Trypanosoma cruzi Trans-sialidase and Neuraminidase Activities Can Be Mediated by the Same Enzymes

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Summary

Trans-sialidase and neuraminidase activities have been detected on the surface membrane of trypomastigotes of *Trypanosoma cruzi*, and both have been implicated in the parasite's invasion of host cells. We show here that these enzymes are structurally related. They are recognized by two independently derived monoclonal antibodies, are anchored to the membrane by glycosylphosphatidylinositol, copurify by ion exchange, molecular sieving, and hydrophobic chromatography, have maximal activities between pH 6.5 and 7.5, and are inactivated by heating at 56°C. Furthermore, the neuraminidase and trans-sialidase reactions are coupled. An increase of the concentration of acceptors of the transfer reaction decreases the amount of free sialic acid released through the neuraminidase reaction. We conclude that a single enzyme can catalyze the transfer or the hydrolysis of macromolecular-bound sialic acid. The predominant direction of the reaction will depend on the availability of appropriate oligosaccharide acceptors of sialic acid.

Trypanosoma cruzi trypomastigotes, the infective forms of the parasite that circulate in the blood, bear on their surface membrane a trans-sialidase (1–3). This unique enzyme catalyzes the transfer of α(2-3)-sialic acid from host glycoconjugates to trypomastigote surface molecules. The sialic acid transfer occurs shortly after the release of trypomastigotes from infected cells, and leads to the assembly of the stage-specific epitope Ssp-3 (3).

Ssp-3 appears to be involved in the invasion of mammalian cells by trypomastigotes. Treatment of the parasites with Fab fragments of mAbs against Ssp-3 blocks attachment to nonphagocytic target cells (4). Moreover, opsonization of trypomastigotes with an anti-Ssp-3 antibody inhibits their internalization by cells transfected with Fc receptors, whereas opsonization with antibodies to other surface membrane epitopes enhances parasite internalization (Schenkman et al., manuscript in preparation). Another finding supports the notion that the infectivity of trypomastigotes is regulated by acquisition of sialic acid from their surface membranes: their infectivity is increased by incubation with sialic acid–containing glycoproteins (5).

A complicating issue for the understanding of the function of the trans-sialidase is that Pereira (6) described a neuraminidase on the surface membrane of blood forms of *T. cruzi*. The enzyme has a negative effect on invasion; that is, when its activity is inhibited by mAbs, or by serum lipoproteins, invasion is enhanced (7, 8). In theory, neuraminidase and trans-sialidase activities could be mediated by the same enzyme, since many glycosidases can transfer glycosidic bonds, if the enzymatic reaction is performed in the presence of appropriate donors and acceptors (9). In this paper, we study some properties of the *T. cruzi* neuraminidase and trans-sialidase in an attempt to clarify their relationship.

Materials and Methods

Parasites. *T. cruzi* trypomastigotes, Y strain (10), were grown in cultures of LLC-MK_2 cells (CCL-7; American Type Culture Collection, Rockville, MD). Usually, 75-cm^2 flasks, with subconfluent cultures of LLC-MK2 cells, were infected with 5×10^6 trypomastigotes. The LLC-MK2 cells were grown in low glucose DME with penicillin and streptomycin (Gibco Laboratories, Grand Island, NY), containing 10% FCS, at 37°C, 5% CO_2. Free parasites were removed 24 h later, and the cultures maintained in 10% FCS-DME. When indicated, the FCS-DME was removed during day 3 postinfection, the monolayers were washed twice with Hanks' solution, and the medium was replaced with DME containing 0.2% BSA (ultrapure; Boehringer Mannheim Biochemicals, Indianapolis, IN) and 20 mM Hepes, pH 7.4 (0.2% BSA-DME). After day 5 postinfection, the trypomastigotes were harvested from culture supernatants. Culture supernatants were collected from parasites grown in FCS (FCS supernatant) or in 0.2% BSA (BSA supernatant).

Immunodepletion Experiments. Frozen pellets of trypomastigotes were lysed in 1% NP-40, 50 mM Tris-HCl, pH 7.4, 1 mM PMSF, 0.1 mM EDTA, and 10 μg/ml of antipain, pepstatin, and leupeptin

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(100 μl/10^8 trypomastigotes), and the lysates were cleared by 5-min centrifugation at 10,000 *g*. Fractions of 60 μl of the lysates were incubated 30 min with 20 μg of mAb 39 or mAb TCN2 preadsorbed on protein A–Sepharose. The mAb 39 (IgG2b) (4) was purified by protein A–Sepharose from ascitic fluids. Tissue culture supernatants of hybridoma cells secreting mAb TCN2 anti-*T. cruzi* neuraminidase were kindly provided by Dr. M. Pereira (Tufts University, Boston, MA). Purified mAb 2C2, which recognizes the Ssp-4 antigen of amastigotes (11), was used as control. At the end of incubations, the beads were removed by centrifugation, washed twice, resuspended in PBS, and assayed for trans-sialidase and neuraminidase activities.

Activity Measurements. Trans-sialidase activity was assayed in a final volume of 50 μl, in 20 mM of the indicated buffers (Sigma Chemical Co., St. Louis, MO) containing sialyllactose and [D-glucose-1-^{14}C]-lactose (60 mCi/mmol) (Amersham Corp., Arlington Heights, IL). The standard assay contained 20 mM Hepes buffer, pH 7.0, 1 mM sialyllactose (50 nmol), and 25,000–40,000 cpm of the radioactive substrate (0.4 nmol). This mixture was incubated 30 min at room temperature, and the reaction terminated by addition of 1 ml of water, followed by passage through 0.5 ml QAE-Sephadex, A25, or A50 column, also equilibrated in water. Activity was expressed in cpm, or as moles of sialyllactose eluted from the column after elution with 0.5 ml of 1 M ammonium formate. In the experiments designed to measure the initial velocity of the reaction, the amounts of the generated [^{14}C]sialyllactose were determined when the formation of the product was linear with respect to time.

Neuraminidase activity was determined by measuring the fluorescence of 4-methylumbelliferone released by the hydrolysis of 1 mM 4-methylumbelliferyl-*N*-acetylneuraminic acid (MuNana).[1] The assays were performed in 50 μl of 20 mM Hepes buffer, pH 7.0, or in other indicated buffers. After 3 h of incubation at 25°C, the reactions were terminated by adding 200 μl of 0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 2 mM $MgCl_2$. The fluorescence was measured at 420 nm using excitation at 365 nm in a Titertek Fluoroskan II (Flow Laboratories Inc., McLean, VA), and was expressed in fluorescence units. Alternatively, neuraminidase activity was assayed by measuring the amount of free sialic acid released from sialyllactose using the thiobarbituric acid method (12).

Enzyme Purification. Trans-sialidase and neuraminidase activities were purified from pellets of parasites stored at −70°C, or from BSA supernatants filtered through a 0.22-μm filter (Millipore Continental Water Systems, Bedford, MA). Pellets containing 5 × 10^9 trypomastigotes were lysed at 4°C in 5 ml of 1% NP-40, 50 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 0.1 mM PMSF, 5 μg/ml of leupeptin, pepstatin, and antipain. The viscous lysate was sonicated three times for 15 s, and the insoluble material was removed by centrifugation (10 min at 10,000 *g*). The supernatant was adjusted to 0.5 M NaCl, 1 mM of $CaCl_2$, $MgCl_2$, and $MnCl_2$, and was incubated with 2 ml of Con A–Sepharose equilibrated with 0.1% NP-40, 0.5 M NaCl, 50 mM Tris-HCl, pH 7.4. After washing with 25 ml of the equilibration buffer, the enzyme was eluted after an overnight incubation with 0.5 M α-methyl-D-mannoside in the same buffer. The eluate was filtered through a G-25 column equilibrated with 20 mM Tris-HCl, pH 8.0, and applied into a a mono-Q FPLC column HR5/5 (Pharmacia-LKB Biotechnology Inc., Piscataway, NJ) preequilibrated in the same buffer. After the absorbance had decreased below 0.002, the enzyme was eluted with a gradient of NaCl.

In another experiment, a sample of the Con A eluate was subjected to sizing chromatography on Superose 12 HR 10/30 and Superose 6 HR 10/30 FPLC columns (Pharmacia-LKB Biotechnology Inc.) connected in series, equilibrated in 50 mM Tris/HCl containing 0.004% BSA, and precalibrated with proteins of molecular masses between 29 and 669 kD (Sigma Chemical Co.).

Trans-sialidase and neuraminidase activities were also purified from BSA supernatants by affinity chromatography on immobilized mAb 39, or by hydrophobic interaction on a phenyl-Superose FPLC column (Pharmacia-LKB Biotechnology Inc.). The affinity chromatography was carried out after concentrating the culture supernatants ~20 times by filtration through membranes (Amicon Corp., Danvers, MA) with a molecular mass cutoff of 300 kD. The concentrated material was then passed through an Affigel-Hz (Bio-Rad Laboratories, Richmond, CA) column containing immobilized mAb 39 (prepared accordingly to the manufacturer's instructions). The column was washed with 0.15 M NaCl, 50 mM Tris-HCl, pH 7.4, 0.05% NP-40, and the enzyme eluted with 3.5 M $MgCl_2$, 20 mM sodium phosphate, pH 7.0. The fractions eluted from the column were immediately filtered through Sephadex-G25 equilibrated in 20 mM Tris-HCl, pH 8.0, to remove the $MgCl_2$, and then subjected to further purification on mono-Q FPLC column HR5/5 as described above.

The hydrophobic interaction chromatography was performed as follows. 14 ml of BSA supernatant was diluted 1:2 with 50 mM phosphate buffer, pH 7, containing 3.4 M ammonium sulfate, and applied to a phenyl-Superose HR 5/5 FPLC column preequilibrated with 50 mM phosphate buffer, pH 7, containing 1.7 M ammonium sulfate. When the absorbance had decreased below 0.002, the bound enzymatic activities were eluted with a gradient of decreasing ammonium sulfate concentration. The collected 0.5-ml fractions were filtered through Sephadex G-25 columns preequilibrated with 50 mM Tris-HCl, pH 7.4, containing 0.02% BSA to remove the ammonium sulfate.

Immunoprecipitation, SDS-PAGE, and Western Blotting. Trypomastigotes (2 × 10^8) were washed in methionine, cysteine-free MEM, containing 10% dialyzed FCS. The parasites were resuspended in 4 ml of the same medium, and after a starvation period of 30 min, incubated with 0.5 mCi of a mixture of ^{35}S-methionine and ^{35}S-cysteine (ICN Biomedicals, Inc., Irvine, CA) for 3 h at 37°C. After washing three times in HBSS, trypomastigotes were lysed with the same buffer used to purify the enzymes. This lysate, as well as culture supernatants, were pretreated with an irrelevant IgG and protein A–Sepharose, centrifuged, and the cleared supernatants incubated for 1 h with 5 μl of ascitic fluid containing mAb 39 or an irrelevant mAb. The immunocomplexes were collected by incubation with 50 μl of a 50% suspension of protein A–Sepharose. The samples were then processed as described (13), and loaded into 6.5% SDS-PAGE gels. The radioactive bands were detected after fluorography using Amplify (Amersham Corp.). Nonradioactive samples were detected on SDS gels by silver staining (14), coomassie blue R250 staining, or Western blotting.

For Western blots of trypomastigote cellular antigens, parasite suspensions were centrifuged in a microfuge, the pellet washed with Hanks' solution, resuspended in SDS-sample buffer, and boiled for 3 min. Samples containing the equivalent of 2 × 10^7 trypomastigotes were loaded onto 6.5% SDS-PAGE gels and subjected to Western blotting. Bound antibodies were detected with anti-mouse IgG conjugated to alkaline phosphatase (Sigma Chemical Co.), followed by incubation with 0.3 mg/ml of nitroblue tetrazo-

[1] *Abbreviations used in this paper:* CRD, crossreactive determinant; GPI, glycosylphosphatydilinositol; MuNana, 4-methylumbelliferyl-*N*-acetylneuraminic acid.

lium and 0.15 mg/ml of 5-bromo-4-chloro-3-indolyl phosphate, in 0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, and 0.005 M $MgCl_2$. In some cases the Western blots of immunoprecipitated FCS supernatants or trypomastigote cellular antigens were revealed with rabbit antibodies to the crossreactive determinant (15, 16), kindly supplied by Dr. M. Davitz (New York University Medical Center).

Results

Activity Depletion by mAbs. To determine whether the trans-sialidase and neuraminidase are antigenically crossreactive, we immunoprecipitated trypomastigote lysates with mAb TCN2 antineuraminidase (8), and with a series of mAbs to other *T. cruzi* surface antigens. We then assayed both the supernatants and the precipitates for enzymatic activity. As shown in Table 1, mAbs 39 and TCN2 (but not the control mAb 2C2) immunoprecipitated both neuraminidase and trans-sialidase from the parasite extracts. Most or all activities were recovered in the pellets, suggesting that the antibodies 39 and TCN2 bind to epitopes outside the enzymatic sites.

Next, we used mAb 39 to immunoprecipitate lysates of trypomastigotes that had been metabolically labeled with ^{35}S-methionine and ^{35}S-cysteine. As shown in Fig. 1, this mAb specifically recognizes several radiolabeled bands ranging between 120 and 220 kD (lane *b*). A similar, complex pattern is seen in Western blots of total extracts of the parasite (lane *c*), except that some bands have different intensity.

Trans-sialidase Is Released by Trypomastigotes. Trypomastigotes were incubated at 37°C in DME-10% FCS, and at different time points, samples were removed and centrifuged. The supernatants and detergent extracts of the pellets were assayed for trans-sialidase activity. As shown in Table 2, there was a progressive increase of enzymatic activity in the supernatant. The activity in the pellets remained constant at least for a few hours, most likely reflecting the biosynthetic activity documented in Fig. 1, lane *b*. After 3 h, about half of the trans-sialidase activity was found in the culture supernatant.

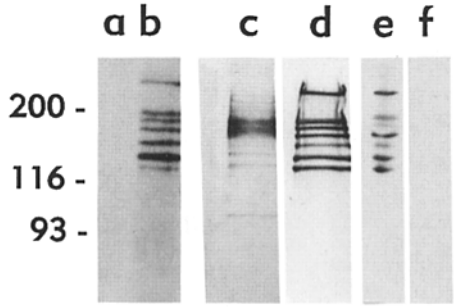

Figure 1. Heterogeneity of the molecules recognized by mAb 39. Lysates made from trypomastigotes labeled for 3 h with [^{35}S]methionine and cysteine were immunoprecipitated with a control antibody (lane *a*) or with mAb 39 (lane *b*). (Lane *c*) Western blot of a total trypomastigote extract, revealed with mAb 39. (Lanes *d*, *e*, and *f*) Western blots of supernatants of cultures of trypomastigotes immunoprecipitated with mAb 39, revealed with mAb 39, with a rabbit antiserum to the crossreactive determinant of the variant surface glycoprotein of African trypanosomes or with a normal rabbit serum, respectively.

The enzymatic activity was not removed by centrifugation of the supernatants for 1 h at 100,000 *g*; that is, the enzyme was released in a soluble form. To determine whether, similarly to the *T. cruzi* neuraminidase (17, 18), the trans-sialidase is linked to the membrane by glycosylphosphatydilinositol (GPI), we revealed a Western blot of the soluble form of trans-sialidase with an antiserum to the crossreactive determinant (CRD) (15, 16). This epitope is characteristic of GPI-anchored proteins, and is only expressed after cleavage of the anchor by a phosphatydilinositol-specific phospholipase C. As shown in Fig. 1, lanes *d* and *e*, the same bands are revealed by mAb 39 and by the antiserum to CRD. If, however, total extracts of trypomastigotes were subjected to

Table 1. *Depletion of Trans-sialidase and Neuraminidase Activity from T. cruzi Trypomastigote Lysates with mAbs*

	Trans-sialidase activity		Neuraminidase activity	
	Pellet	Supernatant	Pellet	Supernatant
	cpm		*fluorescence units*	
mAb 39	7,053 (87%)*	0 (0%)	290 (100%)‡	0 (0%)
mAb TCN2	4,880 (71%)	0 (0%)	270 (93%)	10 (3%)
mAb cont	230 (2%)	6,467 (80%)	0 (0%)	180 (62%)

Frozen *T. cruzi* trypomastigotes were resuspended in 1% NP-40 containing 50 mM Tris-HCl, pH 7.4, 1 mM PMSF, 10 μg/ml of leupeptin, pepstatin, antipain (100 μl/10^8 trypomastigotes), and the lysates cleared by a 5-min centrifugation at 10,000 *g*. Fractions of 60 μl of the lysate were incubated 30 min with 20 μg of the indicated antibody preadsorbed to 30 μl of protein A–Sepharose. At the end of incubations, the beads were removed by centrifugation, washed twice, and resuspended in PBS. Aliquots containing comparable volumes of the initial lysate, the washed beads, and the first supernatant of the immunodepletion reaction were assayed for trans-sialidase reaction with 1 mM sialyllactose for 30 min, or for neuraminidase reaction with 1 mM 4-methylumbelliferyl sialic acid for 3 h at room temperature.

* Percent of trans-sialidase activity measured in the total lysate (8,700 cpm).

‡ Percent of neurominidase activity measured in the total lysate (290).

Table 2. *Release of Trans-sialidase into the Culture Supernatant of T. cruzi Trypomastigotes*

Time	Activity	
	Supernatant	Trypomastigotes
min	*cpm ± SD*	
0	374 ± 24	4,517 ± 166
30	1,146 ± 14	5,482 ± 159
60	1,558 ± 297	5,824 ± 451
120	1,589 ± 41	4,428 ± 245
180	2,058 ± 78	4,340 ± 270

T. cruzi trypomastigotes were washed three times at 4°C with 10% FCS-DME and incubated at 37°C at 5 × 10^7 parasites/ml. At the indicated times, the parasites were centrifuged at 10,000 *g*. Triplicate enzymatic determinations were made in the supernatants and in lysates of the pellets. The pellets were lysed in 1% NP-40 containing 50 mM Tris-HCl, pH 7.4, 1 mM PMSF, and 10 μg/ml of leupeptin, pepstatin, and antipain (1 ml/5 × 10^7 parasites).

Western blotting, no reactivity with the antiserum to CRD was detected.

Purification of the Trans-sialidase and Neuraminidase. The previous results highlight the similarities between the properties of the trans-sialidase and neuraminidase: the enzymes are antigenically crossreactive, are GPI anchored, and are rapidly secreted into the culture medium. Nevertheless, since the immunoprecipitation patterns are very complex, it could still be argued that the trans-sialidase and neuraminidase activities originate from different molecules. In an attempt to resolve this issue, we subjected *T. cruzi* extracts or culture supernatants to various chromatographic procedures. In every instance the two enzymatic activities coincided.

The results of some of these experiments are summarized in Fig. 2, *A–C*. In Fig. 2 *A*, we show the elution pattern from chromatography in a mono Q FPLC column. The input was a sample of a preparation isolated by affinity chromatography on immobilized mAb 39. The purity of the input is documented in Figs. 1 and 2 *A*. Identical bands were observed by Western blotting (Fig. 1) and by coomassie blue or silver staining (inset on the left of the Fig. 2 *A*). The individual fractions eluted from the column were also run on SDS-PAGE and stained with silver (inset on the right side of Fig. 2 *A*). All fractions displayed both enzymatic activities, and contained a family of polypeptides ranging from 120 to 220 kD. Nevertheless, the composition of individual fractions was not the same. Lower molecular mass bands eluted first from the mono Q. Identical results were obtained when we subjected total detergent lysates of trypomastigotes (rather than mAb 39 affinity-purified enzyme) to chromatography on Con A–Sepharose, followed by ion exchange chromatography on a mono Q column (not shown).

On Superose columns (Fig. 2 *B*), which separate proteins on the basis of size, the two activities were detected in a broad peak, in a position corresponding to molecular masses >700 kD. In addition, small peaks of enzymatic activity eluted later in positions corresponding to lower molecular masses. These results suggest that the enzymes form oligomers, as also pointed out by Pereira et al. (19) for *T. cruzi* neuraminidase.

Chromatography on phenyl-Superose columns, which separate molecules on the basis of hydrophobic interactions, also failed to distinguish between the two activities (Fig. 2 *C*).

Effect of pH and Temperature on the Activity of Neuraminidase and Trans-sialidase. Trans-sialidase and neuraminidase activities show maximal velocities between pH 6.5 and 7.5, and very little activity at pH <5.5, or >9.5 (Fig. 3). A similar pH dependence was observed using different preparations of enzyme, that is, total trypomastigote lysates, fractions from the Mono Q column obtained from the purification of trypomastigote lysates, or with the enzymes bound to the immobilized mAbs 39 or TCN2.

Incubation of affinity-purified fractions, or total lysates, for 30 min at 56°C destroys the trans-sialidase and neuraminidase activities. We did not detect any differences in the activities of either enzyme at temperatures between 4°C and 37°C (not shown).

Kinetic Analysis of the Trans-sialidase and Neuraminidase Activities. The experiments illustrated in Figs. 4–7 were performed to determine whether the same or different enzymes transfer and hydrolyse linked sialic acid. Fig. 4 shows that MuNana, the substrate used to assay the *T. cruzi* neuraminidase activity, can also be a sialic acid donor to [^{14}C]lactose; that is, the two reactions can be coupled. On the other hand, 4-methyl-umbelliferone, the fluorescent product of the neuraminidase reaction, is not an acceptor of sialic acid. At concentrations up to 10 mM, it does not inhibit the transfer of sialic acid from sialylactose to [^{14}C]lactose (Fig. 5). These findings raised the possibility that the trans-sialidase can function as a neuraminidase, provided that the reaction mixture does not contain a glycan acceptor (or contains a poor acceptor) of the removed sialic acid.

To study this question further, we measured simultaneously the kinetics of sialic acid release from sialyllactose, and the formation of [^{14}C]sialyllactose, in reaction mixtures containing radioactive lactose. We found that the velocity of transfer of sialic acid was much greater than the velocity of its release. In the presence of 1 mM [^{14}C]lactose and 1 mM sialyllactose (100 nmol of each in a total volume of 100 μl), ~23 nmol of [^{14}C]sialyllactose is produced, but only 0.5 nmol of free sialic acid is released in 1 h. In the absence of lactose, however, the amount of free sialic acid produced increases to ~2.5 nmol (Fig. 6).

In other experiments, we fixed the incubation time (30 min), and measured the effect of increasing the concentration of lactose in the incubation medium. As shown in Fig. 7, the release of free sialic acid decreased from 2 nmol to ~0.5 nmol as the lactose concentration increased from 0 to 100 nmol. The remote possibility that the lactose inhibits neuraminidase activity was excluded by the finding that the release of 4-methyl-umbelliferone from MuNana is not affected by addition of up to 10 mM lactose to the incubation medium (not shown).

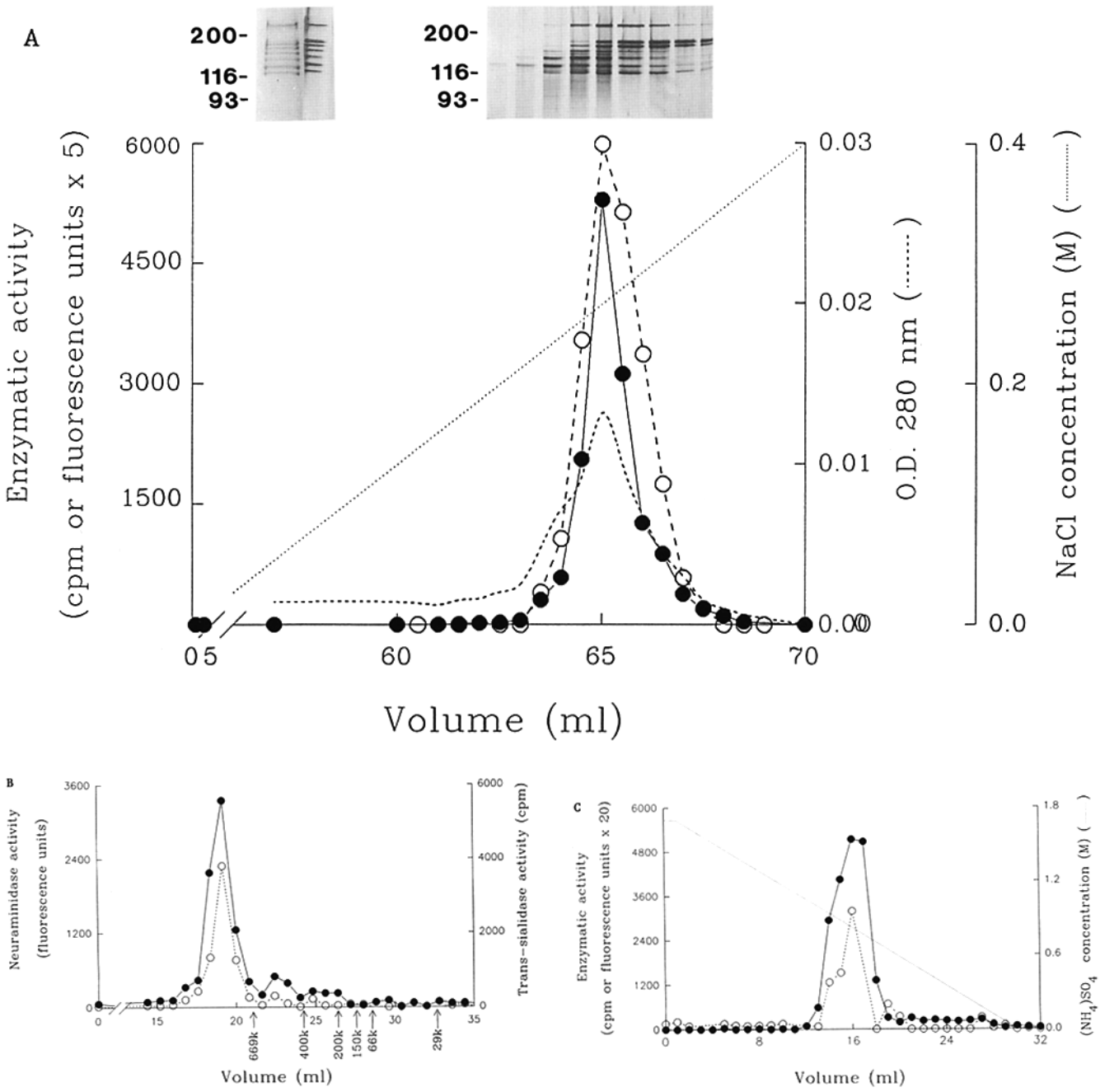

Figure 2. Trans-sialidase and neuraminidase have similar physicochemical properties. Samples of enzyme were subjected to different chromatographic procedures and the fractions analyzed for trans-sialidase (*filled circles*) and neuraminidase (*open circles*). Optical densities at 280 nm and/or salt concentrations are represented by dashed and dotted lines as indicated on the right *y*-axis. (*A*) Enzymatic activities after NaCl elution from a Mono Q FPLC column which had been equilibrated with 20 mM Tris-HCl, pH 8.0. The input was a sample of enzyme purified from culture supernatants by affinity chromatography with mAb 39. The input is analyzed in the two lanes of the inset on the left, showing an SDS-PAGE of the affinity-purified enzyme stained with coomassie blue and silver nitrate. The right inset shows the results of SDS-PAGE and silver staining of the fractions eluted at the corresponding positions on the *x*-axis. (*B*) Enzymatic activities of fractions obtained by gel filtration on Superose 12-Superose 6 FPLC columns run in tandem. The input was an eluate obtained by affinity chromatography of a *T. cruzi* extract on immobilized Con A. On the *x*-axis are shown the positions of eluted protein standards and their molecular masses. (*C*) Enzymatic activities of fractions eluted from an FPLC phenyl-Superose column by decreasing ammonium sulfate concentrations. The input was a sample of supernatant of *T. cruzi* cultures grown in BSA-supplemented medium.

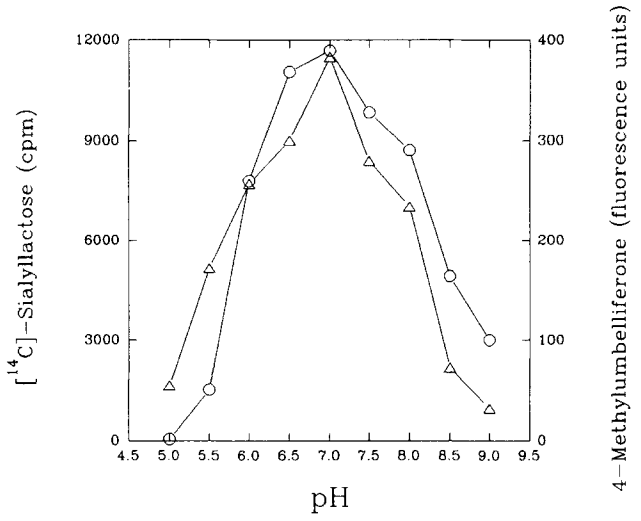

Figure 3. Effect of pH on the activity of trans-sialidase and neuraminidase reaction. Activity of pooled fractions eluting from the mono Q column (Fig. 2 *A*) was measured using sialyllactose and [^{14}C]lactose (*circles*) or MuNana (*triangles*) as substrates, in presence of 20 mM MES buffer (pH 5–6.5), 20 mM Hepes buffer (pH 7–7.5), and 20 mM Tris-HCl buffer (pH 8.0–9.0).

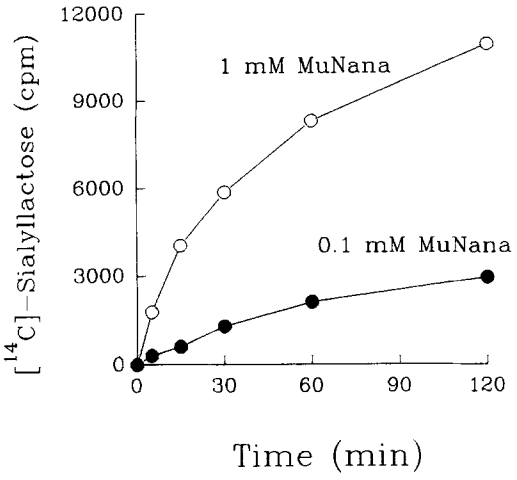

Figure 4. MuNana is a sialic acid donor. Mono Q affinity-purified enzyme was incubated with 1 mM sialyllactose for the indicated times in the presence of 1 mM MuNana (*open circles*), or 0.1 mM MuNana (*filled circles*). The amount of formed [^{14}C]sialyllactose was determined after incubation at 25°C.

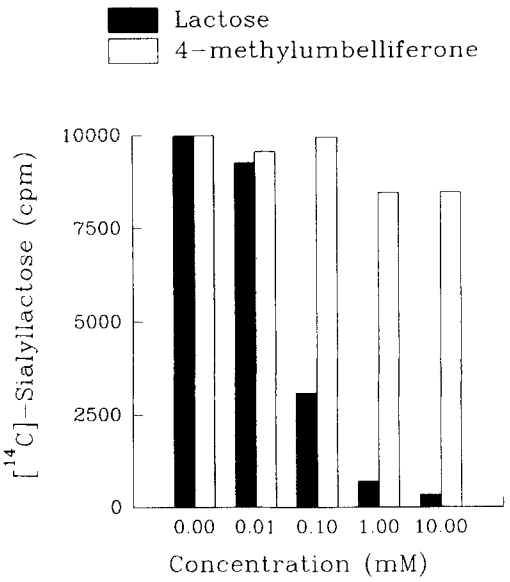

Figure 5. 4-methyl-umbelliferone is not an acceptor of sialic acid. Mono Q affinity-purified enzyme was incubated at 25°C in the presence of 1 mM sialyllactose and 8 μM [^{14}C]lactose in the presence of the indicated concentrations of lactose or 4-methylumbelliferone. After 30 min, the amount of [^{14}C]sialyllactose formed during the reaction was determined.

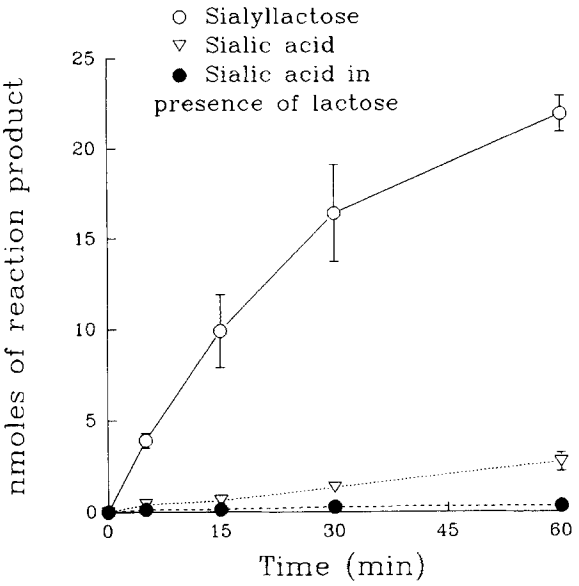

Figure 6. Kinetic analysis of the neuraminidase and trans-sialidase reactions. Affinity-purified enzyme (50 ng protein) was incubated for various periods of time at 25°C in 20 mM Hepes buffer, pH 7.0, in the presence of 100 nmol of sialyllactose and 80 nmol of lactose mixed with [^{14}C]lactose, in a final volume of 0.1 ml. At the end of the reaction, 0.04 ml was used to measure the amount of free sialic by TBA-HPLC method (*closed circles*), and 0.06 ml was used to measure the amount of [^{14}C]sialyllactose produced (*open circles*). The sialic acid produced in the absence of lactose is also indicated (*triangles*). The results are expressed in nmol of sialyllactose or sialic acid produced per 0.1 ml of the reaction.

Discussion

The main conclusion of this paper is that the trans-sialidase and neuraminidase reactions can be catalyzed by the same *T. cruzi* enzyme. This conclusion is based on several observations, which can be summarized as follows. First, both enzymes are recognized by two independently derived mAbs. When trypomastigote extracts are treated with the mAbs immobilized on protein A–Sepharose beads, the trans-sialidase and neuraminidase activities remain with the beads. Second, as previously reported for the *T. cruzi* neuraminidase (17–19),

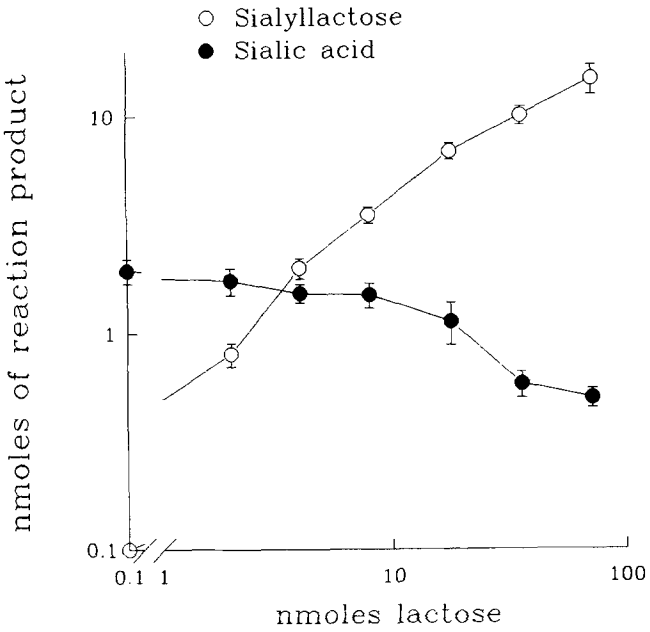

Figure 7. Lactose inhibits the release of sialic acid. Experimental conditions are as described in Figure 6, except that incubation was for 30 min, and final concentrations of lactose mixed with [^{14}C]lactose varied as indicated on the *x*-axis. The synthesis of [^{14}C]sialyllactose (*open circles*) or release of sialic acid (*closed circles*) are indicated.

the trans-sialidase consists of oligomers linked to the membrane through a GPI anchor, is released from the parasite, and accumulates in the tissue culture supernatants. Third, both enzymes bind to Con A, and copurify on ion exchange, sizing, and hydrophobic chromatographies. Fourth, the enzymatic activities are not affected by changes in temperature from 4°C to 37°C, and the enzymatic activities are equally influenced by protonation and deprotonation, suggesting a similar mechanism for both catalyzed reactions. The curves relating activity and pH were bell shaped, with an optimum for both reactions at around pH 7. This is in contrast to the bacterial, viral, and mammalian neuraminidases, which function optimally at acid pH, but is in accord with the observation that the trans-sialidase is active under physiological conditions when the parasites are in the peripheral blood (13) and in tissue culture medium (3).

In addition, the neuraminidase substrate MuNana is a sialic acid donor for the trans-sialidase, and lactose decreases the amount of free sialic acid produced during the overall reaction (Figs. 6 and 7). In fact, as will be described elsewhere, the product of a single *T. cruzi* gene expressed in *Escherichia coli* can display both activities (Uemura et al., manuscript in preparation). Taken together, our findings suggest that the sialic acid donor binds to the trans-sialidase and forms a sialylated intermediate. The bound sialic acid can then be transferred to water in a typical hydrolysis reaction, or transferred to an appropriate oligosaccharide acceptor, such as lactose.

The interpretation of these results is complicated by the fact that the enzyme is very heterogeneous. Trans-sialidase/ neuraminidase from the Y strain migrates on SDS-polyacrylamide gels as a group of 120–220-kD bands. Although the Y strain of *T. cruzi* used in the present studies was not cloned, we eliminated the possibility that the heterogeneity reflects the presence of variants in the population. The SDS-PAGE patterns of the trans-sialidase from six independent Y strain clones were recently analyzed, with identical results (S. Schenkman, unpublished observation). These bands are most likely not degradation products, since the SDS-boiled extracts of the trypanosomes contained several protease inhibitors. Moreover, additional incubation of the extracts at 37°C did not alter the pattern of migration in SDS-PAGE.

Instead, as suggested in reference 19, the bands may be the products of different genes, and/or represent the effect of posttranslational modifications. The trans-sialidase/neuraminidase is part of a gene family. Its sequence is ~80% identical to a polymorphic GPI-anchored antigen named "shed acute phase protein" (SAPA) (20, 21; and Uemura et al., manuscript in preparation). SAPA is glycosylated (21), and Southern blotting of *T. cruzi* chromosomes separated by pulse-field gel electrophoresis and probed with a SAPA clone gave a complicated pattern, indicating that there are several SAPA loci distributed among different chromosomes (22). Each of the SAPA genes contains tandem repeats, and the number of repeats varies among the different genes, further contributing to the heterogeneity of the members of the family (23). In addition, the 120–220-kD products share sequence homology with an antigenically distinct 85-kD family of trypomastigote surface antigens (24–29) and with the bacterial neuraminidases (30).

One group of investigators reported that polyclonal antibodies to a member of the 85-kD family partially removed neuraminidase activity from *T. cruzi* extracts (25). This is in apparent contradiction to the present and previous results (8, 19), which identified neuraminidase and *trans*-sialidase as members of the 120–220-kD family. The reasons for these discordant results are not clear. Perhaps the polyclonal antibodies to the 85-kD proteins crossreacted with the 120–220-kD molecules, or coprecipitated the two groups of molecules. Alternatively, the 85-kD proteins could account for a minor proportion of the total enzymatic activity in the *T. cruzi* extracts. However, no neuraminidase activity has been detected in the in vitro translation product of one 85-kD protein gene (G. B. Takle, personal communication).

In short, the trans-sialidase/neuraminidase is part of a family of *T. cruzi* stage-specific proteins that share sequence motifs with bacterial neuraminidases. This family contains two antigenically distinct group of proteins, one migrating in SDS-PAGE ~85 kD, and the other between 120 and 220 kD. We show here that proteins belonging to the 120–220-kD family have trans-sialidase and neuraminidase activities. It cannot be excluded, however, that some of its members are enzymatically inactive, or display only one of the activities. Whether or not some elements from the 85-kD family have neuraminidase and/or trans-sialidase activity remains to be determined.

It is well known that many glycosidases transfer glycosidic bonds, when appropriate donors and acceptors are provided (9). The kinetics of the transfer vs. hydrolysis reac-

tions are different for individual glycosidases, depending on the relative affinity of the glycosyl residue for the acceptor, vs. its affinity for water. Although it is not clear for most glycosidases whether the transferase reactions have biological relevance, the trans-sialidase reaction predominates on the surface membrane of *T. cruzi* trypomastigotes in vivo. The Ssp-3 epitope, which is expressed only after sialic acid transfer reactions (3), is present on trypomastigotes isolated from blood of infected mice (13). Furthermore, when blood trypomastigotes are isolated from animals whose tissues contain *N*-glycolylneuraminic rather than *N*-acetyl-neuraminic acid, the parasites also contain only *N*-glycolylneuraminic acid (31). While the enzyme can desialylate the membrane of myocardial cells, vascular endothelial cells, and erythrocytes (32), this may in fact have been the outcome of transfer reactions to nonidentified acceptors. Further studies are needed to evaluate the role of these transfer and hydrolysis reactions in the biology of *T. cruzi*, and in mediating host pathology.

While this paper was being prepared for publication, we received from Dr. A. C. C. Frasch a manuscript reaching essentially the same conclusion; that is, that the SAPA antigen has neuraminidase and trans-sialidase activity (Parodi, J. A., G. D. Pollevick, M. Mautner, A. Buschiazzo, D. O. Sanchez, and A. C. C. Frasch, manuscript submitted for publication).

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