Chagas’ disease parasite promotes neuron survival and differentiation through TrkA nerve growth factor receptor

Marina V. Chuenkova and Mercio PereiraPerrin

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

Abstract

TrkA is a receptor tyrosine kinase activated primarily by nerve growth factor (NGF) to regulate differentiation, survival, and other important functions of neurons. Given the critical role TrkA plays in neural maintenance, it may be that microbial invaders of the nervous system utilize this receptor to reduce tissue damage for maximizing host-parasite equilibrium. Candidate pathogens could be those, like *Trypanosoma cruzi*, which may produce relatively little brain or nerve damage in long-lasting infections. We show here that *T. cruzi*, via its neuraminidase, binds TrkA in a NGF-inhibitable manner, induces TrkA autophosphorylation, and, through TrkA-dependent mechanisms, triggers phosphatidylinositol 3-kinase (PI3K)/Akt kinase signaling, cell survival, and neurite outgrowth. Unlike NGF, the neuraminidase does not react with the apoptosis-causing pan-neurotrophin receptor p75NTR. Therefore, these studies identify a novel and unique TrkA ligand in a microbial invader of the nervous system, raising the thus far unsuspected prospect of TrkA underlying clinical progression of an important human infectious disease.

Keywords: Akt kinase, NGF, PDNF, receptor tyrosine kinase, *Trypanosoma cruzi*.


Chagas’ disease, caused by infection of *Trypanosoma cruzi*, is a major cause of cardiac and gastrointestinal (GI) morbidity and mortality in the Western hemisphere. Although patients who die in the chronic stage usually bear degenerated ganglia in their heart and GI tract, the pathogenesis and time-course of the disease suggest nevertheless the existence of neuroregenerative events. In acute disease, *T. cruzi* growth may seriously damage the invaded tissues. However, despite harboring virulent parasites in their CSF, most acute Chagasic patients do not present symptoms and physical signs of brain infection (Hoff et al. 1978; Dias 1984). Histological examination of the brain from patients who died with acute Chagas’ disease reveals morphologically preserved neurons even when they are located within foci of inflammatory cells and adjacent to *T. cruzi* nests (Pittella 1993). Similar evidence of neuroprotection is observed in animals acutely infected with *T. cruzi* (Tafuri 1970; Said et al. 1985; Losavio et al. 1989). Signs of neuroregeneration are also present in patients who progress to chronic indeterminate Chagas’ disease, which may remain asymptomatic for years or decades. For example, neuron counts in both GI and heart ganglia may increase with the age of patients in a trend counter to the age-related physiological reduction in ganglia cells in normal, non-Chagasic individuals (Koberle 1968). Likewise, animals chronically infected with *T. cruzi* show signs of neuroregeneration such as sprouting in sympathetic and parasympathetic nerve fibers in the heart and colon, regenerating fibers in the sciatic nerve, and increased neurotransmitter production (Mott and Hagstrom 1965; Machado et al. 1987). One explanation for neuroregeneration in Chagas’ disease is that *T. cruzi* invasion stimulates the host to release neurotrophic factors, and/or that the parasite itself expresses mimetics of host survival factors.

The hypothesis that *T. cruzi* codes for molecule(s) that stimulate neuroregeneration gained experimental support recently with the discovery that a parasite surface-bound...
and shed neuraminidase (Pereira 1983), also known as trans-sialidase (Schenkman et al. 1994), protects neuronal and glial cells against induced apoptosis through up-regulation of anti-apoptotic bcl-2 gene and activation of PI3K/Akt kinase signaling (Chuenkova and Pereira 2000; Chuenkova et al. 2001). It also stimulates neurite outgrowth in PC12 cells via a mechanism dependent on the sustained activation of the mitogen-activated protein kinase (MAPK)/Erk1/2 kinase cascade (Chuenkova and Pereira 2001). Similar to host growth factors, the neuraminidase can neutralize neurotoxicity of the Parkinsonism-inducing neurotoxin 1-methyl-4-phenylpyridinium for dopaminergic cells (Chuenkova and Pereira 2003). Stimulation of cell survival and differentiation is independent of glycosidase and glycosyl transferase activities of the neuraminidase (Chuenkova and Pereira 2000). Thus, T. cruzi neuraminidase is a parasite-derived mimic of neurotrophic factors (PDNF) (Chuenkova and Pereira 2003).

We show here that, in neuronal cell lines and primary cultures of hippocampal neurons, T. cruzi and PDNF bind and activate TrkA tyrosine kinase, the receptor for the neurotrophins NGF and, to a lesser extent, neurotrophin-3 (NT-3). TrkA regulates vertebrate cell survival, proliferation, axon and dendrite growth and patterning, expression and activity of ion channels and neurotransmitter receptors, and synaptic strength and plasticity (Sofroniew et al. 2001). The results therefore implicate TrkA in T. cruzi-induced neuroprotection. However, we were unable to demonstrate binding of PDNF to the pan-neurotrophin receptor p75NTR, whose biological activities are in some cases synergistic and in other cases antagonistic to those of Trk receptors (Dechant and Barde 2002). The idea that T. cruzi exploits TrkA functions for tissue invasion may reflect a general but so far unsuspected mechanism of host–parasite interactions.

Materials and methods

Materials

PC12<sup>muta</sup> and PC12<sup>αkA</sup> cells were a gift from Dr L. Greene (College of Physicians and Surgeons, Columbia University, NY, USA). ST14A cells were obtained from Dr E. Cattaneo (Institute of Pharmacological Sciences, Milan, Italy). Primary cultures of rat hippocampal neurons were a gift of Dr A. Krantis (QBM Cell Science, Ottawa, Canada). Hoehst 33342, propidium iodide (PI), 4′, 6-diamidino-2-phenylindole (DAPI), nerve growth factor (NGF) and epidermal growth factor (EGF) were from Sigma (St. Louis, MO, USA). Alexa Fluor 594 Protein Labeling kit and Alexa-conjugated anti-mouse antibody were from Molecular Probes (Eugene, OR, USA). Other antibody: anti-trkA (N-terminus specific) from Upstate Biotechnology (Lake Placid, NY, USA) and C-terminus-specific from Santa Cruz Biotechnology (La Jolla, CA, USA), as well as anti-p75<sup>NTR</sup>; anti-P-TrkA (Tyr 490) and P-Akt (Ser473) from Cell Signaling Technology (Beverly, MA, USA), and HRP-conjugated secondary antibody from Chemicon (Tenecula, CA, USA). ECL kit was from PerkinElmer (Life Sciences, Boston, MA, USA). G-Sepharose and streptavidin-Sepharose were from Amersham Biosciences (Piscataway, NJ, USA). HRP-streptavidin and anti-protease cocktail were obtained from Roche Molecular Biochemicals (Indianapolis, IN, USA).

Cell and parasite cultures, cell staining

PC12<sup>muta</sup> and PC12<sup>αkA</sup> cells were grown in RPMI with fetal calf serum (FCS) and horse serum as described earlier (Green et al. 1986; Loeb and Greene 1993). For neurite extension experiments cells were plated at 1 × 10<sup>5</sup> cells/mL on LabTech collagen-coated 16-well slides (Nalge Nunc International, Naperville, IL, USA) in 1% FCS-containing RPMI with or without PDNF, 200 ng/mL, for 24 h. ST14A cell line is derived from the striatum of day 14 (E14) rat embryo by retroviral transduction (Cattaneo and Conti 1998). Cells were grown on DMEM with 10% FCS at 33°C. Rat (E18, 19) hippocampal neurons (QBM Cell Science, Ottawa, Canada) were maintained in Neurobasal medium supplemented with B27 (Invtrogen, Carlsbad, CA, USA). Five days after plating neurons on collagen-coated wells, cells were treated with PDNF (200 ng/mL) or NGF (50 ng/mL) for 10 min, fixed with 4% paraformaldehyde, blocked with 10% goat serum in PBS and probed with P-trkA (Tyr 490) antibody overnight at 4°C. Neurons were stained with Alexa-conjugated anti-mouse antibody and analyzed in an inverted fluorescent microscope. T. cruzi, Tulahuen strain C2, was maintained in Vero cells as previously described (Belen Carrillo et al. 2000). For infection, PC12 cells were plated in growth medium at 3 × 10<sup>4</sup> cells/mL, and after 16 h, growth medium replaced with serum-free medium containing various concentrations of parasites. To identify live and dead PC12 cells 48 h post-infection, serum-free medium was replaced with growth media containing Hoechst 33342 (20 μg/mL) or propidium iodide (PI) 10 μg/mL. After 5 min, the cells were visualized under ultraviolet irradiation at 340–380 nm with an inverted microscope. DAPI staining was performed exactly as described earlier (Chuenkova and Pereira 2000).

Purification of recombinant PDNF and LTR

Recombinant fragment of PDNF, region 1 in the PDNF cartoon in Fig. 7(a), was construct TS-F (Chuenkova et al. 1999), which is the same as cTS (Chuenkova and Pereira 2000). It was expressed in E. coli, purified by Ni-affinity chromatography and FPLC. Recombinant LTR was isolated from insect cells (Chuenkova and Pereira 2000).

Binding of Alexa Fluor 594-labeled PDNF to PC12 cells

PDNF was labeled with Alexa Fluor 594 Protein Labeling kit according to manufacturer’s protocol. Cells were plated in collagen-coated slides, grown in serum-free medium for 24 h, and reacted with Alexa 594-labeled PDNF (200 ng/mL) for 1 h at 37°C. Cells were washed three times with serum-free RPMI and examined by fluorescent microscopy.

Immunoprecipitation and immunoblotting

Cells were plated in collagen-coated wells, grown in serum-free medium for 24 h, challenged with PDNF, NGF or EGF for different times, washed, and lysed with lysis buffer 1 (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM glycerophosphate, 1 mM Na<sub>2</sub>VO<sub>4</sub>, 1 μg/mL...
leupeptin and 1 mM PMSF) on ice for 10 min. Lysates cleared by centrifugation at 10 000 g for 10 min and immunoprecipitated with TrkA antibody at 4°C overnight. Immunocomplexes were collected with protein G-Sepharose for 2 h at 4°C, washed with lysis buffer and resuspended in SDS-sample buffer for PAGE. Proteins were blotted onto nitrocellulose and probed with P-trkA (Tyr 490) antibody. In other experiments, starved cells were stimulated with PDNF or other factors for 5 min, lysed and 30 μg of total protein from each sample were resolved by PAGE, blotted onto nitrocellulose and probed with P-TrkA (Tyr 490) or P-Akt (Ser473) antibody, followed by HRP-conjugated secondary antibody and visualization by ECL kit.

**Biotinylation of PDNF**

PDNF and BSA were biotinylated using sulfo-N-hydroxysulfosuccinimide-biotin or PEO-maleimide activated biotin according to manufacturer’s instructions (Pierce Biotechnology, Rockford, IL, USA). Bio-PDNF and bio-BSA were sterilized at 0.22 μm filters and kept at 4°C until use.

**Co-precipitation**

PC12<sup>TrkA</sup> cells were incubated in serum-free RPMI for 24 h, detached from substratum with 20 mM EDTA in 1 × Hanks solution, washed once in 1 × Hanks medium. Cell lysates were obtained by treating 2 × 10<sup>6</sup> cells on ice with lysis buffer 2 (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, antiprotease cocktail plus 1 mM PMSF). After centrifugation at 10 000 g supernatant was precleared on protein G-Sepharose or streptavidin-Sepharose for 1 h at 4°C with gentle rocking, incubated with bio-PDNF or bio-BSA (2 μg/mL) for 24 h at 4°C, either followed or not by antibody specific to a C-terminus fragment of TrkA (2 μg/mL), and incubation for 18 h at 4°C. Immunocomplexes were captured either on avidin-Sepharose or on protein G-Sepharose, washed with lysis buffer, eluted with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer, and analyzed by immunoblotting with antibody against TrkA receptor or HRP-streptavidin, respectively.

**Cross-linking of biotin-labeled PDNF to cell-surface**

PC12 cells (6 × 10<sup>6</sup> cells per 100 mm Petri dish) (Becton Dickinson Labware, Franklin Lakes, NJ, USA) were grown overnight in RPMI containing 10% FCS, followed by serum-free medium for 24 h, washed twice with binding buffer (PBS, pH 7.4, 1% BSA) and incubated for 90 min at 4°C in binding buffer containing 2 μg/mL of bio-PDNF with and without 100-fold molar excess of unlabelled PDNF, NGF, or EGF, washed with binding buffer and incubated with 2 mM of cross-linker biot(sulfo succinimidyl)isobutyrate BS3 (Pierce Biotechnology) for 30 min. Reaction was stopped by quench solution (50 mM Tris-HCl, pH 7.3, 0.2 mM glycine, 2 mM EDTA). Cells were lysed on ice with lysis buffer 2 for 30 min, cleared by centrifugation at 10 000 g, immunoprecipitated with C-terminus specific anti TrkA antibody and protein G-Sepharose and analyzed by immunoblotting with streptavidin-HRP.

**Ligand blotting assay**

**Version 1**

TrkA receptor was immunoprecipitated from PC12<sup>TrkA</sup> cell extracts, subjected to SDS–PAGE without reducing agents, blotted to nitrocellulose, washed in 5% BSA in 50 mM Tris-buffered saline, pH 7.4, 0.1% Tween-20, incubated overnight at 4°C with bio-PDNF (7 μg/mL) either without or with increasing concentrations of unlabelled PDNF. After extensive washing, TrkA-bio-PDNF complexes were identified by Streptavidin-HRP and chemiluminescence. Positive bands corresponded to TrkA receptor based on its MW of 140 kDa.

**Version 2**

Purified PDNF was subjected to SDS–PAGE, blotted onto nitrocellulose, blocked in 4% BSA, 0.1% Triton X-100 at 4°C, and treated with 1% Triton-solubilized PC12<sup>nnr5</sup> and PC12<sup>TrkA</sup> cells for 14 h at 4°C. For competition experiments, cell extracts were first incubated with different concentrations of PDNF or NGF at 4°C overnight and then used to react with the PDNF blots. Nitrocellulose was washed with PBS, 0.1% Triton X-100 and probed with anti-TrkA antibody or anti-p75<sup>NTR</sup> antibody followed by HRP-conjugated secondary antibody and ECL detection. The intensity of the bands present on the X-ray films after ECL development was quantified by a computer-assisted CS-800 Calibrated Densitometer (Bio-Rad, Hercules, CA, USA) using Quantity One program, version 4.4.0.

**Statistical analysis**

Data are expressed as mean ± SEM of at least three independent experiments. Nonlinear regression curve and apparent affinity constants were generated with GraphPad Prism program, version 4.0.

**Results**

**PDNF binds to TrkA receptors**

Earlier results, showing that the PDNF actions on PC12 cells are additive with the TrkA ligand NGF and synergistic with the non-TrkA ligands ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF) (Chuenkova and Pereira 2000), provided the first suggestion of an interaction between PDNF and TrkA. To explore this hypothesis further, we performed cell and molecular binding analysis using: (i) a 70-kDa N-terminal fragment of the neuraminidase (Fig. 7a) that reproduces the cell survival and neurite outgrowth properties of the native molecule (Chuenkova and Pereira 2000; Chuenkova and Pereira 2001, 2003; Chuenkova et al. 2001) and recombinant long tandem (LTR) repeat of C-terminus of the enzyme; (ii) PC12<sup>nnr5</sup> cells (Green et al. 1986), a NGF-insensitive mutant PC12-derived cell line lacking TrkA receptor (Fig. 1a, insert) but not p75<sup>NTR</sup> receptor; and (iii) PC12<sup>TrkA</sup> cells (Loeb and Greene 1993), the PC12<sup>nnr5</sup> counterpart that responds to NGF due to the stable transfection with the human TrkA gene (Fig. 1a, insert).

Fluorescence microscopy analysis reveals that fluorescent Alexa-594-labeled PDNF binds to PC12<sup>TrkA</sup> cells but not to PC12<sup>nnr5</sup> cells (Fig. 1a). The failure of PDNF to bind PC12<sup>nnr5</sup> cells is therefore restored by transfection with TrkA gene.

This idea that PDNF binds to TrkA receptor was assessed further by ligand blotting, a procedure previously used to...
visualize lipoprotein receptor (Daniel et al. 1983). PDNF was subjected to SDS–PAGE, blotted to nitrocellulose, and incubated with detergent-solubilized PC12trkA and PC12nnr5 cells. TrkA bound to PDNF were detected with antibodies against TrkA followed by peroxidase-labeled second antibody and chemiluminescence. PC12trkA cells, but not PC12nnr5 cells, allow the detection of TrkA bound to immobilized PDNF (Fig. 1b, upper panel). However, similar ligand blotting does not identify the pan-neurotrophin receptor p75NTR bound to PDNF (Fig. 1b, upper panel). In addition, similar blotting experiments did not reveal TrkA binding to the LTR C-terminal long tandem repeat of the native T. cruzi neuraminidase, as it did to PDNF N-terminus Cys-rich domain (Fig. 1b, lower panels). This result is in agreement with earlier finding that the trophic activity of the neuraminidase resides in the N-terminal domain (Chuenkova and Pereira 2000).

The idea that PDNF interacts with TrkA receptors was substantiated further by co-immunoprecipitation experiments. First, biotin-labeled PDNF (bio-PDNF) was incubated with PC12trkA cell lysates, immunoprecipitated on streptavidin-Sepharose (IP, avidin), and analyzed by immunoblotting with antibodies against TrkA (WB, α-trkA) to identify possible co-immunoprecipitated TrkA. The results show that TrkA co-immunoprecipitates with bio-PDNF but not with biotin-labeled BSA (bio-BSA) (Fig. 2a, upper two panels). Second, antibodies against TrkA were used to immunoprecipitate the receptor from bio-PDNF-containing cell lysates, and the immunoprecipitates were analyzed by immunoblotting with antibodies against TrkA and with streptavidin–peroxidase. We found that bio-PDNF, but not control bio-BSA, co-immunoprecipitates with TrkA (Fig. 2a, lower two panels). Similar analysis does not provide evidence for an interaction between PDNF and pan-neurotrophin receptor p75NTR (data not shown).

To define further the PDNF–TrkA receptor interaction, we performed covalent cross-linking analysis. PC12trkA cells were incubated with bio-PDNF without (–) and with 100-fold excess of unlabeled PDNF, NGF, or EGF, with and without the cross-linking reagent BS3. TrkA in cell lysates was immunoprecipitated with TrkA intracellular domain-specific antibodies and analyzed by immunoblotting with streptavidin–peroxidase to identify bio-PDNF in the TrkA-immunoprecipitated material. In the presence of BS3, TrkA and bio-PDNF, but not bio-BSA, formed complexes that migrated on SDS–PAGE as a major band of 215 kDa (Fig. 2b, second lane), close to the expected MW of a TrkA (MW 140 kDa)/bio-PDNF (70 kDa) dimer. We observed minor bands of molecular weight > 215 kDa (Fig. 2b), which might reflect formation of multimers of TrkA receptors and bio-PDNF. The nature of these minor bands was not studied further. A 100× molar excess of unlabeled PDNF blocked the bio-PDNF/TrkA interaction completely, as did 100× molar excess of NGF, but not EGF (Fig. 2b). These
results further suggest that bio-PDNF interacts directly with TrkA receptors and that the interaction is competitively inhibited by excess of unlabeled PDNF and NGF.

The competitive and inhibitable PDNF binding to TrkA was studied in more detail by a ligand blotting procedure used to quantitate calmodulin/EGF receptor interaction (Li and Villalobo 2002). First, TrkA was immunopurified with TrkA antibodies/protein G-Sepharose, blotted to nitrocellulose, and incubated with bio-PDNF without and with various concentrations of unlabeled PDNF. Binding of bio-PDNF to purified TrkA in the absence (100% binding) and presence (residual binding) of unlabeled PDNF was quantitated after reaction with streptavidin-horseradish peroxidase. The results show that bio-PDNF binding to TrkA is competitively inhibited by unlabeled PDNF in a dose-dependent manner (Fig. 3a, insert) with an apparent dissociation constant ($K_d$) of $1.1 \times 10^{-9}$ M (Fig. 3a). This binding is within the range of NGF binding to TrkA on cell surfaces lacking p75NTR receptors (Hempstead et al. 1991).

Second, purified PDNF was blotted to nitrocellulose and incubated with detergent-solubilized PC12<sup>trkA</sup> cells without or with various concentrations of soluble PDNF or NGF. TrkA bound to PDNF was identified and quantitated by
immunoblotting. We find that PDNF, rather than long-tandem repeat (LTR) C-terminal domain of the neuraminidase (Fig. 3b) inhibits PDNF/TrkA interaction, a finding consistent with earlier conclusions that the neurotrophic action of the neuraminidase is restricted to the N-terminal domain of the neuraminidase (Chuenkova et al. 1999; Chuenkova and Pereira 2000, 2001, 2003). Pre-incubating PDNF and NGF with PC12 cell lysates competitively blocks TrkA receptor interaction with nitrocellulose-immobilized PDNF in a dose-dependent manner, with 50% inhibition at $2.5 \times 10^{-9}$ M and $7.2 \times 10^{-9}$ M, respectively (Fig. 3b). Thus, in this assay, PDNF binding to TrkA receptor is also in the low nanomolar range and only three times lower than NGF binding to TrkA in the absence of p75NTR receptors (Hempstead et al. 1991; Sofroniew et al. 2001).

**PDNF causes autophosphorylation of TrkA**

To explore whether PDNF activates TrkA receptors, we stimulated PC12<sup>Trk<sup>A</sup></sup> and PC12<sup>nmr<sup>S</sup></sup> cells with PDNF, NGF (as positive control), and EGF (as negative control), and tested cell lysates by immunoblotting with an antibody P-TrkA (Tyr490) specific for phosphorylated TrkA, a reaction that indicates TrkA autophosphorylation and activation (Segal 2003). We find that treatment of PC12<sup>Trk<sup>A</sup></sup> cells for 5 min with 100 ng/mL of NGF or 200 ng/mL PDNF, but not EGF, induces TrkA activation (Fig. 4a). PC12<sup>nmr<sup>S</sup></sup> cells did not react with the P-TrkA (Tyr490) antibodies (Fig. 4a). Dose–response experiments reveal that PDNF best activates TrkA in relative low doses (200–500 ng/mL or 3.0–7.5 nM) (Fig. 4b).

In addition to PC12 cells, we find that PDNF activates TrkA in striatal ST14A cells (Cattaneo and Conti 1998) after 5–10 min stimulation (Fig. 4b), i.e. with kinetics analogous to PDNF activation of TrkA in PC12 cells. Furthermore, PDNF, like NGF, induces TrkA phosphorylation in primary cultures of hippocampal neurons as demonstrated by immunocytochemistry (Fig. 4c). Thus, PDNF activates TrkA receptors in cell lines and primary cultures of neurons.

**Trophic effects**

We find that *T. cruzi* (trypomastigote) (+ *T. cruzi*, arrowhead) is much more effective in stimulating TrkA-positive PC12<sup>Trk<sup>A</sup></sup> cells to extend neurites (Fig. 5, left upper panels) than TrkA-negative PC12 cells (i.e. PC12<sup>nmr<sup>S</sup></sup> cells) (Fig. 5, left lower panels). Because these results are reproduced by PDNF (Fig. 5, right panels), they confirm the prediction that *T. cruzi* and its PDNF stimulate neuronal differentiation by TrkA receptor-dependent mechanisms.

We also find that infecting PC12<sup>Trk<sup>A</sup></sup> cells, but not PC12<sup>nmr<sup>S</sup></sup> cells, with *T. cruzi* (+ *T. cruzi*) blocks starvation-induced apoptosis compared to uninfected cells (– *T. cruzi*) (Fig. 6a). This result is consistent with the idea that *T. cruzi* can protect neuronal cells from death through TrkA-dependent mechanisms. PDNF reproduces parasite-induced neuroprotection (Fig. 6c, right panels), an effect similar to that of NGF (Fig. 6b, middle panels, and Fig. 6c). Thus, intact parasites and recombinant PDNF promote neurite extension and cell survival by TrkA-dependent mechanisms.

Because NGF-induced survival of neuronal cells requires PI3K/Akt kinase activation (Sofroniew et al. 2001; Dechant and Barde 2002; Segal 2003), we sought to determine whether PDNF also promotes survival through Akt kinase signaling. Indeed, PDNF activates PI3K/Akt kinase in PC12<sup>Trk<sup>A</sup></sup>, but not in PC12<sup>nmr<sup>S</sup></sup> cells (Fig. 6c, insert).

**Discussion**

Pathological alterations in various tissues produced by infectious agents are traditionally viewed to result from...
tissue damage induced directly by the microbial pathogens and/or indirectly by the attraction of excessive host leukocytes (Bloom 1979; Mitchell 1991). Less well developed is the idea that microbial invaders may generate exogenous mechanisms to enhance protective functions and tissue repair in the host. Such actions could promote host-parasite equilibrium and ultimately prolong parasitism.

Here we demonstrate that PDNF of \textit{T. cruzi}, a human parasite that invades the PNS and CNS, is a functional mimic of NGF in that it binds and activates TrkA receptors on neuronal cells to trigger downstream PI3K/Akt kinase (Figs 4 and 6) and MAPK- Erk1/2 signalings (Chuenkova and Pereira 2001). PDNF, like NGF, promotes survival and differentiation of PC12 cells in a TrkA-dependent manner (Figs 5 and 6). PDNF binds to TrkA receptors with an affinity in the low nanomolar range and, thus, similar to NGF–TrkA interaction in the absence or at low abundance of p75NTR receptor (Hempstead \textit{et al.} 1991; Dechant and Barde 2002) (Fig. 3). We could not demonstrate an interaction between PDNF and p75NTR (Figs 1 and 2b and data not shown), a surprising result because p75NTR is a promiscuous receptor that reacts not only with all neurotrophins (NGF, NT-3, brain-derived neurotrophic factor, NT-4/5, and NT-6) but also with several non-neurotrophin ligands, including rabies virus (Tuffereau \textit{et al.} 1998; Dechant and Barde 2002). Given that NGF binding to p75NTR may lead to cell death (Dechant and Barde 2002), the potential for \textit{T. cruzi} to activate TrkA receptor but not p75NTR receptor may reflect the propensity of the parasite to trigger cell survival pathways while avoiding or perhaps even inhibiting cell death-causing signaling mechanisms.

Although our work provides direct evidence that PDNF binds and activates TrkA, it does not indicate that \textit{T. cruzi} exploits the receptor for cell invasion. Neurons in the brain and PNS, particularly in heart and GI tract ganglia, are rarely found to bear intracellular \textit{T. cruzi} (Tafuri 1970; Pittella 1993). However, because TrkA is not widely present in the CNS and PNS (Sofroniew \textit{et al.} 2001; Segal 2003), it is...
likely that TrkA-positive neurons have not been examined for permissiveness to *T. cruzi* invasion. But the survival actions of PDNF, an outer membrane-bound and soluble diffusible peptide (Schenkman et al. 1994), may very well provide an explanation for neuroprotection in Chagas’ disease, like the inverse relation between tissue infection load (parasites + inflammatory cells) and neuron injury, a feature commonly found in humans and animals with Chagas’ disease (Said et al. 1985; Machado et al. 1987; Losavio et al. 1989; Pittella 1993).

PDNF is composed of two domains, the N-terminal catalytic domain, which folds into a six bladed β propeller, connected through a long α helix to a C-terminal domain, which shows a characteristic lectin-like β-barrel structure (Buschiazzo et al. 2002) (see Fig. 7a). In contrast, NGF is a homodimeric molecule formed by twisted β-sheets, a reverse turn at one end and three β-hairpin loops at the other, and cysteine-knot motif (McDonald et al. 1991). However, despite their seemingly distinct architectures, PDNF and NGF may share structural features that might explain, at least in part, PDNF binding to TrkA receptor. NGF binds to the membrane-proximal immunoglobulin-like domain in the extracellular portion of TrkA receptor through two patches (Wiesmann et al. 1999). Patch 1 (in red, Fig. 7b), constitute a binding motif conserved in other neurotrophins, and patch 2 (in green, Fig. 7b), which comprises the N-terminal residues 2–13, is specific for the NGF/TrkA receptor interaction. PDNF fails to promote neurite extension and cell survival when a 20 amino acids stretch is deleted (Fig. 7b; Chuenkova et al. 1999). That stretch contains the sequence Cys-Val present in the conserved NGF patch 1 motif Cys-Val-Cys-Val (Fig. 7b), which establishes contact with TrkA binding site residues (Kullander et al. 1997). Two other conserved NGF patch 1 motifs that contact residues in the TrkA binding site, STHPVF, which is highly homologous (four residues out of six total) to STVPF in the N-terminus of PDNF. The motifs 96QAA and 48VF, also essential for NGF biological activity, are found as similar motifs, 82QIA and 206VF, in PDNF except in a reverse order in the sequence (Fig. 7b). Because some NGF sequences, like specificity patch 2 (green, Fig. 7b), change structure upon interacting with TrkA (Wiesmann et al. 1999), it may be that PDNF will behave correspondingly. Nevertheless, the presence in PDNF of NGF motifs required for NGF–TrkA receptor interaction suggests that *T. cruzi* may react with TrkA in an NGF-like manner.

The presence in PDNF of motifs conserved among other neurotrophin family members (NGF, BDNF, and NT-3) suggests the possibility of the *T. cruzi* ligand to interact with other Trk receptors. Our earlier finding that PDNF is a survival factor for Schwann cells (Chuenkova et al. 2001), which can express TrkB and TrkC but not TrkA (Stark et al. 2001; Yamauchi et al. 2003), also suggests that PDNF may interact with more than one neurotrophin receptor. If this were the case, it would be in keeping with the lack of absolute selectivity of neurotrophin–Trk receptor interactions (Sofroniew et al. 2001; Segal 2003). However, it remains to be demonstrated directly that PDNF reacts with neurotrophin receptors other than TrkA.

In conclusion, we suggest that by mimicking NGF in activating TrkA receptor survival mechanisms on neurons, PDNF helps offset nervous tissue damage caused by *T. cruzi* invasion-promoting molecules, including PDNF itself, which can enhance virulence of *T. cruzi* (Chuenkova and Pereira 1995) and transgenic *Leishmania* (Belen Carrillo et al. 2000). This dual action of PDNF would not be unlike that of NGF, which can cause both survival and death of neurons (Sofroniew et al. 2001; Dechant and Barde 2002; Segal 2003). Our studies may also possibly assist in the development of neurotrophic agents to target not only Chagas’ disease but also unrelated neurodegenerative conditions such as Parkinson’s...
disease, as our exploratory experiments seem to suggest (Chuenkova and Pereira 2003).

Acknowledgements

We are grateful to Drs L. Greene for the generous gift of PC12<sup>TrkA</sup> and PC12<sup>TrkB</sup> cells, A. Cattaneo for ST14A striatal cells, and A. Krantis for primary cultures of rat hippocampal neurons; B. Talamo for comments on the manuscript and G. Taylor for discussing PDNF structure. This work was supported by NIH grants NS40574 and NS42960.

References


