The gut epithelium is an essential interface in insects that transmit parasites. We investigated the role that local innate immunity might have on vector competence, taking Stomoxys calcitrans as a model. S. calcitrans is sympatric with tsetse flies, feeds on many of the same vertebrate hosts, and is thus regularly exposed to the trypanosomes that cause African sleeping sickness and nagana. Despite this, S. calcitrans is not a cyclical vector of these trypanosomes. Trypanosomes develop exclusively in the lumen of digestive organs, and so epithelial immune mechanisms, and in particular antimicrobial peptides (AMPs), may be the prime determinants of the fate of an infection. To investigate why S. calcitrans is not a cyclical vector of trypanosomes, we have looked in its midgut for AMPs with trypanolytic activity. We have identified a new AMP of 42 amino acids, which we named stomoxyn, constitutively expressed and secreted exclusively in the anterior midgut of S. calcitrans. It displays an amphipathic helical structure and exhibits a broad activity spectrum affecting the growth of microorganisms. Interestingly, this AMP exhibits trypanolytic activity to Trypanosoma brucei rhodesiense. We argue that stomoxyn may help to explain why S. calcitrans is not a vector of trypanosomes causing African sleeping sickness and nagana.

Epithelial intestinal innate immunity plays a major role in the control of infectious diseases in vertebrates (1, 2). In invertebrates, data are still fragmentary despite gut epithelium being an essential interface for parasites during their development in insect vectors. Understanding vector biology is a key element in the control of many parasitic diseases. In this context, the comparison of the trypanosome vector Glossina with the sympatric but non-vector Stomoxys calcitrans is particularly interesting. The tsetse fly Glossina spp. is the major vector of the range of trypanosomes that cause African sleeping sickness in humans and nagana in livestock. Stable flies, Stomoxys spp., feed on the same vertebrate hosts as tsetse flies and have a very similar digestive physiology and midgut anatomy. Although Stomoxys is constantly exposed to trypanosomes, it kills them in the midgut within 2–4 days of ingestion (3). So Stomoxys is not a cyclical vector of trypanosomes (although it can act as a mechanical vector, “flying pin”). Why Stomoxys is not a cyclical vector of trypanosomes is unknown. Recent studies have shown that the insect immune system plays a determinant role in the fate of trypanosome infections in tsetse flies (4, 5). Consequently, in this report, we address the possibility that the distinction in vectorial capacity between Glossina and Stomoxys may lie in differences in immune mechanisms.

Trypanosoma vivax matures entirely in the mouthparts of Glossina. The other trypanosomes causing nagana and human disease are ingested into the fly midgut where they multiply first in the endoperitrophic space and later in the ectoperitrophic space tightly sandwiched between the peritrophic matrix and the anterior midgut epithelium (6). These trypanosomes then migrate, entirely within the luminal space of the intestine, to the mouthparts or salivary glands for transmission to a new vertebrate. Consequently it is epithelial rather than systemic immune responses that are likely to have the major bearing on the fate of trypanosome infections. Epithelia constitute the first line of defense in the innate immunity of both vertebrates (reviewed in Ref. 7) and invertebrates (8–10), and AMPs are essential components of this epithelial immunity. For example, the mouse gut epithelium responds to bacterial infection by secretion of α-defensins or cryptdins (reviewed in Refs. 1 and 2), and gut expression of β-defensins is up-regulated by the parasite Cryptosporidium parvum (11). In invertebrates, the S. calcitrans anterior midgut epithelium responds to microbial infection by post-transcriptional up-regulation of secreted defensins, whereas the mosquito midgut responds to Plasmodium infection by up-regulation of a range of immunity factors including several AMP genes (reviewed in Ref. 12). We know that some of these AMPs can be active against trypanosomes (4) and other parasites such as Plasmodium and filarial worms that also spend part of their life in the gut of a blood-sucking insect (13, 14).

Because of the potential importance of epithelial AMPs for determining vectorial capacity, we have looked for novel AMPs in gut extracts of S. calcitrans using biochemical and molecular
approaches. It is already known that the anterior midgut epithelium of *S. calcitrans* produces two defensins (9) that are secreted into the midgut lumen (15, 16). In this study, in addition to these defensins, we identified a novel 42-residue peptide, which we named stomoxyn. Stomoxyn has no sequence homology with other known proteins. This molecule is adult and anterior midgut-specific, where it is constitutively expressed. As shown by a circular dichroism study, stomoxyn adopts an α-helical structure only in the presence of an organic solvent that mimics a phospholipid membrane. Stomoxyn is active against many Gram-negative and Gram-positive bacteria, filamentous fungi, and yeast, and its activity is apparent within a few minutes of exposure. Although stomoxyn has only limited hemolytic activity against bovine red blood cells, it possesses significant trypanolytic effect on trypanomastigotes of *Trypanosoma brucei rhodesiense*. Consequently it may help to explain why *Stomoxys* is not a vector of African trypanosomiasis.

**EXPERIMENTAL PROCEDURES**

**Insect and Tissue Preparation**

*S. calcitrans* were reared as described by O’Brochta et al. (17). Adult male and female insects, both of which feed on blood, were used in the experiments. The artificial blood meal (18) and casein hydrolysate meal were made with high purity water. Flies used for HPLC purification of midgut AMPs were fed from cotton wool soaked swabs on either pig blood or casein hydrolysat (20 g/100 ml). At 24–36 h after feeding, anterior midguts (2,000 consisting of proventriculus, thoracic, and reservoir regions) (19) were dissected in 154 mM NaCl and homogenized at 4 °C in 200 mM sodium acetate at pH 4.5. The homogenate was heated to 100 °C for 5 min and centrifuged at 12,000 × g for 10 min at 4 °C. Pellet and supernatant were stored separately at −20 °C until used.

**Isolation and Structural Characterization of AMPs**

**AMP Purification**—The supernatant of *S. calcitrans* midgut homogenate was subjected to RP-HPLC on an Aquapore OD300 C18 column (220 × 4.6 mm, Brownlee) with a linear gradient of 2–60% acetonitrile in acidified water (0.05% trifluoroacetic acid) over 120 min at a flow rate of 0.8 ml/min at 35 °C. The column effluent was monitored by absorbance at 225 nm, and fractions were hand-collected. After evaporation of the fluorhydric acid by vacuum, the resin was washed with 100% acetonitrile and finally dried under nitrogen. After removal of the fluorhydric acid by vacuum, the fraction was washed with ethyl ether, and the peptide was extracted with acetic acid, water, and acetone. The synthetic peptide was purified to homogeneity by solid-phase extraction and RP-HPLC using a gradient of acetonitrile in acidified water. Peptide purity and integrity were controlled by MALDI-TOF MS.

**Biossays**

**Solid Growth Inhibition Zone Assay**—During the peptide purification, antimicrobial activity was assayed against the two test bacteria, the Gram-positive *Micrococcus luteus* (23) and the Gram-negative *E. coli* K12 RM148 (9). Briefly, 2 μl (40–230 μg/ml, depending on the purification step) of each RP-HPLC fractions were incubated with bacteria overnight at 37 °C. Two AMPs were used as positive controls: *Drosophila* cepaerin A (25 μM) and *Aedes* defensin A (15 μM) for anti-Gram-negative and -positive activities, respectively.

**Liquid Growth Inhibition Assay**—The activity spectrum (minimal inhibitory concentration, MIC) of stomoxyn (concentration range, from 0.2 up to 100 μM) was determined against bacteria and fungi using a liquid growth inhibition assay (23). The strains used were from private and public collections (24, 25). Bactericidal effect was measured by colony-forming unit counting at 24 h. At the antimicrobial activity was assayed in the presence of salts, the appropriate medium was supplemented with 1 mM CaCl2, 50 mM KCl, or 154 mM NaCl.

**Kinetic of Killing against Bacteria and Yeast**—Overnight cultures of *E. coli* K12 RM148, *M. luteus*, and *Cryptococcus neoformans* were diluted in appropriate media and allowed to grow to logarithmic phase. Stomoxyn (10 times the MIC) was incubated with the test microorganisms in a 96-well microtiter plate at 37 °C. At different time points (30, 45, and 150 min), samples were removed, diluted, and plated on Antibiotics (Applied Biosystems Inc., model 473A).

**Library Construction, Screening, and Sequencing**

A *S. calcitrans* adult midgut cDNA library with an estimated complexity of 1.4 × 106 plaque-forming units was constructed in Lambda ZAP (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. The library was plated using Escherichia coli XL-1 Blue. A degenerate sense PCR primer (S1 5′-TATACGCGGNCRTCTTCTT-3′) corresponding to the amino acid sequence KDTAVI of stomoxyn and an M13–20 primer were used in PCR with the cDNA library as template to generate a 336-bp product. This product was cloned using TOPO TA (Invitrogen) and sequenced. A pair of sequence-specific primers were designed from this sequence (S2, 5′-TCTATCCAATCTCATTCA-3′, and S3, 5′-TATGACAGCAGATCTCTT-3′) and used in PCR with the cDNA library as template to generate a 193-bp probe. The probe was 32P-labeled and used to screen the midgut cDNA library. Phagemid clones (BBlueScript) containing the stomoxyn protein cDNA were excised in vitro from the λ vector using ExAssist helper phage and plated using *E. coli* XLOLR (Stratagene) and sequenced.

**Nuclease Protection Assays**

Nuclease protection assays were performed using the Multi-NPA kit (Ambion, Austin, TX). Total RNA was prepared from 10 anterior midguts (proventriculus, thoracic, and reservoir regions), 10 posterior midguts (opaque and lipoid regions), 1 remains of a carcass from adult *S. calcitrans*, 100 eggs, and 2 larvae or 2 pupae using RNeasy Mini Kit (Qiagen, Crawley, UK). For treatment details, see Figure 3 capture. Oligonucleotide probes NPA5 (5′-GGCCTGGTCTGTTGGCGCAAGGACATGACCCAAAACGACCAGAACTACCAGGAATCCACTAA-3′) and NPA4 5′-GGCGTTGGAAATCGGAAATGGGAGTAACATCTTCTCTT-ATAGAT-3′ were used to detect stomoxyn and S14, respectively. Each oligonucleotide probe contained 10 bp of non-complementary sequences on the 3′ end so that full-length probe can be distinguished from the protected fragment.

**Peptide Synthesis**

The peptide was assembled using a multichannel peptide synthesizer adapted to Boc chemistry (22). Classical Boc-protected amino acids were used during the assembly but with histidine residues introduced as 2-(4-hydroxyphenyl)glycine derivatives. Following the last Boc deprotection, the resin was washed five times with dichloromethane and dried under nitrogen. Finally a standard fluorhydric acid procedure was used for deprotection and cleavage of the peptide from the resin. After removal of the fluorhydric acid by vacuum, the resin was washed with ethyl ether, and the peptide was extracted with acetic acid, water, and acetone. The synthetic peptide was purified to homogeneity by solid-phase extraction and RP-HPLC using a gradient of acetonitrile in acidified water. Peptide purity and integrity were controlled by MALDI-TOF MS.
plates were incubated for another 2–4 h. The plates were read in a microplate fluorometer system (Spectramax Gemini, Molecular Devices) using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. Data were transferred into a graphic program (Softmax Pro, Molecular Devices), sigmoidal inhibition curves were determined, and IC₅₀ (drug concentration inhibiting 50% of fluorescence development) values were calculated. The activity against procyclic forms was determined in the same way. Procyclics of the same trypanosome strain were grown in SDM-79 medium (28).

Hemolytic Assay—Bovine red blood cells were washed twice with phosphate buffer saline by centrifugation at 3,000 rpm, 10 min at 4 °C. Elution was performed using a gradient of acetonitrile (dotted line), and the absorbance was measured at 225 nm (full line). Sm2d2 corresponds to the Stomoxys midgut defensin 2 identified previously (9).

For the trypsin stability study, steps were taken according to the procedure described previously (29) except that the stability of stomoxyn (12 μM final concentration) in the presence of 25% bovine serum was tested according to the procedure described previously (29) except that the stability was evaluated by MALDI-TOF MS. For the trypsin stability study, stomoxyn (0.5 mg in a final volume of 20 μl) was incubated with bovine trypsin (Roche Molecular Biochemicals) at an enzyme/peptide ratio of 1:10, according to the manufacturer’s directions. At different time points (5, 15, 30, 60, 150 min, and overnight), an aliquot (0.5 μl) of the sample was removed, and the reaction was stopped with trifluoroacetic acid. The digest products were analyzed by MALDI-TOF MS.

Circular Dichroism Analysis—Circular dichroism spectra of stomoxyn (0.1 mg) were recorded between 180 and 250 nm on a Jasco J-810 spectropolarimeter in 0.5 nm steps at varying concentrations of trifiuoroethanol (TFE) in water (from 0 to 80%, v/v) at pH 4 using a quartz cell of 1 mm. For each experiment, four spectra were averaged, and the baseline was corrected for neat solvent and solvent mixture. All measurements were carried out at 25 and 4 °C.

RESULTS

Stomoxyn Peptide Purification and Characterization

Crude homogenates of anterior midgut of St. calcitrans, 24–36 h after a blood meal, have been shown previously to possess anti-Gram-negative activity. Two insect defensins have already been identified (Sm1d and Sm2d (9)). In this study, screening of HFLC-purified proteins extracted from 2,000 anterior midguts revealed the presence of eight fractions with antimicrobial activities. Two fractions were active against the Gram-positive test organism M. luteus (Fig. 1, peaks 1 and 2), and six were active against the Gram-negative strain E. coli K12 RM148 (Fig. 1, peaks 3–8). After MALDI-TOF MS measurement and partial Edman sequencing, fraction 8 was found to contain Stomoxyn defensin Sm2d2 (data not shown). As the strongest activities were observed in fractions 5 and 7 (Fig. 1), an extensive purification was performed on these fractions, and pure active complexes with molecular masses of 4246.11 MH⁺ and 4415.49 MH⁺ were measured for fractions 5 and 7, respectively (data not shown). For the molecule at 4246.11 MH⁺, the following 33-amino acid partial sequence was obtained, RG-FRKHFNKLVKKHTISETAHVAKDT.

Expression of Stomoxyn mRNA in S. calcitrans

The expression of stomoxyn mRNA in different tissues and at various developmental stages in S. calcitrans was determined by nuclease protection assays (Fig. 3). Stomoxyn is expressed in the anterior midgut (proventriculus, thoracic, and reservoir regions) but not in the posterior midgut (opaque and lipid regions) or the rest of the adult body, eggs, larvae, or pupae of S. calcitrans. The level of CDNA appeared to be unchanged with blood feeding, with and without addition of bacteria and lamellarin. However, it should be noted that other immune peptides in these tissues are strongly regulated at the post-transcriptional level (16).

Activity Spectrum of Stomoxyn

Antimicrobial Activity—To investigate the antimicrobial properties of stomoxyn, the peptide was synthesized using Boc chemistry, purified to homogeneity, and tested against various microorganisms (Gram-negative and Gram-positive bacteria, filamentous fungi, and yeasts). The activity of stomoxyn was compared with a standard α-helical AMP from insects, Drosophila cerropin A. Stomoxyn had marked activity against...
Antimicrobial activity spectrum of stomoxyn (Stom) and Drosophila cecropin A (Cec)

The antimicrobial activity spectrum was established in liquid growth inhibition assays. Two-fold serial dilutions were tested from 100 μM down to 0.19 μM. The activity is reported as MIC values in μM. ND means No Data, and NA means no activity detected at the highest concentration tested (100 μM).

| Microorganisms                  | Stom | Cec |
|---------------------------------|------|-----|
| **Gram-negative bacteria**      |      |     |
| E. coli K12 RM148               | 0.19–0.39 | ND |
| E. coli D22                    | 0.19–0.39 | 0.1–0.25 |
| E. coli D31                    | 0.19–0.39 | 0.1–0.25 |
| E. coli SBS/63                  | < 0.19 | 0.19–0.39 |
| E. coli 1106                   | 0.19–0.39 | 0.25–0.5 |
| Enterobacter cloacae β12       | 3.12–6.25 | 0.78–1.56 |
| E. coli agglomerans            | 7.8–15.6 | 0.78–1.56 |
| Klebsiella pneumoniae           | 0.78–15.6 | 0.39–0.78 |
| Pseudomonas aeruginosa         | 0.39–0.78 | 1–2.5 |
| Salmonella typhimurium          | 0.39–0.78 | 0.5–1 |
| S. marcescens Db 11            | NA    |     |
| Xanthomonas campestris          | 0.39–0.78 | 0.5–1 |
| **Gram-positive bacteria**      |      |     |
| Aerococcus viridans            | 0.78–1.56 | 5–10 |
| Bacillus cereus                | NA    |     |
| Bacillus megaterium            | 0.78–1.56 | 1.56–3.12 |
| B. subtilis                    | 6.25–15.6 | 10–25 |
| Enterococcus faecalis          | 0.78–1.56 | NA |
| L. monocytogenes               | NA    |     |
| M. luteus                      | 0.78–1.56 | 5–10 |
| S. aureus                      | NA    |     |
| Streptococcus pyogenes         | 1.56–3.12 | NA |
| **Filamentous fungi**          |      |     |
| A. fumigatus                   | 50–100 | NA |
| B. bassiana                    | NA    |     |
| Fusarium culmorum              | 0.39–0.78 | 1–2.5 |
| Fusarium oxysporum             | 0.78–1.56 | 1–2.5 |
| Nectria haematococca           | 0.39–0.78 | ND |
| N. crassa                      | 3.12–6.25 | 2.5–5 |
| Trichoderma viride             | 1.56–3.12 | ND |
| Trichophyton mentagrophytus    | 3.12–6.25 | ND |
| **Yeast**                      |      |     |
| C. albicans                    | 25–50 | NA |
| Candida glabrata               | 25–50 | NA |
| C. neoformans                  | 0.78–1.56 | NA |
| S. cerevisiae                  | NA    |     |

Most of the microorganisms tested (Table I). All the Gram-negative bacteria tested, except Enterobacter cloacae (6 μM), were affected by less than 2 μM peptide. No activity could be detected against the entomopathogenic Gram-negative bacteria, Serratia marcescens, up to 100 μM. These activities are comparable with Drosophila cecropin A. Synthetic stomoxyn also showed high activity against most of the Gram-positive bacteria tested (MICs < 3 μM), except for Bacillus subtilis (MIC = 6.25–12.5 μM). No activity was detected against Bacillus cereus, Listeria monocytogenes, or Staphylococcus aureus up to 100 μM peptide. For all the Gram-positive bacteria tested, stomoxyn was more active than Drosophila cecropin A. In addition, stomoxyn was bactericidal on all the bacterial strains affected (data not shown). Most of the filamentous fungi assayed were highly sensitive to stomoxyn (MIC < 6.25 μM), whereas only a few strains were affected by Drosophila cecropin A. The human pathogenic strain Aspergillus fumigatus was only moderately affected by 100 μM stomoxyn, and Beauveria bassiana was unaffected, whatever the doses. However, A. fumigatus sporulation was inhibited by less than 6 μM stomoxyn (Fig. 4A). In contrast to Drosophila cecropin A, which was inefficient on the four yeast strains tested, stomoxyn was highly active against C. neoformans (MIC = 0.78–1.56 μM), moderately effective against Candida albicans and C. glabrata (MIC = 25–50 μM), and inefficient against Saccharomyces cerevisiae up to 100 μM (Table I). Killing assays on the different pathogens (bacteria, filamentous fungi, and yeast) revealed that stomoxyn acted within the first few minutes of incubation (data not shown). The antimicrobial activity of stomoxyn was not affected by various concentrations of different salts such as KCl, CaCl2, or NaCl (data not shown).

Activity of Stomoxyn against Eukaryotic Cells—The antiparasitic activity of stomoxyn was tested against trypanomastigote (vertebrate host forms) and procyclic (insect forms) forms of T. b. rhodesiense. The peptide was found to be lytic at 113 μM with an inhibitory concentration (IC50) of 37 μM on trypons.
The primary structure of stomoxyn indicates the corresponding amino acidic activity of. Trypsin digestion of the digest after 5 min of incubation at 37 °C of stomoxyn (50 μg) with trypsin (5 μg). The labeled peaks (a–l) point out the identified tryptic fragments of stomoxyn. The labeled full lines above and below the primary structure of stomoxyn indicate the corresponding amino acid sequences. The asterisk stands for native stomoxyn.

tigote bloodstream forms (Fig. 4B). Against procyclic T. b. rhodesiense, the peptide was found to be inactive at the highest concentration tested (113 μM; data not shown). Finally, as the anterior part of the midgut is a major site for blood storage and dehydration (30) but not hemolysis (31), stomoxyn was tested for its hemolytic activity. At 10 μM, stomoxyn exhibited hemolytic activity of < 4% on bovine red blood cells, whereas at 100 μM, hemolytic activity did not exceed 10% (Fig. 4C).

Stomoxyn Stability in Bovine Serum and to Trypsin

Because stomoxyn is present in the digestive tract where the blood meal is stored, we evaluated the stomoxyn resistance to bovine serum. To retard the kinetic of degradation and to increase peptide recovery, the stability experiment was performed in diluted serum (25%). After incubation of stomoxyn with bovine serum for 45 min at 37 °C, MALDI-TOF MS did not reveal any significant cleavage product or reduction of the initial amount of native peptide (data not shown). However, after a 2-h incubation, a strong decrease in the amount of stomoxyn was observed through estimation based on the MALDI-TOF MS peak heights. When evaluating the first metabolites at 2 h, only C-terminal fragments of stomoxyn can be detected by MALDI-TOF MS, and no clear signal corresponding to the N-terminal part was detected. After 4–5 h of incubation, no stomoxyn could be detected.

To assay the susceptibility of stomoxyn to trypsin, an enzyme present in abundance in the posterior midgut of the stable fly, a high concentration of peptide (0.5 mM) was incubated with pure trypsin, and the digest products were analyzed by MALDI-TOF MS in a time course experiment. After 5 min of incubation at 37 °C, only traces of native stomoxyn were visible (Fig. 5). Precise mass spectrometric analyses of the crude digest revealed 12 tryptic fragments in the mass range of 400 Da to 3 kDa covering all the primary structure of stomoxyn (Fig. 5), confirming the extreme sensitivity of this peptide to trypsin.

**Circular Dichroism Spectroscopy**

The circular dichroism spectra of stomoxyn were acquired in increasing concentrations of TFE from 0 up to 80% at 4 and 25 °C. In the absence of TFE and at 20% TFE, stomoxyn spectra are characteristic of a random coil conformation. As the TFE concentration increased, a shoulder at 222 nm and a shallow minimum in the vicinity of 208 nm appeared, indicating a transition from a flexible structure to a helical conformation of the peptide in the presence of TFE. A marked effect of the temperature was visible at 30 and 40% TFE. At an equivalent concentration of TFE, the helicity was lower at 25 °C (Fig. 6) than at 4 °C (data not shown).

**DISCUSSION**

The present study reports the identification of stomoxyn, an AMP in the stable fly *S. calcitrans* with no homology with known proteins. Stomoxyn has no cysteine residue and adopts an α-helical structure in an organic solvent mimicking the phospholipidic membranes. As deduced from cDNA cloning, Edman degradation, and mass spectrometry measurement, stomoxyn is post-translationally modified by C-terminal amidation of the threonine residue. A truncated form of stomoxyn (molecule at 4256.11 MH+) lacking the two last C-terminal residues (Ala-Thr) was also isolated, probably corresponding to degradation by exopeptidases. We do not know whether this results from a natural degradation within the gut or from our experimental conditions. Alignment of the prepropeptide sequence of *S. calcitrans* stomoxyn and data base sequences revealed very little identity with any known peptide. The closest identity (p = 0.44) was with a cecropin 3 precursor molecule (swiss protein database accession number Q94558) derived from *Drosophila virilis*, although little identity was found in the mature peptide. The greatest identity was between the first 5 amino acids of the prepropeptide domain of these molecules with the identical sequence, MNFYK. A second region of identity was found in the prepropeptide sequence KYLVVLVVL, which showed some similarity (p = 1.9) to the androrgan precursor (accession number P21663) derived from *Drosophila melanogaster* (32).

As shown by nuclease protection assays, stomoxyn is specifically expressed in the anterior part of the midgut where the blood is stored. Blood is a highly valuable resource, which the fly must protect from microorganisms during storage prior to digestion. It is likely that stomoxyn, with its rapid acting broad range of activity against both bacteria and fungi (Table I), plays an important role in this protection. In previous studies on gut immunity of *S. calcitrans*, two defensins, Smd1 and Smd2, have been described that are also specific to the anterior midgut of adult flies (9, 15). This production of at least three AMPs that are specific to adult midgut tissues illustrates the importance of the gut in the innate immunity of this blood-sucking insect and opens the possibility for the synergistic action of these various AMPs. Using the synthetic stomoxyn as reference, the quantity of native stomoxyn was estimated by μ-RP-HPLC to be ~530 pg/gut. We were not able to quantify the local concentration of stomoxyn. However, given that stomoxyn is likely to be secreted into the very confined space between the gel-like, dehydrated blood meal and the midgut epithelium, we speculate that the local concentration can reach a locally high
level as observed for vertebrate defensins (2). The synthesis of AMPs in the anterior part of the gut seems to be a conserved phenomenon in insects. Gambicin, cecropin, and defensin in Anopheles gambiae (8, 21, 24) and dipterin in D. melanogaster (10) are also secreted in the anterior part of the midgut. Digestive enzymes, secreted in the posterior part of the gut, may inactivate AMPs, as shown by the rapid degradation of stomoxyn by trypsin (Fig. 5).

Stomoxyn is sympatric with Glossina, feeds on many of the same vertebrate hosts, and is thus regularly infected with trypanosomes but kills them efficiently in the gut (3). Glossina only feeds on vertebrate blood, whereas Stomoxyn will feed on a variety of other materials in addition to blood including various sugar sources such as rotting fruit. As a consequence, the midgut of Stomoxyn almost certainly receives a much stronger and more regular microbial challenge than that of the tsetse fly. So we might reasonably predict that the epithelial immune system of S. calcitrans is more highly developed than that found in the tsetse fly. The picture emerging to date is that the midgut of Glossina secretes defensin and dipterin using genes, which are also expressed in fat body (4). In contrast, S. calcitrans has at least three immune genes, which are specialized for exclusive use in the anterior midgut epithelium, two defensins (9) and stomoxyn. To control for the presence of the stomoxyn peptide in the tsetse genome, we carried out PCR experiments using two sets of stomoxyn primers and G. morsitans genomic DNA as template. Although stomoxyn was present in the S. calcitrans midgut cDNA library used, no such gene has been found in either G. morsitans genomic DNA or a G. m. morsitans midgut cDNA library (data not shown). In addition, stomoxyn shows an exceptionally wide spectrum of activities including trypanolytic activity on trypanomastigote bloodstream forms of T. b. brucei. The fact that when α-defensins are secreted into the restricted space of the cryptal well in the vertebrate gut, local concentrations as high as a grams per liter range are estimated to occur (2). We suggest that the presence of stomoxyn in the anterior midgut of S. calcitrans may help to explain why Stomoxyn is not a biological vector of trypanosomes.

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