Biochemical Diversity in the Trypanosoma congolense Trans-sialidase Family

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Abstract

Trans-sialidases are key enzymes in the life cycle of African trypanosomes in both, mammalian host and insect vector and have been associated with the disease trypanosomiasis, namely sleeping sickness and nagana. Besides the previously reported TconTS1, we have identified three additional active trans-sialidases, TconTS2, TconTS3 and TconTS4, and three trans-sialidase like genes in Trypanosoma congolense. At least TconTS1, TconTS2 and TconTS4 are found in the bloodstream of infected animals. We have characterised the enzymatic properties of recombinant proteins expressed in eukaryotic fibroblasts using fetuin as model blood glycoprotein donor substrate. One of the recombinant trans-sialidases, TconTS2, had the highest specific activity reported thus far with very low sialidase activity. The active trans-sialidases share all the amino acids critical for the catalytic reaction with few variations in the predicted binding site for the leaving or acceptor glycan. However, these differences cannot explain the orders of magnitudes between their transfer activities, which must be due to other unidentified structural features of the proteins or substrates selectivity. Interestingly, the phylogenetic relationships between the lectin domains correlate with their specific trans-sialylation activities. This raises the question whether and how the lectin domains regulate the trans-sialidase reaction. The identification and enzymatic characterisation of the trans-sialidase family in T. congolense will contribute significantly towards the understanding of the role of these enzymes in the pathogenesis of Animal African Trypanosomiasis.

Introduction

Trypanosoma congolense (subgenus: Nannomonas) is a major causative agent of the Animal African Trypanosomiasis (AAT) otherwise known as nagana. Other parasites implicated in nagana include T. vivax (subgenus: Duttonella) and T. brucei brucei (subgenus: Trypanozoon). These protozoan parasites are transmitted by several species of hematophagous biting flies of the genus Glossina. Nagana exhibits a severe negative impact on stock farming, milk and meat production [1]. The impact of the disease is thought to be underestimated as most affected areas are remote, limiting access and hence accurate data acquisition. The need for an alternative arsenal against AAT is heightened as existing drugs are either toxic or rapidly becoming ineffective due to drug resistance [2].

The role of TS in Chagas’ disease caused by the South American T. cruzi has been extensively studied [3]. On the other hand, studies on the trans-sialidases from African trypanosomes responsible for the Human African Trypanosomiasis (HAT) as well as AAT are scanty. In T. brucei, TS has been implicated in the cyclical survival of the parasite as evidence supports enhanced survival of parasite in midgut of the insect host [4]. No data exist on T. congolense in this regard. Though scanty, the role of blood stream TS and sialidase in anaemia in animals suffering trypanosomiasis caused by T. congolense [5] [6] and T. vivax [7] has been established.

Multiple copies of TS-like genes exist in Trypanosoma genomes. The highest number occurs in T. cruzi, but most of the over 1000 genes encode enzymatically inactive proteins [8]. In African trypanosomes, the TS-like gene families are much smaller. For example, 9 members have been identified in T. brucei [9] [10] [11]. In T. congolense, at least 17 TS-like genes have been identified [12] [13] [14] [6]. Eleven of these, forming the TconTS1 family, are closely related and share over 95% sequence identity [14]. The key element mediating the functions of TS has been ascribed to the N-terminal catalytic domain (CD) harbouring the active site with characteristic conserved amino acids [15] [16] [17] [9] [18], whereas hardly anything is known about possible functions of the lectin-like domain (LD) at the C-terminus of these enzymes.

Here we report that besides TconTS1, three additional members of the T. congolense TS gene family transfer sialic acids between glycoconjugates, but have much lower sialidase activities. The identification and biochemical characterisation of T. congolense TS genes will enable new studies investigating the role of these genes in nagana disease.

Methods

Unless where stated, all chemicals and reagents used were cell culture and analytical grade. Vibrio cholerae sialidase was purchased...
Author Summary

Trypanosomiasis is a disease also known as sleeping sickness in humans (Human African Trypanosomiasis) and nagana in animals (African African trypanosomiasis). This disease is caused by protozoan parasites of the genus Trypanosoma. Tsetse flies are responsible for the transmission of these parasites. Trypanosoma congolense is the main causative agent of nagana in cattle. The clinical signs of the disease have been linked to the presence of an enzyme called trans-sialidase. Interestingly, the enzyme alternates in different forms in the mammalian and the insect vector. Previous knowledge had shown that the parasite requires the enzyme for survival in the fly vector. Our current work has revealed other forms of the enzyme that could be essential for the persistence of the disease in mammalian and vector hosts. These enzymes, though similar in structural architecture, show differences in their activities that could be key in delineating their individual roles in the pathophysiology of the disease.

from Roche Diagnostics (Mannheim, Germany). Pfu DNA polymerase, HinflIII, XhoI, SpeI, EcoRI and Dpul, PageBlue, molecular weight marker (PageRuler), BCA assay protein kit were all purchased from Thermo Scientific (St. Leon-Rot, Germany). VivaSpin 6 and VivaCell250 ultracentrifugation units were from Sartorius (Gottingen, Germany). Anti-SNAP-tag rabbit polyclonal antibody was from GeneScript (Piscataway, USA) while anti-Strep-tag rabbit polyclonal antibody, Strep-Tactin resin beads and buffers were from IBA (Goettingen, Germany). Polyethyleneimine transfection reagent, glucuronic acid, N-acetyl-neuraminic acid (Neu5Ac), 3’sialyllactose (3’SLL) and lactose were purchased (Sigma, Steinheim, Germany). Hygromycin and Gentamicin were purchased from PAA, (Pasching, Austria). Polyethyleneimine transfection reagent, glucuronic acid, N-acetyl-neuraminic acid (Neu5Ac), 3’sialyllactose (3’SLL) and lactose were purchased from Sigma-Aldrich (Steinheim, Germany). Ex-cell CD CHO media from SAFC, USA, X-ray film, Enhanced Chemiluminescence system, and recProtein-A Sepharose Fast Flow were purchased from GE Healthcare (Uppsala, Sweden). Polycyividine difluoride membrane was from Millipore (Schwabach, Germany).

Cloning, sequencing, expression and purification of trans-sialidase genes

The Basic Local Alignment Search Tool (BLAST) was used to search the shot-gun sequences of T. congolense at the WST1 (http://www.sanger.ac.uk). Using the BLASTN algorithm, the “T. congolense reads” were queried with the partial nucleotide sequences (Genbank Accession numbers TS1: AJ535487 and TS2: AJ535488) previously described [13]. Perfect BLAST hits (smallest sum probability P(N)<10-10) were arranged into contiguous sequences using Contig Express (Invitrogen, Carlsbad, USA). By searching the database with ends of the contiguous sequences, the assembled contigs were expanded until open reading frames (ORF) were obtained. On the basis of the obtained ORFs, primers (Supporting Information, Table S1) were designed to amplify by nested PCR the ORF including flanking regions encoding for TconTS2, TconTS3 and TconTS4 using genomic DNA of T. congolense strain STIB249 [13]. The resulting products were cloned into the pBlueScript KS- vector (Stratagene, Santa Clara, CA, USA) via SpeI and BamHI (TconTS2) or via EcoRI and SmaI (TconTS4) or into the mammalian expression vector pcDNAIII Amp (Invitrogen, Carlsbad, USA) via HinflIII and XhoI (TconTS3) and sequenced (Supporting Information, Table S2).

Cloning and sequencing of T. brucei TS genes followed a similar strategy as described for T. congolense above except that genes were cloned in pJET1.2/blunt vector (Thermo Scientific) following instructions of the manufacturer (for primers see Supporting Information, Table S1).

For the expression of secreted TconTS proteins in mammalian fibroblasts, corresponding DNA sequences without those encoding the signal peptides and GPI anchors were subcloned into a modified pDEF vector providing a 3C protease recognition site, SNAP and Stop tags using SpeI and BamHI restriction sites [14]. For this purpose, the BamHI site in TconTS3 as well as the SpeI and BamHI sites in TconTS4 were removed by site directed mutagenesis without changing the amino acid sequence encoded (for primers see Supporting Information, Table S1). All sequences and mutations were confirmed by Sanger dideoxy DNA sequencing at the Max Planck Institute for Marine Microbiology, Bremen, Germany.

Recombinant TconTS proteins were purified as described [14]. Briefly, CHOLEC1 cells (ATCC CRL-1735) were transfected with polyethyleneimine, transfection reagent (Sigma, Steinheim, Germany) and stably expressing cell lines selected with hygromycin. Expression of recombinant protein was tested from cell culture supernatant by SDS-PAGE and Western blots methods using rabbit anti-Stop and anti-SNAP antibodies. CHOLEC1 cells producing TconTS proteins were subsequently adapted to chemically defined Excel CD CHO media.

Purification of anti-TS1 monoclonal antibody

The 7/23 hybridoma cells [12] were grown for 3 days in RPMI media supplemented with IgG depleted 10% FCS. The tissue culture supernatant was cleared by ultracentrifugation at 105,000 g for 60 min and anti-TconTS antibody was purified using recProtein-A Sepharose Fast Flow and eluted with 0.1 M glycine/HCl pH 3.0. Antibody containing fractions were neutralised with 1M Tris pH 8.0 and dialysed against 10 mM phosphate buffer. Purified antibodies were used in the detection of TconTS proteins in SDS-PAGE and Western Blot analysis as described [14].

Trans-sialidase and sialidase reactions

Purified recombinant proteins were assayed for sialidase and TS activities using Neu5Ac-MU and fetuin as sialic acid donor substrates and lactose as acceptor substrate as described before [14]. In brief, reactions of 50 μL containing substrates and enzymes were incubated at 37°C for the times indicated. Sialidase activity was determined as free sialic acids released from Neu5Ac-MU, 3’SLL or fetuin in the absence and/or presence of an acceptor substrate. TS activity on the other hand was determined as 3’SLL produced in the presence of lactose. Both, free Neu5Ac and 3’SLL were quantified using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using the Dionex system, DX600 (Dionex Sunnyvale, CA, USA) [14]. The curve fit module of Sigmaplot 11 was used to calculate v max and Km employing the Michaelis-Menten equation v = v max×C i /(C i +K m).

Phylogenetic analysis

For the phylogenetic analysis TconTS1b, TconTS2, TconTS3 and TconTS4 were aligned with TS and sialidase sequences from T. brucei, T. vivax, T. cruzi and T. rangeli. As outgroup the sialidase from Vibrio cholerae was used (genes listed in Supporting Information Table S2). Full length protein sequences were first aligned using ClustalW in Geneious and then truncated at the N-terminus. CDs started from the FRIP region to the N-terminus of the conserved α-helix (HL) linking the CD to the LD. The LDs were taken immediately after the α-helix linkage to the C-terminus.
without the stop codon. DNA sequences encoding either full length proteins, the CDs or the LDs were aligned based on the alignment obtained for the amino acid sequences by T-Coffee algorithm in RevTrans, version 2.0 (http://www.cbs.dtu.dk/services/RevTrans2.0/web/). DNA sequences used in the phylogenetic calculations are shown in Supporting Information Files S1, S2 and S3. Best parameters (HKY substitution model with 6 gamma rate categories) for phylogenetic constructions were determined using MEGA5 and applied in the phylogenetic calculations using the “MrBayes” plug-in of Geneious.

Results

T. congolense sialidase/trans-sialidase genes

Partial coding sequences of Tc0nTS1 and Tc0nTS2 genes had been described [13]. From “reads” of the WTSI T. congolense genome sequencing project (http://www.sanger.ac.uk), we assembled the full length sequences coding Tc0nTS1 and Tc0nTS2. Further BLAST hits with smallest sum probabilities (P(N)<1-10) were identified and arranged into contiguous sequences leading to further five genes with sequence similarities. Two of the putative gene products shared over 40% sequence identity leading to Tc0nTS1 and Tc0nTS2 and contained all the conserved amino acids required for transfer reactions [17] [18] [19]. Consistent with the naming of Tc0nTS1 and Tc0nTS2 [13], we refer to them as Tc0nTS3 and Tc0nTS4. The other three genes were distantly related with 20–30% amino acid identity (Table 1) and lack several of the conserved amino acid residues. We assume that these set of genes are likely without sialidase or TS activity and were named Tc0nTS-Like1, Tc0nTS-Like2, and Tc0nTS-Like3.

In order to compare sequence similarities between Tc0nTS genes, we cloned and sequenced full lengths Tc0nTS2, Tc0nTS3 and Tc0nTS4. In an earlier study, we amplified eleven highly similar (about 96% identical amino acids) but clearly different sequences of Tc0nTS1 from T. congolense genomic DNA [14]. Sequencing several clones of Tc0nTS2, Tc0nTS3 and Tc0nTS4 provided no evidence for such heterogeneity of these genes. The alignment of these genes is given in Figure 1.

Amaya et al. [18] identified amino acids in T. cruzi TS in the catalytic and substrate binding. Whereas these amino acids are not conserved in the three Tc0nTS-like gene products, they are almost completely conserved in Tc0nTS1, Tc0nTS2, Tc0nTS3 and Tc0nTS4 (Table 2). Most of these are conservative changes, with the exception of position 293 (numbering in the consensus sequence), where a Tyr is replaced by Pro in Tc0nTS2. As noted that not the same T. cruzi TS orthologues separate from the other African trypanosomes. Tc0nTS-Like2 and Tc0nTS-Like3 form a branch with their T. brucei orthologues separate from all South American TS genes. In contrast, Tc0nTS-Like1 and its T. brucei orthologue appear to be more closely related with the more distant South American branch than the African genes.

Trypanosomal TS contain an N-terminal domain. Besides the phylogenetic analysis with the entire ORFs, analysis were performed using sequences coding for each domain separately. The phylogenetic tree obtained for the CDs resembles that for complete ORFs (not shown). Surprisingly, a different situation was observed for the LDs of Tc0nTS1, Tc0nTS2, Tc0nTS3 and Tc0nTS4 and the T. brucei TS genes (Figure 2B). First, within the Tc0nTS genes, the LD of Tc0nTS2 is most closely related to that of Tc0nTS1, whereas the CD of Tc0nTS3 is more closely related to Tc0nTS2. Second, it should be noted that not the same T. congolense and T. brucei gene group as orthologous pairs, if LDs are compared. Amplification and sequencing ORF of T. brucei TS genes confirmed that the combination of the CDs and LDs were as predicted from the contigs in the databases.

Monclonal anti-TS1 antibody cross-reacts with Tc0nTS2, recognising an epitope on the lectin domain

To biochemically characterise Tc0nTS genes, recombinant proteins were made for Tc0nTS2, Tc0nTS3 and Tc0nTS4 as previously described for Tc0nTS1 [14]. Recombinant Tc0nTS proteins were expressed in CHO<sub>6.1c</sub> [20] and purified by affinity chromatography to obtain pure protein from eukaryotic cells with high mannose-type N-glycans. The apparent molecular masses of the recombinant Tc0nTS proteins including the SNAP and Strep tags are between 110 and 125 kDa as resolved on SDS-PAGE. The generic Strep tag fused to the proteins is recognised by anti-Strep polyclonal Ab in all the recombinant Tc0nTS proteins as shown in Figure 3 (upper panel). Surprisingly, the monoclonal anti-TS antibody [12] reacted with both Tc0nTS1 and Tc0nTS2.

Table 1. Trypanosoma congolense trans-sialidase sequence similarities expressed as percentage of identical amino acids in pairwise alignments.

| Trans-sialidase | Tc0nTS2 | Tc0nTS3 | Tc0nTS4 | Tc0nTS-Like1 | Tc0nTS-Like2 | Tc0nTS-Like3 |
|----------------|--------|--------|--------|-------------|-------------|-------------|
| Tc0nTS1        | 42.2%  | 43.6%  | 46.2%  | 21.1%       | 26.3%       | 29.8%       |
| Tc0nTS2        | -      | 48.3%  | 42.8%  | 20.8%       | 26.2%       | 29.3%       |
| Tc0nTS3        | -      | 48.9%  | 21.1%  | 25.1%       | 29.9%       |             |
| Tc0nTS4        | -      | -      | 21.3%  | 27.8%       | 29.8%       |             |

<sup>Tcon = Trypanosoma congolense</sup>

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but not TconTS3 and TconTS4 (Figure 3; lower panel). This result points to a similar epitope being present in both TconTS1 and TconTS2. Further experiments provided evidence that the epitope is located in the LDs.

**TconTS2, TconTS3 and TconTS4 are trans-sialidases**

In order to investigate the enzymatic activities we used the glycoprotein fetuin as donor and lactose as acceptor substrates. Free sialic acid (the product of sialidase activity) and 3’S-L (the product of TS activity) could be quantified simultaneously from HPAEC-PAD chromatograms of the reactions. Under standard conditions 25 ng TconTS2 produced about 330 pmol/min 3’S-L leading to 200 µM 3’S-L in the reaction mix within 30 minutes (Figure 4A). Product formation by TconTS2 was linear for up to 50 ng enzyme under these conditions. The reaction catalysed by TconTS3 was slower than that of TconTS2, since 500 ng of enzyme generated only 4.2 pmol/min 3’S-L corresponding to the maximum 3’S-L concentration reached. Nevertheless, small amounts of free Neu5Ac were detectable (0.68 µM, 27 pmol) when the 3’S-L concentration had reached almost 600 µM, the maximum 3’S-L concentration reached. Furthermore, incubation did not result in higher 3’S-L concentrations, the amount of free Neu5Ac continuously increased. This observation suggests that TconTS2 releases free Neu5Ac from 3’S-L but not from fetuin. Similar observations were made for TconTS1, but the highest 3’S-L concentration reached was about 300 µM. These data indicated that for TconTS1 and TconTS2 at 300 µM and 600 µM 3’S-L, respectively, the transfer of Neu5Ac between fetuin and 3’S-L has reached an equilibrium. For TconTS3, we could not reach such equilibrium; probably since the maximum 3’S-L concentration obtained was 50 µM due to the low specific activity of this enzyme. Nevertheless, small amounts of free Neu5Ac were detected in prolonged TconTS3 reactions leading to 50 µM 3’S-L. Also in this case, Sia appears to be released from 3’S-L only, since in the absence of lactose no release of free Neu5Ac could be observed.

These observations suggest that TconTS1, TconTS2 and TconTS3 can release free Neu5Ac from glycoconjugates, but the level of product released is too low to be detected under standard TS assay conditions. To address this, higher amounts (500 ng) of TconTS1, TconTS2, TconTS3 and TconTS4 were incubated with 600 µM fetuin-bound Neu5Ac without lactose for 2 h. Under these conditions sialidase activities could also be detected for TconTS1 and TconTS2, but not for TconTS3 (Table 4). However, compared to the corresponding TS activities, the sialidase activities were very low.

**Discussion**

The enzymatic properties of four TconTS were compared using fetuin as a model for a blood glycoprotein donor substrate. Two of these enzymes, TconTS1 and TconTS2, exhibit about 100- or 1000-fold higher specific TS activities than TconTS3 and TconTS4, respectively. The Km values for lactose were around 1 mM (0.6 to 1.8 mM) for all four TconTS and did not correlate with their specific activities. The Km values for the donor fetuin were more different ranging from 0.4 to 6 mM glycoprotein bound sialic acids. The Km for fetuin also did not correlate with the specific activity, since the highest Km was determined for TconTS3, one of the enzymes with low activity, and TconTS4 has a similar Km as TconTS1, but is 1000-fold less active. Since the Km values are lower than the substrate concentrations used, especially for TconTS4, the specific activities given in Table 3 are lower than those to be expected, if the acceptor substrate lactose would be at saturating concentrations.
Table 2. Amino acids in the catalytic domains of TS and TS-Like genes from *T. congolense* involved in enzymatic activities*.

| Consensus | Tcon TS1 | Tcon TS2 | Tcon TS3 | Tcon TS4 | Tcon TS-Like1 | Tcon TS-Like2 | Tcon TS-Like3 |
|-----------|---------|---------|---------|---------|--------------|--------------|--------------|
| catalysis |         |         |         |         |              |              |              |
| 212       | D150    | D135    | D142    | D207    | D110         | E85          | K86          |
| 410       | E324    | E309    | E316    | E381    | N291         | Y257         | S262         |
| 532       | Y438    | Y423    | Y430    | Y493    | F404         | L375         | H382         |
| substrate binding |         |         |         |         |              |              |              |
| 188       | R126    | R111    | R118    | R183    | E86          | H61          | R62          |
| 425       | R339    | R324    | R331    | R396    | A306         | Q272         | S277         |
| 496       | R410    | R395    | R402    | R465    | N375         | L339         | D346         |
| 206       | R144    | R129    | R136    | R201    | R104         | R79          | W80          |
| 270       | D188    | D173    | D180    | D245    | G160         | L122         | P132         |
| 293       | Y211    | P196    | Y203    | Y268    | A183         | G145         | S155         |
| 294       | W212    | W197    | W204    | W269    | L184         | T146         | L156         |
| 374       | Q289    | Q274    | Q281    | Q364    | V255         | E222         | V227         |
| 494       | Y408    | W393    | W400    | Y463    | A373         | E337         | D344         |
| structure |         |         |         |         |              |              |              |
| 411       | A325    | A310    | S317    | A382    | P292         | C258         | S263         |
| 465       | P379    | P364    | P371    | P434    | V344         | G308         | G315         |

*The indicated amino acids have been selected based on structural [18] and mutation [19] [23] studies with *T. cruzi* TS and on the sequence alignment of TconTS1b with *T. cruzi* [14]. Amino acid positions have been numbered based on the consensus of alignment (Figure 1) or starting methionine of each ORF.

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Figure 2. Phylogenetic analysis TS genes. Phylogenetic analyses of DNA sequences were performed as described under Methods using “MrBayes” plug-in of Geneious. Trees are midpoint rooted and nodes supported by posterior probability values and non-parametric bootstraps generated by maximum likelihood analysis in “MrBayes” as described under Methods. TS genes from *T. congolense* and *T. brucei* are marked by blue frames, TS genes from *T. vivax* by red frames. A: Phylogenetic tree for full ORFs, the “African TS-branch” is marked by a grey frame; B: Phylogenetic tree for LDs, the most active TconTS and their orthologues from *T. brucei* are highlighted by a grey box.

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Tiralongo et al. [12] purified two Tc0nTS forms with different TS/sialidase activity ratios from procyclic \textit{T. congolense} cultures. Peptide sequences from these preparations have demonstrated that they contained at least Tc0nTS1 [12] [14]. Recombinant Tc0nTS1 variants expressed in eukaryotic cells had lower specific activities for synthetic substrates than described for the purified enzyme [12] suggesting that these Tc0nTS preparations also contained other enzymes and/or factors influencing the TS/sialidase activities [14]. For example, in those preparations, Glutamic Acid-Rich Protein, GARP, a natural substrate for Tc0nTS was co-purified with one of the TS forms [12]. Although it is unclear what role GARP might have played, its presence, as well as other TS enzymes, might have been responsible for the reported higher specific activities of these TS preparations for synthetic substrates. In summary, it appears that Tc0nTS1 and Tc0nTS2 are responsible for most of the TS activity of \textit{T. congolense}. However, it may well be that Tc0nTS3 and Tc0nTS4 are more active on other donor substrates, such as glycoproteins and/or glycolipids from blood components, the natural substrates for trypanosomal TS.

It has been established that procyclic forms of African trypanosomes express TS [21] and emerging evidences point to expression also in the blood stream forms [6] [7]. So far, no information is available on which TS genes are expressed at what stage of the parasite’s life cycle. Recently, we have identified mRNAs for TconTS1, TconTS2 and TconTS4 in the blood of stage of the parasite’s life cycle. Recently, we have identified information is available on which TS genes are expressed at what months at 37°C for trypanosomal TS.

Lactose was found to suppress the sialidase activity of Tc0nTS4. \textit{In vitro}, lactose appears to be a better acceptor than water (Figure 3B). Therefore, in presence of lactose, the transfer activity of Tc0nTS4 is more active on other donor substrates, such as glycoproteins and/or glycolipids from blood components, the natural substrates for trypanosomal TS.

It appears that TS activity depends on well controlled conformational changes [17] influenced by specific proline residues. This is supported by the potential of the \textit{T. rangeli} sialidase to acquire transfer ability due to a change of Gln to Pro at position 284 [24] and the loss of enzymatic activity in \textit{T. cruzi} TS by the reverse mutation [16]. At the corresponding position 465 Pro is found in all active Tc0nTS (Table 2). The relevance of conformational changes in the enzyme rather than a direct specific interaction of the amino acid were also indicated by mutation of Pro231 to Ala in \textit{T. cruzi} TS [19], corresponding to position 411 in the consensus sequence. Although this mutation led to a significant decrease in \textit{T. cruzi} TS activity, all active Tc0nTS have an Ala or Ser at this position. It would be interesting to see, if higher TS activities can be obtained by introducing a Pro at this position, particularly in Tc0nTS3 or Tc0nTS4, the two enzymes with low TS activities.

Besides these critical amino acids listed in Table 2, other structural features obviously control the ratio of TS versus sialidase activities, since Tc0nTS2 and Tc0nTS4 share identical amino acids at all these positions. Yet, Tc0nTS4 has the highest sialidase to TS ratio amongst the Tc0nTS enzymes, whereas Tc0nTS2 has the lowest ratio (Table 4). Kohler-Brandl et al. [14] observed for Tc0nTS1 that a natural mutation that replaced R144 (206 in sialylation and eventual clearance by the immune system. However, desialylation of parasites is equally possible in presence of lactose due to the action of TS. Along this line it is interesting to note that infusion of lactose in the blood of sheep suffering experimental anaemia from \textit{T. congolense} suppressed anaemia (unpublished observation).

All amino acid residues shown to be involved in the catalytic reaction or interaction with the substrate for \textit{T. cruzi} TS are conserved in the Tc0nTS enzymes (Table 2). Only the two residues interacting with the methylumbelliferyl aglycon of the lactose part of 3’S	extsubscript{L} in the \textit{T. cruzi} TS [18], positions 293 and 494 in consensus sequence (Figure 1), are different in the two most active Tc0nTS1 and Tc0nTS2. This could explain why these enzymes do not use Neu5Ac-MU as substrate (data not shown). Furthermore, these changes could lead to a weaker interaction with the leaving groups and thus facilitate their release during catalysis. In this context it is interesting to note that the most drastic change, Tyr to Pro at position 293, occurs as P196 in Tc0nTS2, the most active enzyme with the highest TS/sialidase ratio (Table 4). Certainly, this modification will reduce the interaction with hydrophobic aglycons or the leaving galactose residue of the donor substrate.

Amaya et al. [18] also provided evidence that in \textit{T. cruzi} TS Y119 (position 293 in the consensus sequence) also contributes to hydrogen bonding with O9 of the covalently bound Sia following a conformational change induced by the reaction. Such an interaction would not be possible in Tc0nTS2, but could be compensated by hydrogen bonding with the conserved side chains W197 and Q274 of Tc0nTS2 corresponding to W120 and Q195 in \textit{T. cruzi} TS, two amino acids contributing to the hydrogen bonding network of O9 in the covalently bound Sia [18].

Similarly, although to a lesser degree, the replacement of a Trp at position 494 (corresponding to W312 in \textit{T. cruzi} TS) with Tyr, as found in Tc0nTS1, is expected to reduce the hydrophobicity of this site leading to a reduced affinity for the leaving group. In \textit{T. cruzi} TS substitution of this Trp (W312) by Ala basically abolished Neu5Ac transfer but only slightly decreased hydrolytic activity for 3’S	extsubscript{L} [23]. Interestingly, in contrast to the wild type \textit{T. cruzi} TS, this mutant was not able to hydrolyse Neu5Ac-MU, similar to Tc0nTS1, which also does not accept Neu5Ac-MU as a substrate [14].

It has been established that procyclic forms of African trypanosomes express TS [21] and emerging evidences point to expression also in the blood stream forms [6] [7]. So far, no information is available on which TS genes are expressed at what stage of the parasite’s life cycle. Recently, we have identified mRNAs for Tc0nTS1, Tc0nTS2 and Tc0nTS4 in the blood of infected goats (data not shown). Also the stability and persistence of shed enzymes in the blood stream has to be taken into account. In \textit{in vitro} Tc0nTS1 and Tc0nTS are the most stable of the four enzymes investigated, retaining full TS activities even after four months at 37°C. Under these conditions Tc0nTS2 lost its activity completely, while Tc0nTS4 retained 40% residual sialidase but no transfer activity (data not shown). It would be interesting to investigate whether this long-term stability correlates with a sustained persistence of enzyme activity in the blood stream.

Lactose was found to suppress the sialidase activity of Tc0nTS4. In \textit{in vitro}, lactose appears to be a better acceptor than water (Figure 3B). Therefore, in presence of lactose, the transfer activity of Tc0nTS4 is more efficient than its hydrolytic activity. Anaemia in animals suffering African \textit{Trypanosoma} infections has been attributed to the effects of sialidases [22] [5] [7]. Desialylation of erythrocytes by sialidases exposes underlying galactose residues and their subsequent degradation. The presence of lactose in mammalian blood would lead to lowered efficiency of parasites.
Diversity of T. congolense Trans-sialidases

A

3'sialyl-lactose (µM) vs TconTS2 (ng)

B

3'sialyl-lactose (µM) vs TconTS3 (ng)

C

3'sialyl-lactose (µM) vs TconTS4 (ng)
consensus) sequence with Cys in the variants TconTS1g (EMBL: HE582290) did not terminate but only reduced TS activity, while increasing relative hydrolytic activity. It can be assumed that a weaker interaction with the hydroxyl group at C4 of sialic acid is responsible for the catalytic properties of TS1g, since the Arg (R53 in T. cruzi TS) is in close contact with the bound sialic acid and probably supports the stabilisation of the enzyme-substrate complex [18].

Conservation and/or substitution of amino acids in the active centre of the catalytic domain did not give clear indications of activity differences between the TconTS enzymes. In this context, an interesting aspect is how the different specific activities of TconTS enzymes correlate with those of related gene products from other African trypanosomes. Phylogenetic analyses have allowed the clear assignment of orthologues for T. brucei, but not for T. vivax, where TS genes clustered exclusively together and away from the TS genes of T. congolense and T. brucei (Figure 2) [7] [25]. A direct comparison of the TS activities between T. congolense and T. brucei is difficult, since limited comparable data for enzymatic activities is available. In T. brucei, TbTS and TbSA C2 have been identified as active TS [9] [10] [11]. This is consistent with the observation that their orthologues (TconTS1 and TconTS2, respectively) are the most active TS in T. congolense and T. brucei [11]. This is lower than what was obtained for T. vivax [6] referred to two highly related TconTS3 genes in the IL3000 strain identified in GeneDB and TrytrypDB databases. However, we could not find evidence for their existence in the STIB294 strain used in this study. Similarly, closely related genes with over 80% sequence identity have been identified for TbSA B and TbSA C in T. brucei [11].

The presence of multiple highly similar TS genes, as described for TconTS1 [14], suggests that these genes undergo active rearrangements, which could lead to strain specific differences. For example, Coustou et al. [6] referred to two highly related TconTS3 genes in the STIB294 strain identified in GeneDB and TrytrypDB databases. However, we could not find evidence for their existence in the STIB294 strain used in this study. Similarly, closely related genes with over 80% sequence identity have been identified for TbSA B and TbSA C in T. brucei [11].

TS and sialidase genes of African trypanosomes are organised in two major domains; the CD and the LD. The LDs of TconTS are more varied (40% pairwise identity) when compared with the CDs (58% pairwise identity). Surprisingly, the phylogenetic relationships between the TS are clearly different, if only the LDs are included in the analysis (Figure 2). Furthermore, the LDs of the two most active enzymes TconTS1 and TconTS2 are more closely related than the CDs, where TconTS2 is most closely related to TconTS3. Interestingly, the monoclonal anti-TS1 antibody also binds TconTS2, recognising an epitope in the LD. First preliminary experiments obtained with recombinant proteins, in which the LDs have been swapped between TconTS, provided supporting evidence that the LD influences TS and sialidase activities of the enzymes (data not shown), while the specific activities of these proteins expressed in bacteria is much lower than those of the proteins expressed in fibroblasts described here.

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**Table 3. Kinetic parameters of TconTS.**

| Donor substrate (fetuin-bound Sia)* | Acceptor (lactose)** |
|-----------------------------------|----------------------|
| Vmax *** (μmol/(min × mg TS)) | Kmax *** (μM) | Vmax *** (μmol/(min × mg TS)) | Kmax *** (μM) |
| TSI1h**** | 7.9±0.3 | 359±45 | 4.3±0.1 | 1683±101 |
| TSI1e-1**** | 7.6±0.5 | 1617±223 | 2.1±0.1 | 727±48 |
| TconTS2 | 17.62±0.13 | 299.00±7.0 | 17.85±0.13 | 602±16 |
| TconTS3 | 0.17±0.02 | 6090.00±1267 | 0.0567±0.0014 | 1104±79 |
| TconTS4 | 0.0067±0.0002 | 949±50 | 0.0075±0.0002 | 1806±112 |

*Approximately 30 nmol Sia per 100 μg fetuin; 2 mM lactose was used as acceptor substrate.
**600 μM fetuin-bound Sia was used as donor substrate.
***Kmax and Vmax were calculated from Michaelis-Menten kinetics (see Supplementary Information, Figure S1) by SigmaPlot. Data points are mean ± standard deviations of three independent experiments, each replicated thrice.
****Values from Koliwer-Brandl et al. [14].

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suggesting that for conclusive interpretation the domain swapped TconTS have to be expressed in eukaryotic cells and that further studies are necessary to optimise the fusion of the two domains. In summary, these data indicate a more significant role for the LD for the TS activities of TS1 and TS2 and thus possibly in the pathogenesis of African trypanosomiasis.

Figure 5. Sialidase activity of TconTS4. A: Recombinant TconTS4 was incubated with 100 μg fetuin (600 μM bound Sia) for the times indicated (see insert for long term reactions) and free Sia determined by HPAEC-PAD as described under Methods. Data points are means ± standard deviations of 3 independent reactions each triplicated. B: Recombinant TconTS4 was incubated for 1440 min with 100 μg fetuin (600 μM bound Sia) in the presence of the lactose concentrations indicated and free Sia determined by HPAEC-PAD as described under Methods. Data points are means ± standard deviations of 3 independent reactions each as triplicates.

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Table 4. Sialidase activities of TconTS.

| TconTS1 | 59.7±2.3 | 4,000±100 | 67 |
| TconTS2 | 73.5±4.0 | 17,850±130 | 243 |
| TconTS3 | n.d.*** | 34±1 | >30 |
| TconTS4 | 2.7±0.07 | 9±0.2 | 3.4 |

* Sialidase activities were determined by incubating 500 ng of the TconTS indicated for 2 h with 100 μg fetuin (600 μM fetuin-bound Sia). Data points are mean ± standard deviations of three replicates.
** TS activity values see Table 3.
*** n.d. not detected.

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