Trialyisin, a Novel Pore-forming Protein from Saliva of Hematophagous Insects Activated by Limited Proteolysis*

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We have characterized a pore-forming lytic protein from the saliva of the hematophagous insect Triatoma infestans, a vector of Chagas disease. This protein, named trialyisin, has 22 kDa and is present in the saliva at about 200 µg/ml. Purified trialyisin forms voltage-dependent channels in planar lipid bilayers with conductance of 880 ± 40 pS. It lyases protozoan parasites and bacteria indicating that it has a role in the control of microorganism growth in the salivary glands. At higher concentrations, but below those found in saliva, trialyisin can also permeabilize and lyse mammalian cells, suggesting that it might also facilitate insect blood feeding by interfering with the cell response of the host. The translated DNA sequence of trialyisin shows a basic, lysine-rich protein in which the N-terminal region is predicted to form an amphipathic α-helical structure with positive charges on one side and hydrophobic amino acids on the opposite side. A synthetic peptide corresponding to this cationic amphipathic α-helix induces protozoan lysis and mammalian cell permeabilization, showing that this region is involved in lytic activity. However, the lytic peptide G6V32 is 10-fold less efficient than trialyisin in lysing protozoa and 100-fold less efficient in permeabilizing mammalian cells. Trialyisin activity is about 10-fold reduced in salivary gland homogenates prepared in the presence of irreversible serine-pro tease inhibitor. Since trialyisin precursor contains an anionic pro-sequence of 33 amino acids contiguous to the cationic amphipathic putative α-helix, we propose that removal of the acidic pro-sequence by limited proteolysis activates trialyisin by exposing this lytic basic amphipathic motif.

Molecules able to form pores in biological membranes have been evolutionarily selected in prokaryotic and eukaryotic organisms where the balance between permeabilization and lytic effect dictates their functions. Over the last decade, several pore-forming peptides, ranging from 12 to 45 amino acid residues, have been identified in animals and plants (1, 2). Most of them are part of an innate defense mechanism against microorganisms. Despite differences in the primary and secondary structure (α-helices and β-sheets), they share a highly basic and amphiphatic feature with a higher selectivity to lyse bacteria than host cells (3).

Another well studied class of pore-forming molecules includes proteins like bacterial toxins (4, 5), which require oligomerization for pore formation and specific membrane targets such as cholesterol (6), glycosylphosphatidylinositol (7), and integrins (8). Crystallographic data showed that α-hemolysin from Staphylococcus aureus is formed by the oligomerization of 33-kDa monomers in a heptameric β-barrel pore (9). Octameric and pentameric pores were found in HlyE hemolysin of Gram-negative bacteria (10) and in Vibrio cholerae cytolsin (11), respectively. In cholesterol-dependent cytolsins produced by more than 20 species of Gram-positive bacteria, the pores can be larger and formed by monomer assembling into rings of 30–50 subunits (12, 13). These cytolsins are bacterial virulence factors, affecting host immune cell function and cytokine induction (14).

Here we describe a new type of pore-forming molecule in the salivary glands of Triatoma infestans (Hemiptera, Reduviidae), an obligate hematophagous insect that transmits the human pathogenic protozoan Trypanosoma cruzi, the agent of Chagas disease, through its feces. It is a 22-kDa protein that shares the properties of the two classes of lytic molecules. It possesses a basic amphiphatic lytic motif in the N-terminal region containing 27 amino acid residues, similar to antimicrobial lytic peptides, and a protein portion that increases the lytic specificity toward eukaryotic cells such as bacterial toxins. This protein, named trialyisin, was purified from the salivary glands of T. infestans. It forms negative voltage-dependent pores in planar lipid bilayers and induces lysis of bacteria, Trypanosoma, and mammalian cells. In addition, we provide evidence that trialyisin is synthesized as a precursor and is processed by limited proteolysis.

**EXPERIMENTAL PROCEDURES**

Insects and Saliva—T. infestans were reared at the Laboratório de Xenodiagnóstico, Instituto Dante Fazzanese de Cardiologia, São Paulo, Brazil, in glass cylinders at 28 °C. The insects fed on ducks during each molt. Male adults were used in this study. One week after feeding, the collector held the insects ventrally and gently blew air at their rostrum to liberate the maxilla with a drop of saliva. This small drop was collected immediately using a glass capillary and stored at −20 °C. Lytic and Permeabilization Assays—The assays were performed with trypanostigate forms of T. cruzi (Y strain) obtained from an infected tissue culture of LLC-MK2 cells maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum at 37 °C with 5% CO2. The parasites were collected by centrifugation and resuspended to the indicated concentrations with Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. After incubation with lytic material, the
parasites were placed on ice, and the number of live parasites was counted in a Neubauer chamber using a phase microscope. Hemolysis was determined by hemoglobin release from human erythrocytes by measuring the absorbance at 405 nm after centrifugation. A 100% rate of hemolysis was achieved by treatment with 2% Triton X-100. Bacterial cell viability was determined by the ability of bacteria to form colonies on LB agar. Permeabilization was detected by incubation of bacteria with the sample containing the lytic activity in the presence of 10% Triton X-100 and 15% methanol for 1 h. The amount of material loaded onto the gel was 0.08% of Hitrap flow through lane b, 0.3% of the phenyl-Superose pool (lane c), and 0.5% of the Mono S pool (lane d). The standards were as in Fig. 1.

**FIG. 1.** Detection of a lytic and pore forming activity in insect saliva. Two aliquots of *T. infestans* saliva were mixed with sample buffer and fractionated by non-reducing 12.5% SDS-PAGE. A, the gel containing one sample (1 μl of saliva) was stained with Coomassie R-250. B, the other piece of gel, containing 2.5 μl of saliva, was treated with 15% methanol for 1 h and with 0.1 M Tris-HCl, pH 7.4, for another 1 h. The gel was cut into slices that were incubated with 1 × 10^6 *T. cruzi* trypomastigotes/ml in 50 μl of Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum. The remaining parasites after 105 min at 37 °C were counted in a Neubauer chamber. Means of triplicate measurements ± S.D. are shown in the figure. This experiment was repeated twice with similar results. The molecular size standards are as follows: bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa; β-lactoglobulin, 18 kDa; and lysozyme, 14 kDa. C, a black planar lipid bilayer (2.5% azolecithin in n-decane) was formed in an orifice 1.0 mm in diameter in 5 mM Tris-HCl and 5 mM KCl, pH 7.4, with a V_d = V_{d_0} = -20 mV. After baseline stabilization, saliva was diluted about 25,000-fold in the cis compartment, and data were recorded.

column equilibrated with 20 mM MES, pH 6.0. Bound proteins were eluted with a linear gradient to 1 M NaCl in the same buffer at 0.5 ml/min. All purification steps were performed using a fast protein liquid chromatography system (Amersham Biosciences) at room temperature. The samples were analyzed by 15% SDS-PAGE after Coomassie Blue or silver nitrate staining.

dNA Library and Cloning Procedures—A cDNA library from *T. infestans* salivary glands was constructed with poly(A)^+ mRNA extracted from D1/D2 salivary glands using the QuickPrep Micro mRNA Purification Kit (Amersham Biosciences). The cDNA was prepared from 3 μg of mRNA using the SuperScript® Lambda System for cDNA Synthesis and inserted into the Lambda ZipLox vector (Lambda Cloning System, Invitrogen) according to the manufacturer’s instructions. The library was packed with the Ready to Go Lambda Packaging System (Amersham Biosciences) and amplified in Escherichia coli Y1090 (ZL).

The library was used as a template for PCR containing a primer based on the N terminus of trialysin, the degenerated oligonucleotide-NT (5’-TTYARATHAARCCNGGNAARG) and the SP6 promoter primer located downstream of the λ vector cloning site. The oligonucleotides-CT (5’-CGGGATCCTTAATCAATTTCAACTTCATC) and the T7 promoter primer were used to amplify the full-length cDNA. Amplified products were purified from agarose gels and cloned into pGEM T-easy (Promega). Plasmids were inserted into *E. coli* DH5α, and clones were sequenced using the Applied Biosystems Inc. 377 Sequencing Apparatus.

**FIG. 2.** Purification of trialysin. A–C respectively show the elution profile of the Hitrap Q, phenyl-Superose, and Mono S columns of a typical purification from 1.2 ml of water-diluted saliva. The trypanolytic activity is represented as open circles, the protein as solid lines, and the salt gradients as dashed lines. To detect the lytic activity the fractions were dialyzed against phosphate-buffered saline and incubated at 37 °C with 1 × 10^6* T. cruzi* trypomastigotes/ml. The number of parasites lysed per min is shown in each graph. Insets show silver-stained SDS-PAGE of total saliva (lane a, 0.1% of the total sample) and of each pool, as indicated by the upper trace. The amount of material loaded onto the gel was 0.08% of Hitrap flow through lane b, 0.3% of the phenyl-Superose pool (lane c), and 0.5% of the Mono S pool (lane d). The standards were as in Fig. 1.

**Lipid Bilayer Measurements**—Planar lipid bilayers were formed using a solution of 2.5% azolecithin (Sigma) in n-decane according to the

The abbreviations used are: MES, 4-morpholineethanesulfonic acid; APMSF, aminophenylmethylsulfonyl fluoride; G6V32, the peptide corresponding to the segment from glycine 6 to valine 32 of trialysin.
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Fig. 3. Effect of trialysin on bacteria, protozoa, and mammalian cells. A, trypanosomes and human erythrocytes (5.0 × 10⁶ cells/ml) were washed in Hank’s glucose containing 0.2% bovine serum albumin and incubated with purified trialysin (8 µg/ml) at 37 °C with shaking. The panels show T. cruzi trypanosomes and human erythrocyte. Bars, 5 µm. This experiment was repeated independently three times with similar results. B, E. coli K12 (5.0 × 10⁹ bacteria/ml) was incubated with ethidium bromide for 40 min at 37 °C in the presence (+) or absence (−) of trialysin (8 µg/ml). Viability of bacteria was assessed by solid medium growth. Bar, 10 µm. C, confluent LLC-MK₂ cells were incubated with ethidium bromide for 50 min at 37 °C in the presence (+), or absence (−) of trialysin (34 µg/ml). After washing the cells, the images were acquired under phase contrast (Phase), and by fluorescence using a Nikon UV2A filter (EtBr). Bars, 20 µm.

technique of Mueller et al. (15). The membranes for macroscopic conductance measurements were 0.8–1.0 mm in diameter, and those for single-channel recordings were 300–500 µm wide. Conductance was measured using a patch clamp amplifier (Dagan Instruments, model 8900) configured in a voltage clamp mode. The data were acquired using the AxoScope 2.0.2 software (Axon Instruments) and analyzed with an AxoScope 8.0 (Axon Instruments). The voltage was simultaneously monitored in a digital oscilloscope (Tektronix TDS 340A).

Peptide Synthesis, Purification, and Quantitation—Peptides were synthesized by the Fmoc (N-(9-fluorenyl)methoxycarbonyl) methodology as described by Hirata et al. (16) using an automated bench top simultaneous multiple solid-phase peptide synthesizer (PSSM) 8 system from Shimadzu, Tokyo. Peptides were purified to homogeneity by high pressure liquid chromatography on a Vydac C₁₈ analytical column, and the sequence was confirmed by Edman degradation (PPSQ 23 system from Shimadzu, Tokyo). Peptide concentration was determined from the sequence was confirmed by Edman degradation (PPSQ 23 system from Shimadzu, Tokyo). Peptide concentration was determined from

RESULTS

We found that saliva of T. infestans killed the infective (trypanosomastigotes) and replicative (epimastigotes) insect stages of the protozoan T. cruzi. Lysis was abolished at 0 °C or when the saliva was heated to 85 °C, suggesting that a protein was involved in parasite lysis (not shown). When the saliva was separated by non-reducing SDS-PAGE, the parasite lytic activity was detected in a gel position corresponding to proteins of 30 kDa (Fig. 1, A and B). Likewise, diluted saliva induced an exponential increase in the conductance of the planar lipid bilayer until disruption of the membrane after 10–20 min (Fig. 1C), suggesting that a pore-forming activity was responsible for the lytic activity.

To purify the lytic molecule, ejected saliva from 800 insects (400 µl) was diluted in water, and the soluble fraction was applied to an anion exchange column (Hitrap Q). Unbound fractions containing the lytic activity (Fig. 2A) were pooled and adjusted to 1.7 M ammonium sulfate, and the pool was chromatographed through a phenyl-Superose column. The lytic activity eluted with ~750 mM ammonium sulfate (Fig. 2B). Fractions with lytic activity were pooled and applied to a cation exchange column (Mono S). The lytic material was eluted with 800 mM NaCl and was separated from most contaminants that did not bind to the column (Fig. 2C). The bound-active fractions contained a protein that migrated as a single band of 22 kDa in reducing SDS-PAGE stained with silver nitrate (Fig. 2C, inset).

Part of the lytic activity did not bind to the Mono S column when the sample was loaded in the presence of 750 mM ammonium sulfate (Fig. 2C). This lytic activity may be trialysin-bound to other proteins because when we dialyzed the phenyl-Superose pool, all the lytic activity bound to Mono S and eluted with 800 mM NaCl, exactly as trialysin. In this case, however, several other proteins bound to the column, contaminating the trialysin fraction. The final fraction yielded a single silver-stained band by SDS-PAGE of a similar size to the original lytic activity found in saliva. From 400 µl of saliva, about 80 µg of trialysin were obtained in a typical purification procedure, indicating that the concentration of this protein in saliva is at least 200 µg/ml or 9 µM. This purification protocol was repeated several times with similar results.

Purified trialysin induced lysis of T. cruzi trypanosomastigotes and human red blood cells at 8 µg/ml (0.4 µM). However, parasite lysis occurred much more rapidly than hemolysis (Fig. 3A). This kinetic difference is probably due to the different membrane composition because both types of cells have similar surface sizes. Trialysin was able to permeabilize (Fig. 3B) and kill E. coli K12 at 8 µg/ml (not shown). Adherent LLC-MK₂ epithelial cells were also permeabilized but required high concentrations of trialysin (1.5 µM), as shown in Fig. 3C.

In an initial attempt to elucidate the mechanism of lysis, we found that saliva generates macroscopic currents in large bilayers. The pore-forming activity of trialysin was then tested in small bilayers where evidence of pore-forming events was obtained, as shown in Fig. 4A. Trialysin added to the aqueous solution at a concentration of 0.2 µg/ml generated a predominant mean current state of 43.77 ± 2.33 pA (n = 100; V<sub>clamp</sub> = V<sub>trans</sub> = −50 mV) with a P<sub>e</sub> of 0.5 and a second current level of 84.82 ± 2.85 pA, indicative of the insertion of two pore-forming complexes in the lipid membrane (Fig. 4, A and B). At positive trans voltages (+30 to +100 mV), previous unitary currents were switched off, with reversal to unitary currents upon declamping to negative trans voltages (Fig. 4C).

Purified trialysin was submitted to N-terminal sequencing,
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Fig. 4. Trialysin generates single channels in bilayers. Black planar lipid bilayers (2.5% azolecithin in n-decane) were formed in an orifice 0.5 mm in diameter in phosphate-buffered saline. A, trialysin was added to the cis compartment (0.2 μg/ml), and data were recorded with a $V_{\text{clamp}} = V_{\text{trans}} = -50$ mV. B, distribution of current events (>30 ms, n = 100) of data recorded in an experiment similar to that shown in A. The numbers above the bars represent the mean ± S.D. current during the respective intervals. C, trialysin was added to the cis compartment (0.2 μg/ml), and data were recorded with $V_{\text{clamp}} = V_{\text{trans}} = \pm 50$ mV. Similar results were obtained with $V_{\text{clamp}} = \pm 30$, ±75, ±90, and ±100 mV.

and the first 20 amino acids were determined. A single sequence was generated (Fig. 5A, bold letters), indicating that the protein was highly purified. From the first eight N-terminal amino acids (underlined in Fig. 5A), a degenerated oligonucleotide primer was designed. This primer and the SP6 promoter primer, located downstream of genes cloned in a directional λ Zlplox library of T. infestans salivary glands, were used to amplify the trialysin cDNA by PCR. Several clones were obtained and sequenced. Seven clones showed the translated sequence of the first 20 amino acids found by protein sequencing. These clones were fully sequenced, presenting similar DNA sequences and almost the same amino acid sequence of 205 amino acids, as shown for clone 30 in Fig. 3A. Some clones had conserved amino acid changes (A28L, clone 31; G45S, M123T, and D183E, clones 17 and 22; F12L and I169V, clone 1), which might be attributed to the fact that we used a population of insects to prepare the cDNA library. Nevertheless, we cannot exclude the possibility that more than one gene encodes trialysin.

The amino acid sequence predicts a protein with a pl of 9.46, compatible with the high retention in Mono S, and a molecular mass of 22.2 kDa, similar to those obtained by reducing SDS-PAGE. No similarity to other proteins from the data bases was found. One striking feature of this molecule was the high percent of lysine residues (16%), even in comparison to the other positively charged amino acids, i.e. arginine (2%) and histidine (0.5%) residues. The repetitive distribution of these lysines in the N-terminal portion suggested that these initial amino acids form an amphipathic α-helical structure. The region between Gly<sup>9</sup> and Val<sup>32</sup> predicts an amphipathic α-helix region by GOR (17) and Eisenberg methods (18) (see Fig. 5B). By plotting this region as a helical wheel (19), the charged amino acids were found predominantly on one side of the helix, whereas the hydrophobic amino acids lay on the opposite side (Fig. 5C). This configuration is similar to that of several other antimicrobial pore-forming lytic peptides such as magainin from frog skin (20), cercopin (21), and sarcotoxin (22) found in insect hemolymph. The G6V32 sequence of trialysin also has a cluster of glycines between the charged and hydrophobic amino acids, as seen in magainin and sarcotoxin.

To verify whether the region between the Gly<sup>9</sup> and Val<sup>32</sup> amino acids of trialysin was involved in the lytic activity, the G6V32 peptide was synthesized and tested for lytic activity. As shown in Fig. 6, the peptide lysed T. cruzi trypomastigotes and permeabilized mammalian cells. The lysis was specific since the peptide was ineffective at 0 °C, and the purified peptide with blocked lysine residues was unable to induce lysis (not shown). Comparatively, trialysin was more efficient than G6V32 in both assays. However, trialysin-induced parasite lysis was about 10-fold higher, and trialysin-induced mammalian cell permeabilization was about 100-fold higher. This result shows that the remaining amino acid residues of the protein, excluding the cationic amphipathic lytic region, increased the activity of trialysin mainly toward mammalian cells.

Since trialysin was efficient in permeabilizing mammalian cells, it could also produce damage in the insect salivary gland if synthesized with full lytic activity. Therefore, we searched for a precursor molecule by amplifying the cDNA library with an anchor primer corresponding to a site upstream of the vector cloning site and a primer corresponding to the C terminus of trialysin. We obtained a PCR product with about 1100 bp, 300 bp longer than the product amplified based on purified trialysin. Its DNA sequence predicts an amino acid sequence that contains a typical signal peptide (23) followed by a 33-amino acid sequence rich in negatively charged amino acids (7 Asp and 8 Glu) separated by 1 arginine located upstream of the N-terminal sequence of the purified trialysin (Fig. 7A). This finding suggests that the mature protein is produced by limited proteolysis and that these acidic amino acids might control the lytic activity by neutralizing the basic amino acids in the adjacent region corresponding to the lytic amphipathic α-helix.

Since we have reported previously (24) the presence of a serine protease in salivary glands of T. infestans that is activated at the time of the bite and specifically cleaves arginines, we tested the lytic activity of salivary gland homogenates prepared in the presence or absence of an irreversible serine protease inhibitor. As shown in Fig. 7B, a 10-fold reduction in lytic activity was obtained in the presence of APMSF, showing that a serine protease is involved in the processing and activation of trialysin. APMSF did not affect T. cruzi lysis when pre-activated saliva was used, excluding the participation of serine proteases in parasite lysis.

DISCUSSION

In the present study we have characterized the cytolytic salivary activity of T. infestans. It is a pore-forming protein, activated by limited proteolysis, that permeabilizes and lyses prokaryotic and eukaryotic cells. This protein was purified to homogeneity, cloned, and named trialysin (from Triatoma infestans cytolysin). We obtained several trialysin cDNA se-
sequences, but all of them predict for proteins with conserved modifications in amino acid sequence.

No significant similarity was found between trialysin and other sequences in data banks. However, the region delimited by Gly° and Val° (30% lysine) is a suitable cationic amphipathic lytic motif and structurally resembles several antimicrobial lytic peptides. The synthetic peptide G6V32, which corresponds to this domain, lyses trypomastigotes and permeabilizes mammalian cells. However, the entire protein is more efficient than the peptide in inducing parasite lysis and particulary in permeabilizing mammalian cells. Compared with trialysin, larger amounts of G6V32 are required to permeabilize mammalian cells. Nevertheless, protozoan parasites and bacteria are more susceptible to trialysin and G6V32 compared with mammalian cells, a fact that could be related to differences in their membrane composition and structure. Saliva generates an exponential increase in conductance leading to disruption of artificial lipid membranes. This effect may be due to the trialysin pore forming activity and not to a detergent-like activity. First, trialysin is found at high concentration in saliva, and in its purified state forms single channels. Second, the lipid membrane remained stable in the presence of trialysin and in absence of a negative potential, and no lytic activity could be detected at 0 °C. Finally, trialysin generated conductance only at negative trans potentials with the channels reversibly closing at positive trans potentials. Events at positive trans voltages were rarely detected, probably due to insertion of trialysin in the trans side of the membrane.

Cytolytic activities need to be tightly regulated in order to avoid self-damage, mainly in storage compartments such as T. infestans salivary glands, where they reach high concentrations (9 μM). One way of controlling the lytic activity is to rely on differential specificity of molecules, as seen with bacterial toxins targeted to specific membrane components of host cells. Another way depends on proteolytic processing, as shown for defensins (25), aerolysin (26), and vibriolysin (27). Our data strongly support the notion that trialysin is synthesized as a precursor molecule that is activated by limited proteolysis. The precursor of trialysin contains a signal peptide sequence followed by an acidic 33-amino acid pro-sequence (45% of Glu and Asp), not found in the mature protein. The anionic region is adjacent to the cationic amphipathic lytic motif (30% of Lys), which is located at the N terminus of the mature protein. This suggests that the anionic pro-sequence controls the trialysin activity by neutralizing the adjacent cationic lytic motif. In addition, the hair pin formed by electrostatic interaction of these opposite charged sequences may organize the site of protein processing (Arg°) in an exposed loop for a proper

FIG. 5. Predicted sequence of trialysin. A, amino acid sequence based on the translated DNA sequence of clone 30 obtained by PCR amplification in a cDNA library of T. infestans salivary glands (GenBank access number AF427486). The bold letters represent the amino acid sequence obtained by Edman degradation of purified trialysin. The underlined region corresponds to the primer position used to amplify the cDNA clones. B, the figure shows plot of α-helical regions using the GOR prediction method with no decision constants (α-region), plot of α-helical amphipathic regions using Eisenberg hydropathic moment (α-amphipathic), and charge (Charge) distribution of the molecule at pH 7.0. C, the figure shows amphipathic helical wheels of trialysin (open box in B) and the indicated pore-forming peptides (45) obtained with the software package Protean 4.0 (DNASTAR).

FIG. 6. Comparison of trialysin and G6V32-induced lysis of T. cruzi and mammalian cells. Trialysin (open circles) and G6V32 (closed circles) were incubated for 50 min at 37 °C with 3.0 × 10° trypomastigotes/ml. Parasite lysis was determined using a Neubauer chamber. Alternatively, trialysin (open triangles) and G6V32 (closed triangles) were incubated for 50 min at 37 °C with attached and confluent LLC-MK2 cells in the presence of 10 μg/ml ethidium bromide. The percentage of permeabilized cells was determined after digital data analysis. Each point represents the mean ± S.D. of triplicate experiments.
cleavage at Arg56 of the trialysin precursor. The proteolytic processing releases the anionic pro-sequence exposing the cationic amphipathic lytic motif of trialysin. B, binding of the activated molecule to membrane phospholipids. The negative charge represents the phosphate group of membrane lipids. C, pore formation under a negative membrane potential (trans side negative) with the organization of G6V32 in an α-helical structure. D, reversible closure of the pore under a positive membrane potential (trans side positive).

The proteolytic attack. A similar anionic pro-sequence of 45 amino acid residues (18% of Glu and Asp) is found in defensin, which is a 30-amino acid peptide that forms cationic amphipathic β-sheets and contains 13% of arginine (28). Interestingly, the ratio of negatively and positively charged amino acids is about 1.5 in both molecules, a finding that suggests a general mechanism of activation control.

We have described previously a serine protease called triapsin, which is present in the salivary glands of *T. infestans* and is released in the ejected saliva (24). Based on the present data, it appears that triapsin is responsible for trialysin activation. First, triapsin is the major proteolytic activity in the salivary glands of *T. infestans*. Second, triapsin is highly specific for cleavage of the arginine bond, and this is the amino acid found before the N terminus of the mature trialysin. Third, APMSF, an irreversible specific inhibitor of triapsin, significantly impairs the activation of trialysin in salivary gland homogenates. Finally, triapsin is activated at the time of saliva ejection, a condition that would prevent self-damage by trialysin.

We propose a simplified model of activation and pore formation, as shown in Fig. 8. The pro-trialysin is stored in the inactive form in salivary glands. At the time of the bite a serine protease is activated (Fig. 8A) and cleaves the exposed Arg<sup>26</sup> of pro-trialysin separating the anionic peptide from the mature pore-forming protein, thus exposing the cationic amphipathic motif of trialysin. This cationic domain of trialysin then binds to the negative head group of membrane phospholipids (Fig. 8B) and inserts the amphipathic α-helix into the lipid bilayer forming an aqueous pore, which has a cis-trans asymmetry (Fig. 8, C and D), being opened by negative trans voltages.

In hematophagous insects, lytic activities have been associated with a digestive hemolytic role (29). However, it is unlikely that trialysin has a nutritional role, since blood is stored in the stomach for weeks without hemolysis, which is performed in the posterior midgut of *T. infestans*. A possible role of trialysin in blood feeding on the mammalian host should also be considered. Saliva is the only interface that promotes shedding of L-selectin in leukocytes, inhibit the lymphocyte function-associated antigen-3 (LFA-3) and induces apoptosis in lymphocytes (41, 42) and in endothelial cells (43), which depend on controlled ionic flux to exert their physiological roles. It has been demonstrated that pore-forming molecules inhibit platelet function (39, 40), induce apoptosis in leukocytes (41, 42) and in endothelial cells (43), promote shedding of L-selectin in leukocytes, inhibit the lymphocyte function-associated antigen-3 (LFA-3) and induces apoptosis in lymphocytes (41, 42) and in endothelial cells (43).
cyte proliferative response, and immunoglobulin production (44) among other cellular activities (14).

In conclusion, our results place trialysin between the lytic pore-forming pepti-des and the lytic pore-forming bacterial pro-teins. The cationic amphiphilic pore-forming peptides are classically characterized as antibacterial molecules because of the resistance of mammalian cells to peptide lysis and are found in the immune system of several unrelated species. This resist-ance could be clearly seen when comparing the permeabilizing activity of peptide G6V32 and the entire protein. On the other hand, there are pore-forming proteins from bacteria that pos-sess target specificity for eukaryotic host cells and in many cases interfere with cell function, thus being important deter-minants of bacterial virulence. Trialysin is able to kill micro-organisms like a lytic peptide and permeabilize mammalian cells like a bacterial toxin. This dual role may allow it to act on blood feeding by interacting with host cells and on microbial growth control in salivary glands. In addition, the lytic mole-cule is activated by proteolytic removal of the anionic pro-sequence contiguous to the cationic lytic motif. Understanding how lytic molecules control their activities and specificities is desirable in order to design cell-specific regulated cytolytic proteins.

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