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Neuroprotective Activity of pDING in Response to HIV-1 Tat

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Abstract

Although neurons are not productively infected with HIV-1, neuronal injury and death are frequently seen in the brains of AIDS patients with neurological and neurocognitive disorders. Evidently, viral proteins including Tat and cellular inflammatory factors released by activated and/or infected microglia, macrophages, and astrocytes contribute to neuronal cell death. Several studies have demonstrated that HIV-1 associated neuronal cell injury is mediated by dysregulation of signaling pathways that are controlled, in part, by a class of serine/threonine kinases. In this study we demonstrate that pDING, a novel plant-derived phosphate binding protein has the capacity to reduce the severity of injury and death caused by HIV-1 and its neurotoxic Tat protein. We demonstrate that pDING, also called p27SJ/p38SJ, protects cells from the loss of neuronal processes induced by Tat and promotes neuronal outgrowth after Tat-mediated injury. Further, expression of pDING prevents Tat-induced oxidative stress and mitochondrial permeability. With its profound phosphatase activity, pDING controls the activity of several kinases including MAPK, Cdk5 and their downstream target protein, MEF2, which is implicated in neuronal cell protection. Our results show that expression of pDING in neuronal cells diminishes the level of hyperphosphorylated forms of Cdk5 and MEF2 caused by Tat and the other neurotoxic agents that are secreted by the HIV-1 infected cells. These observations suggest that pDING, through its phosphatase activity, has the ability to manipulate the state of phosphorylation and activity of several factors involved in neuronal cell health in response to HIV-1.

Keywords

HIV-1; DING; Neural degeneration

INTRODUCTION

Infection of macrophages, microglia and astrocytes by HIV-1 in brain leads to the release of several viral regulatory and structural proteins, as well as various cellular proinflammatory cytokines and chemokines (Gras and Kaul 2010; Yadav and Collman, 2009). Excretion of these viral and cellular proteins, and their unregulated presence in the brain environment can

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trigger a series of biological events in bystander uninfected cells thus dysregulating the normal function leading to their injury and death (Kaul and Lipton 2006). Inhibition of dendrites and axonal growth and synaptic loss are among the pathologic features that are frequently seen in AIDS patients with neurologic diseases, and are believed to be the result of these viral-induced dysregulatory events. Oxidative stress, activation of ROS, permeabilization of mitochondria, activation of MAP kinases and the related signaling pathways involved in the control of cell homeostasis are among the cellular responses that participate in neuronal cell injury and death (Galluzzi et al., 2009; Kaul 2008; Li et al., 2005). Tat is one of the viral regulatory proteins that has received much attention due to its ability to activate transcription of the HIV-1 genome and to dysregulate activity of MAPKs and several other factors involved in the control of cell growth and differentiation (Li et al., 2009; Medders and Kaul 2011; Singh et al., 2005). One important target for MAPK, myocyte-specific enhancer factor 2 also known as MEF2, is a transcription factor whose phosphorylation by MAPK and Cdk5 modulates diverse cellular processes including cell division and death (Cox et al., 2003; Gong et al., 2003; Grégoire et al., 2006; Mao et al., 1999; Ramachandran et al., 2008; Smith et al., 2006; Tang et al., 2005; Zhao 1999). MEF2 also plays an important role in synaptogenesis, neurogenesis, and neuronal degeneration in various CNS diseases (Fiore et al., 2009; Flavell et al., 2006; 2008; Pazyra-Murphy et al., 2009). Accordingly, recent studies have demonstrated that a member of the MEF2 family is dramatically downregulated in neuronal cells of AIDS patients with dementia (Yelamanchili et al., 2010). These observations ascribe an important neuroprotective role for MEF2 whose inactivation induces neurodegeneration. Recently, we have identified a novel member of the DING family of proteins from *H. perforatum*, initially named p27SJ/p38SJ, also called plant DING or pDING, which exhibits strong phosphatase activity. pDING has the capacity to interfere with proliferation of rapidly growing cells such as glioblastoma and interferes with the MAPK signaling pathway (Bookland et al., 2011; Darbinian et al., 2009; 2011; Darbinian-Sarkissian et al., 2006). Here we demonstrate that by hypophosphorylation of MAPK and Cdk5, pDING maintains MEF2 in an active form and that protects neuronal cells from injury and death by Tat in a culture system.

MATERIALS AND METHODS

Cell culture

Human primary cortical neurons were prepared by the method described below. Briefly, after removal of the meninges, intact human fetal brain tissue (embryonic age 16 weeks) was incubated with Tryple Express enzyme (Invitrogen, Carlsbad, CA) and DNase I (10 U/ml; Sigma, St. Louis, MO) at 37 °C for 10-20 minutes, followed by three washes with Hibernate E medium. Tissue trituration was performed in culture medium (Neurobasal medium containing B27 supplement and 0.25 mM Glutamax) using a glass Pasteur pipette, and cells were plated on 60 mm poly-D-lysine coated dishes (Sigma). Cytosine arabinoside Ara-C (Sigma) (final concentration 1 μ M) was added after 16 hours for two days to reduce glial proliferation. Cells were maintained in Neurobasal medium supplemented with antibiotics (10 μ g/ml gentamycin, 100 units/ml penicillin, and 10 μ g/ml streptomycin) and antifungal fungizone (1 μ g/ml; Life Technologies, Inc. Carlsbad, CA) at 37 °C in a humidified atmosphere containing 5% CO₂.

Plasmids

The pEYFP-C1 (Clontech, Madison, WI) and YFP-pDING (YFP-p38SJ) plasmids have been described previously (Darbinian et al., 2011). CFP-Tat and CFP-Tat deletion mutants were created by cloning a BamHI-EcoRI Tat fragment from CMV-Tat into BglII-EcoRI-digested pECFP-C1 expression vector (Darbinian-Sarkissian et al., 2006).

Antibodies

Antibody specific for pDING (anti-GST-p27SJ rabbit polyclonal antibody) was generated against bacterially produced recombinant GST-p27SJ by Lampire Biological Laboratories, Inc. (Pipersville, PA USA). Expression of the YFP-fusion protein, YFP-pDING and CFP-Tat was examined by western blot analysis, using Living Colors full-length monoclonal antibody (BD Biosciences Clontech). Mouse monoclonal anti-Grb2 and anti-Cdk5 antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA USA).

Purification of pDING

For preparation of plant extract, 100 mg of dried callus cultures of 1 month old *H. perforatum* plants were incubated in 1 ml of lysis buffer containing 30 mM Tris (pH 7.4.), 167 mM NaCl; 0.1% Nonidet P-40 and protease inhibitors cocktail (Sigma-Aldrich, St. Louis, MO). Cell debris was removed by centrifugation at 10,000 rpm for 5 min and the supernatant was recovered and fractionated through 3, 30, and 50 kDa Millipore Microcon filters (Millipore, Billerica, MA) to separate the 38 kDa pDING from the low molecular weight proteins and other plant organic components. Protein concentration and size fractionation were performed as described previously (Darbinian-Sarkissian et al., 2006). The purity of the pDING protein was determined by SDS-PAGE.

Transfection of neuronal cells

Human neuronal cells were transfected with expression plasmids (5 µg each) using the Lipofectamine 2000 system (Invitrogen) per the manufacturer's protocol for the transfection of primary cortical neurons

Treatment of neuronal cells

Human cortical neuronal cells were pre-incubated with pDING (300 ng/ml) for 2 h prior to incubation with rTat 101 (ImmunoDiagnostics, Inc. Woburn, MA), (50 ng/ml) for 56 h, or conditioned media (CM) from HIV-1 infected PBMCs for a total of 56 h. The number of neurons and neuronal processes was measured in the presence of pDING, Tat or both.

Infection of peripheral blood mononuclear cells (PBMC) with HIV-1

Approximately 1×10^6 PBMC were incubated with the JR-FL strain of HIV-1 (50 ng of p24-containing virus stock) with serum-free medium for 2 h at 37 °C. PBMC cells were then washed twice with PBS, and fresh medium was added. In parallel, control uninfected cells were prepared six days post-HIV-1-infection. Conditioned media from infected and uninfected PBMC cells were used to treat neuronal cells for 56 hours. Infectivity of the cells with HIV-1 was verified by p24 ELISA assay.

Preparation of protein extracts and immunoblot analysis

For preparation of whole cell extract, cells were washed with cold phosphate-buffered saline (PBS) and solubilized in lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 0.1% Nonidet P-40 and 1% protease inhibitors cocktail (Sigma). Cell debris was removed by centrifugation for 5 min at 4 °C. For Western blot analysis, 50 µg of proteins were eluted with Laemmli sample buffer, heated at 95 °C for 10 min, separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to supported nitrocellulose membranes as described previously (Darbinian-Sarkissian et al., 2006). Proteins were visualized with the enhanced chemiluminescence detection system, ECL+ according to the manufacturer's instructions (Amersham Pharmacia) and exposed to X-ray film. Western blot analysis of extracts from pDING and Tat expressing cells was performed for Cdk5 and MEF2. Grb2 served as a loading control.

Neuronal cell morphology analysis

Neuronal cell complexity and outgrowth were evaluated after three days of transfection or treatment. Neurite outgrowth was analyzed by Sholl method (Braunewell et al., 2011; Yanni and Lindsley 2000). Briefly, the percentages (<50 microns, 50-100 microns, >100 microns), averages and standard deviations are shown. The number of dendritic spines and their distribution in cortical neurons was determined by confocal microscopy and the values were analyzed by Image J software (NIH) (Homma et al., 2003; Sala et al., 2001). In each experiment, 10-50 neurons were randomly selected for spine evaluation and experiments were repeated two times.

Methylthiazolotetrazolium (MTT) Cell Proliferation Assay

For the MTT assay, we used a cell proliferation kit (MTT) according to the manufacturer's protocol (Roche Applied Sciences, Indianapolis, IN USA). Neuronal cells were plated into 96-well plates in triplicate in two sets at a density of 10,000 cells/well and pre-incubated with either pDING protein extract (0.3 µg/ml), or with rTat, at 50 ng/ml. After 24 hours, 10 µl MTT (5 mg/ml) were added to the wells (final concentration, 0.5 mg/ml) for 4 hours, and the reaction was stopped by the addition of 100 µl of solubilization solution. Viable cells with active mitochondria cleave the tetrazolium ring into a visible dark blue formazan reaction product, which was quantified by spectrophotometry in a Dynex MRX Revelation microplate reader (Dynex Technologies, Chantilly, VA) at 570 nm with a reference wavelength of 650 nm. The relative cell viability (percent) was determined as the ratio of average absorbance for treated cells to that for mock, untreated cells.

Caspase 3/7 activity assay

Apoptosis was assessed by analysis of activation of caspase-3 using the substrate DEVD-aminoluciferin from the Caspase-Glo™ 3/7 assay kit (Promega, Madison, WI, USA), according to the manufacturer's instruction. Approximately 1,000 of Tat- or pDING-treated cells, grown in a 24-well tissue culture plate were analyzed in a final volume of 100 microliters culture medium per well. One hundred microliters of Glo reagent (1:1) were added to the culture medium and cell lysis was induced by shaking the cells for 2 min at room temperature. Subsequently, the supernatants of the different wells were transferred to

the microcentrifuge tubes and the luminescent signal was stabilized for 10 min at room temperature. Luminescence was recorded on a Femtomaster FB12 Luminometer (Zylux Corporation). Data were analyzed using Excel software. The histogram shows fold increase of Caspase 3/7 activity and the error bars show the standard deviation from three independent readings.

Superoxide dismutase (SOD) activity assay

To measure SOD activity in primary neurons, cells were transfected with pEYFP-pDING and pECFP-Tat for 56 h, and then cells were analyzed by Superoxide Dismutase (SOD) assay by comparing SOD activity using the OxiSelect SOD Activity Assay kit. Cytosolic fraction was prepared and incubated with Xanthine Oxidase Solution for 1 h at 37 °C. Absorbance was read at 490 nm to generate superoxide anions. The activity of SOD is determined as the inhibition of chromagen reduction. In the presence of SOD, superoxide anion concentration is reduced, yielding less colorimetric signal. SOD activity is shown in % of control.

Mitochondrial bioenergetics

For confocal mitochondrial potential (ψ_m) measurements, primary cortical neurons were transfected with Tat- or pDING-expression plasmids for 56 h, then 50 nM of red-emitting TMRE (tetramethylrhodamine, ethyl ester, perchlorate) was added to cells and incubated for 30 min at 37 °C. Cells were imaged and quantified. The quantitation is based on the visualization of the TMRE leak from the mitochondria to the cytosol in the injured cells. Cells were imaged using a Carl Zeiss 710 two-photon confocal microscope with a W Plan-Apochromat 20×/1.0 DIC M27 75 mm liquid immersion objective, using 2.5× digital zoom, with excitation at 561nm. Images were quantified using ImageJ (National Institutes of Health). The difference in the fluorescence (FAU) intensities of red dye was calculated and compared in control (untransfected, bar 1).

Phospho-kinase array

Human phospho-MAPK array kit was used (R&D Systems, Inc., Minneapolis, MN). Neuronal cells transfected with pEYFP-C1 and YFP-pDING were lysed and lysates were applied and processed over the phospho-MAPK arrays as per the manufacturer's protocol. Quantitative representation of serine/threonine phospho-MAPK protein levels in pDING-expressing neuronal cells was determined by densitometric analysis of each spot. The values were normalized to the levels seen in YFP expressing cells. For tyrosine protein kinases, such as FGF-R2, HGFR, IGF-1R, Jak3, TXK, and the EGF receptors, including Erb3, a Receptor tyrosine kinase phosphorylation array was used per the manufacturer's protocol.

Statistical analysis—Statistical analysis was performed on data imported from Microsoft Excel software t-test.

RESULTS

pDING prevents Tat-induced neuronal cell injury and death

In the first series of experiments, we investigated the neuroprotective activity of pDING in response to neurotoxicity induced by Tat using primary fetal culture of human cortical neuronal cells. As seen in Figure 1 (Panel A), treatment of neuronal cells with Tat substantially changed neuronal cell appearance and reduced the number of living cells. Morphological changes were highlighted by reduced density of the neuronal processes and their complexity. Treatment of those cells with purified pDING prevents the neurotoxicity seen by Tat as evidenced by the number of cells and their neurite extension and complexity. Perhaps it is noteworthy to mention that, similar to Tat, extracellular DING proteins can be taken up by the cells in a biologically functional form (Darbinian et al., 2008). In the next series of experiments, we adapted an in vitro scratch/wound healing assay to evaluate the ability of pDING in promoting regeneration of neuronal processes in the presence of Tat. As shown in Figure 1 (Panel B), Tat inhibited re-growth of the neurites from the injured cells after 5 days, whereas in the presence of pDING, Tat lost its ability to suppress genesis and extension of neuronal processes in these cells. These observations indicated that while Tat prevents recovery of the damaged neurons and neuronal outgrowth, pDING has the ability to effectively block this effect and promote the healing process in the injured cells.

We also examined the cytotoxicity of Tat and the metabolic activity of neuronal cells upon treatment with Tat alone or together with pDING by MTT assay. This assay measures metabolic activity of cells by NAD(P)H-dependent oxidoreductase enzyme, which can reflect the number of viable cells (Berridge et al., 2005). As seen in Figure 1 (Panel C), treatment of the cells with increasing amounts of Tat progressively reduced cellular enzymatic activity and their viability, and pDING was able to restore this activity. Heat inactivated pDING was unable to impact Tat toxicity upon neuronal cells. In an alternative assay, we measured the number of viable cells as determined by the number of ATP, indicative of metabolically active cells by Glo assay (Fig. 1, Panel D). Again, results from this assay show an inhibitory effect of pDING upon Tat-mediated cytotoxicity.

In the next study, we utilized Sholl-plug analysis to quantify the morphological features of neurons as related to dendritic arborization and extension (Braunewell et al., 2011; Yanni and Lindsley 2000). Specifically, we determined the number (Fig. 2B) and length (Fig. 2C) of neurite intersections by superimposing concentric circles around the center of the neuronal soma and gradually increasing radii from 20 to 100 μ M (Fig. 2A). We utilized NIH Image J software for quantification and the results indicated that expression of full-length Tat, but not mutant Tat encompassing amino acid in neuronal cells drastically decreased the range of arborization of dendrites in close proximity of the soma. In the presence of full-length pDING, and to a lesser degree, mutant pDING (1-100), a noticeable improvement in the neurite number and their branchings is observed (Fig. 2B and C).

In a parallel study, we examined formation of dendrite spines, which serve as the primary site of excitatory synaptic transmission, in cells expressing Tat and pDING (Fig. 3A). Quantification of number of spines per dendrites showed a dramatic decrease in the number of spines and alteration in their shape in cells expressing Tat. We also noted that full-length

pDING partially counteracts the effect of Tat as more dendritic spines mushroom-headed, which are seen in healthy neurons, are detected in these cells (Fig. 3B).

Altogether, these observations support the notion that pDING has the capacity to protect neuronal cells against morphological and metabolic alteration caused by HIV-1 Tat.

pDING protects neurons from Tat-induced oxidative stress and mitochondrial hyperpolarization

Mitochondria are a major source of reactive oxygen species and are believed to play an integral role in cellular response to stress. By opening of mitochondria transition permeability, MTP, which allows the release of apoptotic inducing factors, mitochondria triggers apoptosis under stress conditions. Here, we examined the effect of pDING upon Tat-induced oxidative stress by measuring the activity of superoxide dismutase (SOD). As shown in Fig. 4 (Panel A), treatment of neuronal cell culture with Tat decreased SOD activity and that pDING greatly recovered Tat-inhibitory effect. Mutant Tat protein encompassing amino acid residues 1-47 had no effect on neuronal cells. Further, smaller variants of pDING that contain the first 100 amino acids of the protein was less effective in comparison to the full-length protein. To examine mitochondrial permeability, we utilized confocal visualization of tetramethylrhodamine ethyl ester perchlorate (TMRE) upon expression of Tat in neuronal cells. Results from this experiment showed that, in the presence of Tat, TMRE (red-emitting compounds) was leaked from the mitochondria to the cytosol, indicative of the injuries to the cells and the early event of apoptosis. The cytosolic appearance of TMRE in the presence of pDING was less than those seen in Tat. Fig. 4 (Panel B) illustrates quantitative analysis of these observations by Image J software based on the differences in the fluorescence (FAU) intensities of the red dye.

pDING exhibits phosphatase activity

In light of our earlier observations on the phosphatase activity of pDING (Darbinian et al., 2009), and the notion that HIV-1 induces neuronal injury is mediated, in part, by a class of kinases that determines neuronal cell development, differentiation and survival/death, we evaluated the impact of pDING upon serine/threonine and tyrosine kinases. Results from the MAP kinase array revealed that expression of pDING in neuronal cells has a negative effect, ranging between 1.5 to 9.0 fold, on the level of phosphorylation of various members of the serine-threonine MAP kinases (Fig. 5, Panel A). Interestingly, pDING had no significant impact on the level of phosphorylation of receptor tyrosine kinase (Fig. 5, Panel B).

Next, we investigated the effect of pDING on several cellular proteins whose levels and state of phosphorylation are mediated by the MAPK family and play a critical role in survival pathways of neuronal cells. In the first effort, we investigated the state of Cdk5 and the results demonstrated a modest enhanced level of the phosphorylated form of pCdk5, in neuronal cells upon Tat expression. Interestingly, pDING was able to decrease the level of Cdk5, with no impact on the total level of this protein (Fig. 5C). This observation suggests that pDING, either through MAPK and/or independent of MAPK, hypophosphorylates Cdk5. One of the important prosurvival factors that regulates neuronal development and synaptic plasticity is MEF2, a transcription factor, whose level of phosphorylation by Cdk5

and MAPK determines its neuroprotective activity (Flavell et al., 2006; Mao et al., 1999; Smith et al., 2006). Evidently, phosphorylation of MEF2 upon activation of Cdk5 at the specific amino acid residues positioned within the transactivation domain of MEF2, suppresses its activity and induces mature neuronal cell death upon their exposure to neurotoxic agents (Gong et al., 2003). Thus, in the next experiment, we examined the level of MEF2 and the state of its phosphorylation at serine 444, a critical residue for its neuroprotective activity, in neuronal cells expressing Tat, pDING and Tat plus pDING. As illustrated in Fig. 5C, Tat enhanced the level of phospho-MEF2 compared to the control cells and similar to its effects on pCdk5, pDING decreased the level of hyperphosphorylated form of MEF2 in these cells. Expression of Tat was verified by Western blot (data not shown). These observations suggest that due to its phosphatase activity, pDING hypophosphorylates MAPK and Cdk5, which, in turn, initiates a cascade of biological events that potentiate neuroprotective activity of MEF2 and others in neuronal cells.

In a separate set of studies, we utilized human neuronal cell culture and demonstrated that treatment of these cells with conditioned media from the HIV-1 infected cells elevates the level of phosphorylated form MEF2 in neuronal cells, in comparison to that from the neuronal cells treated with the control (HIV-1 negative) conditioned media (Fig. 5D, compare lanes 1 and 3). Again, expression of pDING in these cells decreased the level of phospho-MEF2 (Fig. 5D, compare lanes 3 and 4). Of interest, treatment of neuronal cells with HIV-1 conditioned media decreased the total level of MEF2 in comparison to control (Fig. 5D, compare lanes 1 and 3), while expression of pDING restores its levels (Fig. 5D, compare lane 4 with lane 2). Altogether, these observations highlight the ability of pDING to impact the state of phosphorylation of Cdk5 and its target MEF2, and this event may describe, at least in part, the neuroprotective activity of pDING and cells exposed to HIV-1 Tat and/or the neurotoxic compounds released from the HIV-1 infected PBMCs.

DISCUSSION

Progressive loss of structure and function of neurons that eventually leads to their death is among the pathological hallmarks of the brains of AIDS patients who exhibit neurologic and neurocognitive disorders (Galluzzi et al., 2009; Kaul 2008; Kaul and Lipton 2006; Li et al., 2005). In light of several efforts establishing no evidence for infection of neuronal cells by HIV-1, it is now generally accepted that the observed injuries to neuronal cells in the context of neuroAIDS is initiated by viral and cellular factors secreted from the bystander HIV-1 infected cells, which have neurotoxic activity (Li et al., 2009; Medders and Kaul 2011; Singh et al., 2005). In fact, several studies have demonstrated active release of HIV-1 Tat protein from infected cells and its impact on the function of the neighboring uninfected cells after its uptake (King et al., 2006 Microb Inf). Here, we demonstrate that treatment of primary neuronal cells with Tat has a negative impact on arborization, regeneration of neurites and spine formation, and increases oxidative stress, reduces SOD activity, and increases mitochondrial permeability; all events that are indicative of cell injury and death. Treatment of these cells with pDING prevented neuronal cell damage caused by Tat and allowed re-growth of neuronal processes after the injury. Examination of several kinases that are involved in cell development and differentiation showed that pDING with its strong phosphatase activity can modulate the state of phosphorylation of the MAPK family of

proteins. MAPKs phosphorylate and direct the transcription factor myocyte-specific enhancer factor 2 (MEF2) to multiple signaling pathways, including neuronal cells survival, neuronal differentiation, and synapse development (Flavell and Greenberg, 2008; Zhao et al, 1999; Shalizi et al, 2005; Finegan et al, 2009; McKinsey, 2002; Tian et al, 2010; Jan and Jan, 2010). MEF2 family transcription factors (MEF2, isoforms A-D) are DNA binding proteins that recognize an A/T-rich element within the promoter regions of many genes, and are critical for the development and function of many types of cells, including cardiac, vascular, immune, and nervous systems. In neurons, MEF2 can be activated by neurotrophin stimulation. MEF2A and MEF2C, but not MEF2B or MEF2D, can be phosphorylated and regulated by MAP kinases. In addition to the MAPK family, activation of Cdk5 also contributes to HIV-1 induced neuronal damage and death (Wang J, 2007). Similar to MAPK, Cdk5 is a serine/threonine kinase with a pivotal role in neuronal cell development, regulation of neurotransmission and synaptic function (Cheung et al., 2012; 2006). Our data (presented in Fig. 5) demonstrate that treatment of neuronal cells with Tat and the conditioned media from HIV-1 infected macrophages increased the level of the phosphorylated form of Cdk5 and one of its target proteins, MEF2. MEF2 is a family of transcription factors that are activated by neurotrophin stimulation and its activation controls synapse development. Earlier studies demonstrated that Cdk5-mediated neuronal apoptosis includes phosphorylation of MEF2 at specific serine residues within the transactivation domain of MEF2 that abrogates its neuroprotective activity. Thus, it is conceivable that the neuroprotective activity of pDING, at least in part, involves hypophosphorylation of Cdk5 and MEF2 at serine 444. Once MEF2 at serine 444 is hypophosphorylated it loses its ability to transactivate a subset of cellular genes involved in neuronal survival. Of note, Cdk5 has the capacity to modulate activity of several other prosurvival factors including pRb and p53 (Akay et al., 2011). In fact, recent observations support evidence for the involvement of pRb via Cdk5 kinase activity in HIV-1-induced neuronal cell death. Accordingly, our results show that the state of phosphorylation of pRb in neuronal cells exposed to HIV-1 in the presence of Tat is increased (unpublished data). This observation suggests that hypophosphorylation of Cdk5 by pDING can impact on several neurotoxic pathways that are triggered by HIV-1 and its Tat protein.

In conclusion, our studies indicate that pDING has a strong neuroprotective activity against HIV-1-induced neuronal cell injury, as evidenced by its capacity to promote neurite arborization, enhance superoxide dismutase activity and prevent mitochondrial permeability in the presence of Tat. Further, due to its phosphatase activity, pDING suppresses the activity of several serine/threonine phosphokinases such as the p38MAPK family and Cdk5, hence impacting on the activity of neuroprotective factors such as MEF2 and others. As abnormal regulation of p38MAPK and Cdk5 has been associated with a number of neurodegenerative disorders, one may envision a therapeutic role for pDING in diseases such as Parkinson's disease, Alzheimer's disease and others.

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Figure 1. Effect of Tat and pDING on neuronal cells

A. Phase Imager illustrates the effect of Tat (50 ng/ml) on the appearance of human cortical neurons in the absence or presence of pDING (300 ng/ml). Magnification 200X. B. Scratch-wound healing assay demonstrating genesis and extension of neurite outgrowth after injury in the absence or presence of Tat and Tat plus pDING. Magnification 200X. C. MTT assay illustrating neuronal cell metabolism and activity in the presence of Tat and pDING. The asterisks indicate heat inactivated pDING. The values are derived from three independent experiments. D. Glo assay depicting caspase 3/7 cleavage in cells after treatment as indicated. The values represent the results from these experiments.

Figure 2. Typical morphological illustration of primary human cortical neuronal cells and quantitative analysis of neuronal cell extension and complexity with Tat and pDING expressing cells

A. Images of representative cells transfected with control plasmid (left panels) or plasmids expressing Tat or Tat plus pDING (right panels). Sholl analysis illustrating methods for quantification of neurite extension and arborization at various distances from the soma, which is positioned at the center. B. Quantification of the number of neurites for each cell transfected with control plasmid or Tat variants and Tat plus pDING variants. Ten to fifty cells in each group were randomly selected. C. Shift in dendritic arbor size upon expression of Tat and its correction by pDING. The numbers in each bar represent dendritic arbors of >50 microns, 50-100 microns, and <100 microns as indicated. In each group 10-50 cells were randomly selected.

Figure 3. Illustration of dendritic spines in neuronal cells

A. After transfection of neuronal cells with pEYFP or Tat and Tat plus pDING expressing plasmid, formation of the dendritic spines was analyzed by confocal microscopy. B. Quantitative values of spine formation in the control and neuronal cells expressing Tat and Tat plus pDING. Results were analyzed using Image J Software (NIH).

A

B

Figure 4. Effect of pDING on Tat-mediated oxidative stress and mitochondrial hyperpolarization in cortical neuronal cells

A. Oxidative stress assay in neuronal cells transfected with plasmids expressing Tat (pECFP-Tat) or Tat plus pDING (pEYFP-pDING) and their mutant variants. Fifty-six hours post-transfection, cells were harvested and the levels of superoxide dismutase (SOD) were determined as described in the Materials and Methods. The values represent the results from three assays for each sample. B. Mitochondrial bioenergetic assay as described in the Materials and Methods in cells expressing Tat or Tat plus pDING. The quantification is done using Image J Software (NIH) from the confocal imaging of cells illustrating the leakiness of the mitochondria and diffusion of red-emitting TMRE.

Figure 5. Effect of pDING on phosphorylation of various kinases in neuronal cells

A. Quantitative presentation of results from phosphoMAP kinase array experiment illustrating fold-effect of pDING on serine-threonine in kinases of human cortical neurons. B. Effect of pDING on tyrosine phosphorylation in neuronal cells using receptor tyrosine kinase phospho-array. C. Western blot illustrating expression and state of phosphorylation of various cellular proteins involved in neuroprotection in neuronal cells expressing Tat, pDING and both. D. Examination of MEF2 levels and its phosphorylation status in neuronal

cells treated with conditioned media (CM) from HIV-1 infected macrophages (HIV-1+) or media from uninfected macrophages.