

Pin1-dependent Prolyl Isomerization Modulates the Stress-induced Phosphorylation of High Molecular Weight Neurofilament Protein*

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Aberrant phosphorylation of neuronal cytoskeletal proteins is a key pathological event in neurodegenerative disorders such as Alzheimer disease (AD) and amyotrophic lateral sclerosis, but the underlying mechanisms are still unclear. Previous studies have shown that Pin1, a peptidylprolyl *cis/trans*-isomerase, may be actively involved in the regulation of Tau hyperphosphorylation in AD. Here, we show that Pin1 modulates oxidative stress-induced NF-H phosphorylation. In an *in vitro* kinase assay, the addition of Pin1 substantially increased phosphorylation of NF-H KSP repeats by proline-directed kinases, Erk1/2, Cdk5/p35, and JNK3 in a concentration-dependent manner. *In vivo*, dominant-negative (DN) Pin1 and Pin1 small interfering RNA inhibited epidermal growth factor-induced NF-H phosphorylation. Because oxidative stress plays an important role in the pathogenesis of neurodegenerative diseases, we studied the role of Pin1 in stressed cortical neurons and HEK293 cells. Both hydrogen peroxide (H₂O₂) and heat stresses induce phosphorylation of NF-H in transfected HEK293 cells and primary cortical cultures. Knockdown of Pin1 by transfected Pin1 short interference RNA and DN-Pin1 rescues the effect of stress-induced NF-H phosphorylation. The H₂O₂ and heat shock induced perikaryal phospho-NF-H accumulations, and neuronal apoptosis was rescued by inhibition of Pin1 in cortical neurons. JNK3, a brain-specific JNK isoform, is activated under oxidative and heat stresses, and inhibition of Pin1 by Pin1 short interference RNA and DN-Pin1 inhibits this pathway. These results implicate Pin1 as a possible modulator of stress-induced NF-H phosphorylation as seen in neurodegenerative disorders like AD and amyotrophic lateral sclerosis. Thus, Pin1 may be a potential therapeutic target for these diseases.

The reversible phosphorylation on Ser/Thr-Pro ((S/T)P) motifs governed by Pro-directed protein kinases and phosphatases is a major regulatory mechanism for the control of various cellular processes. The phosphorylation state of a protein reflects the balance between the activation of kinases

and phosphatases, and also of partners, such as isomerases, able to support their activity through conformational changes. Neurofilament proteins are among the most highly phosphorylated proteins in the nervous system (1–3). Extensive phosphorylation of (S/T)P repeats in the tail domain of high molecular weight neurofilament protein (NF-H)² occurs primarily in axons (4, 5). The deregulation of NF-H tail domain phosphorylation is correlated with some pathology seen in neurodegenerative disorders such as Alzheimer disease (AD) and amyotrophic lateral sclerosis (ALS) in which aberrant tail domain phosphorylation occurs in neuronal perikarya (6, 7).

Rat NF-H has 52 KSP repeats in the tail domain, and almost all of them are phosphorylated *in vivo* (8, 9). The multiple repeats of the SP moiety suggest that reconfiguration of NF-H may involve additional factors to catalyze peptidylprolyl isomerization. One such candidate for this is the peptidylprolyl isomerase, Pin1 (protein interacting with NIMA (never in mitosis A)-1). Pin1 catalyzes the *cis* to *trans* isomerization of peptide bonds that link Ser(P) or Thr(P) residues to proline. Although Pin1 has been widely studied in relation to the cell cycle and cancer, little is known about its role in the brain. Recent data suggested that Pin1 might be involved in AD pathogenesis, because it is found in neurofibrillary tangles (10). Pin1 has also been shown to facilitate Tau dephosphorylation by protein phosphatase 2A (10). We have shown recently that Pin1 may also be implicated in post-phosphorylation modulation of pSer/pThr-Pro (KSP) repeats in NF-H, which, when deregulated, may lead to perikaryal motor-neuron inclusions typical of ALS (11).

A growing number of phosphorylated proteins participating in important cellular processes such as RNA polymerase II (12) and CDC25 (13) are regulated through prolyl isomerase-induced conformational changes. Pin1 activity has been reported to influence its target proteins in two completely opposite fash-

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² The abbreviations used are: NF-H, high molecular weight neurofilament protein; AD, Alzheimer disease; Cdk5, cyclin-dependent kinase 5; EGF, epidermal growth factor; Erk1/2, extracellular signal-regulated kinases 1 and 2; JNK, c-Jun N-terminal kinase; Pin1, protein interacting with NIMA (never in mitosis A)-1; p-NF-H, phosphorylated NF-H; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; siRNA, short interfering RNA; ALS, amyotrophic lateral sclerosis; SAPK, stress-activated protein kinase; DN, dominant-negative; GST, glutathione S-transferase; DIC, days in culture; Erk, extracellular signal-regulated kinase; PD, Parkinson disease.

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ions: 1) It can facilitate the dephosphorylation of Tau by protein phosphatase 2A (10). 2) Pin1 is shown to facilitate hyperphosphorylation and inhibit dephosphorylation of RNA polymerase II (12). Several processes are up-regulated by Pin1: some of these involve its actions on Cyclin D1 (14), CK2 (15), c-Jun (16), c-Fos (17), and Gephyrin (18). On the other hand, SRC3 (19), c-Myc (20), and Cyclin E (21) involve processes that are down-regulated by Pin1.

Oxidative stress, an early event in AD that occurs prior to cytopathology, can induce rapid hyperphosphorylation of KSP epitopes of NF-H in cultured neurons. This is likely to be mediated by activation of proline-directed kinases or by protein phosphatase 2A inactivation (22). The role of stress-activated protein kinases such as JNK (SAPK) were reported to be involved in stress-induced NF-H phosphorylation (23, 24). Among the JNK isoforms, JNK3 (SAPK1 β) is the major stress activated protein kinase widely expressed in the nervous system (25), specifically in neuronal cell bodies (26). JNK3 is shown to phosphorylate neurofilament (NF-H) and is activated under glutamate stress (24). Absence of excitotoxicity-induced apoptosis in the hippocampus of JNK3 knockout mice has been reported (27). In this study, we show that Pin1 elevates the NF-H phosphorylation by proline-directed kinases such as Erk1/2, Cdk5, and JNK3 *in vitro*. Both dominant-negative (DN) Pin1 and Pin1 siRNA reduced oxidative and heat stress-induced aberrant NF-H hyperphosphorylation. We show that Pin1 modulates the H₂O₂ and heat stress-induced perikaryal phosphorylation of NF-H via the activation of JNK3.

EXPERIMENTAL PROCEDURES

Homology Modeling—Modeling of the structure of rat Pin1 was performed based on the x-ray structures of the proteins that produced the best E value when using BLAST against the protein in the protein data bank (PDB) data base using the Swiss PDB Viewer package (www.expasy.ch/swissmod/SWISS-MODEL.html) (28). The molecular modeling method used was ProMod II (28). Three-dimensional models of rat Pin1 were predicted using the coordinates of human Pin1 (PDB code 1nmv, determined at 1.45 Å) (29). The Pin1 protein sequence was aligned with the sequence of the homologous Pin1 using the advanced BLAST (www.ncbi.nlm.nih.gov/BLAST/). Refinement of side chains and terminal chains was done using the Molecular Operation Environment software package (Version 2001.01, Chemical Computing Group, Montreal, Canada). The generated model was then energy minimized in SYBYL (Tripos Associates, St. Louis, MO) using a three-stage protocol involving simplex, conjugate-gradient, and Powell minimization methods, by moving side chains alone, to relieve short contacts at the interprotomer interfaces. The quality of the three-dimensional model was evaluated using PROCHECK and Prosa II version 3.0 (30). Improvements in the model were obtained by an iterative sequence-structure alignment procedure, yielding the final sequence alignment between the Pin1 domain and homologous structures. The modeled structure was stable at room temperature during a 140-ps unconstrained full protein molecular dynamics simulation. Three-dimensional models were visualized by RasMol (31), and calculations were performed on Silicon Graphics IRIS 4D/25 workstations.

Primary Embryonic Neuronal Culture—Rat primary cortical neurons were prepared from E17–18-day-old Wistar rat embryos as follows. The brain and meninges were the first to be removed. Cortex was carefully dissected out and mechanically dissociated in culture medium by triturating with a polished Pasteur pipette. Once dissociated and after trypan blue counting, cells were plated at a density of 5×10^5 cells/cm² in poly-D-lysine-coated 6-well plates (BD Biosciences). For dissociation, plating, and maintenance, we used Neurobasal medium supplemented with 2% B27 containing 200 μ M glutamine and 1% antibiotic-antimycotic agent (Invitrogen). The medium was replenished every 3 days and 24 h before cell treatment.

Pin1 and NF-H cDNAs—Dominant-negative Pin1 was produced by making a point mutation to produce an alanine at serine 16, using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Transfections of wild-type and dominant-negative Pin1 constructs were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Wild-type Pin1 was cloned into pGEX-5X-1 obtained from Amersham Biosciences for GST expression studies.

Pin1 siRNA—Employing the HiPerformance Design Algorithm, synthetic 21-mer siRNA (short interfering RNA) was designed and synthesized (Qiagen). Pin1 siRNA (silencing) sense and antisense sequences were: 5'-r(GCUCAGGCCGAGUGUACUA)dTdT-3' and 5'-r(UAGUACACUCGGCCUGAGC)dTdT-3', respectively. All Stars negative control siRNA, a scrambled, non-silencing control siRNA, was obtained (Qiagen). Sense and antisense sequence for non silencing siRNA were: 5'-r(UUCUCCGAACGUGUCACGU)d(TT)-3' and 5'-r(ACGUGACACGUUCGGAGAA)d(TT)-3', respectively. To ensure the absence of possible complementary binding sites in mammalian genomes, the siRNA sequences were extensively checked against the GenBank™ data base using the Smith-Waterman algorithm. This strategy was used to exclude significant sequence homology within the genomes of mammalian species, which may interfere with the target specificity of the siRNA and contribute to unwanted off-target effects in later experiments. The sense and antisense strands were annealed to create the double-stranded siRNA at a 20 μ M concentration. Control siRNA and Pin1 siRNA were dissolved in suspension buffer to obtain a 20 μ M solution and then heated at 90 °C for 1 min. The final transfection step was preceded by a 60-min incubation at 37 °C. Delivery of siRNA was performed as described previously (11). Transfection efficiency of siRNA in HEK293 cells and cortical neurons was judged to be greater than 80% using fluorescent-labeled oligonucleotides. For experiments using cell lines, cells were transfected with siRNA or the indicated constructs using Lipofectamine 2000 (Invitrogen) in Opti-MEM I for 24 h, and then the medium was changed back to growth medium for additional incubation. For experiments using primary cultures, 5 DIC cortical neurons were transfected with siRNA using Lipofectamine 2000 in Opti-MEM I for 1.5 h. Subsequently, the medium was changed back to neurobasal medium (Invitrogen), and neurons were cultured for additional periods of time before further treatment.

HEK293 Cell Culture and Transfection—HEK293 cells were obtained from the American Type Culture Collection, cultured

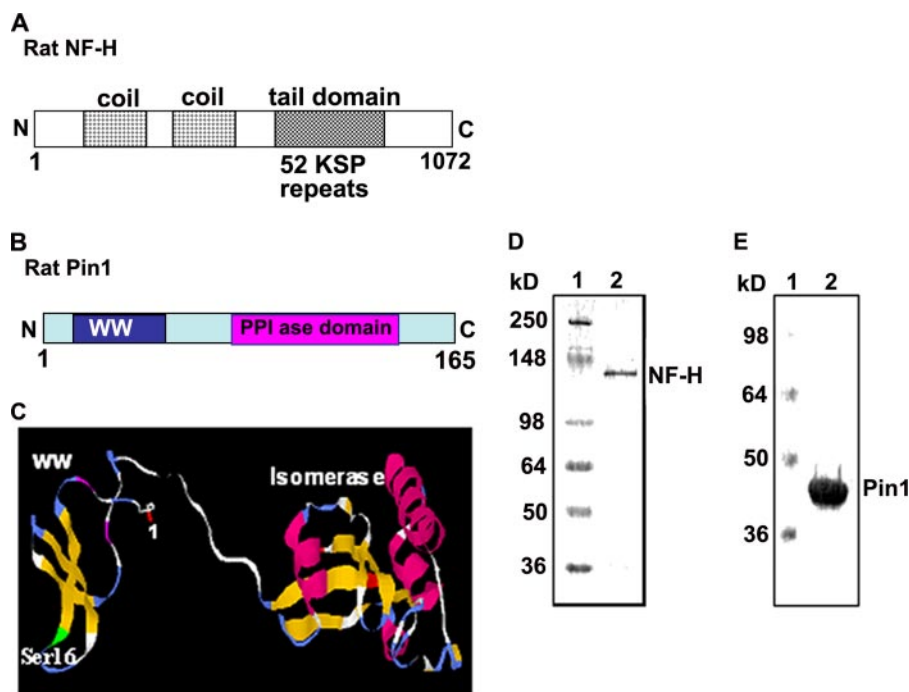


FIGURE 1. *A*, diagram of Rat NF-H showing the multiple KSP repeats in the tail domain. *B* and *C*, rat Pin1 domains and model. Position of Ser-16, WW domain, and the rotamase domain are shown in the model. *D* and *E*, bacterially expressed and purified proteins used in this study. Coomassie Blue staining of 4–12% SDS-PAGE gel of purified rat NF-H (lane 2 (*D*)) and GST-Pin1 (lane 2 (*E*)). Both rat NF-H and Pin1 were bacterially expressed and purified. Sizes of marker proteins are indicated in lane 1 in *D* and *E*.

in Dulbecco's modified Eagle's medium with 10% calf serum, and supplemented with 100 units/ml penicillin and 100 μ g/ml streptomycin. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂. The cells were transiently transfected using Lipofectamine (Invitrogen) according to the manufacturer's instructions. The human NF-H tail domain expression construct, wild-type Pin1, and dominant-negative Pin1 constructs were either transfected independently or co-transfected. Twenty-four hours after transfection, the cells were starved in the presence of 0.2% calf serum overnight (to reduce any background stimulation by serum factors), and then the cells were induced with EGF (10 nM) or PD98059. Finally, the cells were fixed for immunocytochemistry analysis or lysed with lysis buffer for Western blot analysis.

Cell Treatment—Cortical neurons 7 days in culture (7 DIC) or HEK293 cells transfected with NF-H for 2 days were treated with 1 mM H₂O₂ for 1 h, or exposed to 44 °C in a 5% CO₂ incubator for 30 min. Thirty percent stock H₂O₂ (Sigma) was dissolved in 0.2 M phosphate-buffered saline (pH 7.4). JNK inhibitor (SP600125) was purchased from Calbiochem and used at 100 μ M in HEK293 cells and cortical neurons.

Antibodies—Pin1 antibodies were obtained from Cell Signaling Technologies (Beverly, MA) and Oncogene Research Products and used for Western blotting (1:1,000) and immunohistochemistry (1:100), respectively. RT-97 antibody that specifically stains p-NF-H was provided by Drs. Ralph Nixon and Veeranna (Nathan Kline Institute, Orangeburg, NY) and used at 1:500–1000 dilutions for immunofluorescence and 1:5000 dilutions for Western blotting. SMI31 was obtained from Covance (Princeton, NJ) and used at 1:500 for immunofluorescence and 1:2500 for Western blotting. Anti-tubulin

antibody (clone DM1A), total NF-H (clone N52), and 4',6-diamidino-2-phenylindole (Sigma-Aldrich, St. Louis, MO) were used at 1:10,000 for Western blotting and 1:1,000 for nuclear counter-staining, respectively. Anti-phospho-Jun, c-Jun, JNK, and actin antisera were purchased from Cell Signaling (Beverly, MA).

Electrophoresis and Immunoblotting—Equal volumes of cells harvested in Nu-PAGE sample buffer (Invitrogen) were denatured at 100 °C during 5 min, loaded onto 4–12% Nu-PAGE Novex gels (Invitrogen), and transferred to nitrocellulose. Membranes were blocked in Tris-buffered saline, pH 8, 0.05% Tween 20 with 5% skim milk or bovine serum albumin, and incubated with primary antibody. Membranes were incubated with horseradish peroxidase-labeled secondary antibody (goat anti-rabbit or anti-mouse IgGs, Sigma), and bands were visualized by

chemiluminescence (ECL, Amersham Biosciences).

Expression and Purification of the Fusion Proteins—The pGEX recombinant plasmid containing Pin1 was expressed and purified as described earlier (32). Rat NF-H was purified as described (22).

Assay Conditions of Phosphorylation—Bacterially expressed and purified NF-H (2.5 μ g) was incubated in an *in vitro* kinase assay reaction buffer (Tris-HCl, pH 7.4, 5 mM MgCl₂, 1 mM each of vanadate, EGTA, EDTA, and dithiothreitol) 1 mM ATP, 1 mM dithiothreitol. Reaction was performed at 30 °C for 2 h with 1 unit of p42/44 MAPK or Cdk5 or JNK.

In vitro JNK assay was performed using (GST)-cJun-(1–79) (Stratagene) as a substrate. Endogenous JNK from 7 DIC cortical neurons was immunoprecipitated using a polyclonal JNK3-selective antibody (Santa Cruz Biotechnology, Santa Cruz, CA). c-Jun fusion protein at a concentration of 2 μ g/20 μ l reaction was phosphorylated using JNK3 in an *in vitro* kinase assay with 1 \times Kinase Buffer (25 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 5 mM β -glycerophosphate, 0.1 mM Na₃VO₄, 2 mM dithiothreitol, 50 μ M ATP) and 1 mM ATP. After a 30-min assay at 30 °C, the reaction was terminated by the addition of Laemmli sample buffer (33), and phosphorylation was detected by Western blot with phospho-specific c-Jun antibodies (Santa Cruz Biotechnology).

Immunocytochemical Staining of Neuronal Cultures—Primary rat cortical neurons were plated on polylysine-coated glass coverslips and processed for immunocytochemistry as previously described (34). Coverslips were mounted using Gel-Mount (Biomed, Foster City, CA). Double immunostainings were carried out using anti-Pin1 and RT-97 antibodies. Pin1 staining was revealed with a goat anti-rabbit IgG (H+L) anti-

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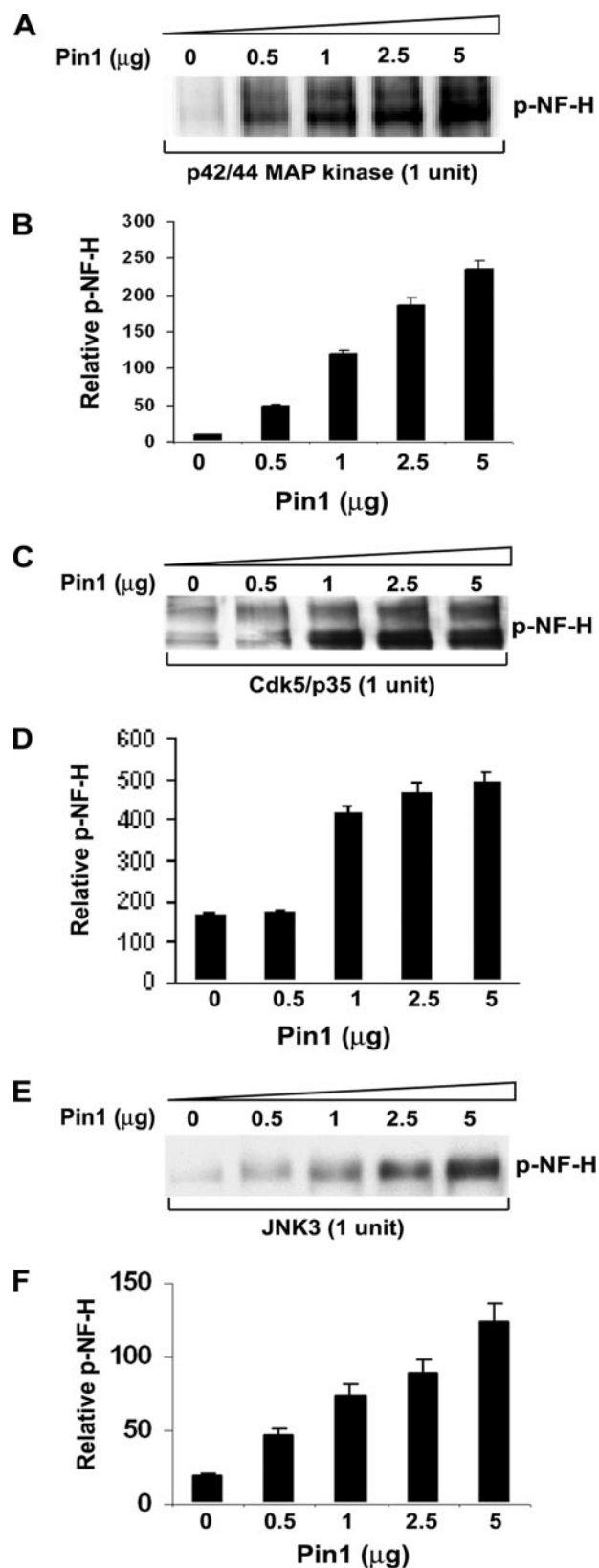


FIGURE 2. Pin1 elevates the NF-H phosphorylation by proline directed Ser/Thr kinases. A, purified NF-H was incubated at 30 °C for 2 h with increasing concentrations of GST-Pin1 (lanes 1–5), in the presence of p42/44 MAPK and 1 mM ATP. Reactions were terminated by addition of SDS loading buffer, and proteins were analyzed by 4–12% SDS-PAGE. Blots were probed with monoclonal antibody RT97 that specifically detects p-NF-H. B, densitometry analysis of p-NF-H with data obtained from A. C, reactions were performed as

body coupled to Alexa Fluor[®]488 and RT-97 with a goat anti-mouse IgG (H+L) antibody coupled to Alexa Fluor[®]568 (Molecular Probes). *In situ* cytotoxicity kits were obtained from Roche Applied Science (Indianapolis, IN) and TUNEL (terminal deoxynucleotidyl transferase-mediated nick end labeling) staining was performed according to the manufacturer's instructions before immunocytochemistry was carried out. Images were captured with an oil immersion 63× objective on a Zeiss LSM510 using LSM Image Software.

Statistics—Each experiment was repeated at least four times. The data were expressed as the means ± S.D. Student's *t* tests were used to compare the effects of all treatments. The differences were considered statistically significant as: *, *p* < 0.01; and **, *p* < 0.001.

RESULTS

The presence of multiple SP repeats in NF-H protein suggests that Pin1 could play a direct role in NF-H function by influencing its phosphorylation pattern (Fig. 1A). Comparative modeling based on human Pin1 model (29) revealed a conserved molecular model for mouse and rat Pin1 (Fig. 1B) with a WW domain on the N terminus and an isomerase catalytic domain on the C terminus (Fig. 1C). We expressed and purified human Pin1 (as a GST fusion protein in *Escherichia coli*) and rat NF-H (recombinant protein in *E. coli*) (Fig. 1, D and E).

Pin1 Stimulates NF-H Phosphorylation by p42/44 MAPK and Cdk5/p35—Initially, we sought to test the effect of Pin1 on the ability of proline-directed kinases to phosphorylate NF-H. Several proline-directed kinases have been identified, and they function under different physiological conditions. Both, MAPKs (Erk1/2 and JNKs) and Cdk5 play pivotal roles in NF phosphorylation and in the development of the nervous system by mediating both neurogenesis and neuronal differentiation. To examine the potential role of Pin1 in NF-H phosphorylation by MAPKs and Cdk5, we first used purified NF-H as a substrate. Recombinant and purified NF-H was incubated with p42/44 MAPK in a kinase assay buffer containing ATP either without or with increasing concentrations of GST-Pin1. The phosphorylated NF-H was run on SDS-PAGE and detected by RT-97 antibody, a monoclonal antibody that detects the p-NF-H. Strikingly, addition of GST-Pin1 increased phosphorylation of NF-H in a dose-dependent manner (Fig. 2, A and B). Addition of GST alone to the reaction mixture did not alter the p-NF-H levels (data not shown). We next tested whether Pin1 might also stimulate phosphorylation of NF-H by Cdk5/p35. Bacterially expressed and purified NF-H was incubated with or without GST-Pin1 and Cdk5/p35. NF-H phosphorylation was monitored by Western blotting with RT-97 antibody. GST-Pin1 significantly stimulated phosphorylation of NF-H by Cdk5/p35 in a concentration-dependent manner (Fig. 2, C and D). In addition, GST-Pin1 elevated the phosphorylation of NF-H in a dose-dependent manner by JNK3 (Fig. 1, E and F). These results suggest that Pin1 facilitates the phosphorylation

in A with Cdk5/p35 at 30 °C for 2 h. D, densitometry analysis of p-NF-H with data obtained from C. E, reactions were performed as in A with JNK3 at 30 °C for 2 h. F, densitometry analysis of p-NF-H with data obtained from E. Data shown are the mean ± S.E. of four different experiments.

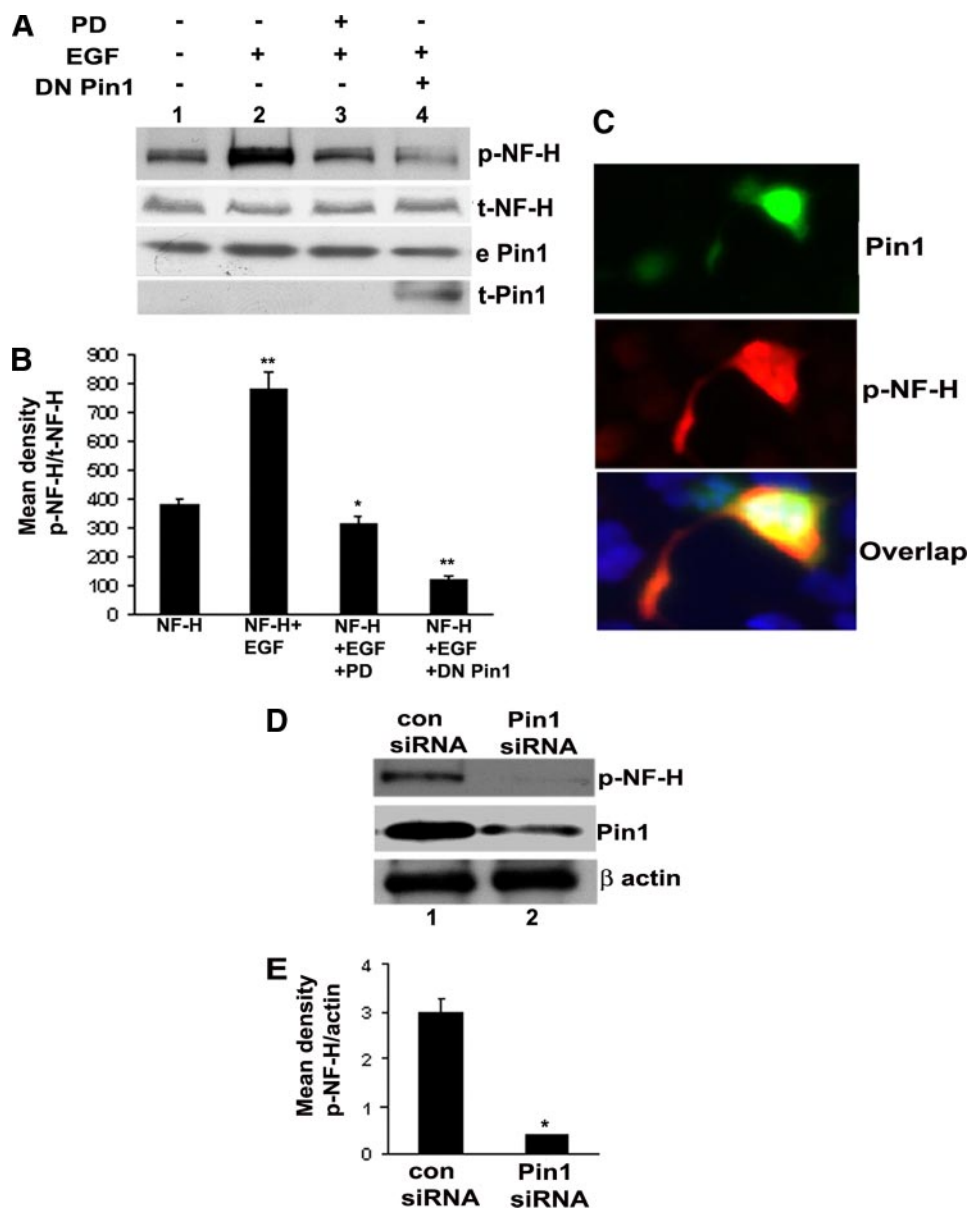


FIGURE 3. DN-Pin1 inhibits the EGF induced p-NF-H in transfected HEK293 cells. *A*, Western blot analysis of NF-H phosphorylation. HEK293 cells were transfected with NF-H (lanes 1–4) or cotransfected with DN-Pin1 (lane 4). After transfection for 24 h, cells were starved overnight and then stimulated with EGF (lanes 2–4) or pretreated with PD98059 (PD) for 1 h and then stimulated with EGF (lane 3). The cell lysates were subjected to Western blot analysis using p-NF-H, Pin1, and total NF-H antibodies. (*t-DNPin1*, transfected green fluorescence protein-Pin1 (50 kDa); *ePin1*, endogenous Pin1 (18 kDa); and *t-NF-H*, total NF-H). Phosphorylated NF-H and total NF-H were detected using RT97 and total NF-H antibodies, respectively. *B*, densitometry analysis of the phosphorylated NF-H obtained from *A*. *C*, colocalization of phosphorylated NF-H and Pin1. HEK293 cells transfected with NF-H were treated with EGF and fixed with 4% paraformaldehyde. Cells were stained with monoclonal RT97 (1:500) and Pin1 antibodies (1:100), followed by fluorescein isothiocyanate-conjugated secondary IgG-conjugated goat anti-rabbit antibody and rhodamine-labeled goat anti-mouse IgG secondary antibody (Sigma, 1:100). Images were obtained using a Zeiss LSM 510 laser scanning confocal microscope. *D*, HEK293 cells were transfected with NF-H and scrambled control siRNA (lane 1), NF-H and Pin1 siRNA (lane 2). Western blot analysis was performed with RT-97 (top row) and Pin1 (middle row) and β -actin (bottom row) antibodies. *E*, densitometry analysis of Western blots of p-NF-H with data obtained from *D*. Data shown are the mean \pm S.E. of three different experiments.

of NF-H by proline-directed kinases such as Erk1/2, Cdk5, and JNK3.

Pin1 Modulates the NF-H Phosphorylation in HEK293 Cells—Because Pin1 has the potential to modulate the NF-H phosphorylation levels *in vitro*, it was important to determine if Pin1 directly modulates the NF-H phosphorylation levels *in situ*. HEK293 cells do not express endogenous NF. We initially

examined the phosphorylation status of NF-H in NF-H-transfected HEK293 cells by Western blotting. EGF stimulation of HEK293 cells transiently transfected with NF-H increased the p-NF-H, suggesting that MAPKs phosphorylate NF-H *in vivo*. The EGF-mediated increase in p-NF-H is inhibited by the MAPK inhibitor, PD98059 (Fig. 3, *A* and *B*). If the function of the Pin1 is to increase the phosphorylation of NF-H, the cotransfection of NF-H and DN-Pin1 should reduce the p-NF-H levels. A DN-Pin1 construct, made by mutating Ser-16 to Ala, inhibits the isomerase activity of Pin1. Transfected DN-Pin1 migrated as a green fluorescence protein fusion protein at \sim 50 kDa, whereas endogenous Pin1 migrated at \sim 18 kDa. Expression of NF-H with transfected DN-Pin1 reduced NF-H phosphorylation in EGF-treated transfected cells (Fig. 3, *A* and *B*). Furthermore, in EGF-treated transfected cells, endogenous Pin1 colocalizes with p-NF-H (Fig. 3*C*). In addition, inhibition of Pin1 by Pin1 siRNA but not scrambled siRNA reduced the p-NF-H expression (Fig. 3, *D* and *E*). These results demonstrate that Pin1 modulates NF-H phosphorylation.

Pin1 Modulates the Oxidative Stress-induced Phosphorylation of NF-H in Transfected HEK293 Cells—We next investigated possible functional consequences of Pin1-dependent hyperphosphorylation of NF-H. Experimental motor neuron disease models are characterized by oxidative stress leading to the accumulation of phospho-NFs within perikarya. Oxidative stress happens to be an early event in AD prior to cytopathology. Shea *et al.* (35) reported that the treatment of DRG neurons with H₂O₂ resulted in the increase of p-NF-H in cell bodies.

We initially sought to test if NF-H phosphorylation is modulated by H₂O₂ stress. Treatment of HEK293 cells with 1 mM H₂O₂ for 1 h in transfected HEK293 cells resulted in \sim 6-fold increase of phosphorylated NF-H (Fig. 4, *A* and *B*). Inhibition of Pin1 by Pin1 siRNA and DN-Pin1 rescued the increase in p-NF-H by H₂O₂ (Fig. 4, *A* and *B*). Phosphorylation of NF-H with heat shock has not been studied earlier. Hence, we tested if

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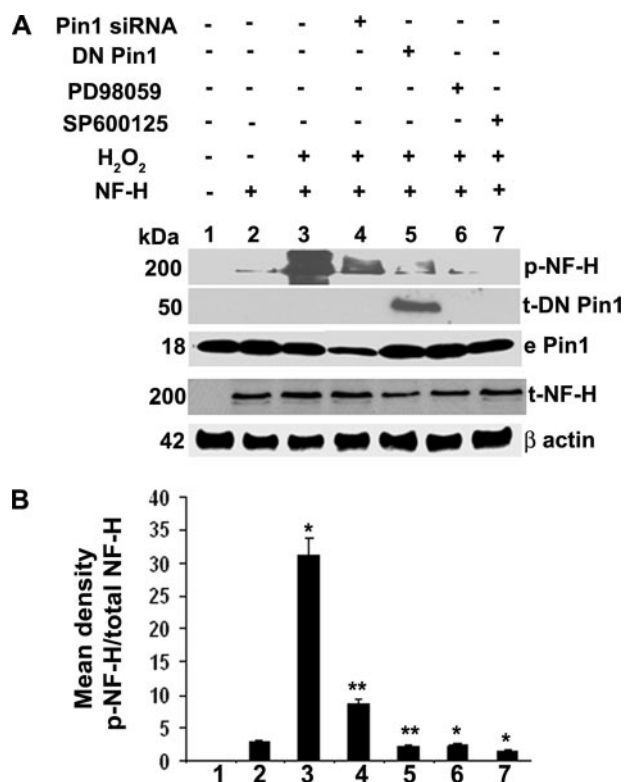


FIGURE 4. Inhibition of Pin1 rescues the H₂O₂ stress-induced phosphorylation of NF-H in transfected HEK293 cells. *A*, Western blots analysis using phospho-dependent (RT-97 and anti-p-NF-H) and total NF-H antibodies. HEK293 cells were treated with 1 mM H₂O₂ for 1 h as described under "Experimental Procedures." *lane 1*, nontransfected; *lanes 2–7*, NF-H transfected; *lanes 3–7*, treated with 1 mM H₂O₂; *lane 4*, transfected with Pin1 siRNA; *lane 5*, transfected with DN-Pin1; *lane 6*, treated with Erk1/2 inhibitor, PD98059; *lane 7*, treated with JNK inhibitor, SP600125. The H₂O₂ stress significantly increased phosphorylated NF-H (*lane 3*). Inhibition of Pin1 with Pin1 siRNA (*lane 4*) and DN-Pin1 (*lane 5*) inhibited H₂O₂ stress-induced NF-H phosphorylation. The H₂O₂ stress-increased phospho-NF-H is inhibited by Erk1/2 inhibitor, PD98059 (*lane 6*) and JNK inhibitor, SP600125 (*lane 7*). *B*, densitometry analysis of Western blots of p-NF-H with data obtained from *A*. Data shown are the mean \pm S.E. of four different experiments.

NF-H phosphorylation levels are regulated by heat shock (Fig. 5, *A* and *B*). Transfected HEK293 cells were subjected to heat shock at 44 °C for 30 min. Phosphorylation of NF-H is increased upon heat shock by ~5-fold, suggesting that p-NF-H levels are indeed regulated by heat stress. Knockdown of Pin1 by Pin1 siRNA as well as DN-Pin1 significantly inhibited the increase in p-NF-H by H₂O₂ and heat shock (Fig. 5, *A* and *B*). Both H₂O₂- and heat stress-induced hyperphosphorylation of NF-H was inhibited by Erk1/2 inhibitor (PD98059) and JNK inhibitor (SP600125) (Figs. 4 and 5).

Inhibition of Pin1 Rescues H₂O₂ Stress-induced Perikaryal Accumulation of p-NF-H in Primary Neurons—Hydrogen peroxide can be neurotoxic, and prolonged treatment can induce apoptosis. The phosphorylation of NF-H and its accumulation in the cell body has also been described as leading to neurotoxicity and apoptosis (36). We studied the effect of Pin1 modulation of hyperphosphorylated NF-H *in vivo*. To this end, we used 7 DIC cortical neurons from E17 dissociated rat cortex. We have used RT-97 antibody that recognize p-NF-H. In unstressed control neurons, p-NF-H is predominantly localized to axonal compartments, with no staining in the cell bodies (Fig. 6*A*, *panel a*). The nonspecific nuclear staining of RT97 and

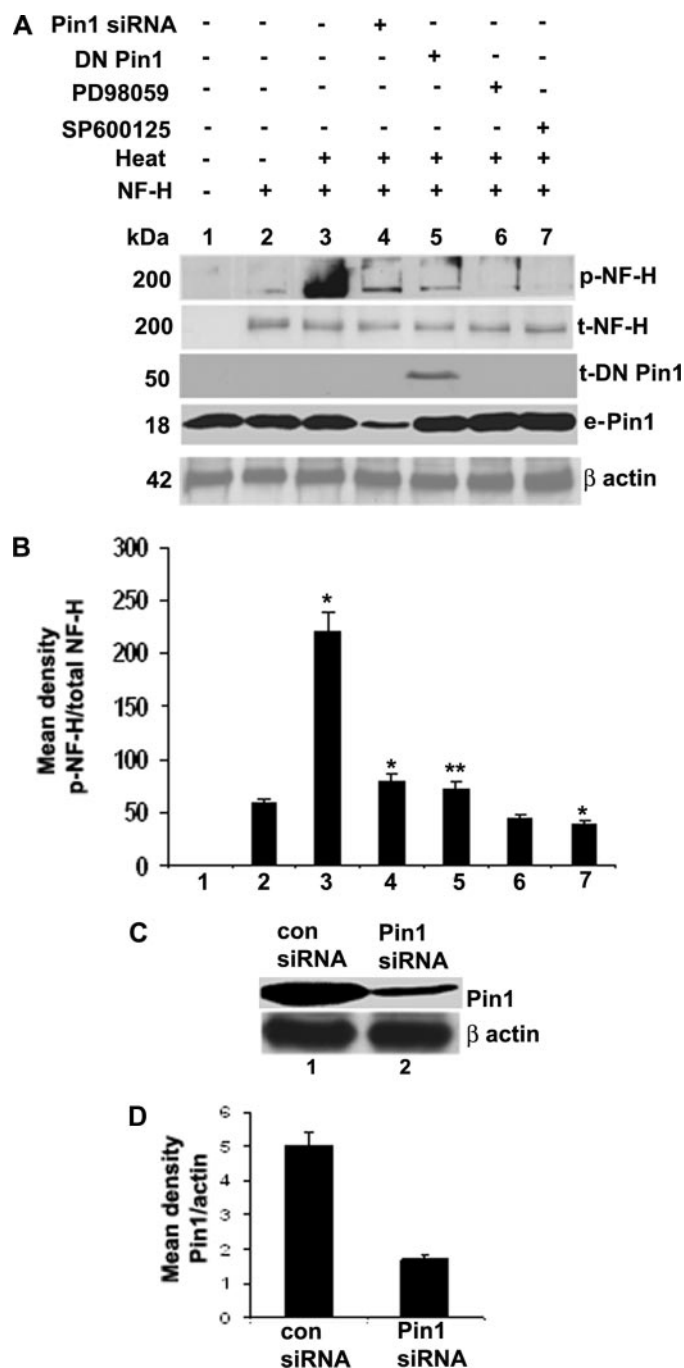


FIGURE 5. Inhibition of Pin1 rescues the heat shock-induced phosphorylation of NF-H in transfected HEK293 cells. *A*, Western blots analysis using phospho-dependent (RT-97 and anti-p-NF-H) and total NF-H antibodies. HEK293 cells were exposed to 44 °C in a 5% CO₂ incubator for 30 min as described under "Experimental Procedures." Heat shock increased phosphorylated NF-H. Inhibition of Pin1 with Pin1 siRNA prevented heat shock-induced NF-H phosphorylation. *Lane 1*, non-transfected; *lanes 2–7*, NF-H transfected; *lanes 3–7*, subjected to heat shock at 44 °C for 30 min; *lane 4*, transfected with Pin1 siRNA; *lane 5*, transfected with DN-Pin1; *lane 6*, treated with Erk1/2 inhibitor, PD98059; *lane 7*, treated with JNK inhibitor, SP600125. *B*, densitometry analysis of Western blots of p-NF-H with data obtained from *A*. Data shown are the mean \pm S.E. of four different experiments. *C*, 7 DIC cortical neurons were transfected scrambled control siRNA (*lane 1*) and Pin1 siRNA (*lane 2*). Western blot analysis was performed with Pin1 and β -actin antibodies. *D*, densitometry analysis of Western blots of p-NF-H with data obtained from *D*. Data shown are the mean \pm S.E. of four different experiments.

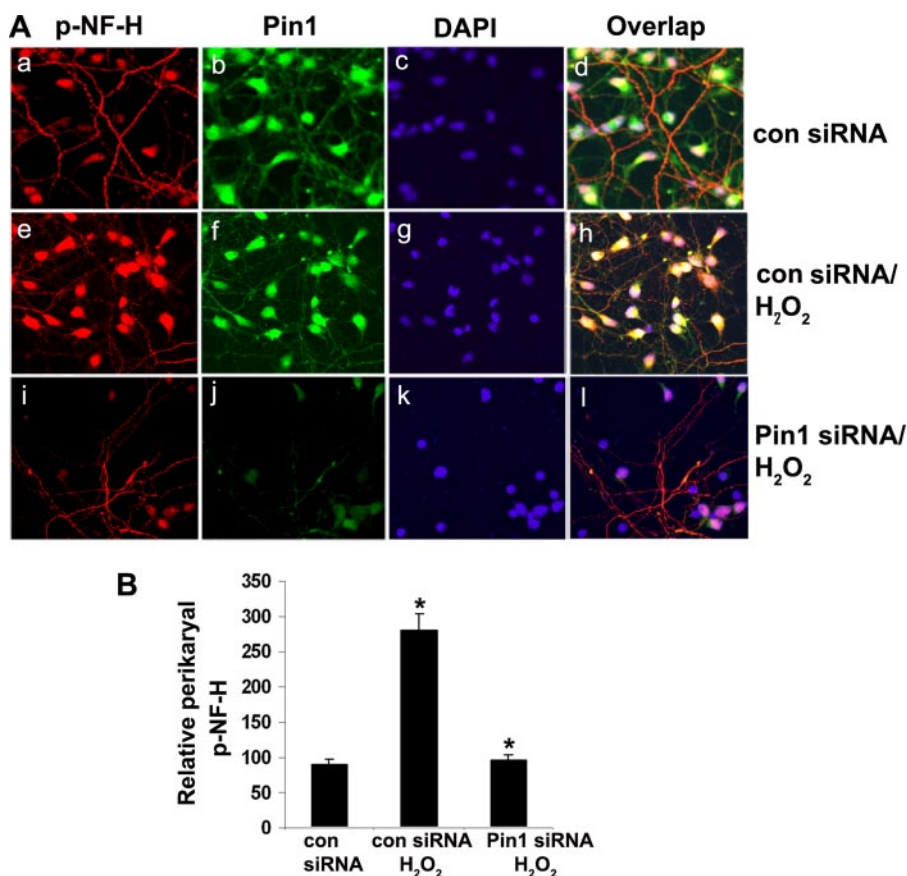


FIGURE 6. Hydrogen peroxide stress-mediated increase in p-NF-H in 7 DIC cortical neurons is reduced by Pin1 knockdown. *A*, 5 DIC cortical neurons were transfected with either control and scrambled siRNA (*a–d* and *e–h*) or Pin1-siRNA (*i–l*) and then treated with 1 mM H₂O₂ (*e–h* and *i–l*) on day 7 for 1 h. Neurons were immunostained, p-NF-H was detected using RT-97 (red), and Pin1 was detected by using Pin1 antibody (green). Neurons transfected with Pin1-siRNA exhibited reduced p-NF-H in the cell body. Non-treated neurons exhibited p-NF-H staining in the processes with little or no staining in the cell body (*a–d*), which is increased upon H₂O₂ treatment (*e–h*). Cell body p-NF-H staining was reduced in Pin1 siRNA-treated neurons (*i–l*). Scale bar, 20 μ m. *B*, representative neurons are treated with H₂O₂ as indicated. Intensity of RT-97 immunoreactivity (p-NF-H) in neuronal cell bodies was analyzed with the ImageJ histogram. The mean signal intensity (total pixel density/number pixels) is shown. Values in graphs are means \pm fluorescence intensity (in arbitrary densitometric units) in perikarya from four different experiments. Note the accumulation of p-NF-H in perikarya in H₂O₂-treated neurons and restoration of normal levels of p-NF-H in Pin1 siRNA-treated cortical neurons.

other phospho-NF antibodies is reported earlier (37–39), which could be the result of an association of phosphorylated NFs with nuclear components. Alternatively, it could be that lamins or other nuclear proteins have a phosphorylated epitopes similar to NFs. Pin1 was immunostained both in cell bodies and axons (Fig. 6*A*, panel *b*). When cortical neurons are subjected to H₂O₂ stress, p-NF-H is predominantly localized in cell bodies (Fig. 6*A*, panels *e–h*). These results are consistent with the reports of p-NF-H staining in DRG neurons subjected to H₂O₂ stress (14). Pin1 was detected in the cell bodies and axons, and there was a strong colocalization of Pin1 and p-NF-H in the cell bodies with H₂O₂ stress. We then monitored the effect of Pin1 in 7 DIC cortical neurons transfected with Pin1 siRNA. Pin1 siRNA resulted in 60% knockdown of Pin1 compared with scrambled control siRNA in 7 DIC cortical neurons (Fig. 5, *C* and *D*). Knockdown of Pin1 by Pin1 siRNA, but not scrambled control siRNA, reduced the Pin1 staining compared with control neurons (Fig. 6*A*, panel *j*). Pin1 siRNA-treated cortical cultures subjected to H₂O₂ stress resulted in reduced p-NF-H in the cell bodies, and p-NF-H staining similar

to that observed in the control, unstressed neurons (Fig. 6*A*, panels *i–l*). The quantitation of perikaryal p-NF-H from untreated and H₂O₂-treated neurons from four independent experiments is shown in Fig. 6*B*. These results suggest that the treatment of cultured neuronal cells with H₂O₂ induces perikaryal accumulation of the NF phospho-epitopes that are normally confined to axons, and these effects are prevented by inhibition of Pin1 with Pin1 siRNA. We also obtained identical results using another well characterized p-NF antibody, SMI31 (data not shown).

Heat Shock-induced Phosphorylation of Perikaryal NF-H Is Rescued by Inhibition of Pin1 in Neurons—Confocal microscopy using RT97 antibody that recognizes p-NF-H reveals that phosphorylated NF-H localizes mainly in the processes of cortical neurons (Fig. 7*A*, panels *a–d*). Pin1 is present in both the cell bodies and axons. Pin1 colocalizes with p-NF-H in the processes. When neurons are subjected to heat shock at 44 °C for 30 min, p-NF-H is enhanced in neuronal perikarya (Fig. 7*A*, panels *e–h*). Confocal images show a strong colocalization of p-NF-H with Pin1 in heat-stressed perikarya. Stress conditions caused by agents such as arsenate, *N*-acetyl-Leu-Leu-norleucinal, and sodium

chloride have been shown to induce perikaryal phosphorylation of neurofilaments in PC12 cells (23), but heat shock-induced perikaryal accumulations of p-NF-H has not been reported previously. When Pin1 siRNA-treated neurons were subjected to heat shock at 44 °C for 30 min, p-NF-H is mainly confined to processes with no staining in the cell bodies (Fig. 7*A*, panels *i–l*). The quantitation of perikaryal p-NF-H from control and heat shock-induced neurons from four independent experiments is shown in Fig. 7*B*. These results suggest that Pin1 modulates the oxidative stress-induced perikaryal accumulations of p-NF-H.

Inhibition of Pin1 Reduces Oxidative Stress-induced Apoptosis—Neuronal cell death plays a central role in several neurodegenerative disorders. To determine whether phosphorylated NF-H accumulations in cell bodies causes neuronal apoptosis in stress conditions and whether knockdown of endogenous Pin1 inhibits cell death, cortical neurons after 7 days in culture were either treated with H₂O₂ or subjected to heat shock. Approximately 10% of untreated cortical neurons were TUNEL-positive (basal level), and this increased to over

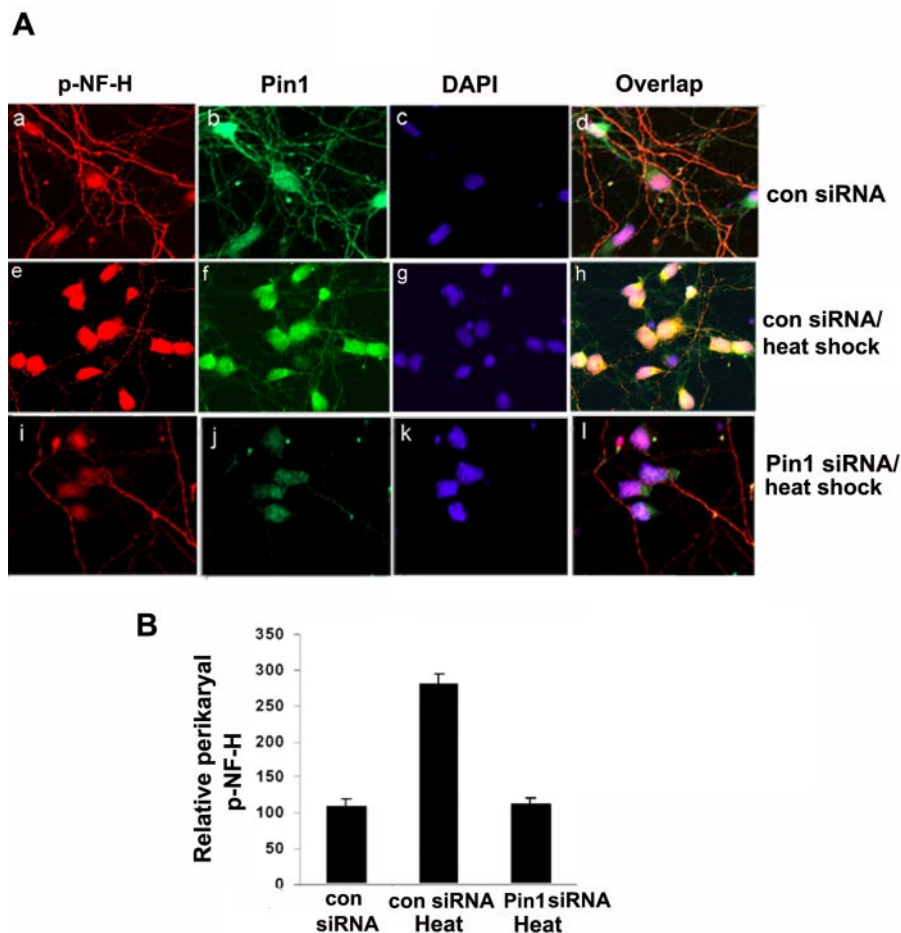


FIGURE 7. Heat shock-induced phosphorylated NF-H is reduced by Pin1 knockdown. A, 5 DIC cortical neurons were transfected with either control siRNA (a–d and e–h) or Pin1-siRNA (i–l) and then exposed to 44 °C in a 5% CO₂ incubator for 30 min (heat shock, e–h and i–l). Neurons were immunostained, p-NF-H was detected using RT-97 (red), and Pin1 was detected by using Pin1 antibody. Scale bar, 20 μm. B, representative neurons are subjected to heat shock as indicated. Intensity of RT-97 immunoreactivity (p-NF-H) in neuronal cell bodies was analyzed with the ImageJ histogram. The mean signal intensity (total pixel density/number pixels) is shown. Values in graphs are means ± fluorescence intensity (in arbitrary densitometric units) in perikarya from four different experiments. Note the accumulation of p-NF-H in perikarya in heat shock-treated neurons and restoration of normal levels of p-NF-H in Pin1 siRNA-treated cortical neurons.

44% upon H₂O₂ stress and heat shock (Fig. 8, A–D). Inhibition of Pin1 by Pin1 siRNA reduced TUNEL-positive neurons to ~16%, approaching numbers comparable to non-treated neurons (Fig. 8, A and B). Representative TUNEL images are shown, where quantification in the bar graph represents TUNEL-positive counts from four separate experiments, where 12 independent fields were counted (Fig. 8). When treatment times were extended, the percentage of TUNEL-positive neurons increased but neurons also began to detach from the cover slips (data not shown).

Pin1 Modulates the Stress-induced Hyperphosphorylation of NF-H via Activation of JNK—The activation of c-Jun is mediated by JNK through the phosphorylation of the N-terminal domain (10). JNK3 is mainly expressed in neuronal cell bodies (29) and is shown to phosphorylate NF-H (24). We used immunoprecipitation kinase assay using JNK3 antibody in 7 DIC cortical cultures and examined the expression of phosphorylated c-Jun by using an antibody raised against c-Jun phosphorylated at serines 63/73. Our results suggest that both hydrogen perox-

ide and heat stresses resulted in the activation of JNK, as observed by the significant increase in the phospho-c-Jun levels (Fig. 9, A–D). Inhibition of JNK by JNK-selective inhibitor SP600125 inhibited the stress-induced JNK activity. Interestingly, both Pin1 siRNA and DN-Pin1 inhibited stress-induced phospho-c-Jun levels. Immune complex kinase assays with no antibody showed the absence of phospho-c-Jun expression (data not shown). These results suggest that JNK is activated under H₂O₂ and heat stresses, and inhibition of Pin1 by Pin1 siRNA and DN-Pin1 inhibits this pathway.

DISCUSSION

Neurofilaments are the major cytoskeletal component of large myelinated axons. The aberrant hyperphosphorylation of NF-H is likely to be one of the main factors in axonal neuropathology. However, the deregulation of NF-H phosphorylation is still a key question in AD and ALS pathogenesis. We have shown recently that Pin1 may also be implicated in the postphosphorylation modulation of pSer/Thr-Pro (KSP) repeats in high molecular weight neurofilament protein (NF-H), which when deregulated, may lead to perikaryal motor neuron inclusions typical of ALS (11). Many phosphorylation sites defined as pathological are phospho-Ser/Thr followed by Pro. In normal neurons, the KSP sites in NF-H/M are phosphorylated within axons but not within cell bodies (40–42). However, in cases of ALS and other neurodegenerative diseases, phosphorylated NFs, particularly NF-H, are found as accumulations in neuronal cell bodies. The neurons displaying this aberrant phosphorylation pattern are then destined to degenerate and die (36). In addition to AD neurons, phosphorylated NF-H accumulations have also been identified in Lewy bodies found in PD patients (43). The NF-H has a remarkable, even unique, primary structure that makes it an exceptional and unusual target for Pin1. Whereas other characterized substrates typically have a small cluster of phosphorylatable (S/T)P dipeptides, the NF-H has a large number of (S/T)P-(43–100), depending upon species, stretched out in an essentially linear array, upon phosphorylation. Pin1 induces conformational changes of these phosphorylated (S/T)P residues. Pin1 may help facilitate such changes, for example, by isomerizing a specific prolylpeptide bond and thereby stabilizing the phosphorylation of nearby Ser and Thr residues followed by Pro.

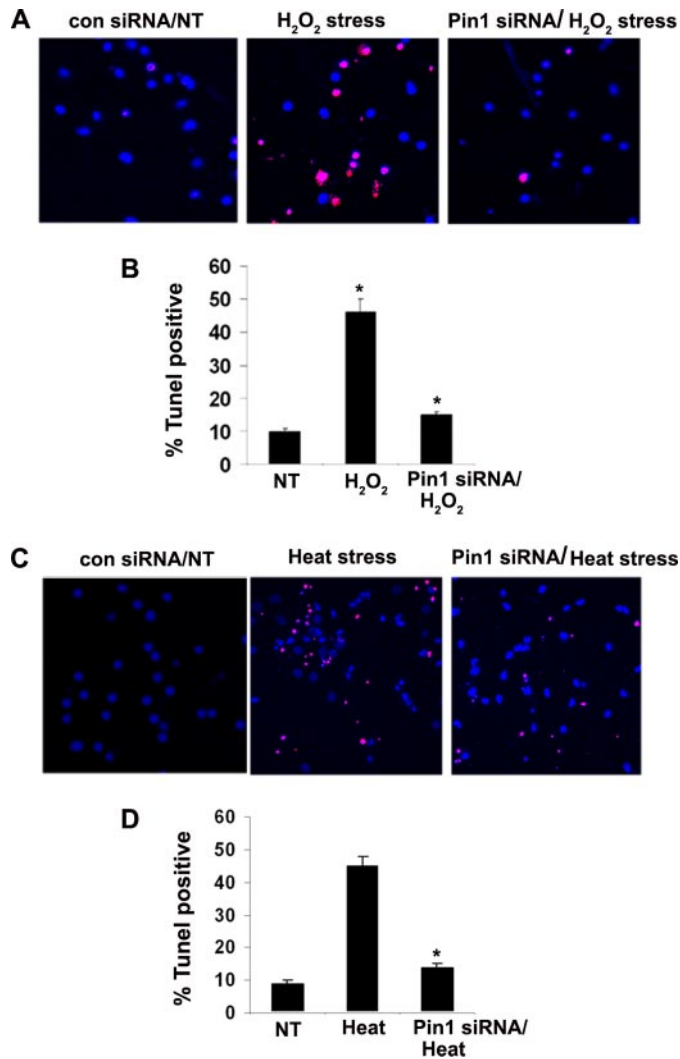


FIGURE 8. Inhibition of Pin1 reduces stress-mediated neuronal cell death. *A*, presence of apoptotic neurons examined by TUNEL-TMR red staining in 7 DIC cortical neurons. Nuclei were counterstained using 4',6-diamidino-2-phenylindole. TUNEL-positive cells were increased upon H₂O₂ (*A*) and heat shock (*C*) and declined in stressed neurons treated with Pin1 siRNA. Scale bar, 20 μm. *B* and *D* show that the neuronal death is increased nearly four times upon exposure to hydrogen peroxide or heat treatment, and this was reduced nearly 2-fold in neurons transfected with Pin1 siRNA and subjected to stress (H₂O₂ and heat). The quantization in the bar graph (*B* and *D*) represents TUNEL-positive counts from four separate experiments, where 12 independent fields were counted.

Recently, a growing number of phosphorylated proteins participating in important cellular processes have been found to be regulated through prolyl isomerization-induced conformational changes. Surprisingly, Pin1 is shown to act on its target proteins in completely opposite fashions. On one hand, Pin1 is shown to facilitate phosphorylation, as in the case of RNA polymerase II (12), neurofilament (11), and transcription factors like c-Jun and c-Fos (16, 17). Likewise, phosphorylation of several cell cycle proteins is up-regulated by Pin1 (16). On the other hand, Pin1 has been shown to facilitate dephosphorylation of Tau at Thr-231 (10). More mechanistic details need to be revealed as to how Pin1 acts on its target proteins. Structural properties, like the position of the phosphorylated residue, number of SP dipeptides, their proximity, or the secondary structure, could play an important role in regulation of target

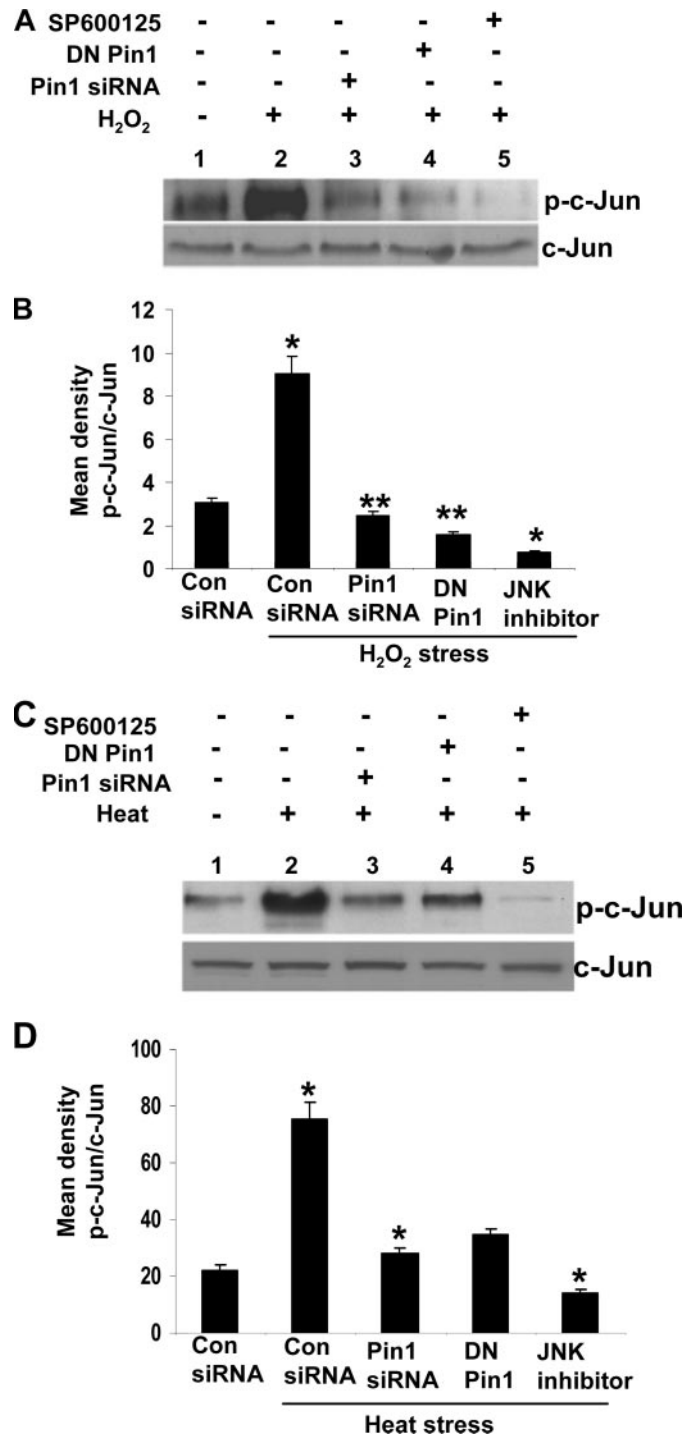


FIGURE 9. Pin1 modulates the stress-induced phosphorylation of NF-H through the activation of JNK. *A*, endogenous JNK3 was immunoprecipitated from 7-day-old primary cortical neurons either untreated (*lane 1*) or treated with 1 mM H₂O₂ for 1 h (*lanes 2–5*), and JNK activity assay was performed using c-Jun as a substrate as described under "Experimental Procedures." The assays show phosphorylation of c-Jun at Ser-63/73. Coomassie Blue-stained gels show equal amounts of substrate in the reaction mix. The H₂O₂ stress-increased c-Jun phosphorylation is inhibited by inhibition of Pin1 by Pin1 siRNA (*lane 3*) and DN-Pin1 (*lane 4*) and JNK inhibitor SP600125 (*lane 5*). *B*, densitometry analysis of phospho-c-Jun with data obtained from *A*. *C*, JNK activity assays were performed with endogenous JNK immunoprecipitated from 7-day cortical cultures either untreated (*lane 1*) or subjected to heat shock (44 °C for 30 min; *lane 2*). Heat shock-induced c-Jun phosphorylation (*lane 2*) and inhibition of Pin1 by Pin1 siRNA (*lane 3*), DN-Pin1 (*lane 4*), and JNK inhibitor SP600125 (*lane 5*) inhibited heat stress-induced c-Jun phosphorylation by JNK. *D*, shown are the results from densitometry analysis of phospho-c-Jun with data obtained from *C*.

Differential Stress-induced NF-H Phosphorylation by Pin1

proteins by Pin1. The data presented here provide biochemical and molecular evidence that Pin1 helps to control the phosphorylation status of the NF-H. Specifically, inhibition of Pin1 rescues the stress-induced aberrant hyperphosphorylated form of NF-H. Another Pin1 target that has multiple SP repeats is RNA polymerase II (52 SP repeats in the C-terminal domain) where Pin1 modulates its activity by stimulating the phosphorylation by cdc2/cyclin B and inhibiting the dephosphorylation by FCP1 (12). We suggest that the proteins with multiple SP repeats in tandem are regulated in a similar fashion by Pin1.

Pin1 seems to have different roles in different neurodegenerative diseases, because a recent report shows that Pin1 activity enhances the formation of cytoplasmic inclusions Lewy bodies, a pathological hallmark found in Parkinson disease (PD) (44). Pin1 was found to be a component of Lewy bodies that form in HEK293T cells after transfection with α -synuclein, whereas overexpression of dominant-negative Pin1 inhibited this formation of inclusions (44). Thus, Pin1 activity impacts target proteins with different results in different neurodegenerative diseases. Recently, Pin1 has been shown to mediate activation of mitochondria-mediated apoptosis specifically in neurons (45). One of the key regulators of BIM_{EL} in neurons is the c-JNK signaling pathway, which is the main pathway activated by numerous apoptotic stimuli, including excitotoxicity. The activation of JNK signaling induces the disassociation of Pin1 from the neuron-specific JNK scaffold protein JIP3, which in turn promotes the binding of Pin1 with phosphorylated BH₃-only protein BIM_{EL}. This stabilizes BIM_{EL} and activates the mitochondrial apoptotic machinery (45). Another report has shown that c-Jun transcription factor is up-regulated by Pin1 (16). Increase in c-Jun and phospho-c-Jun has been determined in postmortem specimens from patients with AD and ALS (46). Masaki *et al.* (47) reported that the p-NF-H accumulations are accompanied by the increase in the apoptosis-specific protein and p-c-Jun.

During the preparation of this report, it was reported that Pin1 promotes cell death in nerve growth factor-dependent neurons through a mechanism requiring c-Jun activity (48). High concentration of H₂O₂ induces cell death via JNK activation (49). Our data suggest Pin1 modulates stress-induced hyperphosphorylation of NF-H via the activation of JNK. Stress-induced neuronal death could be mediated by Pin1-dependent phosphorylation of other neuronal cytoskeletal proteins (*e.g.* Tau and MAPs). In addition to aberrant and hyperphosphorylation of NF-H, oxidative stress causes aberrant phosphorylation of other cytoskeletal proteins (*e.g.* Tau and MAPs) and leads to neuronal death. Pin1 may regulate the phosphorylation of these molecules by stabilizing the (S/T)P phosphorylation. In addition, Pin1 may also regulate the proline-directed phosphatases.

Recent studies have also established that Pin1-catalyzed prolyl isomerization can have a profound impact on many key proteins in diverse cellular processes that include cell growth regulation, genotoxic and other stress responses, immune response, germ cell development, and neuronal differentiation and survival (50). Pin1 is tightly regulated at multiple levels, and its deregulation has a critical role in a growing number of pathological conditions such as cancer, AD, aging, asthma, and

microbial infection. Unlike other known PPLases, Pin1 uses substrate Ser/Thr phosphorylation as an additional level of regulation (50). Only when a substrate is phosphorylated, the *cis-trans* isomerization is accelerated. The prolyl isomerization of pSer/Thr-Pro motifs is especially significant because proline-directed kinases and phosphatases are conformation-specific, acting only on the *trans* conformation.

Juglone, a pharmacological Pin1 inhibitor, was shown to inactivate rotamase activity. However, juglone inhibits numerous parvulins as well as other enzymes comprising Cys residues in their catalytic domains (51). Also, juglone is shown directly to block transcription (52–54), and thus, juglone may not be a specific Pin1 inhibitor. To determine the direct role of Pin1 in NF-H phosphorylation, we have synthesized and used Pin1 siRNA that specifically inhibited the expression of Pin1. We have also used DN-Pin1 that inhibits the isomerase activity of Pin1.

In AD, PD, and ALS diseases, oxidative stress causes the premature phosphorylation of perikaryal NFs leading to their accumulation. Phosphorylated NF protein accumulations in the perikaryon or proximal axon of motor neurons have been shown to block axonal transport of cytoskeletal proteins and mitochondria and eventually cause axonal degeneration (55). The finding that some sporadic ALS patients have NF-H alleles with deletions in the KSP repeat domain suggests that altered phosphorylation may indeed be a cause for neurofilamentous accumulations (56, 57). The evidence provided in this study that oxidative and heat stress causes perikaryal accumulations of p-NF-H could possibly be associated with prevention of axonal transport. Thus, the inhibition of phosphorylated NF-H accumulations in the neuronal cell body could be an attractive avenue for therapeutic intervention in AD, PD, and ALS.

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