecular mass of the mature polypeptide chain 56.5 kDa). Residues Y_{328} to Q_{347} correspond to the N-terminus of a 9 kDa fragment obtained by N-chlorosuccinimide cleavage which was used to deduce an oligonucleotide for cloning of the *lmsap* genes. Alignment of the *Leishmania* sequence with the sequences for acid phosphatases from other organisms revealed some characteristic motifs conserved in this group of enzymes, strongly suggesting that the 100 kDa component of the SAP complex is indeed a phosphomonoesterase (Figure 2; compare Ostanin *et al.*, 1992).

The *lmsap1* gene product (SAP1) has eight sites matching the consensus Asn-X-Thr/Ser-Y sequence for covalent attachment of N-linked glycans. However, based on predictions of Gavel and von Heijne (1990), the two most C-terminal sites (N_{450} and N_{493}) are unlikely to be modified because there is either a proline at position X or the combination of a tryptophan at position X and a proline at position Y. The most remarkable feature of the sequence is a C-terminal acidic serine- and threonine-rich region of 32 amino acids beginning at T_{452} (Figures 2, 3).

The sequence of the *lmsap2* gene yielded an open reading frame of 2664 bp (888 amino acids, molecular mass 91.4 kDa before and 89.1 kDa after signal sequence cleavage). The deduced polypeptide sequence of SAP2 is identical to that of SAP1, apart from the Ser/Thr-rich region, which is 383 amino acids long and comprises 47 repetitive units (Figure 3). Analysis of these repeats led to the identification of two related consensus sequences: type 1 [T][T](S/T)(S/T)(S/T)SSEG and type 2 [T](A/T)(S/T)(S/T)SSD(A/V); the N-terminal Thr-residues are optional. Consensus sequence type 1 also occurs twice in SAP1. Remarkably, the codon usage for Thr and Ser in the repeat region is strongly biased to ACC (100%) and AGC (98.7%), respectively.

Transcription of lmsap genes

Using a probe recognizing both genes, approximately equal amounts of two mRNAs of 4.8 and 8.2 kb were detected in Northern blots of promastigote RNA (Figure 4, lanes 1 and 3). Amastigote RNA, however, contained only the larger message in reduced amounts (lane 2). Probe p1, corresponding to a specific region 2.2 kb downstream of *lmsap1*, reacted only with the 4.8 kb message (lane 4; compare Figure 1B), while probe p2, located 3.1 kb downstream of *lmsap2* hybridized only to the 8.2 kb mRNA. Therefore, the smaller mRNA corresponds to *lmsap1* and the larger to *lmsap2*; both mRNAs extend several kb downstream of the putative translational stop codons of the corresponding genes.

Targeted deletion of the lmsap genes

An unambiguous assignment of the *lmsap* genes and their products is possible by the recently developed technology

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CTGCAGCATTCACAACACATCTCCAGGCCTCCCTGAGTGCGTACCCGAGAGCGGCAAGTA   60
GAGAAAGGAACACAGATACAGCACCATGGCCTCTAGGCTCGTCCGTGTGCTGGCGGCCGC  120
                          M  A  S  R  L  V  R  V  L  A  A  A    12

CATGCTGGTTGCAGCGGCCGTGTCGGTCGACGCGCGCTTCGTGGTGCGCATGGTGCAGGT  180
M  L  V  A  A  A  V  S  V  D  A  R  E  V  V  R  M  V  Q  V     32

GGTACACCGCCACGGTGCGCGCAGCGCACTCATCGACGACAACACGACGGAGATTTGTGG  240
 V  H  R  H  G  A  R  S  A  L  I  D  D  N  T  T  E  I  C  G    52

CACCCTGTACCCGTGCGGTGAGCTGACCGGCGAGGGTGTCGAGATGGTCCGTGCTATCGG  300
 T  L  Y  P  C  G  E  L  T  G  E  G  V  E  M  V  R  A  I  G    72

CGAGTTTGCCCGCAGCCGCTACAACAACCTCTCATTGGTGGAGAGCCCTCTCTTCCCGTC  360
 E  F  A  R  S  R  Y  N  N  L  S  L  V  E  S  P  L  F  P  S    92

GACGCGGTACAACTCCTCTGTCGTGCACACAGCTCCACCCACACCCAGCGCACCATCCA  420
 T  R  Y  N  S  S  V  V  H  T  R  S  T  H  T  Q  R  T  I  Q   112

GAGCGCGACCGCCTTTCTGCGCGGCCTCTTCCAGGACGACTACTTCTACCCGGTGGTGTA  480
 S  A  T  A  F  L  R  G  L  F  Q  D  D  Y  F  Y  P  V  V  Y   132

CTCGACCAACAGAACGACCGAAACGCTGCTCAGCACTGACGCGGTGCCGTCCGTGGTGGG  540
 S  T  N  R  T  E  T  L  L  S  T  D  A  V  P  S  V  V  G   152

CCGTAGCTGGCTCGACAACCCGGCGCTGCACGCCGCCCTCAACCCGGTGATCGATGAGCA  600
 R  S  W  L  D  N  P  A  L  H  A  A  L  N  P  V  I  D  E  H   172

CCTCAGCTGGGACGCCATCCAGAGCGCTGCCAAGGACGCATGGGTCGAGGGCCTGTGCGC  660
 L  S  W  D  A  I  Q  S  A  A  K  D  A  W  V  E  G  L  C  A   192

GGACTACAACGCCCGCACCAACTGCGTCCTCGACATGTACGACGTGGCCGCCGCCTTCGA  720
 D  Y  N  A  R  T  N  C  V  L  D  M  Y  D  V  A  A  A  F  E   212

GGCCGCCGGGCGTCTTGACAATGCCACCAATCTCAAGGCGGTGTATCCCGGCCTTCAGGA  780
 A  A  G  R  L  D  N  A  T  N  L  K  A  V  Y  P  G  L  Q  E   232

GGTGAACGCCGCCTGGTTCAAGTATGTCTTCAGCTGGAACCACACGAGCAAGCTCGATCT  840
 V  N  A  A  W  F  K  Y  V  F  S  W  N  H  T  S  K  L  D  L   252

CACGCAGGGCTCCGCCTCGCAGAACCTTGCGCAGACGGTGCTGGCCAACATCAACGCCCA  900
 T  Q  G  S  A  S  Q  N  L  A  Q  T  V  L  A  N  I  N  A  H   272

CCGCCTCTCTCCGTCGTACAACATGTTCCAGTACAGCGCTCACGACACAACGGTGACTCC  960
 R  L  S  P  S  Y  N  M  F  Q  Y  S  A  H  D  T  T  V  T  P   292

CTTGGCTGTCACGTTCGGTGACCAGGGCGAGACGACGATGCGTCCGCCCTTCGCGGTTAC 1020
 L  A  V  T  F  G  D  Q  G  E  T  T  M  R  P  P  F  A  V  T   312

CATCTTCGTGGAGCTGCTCCAGGACACCGCAGATGCCAGTGGCTGGTACGTGCGCCTCAT 1080
 I  F  V  E  L  L  Q  D  T  A  D  A  S  G  W  Y  V  R  L  I   332

CCGCGGCAACCCTGTGAAGGCAGCCGACGGCACCTATGTCTTCCAGGAGTCTGGTATCAA 1140
 R  G  N  P  V  K  A  A  D  G  T  Y  V  F  Q  E  S  G  I  K   352

GGCATACTGCATCGATGAAGCCGGGAACAAGTACCTCGCACACACCGGCATCTGCCCGCT 1200
 A  Y  C  I  D  E  A  G  N  K  Y  L  A  H  T  G  I  C  P  L   372

GAATAGCTTCCGCCGCATGGTCGACTACTCGCGCCCCGCCGTGGCTGACGGTCACTGCGC 1260
 N  S  F  R  R  M  V  D  Y  S  R  P  A  V  A  D  G  H  C  A   392

CATGACACAGACTCAGTACAGCAACATGGATTGCCCGCGCACTATCGCGGACAACAAGCC 1320
 M  T  Q  T  Q  Y  S  N  M  D  C  P  R  T  I  A  D  N  K  P   412

GGTGCCGTCGCGCTGCTGGCTCTACCGCCACGTTTGCCCTAGCAAGGCATGCCCGGACAG 1380
 V  P  S  R  C  W  L  Y  R  H  V  C  P  S  K  A  C  P  D  S   432
                                      ↓
CTACATTCTCTCCGCGGTCGACCACCAGTGCTACCCCGGGCCCGACGTTACGAACCCCAC 1440
 Y  I  L  S  A  V  D  H  Q  C  Y  P  G  P  D  V  T  N  P  T   452

CAGCAGCAGCAGCAGCGAGGGTACCACCACCAGCAGCAGCAGCGAGGGTACCACCAGCAG 1500
 S  S  S  S  S  E  G  T  T  T  S  S  S  S  E  G  T  T  S  S   472

CAGCAGCAGCAGCAAAAGCACAAGTTCATCGGATGTCCCTTCCTTCAAAAAGCCCGC 1560
 S  S  S  S  K  S  T  S  S  S  D  V  P  S  F  K  K  P  A   492

GAACTGGAGCCCGCGCGTTCTCTCGCCGGAAAGGGGCCGCCACATTGCCGGGGACATCAT 1620
 N  W  S  P  R  V  L  S  P  E  R  G  R  H  I  A  G  D  I  I   512

CCGCCGCGTGACGAACGGTGTTACGATCGGTGCGGGTGTCCGAAAGCACGATGAGTACAG 1680
 R  R  V  T  N  G  V  T  I  G  A  G  V  R  K  H  D  E  Y  S   532

CCGGCACCGCCAACAGTAGCACAACGGCATGTAACTCTTTTGTGCATGTTTGAATGGAGA 1740
 R  H  R  Q  Q                                               537

GGAGGCTTCTGTACAGCGTACATTGTTTCGAGAAGGTATCACAACCGCTCGTTTCACCCC 1800
CGTCATCTTTTCATTTTGATCTCCGTCGTCTCATACTGCCTTTGTGGGCTCTCTCTGGGT 1860
GTGGGCGCTTGTGCGTGTGTCGCTGTAAAGTCGTTGACGCCATCGCTCTTACCTGTGGGC 1920
TATTTTTTTAATTATGGTTTATTATTACTTCCCTCTCTGCGCGTCCCTCTGCAG       1974
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Fig. 2. Nucleotide and deduced amino acid sequence of *lmsap1*. The signal sequence is boxed. The initiating methionine can be predicted with high probability because of an in frame termination codon located 27 bp upstream. Dashed lines mark amino acids determined by peptide sequencing. Residues considered to be important for enzymatic activity are encircled. Underlined residues mark potential N-glycosylation sites. Amino acid residues in italics show the two type 1 repeat consensus sequences of SAP1 (Figure 3). The arrow marks the start of the sequence relevant for the proposed evolution of the Ser/Thr-rich region.

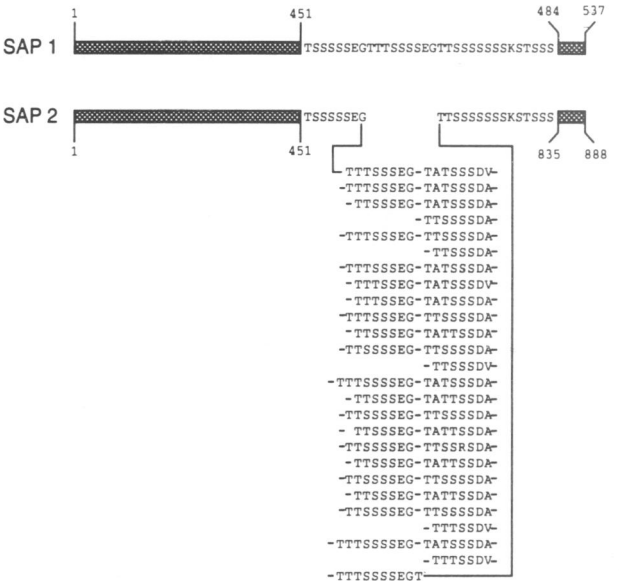

Fig. 3. Alignment of SAP1 and SAP2 proteins showing the Ser/Thr-rich regions. In the shaded parts, the sequences are identical. The Ser/Thr-rich region of SAP2 is arranged in order to indicate the organization of the repeat motifs.

of targeted gene deletion in *Leishmania* (Cruz *et al.*, 1991). To this end, both alleles of either the *lmsap1* or the *lmsap2* gene were sequentially replaced by the selective marker genes conferring neomycin (*neo*) or hygromycin B (*hyg*) resistance, leading to the deletions designated Δ*lmsap1* and Δ*lmsap2*, respectively (Figure 1C). The constructs were designed in a way that assured specific replacement of the respective target genes (Figure 1D). Comparison of the Southern blots shown in parts 1 (wild type), 2 (Δ*lmsap1*) and 3 (Δ*lmsap2*) of Figure 5 demonstrates that Δ*lmsap1* lacks both alleles of *lmsap1* and Δ*lmsap2* is deficient in both copies of *lmsap2*. A third construct was designed so that *lmsap1* and *lmsap2* were simultaneously deleted (Δ*lmsap1/2*). Southern blots of DNA from this mutant probed with the *lmsap1* gene did not give rise to a specific hybridization (not shown) and hybridization with either *hyg* (Figure 5, part 4) or *neo* (part 5) showed the expected fragments (compare Figure 1B). Therefore, the Δ*lmsap1/2* mutant lacks *lmsap2*, *lmsap1*, and the region between the two genes, implying that the *lmsap* genes and the intergenic region are not required for viability and growth of promastigotes *in vitro*.

Figure 6 shows an immunoblot of the concentrated culture supernatant of wild type *L.mexicana* and null mutants probed with mAb LT8.2. This mAb has been shown to recognize a peptide epitope of SAP1 (Ilg *et al.*, 1993), which was subsequently localized on the common C-terminal region of SAP1 and SAP2 next to the Ser/Thr-rich regions (M.Wiese and T.Ilg, unpublished results). The antibody detected a prominent 100 kDa polypeptide and a minor component at ~200 kDa in the concentrated culture supernatant from wild type promastigotes (Figure 6, lane 1). Deletion of *lmsap1* led to the loss of the 100 kDa protein, while synthesis of the 200 kDa protein was retained (compare lanes 1 and 2). Furthermore, this mutant secreted only ~8% of wild type phosphatase activity. In contrast, Δ*lmsap2* formed the 100 kDa protein but was deficient in the secretion of the 200 kDa protein (lane 3). This mutant secreted ~30% of wild type phosphatase activity. Finally, the mutant lacking both genes was totally deficient in the formation of mAb LT8.2-reactive components (lane 4) and acid phosphatase secretion. These results allowed us to assign the 100 kDa protein to the

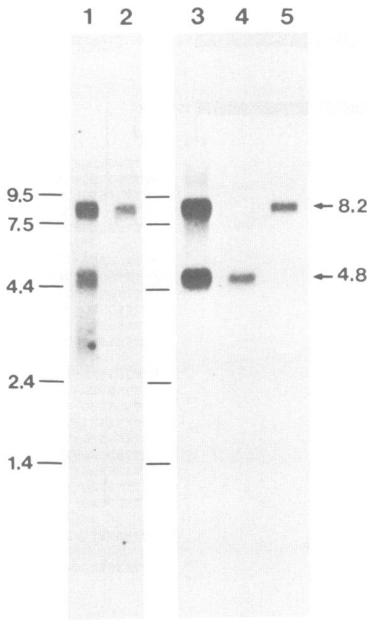

Fig. 4. Northern blot hybridization of 15 μg total RNA of promastigotes (lanes 1 and 3–5) and amastigotes (lane 2) with a ^{32}P-labeled DNA probe carrying either the entire *lmsap2* gene (lanes 1, 2 and 3) or a downstream region of *lmsap1* (lane 4) or *lmsap2* (lane 5), respectively (compare Figure 1B). The size of RNA markers and hybridizing RNAs are indicated in kb.

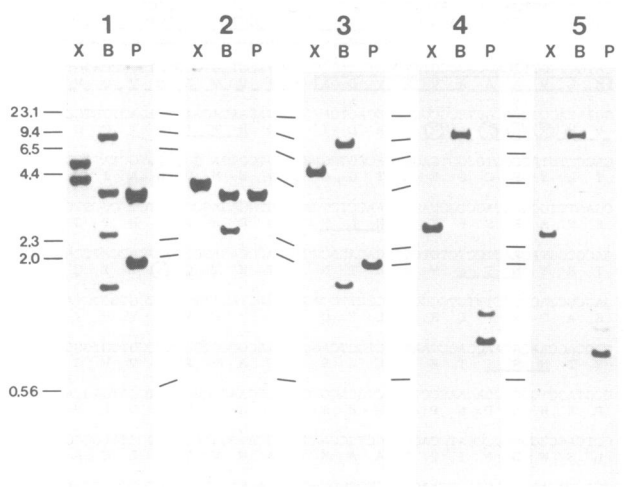

Fig. 5. Southern analysis of genomic DNA. 1, *Leishmania mexicana* wild type; 2, null mutant for *lmsap1* (Δ*lmsap1*); 3, null mutant for *lmsap2* (Δ*lmsap2*); 4 and 5, null mutant for *lmsap1* and *lmsap2* (Δ*lmsap1/2*). DNA (5 μg) was cut with X, *Xho*I; B, *Bgl*I or P, *Pst*I. Digested DNA was electrophoresed on 0.7% agarose gels, blotted onto nylon membranes and probed with DIG-labeled DNA-probes. Blots 1, 2 and 3 were probed with a *lmsap1* coding fragment, blot 4 with a *hyg* coding fragment and blot 5 with a *neo* coding fragment, respectively. Both *neo* and *hyg* contain an internal *Pst*I site. Numbers indicate the approximate size of DNA markers in kb.

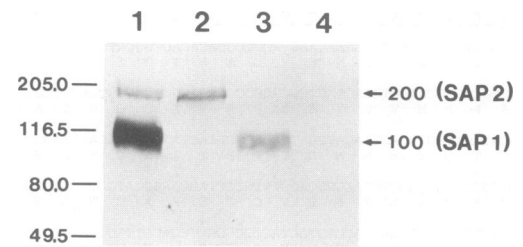

Fig. 6. Immunoblot of secreted products of *Leishmania mexicana* wild type and SAP null mutants with mAb LT8.2. Lane 1, wild type (100% phosphatase activity in culture supernatant); lane 2, Δ*lmsap1* (8%); lane 3, Δ*lmsap2* (30 %); lane 4, Δ*lmsap1/2* (0%). Concentrated culture supernatants of promastigotes (5×10^7 cells/ml) cultured for 30 h in serum-free medium were subjected to SDS−PAGE and blotted to poly(vinylidenedifluoride) membranes (Ilg *et al.*, 1991a). The membranes were probed with mAb LT8.2. The molecular mass of standard proteins and of *lmsap* products in kDa are indicated.

lmsap1 gene and the 200 kDa protein to the *lmsap2* gene, and demonstrate that both genes encode active phosphomonoesterases.

Immunofluorescence of the cells and inspection of the culture supernatant of the three deletion strains by negative staining electron microscopy revealed that mutants deleted for *lmsap1* or *lmsap2*, but not for both, were able to form filaments similar to those described before (Y.-D.Stierhof and M.Wiese, unpublished results; Ilg *et al.*, 1991b; Stierhof *et al.*, 1994). These results suggest that the expression of at least one *lmsap* gene is required for filament formation.

In addition to SAP1 and SAP2, the SAP preparation purified from the culture supernatant of wild type *L.mexicana* promastigotes contains a third component termed proteophosphoglycan (Ilg *et al.*, 1991b, 1994a). This heterogeneous material continues to be secreted by the single and double null mutants and most of it does not bind to an mAb LT8.2 affinity column, which retains the SAP filaments (T.Ilg, unpublished experiments). Therefore, the majority of the co-purified proteophosphoglycan appears not to be an integral part of the SAP filaments.

The Ser/Thr-rich repeats of SAP1 and SAP2 are targets for modification by phosphoglycans

Components of the SAP complex purified by column chromatography (Ilg *et al.*, 1991b) were shown to be covalently modified by oligomannosyl residues and capped phosphosaccharide repeats linked via phosphodiester bonds to serine residues of polypeptides (Ilg *et al.*, 1994a). It appeared likely that the Ser/Thr-rich regions of SAP1 and SAP2 were the targets for these modifications. In order to prove this hypothesis, these regions and their common C-termini were linked to a lysosomal acid phosphatase (LAP) (Menz *et al.*, 1991; Wiese, 1991) lacking its putative C-terminal transmembrane anchor by the expression of suitable gene fusions. These proteins were designated FP2 and FP3, respectively (Figure 7A). As a control, the last 50 nucleotides corresponding to the C-terminus of SAP1 and SAP2 were fused to the truncated *lmlap* gene, the derived fusion protein being designated FP1 (Figure 7A). All three gene fusions were transfected into *L.mexicana* promastigotes after insertion into the pX expression vector (LeBowitz *et al.*, 1990). Culture supernatants of transfected promastigotes were subjected (i) to a two-site ELISA using mAb AP4, which is specific for *L.mexicana* LAP (Menz *et al.*, 1991), as capture antibody and various SAP-specific antibodies recognizing the peptide backbone or phosphoglycan modifications as

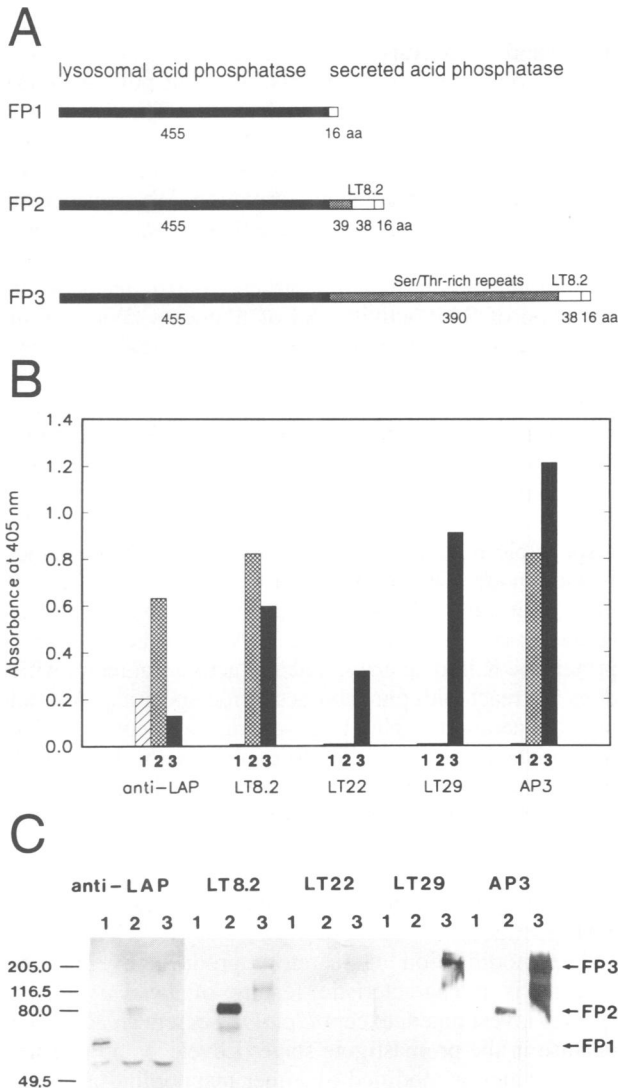

Fig. 7. Antibody binding to fusion proteins of lysosomal acid phosphatase (LAP) and Ser/Thr-rich repeats of SAP1 and SAP2. **(A)** Structure of the fusion proteins. FP1 encompasses the truncated lysosomal acid phosphatase without its membrane anchor but with the last 16 C-terminal amino acids of the secreted acid phosphatase. FP2 and FP3 contain in addition the Ser/Thr-rich repeat regions and the mAb LT8.2 binding site of SAP1 and SAP2, respectively. **(B)** Two-site ELISA with mAb AP4 as capture antibody. The mAbs used for detection are indicated below each group of bars. anti-LAP, rabbit polyclonal antiserum against *L.mexicana* lysosomal acid phosphatase. Absorbance values were normalized to bound acid phosphatase activity. Lanes 1, FP1; lanes 2, FP2; lanes 3, FP3. **(C)** Immunoblot of LAP-SAP fusion proteins. Concentrated culture supernatants of *L.mexicana* promastigotes carrying the leishmanial expression vector pX with either of the three different fusion constructs were immunoprecipitated with mAb AP4 bound to protein G–Sepharose. The bound material was blotted and probed with either anti-LAP antiserum or the indicated mAbs. The molecular masses of standard proteins in kDa are indicated. Lanes 1, FP1; lanes 2, FP2; lanes 3, FP3.

detecting antibodies (Figure 7B) or (ii) to immunoprecipitation with mAb AP4 followed by SDS–PAGE and immunoblot analysis (Figure 7C). The culture supernatant from all three transfectants, but not from untransfected *L.mexicana* promastigotes, contained acid phosphatase activity, which was recognized by mAb AP4. A rabbit polyclonal antiserum against LAP (anti-LAP) recognized FP1 (60 kDa) and FP2 (87 kDa), and also reacted with FP3, albeit rather weakly (240 kDa; left panels in Figure 7B and C). Therefore, the transfected strains secreted lysosomal acid phosphatase activity. Since mAb LT8.2 recognizes an epitope close to the SAP repeat regions, this antibody did not react with FP1. mAb LT8.2 binding to FP2 and a second product of 67 kDa, and FP3 and a minor protein of 130 kDa, confirmed that the fragments of SAP1 and SAP2 were fused in frame to LAP (second panels). mAb LT22 has been shown to recognize phosphotrisaccharide repeats [PO_4-6(Glcβ1–3)Galβ1–4Manα-1-], mAb LT29 reacts with phosphodisaccharide repeats (PO_4–6Galβ1–4Manα-1-), and mAb AP3 is specific for $(Man\alpha1,2)_{1-2}Man\alpha1$-$PO_4$-containing cap structures of *L.mexicana* LPG and SAP (Ilg *et al.*, 1993). Both the two-site ELISA and immunoblots showed that fusion protein FP3 containing the long Ser/Thr-rich region from SAP2 was extensively modified by LPG-type di- and triphosphosaccharide repeat units, and by cap structures (lanes 3 of corresponding panels in Figure 7B and C). The shorter Ser/Thr-rich domain from SAP1 was modified by mAb AP3-reactive cap structures but not by LT22- and LT29-reactive repeats (lanes 2). Finally, the control fusion protein FP1 was modified neither by phosphoglycans nor cap structures, based on the lack of reaction with mAbs LT22, LT29 or AP3 (lanes 1).

These results were corroborated by analysis of the modifications present in SAP1 and SAP2. mAb LT8.2 was used to immobilize the SAP activity released by the deletion mutants Δ*lmsap1* and Δ*lmsap2*. After normalization to the enzyme activity bound to the ELISA plates, it was found that both SAP1 and SAP2 reacted with mAb AP3. In contrast, an anti-phosphosaccharide repeat mAb gave a strong reaction with SAP2 but only marginal binding to SAP1. In summary, these results indicate that the Ser/Thr-rich region of SAP1 appears to be modified only by oligomannosyl cap structures, while the long repeat region of SAP2 contains these same cap structures as well as capped phosphodisaccharide and -trisaccharide repeats.

Discussion

The *lmsap* locus comprises two tandemly arranged genes, *lmsap1* and *lmsap2*, which differ only in the length of the predicted Ser/Thr-rich domains. From an evolutionary point of view, a primordial *lmsap* gene, coding for a single type 1 consensus sequence (TSSSSSEG), and its downstream region may have initially been duplicated. Subsequently, the consensus coding sequence may have been duplicated once to generate *lmsap1*. Consensus sequence type 2 may have evolved from the region immediately preceding the Ser/Thr-rich domain (beginning at nucleotide 1421 in Figure 2). The array of 47 repeat units of SAP2 can be formally generated by duplication of single or multiple repeat units corresponding to the two consensus types followed by point mutations and single codon insertions or deletions.

Both *lmsap1* and *lmsap2* are transcribed in the promastigote stage of the parasite resulting in approximately equal amounts of two corresponding mRNAs, which may be

derived from a polycistronic precursor by trans-splicing and polyadenylation (Laird, 1989). Judged by the ratio of SAP1 and SAP2 (r ~ 10) found in the promastigote culture supernatant (Figure 6), the *lmsap1* mRNA is translated much more efficiently than the *lmsap2* message, suggesting a post-transcriptional control mechanism. Deletion of *lmsap1* produces a strain secreting 8% of wild type phosphatase activity, while deletion of *lmsap2* results in a strain secreting only 30% of wild type activity. The disproportionate reduction of SAP1 formation in Δ*lmsap2* may be the result of a change in the physiology of the strain or, more interestingly, of a polar effect of the upstream replacement of *lmsap2* on the expression of *lmsap1*. Amastigotes contain only mRNA corresponding to the *lmsap2* gene, implying stage-specific regulation of mRNA synthesis. Immunoblots of amastigote lysates probed with mAb LT8.2 gave no reaction (M.Wiese and T.Ilg, unpublished results), and earlier immunoprecipitation experiments using the anti-oligomannosyl cap mAb AP3 suggested that amastigotes are deficient in the synthesis of this enzyme (Menz *et al.*, 1991). Moreover, we have been unable to detect any reaction of mAb LT8.2 by immunofluorescence in macrophages infected with promastigotes *in vitro* or by immunoelectron microscopy of infected macrophages in mouse lesions (M.Wiese, T.Ilg and Y.-D.Stierhof, unpublished results). These results suggest either that *lmsap2* mRNA is not translated in amastigotes or that the protein is synthesized but proteolytically degraded.

The proteins encoded by the two genes can be divided in a common N-terminal domain, the Ser/Thr-rich regions, and a common short C-terminal domain. The presence of some sequence motifs characteristic for acid phosphatases in the N-terminal domain of *L.mexicana* SAP confirms that these proteins are indeed phosphomonoesterases. Particularly noteworthy are the sequence **RHGAR** close to the N-terminus and the **HD** motif in the center of the polypeptide chain (Figure 2; Ostanin *et al.*, 1992). These regions are in close proximity in the three-dimensional structure of phosphomonoesterases such that the histidine residues can take part in the formation of the catalytic center (Schneider *et al.*, 1993).

Modification by phosphoglycans occurs in the acidic Ser/Thr-rich regions of SAP1 and SAP2. The short SAP1 domain appears to be modified exclusively by oligomannosyl cap structures. However, since it remains unknown how many consecutive di- or trisaccharide units are required for antibody recognition, the presence of short repeats on SAP1 or FP2 cannot be excluded. The much longer domain of SAP2 reacts with mAbs directed against phosphodisaccharide and -trisaccharide repeats as well as with the anti-mannooligosaccharide cap mAb AP3. However, a large fraction of FP2 is devoid of phosphoglycan repeats suggesting a low efficiency of the respective enzymatic modification machinery for this construct. It should be noted that consensus sequence type 2 is only present in SAP2. Therefore, this sequence could be a specific target for phosphodisaccharide and -trisaccharide addition.

Wild type *L.mexicana* promastigotes release a single type of SAP filament, as judged by negative staining electron microscopy with or without antibody decoration (Ilg *et al.*, 1991b; Stierhof *et al.*, 1994). This suggests that the filaments may be copolymers of SAP1 and SAP2. The deletion analysis clearly shows that either SAP1 or SAP2 alone can form filaments, and that polymerization is independent of the length of the Ser/Thr-rich repeats and their degree of modification. Formally, there remains the possibility that a gene between *lmsap1* and *lmsap2* may be involved in filament formation. We consider this possibility as unlikely, because either *lmsap1* or *lmsap2*, when expressed in *L.major*, a species deficient in SAP formation (Lovelace and Gottlieb, 1986), leads to the secretion of SAP activity and of filaments (Wiese *et al.*, in preparation). In extension of our previous results (Stierhof *et al.*, 1994), we would like to suggest that during or after release into the endoplasmic reticulum, the N-terminal domains fold, leaving the Ser/Thr-rich regions accessible for phosphoglycan modification, most likely after transfer to the Golgi stacks (Bates and Dwyer, 1987; Bates *et al.*, 1990). The folded N-terminal domain would be responsible for oligomerization and the eventual filament formation after secretion into the flagellar pocket. Interestingly, the non-polymeric SAP secreted by *L.donovani* (Bates *et al.*, 1990; Ilg *et al.*, 1991a) can be cleaved by proteinase K into an enzymatically active fragment, which does not react with phosphosaccharide-specific mAbs, and heterogeneous phosphoglycan-containing peptides (T.Ilg, unpublished results). Therefore, the major part of the polypeptide chain appears to be devoid of phosphoglycans, consistent with a domain structure similar to that of *L.mexicana* SAP. More detailed information on the modification sites in the Ser/Thr-rich repeat regions may be obtained by mutational studies and by the use of suitable SAP peptides.

The modification of secretory products by phosphoglycans is a characteristic feature of *Leishmania*. All species investigated, except *L.major*, secrete an acid phosphatase in the promastigote stage (Lovelace and Gottlieb, 1986), which is modified by either mannooligosaccharide caps, phosphosaccharide repeats, or both (Ilg *et al.*, 1993, 1994b). *L.mexicana* promastigotes and amastigotes secrete proteophosphoglycans without known enzymatic activity (Bahr *et al.*, 1993; Stierhof *et al.*, 1994; Ilg *et al.*, 1995). These proteins may also contain Ser/Thr-rich sequences as targets for O-glycosidic modifications. Inquiry into the importance of these secretory products for parasite survival in the sandfly vector or in mammalian macrophages will be greatly aided by the use of defined deletion mutants.

Materials and methods

Parasites

Promastigotes of *L.mexicana* MNYC/BZ/62/M379 (Lainson and Strangeways, 1963) were grown as described (Menz *et al.*, 1991). Amastigotes were isolated from mouse lesions of BALB/c mice (Bahr *et al.*, 1993).

N-chlorosuccinimide cleavage and amino acid sequencing

The lyophilized SAP complex (Ilg *et al.*, 1991a) was dissolved in 50% acetic acid to a concentration of 0.5 μg/μl and incubated with 15 mM *N*-chlorosuccinimide at room temperature for 30 min. *N*-acetylmethionine (50 mM) was added and the protein was lyophilized, washed with methanol, dissolved in 1× sample buffer [4% SDS, 12% glycerol (w/v), 50 mM Tris, 50 mM DTT, 0.01% Serva blue G, pH 6.8], and neutralized by addition of 200 mM ammonia. Fragments were separated on a 14% tricine gel (Schägger and von Jagow, 1987), blotted onto a Glassybond membrane (Biometra, Göttingen, Germany) according to

Eckerskorn *et al.* (1988), stained with Coomassie blue, excised and sequenced on an Applied Biosystems 477 A gas phase sequencer, equipped with an on-line 120 A PTH amino acid analysis system.

Cloning and sequencing of lmsap1 and lmsap2

The N-terminal sequence of the 100 kDa protein of SAP has previously been determined as XFVVrMVQVVHRHGARS (Menz *et al.*, 1991). Subjecting the complex to *N*-chlorosuccinimide cleavage allowed the determination of the complete N-terminal sequence (RFVVRMVQV-VHRHGARS) on a 57 kDa fragment and an internal sequence (YVRLIRGNPVKAADGTYVFQ) on a 9 kDa fragment. Taking the codon usage of *Leishmania* into account, the following oligonucleotides O1.1 : 5′-TGGT(G/I)CA(G/A)GT(G/I)GT(G/I)CA(C/T)CG(C/I)CA(C/T) GG(A/I)GC(A/I)CG-3′ (31mer), O1.2 : 5′-TGGT(G/I)CA(G/A)GT(G/I) GT(G/I)CA(C/T)AG(G/A)CA(C/T)GG(A/I)GC(A/I)CG-3′ (31mer), O2 : 5′-TG(G/A)AA(C/I)AC(G/A)TA(C/I)GT(G/I)CC(G/A)TC(C/I)GC(C/I) GC(C/T)TT(C/I)AC(C/I)GG(G/A)TT(C/I)CC-3′ (41mer) were used to screen a genomic DNA library in λDASHII/*Bam*HI (Stratagene, La Jolla, CA) constructed from *L.mexicana* using Stratagene's GigapackII Gold packaging extract. Filter lifts were taken twice from every plate and either hybridized with a mixture of O1.1 and O1.2, or with O2. Inserts of double positive phages were cloned into pBluescriptII SK(+) (Stratagene). From these a 7.7 kb *Eco*RI fragment containing *lmsap1* and a 10.8 kb *Bam*HI fragment containing *lmsap2* were subcloned into pBluescriptII SK(+) yielding pB-SAP1 and pB-SAP2 (Figure 1D), respectively (all derivatives of pBluescriptII SK(+) are abbreviated with pB).

Sequence analysis was performed by the dideoxy chain termination method (Sanger *et al.*, 1977) using a T7 Sequencing Kit (Pharmacia-LKB, Freiburg, Germany). For some reactions, deaza T7 sequencing mixtures and single strand binding proteins (United States Biochemical, Cleveland, OH) were used. Suitable DNA fragments were produced by a combination of exonuclease III and S1 nuclease digestions following the procedures given in the Promega Erase-a-Base System manual (Promega Corporation, Madison, WI). Both strands were sequenced. The *lmsap2* repeat coding region contains 22 *Kpn*I restriction sites. Therefore, its sequence could be obtained as follows: A 3.6 kb *Pst*I fragment (Figure 1B) containing the entire *lmsap2* gene was cloned into pBluescriptII SK(+) yielding pB-P1SAP2. This plasmid was linearized by treatment with *Xho*I and then subjected to partial digestion with *Kpn*I. Evaluation of the fragment ladder obtained on a 1.1% Seakem GTG agarose gel together with sequence information of this region allowed an unambiguous determination of the order of the *Kpn*I fragments. The GCG/Wisconsin software (Devereux *et al.*, 1984) was used to analyze the DNA sequences. Nucleic acid isolation and hybridization analyses were basically performed as described by Ziegelbauer *et al.* (1992) with the exception of using digoxigenin labeled probes in DNA−DNA hybridizations according to the manufacturer (Boehringer, Mannheim, Germany).

EMBL Data Library accession numbers for *lmsap1*, *lmsap2* and *lmlap* are Z46969, Z46970 and Z46971, respectively.

Constructs for double targeted gene replacement

A neomycin phosphotransferase gene (*neo*) containing fragment was either prepared by digesting pX63-NEO (LeBowitz *et al.*, 1991) with *Spe*I followed by a fill-in reaction and gel purification yielding a 914 bp *neo*-containing fragment (NEO 914) or by PCR amplification from pX63-NEO using the 25mer 5′-GCCA**TCATGA**GCTCGGCCATTGAAC-3′ to create a *Bsp*HI site at the ATG initiation codon and the 19mer 5′-TTGC**CGATCG**CCTCAGAAG-3′ to create a *Pvu*I site immediately downstream to the TGA termination codon of the *neo* gene. The 19mer 5′-CTAG**TCATGA**AAAAGCCTG-3′ and the 21mer 5′-GGTC**GATAT-**CTACTCTATTCC-3′ were used to amplify a hygromycin B gene (*hyg*) containing fragment from pX63-HYG (Cruz *et al.*, 1991) creating a *Bsp*HI site at the ATG initiation codon and a *Eco*RV site immediately downstream to the TAG termination codon of the *hyg* gene. The amplified DNAs were either digested with *Bsp*HI and *Pvu*I or *Bsp*HI and *Eco*RV yielding a 803 bp *neo*-containing fragment (NEO 803) and a 1034 bp *hyg*-containing fragment (HYG 1034), respectively.

Plasmid pACYC184 was modified by removal of various DNA fragments. It was either cut with *Xba*I and *Eco*RV or with *Sca*I and *Eco*RI, filled in, gel purified and religated yielding pAxe or pAse (all derivatives of pACYC184 are abbreviated with pA), respectively. Alternatively, pACYC184 was cut with *Sca*I and *Bst1107*I, gel purified and religated yielding pAsb.

For replacing *lmsap1* by *hyg* or *neo*, the filled in 5.8 kb *Spe*I fragment from pB-SAP1 was inserted into the *Bst*1107I-digested vector pAse yielding pAse-SAP1 (Figure 1D). *lmsap1* was replaced by *hyg* by digesting the plasmid with *Pvu*I, followed by T4 DNA polymerase treatment to make the 3′-overhangs blunt-ended; after *Nco*I digestion, the larger fragment was purified and ligated to HYG 1034 yielding pAse-HYG1. For replacement by *neo*, pAse-SAP1 was digested with *Pvu*I and *Nco*I, followed by mung bean nuclease treatment to make overhangs blunt-ended; gel purification of the larger fragment and ligation with NEO 914 yielded pAse-NEO1.

Replacement constructs for *lmsap2* were obtained as follows. The 10.8 kb *Bam*HI fragment from pB-SAP2 was inserted into the *Bam*HI site of pAxe yielding pAxe-SAP2 (Figure 1D). The 4.6 kb *Hin*dIII fragment from this construct was then inserted into the *Hin*dIII site of pAsb and designated pAsb-SAP2. *lmsap2* was replaced as described for *lmsap1* yielding pAsb-HYG2 and pAsb-NEO2. The *Hin*dIII inserts from these plasmids were ligated back to the 10.2 kb *Hin*dIII fragment from pAxe-SAP2 resulting in pAxe-HYG2 and pAxe-NEO2, respectively.

For the simultaneous replacement of *lmsap1* and *lmsap2*, the 10.8 kb *Bam*HI insert from pB-SAP2 was ligated into the *Bam*HI site of pACYC184 forming pA-SAP2 (Figure 1D). Then the 9.2 kb *Pvu*I−*Xba*I fragment from pA-SAP2 containing vector sequences and *lmsap2* upstream and protein-coding region was ligated to the 3.5 kb *Pvu*I−*Xba*I fragment from pB-SAP1 containing the downstream region of *lmsap1*, producing pA-SAP2(1) (Figure 1D). The smaller 4.6 kb *Hin*dIII fragment from this hybrid construct was then inserted into the *Hin*dIII site of pAsb yielding pAsb-SAP2(1). *lmsap2* was replaced in pAsb-SAP2(1) by *hyg* using HYG 1034 as described for *lmsap1* yielding pAsb-HYG2(1). Replacement by *neo* was done by digesting pAsb-SAP2(1) with *Pvu*I and *Nco*I, followed by gel purification of the larger fragment and ligation with NEO 803 producing pAsb-NEO2(1). Finally, the *Hin*dIII inserts from these plasmids were ligated back to the 8.0 kb *Hin*dIII fragment from pA-SAP2(1) resulting in pA-HYG2(1) and pA-NEO2(1), respectively.

The replacement plasmids were subjected to digestion with endonucleases that ensured subsequent targeted replacement of the desired gene(s). pAse-HYG1 and pAse-NEO1 were digested with *Spe*I, pAxe-HYG2 and pAxe-NEO2 with *Cla*I and *Xba*I, and pA-HYG2(1) and pA-NEO2(1) with *Cla*I and *Bgl*II (compare Figure 1B). The cleaved plasmid DNAs were precipitated, washed twice with 70% EtOH, resuspended in sterile ddH_2O and used for electroporation.

Expression constructs

The cloned *L.mexicana* lysosomal acid phosphatase gene (*lmlap*; Wiese, 1991) was amplified from a plasmid by polymerase chain reaction introducing *Eco*RV restriction sites immediately in front of the start codon and 12 bp after the stop codon. The amplified 1.6 kb fragment was cloned into *Eco*RV of pACYC184. This construct was cleaved with *Pvu*I and *Xba*I and the larger of two fragments containing 4 kb of vector DNA and a truncated *lmlap* gene lacking 187 bp corresponding to the C-terminus was purified and ligated to a 4.9 kb *Pvu*I−*Xba*I fragment derived from pB-SAP2 containing the last 50 nucleotides of *lmsap2* and its downstream region yielding pA-LSAP2. The fusion construct was excised by *Eco*RV and *Xba*I cleavage and ligated into the *Sma*I and *Xba*I cleaved expression vector pX (LeBowitz *et al.*, 1990) yielding pX-LSAP2. The derived fusion protein was designated FP1 (Figure 7A).

Fragments coding for the Ser/Thr-rich repeats of SAP1 and SAP2 were excised from pB-SAP1 and pB-SAP2, respectively, by cleavage with *Sma*I and *Pvu*I. *Pvu*I linkers (Stratagene) were ligated to the *Sma*I sites and cleaved by *Pvu*I. The purified fragments were ligated to the single *Pvu*I site of pA-LSAP2 yielding constructs coding for the truncated LAP, the Ser/Thr-rich repeats of SAP1 and SAP2, respectively, and the common C-terminus; furthermore, the constructs terminated in the downstream region of *lmsap2*. The ligation products were cloned into pX as described above yielding pX-LrSAP1 and pX-LrSAP2, respectively. Derived fusion proteins were designated FP2 and FP3, respectively (Figure 7A).

Transfection

Late logarithmic phase promastigotes were washed and suspended to 1×10^8 cells/ml in cold electroporation buffer (21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose, pH 7.5). 400 µl cell suspension was either mixed with 30 µg circular or 3–5 µg cleaved plasmid DNA and electroporated in 2 mm BTX disposable cuvettes at 450 V, 500 µF and R1 resistance setting (13 Ω) using a BTX Electro Cell Manipulator 600 (ECM600), generating pulse times of 2.3–2.8 ms. After 10 min on ice, the cells were transferred to HOSMEM-II medium (Berens and Marr, 1978) containing 40 mg/l Tween-80, 3 g/l bovine serum albumin and 15% iFCS and left for 24 h at 26°C. Thereafter,

selection was initiated by plating on HOSMEM-II/1% Bacto-Agar (DIFCO) plates. G418 (Geneticin; Boehringer) was present at 16 μg/ml in plates and up to 50 μg/ml in liquid culture; corresponding concentrations of hygromycin B (Sigma, Deisenhofen, Germany) were 32 and 20 μg/ml.

ELISA

Cationized poly(vinylchloride) microtiter plates were coated with mAb AP4 by incubating the wells with 100 μl antibody solution (20 μg/ml mAb AP4, 50 mM $NaHCO_3$, 100 mM NaCl) for 16 h at 4°C. The plates were washed twice with TBS (50 mM Tris−HCl, 150 mM NaCl, pH 7.5), non-specific binding sites were blocked for 30 min at 37°C with non-fat milk powder in 0.5% Tween-20, 50 mM Tris−HCl, pH 7.5 (200 μl/well) and then 100 μl *Leishmania* promastigote culture supernatants supplemented with 10 mM Tris−HCl, pH 7.5, 0.5% Tween-20 were applied for 2 h at 37°C. The wells were washed three times with TBS and either incubated with blocking solution alone (for measurement of bound acid phosphatase activity) or with saturating concentrations of different biotinylated or non-biotinylated monoclonal antibodies diluted in blocking solution for 1 h at 37°C (100 μl/well) followed by three washings with TBS. 100 μl 2% bovine serum albumin in TBS was applied to all wells destined for determination of bound enzyme activity, while the remaining wells were incubated with 100 μl ExtrAvidin Alkaline Phosphatase (Sigma) diluted 1:3000 or subclass specific antibodies coupled to alkaline phosphatase (dunn Labortechnik GmbH, Asbach, Germany) diluted 1:500 in 2% bovine serum albumin in TBS for 1 h at 37°C. All wells were washed four times with TBS and either developed with 100 mM NaAc, 5 mM *p*-nitrophenylphosphate, pH 5.0, for 1 h at 37°C (100 μl/well) and stopped by addition of 10 μl 2 M NaOH per well for the determination of *L.mexicana* acid phosphatase activity or incubated with 100 μl/well 1 M diethanolamine chloride, 1 mM $MgCl_2$, pH 9.8, containing 5 mM *p*-nitrophenylphosphate for determination of the alkaline phosphatase coupled reagents. The absorbance of released *p*-nitrophenol was determined at 405 nm.

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