Procyclic Trypanosoma brucei Expresses Separate Sialidase and trans-Sialidase Enzymes on Its Surface Membrane*

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The procyclic stage of Trypanosoma brucei in the insect vector expresses a surface-bound trans-sialidase (TbTS) that transfers sialic acid from glycoconjugates in the environment to glycosylphosphatidylinositol-anchored proteins on its surface membrane. RNA interference against TbTS abolished trans-sialidase activity in procyclic cells but did not diminish sialidase activity, suggesting the presence of a separate sialidase enzyme for hydrolyzing sialic acid. A search of the T. brucei genome sequence revealed seven other putative genes encoding proteins with varying similarity to TbTS. RNA interference directed against one of these proteins, TbSA C, greatly decreased the trans-sialidase activity but had no effect on trans-sialidase activity. The deduced amino acid sequence of TbSA C shares only 40% identity with TbTS but conserves most of the relevant residues required for catalysis. However, the sialidase has a tryptophan substitution for a tyrosine at position 170 that is crucial in binding the terminal galactose that accepts the transferred sialic acid. When this same tryptophan substitution in the sialidase was placed into the recombinant trans-sialidase, the mutant enzyme lost almost all of its trans-sialidase activity and increased its sialidase activity, further confirming that the gene and protein identified correspond to the parasite sialidase. Thus, in contrast to all other trypanosomes analyzed to date that express either a trans-sialidase or a sialidase but not both, T. brucei expresses these two enzymatic activities in two separate proteins. These results suggest that African trypanosomes could regulate the amount of critical sialic acid residues on their surface by modulating differential expression of each of these enzymes.

African trypanosomes are protozoan parasites responsible for sleeping sickness in humans and a similar disease in domestic animals called nagana. Their life cycle alternates between the bloodstream of a mammalian host and the tsetse fly vector (Glossina sp.). The surface of the bloodstream form of the parasite is completely covered with 107 copies of a single variant surface glycoprotein (VSG) bearing a glycosylphosphatidylinositol (GPI) anchor. These bloodstream trypanosomes elude the immune response of the mammalian host by periodically switching from one VSG to another immunologically distinct VSG. The bloodstream form of the parasite differentiates into the procyclic form when ingested by the insect vector. This differentiation involves a remodeling of the surface in which the VSG coat is rapidly shed and replaced with a new set of invariant GPI-anchored glycoproteins known as procyclins (1). Procyclins have an unusual GPI anchor that, unlike the GPI anchor of VSGs, is decorated with branched poly-N-acetyllactosamine repeats capped by sialic acid residues (2). Trypanosomes are unable to synthesize sialic acid, but the procyclic form of the African trypanosome Trypanosoma brucei expresses a specific enzyme, trans-sialidase (TbTS), that transfers sialic acid from sialylated glycoconjugates present in the tsetse fly midgut (such as in the blood meal or on the midgut cells) to acceptor molecules on its surface membrane (such as the side chain of GPI-anchored proteins and free GPs) (3). Trypanosomes lacking sialic acids due to a defective GPI-anchored trans-sialidase do not survive in the midgut, indicating that sialic acid residues of the GPI are critical for the survival of the parasite in tsetse flies (4). Trypanosoma cruzi, the agent of Chagas disease in the Americas, has a different life cycle, and its developmental expression of trans-sialidase shows a different pattern. The noninvasive epimastigote form of T. cruzi in the midgut of its hemipterous blood-sucking insect vector expresses trans-sialidase (TcTS) late in this midgut stage. After transmission to the bloodstream of the mammalian host, T. cruzi parasites continue expressing trans-sialidase, although from a different set of genes, and rapidly acquire sialic acid from sialoconjugates in the host (5). Surface sialylation in T. cruzi plays a central role in its evasion of the early complement-mediated immune response (6) and its host cell adhesion/invasion mechanism (7).

The trans-sialidase from T. cruzi is the one that has been

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) DQ 814707.

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better analyzed, including the determination of its three-dimensional structure (8–11). A related American parasite, *Trypanosoma rangeli*, expresses a homologous protein (TrSA) with 70% amino acid identity to TcTS, but this enzyme is devoid of trans-glycosidase activity and is strictly a hydrolase (12). In the case of *T. brucei*, the trans-sialidase has been purified and characterized (13–15), and information on the parasite gene encoding the protein is available (16). It should also be mentioned that the *T. brucei* and *T. cruzi* trans-sialidases have been found to be essentially trans-glycosidases. However, in the absence of acceptors of sialic acid in the milieu, they are able to release free sialic acid and thus are, to a much lower extent, hydrolytic enzymes.

A comparison of the crystal structures of TcTS and TrSA (17) and information derived from several mutagenesis approaches (16, 18, 19) show that trypanosomal sialidases and trans-sialidases share a similar active site architecture in which several amino acid residues critical for enzyme function are conserved. In the *T. cruzi* and *T. rangeli* enzymes, a conserved tryptophan residue (Trp-313) was shown to be implicated in the binding of substrate and to be necessary for the specificity of the enzyme for α-(2,3)-linkage sialic acid (18). Other residues surrounding the active site differ when the structures of sialidase and trans-sialidase are compared. In particular, Tyr-119 (replaced by serine in TrSA) was found to be essential for the transfer reaction and important in the structural environment of the catalytic nucleophile Tyr-342.

*T. cruzi* trans-sialidases and *T. rangeli* sialidases are both encoded by a multigene family (7). *T. cruzi* has ~140 trans-sialidase genes, half of which encode proteins lacking activity due to a substitution of Tyr-342 by a histidine residue (20). In contrast, it has been postulated that *T. brucei* has a much lower number of trans-sialidase genes (16). Recently, two different partial sequences of putative trans-sialidase genes were identified in another African trypanosome species, *Trypanosoma congolense* (21), supporting the hypothesis that the number of gene copies may be lower in African trypanosomes. In this work, we show that, in contrast to all other trypanosome species examined to date, *T. brucei* expresses on its surface different proteins bearing trans-sialidase and sialidase activities, an unusual situation that allows the parasite to regulate sialic acid content on its surface through independent enzymes.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Transfections**—Procyclic *T. brucei* 29-13 cells (7RNAP NEO TETR HYG) or their derivatives were used for all studies. The *T. brucei* 29-13 cells were maintained in SDM-79 medium supplemented with 10% fetal calf serum and were transfected with EcoRV-linearized plasmid (5–10 μg) as described previously (22). Logarithmic phase cells (5 × 10⁶ cells ml⁻¹) were collected by centrifugation, washed with electroporation medium (a 3:1 mixture of Cytomix, 120 mM KCl, 0.15 mM CaCl₂, KH₂PO₄, 25 mM HEPES, 2 mM EDTA, 5 mM MgCl₂, pH 7.6) and phosphate solution (277 mM sucrose, 1 mM MgCl₂, 7 mM KIPO₄, pH 7.4) and suspended in electroporation medium at a concentration of 5 × 10⁷ ml⁻¹. 0.45 ml of cells were mixed with 0.1 ml of linearized DNA in a 0.4-cm electroporation cuvette and subjected to two pulses with a Bio-Rad Gene Pulser electroporator set at 1500 V and 25 microfarads. Stable transformants were selected in 15 μg of G418 ml⁻¹, 50 μg of hygromycin ml⁻¹, and 2.5 μg of phleomycin ml⁻¹. After drug-resistant pooled lines were established, clonal lines were obtained by limiting dilution.

**RNA Interference (RNAi)**—RNAi experiments were performed using the vector p2T7T¹ (23), which allows for stable tetracycline-inducible expression of double-stranded RNA from T7 promoters in procyclic *T. brucei* 29-13 cells. 500- and 1000-bp fragments extending downstream from the initiator methionine codon of the *TbTS* open reading frame were PCR-amplified using the following primers: *Tb* oligonucleotide 14 (5’-ATGGAGCTCCAGCAAAC-3’) and *Tb* oligonucleotide 15 (5’-ATTATACAAAGTGTGCAGTGTG-3’) for the 500-bp fragment and *Tb* oligonucleotide 14 and *Tb* oligonucleotide 16 (5’-GCTCATACGGAGCTCTCCATTCCAC-3’) for the 1000-bp fragment. The PCR-amplified fragments were cloned into pGEM-T easy (Promega), digested with XbaI, and subcloned into XbaI-digested p2T7T¹/GFP to give p2T7T¹/500 bp and p2T7T¹/1000 bp.

The open reading frame of *TbSA C* was amplified using primers *TbSA C* NH (5’-ATAGGCTAGCTGACCTGACCACATGTCGCATGACGTTTCCCATTCCAC-3’) and *TbSA C* stop (5’-TATAGATCTCTATGCTGACAGTAAAC-3’) and cloned into pGEM-*TbSA C*. A fragment of 615 bp of the *TbSA C* open reading frame was excised with EcoRI and BamHI from pGEM-*TbSA C* and ligated into the EcoRI and BamHI sites of pTrcHisC. The resulting plasmid was digested with HindIII and BamHI, and the fragment was subcloned into HindIII/BamHI-digested p2T7¹, yielding p2T7¹/TbSA C.

The open reading frame of *TbSA B* was amplified using primers *TbSA B* 1 (5’-GCTAATCAGATGACCTGAGCCTGATGACGTTTCCCATTCCAC-3’) and *TbSA B* stop (5’-TATAGATCTCTATGCTGACAGTAAAC-3’) and cloned into pGEM-*TbSA B*. A fragment of 850 bp of the *TbSA B* open reading frame was obtained by digestion of pGEM-*TbSA B* with BglII and PstI and was subcloned into pTrcHisC. The resulting plasmid was digested with BamHI and HindIII, and the fragment containing a partial sequence of *TbSA B* was ligated into the corresponding sites of p2T7¹, yielding p2T7¹/TbSA B.

Plasmids p2T7¹/GFPc, p2T7¹/TbTSc, p2T7¹/TbSA Be, and p2T7¹/TbSA Cc were generated by deleting the tetracycline operator regions from p2T7¹/GFP and replacing the GFP gene by the XbaI-digested fragments of *TbTS* or HinDIII/BamHI-digested fragments of *TbSA B* and *TbSA C*, respectively. The tetracycline operator regions were excised with BglII, and the resulting DNA fragments were ligated back together.

**Northern Blot Analysis**—Total RNA was extracted from 2 to 20 × 10⁶ cells (cultures non-induced or tetracycline-induced for 48 h) using the TRizol (Invitrogen) method according to the manufacturer’s instructions. RNA concentration was determined by UV spectrophotometry at 260 nm, and RNA quality was confirmed by gel analysis.

For the Northern blots, 20 μg of total RNA was electrophoresed on formaldehyde-agarose gels (1%) and transferred by capillary action onto Zeta Probe nylon membranes (Amersham Biosciences). After cross-linking, membranes were blocked in hybrid-
Cloning of the TbSA C 5' and 3'-UTRs—RNA was purified using TRIzol reagent (Invitrogen) following the manufacturer’s instructions. To obtain the 5'-UTR sequence, first-strand cDNA was prepared with the Superscript II system using an internal primer (5'-CACACTTAAGCATCCCCCTCGT-3') of TbSA C. Reverse transcription-PCR was carried out with Taq DNA polymerase (Invitrogen) and primers for the T. brucei 39-nucleotide 5'-spliced leader sequence as forward (5'-AAC-GCTATTTAGAAGACTGTTCCTGACT-3') and the one used for first-strand synthesis as reverse. To obtain the 3'-UTR sequence, reverse transcription-PCR was performed with Superscript II (Invitrogen) using the oligonucleotide anchor-(dT)18 (5'-GCCAGCTCCGGCCGGCGGCGG(T)18-3'). PCR was conducted on the first-strand product using the anchor-(dT)18 as the reverse primer. The forward primer was 5'-TACCTTTA-CCTGTTGATGTTGCT-3'. All PCR products were cloned in pGEM-T Easy (Promega) and sequenced using the dideoxynucleotide chain termination method with Sequenase (USB Corp.).

Expression of Sialidase Genes in Bacteria and Protein Purification—In preparation for producing active recombinant proteins, we analyzed TbSA B, TbSA C, and TbTSsh using the iPSORT program (Human Genome Center, Institute of Medical Sciences, University of Tokyo) for predicting the existence of a signal peptide. We also examined the primary sequences of their C-terminal domains to predict the existence of transmembrane regions. Inserts were designed to encode all of the predicted recombinant proteins for these genes.

Plasmids containing the open reading frames of TbSA B and TbSA C (pGEM-TbSA B and pGEM-TbSA C, respectively; see above) were cut with BglII and Nhel, and the DNA fragments corresponding to the genes were ligated into expression vector pTrcHisC (Invitrogen). TbSA B constructs starting at three alternative codons and ending at two alternative stop codons were obtained by PCR on the pGEM-TbSA B plasmid using the following primers: TbSA B NH (5'-GCTAGCATGAAGGCCTGCTGCTAGC-3'; Nhel restriction site is underlined), TbSA B Ma (5'-CGGCTAGCTAAGCTGTGACCACTGTG-3'); TbSA C 2600 (5'-GAGGACTTG-TGCTATGACAGTAAC-3') and primer 270 (5'-GAGGACTTG-TTCAAA-TCTTTCAGG-AGACAGTTTCTGTACTGC-3'), primer TbTS (5'-TATAAGATCTCATGCTGACAGTAAC-3'), primer 360 (5'-GCTTAGCTAGCTTCTTCAGGAGACAAA-3'), and TbSA B STOP5 (5'-TATAAGATCTCTAGTCACAGTAGCTCAGATAC-3'; BglII restriction site is underlined).

A TbSA C construct starting at the codon for lysine 24 was obtained by PCR on the pGEM-TbSA C plasmid using the primers TbSA C ma (5'-GAGGACTTGAAAGAAGGTTGACAATCACCATACTAC-3'; Nhel restriction site is underlined) and TbSA C STOP (5'-TATAAGATCTCTATGCTGACAGTAAC-3'; BglII restriction site is underlined).

After digestion with the corresponding restriction enzymes, the fragments were ligated in the pTrcHisC vector. The His tag encoded in the plasmid vector was used to purify protein. The constructs were introduced in Escherichia coli BL21 (DE3) pLysS cells by the calcium chloride method. Overnight cultures were diluted 1:50 in Terrific Broth and grown at 37 °C to an A660 of 0.8–1.0 with constant agitation at 250 revolutions/min. Bacteria were induced to overexpress recombinant proteins by adding 0.5 mM isopropyl thio-β-galactoside (Sigma), and induction was maintained at 18 °C for 12–16 h. Cells were harvested, washed with NaCl/Tris (20 mM Tris/HCl, pH 7.6, and 50 mM NaCl), and frozen (−80 °C) until needed. After thawing, lysis was carried out in 20 mM Tris/HCl, pH 7.6, 30 mM NaCl, 0.5 mM Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 100 μg of DNase I ml−1.

Supernatants were centrifuged at 21,000 × g for 30 min and subjected to immunoaffinity metal affinity chromatography (IMAC) (Hitrap Chelating, Amersham Biosciences AB) via Ni2+-charged equilibration in 20 mM Pipes, pH 6.9, and 0.5 mM NaCl (buffer IMAC). The column was washed with 30 mM imidazole in buffer IMAC. Elution was achieved using a linear gradient of 30–250 mM imidazole in buffer IMAC.

Site-directed Mutagenesis and Mutant Protein Purification—Site-directed point mutagenesis was performed by amplifying the pTrcHisC vector containing the TbTS gene starting at the codon for leucine 28 (16) with primersYW forward (5'-CAATGTTCAC-GAACGGGTGTTGACACAACGAAAACAGG-3') and YW reverse (5'-CGTTGTTTCTCTTTGCTGGCACCACCCCCTCTGTCATGG-TCATGGG-3'). The PCR product was digested with DpnI, purified, cloned, and introduced in E. coli BL21 (DE3) pLysS cells. Clones were sequenced to confirm the mutation of target sites.

The mutant and the wild-type TbTS recombinant proteins were expressed in bacteria and purified by IMAC (see previous section). After IMAC purification, the activity peak was pooled, dialyzed against 20 mM Bistris, pH 7.4, and further purified by fast protein liquid chromatography anion exchange (Mono Q) equilibrated with the same buffer. The protein was eluted by applying a linear gradient of 0–250 mM trisodium citrate. Purified proteins were analyzed by SDS-PAGE under reducing conditions, stained with Coomassie Blue R250, and quantified with Kodak one-dimensional version 3.0 software using purified bovine serum albumin as the standard.
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**GPI-PLD Treatment**—For GPI-PLD digestion, $5 \times 10^6$ T. brucei procyclic cells were osmotically lysed and the membrane fractions were incubated with GPI-PLD (Boehringer, Mannheim, Germany) at a concentration of 5 units ml$^{-1}$ for 4 h at 37°C in 100 μl of buffer A (150 mM NaCl, 10 mM Heps, NaOH, pH 7) in the presence of 0.1% Nonidet P-40 (24).

**Enzyme Activity Assays**—Neuraminidase activity was determined by measuring the fluorescence of 4-methylumbiliferone released by the hydrolysis of 0.2 mM 2-(4-methylumbelliferyl)-D-N-acetylneuraminic acid (MUNen5Ac, Sigma). The assay was performed in 50 μl in 20 mM Pipes, pH 6.9. After incubation at 37°C for 30 min, the reaction was stopped by dilution in 0.2 M sodium carbonate, pH 10, and fluorescence was measured with a DYNAQuant TM 200 fluorometer (Hoefer Pharmacia, Inc).

**Trans-sialidase activity** was measured in 20 mM Pipes, pH 6.9, using 1 mM Neu5Ac-(2–3)-lactose as the sialic acid donor and 12 μM [d-glucose-1-14C]lactose (55 mCi mol$^{-1}$) (Amersham Biosciences) as the acceptor in a 30-μl final volume at 37°C for 15 min. The reaction was stopped by dilution, and sialyl-[14C]lactose was quantified with a β-scintillation counter as previously described (25).

**Sialic Acid Determination**—The total amount of sialic acid in procyclic parasites was determined after hydrolysis in 0.1 M HCl for 1 h at 80°C using the thiobarbituric acid method and high pressure liquid chromatography analysis (26).

**RESULTS**

**Identification of Genes Coding for Trypanosomal trans-Sialidase-like Proteins**—BLAST searches were performed using sequences corresponding to the catalytic domain of TbTS (AF 310232.1, a T. brucei trans-sialidase) in the T. brucei Genome Data Base (Sanger Centre). The search identified seven T. brucei open reading frames with a blast E value between $1 \times 10^{-167}$ and 0.20. These open reading frames (Fig. 1) were named TbTS for T. brucei trans-sialidase, TbTSsh for a truncated version of TbTS, TbSA B and C for putative T. brucei sialidases B and C, and genes D1, D2, and E (which have much less identity to TbTS, so their products were named TbTS-like proteins). Most of these genes are present as a single copy in the T. brucei Genome Data Base, except TbSA C, which is present in two tandem copies. The differences in these copies are localized in 38 positions. Various sialidase amino acid motifs, such as the FRIP and Asp box motifs, are conserved in the deduced primary structures of proteins encoded by these TbTS family genes (Fig. 1). The six putative non-TbTS proteins have different levels of identity to TbTS. The catalytic regions of TbSA B and TbSA C are 49 and 46% identical, respectively, to the catalytic domain of TbTS. These two proteins have most of the structurally relevant residues displayed in trypanosomal trans-sialidase, including the essential amino acid residues Pro-283 and Tyr-342 (amino acid positions are those of TcTS; see Fig. 2). Point mutations at these positions of TcTS and TbTS completely abolished both sialidase and trans-sialidase activities (16, 19). However, each of these two putative trans-sialidases has a natural mutation in different positions that is relevant for enzymatic activity (Fig. 2).

**TbTS-like**

- **D1**
  - 703
  - 14% identity
  - 703
  - XM 946607

- **D2**
  - 682
  - 13% identity
  - XM 9824113

- **E**
  - 776
  - 10% identity
  - XM 9819357

**FIGURE 1.** Comparison of the primary structures of TbTS and putative members of the TbTS family, TbTSsh, TbSA B, TbSA C, and TbTS-like proteins. The percent identities to TbTS are indicated, and the proteins are shown in decreasing order of identity. Catalytic (open boxes) and lectin-like domains (shaded boxes) are shown. The positions of the FRIP and Asp boxes are indicated with vertical bars inside of the catalytic domain. TcTS superfamily motifs are indicated with dark bars inside of the lectin-like domain. GenBank™ accession numbers are shown.
in position 372, which is equivalent to Trp-312 of TcTS, whereas TbSA B has a phenylalanine residue in this position (Phe-421). The exposed aromatic side chain of this tryptophan favors, in the case of microbial sialidases and trans-sialidases, the high specificity for sialyl-α-(2, 3) substrates (11). Critical amino acid residues are not present in the catalytic domains of deduced sequences coded by the D1, D2, and E genes. The TbTS family genes encode the partially conserved subterminal VTVXNVFLYNR motif (shown in Fig. 1) that, in the case of T. cruzi, defines the trypanosome trans-sialidase/sialidase superfamily of surface proteins. Finally, the deduced protein of the truncated TbTSsh gene has 93% identity to the catalytic domain of TbTS, but codon 428 is a stop codon; therefore, the lectin domain is missing. This protein might not be able to properly fold and may lack enzymatic activity (16). We tested TbSA B, TbSA C, and TbTSsh products for enzymatic activities (see “Experimental Procedures”). None of constructs displayed sialidase/ trans-sialidase activity when expressed in bacteria (data not shown).

RNAi against TbTS Indicates the Presence of a Single Gene Encoding T. brucei trans-Sialidase—RNAi was used to assess TbTS function in procyclic cells. This was performed by cloning 500- and 1000-bp fragments, starting from the initiator methionine codon of the TbTS gene into p2T7Ti, a vector that can be integrated into the T. brucei genome and allows inducible expression of double-stranded RNA (dsRNA) under the control of a tetracycline-inducible T7 promoter (Fig. 3A). The 2956-bp TbTS mRNA is undetectable in procyclic TbTSi cells exposed to tetracycline for 48 h. Neither TbTS mRNA levels in GFPi cells nor tubulin mRNA levels in GFPi and TbTSi cells were affected by tetracycline.

A second approach was used to obtain cells expressing the dsRNAs independent of tetracycline addition. The tetracycline operator regions in the three p2T7Ti constructs mentioned above were deleted (Fig. 3A) and 29-13 procyclic cells were transfected with these constructs producing GFPic and TbTSic products that expressed dsRNA constitutively (Fig. 3C). The TbTS mRNA was undetectable in these constitutive TbTS knockdown cells, and as expected, the trans-sialidase levels in these cells showed a substantive reduction (~20-fold) compared with control cells.

To investigate whether procyclic cells showing reduced trans-sialidase activity have altered levels of sialic acid in their

FIGURE 2. Conserved amino acids among trypanosomal sialidases and trans-sialidases. The amino acid position numbers in TrSA, TcTS, and TbTS are those of the corresponding active recombinant proteins, whereas the same numbers for TbSA B and C are relative to the first methionine of the corresponding open reading frame. Open boxes indicate amino acids conserved in the two trypanosomal trans-sialidases but different in TrSA and the putative T. brucei sialidases. Shadowed boxes indicate unique mutations in the T. brucei putative sialidases.
surface membranes, the total sialic acid content was measured (Fig. 3E). Cells expressing tetracycline-inducible TbTS dsRNA had \( \sim 24\% \) of the sialic acid when tetracycline was included in the growth medium compared with when it was not. The effect was even more dramatic in cells expressing dsRNA constitutively, which showed a reduction to 11% of the total sialic acid content found in control cells. Considering that \( T.\) brucei procyclic cells have \( \sim 3 \times 10^6 \) procyclin molecules on their surface, the observed levels of sialic acid in these parasites correspond to \(< 1 \) sialic acid molecule/procyclin (28). This result is consistent with the earlier observation that sialic acid is not required for the survival of procyclic trypanosomes during growth in vitro (3, 29).

\( T.\) brucei Cells Lacking trans-Sialidase Activity Have a Residual Sialidase Activity—We next measured the sialidase activity in \( T.\) brucei cells lacking trans-sialidase expression via RNAi directed against TbTS. To our surprise, the TbTS knockdown cells did not exhibit a proportional reduction in the levels of sialidase activity as compared with the levels of trans-sialidase activity. TbTSic cells possessed 78% of the sialidase activity and only 4% of the trans-sialidase activity compared with control cells (Fig. 4A). Taken together, these results indicate that at least one additional protein with sialidase activity must be expressed in these cells.

It has been shown previously that TbTS is attached to the \( T.\) brucei membrane by a GPI anchor sensitive to GPI-PLD digestion (14). To separate TbTS from the putative sialidase protein(s), GFPic and TbTSic cells were osmotically lysed and treated with GPI-PLD. Subsequently, the supernatants and pellet fractions were assayed for enzymatic activities. The highest levels of both sialidase and trans-sialidase activities were present in the supernatants of both the control and TbTS knockdown cells (Fig. 4A). These

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**FIGURE 3.** TbTS dsRNA expression in Trypanosoma brucei. A, p2T7\( ^{\text{11}} \) vectors for expression of dsRNA. p2T7\( ^{\text{11}} \)/GFP contains two opposing tetracycline-inducible T7 promoters flanking GFP. p2T7\( ^{\text{11}} \)/TbTS 500 bp and p2T7\( ^{\text{11}} \)/TbTS 1000 bp contain a fragment of 500 and 1000 bp of the TbTS gene, respectively, subcloned between T7 promoters. Black box, tetracycline operators; closed arrows, T7 promoters; open arrow, RNA promoter; vv, T7 terminator; BLE, bleomycin resistance gene. B, Northern blot analysis before and after RNAi. \( T.\) brucei procyclic cells harboring p2T7\( ^{\text{11}} \)/TbTS 500 bp or p2T7\( ^{\text{11}} \)/TbTS 1000 bp (TbSi1 and TbTSi2, respectively) and control cells harboring p2T7\( ^{\text{11}} \)/GFP (GFPi) were induced with 1 \( \mu \)g of tetracycline (Tet) \( \text{ml}^{-1} \) for 48 h, and total RNA was prepared. Membranes were hybridized with a probe encoding a catalytic domain of TbTS and were rehybridized with a tubulin-coding region used as an internal loading control. C, trans-sialidase activity. TbTSi cells were grown in the absence or presence of 1 \( \mu \)g of tetracycline (Tet) \( \text{ml}^{-1} \), and trans-sialidase activity was assayed on the indicated days and the results plotted against days of dsRNA induction. The activity measured in cells grown without the addition of tetracycline was considered to be 100%. After 19 days, the induced cells were washed with phosphate-buffered saline to remove tetracycline. PBS, phosphate-buffered saline. D, Northern blot analysis of RNA from cell lines constitutively expressing dsRNA. The RNAs for the blot were obtained from \( T.\) brucei procyclic cells harboring the p2T7\( ^{\text{11}} \)-GFP constructs (GFPic and TbSic, respectively) and from 29-13 control cells. E, comparison of trans-sialidase activity and sialic acid content of 29-13 cells and RNA-interfered with cells. Trans-sialidase activity and sialic acid were measured as indicated under "Experimental Procedures." Sialic acid content is expressed as nmol of sialic acid/10^7 cells. The values are the mean \( \pm \) S.D. of three independent determinations. The percentage of activity and sialic acid content are relative to the values present in 29-13 cells.
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The sialidase activity was measured using Neu5Ac-(2–3)-lactose and lactose as the sialic acid donor and acceptor, respectively, and expressed in counts/min/10^6 cells. Sialidase activity was measured using MUNen5Ac as substrate and expressed as relative fluorescent units. The values are the mean ± S.D. of three independent determinations.

Specific Silencing of a Putative Sialidase Gene—By comparing the catalytic domain sequences of the different TbTS-like proteins to that of TbTS, the TbSA B and TbSA C genes were identified as the best candidates to code for the putative sialidase protein(s). Analysis of the primary structures of their C-terminal domains was performed using DPGI (The Swiss Institute of Bioinformatics, University of Geneva, Switzerland), and TMHMM programs (Center for Molecular Pathology, Vienna, Austria), and GPI Prediction Server (Research Institute of Molecular Pathology, Vienna, Austria), indicating that neither of them is susceptible to cross-RNAi on the other RNA.

TbSA B has 56% overall identity with TbSA C and 45% with TbTS; therefore, we could not eliminate the possibility of some degradation of TbSA B mRNAs in cells interfered with by dsRNAs corresponding to the other two genes. Because TbSA B expression could be not detected by Northern blot, we tested potential cross-RNAi by analyzing the expression of TbTS and TbSA C genes. As shown in Fig. 5B, neither TbTS mRNA levels in TbSA C knockdown cells nor TbSA C mRNAs levels in TbTS knockdown cells were affected, indicating that TbSA C is not expressed in cell lines expressing TbSA C dsRNA constructs, producing TbSA Ci and TbSA Cic cell lines, respectively. The expression of this gene was analyzed by Northern blot (Fig. 5A). The 2845-bp TbSA C mRNA (DQ 841707) was undetectable in procyclic TbSA Ci cells exposed to tetracycline and in TbSA Ci cells, but it was not altered in control cells by tetracycline. Likewise, tubulin mRNA levels were not affected in the studied cells. To verify the specificity of silencing by RNAi generated by closely related dsRNAs, we tested cross-RNAi production for both the TbTS and TbSA C genes. As shown in Fig. 5B, neither TbTS mRNA levels in TbSA C knockdown cells nor TbSA C mRNAs levels in TbTS knockdown cells were affected.

To determine whether the TbSA B and/or TbSA C genes code for a protein with sialidase activity, we silenced each of them by dsRNA expression. The expression of both mRNAs in T. brucei procyclic cells was analyzed by Northern blot. We did not observe any signal when we hybridized membranes with a TbSA B probe, suggesting the TbSA B gene is not expressed in T. brucei procyclic cells (data not shown). To increase the sensitivity of the search for TbSA mRNA, reverse transcription-PCR assays were performed to detect the TbSA B 5′- and 3′-UTRs, also with negative results (not shown). These results indicate that TbSA B is either not expressed or expressed at a very low level in procyclic cells grown in culture.

To silence the TbSA C gene, 29-13 cells were transfected with tetracycline-inducible and constitutive TbSA C dsRNA constructs, producing TbSA Ci and TbSA Cic cell lines, respectively. The expression of this gene was analyzed by Northern blot (Fig. 5A). The 2845-bp TbSA C mRNA (DQ 841707) was undetectable in procyclic TbSA Ci cells exposed to tetracycline and in TbSA Ci cells, but it was not altered in control cells by tetracycline. Likewise, tubulin mRNA levels were not affected in the studied cells. To verify the specificity of silencing by RNAi generated by closely related dsRNAs, we tested cross-RNAi production for both the TbTS and TbSA C genes. As shown in Fig. 5B, neither TbTS mRNA levels in TbSA C knockdown cells nor TbSA C mRNAs levels in TbTS knockdown cells were affected.

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To silence the TbSA C gene, 29-13 cells were transfected with tetracycline-inducible and constitutive TbSA C dsRNA constructs, producing TbSA Ci and TbSA Cic cell lines, respectively. The expression of this gene was analyzed by Northern blot (Fig. 5A). The 2845-bp TbSA C mRNA (DQ 841707) was undetectable in procyclic TbSA Ci cells exposed to tetracycline and in TbSA Ci cells, but it was not altered in control cells by tetracycline. Likewise, tubulin mRNA levels were not affected in the studied cells. To verify the specificity of silencing by RNAi generated by closely related dsRNAs, we tested cross-RNAi production for both the TbTS and TbSA C genes. As shown in Fig. 5B, neither TbTS mRNA levels in TbSA C knockdown cells nor TbSA C mRNAs levels in TbTS knockdown cells were affected.
A Single Natural Mutation Converts trans-Sialidase into a Sialidase—Some of the amino acids demonstrated to be crucial for the transfer (trans-sialidase) and hydrolysis (sialidase) reactions are conserved in TbTS and TbSA C (Fig. 7A). However, TbSA C has a tryptophan residue in the position structurally homologous to Tyr-191 of TbTS. Previous results (17) indicate that this tyrosine residue is implicated in binding the acceptor carbohydrate in T. cruzi trans-sialidase. Because the RNAi data suggested that TbSA C could be a more efficient sialidase than trans-sialidase, we suspected this natural mutation of TbSA C could be responsible for the different behavior of this enzyme compared with trypanosomal trans-sialidases. Thus, we examined changes in enzymatic activities resulting from a Y191W substitution in a TbTS recombinant protein. The mutant Y191W TbTS recombinant protein was produced and purified using the same criteria described for the wild-type enzyme (Fig. 7B) (16). As shown in Fig. 7B, when Tyr-191 was replaced by a tryptophan residue in TbTS, this mutation practically abolished the sialyltransferase activity and slightly increased the sialidase activity.

To investigate whether procyclic cells with reduced sialidase activity have altered levels of sialic acid in their surface membranes, we measured the total sialic acid content in parasites that express TbSA C RNAi either inducibly with tetracycline or constitutively. We did not observe any significant difference in the levels of sialic acid when tetracycline was included in the growth medium compared with when it was not (TbSA Ci-Tet = 114.8 ± 36.1 ng/10⁷ cells, TbSA Ci + Tet = 115.5 ± 32.2 ng/10⁷ cells). Likewise, we did not observe any difference in the sialic acid content in constitutive TbSA C knockdown cells as compared with the control 29-13 cells (TbSA Cci = 119.5 ± 19.2 ng/10⁷ cells, 29-13 cells = 104.2 ± 9.7 ng/10⁷ cells).

**FIGURE 5. TbSA C dsRNA induction and cross-RNAi analysis.** A, expression of TbTS and TbSA C genes in 29-13 cells containing TbSA C dsRNA. The control cells are TbSA Ci (cells in the absence or presence of tetracycline) and TbSA Cic (cells that express dsRNA constitutively). B, expression of TbTS and TbSA C mRNAs in 29-13 cells in the absence or presence of tetracycline induction of TbTS and TbSA B dsRNA. Membranes were hybridized with the indicated probes and were rehybridized with a tubulin-coding region probe used as an internal loading control. C, schematic representations of the primary sequences of TbTS and TbSA C. Dark bars indicate the position of the region used as the probe for Northern blot analysis.

**FIGURE 6. trans-Sialidase and sialidase activities in TbSA B and TbSA C interfered cells.** TbSA Bi and TbSA Ci were grown in the absence or presence of 1 μg of tetracycline (Tet) ml⁻¹. Sialidase and trans-sialidase activities were plotted against days of dsRNA induction. The activities in cells grown in the absence of tetracycline were considered to be 100%. After 19 days, induced cells were washed with phosphate-buffered saline (PBS) to remove tetracycline.

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The enzymatic activities were measured in TbTSic, TbSA Bic, and TbSA Cic cells as indicated under “Experimental Procedures.” The percentages of sialidase and trans-sialidase activities are relative to those of 29-13 control cells. The values are the mean ± S.D. of three independent determinations.

| Clone     | Ts activity | Sa activity |
|-----------|-------------|-------------|
| 29-13 cells | 100         | 100         |
| TbTSic    | 5 ± 2       | 97 ± 8      |
| TbSA Cic  | 95 ± 1      | 33 ± 3      |
| TbSA Bic  | 87 ± 4      | 89 ± 7      |

**FIGURE 7. Site-directed mutagenesis on TbTS.** A, locations of amino acids in TbSA C that are likely equivalent to amino acids involved in TbTS trans-sialidase activity. B, recombinant proteins were expressed and purified as indicated under “Experimental Procedures.” Sialidase activity was measured using MUNen5Ac as substrate and trans-sialidase activity was measured using sialyl-lactose for the appropriate accommodation of donor sialic acid into the active site (Fig. 2). These residues include the arginine triad that forms electrostatic interactions with sialic acid carboxylate (Arg-84, -298, and -375 of TbSA C) and the tryptophan residue that forms a weak H-bond with the glycerol side chain of sialic acid (Trp-171), as well as Arg-102 and Asp-146 that are involved in H-bond formation with the 4-OH group of sialic acid. TbSA C also has the aspartic residue that serves as a proton donor in the reaction (Asp-108), two residues Glu-283 and Tyr-402 that probably stabilize the transition state intermediate, and the tryptophan that determines substrate specificity (Trp-372).

Considering the amino acids that are invariant between TbTS and TcTS but differ in TrSA, we observed that the novel *T. brucei* sialidase shows higher identity in these positions with trypanosomal trans-sialidases than with *T. rangeli* sialidase (Fig. 2). TbSA C conserves the sequence Pro–Glu–Ser at positions 342–344, the structural homologs of positions 283–285 in TcTS, whereas this sequence is substituted by Gln–Asp–Cys in TrSA. Furthermore, trypanosomal sialidase and trans-sialidase differ in three other positions in the neighborhood of the active site; Met-96, Phe-114, and Val-180 in TrSA are substituted by valine, tyrosine, and alanine in the trans-sialidases, respectively. TbSA C conserved Val-232 (equivalent to Val-180 in TrSA) but has the same residues as trans-sialidase in the other two positions (Fig. 2). The most significant difference between TbSA C and trypanosomal trans-sialidases is the substitution of a tyrosine residue by tryptophan in position 170 of TbSA C (Y170W). We have previously reported the effect of the substitution Y191S in TbTS recombinant protein, the structural homolog to Trp-170 in TbSA C. This mutation suppressed both activities in *T. brucei* trans-sialidase (16). Nevertheless, when we assayed the activity of TbTS recombinant protein bearing the Y191W substitution, we observed that this amino acid replacement almost completely abolished trans-sialidase activity, although slightly increasing the sialidase activity. A similar effect was observed by replacing Tyr-119 with serine in TcTS (17, 18), indicating that this residue is critical for the trans-sialylation reaction in both trypanosomal trans-sialidases. Watts *et al.* (30) postulate that the replacement of the side chain of Tyr-119 of *T. cruzi* trans-sialidase by Ser-120 in *T. rangeli* sialidase could be responsible for the difference in the positioning of the glycerol of sialic acid observed by comparing the covalent intermediates of both enzymes. Tyr-119 in TcTS occupies two alternative conformations. In the absence of suitable acceptors for the transfer reaction, the mobile conformation corresponds to one that forms the acceptor binding site in the trans-sialylation reaction, whereas the more rigid conformation (where the OH group of Tyr-119 establishes two hydrogen bonds with O-7 and O-9 of the glycerol) constrains the sialyl moiety. The presence of a serine in this position in *T. rangeli* sialidase eliminates this constraint, allowing the free glycerol group to form intramolecular hydrogen bonds, and the glycerol chain is less buried in the pocket. The fact that TbSA C has a tryptophan in this position suggests the side chain of tryptophan could have the same effect as the side chain of Tyr-119 of TcTS in the positioning of the glycerol group. Nevertheless, the absence of an OH group in tryptophan would eliminate the H-bonds with oxygen atoms of
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glycerol, consequently, the glycerol side chain of sialic acid could be less constrained in the pocket. This difference could affect the ability of TbSA C to transfer sialic acid residues compared with that of trypanosomal trans-sialidases.

We have identified an additional gene, the TbsA B gene, which encodes a protein with high homology to TbTS (higher than TbSA C) and conserves most of the important amino acids in the active site described above. The putative TbsA B protein might have sialidase or trans-sialidase activity, but we could not detect the expression of this gene in procyclic T. brucei. TbsA B has a mutation in a tryptophan important in the specificity for α-(2–3) substrates. This substitution, W421F, could change the substrate specificity of TbsA B to different α-linkages. One possibility might be that TbsA B is expressed in the bloodstream form of T. brucei despite the fact that neither trans-sialidase nor sialidase activity on the substrate MUNen5Ac (14) could be detected in this stage. We suggest that sialidase activity of this protein against MUNen5Ac could be diminished or abolished as a result of the substitution W421F (16, 17). Further work will be required to assess the enzymatic properties and functional role of TbsA B. We also identified three other genes related to TbTS in T. brucei and describe them as encoding TbTS-like proteins, because they do not have the relevant residues for trans-sialidase and sialidase activities. One, the D1 gene, has been previously described and characterized (16). Finally, we detected the TbTSsh gene, which encodes a truncated form of TbTS that might be unable to fold to form the complete active trans-sialidase site. This gene is expressed at low levels in procyclic T. brucei (data not shown).

The most surprising finding described here is that procyclic T. brucei expresses sialidase activity and trans-sialidase activity on separate proteins, whereas the related trypanosomes T. cruzi and T. rangeli express only a trans-sialidase protein or only a sialidase protein, respectively. The key question is why T. brucei needs to express these enzymatic activities in different proteins if, in fact, trans-sialidases also have sialidase activity in the absence of suitable sialic acid acceptors. We suggest these two enzymes might be expressed at different times in vivo as procyclic cells migrate through the midgut of their tsetse fly vector. This differential expression in vivo could constitute a mechanism to regulate the amount of sialic acid on the procyclic surface membrane, allowing the parasite to hide or to show specific carbohydrate residues involved in the attachment with specific molecules in its vector. Similar mechanisms have been proposed for the attachment of T. rangeli to the salivary glands of its vector Rhodnius prolixus (31) and another protozoan parasite Leishmania major with its sand fly vector Phlebotomus papatasii (32). Trans-sialidase might also have other relevant functions in the survival of T. brucei in the insect vector (4). For example, sialic acid could contribute to a resistance mechanism of trypanosomes against trypanocidal lectins and digestive enzymes present in the midgut (33). Further experimental work will indicate if TbTS, TbSA C, and/or both enzymes are targets to control disease transmission by the insect vector.

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