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Enzymically inactive members of the *trans*-sialidase family from *Trypanosoma cruzi* display β-galactose binding activity

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***trans*-sialidase is a unique sialidase in that, instead of hydrolizing sialic acid, it preferentially transfers the monosaccharide to a terminal β–galactose in glycoproteins and glycolipids. This enzyme, originally identified in *Trypanosoma cruzi*, belongs to a large family of proteins. Some members of the family lack the enzymatic activity. No function has been yet assigned to them. In this work, the gene copy number and the possible function of inactive members of the *trans*-sialidase family was studied. It is shown that genes encoding inactive members are not a few, but rather, are present in the same copy number (60–80 per haploid genome) as those encoding active *trans*-sialidases. Recombinant inactive proteins were purified and assayed for sialic acid and galactose binding activity in agglutination tests. The enzymatically inactive *trans*-sialidases were found to agglutinate de-sialylated erythrocytes but not untreated red blood cells. Assays made with mouse and rabbit red blood cells suggest that inactive *trans*-sialidases bind to β, rather than α, terminal galactoses, the same specificity required by active *trans*-sialidases. A recombinant molecule that was made enzymatically inactive through a mutation in a single amino acid also retained the galactose binding activity. The binding was competed by lactose and was dependent on conservation of the protein native conformation. Therefore, at least some molecules in the *trans*-sialidase family that have lost their enzymatic function still retain their Gal-binding properties and might have a function as lectins in the parasite–host interaction.**

Key words: T.cruzi/sialidase/enzymatic function/*trans*-sialidase

Introduction

Sialic acid has been described as an important mediator involved in the interaction of numerous pathogenic agents with their receptor cells (Weis *et al*., 1988). *Trypanosoma cruzi*, the agent of Chagas' disease, is unable to synthesize sialic acid *de novo* (Schauer *et al*., 1983). The parasite employs the *trans*-sialidase (TS) to transfers terminal α(2–3) linked sialic acid residues to terminal β-linked galactoses in glycoproteins or glycolipids (Previato *et al*., 1985; Zingales *et al*., 1987). In *T.cruzi*, mucins have been shown to be the main sialic acid acceptors in the reaction catalyzed by this enzyme (Schenkman *et al*., 1993) and were suggested to be involved in the invasion of host cells by the parasite (Yoshida *et al*., 1989; Franchin *et al*., 1997), an essential step for parasite multiplication and survival in mammals. TS activity is present in several members of a large protein family that was originally identified as a group of surface molecules containing the antigen SAPA (for Shed Acute Phase Antigen). SAPA is highly antigenic during natural and experimental infections (Reyes *et al*., 1990; Parodi *et al*., 1992).

Members of the TS family have two major domains (Cazzulo and Frasch, 1992). The amino terminal 680 amino acids domain contains four short sequences nearly or completely identical to the Asp consensus boxes (Ser-X-Asp-X-Gly-X-Thr-Trp) also found in bacterial and viral neuraminidases (Roggentin *et al*., 1989). This domain contains the catalytic activity (Campetella *et al*., 1994; Schenkman *et al*., 1994a). The C-terminal region is made up of an array of 12 amino acid repeats (SAPA repeat) followed by a GPI-anchoring signal (Pollevick *et al*., 1991). These SAPA repeats are included only in the TS expressed in the infective trypomastigote form of the parasite (Affranchino *et al*., 1991). The TS purified from epimastigotes, the parasite replicative form in the insect, lacks the SAPA domain (Briones *et al*., 1995).

Some of the TS family members have enzymatic activity while others are completely inactive (enzymatically inactive *trans*-sialidase or iTS; Uemura *et al*., 1992; Cremona *et al*., 1995). Comparison of the deduced amino acid sequences of enzymatically active and inactive members showed that the differences are restricted to about 20 amino acid positions, only one being essential for the enzymatic activity (Cremona *et al*., 1995). All the genes analyzed until now (Pereira *et al*., 1991; Uemura *et al*., 1992; Cremona *et al*., 1995) show that those coding for active TS predict a Tyr at position 342 while members that encode iTS predict a His at the same position. This change is the cause for the total loss of TS activity in naturally occurring molecules (Cremona *et al*., 1995). The Tyr to His mutation is due to a transition T to C in a highly conserved region.

Two basic questions were addressed in this work concerning the iTS, the redundancy of their gene family and their possible biologic function. We now show that the pool of genes encoding iTS is indeed large and that these proteins display galactose-binding activity.

Results

Number and genomic organization of the genes encoding inactive members of the trans-sialidase family

To identify genes encoding TS and iTS, two oligonucleotides of 18 bp were constructed. They differ in a single base in the codon for Tyr (Tyr probe for active TS) or His (His probe for iTS), respectively (Figure 1A). Under the hybridization conditions used (see *Materials and methods*), Tyr and His probes specifically detect genes encoding enzymatically active and inactive proteins having a Tyr or a His at position 342, respectively (Figure 1B). A series of spots with increasing amounts of genomic DNA from *T.cruzi* were hybridized with the two oligonucleotides. Copy

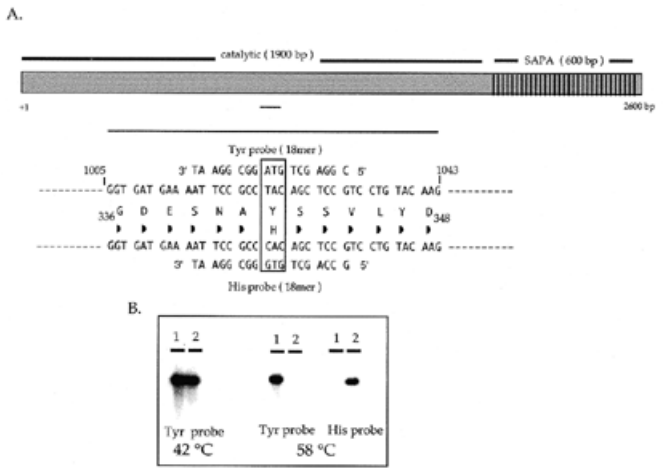

Fig. 1. (**A**) Schematic representation of the probes used in hybridization experiments. The bar represents one of the genes belonging to the *trans*-sialidase family. Below the bar the sequence that surrounds the codon for the amino acid 342 of the mature protein and the sequences of the oligonucleotides (His probe and Tyr probe) designed to identify the two groups of genes of the TS family are detailed. The amino acid residue changed between iTS and TS proteins is boxed. The catalytic domain (CD) probe comprises the region of the gene that codes for the N-terminal portion of the protein, which is the only one necessary for the enzymatic activity, and the SAPA repeats (SR) probe comprises the region that codes for the twelve amino acid repetitions that form the antigenic C-terminal domain. (**B**) Hybridization of the oligonucleotides with cloned genes coding for a TS (lane 1) and a iTS (lane 2) in the presence of 3M TMAC, at 42 °C (right panel) or 58 °C (center and left panel), temperature at which each probe differentially identifies its complementary sequence.

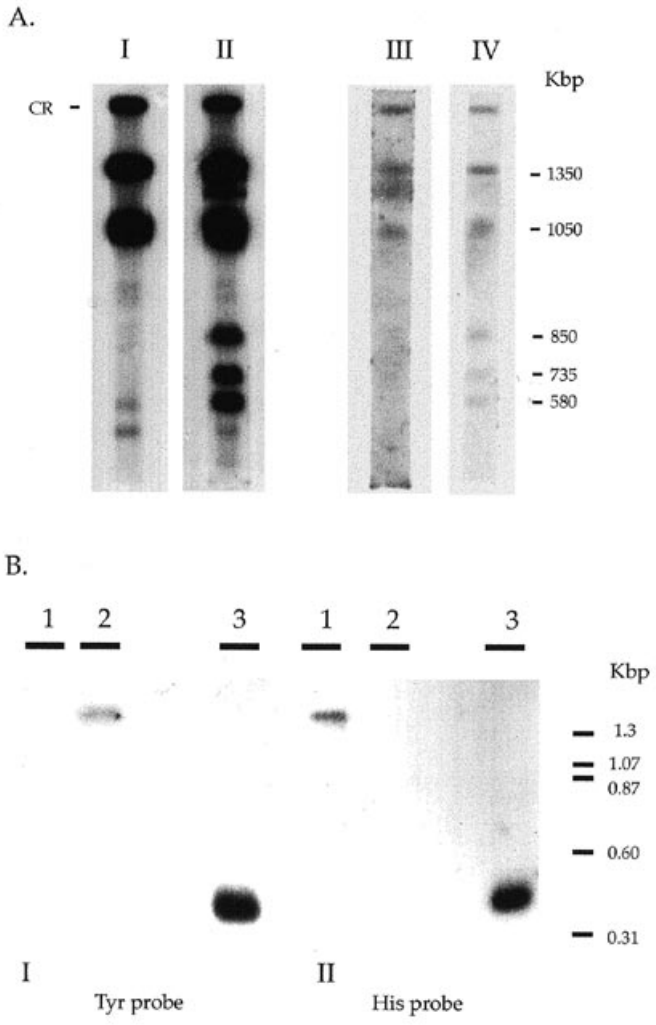

Fig. 2. (**A**) A Southern blot obtained after separation by PFGE of chromosomes from CL Brener strain was hybridized with the four probes described in Figure 1 to obtain information on the chromosomal distribution of the TS family. (I) SR probe hybridization, (II) CD probe hybridization, (III) His probe hybridization, (IV) Tyr probe hybridization. CR, Compression region. (**B**) RT-PCR performed on total RNA from trypomastigote stage of *T.cruzi* RA strain. A region encompassing the catalytic domain was amplified using the primers described in *Materials and methods*. The product of the reaction was blotted and hybridized with the oligonucleotide probes following the protocol described earlier. (I) hybridization with Tyr probe; (II) hybridization with His probe. Lanes: 1, DNA of a recombinant clone encoding iTS; 2, DNA of a recombinant clone encoding TS; 3, RT-PCR product (414 bp).

number of genes was estimated by comparison with internal controls made of known amounts of DNA from clones for active and inactive TS (Cremona *et al*., 1995). Using two different *T.cruzi* stocks (the RA strain and CL Brener clone), 64 ± 20 and 80 ± 20 genes per haploid genome encoding His^{342} were detected, respectively. Genes encoding Tyr^{342} were found to be present in 62 ± 18 and 56 ± 20 copies per haploid genome for RA and CL Brener, respectively. Hence, the genes that putatively encode enzymatically inactive and active TS proteins are represented in similar amounts in the parasite genome. The gene encoding the α-subunit of the pyruvate dehydrogenase from *T.cruzi*, known to be present once per haploid genome (Buscaglia *et al*., 1996), was used as control. This gene produced a signal equivalent to 0.9 ± 2 copies per haploid genome.

To analyze the distribution of genes encoding TS and iTS, His and Tyr probes were used in hybridization experiments on PFGE-separated chromosomes. Both, the His and Tyr probes, together with probes containing the sequences encoding the N-terminal catalytic domain (CD) and the C-terminal SAPA repeats (SR) detected two bands of 1050 and 1350 kbp (Figure 2). The Tyr and CD probes, but not the His and the SR probes, detected three additional bands of 580, 735, and 850 kbp. These results suggest that genes coding for iTS and active TS are placed on the same chromosomes and that iTS might contain SAPA repeats, since His probe detect the same chromosomal bands where the repeats are placed. To further confirm these findings, the same probes were hybridized to an ordered cosmid library made up of 36,864 original clones from CL Brener strain (Hanke *et al*., 1996). All cosmid clones detected by the SAPA repeats were also detectable by the Tyr probe alone or by both Tyr and His probes. Conversely, clones lacking the sequences encoding the SAPA repeats hybridized only with the Tyr but not by the His probe. Therefore, it is likely that iTS coding genes contain the sequence for SAPA repeats, as is the case of the ones that code for the TS expressed in the infective trypomastigote form (Affranchino *et al*., 1991).

Genes coding for iTS are transcribed in trypomastigotes

To determine if the genes coding for iTS proteins are transcribed, a PCR with specific oligonucleotides was performed on cDNA obtained from total RNA from trypomastigotes. This PCR rendered amplification from mature mRNAs as it was performed with a primer that contains the splice-leader sequence which is added in a *trans*-splicing reaction to the 5′-end of most mRNAs in a number of trypanosomatids: this process generates mature

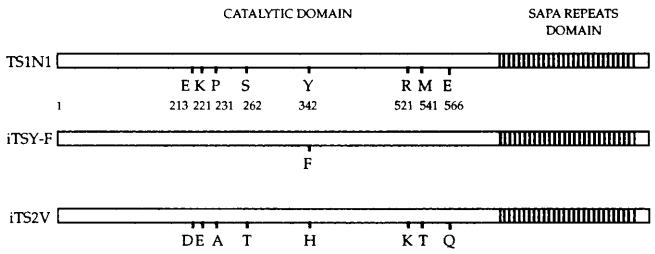

Fig. 3. Comparison of the deduced amino acid sequences of iTS proteins used in the RBC agglutination experiments (iTSY-F and iTS2V) respect to active TS (TS1N1). Only the amino acids that differ between proteins are indicated.

mRNAs from polycistronic units (Nilsen, 1995). The product of the PCR reaction was blotted and hybridized with Tyr and His probes (Figure 2B, lane 3), as well as with DNA from clones coding for active (Figure 2B, lane 2) and inactive TS (Figure 2B, lane 1). Both the His and the Tyr probe detected PCR products, indicating that genes encoding iTS, as well as those for active *trans*-sialidases, are transcribed and processed in the parasite.

Inactive trans-sialidase proteins recognize terminal galactoses

In the previous section we showed that iTS are encoded by a large number of genes and that their mRNA are detectable in the trypomastigote form of the parasite. It is possible that these molecules could have a function related to TS, for example, having affinity for the natural substrates of the enzyme. An hemagglutination (HA) assay with red blood cells (RBC) from mice was used to test the capacity of iTS to bind α-linked sialic acid or to β-Gal. iTS2V is an inactive protein derived from a natural genomic clone (TcTS2V0; Cremona *et al*., 1995) that contains the SAPA repeats (Figure 3). To induce agglutination, an anti-SAPA repeats antibody was required to link two iTS molecules binding to different RBC. Anti-SAPA serum in the absence of iTS failed to agglutinate RBC (Table I, panel A). Mouse RBC were used as such or after desialylation with *Vibrio cholerae* sialidase to expose terminal galactoses.

Hemagglutination activity of iTS2V was detected on mouse RBC treated with sialidase, but not with untreated RBC (Table I, panel A). Five different concentrations of iTS (from 5 to 250 μg · ml^{-1}) were tested and positive agglutination was observed throughout this range. The quality of the RBC used and the effectiveness of the treatment with sialidase were tested with lectins from *Maackia amurensis* (for sialic acid) and from *Arachis hypogaea* (for galactose) which specifically agglutinated untreated and sialidase-treated RBC, respectively (Table I, panel A). iTS2V differs at eight amino acid positions from active TS, including the one at position 342 (Cremona *et al*., 1995). Therefore, it was investigated whether subtle changes in the region that determine the enzymatic activity may cause the transition between the catalytic and the Gal-binding activity. The Tyr residue at position 342 of a TS protein was changed to Phe by site directed mutagenesis on a gene encoding an active TS, causing the loss of the enzymatic activity (iTSY-F, Figure 3). The mutant protein iTSY-F was tested in a HA assay on mouse RBC (Table I, panel A) and shown to retain its capacity to bind neuraminidase-treated RBC.

Table I. Agglutination of red blood cells by inactive TS

	Neuraminidase treatment of RBC	Agglutination
A. Mouse erythrocytes		
iTSY-F	-	NEG[a]
	+	10 μg·ml^{-1} [b]
iTS2V	-	NEG
	+	75 μg·ml^{-1}
iTSY-F denatured	-	NEG
	+	NEG
iTS2V denatured	-	NEG
	+	NEG
Maackia amurensis lectin	-	0.4 μg·ml^{-1}
	+	NEG
Arachis hypogaea lectin	-	NEG
	+	0.5 μg·ml^{-1}
Anti-SAPA serum 1:10	-	NEG
	+	NEG
B. Rabbit erythrocytes		
iTSY-F	-	NEG
	+	NEG
iTS2V	-	NEG
	+	NEG
Bandeirae simplicifolia lectin	-	NEG
	+	80 μg·ml^{-1}
Anti-SAPA serum 1:10	-	NEG
	+	NEG
C. Mouse erythrocytes		
	Lactose (μg · ml^{-1})	Agglutination
iTSY-F (10 μg · ml^{-1})		
	0	+
	0.1	+
	1	+
	10	NEG
	100	NEG

[a]NEG, Agglutination not detected.
[b]The concentrations shown are the smallest ones that produced a positive agglutination assay.

The specificity of the interaction of iTS recombinant molecules with Gal residues was analyzed by competing with lactose the agglutination of desialylated RBC by iTSY-F. The presence of 10 μg · ml^{-1} (i.e., 29 μM) of lactose inhibited the HA activity of 0.14 μM of iTSY-F (Table I, panel C). In addition, the binding of both iTS to terminal galactoses is dependent on the native conformation of the site. No agglutination, either on sialylated or desialylated RBC, was detected when the iTS used in the assay was previously denatured by heat (Table I, panel A).

trans-sialidases are known to use β-terminal galactoses, but not α-terminal galactoses, as acceptors of sialic acid in the *trans*-glycosidation reaction (Ferrero Garcia *et al*., 1993). If the galactose binding site in iTS is the same as the one present in

active TS, they should have this same specificity. To test this alternative, we made use of rabbit RBC in agglutination assays with iTS. Rabbit RBC, unlike mouse RBC, are known to contain abundant terminal α-Gal and very low levels of sialic acid residues on its membrane glycoconjugates (Galili *et al*., 1987). Neither of the two iTS proteins tested was able to react with rabbit RBC, either untreated or after treatment with sialidase (Table I, panel B). The lectin from *Bandeirae simplicifolia*, which recognize α-galactoses, was used as positive control (Table I, panel B).

An inactive TS protein generates antibodies that inhibit TS activity

During *T.cruzi* infections, TS antibodies able to inhibit the TS activity directed against conformational epitopes in the catalytic domain are detectable in serum (Leguizamon *et al*., 1994). Therefore, if a spatial conformation is conserved in active and inactive *trans*-sialidases providing a common binding site for β-terminal galactose, antibodies raised against iTS should inhibit the TS activity of a recombinant active enzyme. Three mice were immunized by the intraperitoneal route (ip) with purified iTS2V protein absorbed onto alumin, an adjuvant that allows retention of the native structure (Buscaglia *et al*., 1998). As shown in Table II, all mice immunized with iTS2V protein elicited a response that neutralized the activity of a recombinant TS.

Table II. Neutralization of TS activity (TIA assay) by sera from C3H/HeN mice inoculated with inactive TS protein (iTS2V)

	Remnant TS activity			
	1st booster		2nd booster	
Mouse	(cpm)	(%)	(cpm)	(%)
Normal	2981	100	3131.5	100
M1	494	11.4	207.4	6.62
M2	1597.7	50.7	503.3	16.1
M3	2086.9	74.3	886.9	28.3

TIA values are the means of duplicate assays. Percentage of inhibition is based on values obtained with a pool of normal sera being considered 100% of TS activity.

Discussion

Previous work has shown that TS is encoded by a large gene family and that some of its members are enzymatically inactive (Pollevick *et al*., 1991; Parodi *et al*., 1992; Uemura *et al*., 1992). Between 30 and 120 gene copies for the catalytic region of the TS were shown to be present in *T.cruzi* (Egima *et al*., 1996). However, the proportion of genes encoding inactive proteins was not known. To answer this question, we took advantage of the fact that all genes for active and inactive proteins identified so far encode a Tyr or a His at position 342, respectively (Uemura *et al*., 1992; Cremona *et al*., 1995). Oligonucleotides specific for these codons allowed to show that genes coding for His^{342} are not few, but rather, are present in a similar number in the parasite genome as those encoding active TS. The other result derived from this study indicates that most if not all iTS belong to the group of molecules having SAPA repeats. This group of proteins, at variance with those lacking SAPA repeats (Briones *et al*., 1995), are expressed in the infective trypomastigote form, suggesting a possible role of iTS in the bloodstream of the mammal. In this host, SAPA repeats are required to stabilize the enzymatic activity in the bloodstream (Buscaglia *et al*., 1998).

The iTS were found to be galactose-binding proteins. The RBC agglutination assays strongly suggest that iTS binds to terminal galactoses linked in β but not in α, the same specificity required by active TS in the acceptor molecule (Ferrero Garcia *et al*., 1993). Since iTS mutant molecules would failed to bind to sialic acid as shown in Table I, it is possible that Tyr^{342}, in addition to its role in stabilizing a carbonium ion transition state intermediate as described in bacterial sialidases (Crennell *et al*., 1993), could have a function in the binding to sialic acid in the donor molecule.

Two models have been proposed for the catalytic activity of TS (Schenkman *et al*., 1994b). In one of them, a single site for the sialic acid donor was proposed, in which both, the hydrolysis and the transfer to the galactose acceptor take place. In a second model, two different sites, one for the sialic acid donor and another for the terminal galactose in the acceptor molecule were suggested. Recent work supports the second model. Temperature differences for the *trans*-sialylation and hydrolysis reaction were interpreted as favoring the existence of an independent binding site for galactosyl residues (Riberao *et al*., 1997). In another study, hybrid molecules having regions from *T.cruzi* TS and *T.rangeli* sialidase, which is 70% identical to TS (Buschiazzo *et al*., 1993, 1997), were constructed and analyzed (Smith *et al*., 1996). Chimeric molecules showed that there are regions and amino acid residues that are required for TS but not for sialidase activity, thus pointing to the presence of an independent galactose binding region (Smith *et al*., 1996). Our results show that iTS conserves this binding site despite its apparent inability to bind to sialic acid. The galactose binding site detected in iTS might be equivalent to the one present in active TS, but their structure awaits detailed crystallographic studies. The fact that it is possible to have molecules with Gal-binding activity in the absence of TS activity, raise the possibility that TS were originated from a sialidase that has acquired a Gal-binding region. Further loss of the sialidase activity might generate lectin-like molecules from TS. Related molecules having either glycosidase or lectin activity has been described in other cell types (Hinek, 1994; Battelli *et al*., 1997). Among them are the family of chitinases in mammals, where some of their members are glycosyl hydrolases while others lacking enzymatic activity are chitin-specific lectins (Renkema *et al*., 1998).

In *T.cruzi*, 60–70 kDa galactose and mannose-mediated carbohydrate binding proteins were identified (Bonay and Fresno, 1995). A partial amino acid sequence obtained from the mannose binding proteins (Bonay and Fresno, 1995) have no homology with the sequence in the iTS family. Furthermore, the iTS we have identified as galactose-binding protein should be much larger in size (from 140 to 200 kDa; Parodi *et al*.,1992) than the one previously reported. It might not be surprising, however, if *T.cruzi* has a number of different molecules used to interact with different cell types (Zingales and Colli, 1985) since this parasite is able to invade almost all mammalian cell types.

Materials and methods

Parasites

The RA strain and CL Brener clone (Brener, 1965) of *T.cruzi* were used. Epimastigote forms were grown in BHT medium containing 10% of fetal bovine serum at 28°C (Cazzulo *et al*., 1985). Trypomastigotes from the RA strain were obtained from infected

Vero cells grown in low glucose Dulbecco's MEM containing 10% fetal bovine serum at 37°C, 5% CO_2.

Probes

The catalytic domain (CD) and SAPA repeat (SR) probes (Figure 1A) were obtained by PCR with ^{32}P-dCTP using specific primers. For the CD probe, the oligonucleotides employed were the LAP and TGA primers (Cremona *et al*., 1995). For the SR probe the oligonucleotides used were 5′SAPA/Eco and 3′SAPA/Eco primers (Buscaglia *et al*., 1998). The 18-mer Tyr and His oligonucleotide probes were designed from the sequences for the TS genes from Cremona *et al*. (1995). Tyr probe: 5′C GGA GCT GTA GGC GGA AT3′ contains the codon for a tyrosine at position 342 (underlined) of the mature protein. His probe: 5′C GGA GCT GTG GGC GGA AT3′ contains the codon for a histidine at the same position. A search on the GenBank+EMBL+DDBJ+PDB data bank using the BLAST Program (Altshul *et al*., 1990) was performed showing that these oligonucleotides recognize only members of the TS family and are specific to each group. The oligonucleotide probes were end-labeled using T4 polynucleotide kinase and ^{32}P-ATP (New England Biolabs Inc., Beverly, MA), following the supplier's protocol. The specificity of the oligonucleotides was tested on dot blots of DNA belonging to recombinant clones coding for active (1N1) and inactive TS (2V0) (Cremona *et al*., 1995) (see below). These clones were processed in parallel with all experiments done later as a control for hybridization specificity.

Base composition-independent hybridization in tetramethylammonium chloride (TMAC)

An initial nonstringent hybridization with the radiolabeled oligonucleotide probes was followed by washing with 3M TMAC (Sigma, St. Louis, MO) to control the stringency of hybridization. Washing buffer containing TMAC abolishes the differential melting of AT and GC base pairs (Woods *et al*., 1985). The filters were prehybridized in 6× SSC (1× SSC: 0.15 M NaCl,0.015 M Na citrate), 10 mM Na phosphate pH 6.8, 5× Denhardt's solution and sonicated salmon sperm DNA 0.1 mg/ml for 4 h at 42°C and then hybridized overnight at 42°C in the same solution with the different oligonucleotide probes at a concentration of 10^6 c.p.m./ml. The filters were washed three times with 6× SSC at room temperature and then rinsed with TMAC wash solution (3 M TMAC/2 mM EDTA/SDS 0.1%/50 mM Tris–HCl, pH 8.0) at room temperature to remove the Na^+ as it competes for TMAC binding. The washing temperature in 3 M TMAC was progressively raised from 42°C to 60°C. The stringent wash condition at which the 18-mer oligonucleotide probes only match with their complementary sequences was determined to be 58°C for 30 min.

Dot blot experiments

DNA was isolated following the standard phenol/chloroform protocol (Sambrook *et al*., 1989). For dot blot experiments, increasing amounts of genomic DNA ranging from 0.01 to 25 μg were denatured in a 0.25 M NaOH, 0.5 M NaCl solution, blotted to Hybond-N nylon membrane (Amersham, Little Chalfont, Buckinghamshire, UK) using a Bio-Dot SF Microfiltration Apparatus (Bio-Rad Chemical Division, Richmond, CA) and then fixed by incubating the filters for 2 h at 80°C or UV-cross-linked (Strategene, La Jolla, CA). When PCR-labeled probes were used, the filters were prehybridized in 6× SSC, 0.5% SDS, 5× Denhardt solution, 0.1 mg/ml salmon sperm DNA at 60°C for 2 h and hybridized overnight with 10^6 c.p.m./ml at the same temperature. Filters were washed in 0.1× SSC, 0.1% SDS at 65°C and exposed to x-ray films. When using oligonucleotide probes the hybridization was performed using the TMAC procedure described earlier. The results of the dot blot hybridization were scanned using a Scan Jet 4C (Hewlett Packard) and quantified using Image Quantifier program. Data from reference and tested DNA were plotted and linear curves were fitted by using the KaleidaGraph 2.1 program for Macintosh. Results are the mean and SD of four independent assays.

Pulse field gel electrophoresis

PFGE blots were kindly provided by Mario Galindo and Norber Galanti from the University of Chile. Briefly, the conditions used for separation in the size range of 450–750 kbp were a linear ramping with initial pulse of 52.22 sec and final pulse of 1 min 8.67 s, a voltage angle of 120° (60° and –60°) at 4 V/cm for 48 h.

Reverse polymerase chain reaction

Total RNA was extracted from trypomastigote stage (~10^9 cells) using Trizol (Life Technologies Inc., Gaithersburg, MD) and first strand of cDNA was synthesized using the standard protocol for Superscript II Reverse Transcriptase (Life Technologies Inc.) in the presence of RNAsin (Promega, Madison, WI). The reactions were stopped by heating at 95°C for 5 min and then diluted to 200 μl with distilled water. Ten microliters of each reaction were used in the two sequential polymerase chain reactions (semi-nested PCRs) using *Taq* DNA polymerase (Life Technologies Inc.) and 0.2 μg of each primer: 5′AACTAACGCTATTATTGATA3′ (primer miniexon) and 5′GCAGCGGTACGCATCCTCCCAT3′ (primer 1300) for the first PCR and 5′GCTCTTCACACACCCGCTGAA3′ (primer 900) and primer 1300 for the second PCR. All these sequences are conserved among the members of the family analyzed so far. The PCR reactions were performed in a final volume of 50 μl with a denaturing temperature of 94°C, 30 sec, annealing of 44°C (for the first PCR) and 58°C (for the second PCR), 1 min and elongation of 72°C, 1 min. The product of the PCR and the appropriate controls were loaded in a 1.2% agarose gel and transferred to a nylon membrane to hybridize with the end-labeled oligonucleotide probes.

Expression and purification of recombinant iTS

iTS2V clone were obtained by replacement of the *Bgl*II–*Kpn*I restriction fragment from a genomic clone coding for inactive TS (Cremona *et al*., 1995) on TS-SAPA clone in pTrcHisA vector (Invitrogen Corporation, San Diego, CA) (Buscaglia *et al*.,1998), and iTSY-F was obtained by site directed mutagenesis on TS-SAPA clone as described in Cremona *et al*., 1996. The constructions were sequenced to confirm the replacements. Recombinant proteins were expressed and purified as described by pTrcHis manufacturer«s guide using iminodiacetic acid metal affinity column Ni^{2+} charged. The presence of the proteins were tested by Western blot of bacterial extracts using polyclonal serum raised against the SAPA repeat domain in rabbit.

Hemagglutination tests

Dilution of purified recombinant proteins were prepared in hemagglutination buffer (150 mM NaCl, 20 mM Tris–HCl pH 7.6) and mixed with 50 μl of a 2% suspension of washed mouse RBC in U-well microtiter plates. An anti-SAPA rabbit polyclonal

serum diluted 1:10 was used to detect the agglutination sheet. Agglutination was scored after incubation for 1 h at room temperature in a final volume of 100 µl. Rabbit RBC that contain no sialylated glycoconjugates were employed under the same conditions. Agglutination control were done using the appropriate lectin diluted in the same buffer. The inhibitory effect of lactose was tested by adding serial dilutions in hemagglutination buffer of the purified disaccharide (Calbiochem, San Diego, CA), ranging from 10 ng to 10 µg, to a similar assay as described before.

Desialylation of erythrocytes

Mouse RBC were washed in hemagglutination buffer, resuspended in 150 mM NaCl 20 mM Tris–HCl pH 6.8 containing 0.5 U ml^{-1} of *Vibrio cholerae* sialidase (Sigma) and incubated for 60 min at 37°C. Erythrocytes were then washed twice and resuspended in hemagglutination buffer at the required concentration.

Detection of antibodies inhibiting trans-sialidase

Adult 60–90-day-old male C3H/HeN mice were immunized by the intraperitoneal route with 10 µg of iTS2V protein absorbed onto alumin as described (Buscaglia *et al*., 1998). Sera from these mice were processed and tested for the presence of inhibitory antibodies in a *trans*-sialidase inhibition assay (TIA) by mixing with active TS and testing for remaining enzymatic activity as described (Leguizamon *et al*., 1994). TIA values are expressed as means of duplicate assays.

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Abbreviations

kbp, kilobase pairs; TMAC, tetramethyl ammonium chloride; SDS, sodium dodecyl sulfate; PFGE, pulse field gel electrophoresis; PCR, polymerase chain reaction; RT-PCR, reverse transcription coupled to PCR; mRNA, messenger RNA.

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