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Trypanosoma rangeli sialidase lacks trans-sialidase activity

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Extracts and tissue culture supernatants of axenic forms of *T. rangeli* were assayed for the presence of sialidase and trans-sialidase activities. Using sialyl(α2–3)lactose, sialyl(α2–6)lactose, poly(α2–8)*N*-acetylneuraminic acid, fetuin and 4-methylumbelliferyl-*N*-acetylneuraminic acid as sialic acid donors, and lactose as a sialic acid acceptor, no trans-sialidase activity was detected. Nevertheless, *T. rangeli* lysates and culture supernatants contain a sialidase that hydrolyzes sialyl(α2–3)lactose, and much less efficiently sialyl(α2–6)lactose, but not poly(α2–8)*N*-acetylneuraminic acid. *T. cruzi* trans-sialidase hydrolyzed only sialyl(α2–3)lactose under the same conditions. The *T. rangeli* and the *T. cruzi* enzymes differ antigenically and in their pH optimum for hydrolase activity.

Key words: *Trypanosoma rangeli*; Sialidase; Neuraminidase; Trans-sialidase

Introduction

Pereira and coworkers have documented the presence of sialidases in *Trypanosoma cruzi* [1] and *Trypanosoma rangeli* [2], and suggested that this enzyme plays a role in modulating the initial interactions of *T. cruzi* trypomastigotes with host cells, and in the pathogenesis of Chagas' disease [1]. More recently, the *T. cruzi* enzyme was shown to function principally as a trans-sialidase (TS) [3–5]: it transfers α(2,3)-linked sialic acid to terminal β-linked galactopyranosyl residues [6–8, reviewed in 9,10]. An enzyme with similar donor and acceptor specificities was also described in procyclics of *T. brucei*, an insect form of the parasite [11]. The *T. cruzi* TS appears to be involved in the attachment to and penetration of host cells by trypomastigotes, presumably through the recognition/generation of sialylated ligands on the surface membrane of target cells and of the parasite [6,12,13]. Other findings suggest that the *T. cruzi* TS facilitates the escape of trypomastigotes from the phagocytic vacuoles into the cytoplasm of the host cells [14].

The two trypanosomes *T. cruzi* and *T. rangeli* share several antigens [15,16] and are transmitted by blood-sucking insect vectors of the same genera, *Triatoma, Rhodnius* and *Panstrongylus* [17–19]. However, the two parasites differ widely in their development in the mammalian host. *T. cruzi* is pathogenic and multiplies intracellularly [17,18], whereas *T. rangeli* does not invade cells in vitro. Very few *T. rangeli* are ever found in the circulation of infected experimental animals or humans [19], and infection is asymptomatic. Since *T. cruzi* interactions with host cells appear to involve TS-mediated recognition events, here we compare the properties of the sialidase/TS of T.*cruzi* with the *T. rangeli* enzyme.

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Abbreviations: Con A, concanavalin A; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; NP-40, Nonidet P-40; Tris, tris(hydroxymethyl)aminomethane; TS, trans-sialidase.

Materials and Methods

Parasites and lysates. Two different *T. rangeli* strains were studied: SC-58 [20], kindly supplied by Dr. M. Deane, Departamento de Protozoologia, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil, and ATCC 30032 (American Type Culture Collection, Rockville, MD), kindly supplied by Dr. J.E. Donelson, Department of Biochemistry, University of Iowa, USA. Parasites were cultured in liver infusion-tryptose medium (Difco Laboratories, Detroit, MI) [21] containing 10% fetal calf serum (Hyclone Laboratories Inc., Logan, UT) at 27°C. ATCC 30032 *T. rangeli* cultures yielded 50% to 60% long epimastigotes at high parasite density, whereas SC-58 *T. rangeli* cultures yielded 85% to 95% long epimastigotes at high parasite density. The Y strain [22] of *T. cruzi* trypomastigotes were obtained by infecting LLC-MK2 cells (CCL-7; American Type Culture Collection) [6]. Epimastigotes of *T. cruzi* were obtained in liver infusion-tryptose medium at 27°C. *T. brucei brucei* procyclic trypomastigotes (TREU 667-stock) [23], kindly supplied by Dr. E.J. Bienen, Department of Medical and Molecular Parasitology, New York University Medical Center, New York, USA, were grown in buffered semi-defined medium containing 10% fetal calf serum at 27°C [24]. Parasite lysates were prepared by incubating 10^9 washed parasites with 1 ml of 50 mM tris(hydroxymethyl)-aminomethane (Tris)-HCl, pH 7.4, containing 1.5% Nonidet P-40 (NP-40), 1 mM phenylmethanesulfonylfluoride, 5 μg ml^{-1} of antipain, pepstatin and leupeptin (Sigma Chemical Co., St. Louis, MO) at 4°C. The lysates were cleared by centrifugation at 10 000 *g* for 5 min at 4°C.

Enzymatic assays and sialic acid measurement. Trans-sialidase activity in *T. rangeli* lysates and supernatants was usually assayed by incubation with 1 mM α(2–3)-sialyllactose (Boehringer Mannheim Biochemicals, Indianapolis, IN) and 7.2 mM [D-glucose-1-^{14}C]-lactose (60 Ci mol^{-1}; Amersham Corp., Arlington Heights, IL), diluted in a final volume of 50 μl of 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Sigma), pH 7, for 50 min at room temperature [6]. Under these conditions the trans-sialidases of *T. cruzi* and *T. brucei* transfer sialyl residues from the sialyllactose to the radioactive lactose [6,11]. The generated radioactive sialyllactose is subsequently separated from free lactose by retention on 0.5 ml QAE-Sephadex A50 columns. Results are expressed as cpm eluted from the columns with 0.5 ml of 1 M ammonium formate [6]. In some assays sialyl(α2–6)lactose (Oxford Glycosystems Inc., Rosedale, NY), poly(α2–8)*N*-acetylneuraminic acid (colominic acid), fetuin or 4-methylumbelliferyl-*N*-acetylneuraminic acid (Sigma) were substituted for the sialyl(α2–3)lactose.

Sialidase activity was assayed using 4-methylumbelliferyl-*N*-acetylneuraminic acid as substrate, and measuring the fluorescent product 4-methylumbelliferone at 420 nm, after excitation at 365 nm, in a Titertek Fluoroskan II (Flow Laboratories Inc., McLean, VA). Results are expressed in fluorescence units [3]. In general, the samples were assayed in 50 mM HEPES, pH 6.6, but buffers with different pH, identified in the legend of Fig. 4, were also used.

The comparison between the substrate specificities of the *T. rangeli, T. cruzi* and *Vibrio cholerae* sialidases was performed as follows. A *T. rangeli* culture supernatant was extensively dialyzed against 0.15 M phosphate-buffered saline, pH 7.2, in order to remove free sialic acid molecules. Ten microliters of the dialyzed supernatant, 10 μl of a solution of *T. cruzi* trans-sialidase purified by affinity chromatography on concanavalin A (Con A)-Sepharose (Pharmacia-LKB Biotechnology Inc., Piscataway, NJ) [6], or 10 μl of 4 U. ml^{-1} *V. cholerae* sialidase (Boehringer Mannheim Biochemicals) were incubated with 40 ml of 7.5 mM sialyl(α2–3)lactose, sialyl(α2–6)lactose, or a pentamer of (α2–8)-linked *N*-acetylneuraminic acid (EY Laboratories Inc., San Mateo, CA). The incubation was carried out at the optimum pH for each enzyme, i.e., in 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES; Sigma), pH 5.5 (*T. rangeli* supernatant and *V. cholerae* sialidase) or in 50 mM HEPES,

pH 6.6 (*T. cruzi* TS). The degree of purity of these saccharides was assessed by thin-layer chromatography on silica gel 60 plates (Macherey-Nagel, Düren, Germany) using ethanol/*n*-butanol/pyridine/water/acetic acid [100:10:10:30:3 (v/v)] [6]. The amount of free sialic acid present in 7 μl of the reaction mixtures was measured after 0.8, 3.5, 20 or 72 h of incubation at room temperature by the thiobarbituric acid method [25], and expressed as nmol of free sialic acid present in the total reaction mixture. The HPLC-thiobarbituric acid method [26] was used to investigate the presence of sialic acid in *T. rangeli*. Parasites were washed six times with cold DME containing 0.05% bovine serum albumin (Ultrapure, Boehringer Mannheim Biochemicals) and incubated in 0.1 M sulfuric acid for 1 h at 80°C before the assay.

Fractionation of T. rangeli lysates. Lysate and culture supernatants were subjected to anion-exchange and sizing chromatographies on Mono-Q and Superose 6-12 FPLC columns (Pharmacia-LKB Biotechnology Inc.), as described [3,6]. Some samples were also subjected to affinity chromatography on Con A-Sepharose [6].

Immunoabsorption. T. rangeli lysates were incubated with increasing volumes of protein A-agarose (Sigma) bearing antibodies against *T. cruzi* trypomastigote TS. The following antibodies were bound to different batches of protein A-agarose: monoclonal antibody 39 [27], rabbit IgG against purified TS, rabbit IgG against a synthetic peptide corresponding to the first 19 amino-terminal amino acid residues of TS [11], and rabbit IgG against the repeat

TABLE I

Comparison of trans-sialidase and sialidase activities of *T. rangeli, T. cruzi* and *T. brucei*

Parasite species (strain /stage)	Enzyme source	Amounts[a]	Enzymatic activity	
			Trans-sialidase[b]	Sialidase[c]
T. rangeli (SC-58)	Lysate	5×10^5	9[d]	718[e]
		10^7	43	ND[f]
	Supernatant	1	−4	3,503
		30	3	ND
T. rangeli (ATCC 30032)	Lysate	5×10^5	0	1,788
		10^7	−23	ND
	Supernatant	1	−14	7,204
		30	−1	ND
T. cruzi (trypomastigotes)	Lysate	10^4	215	2
		10^5	986	−3
		5×10^5	6,550	418
T. cruzi (epimastigotes)	Lysate	10^5	148	0
		5×10^5	1,036	32
		10^7	10,731	696
T. brucei (procyclics)	Lysate	5×10^5	3,429	473

[a]Total numbers of lysed parasites in lysate samples or volumes in μl of supernatants from liver-infusion tryptose cultures containing 2.8×10^7–3×10^7 parasites/ml.

[b]Trans-sialidase activity was measured by incubating the indicated amounts of supernatant or lysates with 1 mM (α2-3)sialyllactose and 7.2 μM radiolabelled lactose, in 50 μl of 50 mM Hepes, pH 7, for 50 min. at room temperature, as described in the text. The radiolabeled sialyllactose formed was separated by anion-exchange chromatography and quantified in a beta-counter.

[c]Sialidase activity was assayed by incubating the lysates or supernatants with 1 mM 4-methylumbelliferyl-*n*-acetylneuraminic acid in 50 μl of 50 mM HEPES buffer, pH 6.6, for 2 h at room temperature. The amount of fluorescent 4-methylumbelliferone formed was measured at 420 nm using excitation at 365 nm in a Titertek Fluoroskan II (Flow Laboratories Inc.).

[d]Mean cpm of duplicates; the background value (109–136 cpm, obtained in the absence of trans-sialisase) was subtracted. Variation between duplicate values was less than 12% of the mean.

[e]Mean number of fluorescence units of duplicates; the background value (653-1144 fluorescence units, obtained in the absence of sialidase) was subtracted. Variation between duplicate values was less than 17% of the mean.

[f]Not done.

sequences from the carboxy-terminal half of TS [4,5]. The antibodies against synthetic peptides were from rabbits immunized with peptides coupled to keyhole limpet hemocyanin (Sigma) [11].

Results and Discussion

Using sialyl(α2–3)lactose as a sialic acid donor and lactose as the acceptor, no TS activity was detected in extracts or culture supernatants from two different strains of *T. rangeli*. The *T. rangeli* lysates contained 100 to 1000 times more lysed parasites than the TS-positive *T. cruzi* lysates which were simultaneously tested as positive controls (Table I). It is unlikely that *T. rangeli* contains a TS differing in specificity from the *T. cruzi* and *T. brucei* enzymes [7,8,11], since no TS activity was detected when we used other potential sialic acid donors, i.e., sialyl(α2–6)lactose, poly(α2–8)*N*-acetylneuraminic acid, fetuin or 4-methylumbelliferyl-*N*-acetylneuraminic acid (data not shown).

Although *T. rangeli* lysates and supernatants lacked TS activity, they had more sialidase activity than samples of *T. cruzi* and *T. brucei* lysates derived from equivalent numbers of parasites (Table I). To exclude the possibility that the high sialidase activity in *T. rangeli* hindered the detection of a TS

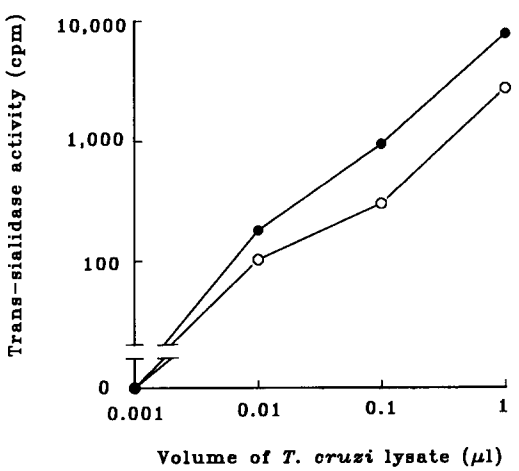

Fig. 1. *T. cruzi* trans-sialidase activity in the presence of *T. rangeli* sialidase. Different volumes of a lysate from *T. cruzi* trypomastigotes were tested for trans-sialidase activity in the presence (○) or absence (●) of 10 μl volumes of a *T. rangeli* lysate. The number of lysed *T. cruzi* or *T. rangeli* per μl of lysate was 10^6.

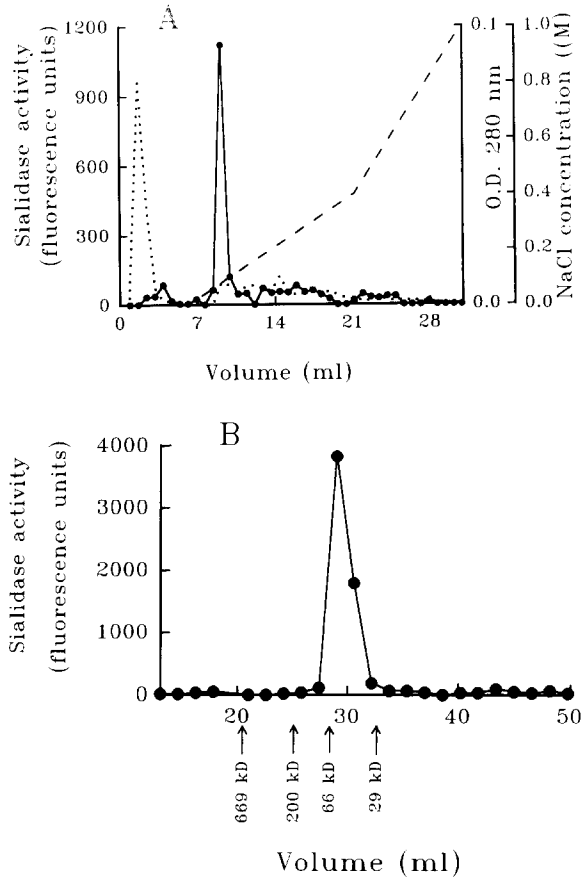

Fig. 2. Purification of sialidase activity from lysates of *T. rangeli* (ATCC 30032). Sialidase activity in the chromatographic fractions was followed by the 4-methylumbelliferyl-*N*-acetylneuraminic acid assay. The results, after subtracting the background values (790–1033 fluorescence units, in the absence of sialidase), are represented by the solid lines. No trans-sialidase activity was detected in any of the fractions, using radiolabeled lactose as acceptor, and sialyl(α2–3)lactose, sialyl(α2–6)lactose, poly(α2–8)*N*-acetylneuraminic acid, 4-methylumbelliferyl-*N*-acetylneuraminic acid, and fetuin as donors (not shown). (A) Fractions eluted from a Mono Q column with an NaCl gradient in 20 mM Tris/HCl containing 0.1% NP-40. The optical densities at 280 nm are represented by the dotted line. The broken line represents the NaCl concentrations in the gradient. (B) Fractions eluted from Superose 12-Superose 6 columns run in tandem. The chromatography was performed in the presence of 0.4% bovine serum albumin and 0.1% NP-40 to minimize non-specific interactions with the gel matrix. The positions where protein standards are eluted, and their molecular weights are shown in the x-axis.

present in the same sample, a *T. rangeli* lysate was tested for its ability to inhibit *T. cruzi* TS activity. Dilutions of a *T. cruzi* lysate were mixed with a fixed volume of *T. rangeli* lysate and tested for TS activity in the presence of 4 mM sialyllactose in a 40-min assay. As shown in Fig. 1, 10 μl of the *T. rangeli* lysate (corresponding to 10^7 parasites) barely inhibited the activity of TS contained in as little as 0.01 μl of *T. cruzi* lysate (corresponding to 10^4 parasites). Furthermore, when *T. rangeli* lysates were subjected to anion exchange or molecular sieving chromatographies, no TS activity was uncovered in the fractions. Instead, a single peak of sialidase activity was detected with either chromatographic procedure (Fig. 2). We conclude that culture forms of *T. rangeli* do not express a TS and that their sialidase is strictly hydrolytic, while TS activity predominates over sialidase activity in *T. cruzi* and *T. brucei* enzymes [3,4,11].

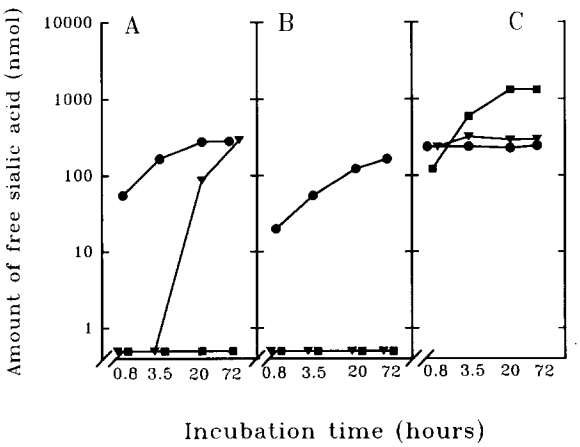

Fig. 3. Substrate specificity of the *T. rangeli* sialidase, and of the hydrolase activity of the *T. cruzi* trans-sialidase. About 300 nmol of sialyl(α2–3)lactose (circles), sialyl(α2–6)lactose (triangles), or a pentamer of (α2–8)-linked *N*-acetylneuraminic acid (squares) were incubated with: (A) 10 μl of a *T. rangeli* culture supernatant, pre-dialyzed against 0.15 M phosphate-buffered saline, pH 7.2; (B) *T. cruzi* TS purified by affinity chromatography on concanavalin A-Sepharose; or (C) 0.04 U of *V. cholerae* sialidase. The reactions were carried out in a total volume of 50 μl 50 mM MES, pH 5.5, to assay for the *T. rangeli* and *V. cholerae* sialidases, or in 50 mM HEPES, pH 6.6, to assay for the *T. cruzi* sialidase activity). After different incubation times, the amount of free sialic acid present in 7 μl of the reaction mixture was measured by the thiobarbituric method, and expressed as nmols/total reaction volume.

On the basis of its elution pattern from Superose columns, the *T. rangeli* sialidase has a molecular weight of approximately 58 kDa, a result which is in agreement with the findings of Pereira and Moss [1]. In contrast, the *T. cruzi* trypomastigote TS is a multimer of about 700 kDa, with subunits varying from 120–240 kDa [3], and the *T. cruzi* epimastigote TS has a molecular weight of 90 [28]. The sialidase of *T. rangeli* could be further purified by affinity chromatography on Con A-Sepharose, following elution with an excess of α-methyl-mannopyranoside (not shown). In this respect it resembles the TSs of *T. brucei* [11], *T. cruzi* trypomastigotes [6] and *T. cruzi* epimastigotes [28]. As might have been predicted from the high sialidase content and lack of TS activity in the supernatants of *T. rangeli* cultures, the parasites did not contain sialic acid, as measured in this paper by the HPLC-thiobarbituric acid method (not shown). This confirms previously reported data obtained with the less sensitive thiobarbituric acid method [29].

In the next series of experiments we compared the specifities of the sialidases from the different species of trypanosomes. Extracts of parasites were incubated for different periods of time with 300 nmol of various sialylated saccharides. After 3.5 h of incubation with 6 mM sialyl(α2–3)lactose, the *T. cruzi* TS, *T. rangeli* and *V. cholerae* sialidases released about 55, 165 and 240 nmol of sialic acid respectively. Neither the *T. cruzi* nor the *T. rangeli* enzymes hydrolysed poly(α2–8)*N*-acetylneuraminic acid or sialyl(α2–6)lactose during the first hours of incubation. Nevertheless, after 20 h, the *T. rangeli* sialidase had hydrolysed 86 nM of sialyl(α2–6) lactose. Although we detected by thin-layer chromatography small amounts of contaminant sialyl(α2–3)lactose in the sialyl(α2–6)lactose preparation, this could not account for the large amounts of sialic acid released by the *T. rangeli* sialidase. These results are in agreement with Reuter and collaborators' finding that the *T. rangeli* sialidase preferentially cleaves sialyl(α2–3)lactose in relation to sialyl(α2–6)lactose [30]. On the other hand, the *T. cruzi* TS did not hydrolyse sialyl(α2–6)lactose, even

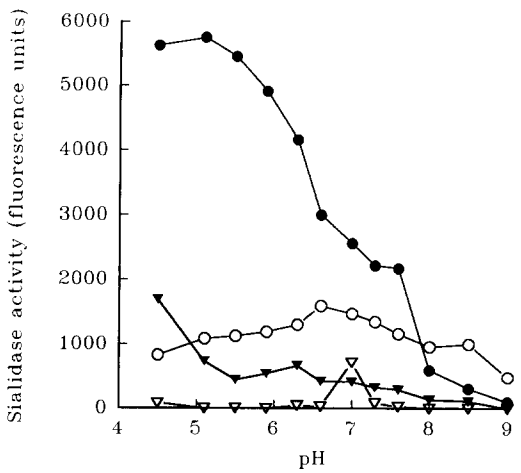

Fig. 4. Optimum pH activity of the *T. rangeli* and the *T. cruzi* sialidases. Samples of *T. rangeli* culture supernatant (closed circles), *T. rangeli* lysate (closed triangles), *T. cruzi* trypomastigote TS, partially purified by affinity chromatography on Con A-Sepharose (open circles), or *T. cruzi* epimastigote lysate (open triangles) were tested for sialidase activity in the 4-methylumbelliferylneuraminic acid assay. The enzymes were diluted in 50 mM of the following buffers: acetate, pH 4.5; MES, pH 5.1–6.3; HEPES, pH 6.6–7.3; and Tris, pH 8.0–9.0.

after 72 h of incubation. However, it appears that at high concentrations sialyl(α2–6)lactosamine can donate sialic acid to lactose in a reaction catalysed by the *T. cruzi* TS [6].

Although the substrate specificities of the *T. cruzi* and *T. rangeli* enzymes are similar, the optimum pH's for their hydrolytic activities differ somewhat. Both enzymes are active over broad pH ranges, but the *T. rangeli* sialidase is more efficient at lower pH (around 5), as also reported by Pereira and Moss [1]. The TSs of *T. cruzi* trypomastigotes and epimastigotes are more active at neutral pH (Fig. 4).

The *T. rangeli* and *T. cruzi* trypomastigote enzymes are antigenically distinct. We could not deplete *T. rangeli* sialidase activity from culture supernatants by incubation with agarose-protein A bearing monoclonal and polyclonal antibodies to *T. cruzi* trypomastigote TS. The polyclonal antibodies were against the repeats and the N-terminal region of TS [5,11].

The functions of the *T. cruzi* epimastigote TS and of the *T. rangeli* sialidase are not known. One possibility is that they have specific roles in the development of the parasites in the insect vector. For example, sialic acid and/or lectins on the epithelial lining of the intestinal tract of the insect may influence the migration and/or attachment of the parasites. Whether the hematophagous vectors of these parasites synthesize sialic acid is not known, but if the appropriate saccharide acceptors are available the *T. cruzi* TS may sialylate them using sialic acid from the glycoconjugates in the ingested blood.

Although the *T. cruzi* and *T. rangeli* enzymes are antigenically distinct, they are structurally related (D. Eichinger, in preparation), and, as shown here, their substrates specificities are very similar. It is conceivable that a limited number of mutations in the *T. rangeli* sialidase will transform it into a TS. If this hypothesis is correct this could have obvious implications for the understanding of the origin and evolution of parasitism. Although clearly speculative, this idea can be tested experimentally. One prediction is that the *T. rangeli* sialidase can be converted into a TS by site-directed mutagenesis. In addition, following transfection of TS into *T. rangeli*, the parasites should adhere to mammalian cells, provided that the enzyme is expressed on the plasma membrane.

While the present studies were being prepared for publication, an abstract reporting the inability of the *T. rangeli* sialidase to act as a trans-sialidase was brought to our attention (M. Engstler, G. Reuter and R. Schauer, Biol. Chem. Hoppe-Seyler, 373, 843, 1992).

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