# The Trypanosoma cruzi Neuraminidase Contains Sequences Similar to Bacterial Neuraminidases, YWTD Repeats of the Low Density Lipoprotein Receptor, and Type III Modules of Fibronectin

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## Summary

Trypanosoma cruzi expresses a developmentally regulated neuraminidase (TCNA) implicated in parasite invasion of cells. We isolated full-length DNA clones encoding TCNA. Sequence analysis demonstrated an open reading frame coding for a polypeptide of 1,162 amino acids. In the N-terminus there is a cysteine-rich domain containing a stretch of 332 amino acids nearly 30% identical to the Clostridium perfringens neuraminidase, three repeat motifs highly conserved in bacterial and viral neuraminidases, and two segments with similarity to the YWTD repeats found in the low density lipoprotein (LDL) receptor and in other vertebrate and invertebrate proteins. This domain is connected by a structure characteristic of type III modules of fibronectin to a long terminal repeat (LTR) consisting of 44 full length copies of twelve amino acids rich (75%) in serine, threonine, and proline. LTR is unusual in that it contains at least 117 potential phosphorylation sites. At the extreme C-terminus is a hydrophobic segment of 35 amino acids, which could mediate anchorage of TCNA to membranes via a glycosylphosphatidylinositol linkage. This is the first time a protozoan protein has been found to contain a YWTD repeat and a fibronectin type III module. The domain structure of TCNA suggests that the enzyme may have functions additional to its catalytic activity such as in protein-protein interaction, which could play a role in T. cruzi binding to host cells.

**T**rypanosoma cruzi causes Chagas disease, a chronic debilitating, incurable disease that afflicts millions of people in Latin America. *T. cruzi* exists in three developmental forms: amastigotes and epimastigotes which are multiplying forms, and trypomastigotes, which do not multiply but instead transmit infection from insects to mammals, and vice-versa (1). The molecular mechanism underlying trypomastigotehost cell interaction is beginning to be understood, and it appears that specific recognition molecules mediate *T. cruzi* invasion of cells (2).

One protein suggested to play a role in *T. cruzi*-host cell interaction is the parasite neuraminidase  $(TCNA)^1$  (3, 4). Polyclonal and monoclonal antibodies raised against TCNA enhance infection at concentrations that inhibit enzyme activity (5, 6). Enhancement of infection is probably a consequence of reduced TCNA activity because it can be abrogated by exogenous neuraminidase (e.g., Vibrio cholera), whose activity is not inhibited by the TCNA antibodies (5, 6). T. cruzi infection is also augmented by high density lipoprotein (HDL) and low density lipoprotein (LDL), two inhibitors of TCNA activity (7, 8). Enhancement of infection by HDL and LDL is blocked by V. cholera neuraminidase, an enzyme that is not inhibited by HDL or LDL. It is not clear how neutralization of endogenous TCNA keeps T. cruzi infection in check, but it seems that desialylation removes the receptor for the trypomastigote adhesin (2).

TCNA is developmentally regulated, with maximal activity in trypomastigotes, minimum in epimastigotes, and not detectable in amastigotes (3, 4). The enzyme is located on the surface of the parasite (9, 10) where it can chemically modify, by desialylation, the surfaces of myocardial and vascular endothelial cells (11), and of erythrocytes (12). After transformation from amastigotes intracellularly, all trypomastigotes express neuraminidase (i.e., they are of the NA<sup>+</sup> phenotype), as they do upon exiting ruptured host cells into the culture medium (13). Once in the extracellular millieu, NA<sup>+</sup> trypomastigotes differentiate into NA<sup>-</sup> trypomastigotes (i.e., parasites that do not express enzyme activity) to reach an

<sup>&</sup>lt;sup>1</sup> Abbreviations used in this paper: BNR, bacterial neuraminidase; EGF, epidermal growth factor; GPI, glycosylphosphatidylinositol; HDL, high density lipoprotein; LDL, low density lipoprotein; LTR, long-terminal repeat; ORF, open reading frame; SAPA, shed acute phase antigen; TCNA, *Trypanosoma cruzi* neuraminidase.

equilibrium in which  $\sim 20\%$  of the population is NA<sup>+</sup> and  $\sim 80\%$  is NA<sup>-</sup> (3).

TCNA is highly polymorphic. The enzyme from metacyclic trypomastigotes has an apparent molecular weight of 66,000 (14), as does the NA from epimastigotes (Rosenberg, I., R.P. Prioli, and M.E.A. Pereira, unpublished observation), and, as assessed by immunoblotting using mono- and polyclonal antibodies, the TCNA from tissue culture trypomastigotes consists of a set of apparently glycosylated polypeptides whose range in size depends on the strain or clone analyzed (6). For example, in the Silvio X-10/4 clone, the enzyme is comprised of bands of apparent mol wt 120,000, 160,000, and a 200,000 doublet; and in the Y-H6 and MV-13 clones, TCNA is made up of bands ranging from 130,000 to 222,000. Under nondenaturing conditions, these polypeptides are held together as trimers by noncovalent bonds (Prioli, R.P., J.S. Mejia, and M.E.A. Pereira, manuscript in preparation).

The primary structure of TCNA is unknown. Here we report the isolation and sequencing of the DNA encoding one of the TCNA isoforms from tissue culture trypomastigotes. The deduced amino acid sequence reveals a catalytic domain in the N-terminus, which is structurally similar to bacterial neuraminidases, in particular the *Clostridium perfringens* enzyme, that contains two YWTD sequence motifs; a domain similar to fibronectin III modules containing GTPbinding consensus sequences; a long terminal tandem repeating structure rich (75%) in serine, threonine and proline residues; and, a hydrophobic stretch of 35 amino acids at the extreme C-terminus which could mediate anchorage of TCNA to the cell surface by a glycosylphosphatidylinositol (GPI) linkage.

#### Materials and Methods

Parasites. All studies were performed with the cloned Silvio X-10/4 and Montalvania-13 strains of T. cruzi (4). Epimastigotes were grown in liver-infusion medium containing 10% FCS, and trypomastigotes were propagated in Vero cells as previously described (4, 11).

Antibodies. TCNA mAbs TCN-1 and TCN-2 were isolated from mice infected with a clone of Montalvania-13 that expresses high enzyme activity (4). These antibodies inhibit neuraminidase activity and deplete TCNA from trypomastigote lysates after addition of protein A-Sepharose, and are specific for the TCNA from tissue culture trypomastigotes, as they do not react with the enzyme from epimastigotes.

Antibodies against 7F, a recombinant TCNA clone, were obtained as a modification of our previously published procedure (5). Bacterial lysates containing recombinant TCNA from 7F insert (see below) were run on a 10% SDS-PAGE, the lanes in the right and left corners cut out with a scalpel, blotted on nitrocellulose and probed with TCN-2 to visualize the relative position of the TCN-2 immunoreactive protein. The blots were then aligned with the polyacrylamide gel, and the portion of the gel corresponding to the immunoreactive band cut out, frozen at  $-70^{\circ}$ C, lyophilized, and injected weekly into mice with Freund's adjuvant. Three injections of gel slices corresponding to those shown in Fig. 2 (lane 8 from left) sufficed to elicit antibodies that reacted with endogenous TCNA (Fig. 2, lane 4 from left). Similar results were obtained by eliciting antibodies against the recombinant TCNA from clone 10C. Immunoblots. Endogenous TCNA was obtained by lysis of trypomastigotes and epimastigotes in 0.01 M PBS, pH 7.0, containing 1% Triton X-100 and protease inhibitors (5  $\mu$ M pepstatin and leupeptin, 10 mM EDTA, 5 mM iodoacetamide, and 10  $\mu$ g/ml soybean trypsin inhibitor), centrifuged at 1,000 g to remove nuclei and debri, and cleared at 100,000 g for 1 h. Amastigotes and epimastigotes were treated similarly as trypomastigotes. Such lysates (~10  $\mu$ g/lane) were run on 10% SDS-PAGE, blotted to nitrocellulose, and allowed to react with TCN-2 or 7F antibodies, followed by an anti-mouse antibody conjugated to alkaline phosphatase, as previously described (6).

Recombinant TCNA was obtained from *Escherichia coli* XL-1 Blue that had been transformed with pBluescript containing TCNA inserts (7F, 10C, 16C, 19Y, and 20G), or without inserts. Bacteria were grown to an OD550 = 1.0, washed once with PBS, lysed by freezing in liquid nitrogen in the presence of protease inhibitors (see above) and thawing at 37°C for 15 min, and centrifuged in a microfuge for 10 min. The supernatant was immediately analyzed by immunoblotting, sieving chromatography, or neuraminidase activity (see below). Preliminary experiments showed most recombinant TCNA to be present in supernatants of the lysed bacteria and very little in bacterial pellets. It should be emphasized that recombinant TCNA is highly susceptible to degradation, and only fresh samples were analyzed. Even storage of the enzyme in crude lysates at  $-20^{\circ}$ C for several days gives rise to low mol wt bands in the immunoblots and to reduced enzyme activity.

Sieving Chromatography. Supernatants (0.2 ml) from lysates of bacteria transformed with 7F (prepared as above) were applied to an Ultrogel AcA-22 (Pharmacia-LKB; Uppsala, Sweden) column ( $40 \times 1$  cm) equilibrated with PBS at 4°C that had been calibrated with dextran (void volume), thyroglobulin (660 kd), ferritin (440 kd), catalase (230 kd) and aldolase (158 kd). Aliquots (0.02 ml) of each fraction (0.8 ml) were electrophoresed on 10% SDS-PAGE, blotted to nitrocellulose, and probed with TCN-2, followed by an alkaline phosphatase-conjugated goat anti-mouse IgG (6). The amount of immunoreactive material in the blots was quantitated with a laser scanning densitometer.

Neuraminidase Activity. Lysates of bacteria prepared as above were analyzed for neuraminidase activity by hydrolysis of the fluorogenic substrate 4 MU-NANA as previously described (5, 6). Lysates were also incubated with human erythrocytes and the degree of cell dehydration ascertained by peanut lectin hemagglutination (12).

Library Construction and Screening. A tissue culture trypomastigote (Silvio X-10/4) genomic DNA library was constructed in  $\lambda$ ZAP by Stratagene (La Jolla, CA), after shearing and sizing of the DNA to produce inserts of 4–9 kbp. The library was screened unamplified with mouse polyclonal TCNA antibodies and TCN-2 mAb (see above) as described by Huynh et al. (15). Clones 7F and 10C were isolated with the polyclonal antibody, and clones 16C, 19Y and 20G with TCN-2.

DNA Sequencing and Analysis. DNA inserts were automatically excised and subcloned into pBluescript as recommended by Stratagene. Clone 7F insert, despite being the largest of the isolated inserts (Fig. 1 B), was chosen for DNA sequencing because it consistently produced highest TCNA activity and a recombinant protein that reacted the strongest with TCN-1, TCN-2 (Fig. 2), and rabbit and mouse TCNA antibodies. Nucleotide sequence of 7F insert was determined by the chain-termination method of Sanger et al. (16) using double-stranded DNA as templates for T7 DNA polymerase (United States Biochemicals [Cleveland, OH] and Pharmacia-LKB [Uppsala, Sweden]). 7F templates were obtained by exonuclease III/S1 nuclease unidirectional digestion (17) starting from the 3' end of the insert. Ambiguities and gaps were corrected by using oligonucleotide primers designed from previous sequences. Nucleotide sequence of the other clones was determined in their 3'- and 5'- ends using KS and SK primers (Stratagene), and in some specified regions (see Fig. 1 A), with oligonucleotide primers based on the 7F sequence.

Sequences were analyzed on an Apple MacIntosh computer using the DNA Strider program (18) or programs available from the Molecular Biology Computer Resource Center (MBCRR) at Dana-Farber Cancer Institute, Harvard University (Boston, MA); and from University of Wisconsin (Madison, WI) Genetics Computer Group's sequence analysis software package (GCG) (19) (GCG use was a courtesy of the GRASP Core Center at the New England Medical Center, Boston, MA). Databases searched were Genbank Release 65, Protein Identification Resource Release 25, and Swiss-Prot-16. Optimal alignments between two sequences were determined with the FASTA program (20). Statistical significance of comparisons was computed with the program ALIGN (21).

RNA and DNA Blot Hybridization. Total RNA was prepared from various developmental forms of *T. cruzi* and Vero cells using LiCl/guanidinium isothiocyanate (22). RNA transfer was performed as described (23). Probes were derived from the coding region of TCNA (fragment 2 was an Exo III/S1 deletion mutant equivalent to bp -92/537 and fragment 1 a Pst1/Pvu II digest of 7F, bp 1800/3751; see Fig. 1 B and Fig. 3), labeled by random priming (24), and hybridized under standard conditions. DNA (Southern) blots were performed exactly as described by Sambrook et al. (23). Markers for the RNA blot was the RNA ladder (BRL) and for the DNA blot, a Hind III digest of phage  $\lambda$  (New England Biolabs, Beverly, MA).

## Results

Isolation and Nucleotide Sequence of DNA Encoding TCNA. Mouse polyclonal antibody against TCNA (6) was used as a probe to screen a trypomastigote genomic DNA library constructed in the  $\lambda$ ZAP expression vector. Approximately 10<sup>5</sup> clones from the unamplified library were screened, and two immuno-reactive plaques containing ~9.0- and 8.5-kb inserts (7F and 10C, respectively) were isolated. Additional clones (20G, 19Y and 16C) were isolated using the TCNAspecific mAb TCN-2 (6) (see Fig. 1 B).



Figure 1. Hydrophobicity plot of the TCNA sequence and schematic representation of the TCNA DNA. (A) Hydrophobicity of the TCNA sequence. The plot is colinear with the TCNA DNA sequence, and was generated according to Kyte and Doolittle (26). Positive values indicate hydrophobic amino acids. The plot clearly shows the hydrophilic LTR and the hydrophobic segment in the C-terminus of TCNA, and the absence of N-terminal peptide sequence. (B). Plasmids and DNA fragments used for sequencing and Northern and Southern blots. Heavy lines indicate the region of each DNA clone that was sequenced. The fragments used for Northern and Southern blots are shown below the restriction map of 7F. Restriction sites marked are: Pv, Pvu II; Ps, Pst I; X, Xho I.

Inserts were subcloned into the plasmid Bluescript (pBlue) and expressed in the *E. coli* XL-1 Blue. By immunoblotting, 7F, 10C and 20G produced proteins (180 to 170 kD) that were reactive with TCN-2 (Fig. 2 A) and with mouse (6) and rabbit (5) polyclonal antibodies specific for TCNA (data not shown). These clones contain full-length copies of the TCNA gene (Fig. 1). Clone 16C, which has only part of the TCNA gene (Fig. 1 B), displayed a band of 60 kd recognized by the TCNA antibodies (Fig. 2 A). Gel slices containing the recombinant 7F protein were injected into mice

## A) Immunoblot



Figure 2. Expression of TCNA. (A) Immunoblot analysis of endogenous (left) and recombinant (right) TCNA. Lysates of trypomastigotes (tryp), epimastigotes (epi), and amastigotes (ama) were electrophoresed in a 10% polyacrylamide gel, and probed with TCN-2 or anti-7F antibodies as indicated. Lysates of *E. coli* transformed with TCNA clones 7F, 20G, 10C, and 16C, and with Bluescript alone were electrophoresed as above and probed with TCN-2. (B) Sieving chromatography on a Ultrogel AcA22 column of lysates of *E. coli* transformed with 7F. The column was equilibrated with PBS and run at 4°C. Aliquots (20  $\mu$ l) of each fraction was electrophoresed on SDS-polyacrylamide gel, blotted to nitrocellulose, probed with TCN-2, followed by alkaline phosphatase conjugated goat anti-mouse IgG. The amount of TCN-2 reactive material was quantitated with a laser scanning densitometer. Arrows indicate the void volume (Vo), the estimated mw of TCNA (660 Kd) and ferritin (440 Kd). (C) Neuraminidase activity of lysates of *E. coli* transformed with Bluescript containing 7F ( $\odot$ ) or without insert (O).

#### 182 The Trypanosoma cruzi Neuraminidase Contains Sequences

to generate anti-7F antibodies. These antibodies reacted with trypomastigote lysates, giving a pattern similar to that produced by TCN-2 (Fig. 2 A). Sieving chromatography of 7Fcontaining bacterial lysates, under nondenaturing conditions, showed the TCN-2 immunoreactive material to have an apparent Mr of 660 kD (Fig. 2 B). Since the mol wt of the 7F monomer is 180 kD (Fig. 2 A), the 7F recombinant protein appears to be a trimer in its native state, consistent with conclusions about the endogenous TCNA (Prioli, R.P., J.S. Mejia, and M.E.A. Pereira, manuscript in preparation). Lysates of bacteria transformed with 7F had neuraminidase activity, as measured by their ability to hydrolyze a fluorogenic substrate (Fig. 2 C) and to induce human erythrocytes to be agglutinated by peanut lectin (data not shown) (12). Lysates of bacteria transformed with pBluescript alone did not have detectable neuraminidase activity (Fig. 2 C). Similarly, plasmids 10C, 20G, and 19Y also induced neuraminidase activity in E. coli (data not shown). Taken together, these findings establish that the clones isolated with the TCNA antibodies encode the trypomastigote neuraminidase.

Α	
-483	aaagaccgttggaagaagaaagaaggttccggagcgtggccaccaccaacgatgaactgccacaattgcgtgctgtccgcgggcgg
-393	cggcgctttgagcccacggcgacttgtgtgttcccctttctctcccactttctccgcggcaatccccctgcaaagagacgatcttgaca
-303	ccattgttttaggcataatagaagttctacaaacgaccgaaggacacacaggcagg
-213	ggccagtaggatgttctggctaatgtttttcgtgccgcttcttctggatctgcccagtagcccggatgcccggatgcccggatgcccggatg
-123	qageegaqttgagggtttaagegtaagaattegatggtgtugttaagaatagutggutggtaagegtagtagtagtugtugtgag
	HVAIADARYETSSENSLID
58	ACCETIGECERAGTACAGCSTCGACGATGGGGAGACBTGGGGAGACCTAATTGECATCAAGAACAGCCGTGTATCGTCTATCTTTTTTTTTT
20	IV A KIGVODGEOORIJVI AIKSKUUUUU
148	CTGGATCCCACCCTGATTGTGAAGGGCAACAAGCTTTACGTCCTGGTTGGAAGCTACTATAGTTCGAGAAGCTACTGGTCGTCGTCGCATGGT
50	V D P T V I V K G N K L Y V L V G S Y Y S S R 5 Y W S S H G
738	CATCCCACAGAGTCTGGGATATTCTGCTTGCCGTGGCGAGGTCACGAGGCGCGCGGCGGCGAGATACGCGAGTATCAAATGGGGG
80	DARDWDILLAVGEVIKSTAGGKITASIXWG
328	AGCCCCGTGTCACTGAAGAAGTTTTTTCCGGCAGAAATGCAATGCACGCATGCACAAATCAATTCTTGGCGGCGCGCGGCGCGGGCCAGAATGAA
110	SPVSCKKFFPKEREGRAINOFEGGAGV <del>G</del>
418	GCGTCCAACGGGAATCTTGTGTACCCTGTGCAGGTTACGAACAAAAGGAAGCAAGTTTTCTCCCAAGATCTTCTACTCGGAAGATGATGGC
140	ASNGNLVYPVQVTNKRKQVFSKIFY(S)E(O)D(G)
170	A CACCING A GIT BOOK CONTROL CONTINUE OF A CONTROL OF A
110	
596	COASTTEACTEGEAAACGCCGTCTGCTGCTGCACGAGTCCACGCAGAGAAACCGTCGCGTGCGGAACCGTCTCGCGTCTGTGG
200	RVDWKRRLVYE(S)SDHEKP(#VE AVGTVSRVW
688	GGCCCCTCACCAAAATCGAACCAGCCCGGCAGTCACACSAGCTCACTGCCGTGACCATCGAAGGAATGCGTGTGATGCTCTTCACALAC
230	C b S b K S N G b C S G T S B T K A T T E G M A M E F F M
778	CCCCTGAATTTTAAGGGAAGGTGCGTGCGCGACCGACCGA
260	PLNFKGRC VRDRLNLWLTDNQRIYNVGQV5
	-
868	ATTGGTGATGAAAATTCCGCCTACAGCTCCEGTCCTGTACAAGCATGATAAGCTGTACTUTTGCATGAGATCAACAGCACGAGCGAGTCTAC
290	I G D E N S A Y S S V L I K D V L I (C L I Z I N I O Z V I
958	AGCCTTGTTTTTGCACGCCTGGTTGGCGAGCTACGGATCATTAAATCAGTGCTGCGGTCCTGGAAGAATTGGACAGCCACCTGTCCAGCA
320	SLVFARLVGELRIIKSVLRSWKNWTA,TC)PA
1048	THE ACCOUNT ALL AND A THE BOOK AND A THE AND A
330	
1138	CAACGCCTCCCAAAACGTATGGGAGGATCGTACCGCTGCGTCAACGCAACGGCAAATGCGGAGAGGGTTCGGAACGGTTTGAAGTTT
380	QRLPXRMGGSYR©V <u>NAS</u> TANAERVRNGLKF
	-сно-
1228	GCGGGGGTTGGCGGAGGAGCGCTTGGCCGGTGAGCCAGCAGGGCCAGAATCAGCGGTATCGTTTGCAAACCACGCGTTCACGCTGGTG
410	A G V G G G A L W P V S Q Q G Q N Q R Y R F A N H A F T L V
1710	CONTRACTOR ACCONTRACTOR ACCORDING ACCOUNTING TOGOTOGOLOGICAL ACCOUNTING TOGOLOGICAL ALABAMETECTICSG
440	A S V T I H E A P R A A S P L L G A S L D S S G G K K L L G
1408	CTCTCGTACGACGACGACGACGACGACGACGACGACGACGACGACGGCGACGGGATCGTGGGAGACGGGTAAAAGGTAC
470	LSYDERHQ@QPIYGSTPVTPTGSWETGKRY
500	La transmission and the transmission and transmi
300	
1588	666A66ACCACCACCACCTCCACCTCCACCTCGCC666CACAAAA66ACCAAACCAA
530	G R T P D I S H F Y Y G G Y K R S D. M P T I S H V T V N N V

The nucleotide sequence of 7F contains a single long open reading frame (ORF) starting with a methionine codon at nucleotide 483 in a favorable context (7 out 13 nucleotides) for translation initiation (25) and ends with a TGA stop codon at nucleotide 3486 (Fig. 3). Nucleotides upstream of nt -92 were derived from clone 19Y and all others from 7F. Methionine codons are not found in-frame 5' of the putative start site, and sequences upstream of the start site contain stop codons in all three frames. The ORF encodes a protein of 1162 amino acids with a calculated mass of 119,959 daltons, in contrast to the observed mol wt of 180 kD for the 7F protein subunit as assessed by immunoblotting (Fig. 1 A). The apparent increase in size after SDS-PAGE and blotting to nitrocellulose could be attributed to anomalous migration of the protein due to a long tandem repeat (LTR) domain (see below). The mass of the endogenous enzyme can be slightly increased by glycosylation since there are only two N-glycosylation sites (Fig. 3), which probably are glycosylated in vivo because of a decrease of  $\sim$ 5.0 kd in the mol wt of TCNA polypeptides after digestion with N-Glycanase (10).

B		
1678	CTTCTTTACAACCGACGACGACGACGAGGAGGAGGAGGACGTCAGGACCTTGTGGGCCAGGACCTTATTGGCACGGAAGCACACCCTGGAC	
560	LLYNRRQLNTEEIRTLFLSQDLIGTEAHMD	
1768	AGCAGCAGCACCAGCACCAGCACCACCACCACCCCCCCC	
590	S S S S S S R S T P S T P N D S S N H S T P S T P V D S S	
1858	GCCCACAGTACGCCCTCGACTCCCGCTGACAGCAGTGCCCACGGTACGCCCTCAACTCCCGTTGACAGCAGTGCCCACGGTACGCCCTCA	٩.
620	A H S T P S T P A D S S À H G T P S T P V D S S À H G T P S	
1948	ACTCCCCCTGACAGCAGTACCCCACGGTACCCCCTCAACTCCCGTTGACAGCAGTACGCCCACAGTACGCCCTCAACTCCCCGTTGACAGCAGT	1
650	TPADSSANGTPSTPVDSSANSTPSTPVDSS	
2038	GCCCACAGTACGCCCTCAACTCCCGTTGACAGCAGTGCCCCACGGTGCGCCCTCAACTCCCGCTGACAGCAGTGCCCACGGTACGCCCTCA	;
680	A H S T P S T P V D S S A H G A P S T P A D S S A H G T P S	
2128	ACTCCCGTTGACAGCAGTGCCCACGGTACGCCCTCGACTCCCGCTGACAGCAGTGCCCACAGTACGCCCTCGACTCCCGCTGACAGCAG	1
710	T P V D S S A H G T P S T P À D S S À H S T P S T P À D S S	
2218	GCCCACAGTACGCCCTCGACTCCCCCCGCTGACAGCAGTGCCCACAGTACGCCCCCCCC	;
740	A H S T P S T P A D S S A H S T P S T P V D S S A H G T P S	
2308	ACTCCCCCTGACAGCAGTGCCCACAGTACGCCCTCGACTCCCGCTGACAGCAGTGCCCACAGTACGCCCTCGACTCCCGTTGACAGCAGT	1
770	TPADSSARSTPSTPADSSAHGIPSTPVDSS	
2398	GCCCACAGTACGCCCTCGACTCCCGTTGACAGCAGTGCCCACGGTACGCCCTCAACTCCCGTTGACAGCAGTGCCCACAGTACGCCCTC	•
800		
2488	ACTCCCGTTGACAGCAGTGCCCACGGTACGCCTCAACTCCCGTTGACAGCAGTGCCCACAGTALGUCTCGACTCCUCLGACAGCAG	1
830	TPVDSSAHGIPS (PVDSSAAS) FSIFAGS	
2578	GCCCACAGTACGCCCTCAACTCCCGCTGACAGCAGTGCCCACGGTACGCCCTCAACTCCCGTTGACAGCAGTGCCCACAGTACGCCCTCA	,
860	ARS PSTPADS SANGTPS I PUSSANS LES	
2668	ACTCCCCCCTGACAGCAGTCCCCCACAGTACCCCCTCAACTCCCGTTGACAGCAGTACCCCCCACAGTACCCCCCACAGTACCCCCCCACAGTACCCCCCCACAGTACCCCCCCACAGTACCCCCCCC	6
a 90 .		
2758	GCCCACGTACGCCCCTCAACTCCCGTTCACAGCAGTCCCCACGGTACGCCCTCGACTCCCGCTGACGCCGTGACGCCCTCACGTAGCCCCCC	'
920		
2848	$\mathbf{A} = \mathbf{C} = $	'
930		
980	A H S T P S T P A D S S A R S T P S T P A D S S A H S T P S	
1018	ACTOPROTOSCARCE CONCERCING ACTOPROTOSCARCE ACTOPROTOS	
1010	TPADSSARSTPSTPVDSSAHSTPSTPADSS	
1118	CCC+ACCTACTACTACTACTACTACTACTACTACTACTACTACT	;
1040	A H G T P S T P A D S S A H S T P S T P V D S S A H S T P S	
•		
3208	ACTECCGCTGACAGEGCGCCCGGGTACGCCTCGACTCCCGCTGACAGEAGTGCCCACTALGCCTCGACTACCGCTGACAGEAGTGCCCCGGCTGACAGEAGTGCCCGCCGGGTGCGGCTGGCCGGGTGGCGGGGGGGGG	1
10.40	1 F M D S M M G I F S I F M D S M M S I F S I F M D S G	
3298	CCCCACGGTACGCCCTCGACTCCCGCTGACAGCAGTGCCCACAGTACGCCCTCAACTCCCGCTGGCAGCAGCGCCCAATGGTACGGTTTC	G
1100	AHGTPSTPADSSADSTPSTPAGSSA <u>BGT</u> VG -CHO-	
3368	ATTTTGCCCSATGGCGCTGCACTTTCGACCTTTTCGGGCGGAGGCCCTCTTCTGTGCGGGGCTTTGCTGCTGCACGTGTTTTTTAT	•
1130	ILPDGAALSTPSGGGLLL(C)ACALLLHVFFM	

С

Figure 3. DNA and predicted amino acid sequence of TCNA. Numbering of amino acids starts at the first in-frame methionine. The repeats conserved in BNR are boxed, and the amino acids invariant in the consensus sequence are circled. Cysteine residues are also circled, and potential N-glycosylation sites are underlined and indicated by -CHO-. These sequence data are available from EMBL/GenBank/DDBJ under accession number R61732. Of the 8,885 nt of 7F only 5,404 are displayed in Fig. 3 because the remaining 3,481 nt in the 3'-end of the insert contains an ORF of 1,061 nt that is transcribed in the three stages of *T. cruzi* (data not shown). The deduced amino acid sequence of this ORF is unrelated to TCNA. Details about the remaining 3,481 nt of 7F will be published elsewhere.

Hydrophobicity and RNA Blot Analysis. Hydrophobicity analysis (26) of TCNA does not reveal typical N-terminal signal peptide sequences (Fig. 1 A). Although most proteins do contain a peptide leader sequence (27), TCNA is not unique in lacking a signal peptide because several proteins such as human IL-1 (28) and bovine scavenger receptor (29) are synthesized without a leader peptide. Therefore such peptide is not an absolute requirement for proteins to be exported to the surface or the environment.

Hydrophobicity analysis also illustrates a hydrophilic LTR in the C-terminus, followed by a nonpolar region of 35 amino acids at the extreme C-terminus. This region is characteristic of proteins anchored to the cell surface by a GPI linkage (30, 31), suggesting that TCNA is GPI-anchored to the trypomastigote surface. This interpretation is consistent with findings showing that TCNA is released spontaneously into the culture medium (5) or by GPI-specific lipases (32).

RNA blotting (Fig. 4) shows expression of the cloned gene in trypomastigotes, epimastigotes, and amastigotes: three major bands of 5.3, 4.5, and 4.0 kb, and three minor bands of 3.1, 2.8, and 2.4 kb hybridized to a probe derived from the catalytic domain (Fragment 2, see Fig. 1 A). Hybridization was more intense with trypomastigotes than with epiand amastigotes, in accordance with immunoblot analysis (see Fig. 2 A) and neuraminidase activity assays of the developmental forms of T. cruzi (3, 4). No hybridization was observed with Vero cells RNA (Fig. 4).

Primary Structure Analysis. The predicted protein sequence of TCNA (Fig. 3) can be considered in four domains (Fig. 5): (I) a N-terminal cysteine-rich region (residues 1-457) which should contain the catalytic site of the enzyme; (II) a fibronectin (FN) type III module (residues 458-588); (III) a C-terminal long tandem repeat (residues 589-1127); and (IV) a hydrophobic segment (residues 1128-1162) at the extreme C-terminus.

Domain I: The Cysteine-Rich Region. The N-terminus of TCNA contains six cysteine residues and exhibits 27% identity with the C. perfringens neuraminidase (33) in an alignment extending for about 90% of the bacterial enzyme (Fig. 6). Support for the probable homology between the cysteinerich domain of TCNA and the C. perfringens neuraminidase is provided by statistical analysis using the program ALIGN (21), which gave a score of 10.33 standard deviations above the mean obtained by 100 random permutations of the two sequences. The TCNA domain includes three consensus sequences (X-X-S-X-D-X-G-X-T-W-X-X) (Figs. 3 and 6) which are highly conserved in bacterial and viral neuraminidases (34). These bacterial neuraminidase repeats (BNR) are located in separate regions of TCNA, similar to their distribution in bacterial neuraminidases (34). Because of its structural similarity with the C. perfringens neuraminidase and with the



Figure 4. RNA blot analysis of TCNA transcripts. Total RNA  $(30 \ \mu g)$  was separated on a 1.2% agarose-formaldehyde gel, blotted to a nylon membrane, and probed with fragment 2 (Fig. 1 B) derived from the catalytic domain, which was labeled with <sup>32</sup>P by random priming. RNA sources were: trypomastigotes (lane A), epimastigotes (lane B), amastigotes (lane C), and Vero cells (lane D).



Figure 5. Schematic model of the predicted monomeric structure of TCNA. ( $\bigcirc$ ), BNR repeats; ( $\bigcirc$ ) YWTD repeats; ( $\prec$ ), N-glycosylation sites; ([]), type III module; (+++), LTR domain.

TCNA		1	MVAIADARYETSSENSLIDTVAKYSVDDGETWETQIAIKNSRVSSV-SRV
<u>C. perfringens</u>	NA	50	ILTFSDIRYNGPDDHAYIDIASARSTDFGKTWSYNIAMKNNRIDSTYSRV
TCNA		50	VDPTVIVKGNKLYVLVGSYYSSRSYWSSHGDARDWDILLAVGEVTKST
<u>C. perfringens</u>	NA	101	MDSTTVITNTGRIILIAGSWNTNGNWAMTTSTRRSDWSVQMIYSD
TCNA		98	AGGKITASIKWGSPVSL-KKFFPAEMEGMHTNQFLGGAGVAIVASNGNLV
<u>C. perfringens</u>	NA	146	DNGLTWSNKIDLTKDSSKVKNQPSNTIGWLGGVGSGIVMDDGTIV
TCNA		147	YPVQVTNKRKQVFSKIFYSEDDGKTWKFGKGRSDFGCSEPVALEWEG
<u>C. perfringens</u>	NA	192	MPAQISLRENNENNYYSLIIYSKONGETWTMGNKVPNSNTSENMVIELDG
TCNA		194	KLIINTRVDWKR-RLVYESSDMEKPWVEAVGTVSRVWGPSPKSNQPGSQT
<u>C. perfringens</u>	NA	243	ALIMSTRYDYSGYRAAYISHDLGTTW-EIYEPLN-~-GKILTGKGSGCQG
TCNA		243	SF-TAVTIEGMRVMLFTHPLNFKGRCVRDRLNLWLTDNQRIYN-VGQVSI
<u>C. perfringens</u>	NA	290	SFIKATTSNGHRIGLISAPKNTKGEVIRDNIAVYMIDFDDLSKGVQEICI
TCNA		291	GDENSAYSSVLYKDDKLYCLHEINTDEVYSLVFARLVGELRI
C. perfringens	NA	341	PYPEDGNKLGGGYSCLSFKNNHLGIVYEANGNIEYODLTPYYSLINKO

Figure 6. Alignment of the N-Terminus domain of TCNA with the *C. perfringens* neuraminidase. Identities are marked by vertical lines and conservative substitutions (D,E; K,R; L,I,V,M,C; Y,F,W; S,T) by colons. Gaps are indicated by (-).

BNR motifs, the cysteine-rich domain is assumed to contain the catalytic site of the enzyme.

The cysteine-rich domains also includes two motifs homologous to the YWTD repeats present in the LDL receptor (35), the LDL-related lipoprotein receptor (36), the epidermal growth factor (EGF) precursor (37), the basement membrane protein nidogen (38), and the sevenless protein (a tyrosine kinase) of *Drosophila* (39). Five and four consensus residues of the YWTD module are aligned in the two TCNA repeat sequences, respectively (Fig. 7), compared to four in module E of the LDL receptor, and eight and seven in modules A and B of sevenless (reference 39; see Fig. 7). The YWTD repeats of TCNA were discovered by scanning protein data bases with the cysteine-rich domain using FASTA (20), which revealed 34% identity between residues 184-230 of TCNA and residues 1465-1511 of sevenless, which were proposed to form a YWTD motif (39).

The domain has 10 potential protein kinase substrate sites (40): 4 for the cGMP-dependent protein kinase (XSRX; residues 40-43, 46-49, 70-73, and 225-228), 1 for the S6 kinase (XRXXSX; residues 448-453), 1 for casein kinase II (XSXXSX; residues 231-236), and 4 for the glycogen synthase kinase (XSXXXSX; residues 11-17, 66-72, 72-78, and 334-340). One N-glycosylation site separates the BNR and YWTD like repeats from the next domain, the FNIII module (Figs. 3 and 5).

Domain II: The FNIII Module FNIII modules were defined originally as segments of 71-109 amino acids repeated 15 times in fibronectin (41). Their characteristics include conservation of certain aromatic residues and adjacent hydrophobic amino acids at defined positions, with no conserved cysteines (39, 41). Sequence similarity among the fibronectin modules ranges from 5 to 39% (41). Sequences similar to FNIII repeats were subsequently found in other vertebrate proteins including cytotactin (42), contactin (43), leukocyte common antigenrelated protein LAR (44), the neural adhesion molecule

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Figure 7. Alignment of the TCNA repeats with 'YWTD' motifs from human LDL receptor, epidermal growth factor precursor, and sevenless protein. Sequences for the human LDL receptor (LdIR) and epidermal growth factor precursor (EgfP) and sevenless were taken from Norton et al. (39), as was the consensus element, which was derived by the alignment of 55 YWTD repeats. Amino acid residues for the TCNA YWTD motifs A and B are 54-92 and 189-220, respectively.

N-CAM (45), tenascin (46), growth hormone/prolactin receptors (47), and in the invertebrate proteins fascilin II (48) and the sevenless gene product (39).

Consensus sequences for the FNIII repeats present in hormone receptors (reference 47; Receptor in Fig. 8), sevenless protein (reference 39; Sevenless in Fig. 8), and fibronectin, LAR and tenascin (reference 39; FN in Fig. 8) have been derived by multiple alignment programs. Comparison of the three consensus sequences with TCNA segment 458–588, which is devoid of cystine residues, reveals conserved aromatic residues (tryptophan and tyrosine; denoted by  $\phi$  in Fig. 8) and nearby hydrophobic residues (highlighted by (\*) in Fig. 8): 7 of 16 residues typical of the sevenless protein type III module are conserved in the TCNA sequence. Relatedness of TCNA with the fibronectin type III repeat was initially detected by scanning the Protein Identification Resource and Swiss-Prot data bases with the program FASTA (20).

The FNIII domain has 1 BNR motif (residues 469–480) and 5 recognition sites for protein kinases (40): 3 for prolinedependent protein kinase (XTPX; residues 482–485, 487–490, and 531–534), 1 for casein kinase II (XSXXEX; residues 470–475), and 1 for glycogen synthase kinase (XSXXXSX; residues 457–462).

The domain also contains three stretches of amino acid sequences, G-S-W-E-T-G-K (residues 491-497), D-L-I-G (residues 580-583), and N-K-I-G (residues 497-500), that fit (with one amino acid mismatch) the consensus elements required for GTP-binding proteins: G-X-X-X-G-K, D-X-X-G, and N-K-X-D (49, 50). A fourth additional consensus sequence, E-X-S-A, required by ras and ras-related proteins for GTPbinding (51), is present in domain I (residues 293-296, E-N-S-A). However, while the order and spacing of two of the GTP-binding motifs correctly match with TCNA sequence, two do not.

Domain III: The Long Terminal Repeat. Contiguous to the FNIII module is a stretch of 535 amino acids composed of 44 units of 12 amino acids, plus a degenerate unit of 7 amino acids in the C-terminus of the LTR. The truncated unit contains one potential N-glycosylation site separating LTR from the hydrophobic segment (Domain IV; see Figs. 3 and 5). LTR constitutes almost half (46%) of the entire TCNA se-

	* * * * *		Figure 8. Alignment of TCNA (res-
FN	PLTV-WP4-8aa.11-V-1	5-14aa1-GL-PGIEI-V-V-A	idues 458-592) with sequence motifs
Receptor	D-PL-LNV-WPN.8aa.IY-L-Y	19-26aaL-~LYVR~RWS-WSE	from fibronectin type III domains. Con-
Sevenless	Y-LLWP3-10aaY-L-L	10-33aaL-~LQP-T-Y-LRL	sensus sequences were derived from type
TCNA	Φ Φ SLDSSGGKKLLGLSYDEKHQWQPIYGS.llaa.GKRYHLVLTMANKIGS	Φ SV40aaHVTVNNVLL <u>Y</u> NR <u>R</u> QLNTEEIRTLFLSQDLIG <u>T</u> EAHMDSS	III repeats found in fibronectin (FN)
			(39), growth hormone/prolactin re-

ceptors (Receptor) (47), and sevenless (39). Conserved hydrophobic residues are marked by asterisks, aromatic residues by  $\phi$ , and amino acids conserved in at least one the three consensus sequences are underlined.

quence. Each unit is rich (75%) in serine, threonine and proline, with a consensus sequence (D-S-S-A-H-X-T-P-S-T-P-X) remarkably conserved in the entire LTR domain (Fig. 9).

The LTR domain is predicted to be very hydrophilic (Fig. 1 A) because of its high content (75%) of polar and charged amino acids (serine, threonine, aspartic acid and histidine). The domain contains the motif X-T-P-X, which is a recognition sequence for the proline-dependent protein kinase (40), repeated twice in each unit (except unit 9 in which one T-P is replaced by A-P) and thus 87 times in LTR, and the motif X-S-X-X-S-P-X, a substrate for glycogen synthase kinase, is repeated 30 times. The domain therefore contains at least 117 potential phosphorylation sites. When the LTR domain was compared with proteins in the Protein Information Resource and Swiss-Prot data bases using FASTA (20), best similarity was with the carboxyl-terminal domain of the mouse RNA polymerase II largest subunit, which consists of 52 repeats of seven amino acid blocks rich in serine, threonine and proline (52); and the serines from the repeats are phosphorylated in vivo by kinase such as cdc2 (53).

To determine whether LTR is polymorphic or not, genomic DNA from T. cruzi Silvio X-10/4 was digested with Pvu II and Pst I enzymes (whose recognition sites are uniquely flanking the 5'- and 3'- ends of the domain, respectively, see Fig. 1 B), electrophoresed in 1% agarose and analyzed by Southern blotting using <sup>32</sup>P-labeled fragment 1 DNA derived from the repeat. At least seven bands (3.7 to 0.6 kb) hybridized to fragment 1 (Fig. 10) suggesting that LTR has variable lengths within the T. cruzi genome, which could account in part for the polymorphism of the TCNA protein (Fig. 2 A).

Domain IV: The Hydrophobic Segment. A functionally unrelated group of at least 30 proteins is known to be attached to the plasma membrane by a GPI lipid anchor (30-32). A common feature among these proteins is the presence of a hydrophobic domain in their C-terminus, which is proteolytically processed in the endoplasmic reticulum to be replaced by the GPI anchor. TCNA contains a stretch of 35 amino acids at the extreme C-terminus (Figs. 1 B, 3, and 5), raising the possibility that it is anchored to the outer membrane by a GPI mechanism. This is likely to be the case because TCNA from tissue culture trypomastigotes can be released into the supernatant by digestion with phosphatidylinositol-specific phospholipase C (PIPLC), an enzyme that cleaves the GPI anchor specifically (31, 32). If so, the hydrophobic segment of TCNA must be processed to allow insertion of the GPI and thus it must be absent from the mature enzyme located on the trypomastigote surface. Although the C-terminus of GPI-anchored proteins contains information required for attachment of the anchor, the nature of and consensus sequence for the signal that directs processing of hydrophobic tails is unknown. Therefore it remains to be determined if residual amino acids from the hydrophobic segment will bridge the LTR domain to the GPI anchor.

### Discussion

Neuraminidases catalyze the hydrolysis of sialic acid from glycoconjugates in solution or on cell membranes and are widely spread in vertebrates, where they perform important functions such as in reproduction and blood clot formation (54, 55). Their absence in humans causes inborn error metabolism (56). They are absent from invertebrates and plants but present in some microorganisms, in particular in those that are pathogenic to vertebrates (54). However, many parasites do not have detectable enzyme activity.

The neuraminidase from influenza virus was the first to be cloned and it remains one of the best studied proteins, and much is known about its crystal structure, structurefunction relationships, antigenicity, and role in viral entry and exit from host cells (57). The genes for several bacterial neuraminidases have been cloned and characterized in the last three years (34, 58), and progress about their structure-function relationships is anticipated in the near future. In contrast, nothing is known about the primary structure of protozoan neuraminidases and, surprisingly, of vertebrates, including man. The sequence and domain structure of TCNA presented here is therefore the first for a neuraminidase derived from eukaryotes.

 

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Figure 9. Amino acid sequence of the LTR domain of TCNA. Numbers to the left refer to positions within the repeat and numbers on the top refer to the number of the repeat. The only variable positions are 6 and 12, except repeat 9, in which A replaces T in position 7.

186 The Trypanosoma cruzi Neuraminidase Contains Sequences



Figure 10. Southern blot of the LTR domain of TCNA. Trypomastigote genomic DNA (Silvio X-10/4) was digested with Pvu II and Pst I, electrophoresed on 1% agarose gel, transferred to a nylon membrane and probed with <sup>32</sup>P-labeled fragment 1 (Fig. 1 B) from the LTR domain.

Cysteine-Rich Domain. TCNA has structural features shared by other neuraminidases, the most striking of which is the presence of repeats conserved in bacterial and viral neuraminidases. All bacterial neuraminidases whose primary sequence is known have four repeats of consensus X-X-Ser-X-Asp-X-Gly-X-Thr-Trp-X-X, which are spaced from one another by 40-80 residues (34). TCNA also has four such BNRs (three in domain I and one in domain II) (Figs. 6 and 8) with consensus spacings similar to those in bacterial enzymes. In addition, domain I of TCNA is about 30% identical in sequence to the C. perfringens neuraminidase with an ALIGN score of 10.33 standard deviations above the mean, supporting the argument that TCNA domain I and the C. perfringens enzyme are homologous to one another. TCNA, like the *C. perfringens* enzyme, does not have common amino acid sequence with bacterial neuraminidases, except for the BNRs.

TCNA has structural motifs which are unique when compared to other neuraminidases. In the cysteine-rich domain there are two repeats similar to the YWTD motifs found in vertebrate and invertebrate proteins. Little is known about their function, but mutagenesis of the YWTD repeats and flanking cysteine-rich motifs of the LDL receptor produces mutant receptors unable to bind LDL, to release  $\beta$ -VLDL at acid pH, and to recycle properly (59). It is of interest that HDL and LDL are specific inhibitors of TCNA activity (7, 8), and that binding of <sup>125</sup>I-HDL to the developmental forms of *T. cruzi* correlates with TCNA activity, suggesting that TCNA can serve as receptors for lipoproteins. If so, it could be that the YWTD motifs of TCNA are involved in binding to host lipoproteins.

FNIII Domain. The FNIII motif of TCNA connects the cysteine-rich domain to LTR (Fig. 5). Like the YWTD repeats, FNIII molecules have not been previously observed in microorganisms. They are found in multiple copies in several developmentally regulated proteins that participate in activities such as binding to cell surface integrins and to heparin. It is therefore possible that the FNIII domain contributes to TCNA binding to host cell-surface proteins during T. cruzi-host cell interactions. This hypothesis is consistent with the alteration of T. cruzi infection by TCNA antibodies (5, 6). Alternatively, the FNIII domain could mediate TCNA binding to endogenous T. cruzi proteins to form ligand-enzyme complexes. This hypothesis is in keeping with recent findings which reveal a protein of 110-115 kd that co-precipitates with TCNA during immunoprecipitation with monoclonal antibodies specific for the T. cruzi enzyme but not with isotypematched control antibodies (Mejia, J.S., R.P. Prioli, E. Ortega-Barria, and M.E.A. Pereira, unpublished observations).

Nucleotide binding consensus motifs have been derived by sequence comparison of many functionally distinct GTPbinding protein families (49-51). The importance of these motifs was confirmed by the X-ray structure of the GTPbinding domain of the elongation factor Tu (60) and of ras protein (61). These studies showed that the motifs GXX-XXGK and DXXG, separated from one another by 40-80 amino acids, are involved in binding to phosphate of GTP, whereas the motif NKXD mediates interaction with the keto group of the guanine ring. The FNIII module of TCNA contains the phosphate binding motifs in correct spacing and order (residues 491-497 and 580-583; respectively), and, although it contains the guanine-ring binding motifs (residues 497-500 and 293-296), they are not spaced properly. Thus, although it is unlikely that the FNIII domain binds GTP, the conservation of GXXXXGK and DXXG raises the intriguing possibility that TCNA binds phosphate, either as sugar-phosphate or as nucleotides (other than GTP).

LTR Domain. The LTR domain is unique among known neuraminidases, but it is a common feature of parasitic protozoan proteins whose primary structures are known (62). Proteins with long tandem repeats usually have anomalous migration in SDS-PAGE, and the differences between the value predicted by DNA sequence and the one obtained experimentally can vary by as much as 100% (62). The LTR domain helps therefore explain the discrepancy between the predicted mol wt of TCNA (119,959 kD) and the one observed by immunoblotting of recombinant TCNA (180,000 kD).

The function of LTRs in protozoan proteins has not been elucidated, but it has been suggested that tandem motifs, due to their intrinsic repeated structure and consequent enhanced immunogenicity, may form a "smokescreen" against the host immune response to protect domains involved in functions such as in binding to host cells or catalytic activity (62). This could well be the case for TCNA, because the repeat unit of LTR contains epitopes for monoclonal and polyclonal antibodies raised against the intact enzyme. For example, we have found that binding of the mAb TCN-2 to TCNA is competitively inhibited by the soluble synthetic peptide Asp-Ser-Ser-Ala-His-Gly-Thr-Pro-Ser-Thr-Pro-Val, that is repeated many times in LTR, and that TCN-2 and Chagasic sera bind to the peptide coupled to a protein carrier (ovalbumin) (Prioli, R.P., E. Ortega-Barria, J.S. Mejia, and M.E.A. Pereira, unpublished observations). TCN-2 epitopes present in the C-terminus is consistent with the finding that the antibody neutralizes desialylation of erythrocytes but not of soluble small molecules (6). We have not yet found antibodies, monoclonal or polyclonal that react with synthetic peptides from domain I (Fig. 5) or with the intact domain.

The amino acid composition of LTR contains a high content of  $\alpha$ -helix- and  $\beta$ -sheet-breaking amino acids (serine, threonine, and proline) in invariant positions (Fig. 9) (63). Empirical predictive methods indicate that the probability of  $\alpha$ -helix and  $\beta$ -sheet is less than 1%, and for  $\beta$ -turn more than 99% (64). TCNA may belong to a group of proteins such as RNA polymerase II and rhodopsin, that contain tandem repeats having 20–60% Pro with abundant Gly, Gln, and Ser, which can give rise to a novel secondary structure, polyproline  $\beta$ -turn helix (65). Many of these structures are consistent with a series of turns in an extended conformation.

LTR is similar to the C-terminal sequence of shed acute phase antigen (SAPA) of T. cruzi, which contains tandem units of 12 amino acids rich in Ser, Thr, and Pro, ending with a hydrophobic segment (66). SAPA has only 14 tandem repeats, compared to 44 for TCNA. The N-terminus sequence of SAPA is now known, and it is about 80% identical to that of TCNA (A.C.C. Frasch, personal communication). It is of interest that TCNA activity was detected in the serum of a patient accidentally infected with T. cruzi, but only in the acute phase of Chagas' disease (67). Since TCNA activity was present before noticeable parasitemia, neuraminidase activity assays were proposed to serve as an aid for the diagnosis of acute Chagas' disease, similar to what has been suggested for SAPA (66). SAPA and corresponding antibodies also appear to be extremely useful reagents to confirm congenital cases of Chagas' disease (68). Although it is not yet known whether SAPA has neuraminidase activity, it is possible that it belongs to the TCNA family. Differences in their sequences could be attributed to strain variations, to the fact that the TCNA and SAPA sequence were derived from trypomastigote and epimastigote genomes, respectively, or to isoform variations.

TCNA Polymorphism. TCNA from tissue culture trypomastigotes is comprised of a set of high mol wt polypeptides that differ in size and number according to strains and clones of T. cruzi. Within a given cloned T. cruzi strain, the size of the polypeptides may vary by more than 80 kd (5, 6). The mechanism underlying such polymorphism is not clear, but postranslational modifications may not be one them because pulse-chase experiments failed to show a time-dependent interrelationship in the generation of the polypeptides (Mejia, J.S., E. Ortega-Barria, R.P. Prioli, and M.E.A. Pereira, unpublished observations). Alternatively the polypeptides could arise from distinct TCNA genes or by cotranslational modifications: RNA blots hybridized with DNA derived from the catalytic domain (Fragment 2, Fig. 1 B) show at least 7 bands (Fig. 4). Southern blotting experiments using DNA from the LTR domain as a probe, and genomic DNA digested with Pvu II and Pst I, that cut the repeated structure in its 5' and 3' ends, respectively (Fig. 1 A), reveal a multiple banding pattern. Thus, Southern blotting results are consistent with the notion that variable length polymorphism of the LTR DNA is a mechanism for the multiple banding of TCNA polypeptides.

TCNA polymorphism can also be rationalized on the basis of protein synthesis initiation at different methionine codons. Proteins can be synthesized from overlapping reading frames if the first AUG codon does not lie in an optimum context for ribosomal subunit recognition, such as with many viral proteins (69). Assuming that the initiation signal form high eukaryotes (GCCGCCAGCCAUGG) holds for T. cruzi, the first AUG codon of TCNA is in a good but not perfect context (7 out of 13 nucleotides). If the ribosomal subunit bypass the first AUG, it may stop and initiate protein synthesis at the next in-frame AUG (residues 364-366), which is an even better context (8 out of 13 nucleotides). And it is possible that some TCNA isoforms contain AUG initiation codon upstream of the one shown in Fig. 3. But whatever the mechanism for the polymorphism of TCNA may be, it does not seem to abolish enzyme activity since each polypeptide from Silvio X-10/4 (Fig. 2 A) exhibits neuraminidase activity after isolation by sieving chromatography under nondenaturing conditions (Prioli, R.P., J.S. Mejia, and M.E.A. Pereira, manuscript in preparation).

In conclusion, the structure of TCNA as deduced from DNA sequence suggests that the enzyme, in addition to catalyzing hydrolysis of sialic acid from glycoconjugates, may have other important functional properties, including proteinprotein interactions and binding to nucleotides. The availability of TCNA DNA clones provides experimental tools that should be useful in further dissecting the structure and function of the enzyme. We thank Jacqueline Sharon for critical reading of the manuscript and for useful suggestions, and Carlos Frasch and George Cross for communicating unpublished findings and for helpful discussions.

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189 Pereira et al.

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