A Synthetic Peptide Modeled on PDNF, Chagas' Disease Parasite Neurotrophic Factor, Promotes Survival and Differentiation of Neuronal Cells through TrkA Receptor

Marina V. Chuenkova and Mercio PereiraPerrin*

Parasitology Research Center, Department of Pathology, Tufts University School of Medicine, 150 Harrison Avenue, Boston, Massachusetts 02111

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ABSTRACT: The human parasite *Trypanosoma cruzi*, the agent of Chagas' disease, expresses a membrane-bound neuraminidase/*trans*-sialidase, also known as parasite-derived neurotrophic factor, PDNF, because it binds and activates nerve growth factor (NGF) receptor TrkA in neuronal cells. Here, we identify a 21 amino acid region (^{425}GNASQNVWEDAYRCVNASATAN^{445}) of PDNF that reproduces its neurotrophic activities. Synthetic peptide Y_{21}, modeled on this sequence, induces survival and neurite outgrowth in primary dorsal root ganglion neurons. Y_{21} but not other PDNF-based peptides promotes survival and neurite extension in TrkA-expressing but not in TrkA-deficient PC12 cells. Y_{21} also enhances phosphorylation of TrkA in PC12 cells and activation of Erk1/2 and Akt kinases with kinetics distinct from that of PDNF. In addition, Y_{21} stimulates phosphorylation of cAMP response element-binding protein, CREB. Peptide Y_{21}, therefore, reproduces several TrkA-dependent activities of PDNF and NGF. However, Y_{21} inhibits the binding of PDNF but not NGF to TrkA. Similarly, Y_{21} blocks PDNF- but not NGF-dependent phosphorylation of Erk1/2. These findings raise the possibility that Y_{21} reacts with a TrkA site required for the binding of PDNF but not NGF. The functioning of Y_{21} as TrkA agonist reproducing TrkA-dependent biological activities of PDNF should help elucidate the mechanism of PDNF activation of TrkA-expressing cells and the design of small drugs for the treatment of Chagas' and other neurodegenerative diseases.

Trypanosoma cruzi causes Chagas' disease, a chronic debilitating condition afflicting millions of people in the Americas. *T. cruzi* may seriously damage and destroy tissues that it infects, including the nervous system, either directly through parasite load or indirectly by eliciting a strong inflammatory response. It is therefore surprising that most patients in acute Chagas' disease, characterized by the thriving of the parasite in central and peripheral organs, rarely present symptoms and physical signs of brain infection, even when trypanosomes inhabit the cerebrospinal fluid (*1*). Furthermore, patients who die with acute Chagas' disease usually reveal morphologically preserved neurons located within foci of inflammatory cells or adjacent to *T. cruzi* nests (*2*). Similar to humans, animals infected with *T. cruzi* may display similar evidence of neuroprotection (*2*), as do patients who progress from acute to chronic asymptomatic disease, where 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 in normal, non-Chagasic individuals (*3*). Thus, the pathogenesis and time course of Chagas' disease suggest the existence of neurore-generative events.

One explanation for these events is that *T. cruzi* promotes the healing of the nervous system by secreting and/or stimulating the host to secrete neurotrophic factors to help repair infected and uninfected cells damaged by the invasive process. This hypothesis gained credence with the discovery that trypomastigote, the invasive form of *T. cruzi*, expresses a GPI-anchored PDNF (parasite-derived mimic of neurotrophic factors)[1] that directly promotes the survival of serum-deprived neuroblastoma cells, primary cultures of neurons, and Schwann cells (*4*, *5*). PDNF, originally discovered for its ability to release sialic acid from glycoconjugates into aqueous solution (neuraminidase) (*6*) and to transfer sialic acid to β-galactosyl acceptors (sialyl transferase) (*7*−*9*), promotes neuronal and glial cell survival independent of sialic acid-binding activities (*4*). In addition, PDNF can stimulate human intestinal microvascular endothelial cells and peripheral blood mononuclear cells to secrete biologically active interleukin-6 (IL-6), which could trigger survival mechanisms in neurons and other cells (*10*). PDNF has also been reported to cause apoptosis in some organs of the immune system (*11*), consistent with its functional mimicry of host survival factors, which can cause cell death under certain circumstances (*12*).

PDNF-induced survival requires activation of the PI3K (phosphatidylinositol 3-kinase)/Akt kinase signaling pathway and upregulation of the anti-apoptotic *bcl-2* gene (*4*, *5*), whereas PDNF-induced neuronal differentiation depends

* To whom correspondence should be addressed. Telephone: 617-636-2933. Fax: 617-636-6849. E-mail: mercio.perrin@tufts.edu.

[1] Abbreviations: PDNF, parasite-derived neurotrophic factor; TrkA, tropomyosin-related kinase A; NGF, nerve growth factor; MAPK/Erk, mitogen-activated protein kinase/extracellular-regulated kinase; CREB, c-AMP response element-binding protein; DRG, dorsal root ganglion.

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upon the activation of the Ras-dependent MAPK/Erk (mitogen-activated protein kinase/extracellular signal-regulated kinase) signaling (*13*). PDNF exploits MAPK/Erk signaling to inhibit caspase activation, free-radical formation, and death of dopaminergic cells exposed to the Parkinsonism-inducing neurotoxin MPP+ (1-methyl-4-phenylpyridinium) (*14*). PDNF binds and activates the NGF receptor TrkA to trigger PI3K/Akt and MAPK/Erk signalings in several types of neuronal cells (*15*, *16*).

The ability of PDNF to promote survival and differentiation of neurons have been mapped to its N-terminal region of 632 amino acids (*4*, *5*), made-up of a domain bearing the neuraminidase/sialyltransferase catalytic sites and another one having a lectin-like structure (*4*, *5*, *17*). In contrast, PDNF-induced stimulation of IL-6 secretion and lymphocyte activation has been assigned to its C-terminal region (*10*, *18*), composed of a long tandem repeat of a 12 amino acid unit (peptide TR) (*19*). A peptide-mapping approach revealed that a bacterially expressed truncated PDNF corresponding to amino acids 1−445 promoted survival and differentiation of TrkA-positive PC12 cells, while mutant PDNF equivalent to amino acids 1−425 did not (*4*). This finding raised the possibility that the amino acid sequence matching PDNF segment 425−445 may reproduce TrkA-dependent neurotrophic properties of the trypanosomal protein.

We show here that synthetic peptide Y_{21}, a 21-mer corresponding to PDNF amino acid sequence 425−445, is a peptidomimetic of PDNF and a TrkA agonist. In addition to possibly facilitating the dissection of mechanisms underlying neurotrophic actions of PDNF, Y_{21} could offer a novel therapeutic strategy to selectively target neuronal and other TrkA-bearing cells.

MATERIALS AND METHODS

Materials. $PC12^{nnr5}$ and $PC12^{trkA}$ cells were a gift from Dr. L. Greene (College of Physicians and Surgeons, Columbia University, New York). Anti-TrkA antibody was purchased from Abcam (Cambridge, MA), while antibodies against phospho-TrkA (Tyr 490), phospho-p42/44 MAPK, phospho-Akt (Ser473), phospho CREB, p42/44MAPK, Akt, and CREB were from Cell Signaling Technology (Beverly, MA). HRP-conjugated secondary antibody was from Chemicon (Tenecula, CA). Anti-neurofilament NF200 antibody was obtained from Sigma (St. Louis, MO). ECL kit was from Perkin−Elmer (Life Sciences, Boston, MA). Protein G-Sepharose was purchased from Amersham Biosciences (Piscataway, NJ). Anti-protease cocktail was obtained from Roche Molecular Biochemicals (Indianapolis, IN). Synthetic peptides were obtained from Tufts University Core Facilities (Tufts University, Boston, MA). PDNF refers here to the Cys-rich region of 632 amino acids of clone 19Y composed of catalytic and lectin-like domains but without the C-terminal tandem repeat (*20*); recombinant PDNF was expressed in *Escherichia coli* and purified by Ni-chelate chromatography and FPLC, as described earlier (*4*, *20*).

PC12 Cultures and Assays for Neurotrophic Activity. $PC12^{nnr5}$ and $PC12^{TrkA}$ cells were grown in RPMI with 10% FCS and 5% horse serum as described earlier (*4*, *21*). Two approaches were used to study neurite extension: (1) plating cells (1×10^5 cells/mL) on 16-well slides (LabTech, Nalge Nunc International, Naperville, IL) coated with synthetic peptides (1 mM in water, 4 °C, overnight), bovine serum albumin (BSA), or rat tail collagen (1 mg/mL PBS, 4 °C, overnight); (2) adding peptides in solution to cells plated on uncoated substratum. Cells were grown in 1% FCS-containing RPMI for 72 h and reacted with 0.5 mg/mL MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma, St. Louis, MO) in serum-free medium for 1 h at 37 °C. Light microscopy was used to visualize viable cells (i.e., containing reduced insoluble formazan) with or without extended neurites. Only cells with processes greater than 10 μm in length were counted as neurite-bearing. For each culture condition, at least 300 cells were scored.

Cell viability was also determined by the CellTiter 96 assay (Promega, Madison, WI) as follows: cells, plated at 1×10^5/mL, were treated with peptides in serum-free medium for different times and exposed to CellTiter 96 Aqueous One Solution for 1 h at 37 °C; absorbance at 490 nm was read using SpectraMax 340 Microplate Spectrophotometer (Molecular Devices, Sunnyvale, CA). Apoptotic cells were identified by staining with DAPI (250 ng/mL) (Sigma) 48 h after cells were exposed to serum-free medium with or without additives in solution, as described earlier (*4*).

DRG Cultures and Immunocytochemistry. Dorsal root ganglion (DRG) neurons derived from neonatal rats were purchased from QBM Cell Science (Ottawa, Canada). Isolated DRG neurons were plated onto poly-L-lysine/laminin-coated plastic dishes in Neurobasal (NB) medium supplemented with 2% B27, 2 mM L-glutamine and penicillin/streptomycin (100 units/100 μg/mL, respectively) (Gibco), and mitotic inhibitors (17.5 μg/mL uridine and 7.5 μg/mL 5-fluoro-2-deoxyuridine) (Sigma). Peptides were added to DRG cultures 24 h after plating, and neurite outgrowth was examined 24 h afterward by MTT staining and measured using Spot Camera imaging software (Diagnostic Instruments, Sterling Heights, MI).

For survival experiments, neurons were deprived of the B27 supplement on the 5th day of culture and cell morphology was analyzed in 18 h by immunofluorescence: DRG neurons were fixed in 4% paraformaldehyde, permeabilized in 0.2% Triton X-100 in PBS, and background-labeling blocked with 10% goat serum. Cells were incubated with anti-neurofilament (NF) 200 monoclonal antibody overnight at 4 °C, followed by Alexa-Fluor 568-conjugated secondary antibody (Molecular Probes). Cells were also stained with DAPI (described above) to monitor the number of neuron bodies in the field.

Peptides Cross-Linking to BSA. Peptides were cross-linked to BSA with sulfosuccinimidyl 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate, sulfo-SMCC (Pierce, Rockford, IL) according to protocol of the manufacturer. Briefly, peptides were reduced using 2-mercaptoethylamine to create free sulfhydryl groups; at the same time, BSA was activated with sulfo-SMCC. Conjugation was performed by mixing the reduced peptide and activated BSA followed by incubation for 2 h at 4 °C. Conjugates were dialyzed against PBS, sterilized at 0.22 μm filters, and stored at −20 °C.

Western Blotting and Immunoprecipitation. The activation state of TrkA, Erk1/2, and Akt kinases and transcription factor CREB was analyzed by testing phosphorylation by Western blot with appropriate phospho-specific antibodies. For this, cells were plated in complete medium at a density of 3×10^5 cells/mL overnight on 6-well plates (Becton

Dickinson Labware, Franklin Lakes, NJ). Medium was changed to serum-free for another 24 h to reduce background phosphorylation. Then, monolayers were stimulated with peptides, PDNF, or NGF for indicated times, washed with ice-cold PBS at pH 7.4, and lysed with lysis buffer [(20 mM Tris at pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM glycerophosphate, 1 mM Na_3VO_4, protease inhibitors leupeptin (10 μg/mL), and PMSF (1 mM)]. Lysates were cleared by centrifugation at 14 000 rpm for 30 min, and the protein concentration of supernatants was determined by the Bradford assay (Bio-Rad, Richmond, CA). For Western blotting, 50 μg of cell lysate was resolved by SDS−PAGE, transferred to nitrocellulose, blocked with 5% nonfat milk, and reacted with polyclonal phospho-p42/44 MAP antibody to detect activated Erk1/2 or polyclonal antibody specific for phospho-Ser-473-Akt to identify activated Akt kinase or phospho-Ser133-CREB antibody to detect activated transcription factor CREB. The antibody reaction was visualized by horseradish peroxidase (HRP)-conjugated secondary antibody and enhanced chemiluminescence kit (ECL). Some blots were stripped in appropriate buffer (50 mM Tris-HCl at pH 7.8, 2% SDS, and 1% β-mercaptoethanol) at 50 °C for 60 min and reprobed with p42/44MAPK, Akt, or CREB antibody to ensure equal loading of proteins. For immunoprecipitation experiments, cell lysates were preadsorbed on protein G-Sepharose for 1 h at 4 °C, and after brief centrifugation, supernatants were incubated with anti-TrkA antibody for 24 h at 4 °C with gentle rocking. Formed immunocomplexes were captured on protein G-Sepharose, eluted with SDS−PAGE sample buffer, and analyzed by immunoblotting with antibody against phospho-TrkA receptor.

Ligand-Blotting Assay. Purified recombinant PDNF (*4*) was subjected to SDS−PAGE, blotted onto nitrocellulose, and blocked in 4% BSA and 0.1% Triton X-100 at 4 °C overnight. Strips of nitrocellulose containing immobilized PDNF were incubated with TrkA-ECD, (recombinant extracellular domain of human TrkA receptor fused to the FC region of human IgG) (R&D Systems, Minneapolis, MN) alone or with increasing concentrations of peptide overnight at 4 °C. Strips were washed with 0.1% Triton X-100 in PBS and probed with anti-human HRP-conjugated antibody (Promega, Madison, WI). 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) using Quantity One program, version 4.4.0.

Transfections and Reporter Assay. The plasmids for the reporter assay were a generous gift of Dr. Dona M. Chikaraishi (Duke University Medical Center, Durham, NC). Two plasmids were used: pFLuc, which contained rat tyrosine hydroxylase promoter followed by firefly luciferase sequence of pGL2-Basic plasmid (Promega), and pCRE$^-$, where the CRE site at −45 bp of native TH promoter was mutated (*22*). pGL4.74 plasmid (Promega) with the *Renilla* luciferase gene was used as an internal control.

For the reporter assay, cells were plated at 4×10^5 cells/mL in 48-well plates 20 h before transfection. Transfection was performed using 1 μg of Lipofectamine2000 (Invitrogen, San Jose, CA) and 0.65 μg of total DNA/well. The DNA mixture was composed of either pFLuc or pCRE$^-$ mixed with pGL4.74 (internal transfection control) at a ratio of 40:1. Transfected cells were incubated for 20 h, and the media was changed to a fresh one with or without additives. Cells were cultured for another 10 h before harvesting for the reporter assay. Cell lysates were assayed for luciferase activity with Dual-Luciferase Reporter Assay system (Promega) using Optocomp I Luminometer (MGM Instruments) according to instructions of the manufacturer. The protein concentration of cell lysates was determined with the Bradford Reagent (Bio-Rad).

All of the results are representative of at least three independent experiments.

Statistical Analysis. All reported data analyses are based on a minimum of three independent experiments for each manipulation. Data are plotted as mean ± standard error.

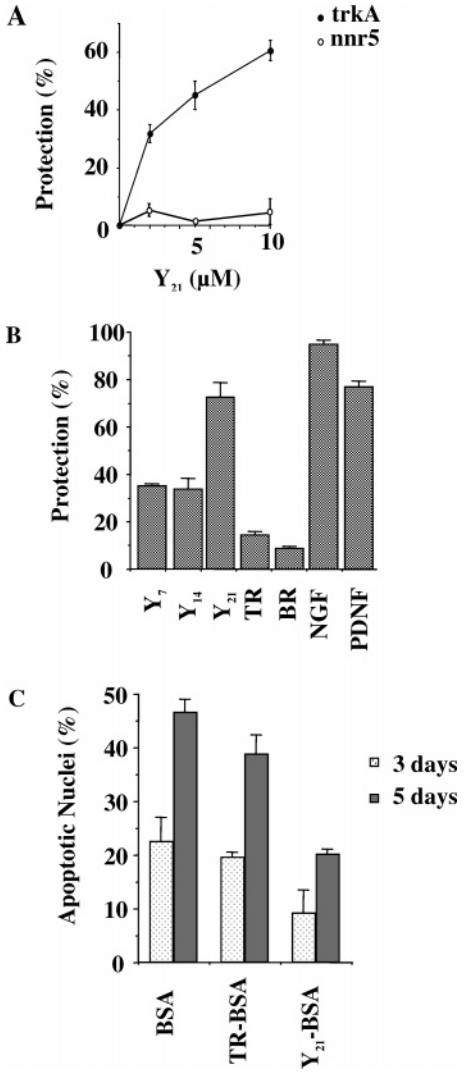

FIGURE 1: Synthetic peptide Y_{21} promotes survival of PC12 cells. (A) Y_{21}-induced cell survival. PC12 cells were cultured in serum-free medium without (0% protection) or with the indicated concentrations of peptide Y_{21}. Cell viability was assessed using a MTT colorimetric assay as described in the Materials and Methods. (B) Specificity of Y_{21} responses. $PC12^{trkA}$ were starved in serum-free medium without or with synthetic PDNF-derived peptides (Y_{21}, Y_{14}, Y_7, TR, and BR) (10 μM), PDNF (500 ng/mL), or NGF (30 ng/mL) for 48 h. Apoptosis was determined by fluorescence microscopy after staining with DAPI. (C) Y_{21}, coupled to a macromolecular carrier, preserves its activity. Peptides Y_{21} and TR were cross-linked to BSA and assayed for promotion of $PC12^{trkA}$ cell survival for 3 and 5 days. Apoptotic cells were counted by fluorescence microscopy after staining with DAPI.

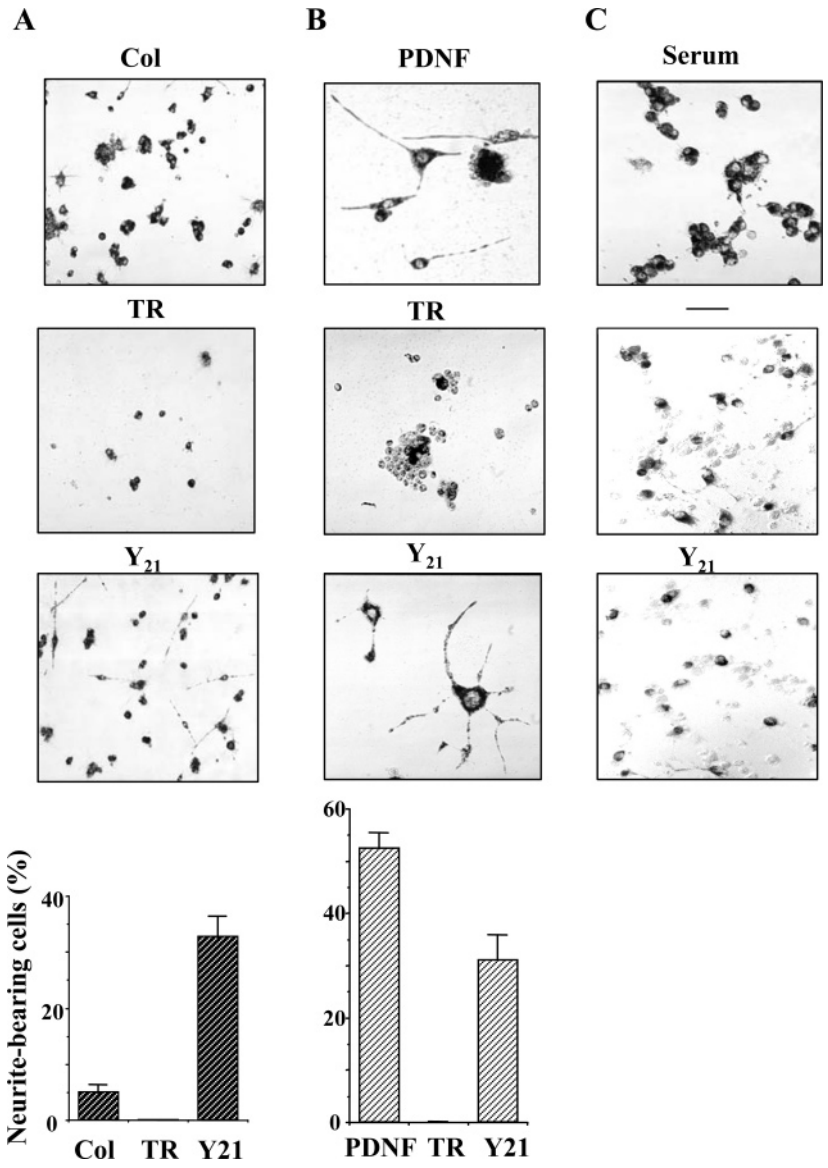

FIGURE 2: Peptide Y_{21} promotes differentiation of PC12 cells expressing TrkA. (A) $PC12^{TrkA}$ cells in DMEM/1% FCS were cultured for 3 days in plates coated with Y_{21}, TR, or collagen (Col). Live cells (containing dark insoluble formazan) were visualized by Nomarski microscopy after staining with MTT. (B) $PC12^{TrkA}$ cells were maintained for 5 days in RPMI/1% FCS medium without or with peptides Y_{21}, TR (10 μM), or PDNF (500 ng/mL), added to the medium. Graphs show the response of PC12 cells to different incubation conditions quantified as the percent of neurite-bearing cells. (C) $PC12^{nnr5}$ were cultured as above without (—) or with Y_{21} (10 μM) or 5% FSC (serum), as a positive control. Morphological differentiation of PC12 cells was determined by Nomarski microscopy (400×) after MTT staining.

RESULTS

Synthetic Peptide Y_{21} Promotes Survival and Differentiation of PC12 Cells Expressing TrkA ($PC12^{TrkA}$) but Not of PC12 Cells Lacking TrkA ($PC12^{nnr5}$). A 21 amino acid region of the PDNF lectin-like domain was responsible for 80% of PDNF neurotrophic activity (*4*), which depended upon TrkA signaling (*15*). We hypothesized that TrkA-related trophic effects of PDNF could be reproduced by a synthetic peptide Y_{21} (^{425}GNASQNVWEDAYRCVNASTAN^{445}) corresponding to this region. To test this, we used two types of cells, parental $PC12^{nnr5}$ lacking TrkA receptor (*21*), and $PC12^{trkA}$, parental cells stably transfected with the human TrkA gene (*23*). Cells were treated with Y_{21} and other PDNF-derived synthetic peptides in serum-free medium. Deprived of trophic support (serum), PC12 cells normally die of apoptosis, but Y_{21} rescued TrkA-expressing cells from death in a dose-dependent manner (Figure 1A). In the same conditions, however, Y_{21} did not promote viability of parental TrkA-negative $PC12^{nnr5}$ cells. Y_{21} also significantly decreased the number of cells with apoptotic (fragmented) nuclei, detected by DAPI staining: 70% of $PC12^{trkA}$ were protected from apoptosis by 10 μM Y_{21}, comparable to the survival activity of PDNF at 7 nM (Figure 1B). Truncated variants of the peptide, Y_{14} (^{425}GNASQNVWEDAYRC^{438}) and Y_7 (^{439}VNASTAN^{445}), had very low, if any, antiapoptotic effect (Figure 1B). Consistent with previous observations, peptide TR (^{639}DSSAHGTPSTPA^{650}), deduced from the PDNF sequence of the C-terminus tandem repeat (LTR), did not protect PC12 cells from apoptosis (*4*, *15*) and neither did peptide BR (bacterial repeat) (^{70}YSVDDGETWE^{80}) from PDNF N-terminal Asp-box motif, common also for bacterial sialidases (*24*, *25*) (Figure 1B).

In the next experiment, we used Y_{21} conjugated to macromolecular carrier BSA to determine whether Y_{21} can

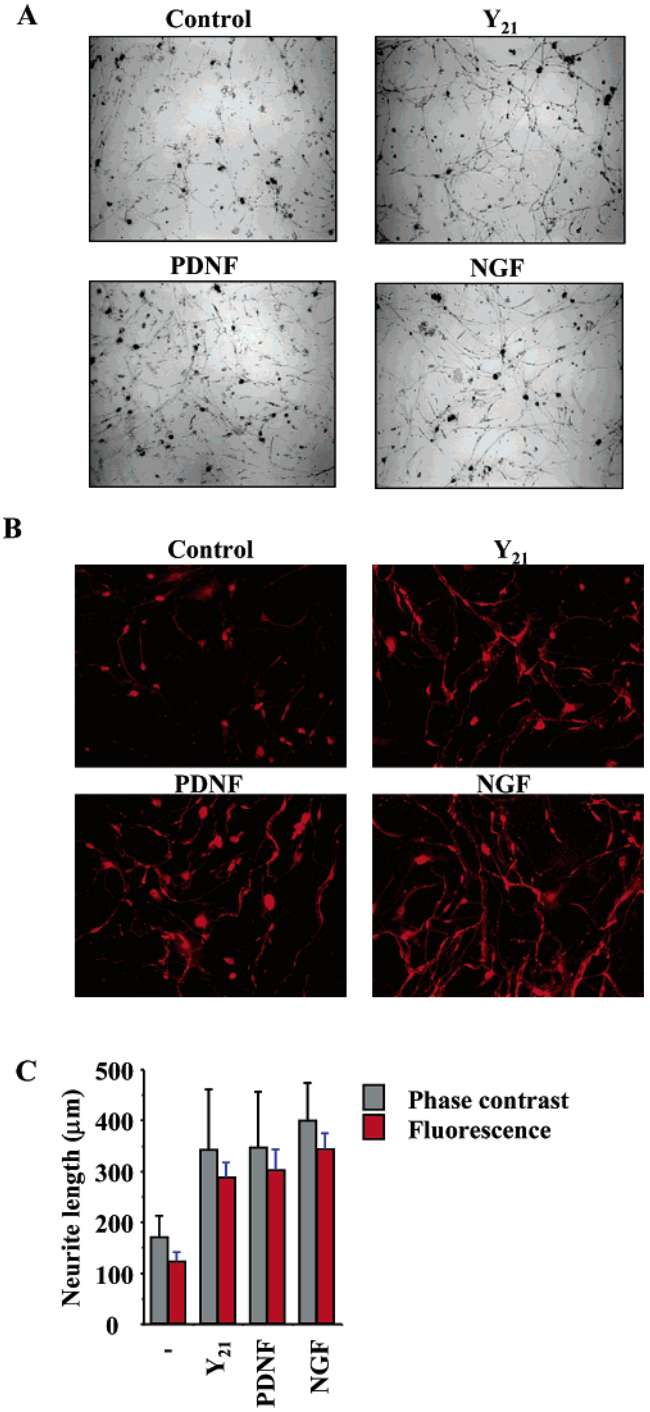

FIGURE 3: Peptide Y_{21} induces neurite outgrowth and survival of primary DRG neurons. (A) DRG neurons cultures derived from DRG ganglia of neonatal rats were maintained in complete NB medium for 24 h and then with Y_{21} (10 μM), PDNF (500 ng/mL), NGF (50 ng/mL), or medium alone (control) for another 24 h before staining with MTT. Phase contrast optics, 200×. (B) DRG neurons were cultured in complete NB medium for 5 days before changing to B27 growth supplement-free medium with Y_{21}, PDNF, NGF, or no additives (control) for 18 h. Cell morphology was analyzed under a fluorescent microscope (400×) after immunostaining with NF 200 antibody. (C) Neurite outgrowth was measured using Spot Camera imaging software. For each condition, a neurite length of at least 50 neurons was analyzed.

confer its survival-promoting activity to otherwise inactive protein. Conjugates were purified by MW exclusion to prevent contamination of the macromolecular fraction with free (unbound) peptides. As shown in Figure 1C, Y_{21}-BSA

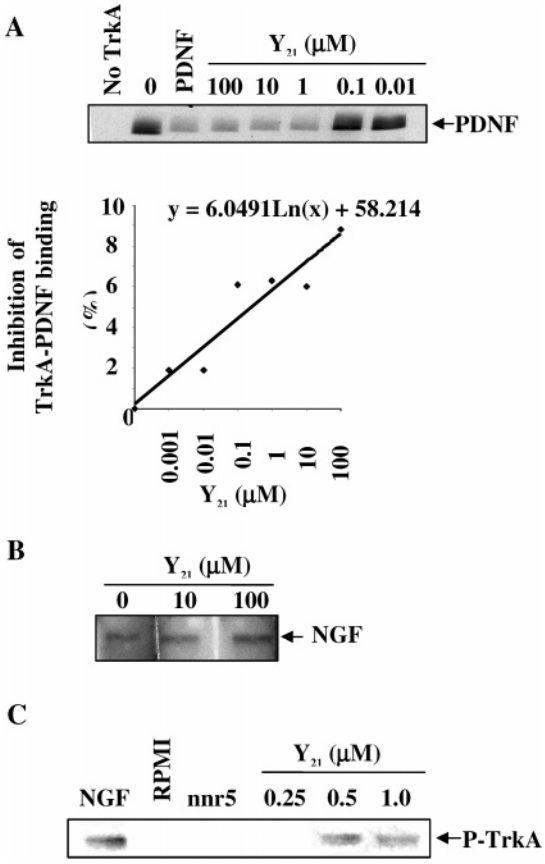

FIGURE 4: Synthetic peptide Y_{21} inhibits PDNF binding to TrkA. (A) Purified recombinant PDNF was subjected to SDS/PAGE, transferred to nitrocellulose, and reacted with the extracellular domain of human TrkA receptor (TrkA-ECD) fused to the Fc domain without (0) or after preincubation with 100 nM PDNF (PDNF) or with the indicated concentrations of Y_{21}. Binding of TrkA to PDNF was detected with anti-human HRP-conjugated antibody. The relative amount of TrkA-ECD bound to PDNF was determined by scanning densitometry and plotted using GraphPad Prism (graph). (B) Protocol similar to A, except NGF rather than PDNF, was immobilized on nitrocellulose. (C) $PC12^{TrkA}$ and $PC12^{nnr5}$ cells were starved in serum-free medium for 24 h, stimulated with NGF (30 ng/mL) and the indicated concentrations of Y_{21} for 5 min. Cells were lysed, immunoprecipitated with anti-TrkA antibody, and subjected to SDS/PAGE and Western blotting with phospho-TrkA antibody to identify phospho-TrkA (arrow).

was almost as efficacious in inhibiting apoptosis of PC12-TrkA as the 21-mer peptide, reducing the number of cells with fragmented nuclei by 50%, compared to the control (BSA) and TR-BSA conjugate (Figure 1C).

To determine whether Y_{21}-elicited trophic response in $PC12^{trkA}$ was initiated on the cell membrane, peptides were immobilized on the plastic surface of tissue culture dishes and $PC12^{trkA}$ were plated there in low (1%) serum medium. After 3 days, when cells morphology was evaluated by MTT staining, few cells remained alive and attached to BSA (negative control, data not shown) or TR peptide-coated wells; at the same time, surface-coated collagen (positive control) and peptide Y_{21} supported adhesion and survival of $PC12^{trkA}$ (Figure 2A), indicating a membrane-initiated effect (Figure 1C). Interestingly, in comparison to collagen, which primarily enhanced cell survival, Y_{21} also induced many (up to 35%) $PC12^{trkA}$ cells to differentiate with the extension of long processes (graph in Figure 2A).

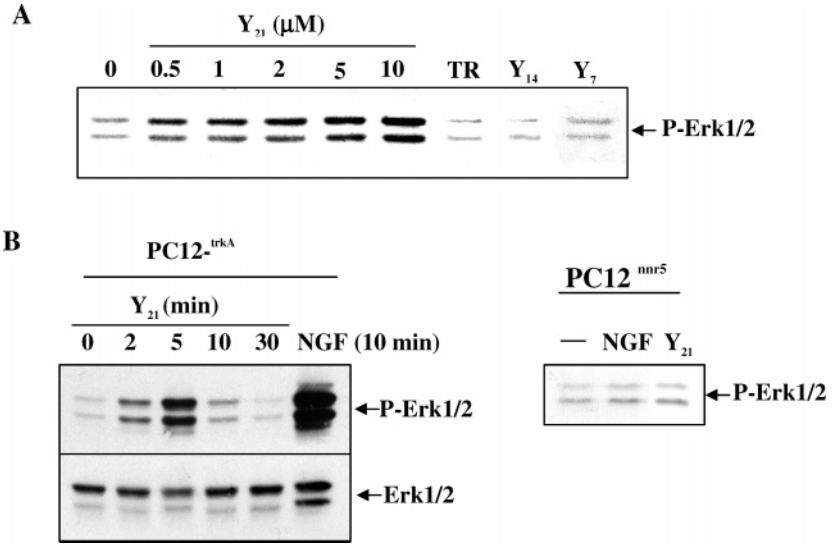

FIGURE 5: Peptide Y_{21} activates MAPK/Erk signaling. (A) $PC12^{trkA}$ cells were incubated in serum-free medium for 24 h before stimulation for 5 min with the indicated concentrations of Y_{21} or 10 μM peptides TR, Y_{14}, and Y_7. Cell lysates were analyzed by Western blotting with anti-phospho-Erk antibody and HRP-labeled second antibody. (B) (Left panel) $PC12^{trkA}$ cells were stimulated with Y_{21} (10 μM) for indicated times or with NGF (30 ng/mL) for 10 min and analyzed by Western blotting with antibodies against P-Erk and Erk. (Right panel) $PC12^{nnr5}$ cells were untreated (0) or treated with NGF for 10 min or Y_{21} for 5 min and probed with P-Erk antibody by Western blotting.

The effect of substratum-bound Y_{21} was reproduced with the peptide in solution, as demonstrated by multiple neurites in $PC12^{trkA}$ cells exposed to 10 μM soluble Y_{21} (Figure 2B). This response to soluble Y_{21} was comparable to that of the original PDNF (7 nM) (graph in Figure 2B). Y_{21} was inactive on TrkA-deficient $PC12^{nnr5}$ cells (Figure 2C).

Peptide Y_{21} Enhances Neurite Growth and Survival of DRG Neurons. It is well-established that *T. cruzi* infection affects peripheral nervous system functions through unknown mechanisms (*26*). Because *T. cruzi* do not commonly invade neurons (*27*), parasite-released factors could possibly contribute the denervation/reinnervation of Chagas' disease (*1*, *2*, *28*).

To further investigate neurotrophic effects of a parasite-derived peptide, we used primary cultures of rat DRG neurons. DRG neurons treated with Y_{21} or PDNF for 24 h exhibited a neurite growth pattern different from the control, untreated cells (Figure 3A). Although neurons in control cultures extended neurites, these were shorter (Figure 3C) and had a less complex network (Figure 3A) than in the neurons treated with Y_{21}, PDNF, or NGF.

DRG neurons deprived of growth factors (B27 supplement) detached from substratum in 18 h and showed signs of degeneration (control in Figure 3B). Such detachment and degeneration of the neurite network were abrogated or reduced by Y_{21}, PDNF, and NGF (parts B and C of Figure 3).

Peptide Y_{21} Competes with PDNF for Binding to the TrkA External Domain and Induces TrkA Intracellular Tyrosine Kinase Activity. Because Y_{21} reproduced neurotrophic effects of PDNF, we sought to determine whether the peptide bound and activated the TrkA receptor. Thus, PDNF was immobilized on nitrocellulose after PAGE and incubated with the recombinant human extracellular TrkA-Fc domain without and with different concentrations of Y_{21}. Binding of TrkA to PDNF was quantitated by densitometry after the reaction with anti-human-HRP-conjugated antibody. The results showed that PDNF binding to TrkA, set at 100%, was competitively inhibited by Y_{21} (Figure 4A). Taking into account the fact that Y_{21} even at a high concentration of 100 μM did not inhibit NGF binding to TrkA extracellular domain (Figure 4B), we conclude that the peptide binds a site within TrkA bound by PDNF but not by NGF.

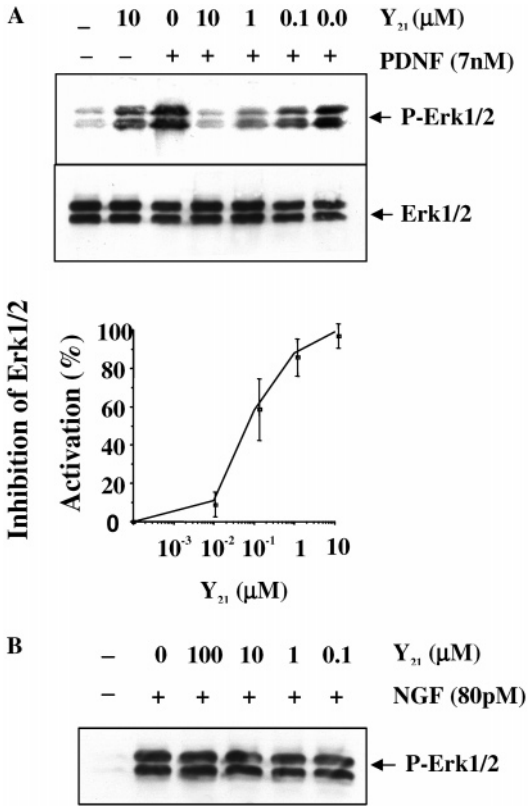

FIGURE 6: Peptide Y_{21} blocks PDNF- but not NGF-dependent stimulation of P-Erk. (A) PC12 cells were serum-starved for 24 h, treated with an indicated concentration of Y_{21} for 5 min, followed by 7 nM PDNF for 10 min, lysed, and probed with anti-phospho-Erk antibody or anti-Erk antibody by Western blotting (panel). P-Erk was estimated by scanning densitometry (line graph), with the median of triplicate experiments. (B) PC12 cells were treated as in A followed by 80 pM NGF for 5 min, lysed, and probed with P-Erk antibody.

PDNF induced auto-transphosphorylation of Tyr 490 in the TrkA intracellular activation domain (*15*); such autophosphorylation is required for signaling to MAPK and PI3K pathways (*29*). Because Y_{21} binds to the extracellular portion of the TrkA receptor, we examined whether it was also able to induce TrkA kinase activation, which should result in autophosphorylation at Tyr490.

After PC12 cells were stimulated with either 30 ng/mL NGF (positive control) or various concentrations of Y_{21} for 5 min, TrkA receptors were immunoprecipitated and Tyr490 phosphorylation was determined by Western blotting with an anti-P490-TrkA antibody (Figure 4C). Treatment of $PC12^{trkA}$ cells with as low as 5 μM Y_{21} resulted in an increase in Tyr 490 phosphorylation of TrkA in 5 min.

When the data presented on Figure 3 are taken together, they suggest that Y_{21} binds to and activates TrkA.

Y_{21} Promotes Phosphorylation of MAPK/Erk and Akt Kinases. As a TrkA ligand, PDNF activates the PI3K/Akt pathway essential for survival (*4*), as well as the MAPK/Erk cascade (*13*), implicated both in survival and differentiation (*30*). It was therefore of interest to determine whether Y_{21} activated MAPK/Erk and Akt. As shown in Figure 4, Y_{21} induced Erk1/2 phosphorylation in a time- and dose-dependent manner. In contrast, other PDNF-derived synthetic peptides, Y_7, Y_{14}, and TR, did not enhance Erk1/2 activation compared to the control, unstimulated $PC12^{trkA}$ (Figure 5A). NGF and Y_{21} did not promote Erk activation in TrkA-deficient $PC12^{nnr5}$ cells (right panel in Figure 4B), reinforcing the conclusion that Y_{21} induced Erk1/2 phosphorylation via TrkA.

Erk1/2 activation in $PC12^{trkA}$ cells by Y_{21} started at 2 min, peaked at 5 min, and waned after a longer incubation (Figure 5B); such kinetics was different from that of the original PDNF, which produced a sustained activation of 30−60 min (*13*). Rapid desensitization of TrkA in response to Y_{21}, resulted in the functional uncoupling of the MAPK/Erk activation pathway, rendering the receptor also unresponsive to stimulation with parental PDNF. Continuous exposure of $PC12^{trkA}$ to Y_{21} inhibited PDNF-elicited Erk1/2 phosphorylation in a dose-dependent manner (Figure 6A). In contrast, when Y_{21}-treated cells were stimulated with NGF, no inhibition in Erk1/2 phosphorylation was detected, compared to cells not treated with the peptide (Figure 6B). Thus, the Y_{21}-occupied TrkA receptor appeared to be inaccessible for PDNF but still responsive to NGF signaling.

Similar to PDNF (*4*), Y_{21} but not other tested peptides also induced Akt phosphorylation in $PC12^{trkA}$ that peaked sharply at 5 min (Figure 7A). The effect was concentration-dependent (Figure 7B) and obviously occurred via TrkA signaling, because TrkA-negative $PC12^{nnr5}$ cells did not respond by Akt phosphorylation either to Y_{21} or PDNF and NGF (data not shown).

Synthetic Peptide Y_{21} Activates the Transcription Factor CREB. NGF activation of TrkA initiates intracellular signaling cascades that propagate to the nucleus to target transcription factors, particularly the cAMP response element-binding protein, CREB. Upon NGF-induced phosphorylation at regulatory site Ser133, CREB activates many genes, including other transcription factors such as *c-fos* (*31*, *32*). CREB is a major downstream target of Erk1/2 signaling in the initiation of neuronal differentiation (*31*) and of Akt kinase in the regulation of neuron survival (*30*, *33*).

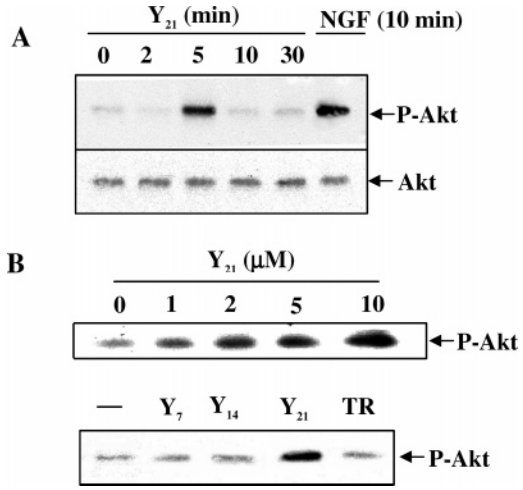

FIGURE 7: Peptide Y_{21} induces phosphorylation of Akt tyrosine kinase. (A) $PC12^{trkA}$ cells were stimulated with Y_{21} (10 μM) for different times or with NGF (30 ng/mL, 10 min). Identification of P-Akt was by Western blotting. (B) Cells were stimulated for 5 min with various concentrations of Y_{21} (upper panel) or with 10 μM indicated peptides (lower panel). Total cell proteins were subjected to PAGE, followed by immunoblotting with anti-phospho-Akt antibody.

Because CREB activation is the point that converges signals from two pathways activated by Y_{21} and PDNF, we determined whether Y_{21}-mediated signaling resulted in activation of CREB.

To test whether Y_{21} increased CREB phosphorylation at Ser133 in $PC12^{trkA}$, we performed P-CREB immunoblot experiments using extracts of untreated cells or treated with Y_{21}, PDNF, or NGF. We found that exposure of $PC12^{TrkA}$ cells to PDNF and Y_{21} enhanced the formation of phosphorylated CREB, an effect similar to that of NGF (Figure 8A).

To further substantiate this observation and to determine whether CREB phosphorylation induced by Y_{21} in fact resulted in transcription activation, a luciferase reporter assay was performed. PC12 cells were transfected with two reporter plasmids: pFLuc, containing tyrosine hydroxylase promoter, which had a canonical CRE site and was linked to firefly luciferase reporter gene, and $pCRE^{-}$, where the CRE element of the pFLuc promoter was mutated (*22*) (inset of Figure 8B). The third plasmid with the *Renilla* luciferase gene was used as an internal control. Transfected cells were then cultured in the presence or absence of inducing stimuli, and luciferase expression was assayed and calculated as a ratio between firefly and *Renilla* luciferase activity.

PC12 cells, transfected with pFLuc, responded to Y21, PDNF, and NGF with increased firefly luciferase activity. However, the mutation of the CRE site in the pFLuc promoter rendered cells transfected with $pCRE^{-}$ unresponsive to either cAMP stimulation (data not shown), PDNF, or Y_{21} (Figure 8B). The response to NGF was significantly reduced but not completely abolished because NGF could also induce transcriptional activation via the AP1 response element (*34*), also present in the TH promoter region (inset of Figure 8B).

These results further confirm, at the transcriptional level, that synthetic peptide Y_{21} is a functional mimic of PDNF.

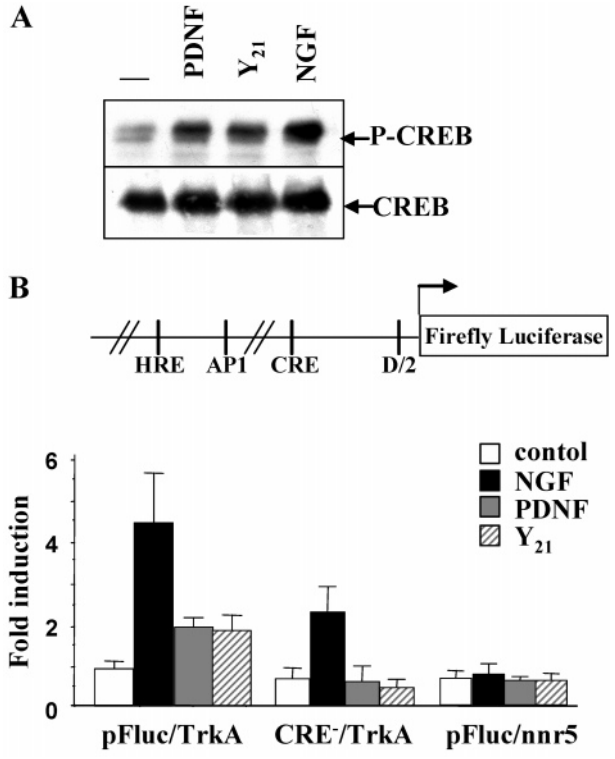

FIGURE 8: Peptide Y_{21} activates cAMP response element-dependent transcription. (A) $PC12^{trkA}$ cells were treated with PDNF (500 ng/mL) for 10 min or Y_{21} (10 μM) or NGF (30 ng/mL) for 5 min. P-CREB and CREB were detected by Western blotting with an antibody specific for P-CREB and CREB, respectively. (B) (Inset) pFLuc reporter plasmid with TH promoter (enhancer/promoter elements marked) driving a luciferase reporter (adopted from ref *22*). (Graph) PC12 cells transfected with pFLuc (pFluc/TrkA and pFluc/nnr5) or with $pCRE^{-}$ (CRE^{-}/TrkA) were treated with the control medium (control), NGF, PDNF, or Y_{21} for 10 h. Data were normalized as the fold of firefly luciferase expression in $PC12^{trkA}$ under control conditions.

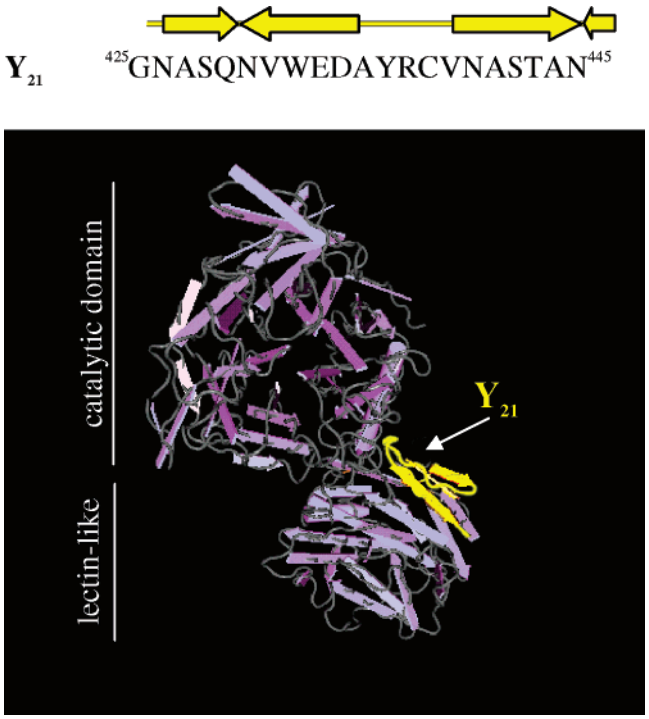

FIGURE 9: Location and secondary structure of peptide Y_{21} in the 3D structure of PDNF. Amino acid sequence of peptide Y_{21} depicting secondary structures (yellow arrow ribbons represent β strands, and the line connecting antiparallel β strands denotes a β-hairpin turn) that occur in the 3D structure of intact PDNF. The location of Y_{21} on the 3D structure of N-terminal region of PDNF (*17*) and the secondary structure of Y_{21} were determined with Cn3D program 4.1 (National Center for Biotechnology Information, NIH). Note that peptide Y_{21} is located in the lectin-like domain of PDNF.

DISCUSSION

The results presented here show that synthetic peptide Y_{21}, a 21-mer modeled on the amino acid sequence 425−445 of *T. cruzi*-derived PDNF, can promote survival and neurite outgrowth of primary DRG neurons and PC12 cells in a TrkA-dependent manner. The trophic actions of Y_{21} correlate with its ability to activate pro-survival signaling pathways (PI3K and MAPK/Erk) and transcription factor CREB. In addition to functioning as a peptidomimetic of PDNF, synthetic peptide Y_{21} can competitively inhibit PDNF binding to TrkA and, consequently, PDNF activation of the TrkA receptor and MAPK/Erk signaling pathway. Our results, therefore, support the hypothesis that the amino acid sequence corresponding to Y_{21} forms a site critical for PDNF to bind and activate TrkA receptor and, consequently, enhance survival and differentiation of neuronal cells.

The location of Y_{21} on the PDNF three-dimensional structure is consistent with a role of mediating receptor binding. The 3D structure of the N-terminal region of the PDNF isoform (clone pTrcTS611/2) (*17*) reveals a pattern commonly found in other microbial neuraminidases (*25*): one domain with a β-propeller structure containing catalytic sites for neuraminidase and sialyl transferase activities, connected with an α-helical segment to another domain having a β-barrel lectin-like structure (*17*). This lectin-like domain bears on its surface the 425−445 amino acid region (white arrow in the lower panel of Figure 9), which consists of antiparallel β strands and a reverse turn (upper panel of Figure 9) and is exposed on the surface. The structure, if assumed by Y_{21} in the PDNF isoform used here (clone 19y) (*19*, *20*), is consistent with the segment 425−445 mediating PDNF binding to TrkA; as such, surface loops often participate in the protein interaction with specific receptor sites, including NGF binding to TrkA (*35*, *36*).

The structure of PDNF bears little resemblance to that of NGF, which is a homodimeric molecule formed by twisted β sheets and reverse turn in one end and three reverse turns and cysteine-knot motif in the other end (*37*). NGF binds to the membrane-proximal immunoglobulin-like domain in the extracellular portion of TrkA through two patches, patch 1 conserved in other neurotrophins and patch 2 specific for the NGF/TrkA interaction (*35*). Several residues in both patches are essential for the neurotrophin/Trk receptor interaction, including the NGF C-terminal motif ^{109}VCV, present in peptide Y_{21} as a CV motif in the transition between the reverse turn and β strand (Figure 9). Future studies should aim to determine whether the CV motif is required for the trophic actions of Y_{21}, as it is for NGF (*38*).

Synthetic peptide Y_{21} competitively inhibits PDNF but not NGF, binding to TrkA (Figure 4). Given that PDNF binding to TrkA is inhibitable by NGF and vice versa (*15*), we assume that Y_{21} reacts with a TrkA site dispensable for NGF binding. The extracellular domain of TrkA comprises three leucine-rich motifs, flanked by two cysteine-rich clusters, followed by two Ig-like domains. Although NGF reacts with

the membrane-proximal Ig-like motif to activate TrkA (*39*, *40*), the other extracellular domains of TrkA also play a role in receptor activation (*41*). Thus, given the inability of Y_{21} to inhibit NGF binding to TrkA, presumably to the proximal Ig-like domain, it is possible that Y_{21}, as a synthetic peptide or on PDNF, reacts preferentially with a TrkA domain other than the NGF-preferred proximal Ig-like domain.

The situation is similar to that described for NGF and its peptidomimetic D3. D3 competes for binding to TrkA with TrkA agonist mAb, which blocks most of the NGF-binding sites on TrkA-expressing cells; however, D3 itself does not block NGF binding to TrkA (*42*).

In conclusion, Y_{21} reproduces TrkA-dependent trophic activity of the original protein, PDNF, by binding and activating TrkA at concentrations (1−10 μM) equaling or lower than those for short peptide mimetic agonists of TrkA (*42*, *43*).

It may very well be that peptide Y_{21}, in addition to its possible usefulness in ascertaining the molecular mechanism of PDNF activation of TrkA, can also serve as a basis for the development of small therapeutics to treat disorders where Trk receptors play a role such as peripheral diabetic neuropathies (*44*) and neuronal damage (*45*).

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