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PIN: A Novel Protein Involved in IFN- γ Accumulation of NOS-1 in Neurons

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Abstract

In this study we investigate the role of the protein inhibitor of NOS-1 (PIN) in the interferon- γ (IFN- γ)–mediated posttranscriptional accumulation of nitric oxide synthase-1 (NOS-1) and the anti-vesicular stomatitis virus response in neuronal cells. IFN- γ –induced enhancement of NOS-1 activity is crucial for its antiviral activity in the central nervous system. IFN- γ treatment of neuronal cells results in an increase of total NOS-1 and decrease of total PIN proteins without alteration in their respective mRNA levels. PIN/NOS-1 complexes decreased after IFN- γ treatment. Transfection of cells with small interfering RNA (siRNA) for PIN results in a higher constitutive activity of NOS-1 and inhibition of viral replication. IFN- γ treatment did not change the amount of NOS-1 detectable by Western blot, when PIN is diminished by RNAi treatment. Overexpression of PIN results in lower constitutive NOS-1 expression and activity, and diminishes activation of NOS-1 by IFN- γ . Our findings indicate that in neurons, IFN- γ upregulates NOS-1 through proteasomal degradation of PIN.

Introduction

Interferon- γ (IFN- γ) plays a crucial early antiviral role in protection against viral infection in the central nervous system (CNS) (Komatsu *et al.*, 1999; Rodriguez *et al.*, 2003). IFN- γ treatment results in nitric oxide synthase-1 (NOS-1) accumulation posttranscriptionally. Viral clearance from neurons is associated with increased NO production both *in vitro* and in the CNS (Bi and Reiss, 1995; Komatsu *et al.*, 1996; Chesler *et al.*, 2004). The regulation and the mechanism of this phenomenon largely remain unknown. Protein inhibitor of NOS-1 (PIN) is degraded following IFN- γ treatment; more ubiquitin-modified PIN and a shorter half-life are observed in IFN- γ –treated neuronal cells (Yang *et al.*, 2006). We propose that PIN is centrally involved in the IFN- γ –mediated accumulation of NOS-1 and antiviral responses in neurons.

Localization and activation of NOS enzymes are regulated via protein–protein interactions (Alderton *et al.*, 2001; Dedio *et al.*, 2001). Of the three NOS isoforms, NOS-1 is the largest due to a 300-amino-acid PDZ (PSD-95/Discs large/zona occludens-1) domain insertion at the N terminus, targeting the protein to the postsynaptic density (PSD). Several proteins have been identified to interact with NOS-1 in neurons and modulate its activity. The PSD protein PSD-95 binds and anchors NOS-1 in a complex with the N-methyl-D-aspartic acid receptor (NMDAR)

(Christopherson *et al.*, 1999). Carboxy-terminal PDZ ligand of nNOS (CAPON) competes with PSD-95 for interaction with NOS-1 in the PSD of synaptic spines (Jaffrey *et al.*, 1998), and is thought to reduce the capability of NOS-1 to produce NO. Nitric oxide synthase-interacting protein (NO-SIP) interacts and colocalizes with NOS-1 in synaptic spines and inhibits NOS activity (Dreyer *et al.*, 2004). In the CNS, NOS-1 is activated by Ca^{2+} influx through the NMDA glutamate receptor, triggering of the NMDAR kinase (Putzke *et al.*, 2000).

A 10 kDa protein that physically interacts with and inhibits the activity of NOS-1 was identified using a yeast two-hybrid screen. The protein was named Protein Inhibitor of Neuronal NOS (Jaffrey and Snyder, 1996a). PIN and NMDAR colocalize with NOS-1 in penile nerves (Ferrini *et al.*, 2003). The PIN-binding region of NOS-1 maps to a 17-residue peptide fragment from Met-228 to His-244 of NOS-1 (Fan *et al.*, 1998). PIN binding destabilizes the NOS-1 homodimer, a conformation necessary for enzymatic activity (Dunbar *et al.*, 2004). Prostaglandin E2, an inhibitor of NOS-1, induces PIN expression in neurons; CelecoxibTM treatment, which enhances NOS-1 activity, inhibits PIN expression (Chen and Reiss, 2002). In addition, PIN inhibits NO and superoxide production by purified NOS-1 (Xia *et al.*, 2006).

IFN- γ treatment prolongs the half-life of NOS-1 (Yang *et al.*, 2006). In neurons, PIN is posttranscriptionally downregulated by IFN- γ treatment; more ubiquitination and faster degradation of PIN are detected in IFN- γ -treated samples (Yang *et al.*, 2006). In this study, we examine the turnover and interaction of PIN and NOS-1 following IFN- γ treatment. To assess this, RNA interference (RNAi) and overexpression techniques were employed. The results demonstrate that in neuronal cells, IFN- γ enhances NOS-1 and antiviral activity through down-regulation of PIN.

Materials and Methods

Cell cultures and reagents

NB41A3 cells and L929 cells were purchased from ATCC (Manassas, VA). NB41A3 cells were maintained in F-12K medium containing 15% horse serum, 2.5% fetal bovine serum (FBS), and 1% penicillin/streptomycin at 37°C and 5% carbon dioxide (CO_2) (Komatsu *et al.*, 1996). L929 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) low-glucose media containing 10% FBS, 1 mM L-glutamine, HEPES buffer, and 1% penicillin/streptomycin at 37°C and 5% CO_2 (Reiss *et al.*, 1983). Unless otherwise stated, all tissue culture reagents were obtained from Mediatech (Herndon, VA). Anti-vesicular stomatitis virus (VSV), Indiana serotype, San Juan strain, originally obtained from Alice S. Huang (then at the Children's Hospital, Boston, MA) was used in all viral studies. VSV was propagated in Chinese hamster ovary cells and purified over a sucrose gradient as previously described (Chang *et al.*, 1994). Recombinant murine IFN- γ was purchased from R&D Systems (Minneapolis, MN) and used at a concentration of 20 ng/mL in all experiments.

Antibodies

Mouse anti-mouse NOS-1 monoclonal antibody (mAb) (1:2000 for Western blot) and rabbit anti-mouse NOS-1 polyclonal antibodies (pAbs) were purchased from BD Pharmingen (San Jose, CA). Rat anti-mouse PIN mAb (1:500 for Western blot, 1:50 for IP) was purchased from Axxora (San Diego, CA). Anti-GAPDH mAb (1:100,000 for Western blot) was purchased from Abcam (Cambridge, MA). Anti-Xpress mAb was purchased from Invitrogen (Carlsbad, CA). Sheep anti-VSV pAb was a generous gift from Dr. Alice S. Huang (when at the Children's Hospital, Boston, MA). Horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody (1:2000) was purchased from Vector (Southfield, MI). Donkey anti-sheep (1:4000) and donkey anti-rabbit (1:5000) secondary antibodies, both HRP conjugated, were purchased

from Jackson Immunologicals (West Grove, PA) and Zymed (South San Francisco, CA), respectively.

Plaque assay

Viral titers were determined in triplicates using serially diluted culture supernatants on monolayers of L929 cells as previously described (Komatsu *et al.*, 1996). The geometric mean and geometric mean error of the titer were calculated, and the groups were compared statistically using analysis of variance (ANOVA).

RT-PCR

mRNA levels were determined by multiplex RT-PCR using the Qiagen One-Step RT-PCR kit (Valencia, CA). Total RNA (100 ng) was used in each reaction. The following protocol was used to carry out the reaction: 50°C for 30 min, 95 °C for 15 min, 36 cycles of 94°C for 1 min, 58°C for 30 s, 72°C for 1 min, then 72°C for 10 min, and 4°C hold. The primers used in these reactions are listed in Table 1. Products were resolved by 1% agarose gel electrophoresis, stained using ethidium bromide (Bio-Rad, Hercules, CA) or Sybr-Gold dye (Invitrogen), and visualized using a UV transilluminator. Results were digitally photographed using a Kodak DC290 camera controlled by the Kodak 1D analytic software (Kodak, Rochester, NY). DNA band densities were determined using the UN-SCAN-IT software.

Griess assay

The concentration of NO₂⁻ in culture supernatants was determined by assaying the stable end product of NO (Hewett, 1999).

Western blot

Cells were lysed, and proteins were analyzed by Western blot (Chesler *et al.*, 2004). Protein band densities were quantitated by UN-SCAN-IT software.

Sandwich enzyme-linked immunosorbent assay

Flat 96-well plates were coated with capture antibody (anti-NOS mAb 500 ng/well, or anti-PIN mAb 100 ng/well) diluted in 100 µL coating buffer (0.1 M sodium bicarbonate, pH 9.6) and incubated at 37°C for 1 h. The plates were washed five times with phosphate-buffered saline (PBS) containing 0.1% Tween-20 and air dried. Two hundred µL (0.5 mg/mL) of the test sample was added to duplicate wells and incubated overnight at 4°C. Wells were washed five times as above and blocked with 100 µL 2% dried milk powder/PBS (BLOTTO) for 1 h at 37°C. Following another five washes, detecting antibody (anti-NOS mAb 10 µg/mL, or anti-PIN mAb 10 µg/mL) diluted in 100 µL BLOTTO was added to plates and incubated at 37°C for 1 h. Wells were washed and incubated with secondary antibody for 30 min (HRP-conjugated anti-mouse IgG 1:1000 or anti-Rat IgM 1:500). TMB substrate was added, and 15 min later the reaction was stopped by TMB Stopping Solution (Sigma, St. Louis, MO). The reaction produces a yellow-to-orange color, which was quantified at 450 nm by microplate reader.

RNAi

siRNA targeting PIN, a negative control siRNA, and the siPORT™ Amine® transfection reagent were purchased from Ambion (Austin, TX). The siRNA was transfected into NB41A3 cells according to the instruction of the manufacturer's manual (Ambion). Briefly, the transfection was carried out using siR-NA (final concentration 50 nM) diluted in 50 µL of OPTI-MEM media and 1.5 µL of the Amine reagent diluted in 47.5 µL OPTI-MEM media. Ten minutes later, the siRNA was mixed with the transfection reagent and incubated for another 10 min at room temperature (RT) for complex formation. The 100 µL siRNA–Amine complex

was mixed with 900 μ L regular NB41A3 media containing 1×10^5 NB41A3 cells and plated into 12-well plates. Total RNA was extracted at 24 h posttransfection, and protein isolation or VSV infection was conducted 48 h posttransfection.

Plasmid construction and mutagenesis

The plasmid containing cDNA of PIN, PGEX-4T2-PIN, was a gift from Dr. Samie Jaffrey at Weill Cornell Medical School (Jaffrey and Snyder, 1996b). The PIN cDNA fragment was cloned into PcDNA3.1/His A vector by restricting enzyme cutting and ligation (EcoRI and NotI), generating PcDNA3.1/His A/PIN. Mutagenesis of this new plasmid was conducted by constructing a one-nucleotide insertion in the PIN sequence, using Stratagene QuickChange kit (La Jolla, CA). Primers used for mutagenesis are listed in Table 1. The PCR was programmed as follows: 95°C for 30 s, 18 cycles of 95°C for 30 s, 55°C for 30 s, 68°C for 6 min, and then 4°C hold. PCR products were digested with Dpn to remove original template and purified with a Qiagen gel extraction kit. Successful mutagenesis was confirmed by sequencing.

Transient and stabilized transfection

NB41A3 cells were transfected with the PcDNA3.1/His A/PIN vector using the Lipofectamine2000[®] transfection reagent, following manufacturer's instructions (Invitrogen). Briefly, 2×10^5 NB41A3 cells were plated in each well and grown overnight to reach 50% confluency in six-well plates (Nunc, Rochester, NY). The transfection was carried out using 6 μ g of plasmid diluted in 75 μ L of DNA diluent and 15 μ L of the Lipofectamine2000 reagent diluted in 60 μ L serum-free DMEM. The DNA was then added to the transfection reagent and incubated for 10 min at RT for complex formation.

The PcDNA3.1/His A/PIN–Lipofectamine2000 complex was placed on the cells and incubated for 48 h at 37°C and 5% CO₂. For transient transfection, cells were lysed on 24 h for RNA expression and 48 h for protein detection. For stabilized transfection, 48 h posttransfection the media were replaced with standard growth media containing 400 μ g/mL G418. Mock-transfected NB41A3 cells and NB41A4 cells transfected with PcDNA3.1/His A/MUTA were also treated with G418 as a control for selection. After 4 days of culture, the mock-transfected NB41A3 cells were completely eliminated, while the PcDNA3.1-transfected cells were healthy. These cells were split under selection conditions and plated for further experiments.

Statistical methods

To determine if there was a significant difference among the groups, mean $\pm$ SEM of each group was compared using a parametric ANOVA. The results were considered significant for a *p*-value less than 0.05. Pairwise comparison among the groups was done using the Tukey's *post hoc* test.

Results

The constitutive ratio of PIN:NOS-1 and complexes between these proteins are affected by IFN- γ treatment

To investigate if the interaction between PIN and NOS-1 was altered by IFN- γ treatment, we collected total protein from IFN- γ -treated and control cells and determined the amount of PIN/NOS-1 complexes as well as free PIN and NOS-1 by sandwich enzyme-linked immunosorbent assay (ELISA). We had earlier attempted to characterize this interaction using standard co-immunoprecipitation (co-IP), but we found that the association of PIN and NOS-1 is weak and easily disrupted (data not shown). To resolve this problem, we combined bivalent reversible

cross-linking with co-IP; however, two cross-linkers aggregated too many proteins and the gels were uninterpretable (data not shown).

Using a sandwich ELISA, equal amounts of fresh cell lysates from cells treated with media or IFN- γ at different time points were added to plates precoated with the indicated capture antibody, and then incubated with primary detection and secondary antibodies for signal production (Fig. 1). Consistent with Western blot data (Yang *et al.*, 2006), IFN- γ treatment resulted in a sixfold increase of total NOS-1 and 75% decrease of total PIN. A transient increase followed by a decrease of PIN/NOS-1 complexes was observed with IFN- γ treatment in neuronal cells (Fig. 1). This suggests that there is more free PIN than NOS-1 in NB41A3 cells.

RNAi of PIN expression results in a higher constitutive expression and activity of NOS-1 and diminishes enhancement of NOS-1 by IFN- γ

To further characterize the involvement of PIN in the IFN- γ -mediated accumulation of NOS-1 and the antiviral response, we suppressed expression of PIN by RNAi. PIN siRNA and the negative control siRNA were introduced to NB41A3 cells.

One concern we had using this technique is that RNAi may induce an IFN- β response (Sledz *et al.*, 2003) because of double-stranded RNA (dsRNA) segments. Although reports suggested that siRNA smaller than 30 nucleotides was less likely to induce this response (Zhou *et al.*, 2003a), we wanted to make certain that IFN- β production was not triggered by introduction of siRNA. We tested for expression of the IFN- β mRNA in our samples by RT-PCR and confirmed that siRNA treatment did not induce IFN response in NB41A3 cells (Supplemental Fig. 1, available online at www.liebertpub.com).

siRNA-transfected cells and mock-transfected cells were treated with IFN- γ for 24 h, and the suppression of PIN expression by siRNA was validated by Western blot (Fig. 2A). Expression of NOS-1 protein and NO₂⁻ production were measured. Figure 2A shows that when PIN is diminished, a higher constitutive NOS-1 level is observed and IFN- γ treatment does not change the amount of NOS-1. NO production is consistent with the protein expression data (Fig. 2B).

RNAi suppression of PIN results in increased inhibition of viral replication

Following siRNA transfection and IFN- γ treatment, we infected NB41A3 cells with VSV and examined the expression of VSV proteins by Western blot and viral replication by plaque assay. RNAi inhibition of PIN results in ~25% of the amount of M protein synthesized compared to the control, and more than 10-fold increased inhibition of release of infectious viral progeny (Fig. 3). We interpret these data to be consistent with the decrease of PIN results in increased functional NOS-1 and decreased unstable PIN/NOS-1 complexes. As a consequence, the half-life of NOS-1 is prolonged, higher constitutive NOS-1 expression is observed, accompanied by increased NOS-1 activity, and enhanced inhibition of VSV replication results.

Overexpression of PIN results in lower constitutive NOS-1 levels and diminished activation of NOS-1 by IFN- γ

We overexpressed PIN in NB41A3 cells to examine its effect on IFN- γ -mediated accumulation of NOS-1 and inhibition of viral replication. cDNA for PIN from PGEX-4T2-PIN (Jaffrey and Snyder, 1996a; Zhou *et al.*, 2003b) was excised and inserted into a cytomegalovirus (CMV)-driven eukaryotic expression vector PcDNA3.1/His A. The newly constructed PcDNA3.1/His A/PIN encodes cDNA with an N-terminal XpressTM tag fused to PIN. Transfected cells were visualized by antibody staining and fluorescence microscopy (Supplemental Fig. 2A, available online at www.liebertpub.com). A 50–70% transfection efficiency was achieved. Expression of Xpress-PIN mRNA was determined by RT-PCR (Supplemental Fig. 2B, available online at www.liebertpub.com).

A negative control plasmid PcDNA3.1/His A/MUTA was constructed using PcDNA3.1/His A/PIN as template; mutagenesis was performed by PCR to introduce a one-nucleotide insertion within the open-reading frame of Xpress-PIN. With the frameshift on the open-reading frame, the cells transfected with PcDNA3.1/His A/MUTA did not express Xpress-PIN (Fig. 4A).

To characterize the consequence(s) of PIN overexpression on the IFN- γ -mediated anti-VSV response in the neuronal cells, NB41A3 cells were transfected with PcDNA3.1/His A/PIN or PcDNA3.1/His A/MUTA. Cell lysates were collected, and overexpression of Xpress-PIN was confirmed by co-IP of Xpress and PIN (Fig. 4A). The interaction of Xpress-PIN and NOS-1 was determined by sandwich ELISA (Fig. 4B). Expression of NOS-1 protein and NO₂⁻ production were also measured (Fig. 4C, D). When PIN is overexpressed, we observed increases in PIN/NOS-1 complexes, lower constitutive NOS-1 expression and activity, and diminished activation of NOS-1 by IFN- γ (Fig. 4). While IFN- γ downregulates Xpress-PIN by ~25%, in PIN overexpressing cells, there was an excess pool of endogenous PIN remaining in the cells that inhibits NOS-1 accumulation and activity as effectively as in control cells.

The effect of PIN overexpression on viral replication

In a parallel experiment, neuronal cells were mock treated or IFN- γ treated for 24 h and infected with VSV. Viral proteins were detected by Western blot, and viral titer in supernatants was determined by plaque assay (Fig. 5A and B, respectively). More abundant viral proteins are produced, and increased viral replication is observed in NB41A3 cells that over-expressed PIN. IFN- γ treatment inhibits viral replication about threefold in PIN-overexpressing neuronal cells, while more than 15-fold inhibition is observed in the control IFN- γ -treated cells (Fig. 5). These data indicate that PIN is centrally involved in the IFN- γ regulation of NOS-1 activity.

Discussion

Posttranscriptional accumulation of NOS-1 by IFN- γ treatment appears to be critical in the inflammatory cytokine-mediated antiviral response in the neurons (Chesler *et al.*, 2004). The mechanism of this posttranscriptional increase in NOS-1 and enhanced viral clearance in neurons were investigated in this study. We have concentrated on the role(s) of PIN in VSV infection in the neuronal cells.

Following IFN- γ treatment of neuronal cells, we observed a transient increase followed by a decrease of PIN/NOS-1 complexes (Fig. 1). This suggests that there are more free PIN than NOS-1 in the cytosol. When expression of PIN is reduced, higher constitutive NOS-1 expression levels and activity are observed (Fig. 2 and Supplemental Fig. 1). In PIN-overexpressing neuronal cells, we observed lower constitutive NOS-1 activity, and diminished activation of NOS-1 by IFN- γ compared with the control cells (Figs. 4 and 5, and Supplemental Fig. 2). While the conditions we have used are not physiological, they may give insights into responses *in vivo*.

In the CNS, IFN- γ 's functions are related to the modulation of the immune system and the control of the infectious diseases. Elevated expression of IFN- γ was observed in the human immunodeficiency virus (HIV-1)-infected brain and may play an important role in neuropathological pathways of HIV-related encephalopathy (Koedel *et al.*, 1999; Shapshak *et al.*, 2004). IFN- γ -producing $\gamma\delta$ T cells may help control murine West Nile virus infection (Wang *et al.*, 2003). The murine coronavirus, JHMV, infects a variety of CNS cell types during the acute phase of infection. In IFN- γ KO mice, increased morbidity and mortality were associated with persistent virus replication, indicating a role of the cytokine in control of viral replication (Parra *et al.*, 1999). These diverse viral infections illustrate the importance of IFN- γ in the immune modulation and clearance of viruses from the CNS.

NO-dependent antiviral activity has been found in a variety of neurotropic virus infections, including CMV (Kosugi *et al.*, 2002), HIV (Hori *et al.*, 1999), herpes simplex virus 1 (HSV-1) (Kodukula *et al.*, 1999), mouse hepatitis virus (MHV) (Lane *et al.*, 1997), poliovirus (Komatsu *et al.*, 1996), and reovirus (Goody *et al.*, 2005). Several studies have suggested possible mechanisms by which viral replication is inhibited by NO. Peroxynitrite, ONOO(−), was reported to react with tyrosines, serines, and cysteines of proteins, and results in NO₂Y, NO₂S, and NO₂C (Gu *et al.*, 2002). Such modifications could alter the activity of a viral polymerase or other viral proteins that are critically involved in virus life cycle, or affect host proteins required for virus assembly to attenuate viral infection (Simon *et al.*, 1996; Saura *et al.*, 1999).

PIN was reported to inhibit nNOS activity in cell lysates through disruption of enzyme dimerization (Dunbar *et al.*, 2004). In both *in vitro* and *in vivo* studies, dimer stabilization of NOS-1 protects the enzyme from proteolysis (Bender *et al.*, 2000; Dunbar *et al.*, 2004). However, the effect of PIN on degradation and NO production of NOS-1 in the neurons has not been examined. Utilizing overexpression and siRNA, as well as the sandwich ELISA technique, we not only explore the role of PIN in the IFN- γ -mediated accumulation and activation of NOS-1, but also quantitate the PIN:NOS ratio over the course of IFN- γ treatment. Our findings provide important insights into IFN- γ -mediated antiviral responses in neurons.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIG. 1.

IFN- γ treatment in neuronal cells results in an increase followed by decrease of PIN/NOS-1 complexes. NB41A3 cells were treated with media or IFN- γ for 8, 16, or 24 h. Amounts of NOS-1, PIN, or NOS-1/PIN complex were determined by double-antibody sandwich ELISA. HRP-conjugated secondary antibody and TMB substrate were used in all reactions. **(A)** Expression of NOS-1 increases with IFN- γ treatment. Ninety-six-well plates were coated with anti-NOS-1 pAb (Capture Ab). Equal amounts of cell lysates from above treated samples were added in triplicate into precoated well and detected with anti-NOS-1 mAb. **(B)** Expression of PIN decreases with IFN- γ treatment. Anti-PIN antibody was used for both capturing and detecting. **(C)** An increase followed by decrease of NOS-1/PIN complexes was observed along with IFN- γ treatment in neuronal cells. Anti-NOS-1 antibody was used in capturing steps, and anti-PIN antibody was used in detecting steps. Same results were observed when using anti-PIN antibody for capturing and anti-NOS-1 antibody for detecting. Results are representative of three independent experiments (* $p < 0.01$; ** $p < 0.05$).

FIG. 2.

IFN- γ treatment does not change the amount of NOS-1 when PIN is knocked down by RNAi. siRNA of PIN or negative control siRNA (Neg, random sequence that does not target any genes) was introduced to NB41A3 cells using siPORT Amine transfection reagent. siRNA-transfected cells and mock-transfected cells were treated with IFN- γ for 24 h. **(A)** Expressions of PIN and NOS-1 protein were determined by Western blot. **(B)** NO_2^- production was measured by Griess assay. Values are mean $\pm$ SE of three independent experiments (* p > 0.05; ** p < 0.01; *** p < 0.05).

FIG. 3.

RNAi of PIN in NB41A3 cells results in inhibition of viral replication. siRNA of PIN or Neg siRNA was introduced to NB41A3 cells using siPORT Amine transfection reagent. siRNA-transfected cells and mock-transfected cells were treated with IFN- γ for 24 h and infected with VSV at M.O.I = 3. Cell lysates were extracted at 5 h postinfection. Supernatants were collected at 7 h postinfection. (A) Expression of the VSV M protein relative to GAPDH was determined by Western blot. (B) Viral replication was measured by plaque assay. Values are mean $\pm$ SE of three samples. Results are representative of three independent experiments (* p > 0.05; ** p < 0.01; *** p < 0.001).

FIG. 4.

Overexpression of PIN results in lower constitutive NOS-1 expression and activity, increased PIN/NOS-1 complexes, and diminished activation of NOS-1 by IFN- γ . NB41A3 cells were plated in six-well plates and cultured overnight prior to transfection with PcDNA3.1/His A/PIN or PcDNA3.1/His A/MUTA. The untransfected control NB41A3 cells were treated with the same amount of transfection reagent. Twenty-four hours posttransfection, cells were treated with media or IFN- γ for indicated time. (A) Overexpression of Xpress-PIN was confirmed by co-IP of Xpress and PIN. Anti-PIN antibody was used at IP step, and anti-Xpress antibody was used for Western blot (or vice versa, data not shown). Original lysates (no IP step involved) collected from NB41A3 cells transfected with pcDNA3.1/His/LacZ plasmid (Invitrogen) served as a positive control for anti-Xpress antibody in the Western blot. (B) Amount of NOS-1/PIN complex was measured by sandwich ELISA using original lysates. (C) Expression of NOS-1 protein was determined by Western blot. (D) In a parallel experiment, NB41A3 cells were transfected with PcDNA3.1/His A/PIN or PcDNA3.1/His A/MUTA, and then treated with media or IFN- γ for 48 h. Supernatant medium was collected, and production of NO $_2^-$ was measured by Griess assay. Values are mean $\pm$ SEM of three independent experiments (* $p > 0.05$; ** $p < 0.01$; *** $p < 0.05$).

A **B** **FIG. 5.**

PIN overexpression resulted in increased viral replication in NB41A3 cells. In PIN overexpressed cells, IFN- γ treatment did not inhibit viral replication as significantly as the control group. NB41A3 cells were plated in 12-well plates (2.5×10^5 cells/well) and cultured overnight prior to transfection with PcDNA3.1/His A/PIN or PcDNA3.1/His A/MUTA. The untransfected control NB41A3 cells were treated with the same amount of transfection reagent. Twenty-four hours posttransfection, cells were treated with media or IFN- γ . Following 24 h IFN- γ treatment, cells were infected with VSV at M.O.I = 3. Supernatant and cell lysates were collected at 5 h postinfection. (A) VSV M protein was measured by Western blot with GAPDH

as housekeeping control. **(B)** The viral titer was determined by plaque assay. This experiment is representative of three independent experiments (* $p < 0.01$; ** $p > 0.05$).

Table 1

Primers Used for PCR/RT-PCR and Size of Products

	Sense 5'–3'	Antisense 5'–3'	Size (bp)
Xpress-PIN	tagcatgactgggtggacagc	ggatcactgggtgtttggca	490
GAPDH	atcacaaggcagctcaagg	tggatgcagggatgatgttc	467
IFN- β	catcaactataagcagctcca	ttcaagtggagagcagttgag	353
PcDNA3.1/His A/MUTA	ccagcacagtggcggccgcgtgtc	gacagcgcggccgccactgtgctgg	6000