Molecular Biology of the Cell
Vol. 11, 1487–1498, April 2000

A Trypanosomal Protein Synergizes with the Cytokines Ciliary Neurotrophic Factor and Leukemia Inhibitory Factor to Prevent Apoptosis of Neuronal Cells

Marina V. Chuenkova and Miercio A. Pereira*

Parasitology Research Center, Department of Pathology, Tufts University School of Medicine, Boston, Massachusetts 02111

Submitted November 3, 1999; Revised January 27, 2000; Accepted February 7, 2000
Monitoring Editor: Martin Raff

Despite the neuronal degeneration in the chronic stage of Chagas' disease, neuron counts actually increase in the preceding, asymptomatic stage, in contrast to the age-related decrease in neuron counts in age-matched normal individuals. Relevant to this observation, we found that the *trans*-sialidase (TS) of *Trypanosoma cruzi*, the etiologic agent of Chagas' disease, induces neurite outgrowth and rescues PC12 cells from apoptotic death caused by growth factor deprivation. These properties, novel for a parasite protein, were independent of catalytic activity and were mapped to the C terminus of the catalytic domain of TS. TS activated protein kinase Akt in a phosphoinositide-3 kinase-inhibitable manner, suggesting a molecular mechanism for the TS-induced neuroprotection. TS also triggered bcl-2 gene expression in growth factor-deprived cells, an effect consistent with TS protecting against apoptosis. Ciliary neurotrophic factor and leukemia inhibitory factor, two cytokines critical to the repair of injured motor neurons, specifically potentiated the TS action. The results suggest that TS acts in synergy with host ciliary neurotrophic factor or leukemia inhibitory factor to promote neuronal survival in *T. cruzi*-infected individuals.

INTRODUCTION

Chagas' disease, produced by the obligate intracellular protozoan *Trypanosoma cruzi*, affects millions of people in Latin America and is an important cause of cardiac and gastrointestinal (GI) morbidity and mortality. For the first few months after infection, parasites circulate in the bloodstream as a result of their invasion of and rapid replication in a variety of cell types, in particular muscle cells in the heart and GI tract and glial cells in the nervous system (acute infection). This robust parasite growth may lead to serious damage of the nervous system, and humans with acute disease, particularly young children, may suffer fulminating encephalitis, as first observed by Carlos Chagas more than 80 years ago (Chagas, 1916).

Most patients, however, survive the acute infection to enter a subclinical, asymptomatic stage that lasts years or decades (the indeterminate phase). The vast majority of patients in the indeterminate phase (~90%) show no signs of peripheral neuropathy (Genovese at al., 1996). In addition, a study of >200 autopsied chagasic cases revealed relatively few lesions in the autonomic nervous system of the heart and GI tract (Köberle, 1968). This study also showed that, although the number of neurons in patients in the indeterminate phase is lower than in age-matched normal individuals, the average number of neurons in both cardiac and GI ganglia actually increases with the age of chagasic patients. This trend is contrary to the age-related physiological reduction in ganglion cells in nonchagasic individuals (Köberle, 1968; Meciano Filho *et al.*, 1995). This effect is consistent with histological and electrophysiological findings in rodents infected with *T. cruzi*, which show signs of neurite development, axon regeneration, and sprouting in sympathetic and parasympathetic nerve fibers of the heart and colon, despite some neuronal degeneration (Machado *et al.*, 1987; Losavio *et al.*, 1989).

The findings in the indeterminate phase are contrary to the extensive destruction of the autonomic nervous system in the heart and GI tract of patients with chronic Chagas' disease. Neurons in the heart shrink and disintegrate, with or without perineural and intraneural inflammation, and are likely to contribute to the generation of cardiomegaly (Andrade, 1983; Oliveira *et al.*, 1985). In the GI tract, myenteric (Auerbach's) and submucosal (Meissner's) ganglia may be >95% destroyed (Köberle, 1968), providing one explanation for the tremendous enlargement of the esophagus and colon

* Corresponding author. E-mail address: mperei01@emerald.tufts.edu.

(megaesophagus and megacolon) of chronic Chagas' disease (Adad *et al.*, 1991).

Most published work on *T. cruzi*–neuron interaction has investigated the still unknown mechanism underlying the neuronal destruction in chronic disease (Said *et al.*, 1985; Van Voorhis and Eisen, 1989). However, no attempts have been made to understand the mechanism underlying neuronal survival in Chagas' disease, a critical event for the long-lasting equilibrium of *T. cruzi* parasitism of human hosts.

One intriguing possibility is for *T. cruzi* to secrete a factor(s) that promote(s) regeneration and survival of neurons. Such a factor(s) could help neurons counterbalance neurotoxic insults resulting from the infectious process and might act in synergy with host neurotrophic factors. We found the *T. cruzi trans*-sialidase (TS) to be very potent in promoting neurite outgrowth and survival of the neurons. In addition, TS synergized specifically with ciliary neutrophic factor (CNTF) and leukemia inhibitory factor (LIF) to rescue neurons from death.

TS was originally discovered by its ability to catalyze the release of sialic acid from glycoconjugates in solution or on cell surfaces (i.e., neuraminidase activity) (Pereira, 1983). Subsequently, it was demonstrated that TS can transfer sialic acid to β-galactosyl acceptors (sialyl transferase activity) (Parodi *et al.*, 1992; Schenkman *et al.*, 1992, 1994; Scudder *et al.*, 1993). TS is attached to the trypomastigote outer membrane through a glycosylphosphatidylinositol anchor (Pereira *et al.*, 1991) and is present in the extracellular milieu as a soluble factor (Cavalesco and Pereira, 1988). It is therefore strategically located for its role in mediating trypanosome–host interactions, such as parasite attachment to cells (Ming *et al.*, 1993; Schenkman *et al.*, 1993), alteration of immune cell function to enhance parasitism (Chuenkova and Pereira, 1995), and promotion of neuronal survival.

CNTF and LIF, which we now find to synergize with the *T. cruzi* neuraminidase to rescue neurons from death, are neurocytokines of the interleukin-6 (IL-6) family produced by Schwann cells in the peripheral nervous system (PNS) and by astrocytes in the CNS (Sendtner *et al.*, 1994; Ip and Yancopoulos, 1996). Upon release into the extracellular environment, usually after trauma, CNTF initiates signaling events that may result in the repair and survival of an injured neuron.

Our present findings support the hypothesis that the *T. cruzi* neuraminidase, in collaboration with CNTF or LIF, serves to enhance neuron survival in *T. cruzi*-infected individuals. Such neuroprotection would be advantageous both to the host and to the protozoan parasite, which can inhabit humans for decades without producing significant neuronal degeneration.

MATERIALS AND METHODS

Growth Factors, Cytokines, Cruzipain, and Penetrin

Mouse nerve growth factor (NGF) 2.5S was purchased from Collaborative Biomedical Products (Bedford, MA); human CNTF was a gift of Dr. E. Granowitz (New England Medical Center, Boston, MA); human and rat recombinant CNTF and *Vibrio cholera* neuraminidase (VCNA) were from Calbiochem (La Jolla, CA). Recombinant human IL-11, LIF, and oncostatin-M (OSM) were from Sigma (St. Louis, MO), and recombinant human IL-6 was from Endogen (Woburn, MA). Cruzipain and penetrin were gifts from Drs. Julio Scharfstein (Univeridade Federal do Rio de Janeiro, Rio de Janeiro, Brazil) (Murta *et al.*, 1990) and Macario Herrera (Tufts University, Boston, MA) (Ortega-Barria and Pereira, 1991), respectively.

Purification of TS

TS was affinity purified as previously described (Scudder *et al.*, 1993). Briefly, conditioned supernantants of Vero cell cultures infected with the *T. cruzi* strain Silvio-X10/4 were applied to affinity columns of mAb TCN-2 adsorbed to protein G-Sepharose; the bound enzyme was eluted with synthetic peptide TR (DSSAHGTP-STPA, 10 mg/ml), which was separated from the enzyme by Centricon-10 ultrafiltration (Amicon, Beverly, MA).

TS Assay

The TS assay was performed by measuring the amount of sialic acid transferred from fetal calf serum glycoproteins to [^{14}C]*N*-acetyllactosamine as previously described (Scudder *et al.*, 1993). The sialylated product was quantitated by scintillation counting.

Cloning and Expression of Recombinant Fragments of TS

The DNA fragments corresponding to various regions of TS were amplified by PCR using as templates TS clones 19Y and 7F derived from a genomic library of *T. cruzi* trypomastigote (Pereira *et al.*, 1991). The full catalytic domain, TS-F, and TS-CC-46 were obtained as described (Chuenkova *et al.*, 1999). DNA fragment TS-Cat11–46 was amplified using clone 7F DNA with MP13 (5′-GGGAATTCG-GTTGCCAATCGCGGACGCTC-3′) as a forward primer and MP11 (5′-CCCCTCGAGCCGACAAAAAGCCAACAAAGAC-3′) as a reverse primer. The amplified fragments were cloned into pET 23b (Novagen, Madison, WI) and expressed in the BL21 DE3 strain of *Escherichia coli.* Expressed polypeptides were purified by Ni-nitrilotriacetic acid affinity chromatography as recommended (Novagen). In the lysates containing TS-F-46, TS-CC-46, and TS-Cat11–46, urea was added to 8 M to facilitate solubilization. Refolding of the urea-soluble proteins was performed as described (Marti *et al.*, 1994). TS-F polypeptide was further purified by fast protein liquid chromatography on the anion exchange column MonoQ HR (Pharmacia, Piscataway, NJ). The full-length C-terminal long tandem repeat (LTR fragment) of TS was generated by Wenda Gao (Saavedra *et al.*, 1999).

To determine the relative amount of the recombinant proteins, bacterial proteins were separated on 10% SDS-PAGE and stained with Pro Blue (Integration Separation Systems, Natick, MA) in the polyacrylamide gels. Alternatively, the recombinant proteins were blotted to nitrocellulose membranes and visualized with the anti-His T7.Tag antibody (Novagen) or with a mouse polyclonal antibody against TS. The relative amounts of proteins were quantitated in a Gel Doc 1000 apparatus (Bio-Rad, Hercules, CA) using various concentrations of BSA as standard.

Cell Cultures

PC12 cells were obtained from Drs. Arthur Tischler (Tufts University School of Medicine) (Greene and Tischler, 1976) and G. Cooper (Boston University, Boston, MA) (Yao and Cooper, 1995) and from American Type Culture Collection (Manassas, VA). They were cultured on collagen-coated dishes in RPMI-1640 supplemented with 10% horse serum and 5% fetal bovine serum (Greene and Tishler, 1976). For neurite extension experiments, PC12 cells were pretreated with NGF (50 ng/ml) for 2 wk and mechanically deprived of their processes (Greene *et al.*, 1986). Cells were replated in 1% FCS together with NGF (50 ng/ml) or TS (100 ng/ml). After 24 h the cells with neurites were counted in no less than 300 cells. For survival experiments, PC12 cells were washed three times in serum-free RPMI and plated in the same medium in collagen-coated plastic dishes at 2×10^5 per ml, without and with growth factors, cyto-

kines, TS, and recombinant proteins for the time and with the concentrations indicated in the figures.

Immunodetection of Activated Akt

PC12 cells were deprived of serum for 24–48 h, stimulated with different agents for the times indicated in Figure 6B, and immediately lysed with 2% SDS. In the experiments with phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002), cells were pretreated with the phosphoinositide-3 kinase (PI-3 kinase) inhibitor (1 μM) for 30 min before the addition of TS-F or TS. The proteins in the cell lysate were separated in SDS-10% polyacrylamide gels and transferred to a nitrocellulose membrane (Bio-Rad), and the phosphorylated form of protein kinase Akt and total Akt were detected with phospho-Akt (Ser-473) antibody and Akt antibody, respectively (New England BioLabs, Beverly, MA), followed by alkaline phosphatase-conjugated secondary antibody (Promega, Madison, WI). Bands corresponding to phospho-Akt (60 kDa) were quantified using a scanning densitometer (Bio-Rad).

PKB/Akt In Vitro Kinase Assay

Akt kinase assays were performed by the protocol provided by New England Biolabs. Cells were lysed in ice-cold lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium phosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, and 1 μg/ml leupeptin). After clearing by centrifugation at 12,000 rpm at 4°C for 10 min, 400 μl of the lysates were immunoprecipitated with Akt antibodies coupled to agarose beads for 3 h at 4°C. The resulting immunoprecipitates were then incubated with GSK-3 fusion protein as a substrate in the presence of 200 μM ATP and kinase buffer (25 mM Tris, pH 7.5, 5 mM β-glycerolphosphate, 2 mM DTT, 0.1 mM Na_3VO_4, and 10 mM $MgCl_2$) at 30°C for 30 min. Phosphorylation of GSK-3 was measured by Western blotting using a phospho-GSK-3α/β (Ser-21/9) antibody, HRP-conjugated secondary antibody, and LumiGLO chemiluminescent reagent (New England Biolabs).

Neurite Outgrowth Assay of N18 Cells

Ninety-six-well microtiter plates were coated overnight at 4°C with the compounds indicated in Figure 1E at 500 μg/ml. After removing the compounds, the plates were further incubated with 1% BSA for 1 h at room temperature and immediately used as substratum for N18 cells in serum-free RPMI. Neurite outgrowth was measured in a phase-contrast microscope 17 h later. Cells exhibiting neurite outgrowth were those having one or more cytoplasmic extension >2 μm in length.

Assays for Cell Survival

Cell Staining with DAPI. Cells were fixed with 4% formaldehyde in PBS for 5 min, washed with PBS, stained with 10 μg/ml DAPI (Sigma) for 2 min, washed with PBS, and visualized under UV light in a fluorescent microscope to determine cells with fragmented (apoptotic) nuclei. Approximately 300–400 cells were examined under the microscope to determine the percentage of apoptotic cells.

Cell DNA Nick End Labeling. A terminal deoxynucleotidyl transferase-mediated biotinylated dUTP nick end-labeling (TUNEL) (Gavrieli *et al.*, 1992) assay was performed as described by the manufacturer (Boehringer Mannheim, Indianapolis, IN).

Protection against apoptosis of PC12 cells in serum-free medium by peptide N (TS, TS fragments, NGF, or other growth factors) was calculated by the formula 100% − [(apoptosis (%) of cells kept in RPMI containing N ÷ apoptosis (%) of cells kept in RPMI) × 100%]. Testing the effect of wortmannin (Sigma) and LY249002 (Sigma) in inhibiting the neuroprotection of TS was performed following the protocol described by others (Ui *et al.*, 1995; Yao and Cooper, 1995).

Reverse Transcription (RT)-PCR of Bcl-2 Gene Transcripts

Total RNA was extracted from PC12 cells by the acid guanidinum isothiocyanate method using Tri Reagent (Molecular Research Center, Cincinnati, OH). cDNA synthesis was performed according to the instructions of the manufacturer (Life Technologies, Gaithersburg, MD). PCR reactions were performed using an amount of cDNA synthesized from 100 ng of total RNA, as a template, 100 μM deoxynucleotides, 2.5 mM $MgCl_2$, 10 mM DTT, 10 pM Bcl-2 primers, and 2 U of *Taq* DNA polymerase (Life Technologies). The primers of the rat Bcl-2 were 5′–AGATGAAGACTCCGCGCCCCTCAGG-3′ and 3′-GTAGTGAGACCCACGTATGGACC-5′ to give a PCR product of 566 bp. glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (Wong *et al.*, 1994) gave a PCR product of 306 bp. Amplifications were carried out in a MiniCycler (MJ Research, Watertown, MA) using the following conditions: 98°C for 5 min; three cycles of 94°C for 1 min, 63°C for 1 min, and 72°C for 1.5 min; three cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1.5 min; and 29 cycles of 94°C for 1 min, 61°C for 1 min, and 72°C for 1.5 min, followed by 72°C for 10 min. The second pair of primers for GAPDH (10 pM) was added at cycle 7 by the "primer-dropping" method (Wong *et al.*, 1994). Aliquots of PCR reaction products were analyzed by agarose gel electrophoresis.

RESULTS

TS Promotes Neurite Outgrowth in PC12 and N18 Neuronal Cells

The rat pheochromocytoma PC12 cells (Greene and Tischler, 1976) and other neuroblastoma cell lines (Prasad, 1991) are widely used as a model system of neuronal cell differentiation. Upon stimulation with NGF, PC12 cells exhibit several characteristics of neurons, such as neurite outgrowth and electrical excitability. We first used the PC12 cells and the mouse neuroblastoma N18 cells to determine whether *T. cruzi* promotes neurite outgrowth. In exploratory experiments we found that undifferentiated PC12 and N18 cells extended multiple neurites when live *T. cruzi* trypomastigotes, a mobile, invasive form that shuttles from peripheral tissues and internal organs to the circulation and vice versa, were added to the liquid overlaying cultures of the neuronal cells (our unpublished data). In addition, a conditioned medium prepared by incubating live trypomastigotes with RPMI at 4°C for 24 h (Cavalesco and Pereira, 1988) also induced neurite outgrowth in both PC12 and N18 cells.

The trypomastigote-conditioned medium comprises TS (Cavalesco and Pereira, 1988), proteases such as cruzipain (Murta *et al.*, 1990), the adhesion molecule penetrin (Ortega-Barria and Pereira, 1991), and other factors thought to mediate *T. cruzi* infection (Pereira, 1994). In an attempt to identify the differentiating factor present in the trypomastigote extract, we sought to determine whether the above mediators of *T. cruzi* invasion would reproduce the effect of the crude extract on PC12 and N18 cells.

PC12 cells differentiate into cells similar to sympathetic neurons after treatment with NGF for several days (Greene and Tischler, 1976; Mesner *et al.*, 1992). These cells, after mechanical deprivation of their processes, exhibit NGF-dependent neurite regeneration within 24 h (Figure 1, B and D; Greene *et al.*, 1986). Replacing NGF with TS likewise

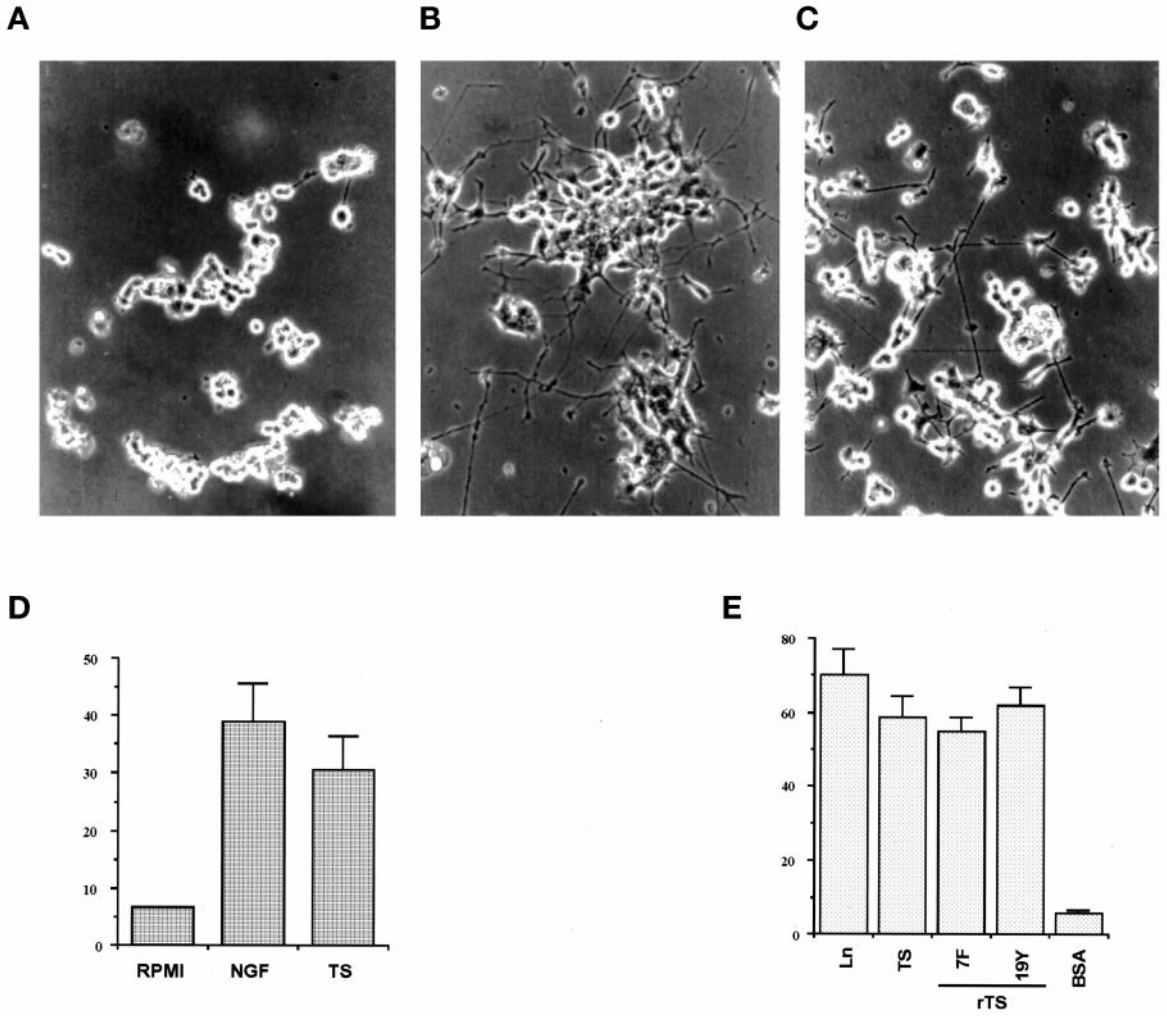

Figure 1. Neurite outgrowth in PC12 and N18 cells in response to TS. (A–D) PC12 cells, mechanically stripped of their neurites, were seeded in serum-free RPMI medium on collagen-coated plastic dishes with no addition (A), 50 ng/ml NGF (B), or 100 ng/ml TS (C). Pictures were taken 24 h after addition of the reagents. Magnification, 400×. (D) Quantitation of PC12 cells exhibiting neurite outgrowth (triplicate samples) from an experiment similar to that shown in A–C. (E) Neurite outgrowth of N18 neuroblastoma cells by TS and rTS-F. Microtiter wells were coated overnight in triplicate with laminin (Ln), TS, rTS clones 7F and 19Y, and BSA and used as substratum for attachment of N18 cells. Cells exhibiting neurite extension were counted 17 h later. The values are the mean ± SEM of three separate experiments.

caused neurite outgrowth in the NGF-differentiated cells (Figure 1, C and D). Recombinant TS (rTS) caused neurite extension in PC12 cells (our unpublished data) and in N18 cells (Figure 1E), demonstrating that the observed activity could not be due to a contaminant that might have copurified with native TS.

The action of TS on neurons was specific to TS because other *T. cruzi* proteins such as the protease cruzipain (Murta *et al.*, 1990) and the heparin-binding penetrin (Ortega-Barria and Pereira, 1991) did not induce neurite extension when added to PC12 cells at 25, 100, and 200 ng/ml (our unpublished data). Likewise, the neuraminidase (sialidase) from the bacterium *Vibrio cholera* (VCNA) did not cause measurable neurite outgrowth in PC12 cells (our unpublished data).

TS Protects PC12 Cells from Death Caused by Trophic Factor Deprivation

In addition to regulating the differentiation and maintenance of the nervous system, neurotrophic factors are critical to the survival of neuronal cells. Depriving neuronal cells such as PC12 of neurotrophic factors results in the induction of apoptosis (Mesner *et al.*, 1992; Yao and Cooper, 1995; Pettmann and Henderson, 1998).

In an attempt to determine whether TS can rescue neurons from apoptotic death caused by serum deprivation, we grew PC12 cells in serum-free medium without and with various concentrations of TS for various times. Apoptosis was measured by counting cells with nuclear fragmentation after staining with DAPI (Figure 2A) or with antibodies to free 3′-OH termini labeled with modified nucleotides (TUNEL

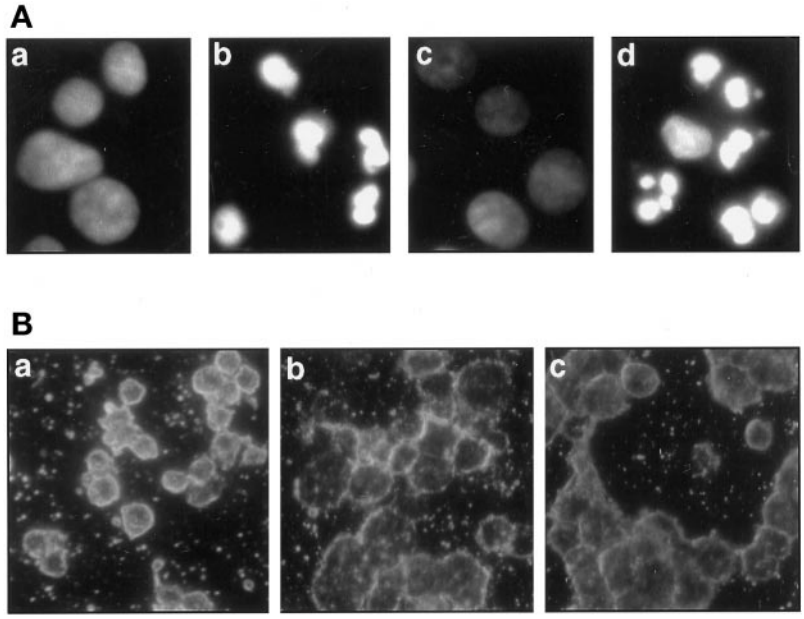

Figure 2. Inhibition of apoptosis in PC12 cells by TS. PC12 cells in serum-free RPMI were plated in collagen-coated plastic dishes in the presence of various reagents for 17 h and stained with DAPI (A) or TUNEL (B). The additions in A were (a) 0.1 μg/ml NGF; (b) no addition; (c) 0.1 μg/ml TS; and (d) 0.2 μg/ml VCNA. Magnification, 1000×. Additions in B were (a) no addition; (b) 0.1 μg/ml NGF; and (c) 0.1 μg/ml TS. Magnification, 400×.

assay; Figure 2B). We found that TS effectively promoted survival of PC12 cells in the low picomolar (nanograms per milliliter) range under conditions in which VCNA did not (Figures 2A and 3A). Dose–response analysis revealed that a TS concentration as low as 20 pM, equivalent to 4.0 ng/ml, protected 48 ± 3.3% of PC12 cells from death in serum-free medium (Figure 3A). The time course of the protection showed that ~55% of the PC12 cells kept in RPMI were apoptotic within 1 d, and almost all cells died after 3 d (Figure 3B), in agreement with published results (Mesner *et al.*, 1992). However, most PC12 cells maintained in serum-free medium supplemented with TS (0.5 nM) or NGF (4.0 nM) remained viable (i.e., without nuclear fragmentation) for at least 3 d (Figure 3B).

Identification of a TS Domain responsible for Protecting PC12 Cells from Apoptosis

The TS of trypomastigotes is composed of a Cys-rich catalytic domain of 633 amino acids in the N terminus and of a long 12-aa tandem repeat domain in the C terminus (Chuenkova *et al.*, 1999). To identify a region of the TS molecule that induces survival of neurons, we generated, by PCR, various poly-His-tagged fragments of TS DNA (Pereira *et al.*, 1991; Figure 4A), expressed them in *E. coli*, purified them on a Ni^{2+}-agarose column, and tested them for antiapoptotic activity. Fragment LTR, which corresponds to the C-terminal tandem repeat, did not protect PC12 cells from apoptosis (Figure 4A). In contrast, the enzymatically active fragment TS-F, which represents the full-length catalytic domain of TS, protected cells to about the same extent as intact TS (Figure 4, A and B). However, the inherent enzymatic activity of TS was not essential for protection, because fragment TS-F-46, generated by deleting 46 amino acids in the N terminus of fragment TS-F, was enzymatically inactive and yet as good as the native enzyme in promoting neuroprotection (Figure 4, A and B). Also, the sequence of 188 amino acids in the C terminus of the catalytic domain was apparently not required for protection, because deletion of this sequence from TS-F-46, generating fragment TS-CC-46, did not substantially reduce neuroprotective activity (Figure 4, A and B). However, a C-terminal deletion of 21 amino acids in TS-CC-46 produced fragment TS-Cat11–46, which was inactive in promoting cell survival (Figure 4, A and B). Therefore, the 21-amino-acid sequence that distinguishes fragments TS-CC-46 from TS-Cat11–46 defines one domain responsible for the TS-induced neuroprotection.

The ability of TS-derived recombinant fragments to rescue PC12 cells from apoptotic death mirrored their performance in neurite extension. For example, fragments TS-F-46 and TS-CC-46, which were active in preventing apoptosis of PC12 cells, were also active in stimulating neurite outgrowth, whereas fragment TS-Cat11–46 did not promote neuronal survival or neurite outgrowth (Figure 4A).

TS Synergizes with CNTF and LIF to Promote Survival of PC12 Cells

It is well known that neurotrophic molecules show specific synergy with other trophic molecules in stimulating neurite outgrowth and neuron survival (Ip and Yancopoulos, 1996). To test whether TS synergizes with conventional neurotrophic factors, we grew PC12 cells in serum-free medium without and with TS alone or in combination with conventional neurotrophic factors, all at concentrations that produce modest or no neuroprotective response. We first tested the combination of TS with NGF. Such coadministration did not produce substantial augmentation of neuron survival compared with the effect of individual agonists. For example, TS at 2.5 ng/ml and NGF at 0.5 ng/ml each protected ~15 and 18% PC12 cells from apoptotic death, respectively, whereas coadministration of TS and NGF at the same concentrations protected 35% of the PC12 cells. Such additive response was observed in the coadministration of other TS

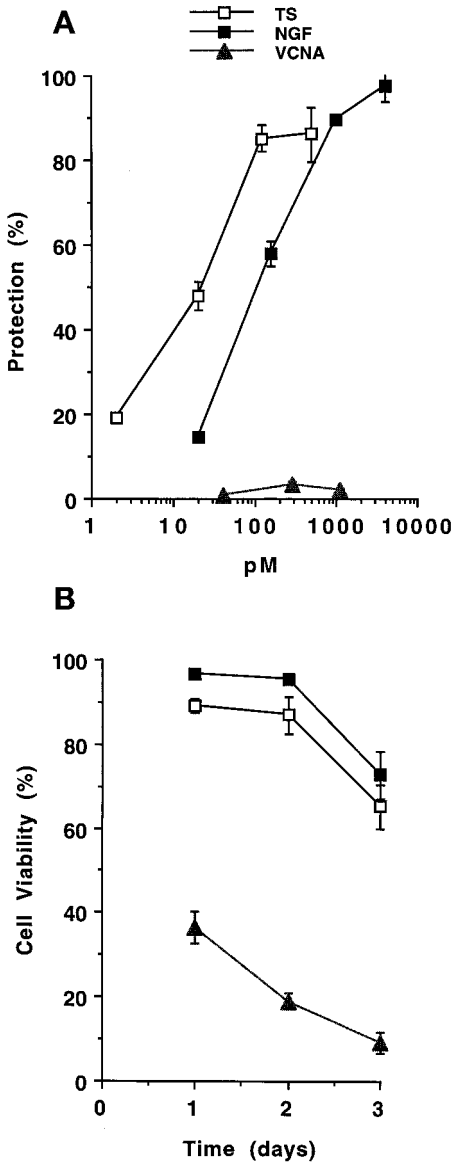

Figure 3. Dose response and kinetics of TS inhibition of apoptosis in PC12 cells. (A) Dose response. PC12 cells were grown in triplicate in serum-free RPMI in the presence of the indicated concentrations of TS, NGF, and VCNA for 17 h. Apoptosis was measured by counting cells with nuclear fragmentation after DAPI staining. Protection against apoptosis was measured by the formula described in MATERIALS AND METHODS. Data are representative of an experiment repeated three times with similar results. The molar concentration was based on a molecular mass of 200 kDa for TS, 26 kDa for NGF, and 90 kDa for VCNA. (B) Kinetics. PC12 cells in serum-free RPMI medium were grown in the absence (RPMI) and in the presence of 4.0 nM NGF and 0.5 nM TS for the times indicated. Cell viability was measured by determining the number of cells without nuclear fragmentation relative to those with nuclear fragmentation (DAPI staining) in a sample of 300–400 cells. The average of two experiments is shown, each point in triplicate.

concentrations (5, 11.5, and 30 ng/ml) with NGF (0.5 ng/ml) (our unpublished data).

Next we tested the response of PC12 cells to the combination of TS with neurotrophic factors of the IL-6 family, namely IL-6, IL-11, CNTF, LIF, and OSM (Ip and Yancopoulos, 1996). Although TS at 2.5 ng/ml promoted survival in 13% of PC12 cells grown in serum-free medium, coadministration of TS with a subthreshold concentration of CNTF (50 ng/ml) or LIF (0.5 ng/ml) dramatically increased neuron survival by 25- to 20-fold to 61 and 45%, respectively (Figure 5A). Human CNTF and recombinant human and rat CNTF were equally effective in potentiating the TS action on PC12 cells. Such synergy was also observed at the neurite outgrowth level (our unpublished data) and was specific for CNTF and LIF, because the combination of 2.5 ng/ml TS with any of the other IL-6 family members at their threshold concentrations did not substantially increase neuronal survival (Figure 5A). Dose–response experiments revealed that the TS/CNTF or LIF synergy was most striking at subthreshold or threshold concentrations of TS or LIF (Figure 5, B and C). Similar dose-response experiments of TS with the other IL-6 family members, including IL-6, did not reveal synergy in death protection (our unpublished data).

Reversal of TS-induced Neuroprotection by Inhibitors of PI-3 Kinase

Induction of survival in PC12 cells by NGF or in cerebellar granule cells by insulin-like growth factor 1 requires signaling through PI-3 kinase, as demonstrated by the use of specific pharmacological inhibitors (Yao and Cooper, 1995; D'Mello *et al.*, 1997). Wortmannin inhibits PI-3 kinase both in vitro and in vivo (Ui *et al.*, 1995). Addition of wortmannin to PC12 cells maintained for 24 h in serum-free medium supplemented with TS-F, the catalytic domain of TS (see Figure 4A), reduced neuronal viability in a dose-dependent manner (Figure 6A). This reversal was quantitatively similar to the inhibition of NGF-induced protection in PC12 cells (Figure 6A), as previously reported (Yao and Cooper, 1995).

Wortmannin also reversed protection against apoptosis induced by the coadministration of TS-F and CNTF, although to a lesser extent than that of TS-F alone (Figure 6A). For example, when PC12 cells were cotreated with wortmannin (200 nM) and TS-F or TS-F plus CNTF, neuronal viability was 57 $\pm$ 2 and 80 $\pm$ 3% of that observed with TS-F or TS-F plus CNTF without wortmannin, respectively (Figure 6A). Because CNTF signaling does not appear to require PI 3-kinase activation (Inoue *et al.*, 1996), the reduced efficiency of wortmannin to inhibit neuroprotection produced by the TS-F–CNTF combination, compared with TS-F alone, is consistent with TS signaling in PC12 cells through PI-3 kinase activation. In addition, in agreement with published results (Zhong *et al.*, 1994), we found that CNTF by itself produced little, if any, protection of PC12 cells from undergoing apoptosis in serum-free medium (Figure 6A).

The inhibition of TS-F-induced neuroprotection by wortmannin was confirmed by experiments with LY294002, another PI-3 kinase inhibitor (Vlahos *et al.*, 1994). LY294002 induced apoptosis in PC12 cells maintained in TS-F or NGF in a concentration range (Figure 6B) similar to the one effective in causing death of insulin-like growth factor 1-stimulated cerebellar granule neurons (D'Mello *et al.*, 1997). As with wortmannin, LY294002 was

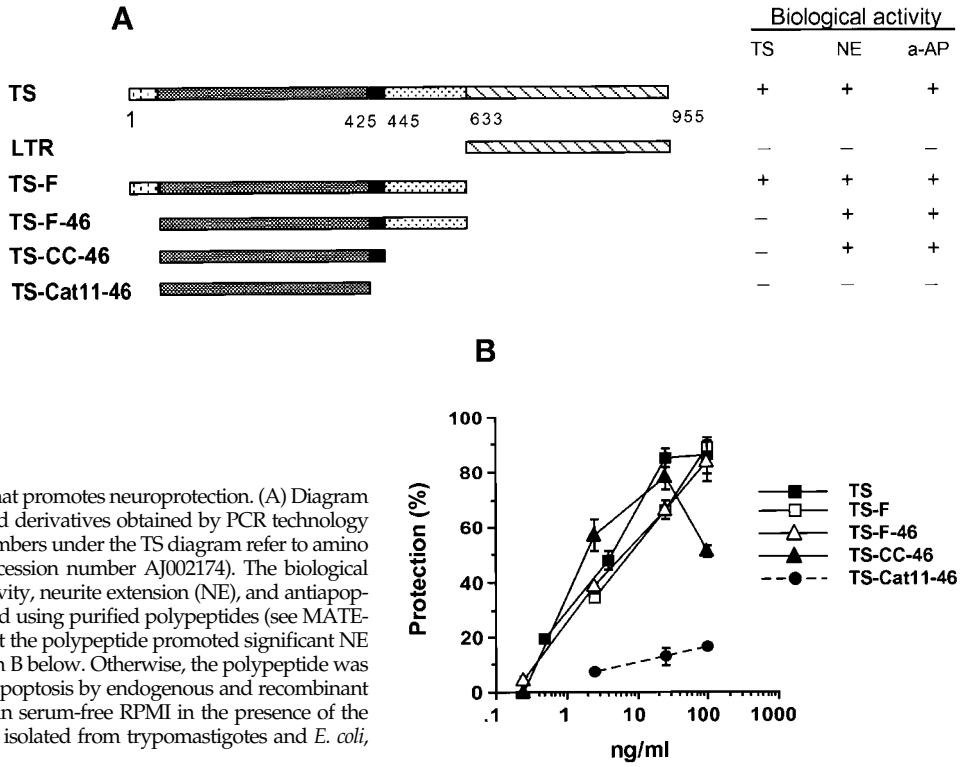

Figure 4. Identification of a TS domain that promotes neuroprotection. (A) Diagram of the linear structure of rTS and truncated derivatives obtained by PCR technology (see MATERIALS AND METHODS). Numbers under the TS diagram refer to amino acid positions in clone 19Y (GenBank accession number AJ002174). The biological activities indicated on the right are TS activity, neurite extension (NE), and antiapoptose (a-AP). These activities were measured using purified polypeptides (see MATERIALS AND METHODS). + indicates that the polypeptide promoted significant NE or a-AP in a dose-dependent manner, as in B below. Otherwise, the polypeptide was considered inactive (−). (B) Inhibition of apoptosis by endogenous and recombinant fragments of TS. PC12 cells were grown in serum-free RPMI in the presence of the indicated concentrations of TS and rTS-F, isolated from trypomastigotes and *E. coli*, respectively.

less effective in reversing the protection of TS-F plus CNTF than of TS-F only (Figure 6B).

Activation of Protein Kinase Akt by TS

Lipid products of PI-3 kinase activity directly activate the serine/threonine kinase Akt, which then becomes phosphorylated at threonine 308 and serine 473 by the protein kinase PDK1 and an unknown kinase, respectively (Downward, 1998; Franke *et al.*, 1997). Activated Akt phosphorylates the proapoptotic Bcl-2 family member BAD, preventing it from complexing with the prosurvival Bcl-2 and Bcl-x proteins, and also the Forkhead transcription factor—events leading to cell survival (Datta *et al.*, 1997; Brunet *et al.*, 1999).

To determine whether Akt is activated in response to TS, we attempted to detect Akt phosphorylation in TS-stimulated PC12 cells using an antibody specific for the Akt serine 473 epitope. The immunoblot displayed in Figure 7A shows that Akt becomes activated after a brief (2- to 5-min) exposure of PC12 cells to TS. The extent of TS-dependent Akt phosphorylation was similar to the phosphorylation produced by exposing the cells to 20% fetal calf serum (Figure 7A). The catalytic domain of TS (fragment TS-F) was as effective in activating Akt as affinity-purified natural enzyme, whereas the C-terminal tandem repeat LTR fragment of TS was not (Figure 7B), consistent with PC12 cell survival being induced by TS-F and not by LTR (Figure 4, A and B). Immunoprecipitation of TS-F-activated Akt and after in vitro kinase assay showed a dramatic increase of phosphorylation of an Akt-specific substrate, thus demonstrating induction of Akt kinase activity (Figure 7C).

Furthermore, the PI 3-kinase inhibitor LY294002 completely blocked TS-F-induced Akt phosphorylation (Figure 7B), in keeping with a role for a TS-dependent PI 3-kinase activation of Akt and analogous to the PI-3 kinase/Akt kinase activation by NGF, platelet-derived growth factor, IL-3, and other growth factors (Downward, 1998; Franke *et al.*, 1997).

TS Induces Bcl-2 Gene Expression in PC12 Cells

NGF promotes survival of PC12 cells by inducing overexpression of the antiapoptotic bcl-2 gene (Mah *et al.*, 1993; Merry and Korsmeyer, 1997; Katoh *et al.*, 1999). To determine whether TS-induced cell survival correlates with up-regulation of the Bcl-2 gene, we measured Bcl-2 transcripts by RT-PCR in PC12 cells grown in serum-free medium with or without TS. Like NGF, TS at low concentrations dramatically increased Bcl-2 mRNA (Figure 8A). These data are consistent with the notion that TS, like NGF, enhances the survival of PC12 cells by up-regulating Bcl-2 (Katoh *et al.*, 1999). The synergy between TS (2.5 ng/ml) and CNTF (50 ng/ml), which rescued PC12 cells from death, also led to a great increase in Bcl-2 gene transcripts (Figure 8B), providing further support to a cause–effect relationship between Bcl-2 expression and TS-induced survival of PC12 cells.

DISCUSSION

Two unique findings presented here may relate to the regeneration and protection of neurons in Chagas' disease. The first was that trypanosome-derived and recombinant TS

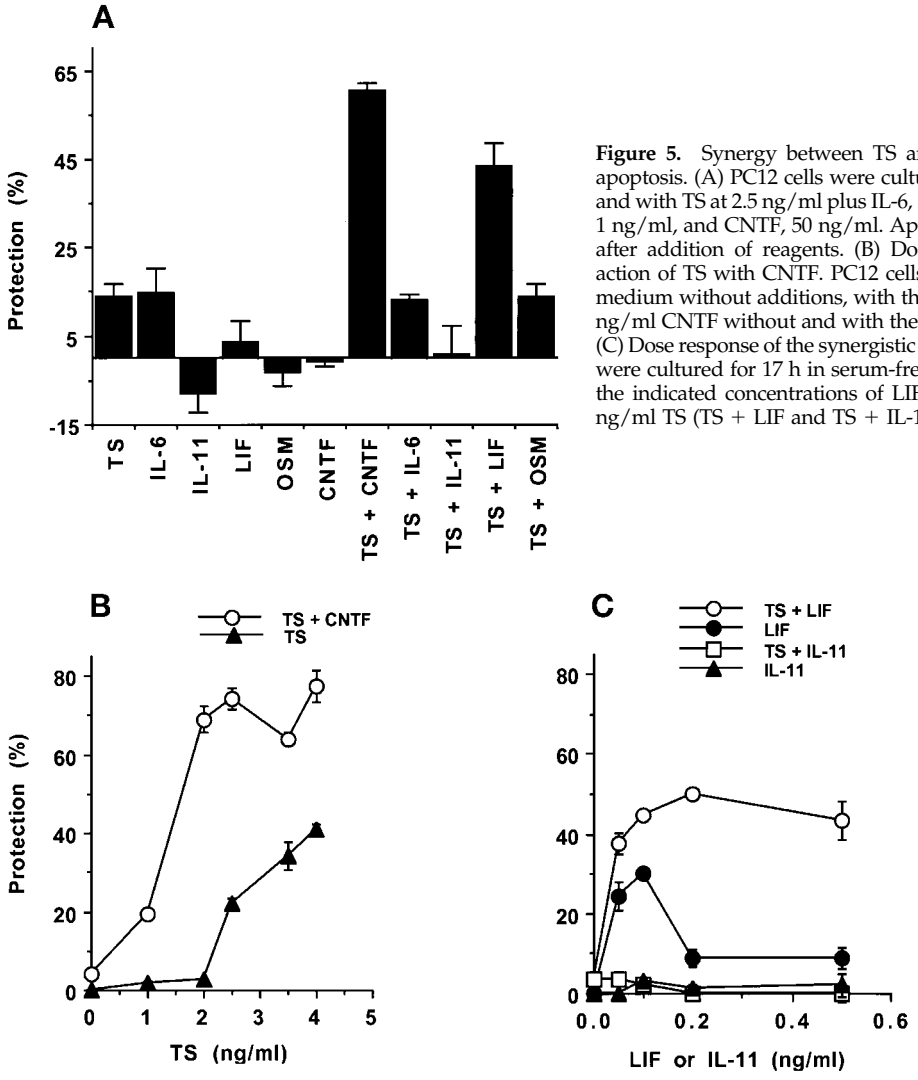

Figure 5. Synergy between TS and CNTF or LIF to rescue PC12 cells from apoptosis. (A) PC12 cells were cultured in serum-free RPMI for 17 h without TS and with TS at 2.5 ng/ml plus IL-6, 10 ng/ml, IL-11, 1 ng/ml, LIF, 1 ng/ml, OSM, 1 ng/ml, and CNTF, 50 ng/ml. Apoptosis was measured by DAPI staining 48 h after addition of reagents. (B) Dose response of the synergistic antiapoptotic action of TS with CNTF. PC12 cells were cultured for 17 h in serum-free RPMI medium without additions, with the indicated concentrations of TS, and with 50 ng/ml CNTF without and with the indicated concentrations of TS (TS + CNTF). (C) Dose response of the synergistic antiapoptotic action of TS with LIF. PC12 cells were cultured for 17 h in serum-free RPMI medium without additions and with the indicated concentrations of LIF or IL-11 without (LIF or IL-11) or with 2.5 ng/ml TS (TS + LIF and TS + IL-11, respectively).

promoted sprouting (Figure 1) and survival (Figures 2–5) of neuronal cells under conditions in which the cells would otherwise die of growth factor starvation. TS induced survival of the neuronal cell line PC12 (Figure 2) and of primary cultures of rat cerebellar granule neurons (M.V. Chuenkova and M.A. Pereira, unpublished observations). Importantly, these TS actions became evident at remarkably low concentrations, in the low picomolar range (Figures 3A and 4B). In fact, TS was more potent on a molar basis than NGF (Figure 3A). The low TS concentrations required to protect PC12 cells are easily achievable in vivo and thus likely to be physiologically relevant.

It is interesting that promotion of PC12 survival was independent of the enzymatic activity of TS, because enzymatically inactive TS polypeptides were as good as the native enzyme in protecting PC12 cells (Figure 5, A and B). TS, through its catalytic activity, is generally thought to mediate adhesion (Ming *et al.*, 1993; Schenkman *et al.*, 1993), protection against complement-mediated lysis (Tomlinson *et al.*, 1994), and other parameters of *T. cruzi* invasion (Schenkman *et al.*, 1992). But the results presented in Figure 5, A and B, suggest that, in addition to enzymatically active TS, homologues of TS lacking both neuraminidase and TS activities, which are present on multiple chromosomes of *T. cruzi* (Parodi *et al.*, 1992; Uemura *et al.*, 1992), may serve to prolong parasitism in mammalian host by promoting neuronal survival. The results with the truncated derivatives of TS also suggest that synthetic peptides modeled on the TS sequence that distinguishes fragments TS-CC-46 from TS-Cat11–46 (Figure 4, A and B) should mimic the neuroprotection of TS. Current experiments suggest that this may indeed be the case (M.V. Chuenkova and M.A. Pereira, unpublished observations).

The second unique finding was the synergy of a trypanosome protein, TS, with two mammalian cytokines, CNTF and LIF (Figure 6). In the presence of CNTF or LIF, TS significantly promoted neuronal survival at concentrations otherwise ineffective. Although the collaboration of a human cytokine (i.e., CNTF and LIF) with a ligand from a pathogenic microbe (i.e., the *T. cruzi* neuraminidase) is a novelty,

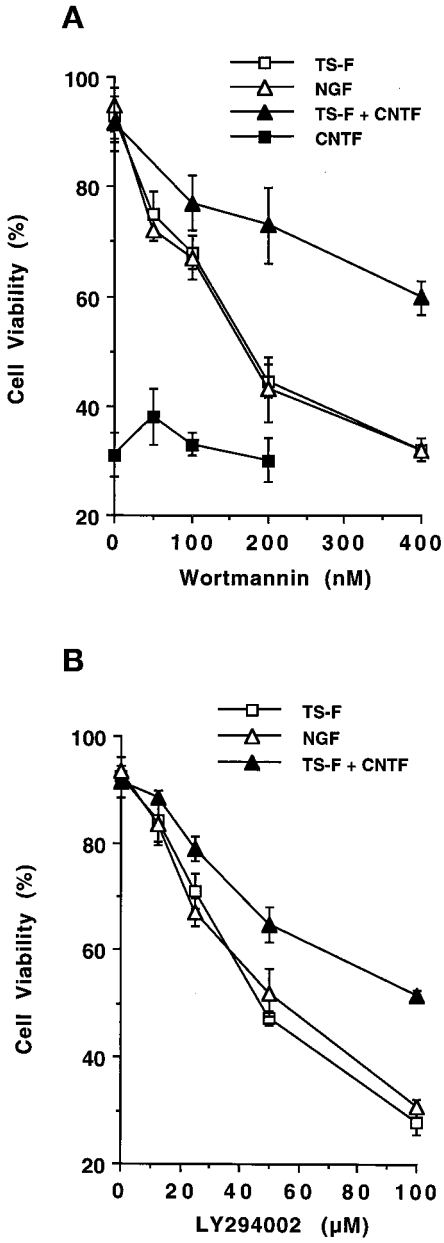

Figure 6. Reversal of the survival-promoting activity of TS by the PI-3 kinase inhibitors. PC12 cells were switched to serum-free RPMI without and with TS-F (100 ng/ml), NGF (100 ng/ml), CNTF (50 ng/ml), and TS-F (2.5 ng/ml) plus CNTF (50 ng/ml). After 24 h, wortmannin (A) or LY294002 (B) was added to the cultures at the indicated concentrations. Neuronal viability was measured by DAPI staining 24 h after addition of wortmannin or LY294002. Viability of PC12 cells in RPMI alone (33%) was similar to the viability of the cells in RPMI 1640 plus CNTF. Values are from a representative experiment (each point in triplicate) repeated three times, with similar results.

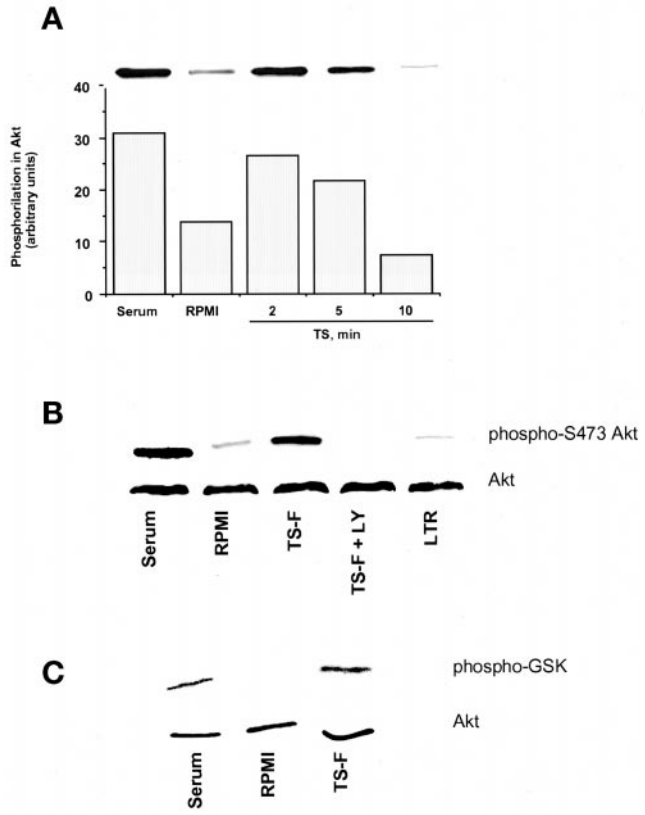

Figure 7. Activation of signaling pathways in PC12 cells by TS. (A) TS induces serine phosphorylation of Akt kinase. PC12 cells were kept in RPMI containing 0.1% FCS for 2 d. Then some monolayers were switched to 20% FCS (Serum) and others to RPMI without (RPMI) and with 100 ng/ml TS (TS) for the indicated times. Cells were lysed in 2% SDS, and proteins in the lysate were resolved by SDS-PAGE on 10% gels and stained with anti-phospho-Akt antibody. The bar graph represents the quantitation by scanning densitometry of the corresponding bands in the immunoblot (inset). (B) TS-induced phosphorylation of Akt kinase is inhibited by the PI-3 kinase inhibitor LY294002. The protocol was similar to the one in A, except that, where indicated, cells were preincubated with 1 μM LY294002 (TS-F + LY) before the addition of 100 ng/ml catalytic domain of TS (TS-F). Note that LY294002 completely blocked TS-F-induced phosphorylation of Akt kinase. LY294002 was similarly effective in inhibiting Akt phosphorylation induced by full-length TS (our unpublished data). (C) TS-F induces PKB/Akt kinase activity. PC12 cells were treated as described in A. Then some were switched to 20% FCS (Serum) and others to RPMI without (RPMI) and with 100 ng/ml TS-F (TS-F) for 2 min. Cell lysates were immunoprecipitated with Akt antibodies coupled to agarose beads The resulting immunoprecipitates were incubated with GSK-3 fusion protein as an Akt kinase substrate. Phosphorylation of GSK-3 was measured by Western blotting using a phospho-GSK-3α/β (Ser-21/9) antibody.

the synergism of CNTF with conventional neurotrophic factors is not. For example, it is known that CNTF collaborates with NGF to enhance neurite outgrowth in PC12 cells (Zhong *et al.*, 1994) and with BNDF to arrest motor neuron disease in *wobbler* mice (Mitsumoto *et al.*, 1994) in a cross-talk pattern common among neurotrophic factors (Ip and Yancopoulos, 1996). Thus, given that TS triggers signals, at least in the cell lines PC12 and N18 (Figure 1–7) and in primary cultures of rat cerebellar granule neurons (M.V. Chuenkova and M.A. Pereira, unpublished observations), it is not en-

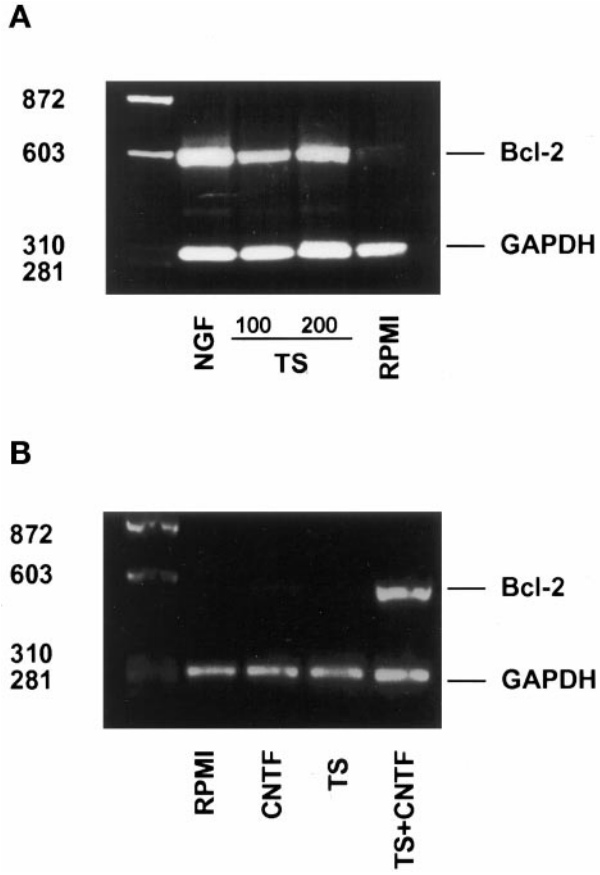

Figure 8. Induction of Bcl-2 Expression in PC12 cells. Bcl-2 expression in total RNA was isolated 17 h after addition of the growth factors and was assessed by RT-PCR using primers specific for Bcl-2 and for control GAPDH. Molecular weight markers (in base pairs) are indicated. (A) Bcl-2 expression in PC12 cells cultured in serum-free medium without (RPMI) and with NGF (100 ng/ml) or TS (100 and 200 ng). (B) Bcl-2 expression induced by TS (2.5 ng/ml) plus CNTF (50 ng/ml). Bcl-2 transcripts were not detected when TS (2.5 ng/ml) and CNTF (50 ng/ml) were added separately to PC12 cells in serum-free medium (RPMI).

tirely surprising that the *T. cruzi* neuraminidase synergized with the cytokines CNTF and LIF.

CNTF and LIF are IL-6 family members that promote survival of several types of neurons, including hippoccampal neurons in the CNS (Ip and Yancopoulos, 1996) and motor neurons (Arakawa *et al.*, 1990) in the PNS. CNTF plays a major role in the response of the nervous system to injury, as exemplified by the CNTF-induced prevention of motor neuron degeneration after axotomy and by the dramatic reduction of clinical symptoms in mice with progressive motor neuropathy (Sendtner *et al.*, 1992). Although a null mutation of the CNTF gene in humans does not seem to be associated with neurological diseases (Takahashi *et al.*, 1994), disruption of the CNTF gene in mice results in motor neuron degeneration (Masu *et al.*, 1993). CNTF is synthesized in the neuroglia by Schwann cells in the PNS and astrocytes in the CNS. After nerve injury CNTF is released from the cytoplasm of the glial cells into the extracellular environment, where it may initiate the repair process by binding to a protein-tyrosine kinase receptor on the neuron surface (Sendtner *et al.*, 1994; Ip and Yancopoulos, 1996).

The cellular sources of CNTF, Schwann cells and astrocytes, are precisely the cells most susceptible to *T. cruzi* invasion in the nervous system. These glial cells, but not neurons, are frequently loaded with the dividing form of *T. cruzi* (amastigote) in experimental models of, and in humans with, acute Chagas' disease (Tafuri, 1970; Brown and Voge, 1982). Schwann cells and astrocytes may release CNTF after a *T. cruzi* insult, which surely happens in the intracellular cycle of the parasite (Pereira, 1994; Burleigh and Andrews, 1995). On the other hand, the invasive trypomastigote, a major source of neuraminidase (Pereira, 1983), releases copious amounts of the enzyme into the extracellular environment in vitro or in vivo (Cavalesco and Pereira, 1988; de Titto and Araujo, 1988). Thus, CNTF and TS will most likely coexist in the vicinity of glial cells, where they could collaborate to trigger antiapoptotic events in nearby neurons. CNTF and TS do not need to diffuse far to reach a neuron, which is separated from the glial cells by a very short distance, ~20 nm in the case of astrocytes (Bear *et al.*, 1996).

The molecular basis of TS-induced neuroprotection in not understood, but it may well be through a mechanism dependent on activation of the PI 3-kinase and Akt kinase signaling pathway, as judged by pharmacological, biochemical, and enzymatic evidence. Inhibitors of PI 3-kinase effectively blocked TS-induced death protection in PC12 cells (Figure 6). In addition, TS induced phosphorylation of Akt kinase at Ser-473, an effect inhibited by the PI 3-kinase inhibitor LY294002 (Figure 7). Also, the TS N-terminal domain that induced neuronal survival (TS-F), but not the C-terminal domain (LTR) that was ineffective in promoting survival (Figure 4), was precisely the polypeptide fragment that activated Akt of PC12 cells (Figure 7, B and C). These findings are significant because activation of PI 3-kinase leads to cell survival through the activation of Akt kinase, which then phosphorylates and deactivates the proapoptotic Bcl-2 family member BAD (Datta *et al.*, 1997; Franke *et al.*, 1997) and the Forkhead transcription factor (Brunet *et al.*, 1999).

Given that TS-induced neuroprotection was additive with NGF and synergistic with the cytokines CNTF and LIF, it is tempting to speculate that TS recognizes receptors akin to those for NGF. However, this may not be the case, because TS also activates the PI-3K/Akt pathway in cells that are unresponsive to NGF, such as fibroblasts (Vero cells) and epithelial cells (mink lung epithelial Mv1Lu cells) (M.C. Chuenkova and M.A. Pereira, unpublished observations). Thus, at present the nature of the TS receptor(s) triggering survival of neuronal cells remains speculative.

Nevertheless, on the basis of the results presented here, we propose an experimentally testable model, in which TS reacts with a yet-to-be-determined receptor protein on PC12 cells, cerebellar granule neurons, and perhaps other neurons, to activate kinases such as PI3K and Akt, leading to deactivation of proapoptotic and induction of the prosurvival members of the Bcl-2 family. This hypothesis is based on current concepts of protection against apoptosis, in which a link exists between PI3K/Akt activation and Bcl-2 gene expression (Downward, 1998; Ahmed *et al.*, 1997; Franke *et al.*, 1997; Brunet *et al.*, 1999; and Sonoda *et al.*, 1999).

Finally, the results presented here raise the intriguing possibility that the neuroprotective actions of TS, particularly the collaboration with CNTF and LIF, may facilitate the design of compounds to prevent or treat Chagas' disease. TS or an active synthetic peptide, alone or together with CNTF or LIF, could be used to accelerate neuronal regeneration in the acute to indeterminate phase transition of Chagas' disease. Likewise, it may be possible that administration of TS/CNTF or LIF will reduce neuronal degeneration during progression to the chronic phase. Such an approach would provide the opportunity to prevent or delay pathological manifestations rather than just treating symptoms.

In addition, the TS/CNTF or LIF combination may be exploited to develop strategies for the treatment of other types of motor neuron diseases, particularly those responsive to CNTF. Because administration of CNTF and LIF alone produces undesirable side effects, such as cachexia, anorexia, and muscle atrophy (Miller *et al.*, 1996), the TS/CNTF combination would be particularly useful if it lowered the threshold response of motor neurons to CNTF.

ACKNOWLEDGMENTS

We thank Lewis Cantley for advice on the Akt experiments, Jacqueline Sharon, Barbara Talamo, and James Wang for reviewing the manuscript and for many useful suggestions, Wenda Gao for the LTR fragment, Arthur Tischler and Geoffrey Cooper for the PC12 cells, Julio Scharfstein for the purified cruzipain, Macario Herrera for the purified penetrin, and Anne Kane (GRASP Center, National Institute of Diabetes and Digestive and Kidney Diseases grant P30DK-34928) for the *E. coli* expressing rTS-F. This work was supported by National Institutes of Health grant AI-18102. Dedicated to Prof. José Murilo Martins, a superb and compassionate physician–scientist and an inspiring teacher at the Faculdade de Medicina da Universidade Federal do Ceará (Ceará, Brazil) on the occasion of his 70th birthday.

REFERENCES

Adad, S.J., Andrade, D.C., Lopes, E.R., and Chapadeiro, E. (1991). Pathological anatomy of chagasic megaesophagus. Rev. Inst. Med. Trop. São Paulo *33*, 443–450.

Ahmed, N.N., Grimes, H.L., Bellacosa, A., Chan, T.O., and Tsichlis, P.N. (1997). Transduction of interleukin-2 antiapoptotic and proliferative signals via Akt protein kinase. Proc. Natl. Acad. Sci. USA *94*, 3627–3632.

Andrade, Z.A. (1983). Mechanisms of myocardial damage in *Trypanosoma cruzi* infection. Ciba Found Symp. *99*, 214–233.

Arakawa, Y., Sendtner, M., and Thoenen, H. (1990). Survival effect of ciliary neurotrophic factor (CNTF) on chick embryonic motorneurons in culture: comparison with other neurotrophic factors and cytokines. J. Neurosci. *10*, 3507–3515.

Bear, M., Connors, B.W., and Paradiso, M.A. (1996). Neurons and glia. In: Neuroscience: Exploring the Brain, ed. T.S. Satterfield, Baltimore: Williams & Wilkins, 22–45.

Brown, W.J., and Voge, M. (1982). Trypanosomiasis. In: Neuropathology of Parasitic Infections, Oxford: Oxford University Press, 60–85.

Brunet, A., Bonni, A., Zigmond, M.J., Lin, M.Z., Juo, P., Hu, L.S., Anderson, M.J., Arden, K.C., Blenis, J., and Greenberg, M.E. (1999). Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell *96*, 857–868.

Burleigh, B.A., and Andrews, N.W. (1995). The mechanisms of *Trypanosoma cruzi* invasion of mammalian cells. Annu. Rev. Microbiol. *49*, 175–200.

Cavalesco, R., and Pereira, M.E.A. (1988). Antibody to *Trypanosoma cruzi* neuraminidase enhances infection in vitro and identifies a subpopulation of trypomastigotes. J. Immunol. *140*, 617–625.

Chagas, C. (1916). Trypanosomíase Americana; forma aguda da molestia. Mem. Inst. Oswaldo Cruz *8*, 37–60.

Chuenkova, M., and Pereira, M.E.A. (1995). *Trypanosoma cruzi trans*-sialidase: enhancement of virulence in a murine model of Chagas' disease. J. Exp. Med. *181*, 1693–1703.

Chuenkova, M., Pereira, M., and Taylor, G. (1999). Trans-sialidase of *Trypanosoma cruzi*: location of galactose-binding site(s). Biochem. Biophys. Res. Commun. *262*, 549–556.

Datta, S.R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg. M.E. (1997). Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell *91*, 231–241.

de Titto, E., and Araujo, F.G. (1988). Serum neuraminidase activity and hematological alterations in acute human Chagas' disease. Clin. Immunol. Immunopathol. *46*, 157–151.

D'Mello, S.R., Borodez, K., and Soltoff, S.P. (1997). Insulin-like growth factor and potassium depolarization maintain neuronal survival by distinct pathways: possible involvement of PI-3 kinase in IGF-1 signaling. J. Neurosci. *17*, 548–1560.

Downward, J. (1998). Mechanisms and consequences of activation of protein kinase B/Akt. Curr. Opin. Cell Biol. *10*, 262–267.

Franke, T.F., Kaplan, D.R., and Cantley, L.C. (1997). PI3K: downstream AKTion blocks apoptosis. Cell *88*, 435–437.

Gavrieli, Y., Sherman, Y., and Ben-Sasson, S.A. (1992). Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J. Cell Biol. *119*, 493–501.

Greene, L.A., Drexler, S.A., Connolly, J.L., Rukenstein, A., and Green, S. (1986). Selective inhibition of responses to nerve growth factor and of microtubule-associated protein phosphorylation by activators of adenylate cyclase. J. Cell Biol. *103*, 1967–1978.

Greene, L.A., and Tischler, A.S. (1976). Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc. Natl. Acad. Sci. USA *73*, 2424–2428.

Inoue, M., Nakayama, C., and Noguchi, H. (1996). Activating mechanism of CNTF and related cytokines. Mol. Neurobiol. *12*, 195–209.

Ip, N.Y., and Yancopoulos, G.D. (1996). The neurotrophins and CNTF: two families of collaborative neurotrophic factors. Annu. Rev. Neurosci. *19*, 491–515.

Katoh, S., Mitsui, Y., Kitani, K., and Suzuki, T. (1999). Hyperoxia induces the neuronal differentiation phenotype of PC12 cells via a sustained activity of mitogen-activated protein kinase induced by Bcl-2. Biochem. J. *338*, 465–470.

Köberle, F. (1968). Chagas' disease and Chagas' syndromes: the pathology of American trypanosomiais. Adv. Parasitol. *6*, 63–71.

Losavio, A., Jones, M.C., Sanz, O.P., Mirkin, G., Gonzalez Cappa, S.M., Muchnik, S., and Sica, R.E. (1989). A sequential study of the peripheral nervous system involvement in experimental Chagas' disease. Am. J. Trop. Med. Hyg. *41*, 539–547.

Machado, C.R., Gomez, M.V., and Machado, A.B. (1987). Changes in choline acetyltransferase activity of rat tissues during Chagas' disease. Braz. J. Med. Biol. Res. *20*, 697–702.

Mah, S.P., Zhong, L.T., Liu, Y., Roghani, A., Edwards, R.H., and Bredesen, D.E. (1993). The protooncogene bcl-2 inhibits apoptosis in PC12 cells. J. Neurochem. *60*, 1183–1186.

Marti, D., Schaller, J., Ochensberger, B., and Rickli, E.E. (1994). Expression, purification and characterization of the recombinant kringle 2 and kringle 3 domains of human plasminogen and analysis of their binding affinity for the ω-aminocarboxylic acids. Eur. J. Biochem. *219*, 455–462.

Masu, Y., Wolf, E., Holtmann, B., Sendtner, M., Brem, G., and Thoenen, H. (1993). Disruption of the CNTF gene results in motor neuron degeneration. Nature *365*, 27–32.

Meciano Filho, J., Carvalho, V.C., and de Souza, R.R. (1995). Nerve cell loss in the myenteric plexus of the human esophagus in relation to age: a preliminary investigation. Gerontology *41*, 18–21.

Merry, D.E., and Korsmeyer, S.J. (1997). Bcl-2 gene family in the nervous system. Annu. Rev. Neurosci. *20*, 245–267.

Mesner, P.W., Winters, T.R., and Green, S.H. (1992). Nerve growth factor withdrawal-induced cell death in neuronal PC12 cells resembles that in sympathetic neurons. J. Cell. Biol. *119*, 1669–1680.

Miller, R.G., Bryan, W.W., Dietz, M.A., Munsat, T.L., Petajan, J.H., Smith, S.A., and Goodpasture, J.C. (1996). Toxicity and tolerability of recombinant human ciliary neurotrophic factor in patients with amyotrophic lateral sclerosis. Neurology *47*, 1329–1331.

Ming, M., Chuenkova, M., Ortega-Barria, E., and Pereira, M.E.A. (1993). Mediation of *Trypanosoma cruzi* invasion by sialic acid on the host cell and trans-sialidase on the trypanosomes. Mol. Biochem. Parasitol. *59*, 243–252.

Mitsumoto, H., Ikeda, K., Klinkosz, B., Cedarbaum, J.M., Wong, V., and Lindsay, R.M. (1994). Arrest of motor neuron disease in wobbler mice co-treated with CNTF and BDNF. Science *265*, 1107–1110.

Murta, A.C., Persechini, P.M., Padron, T.d.S., de Souza, W., Guimarães, J.A., and Scharfstein, J. (1990). Structural and functional identification of GP57/51 antigen of *Trypanosoma cruzi* as a cysteine proteinase. Mol. Biochem. Parasitol. *43*, 27–38.

Oliveira, J.S.M., Monteiro dos Santos, J.C., Muccillo, G., Ferreira, A.L., and Preto, R. (1985). Increased capacity of coronary arteries in chronic Chagas' heart disease: further support for the neurogenic pathogenesis concept. Am. Heart J. *109*, 304–308.

Ortega-Barria, E., and Pereira, M.E.A. (1991). A novel *T. cruzi* heparin-binding protein promotes fibroblast adhesion and penetration of engineered bacteria and trypanosomes into mammalian cells. Cell *67*, 411–421.

Parodi, A.J., Pollevick, G.D., Mautner, M., Buschiazzo, A., Sanchez, D.O., and Frasch, A.C.C. (1992). Identification of the gene(s) coding for the trans-sialidase of *Trypanosoma cruzi*. EMBO J. *11*, 1705–1710.

Pereira, M. (1994). Intracytosolic survival of *Trypanosoma cruzi*. In: Baillière's Clinical Infectious Diseases, ed. D. Russell, London: Baillière Tindall, 305–334.

Pereira, M.E.A. (1983). A developmentally regulated neuraminidase activity in *Trypanosoma cruzi*. Science *219*, 1444–1447.

Pereira, M.E.A., Mejia, J.S., Ortega-Barria, E., Matzilevich, D., and Prioli, R.P. (1991). The *Trypanosoma cruzi* neuraminidase contains sequences similar to bacterial neuraminidases, YWTD repeats of the low-density lipoprotein receptor, and type III modules of fibronectin. J. Exp. Med. *174*, 179–191.

Pettmann, B., and Henderson, C.E. (1998). Neuronal cell death. Neuron *20*, 633–647.

Prasad, K.N. (1991). Differentiation of neuroblastoma cells: a useful model for neurobiology and cancer. Biol. Rev. Camb. Philos. Soc. *4*, 431–451.

Saavedra, E., Herrera, M., Gao, W., Uemura, H., and Pereira, M.A. (1999). The *Trypanosoma cruzi* trans-sialidase, through its COOH-terminal tandem repeat, up-regulates interleukin-6 secretion in normal human intestinal microvascular endothelial cells and peripheral blood mononuclear cells. J. Exp. Med. *190*, 1825–1836.

Said, G., Joskowicz, M., Barreira, A.A., and Eisen, H. (1985). Neuropathy associated with experimental Chagas' disease. Ann. Neurol. *18*, 676–683.

Schenkman, R.O.S., Vandekerckhove, F., and Schenkman, S. (1993). Mammalian cell sialic acid enhances *Trypanosoma cruzi* invasion. Infect. Immun. *61*, 898–902.

Schenkman, S., Eichinger, D., Pereira, M.E.A., and Nussenzweig, V. (1994). Structural and functional properties of *Trypanosoma* trans-sialidase. Annu. Rev. Microbiol. *48*, 499–523.

Schenkman, S.L., de Carvalho, P., and Nussenzweig, V. (1992). *Trypanosoma cruzi* trans-sialidase and neuraminidase activities can be mediated by the same enzyme. J. Exp. Med. *175*, 567–575.

Scudder, P., Doom, J.P., Chuenkova, M., Manger, I.D., and Pereira, M.E.A. (1993). Enzymatic characterization of the 36-D-galactoside α2,3-trans-sialidase from *Trypanosoma cruzi*. J. Biol. Chem. *268*, 9886–9891.

Sendtner, M., Carroll, P., Holtmann, B., Hughes, R.A., and Thoenen, H. (1994). Ciliary neurotrophic factor. J. Neurobiol. *25*, 1436–1453.

Sendtner, M., Schmalbruch, H., Stockli, K.A., Carroll, P., Kreutzberg, G.W., and Thoenen, H. (1992). Ciliary neurotrophic factor prevents degeneration of motor neurons in mouse mutant with progressive motor neuropathy. Nature *358*, 502–504.

Sonoda, Y., Watanabe, S., Matsumoto, Y., Aizu-Yokota, E., and Kasahara, T. (1999). FAK is the upstream signal protein of the phosphatidylinositol 3-kinase-Akt survival pathway in the hydrogen peroxide-induced apoptosis of a human glioblastoma cell line. J. Biol. Chem. *274*, 10566–10570.

Tafuri, W.L. (1970). Pathogenesis of lesions of the autonomic nervous system of the mouse in experimental acute Chagas disease. Am. J. Trop. Med. Hyg. *19*, 405–417.

Takahashi, R., Yokoji, H., Misawa, H., Hayashi, M., Hu, J., and Deguchi, T. (1994). A null mutation in the human CNTF gene is not casually related to neurological diseases. Nat. Genet. *7*, 79–84.

Tomlinson, S., Pontes de Carvalho, L.C., Vandekerckhove, F., and Nussenzweig, V. (1994). Role of sialic acid in the resistance of *Trypanosoma cruzi* trypomastigotes to complement. J. Immunol. *153*, 3141–3147.

Uemura, H., Schenkman, S., Nussenzweig, V., and Eichinger, D. (1992). Only some members of a gene family of *Trypanosoma cruzi* encode proteins that express both trans-sialidase and neuraminidase activities. EMBO J. *11*, 3837–3844.

Ui, M., Okada, T., Hazeki, K., and Hazeki, O. (1995). Wortmannin as a unique probe for an intracelluar signaling protein, phosphoinositide 3-kinase. Trends Pharmacol. *20*, 303–307.

Van Voorhis, W.C., and Eisen, H. (1989). Fl-160: a surface antigen of *Trypanosoma cruzi* that mimics mammalian nervous tissue. J. Exp. Med. *169*, 641–648.

Vlahos, C.J., Matter, W.F., Hui, K.Y., and Brown, R.F. (1994). A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). J. Biol. Chem. *269*, 5241–5248.

Wong, H., Anderson, W.D., Cheng, T., and Riabowol, K.T. (1994). Monitoring mRNA expression by polymerase chain reaction: the "primer-dropping" method. Anal. Biochem. *223*, 251–258.

Yao, R., and Cooper, G.M. (1995). Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. Science *267*, 2003–2006.

Zhong, W., Zaheer, A., and Lim, R. (1994). Ciliary neurotrophic factor (CNTF) enhances the effects of nerve growth factor on PC12 cells. Brain Res. *661*, 56–62.