Inhibition of Pin1 Reduces Glutamate-induced Perikaryal Accumulation of Phosphorylated Neurofilament-H in Neurons

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Under normal conditions, the proline-directed serine/threonine residues of neurofilament tail-domain repeats are exclusively phosphorylated in axons. In pathological conditions such as amyotrophic lateral sclerosis (ALS), motor neurons contain abnormal perikaryal accumulations of phosphorylated neurofilament proteins. The precise mechanisms for this compartment-specific phosphorylation of neurofilaments are not completely understood. Although localization of kinases and phosphatases is certainly implicated, another possibility involves Pin1 modulation of phosphorylation of the proline-directed serine/threonine residues. Pin1, a prolyl isomerase, selectively binds to phosphorylated proline-directed serine/threonine residues in target proteins and isomerizes *cis* isomers to more stable *trans* configurations. In this study we show that Pin1 associates with phosphorylated neurofilament-H (p-NF-H) in neurons and is colocalized in ALS-affected spinal cord neuronal inclusions. To mimic the pathology of neurodegeneration, we studied glutamate-stressed neurons that displayed increased p-NF-H in perikaryal accumulations that colocalized with Pin1 and led to cell death. Both effects were reduced upon inhibition of Pin1 activity by the use of an inhibitor juglone and down-regulating Pin1 levels through the use of Pin1 small interfering RNA. Thus, isomerization of lys-ser-pro repeat residues that are abundant in NF-H tail domains by Pin1 can regulate NF-H phosphorylation, which suggests that Pin1 inhibition may be an attractive therapeutic target to reduce pathological accumulations of p-NF-H.

INTRODUCTION

The functions of most proteins are regulated by posttranslational modifications, of which phosphorylation is probably the most common. In neurons, phosphorylation of cytoskeletal proteins is tightly regulated and compartmentalized. Although proline-directed kinases are found in both cell bodies and axons, the multiple-repeat lysine-serine-proline (KSP) sites in the neurofilament (NF) tail domains are known to be almost exclusively phosphorylated in the axonal compartment of mammalian and squid neurons (Julien and Mushynski, 1982; Lee *et al.*, 1987; Nixon *et al.*, 1994; Grant and Pant, 2004). Defects in the relative kinase and phosphatase activities and/or deregulation of compartment-

specific phosphorylation result in neurodegenerative disorders: Aggregated forms of hyperphosphorylated tau and phosphorylated NF (p-NF) are found in pathological cell body accumulations in the CNS of patients suffering from Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS). Antibodies against phosphorylated neurofilament heavy/middle chain NF-H/M recognize epitopes found in perikaryal inclusions, neurofibrillary tangles, and paired helical filaments of AD (Cork *et al.*, 1986; Wang *et al.*, 2001), in Lewy bodies (Forno *et al.*, 1986; Pollanen *et al.*, 1993), and in inclusions in spinal cord motor neurons (Sobue *et al.*, 1990; Itoh *et al.*, 1992; Al-Chalabi and Miller, 2003).

Within the last decade, a novel level of modulation of protein phosphorylation has emerged, namely, the factors that regulate the structural conformation and stability of proteins, particularly those phosphorylated at Ser/Thr-Pro (S/T-P) sites by proline-directed kinases. Most proline-directed kinases (Weiwad *et al.*, 2004) and phosphatases (Wulf *et al.*, 2005) are highly selective for *trans* S/T-P bonds. Peptidyl-prolyl *cis/trans* isomerases, such as Pin1, specifically target phosphorylated S/T-P sites and, by virtue of the proline residue, can "toggle" an inactive *cis* isomer to the more stable *trans* form, often with altered function. Pin1 plays a key role in diverse cellular functions, including the

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Abbreviations used: Cdk5, cyclin-dependent kinase 5; p-NF-H, phosphorylated neurofilament-H; Pin1, prolyl isomerase 1.

cell cycle, differentiation, cancer, neurodegeneration, DNA damage response, and apoptosis (Lu *et al.*, 1996; Liou *et al.*, 2003; Lu, 2004; Thorpe *et al.*, 2004; Wulf *et al.*, 2005). Pin1 is localized in nuclei of most cells, where it modulates the functions of several mitotic proteins such as cyclin D1, cdc2, and transcription factors such as p53, c-Jun N-terminal kinase (c-JNK), β -catenin, and tau, a regulator of microtubule dynamics in mitosis (Lu *et al.*, 1996). Pin1 also regulates structure and function of RNA polymerase II during transcription (Xu *et al.*, 2003). It is overexpressed in transformed cells and is thus implicated in oncogenesis (Bao *et al.*, 2004).

In neurons, however, Pin1 is distributed in both nucleus and cytoplasm and increases during neuronal differentiation, and its expression correlates with the phosphorylation of tau, although all potential phosphorylation sites are not equally phosphorylated (Hamdane *et al.*, 2006). Phosphorylation at one Pin1 binding site Thr 231, which activates tau dephosphorylation in vitro (Lu *et al.*, 1999; Zhou *et al.*, 2000), is down-regulated during differentiation, suggesting that Pin1 regulates tau dephosphorylation by inducing conformational changes, at least at this site. In vitro, Pin1 specifically binds to tau proteins that are phosphorylated on their Thr 231 residue (Wintjens *et al.*, 2001; Lu *et al.*, 2002; Hamdane *et al.*, 2006). Significantly, the Thr 231 phosphoepitope of tau is exclusively found in mitotic cells, is a marker of abnormal tau hyperphosphorylation (Hamdane *et al.*, 2003), and is specifically detected in a conformation-dependent manner in neurofibrillary tangles (NFT) in AD brains, thus linking Pin1 to neurodegeneration (Lu *et al.*, 1999; Augustinack *et al.*, 2002a). This link was dramatically confirmed by Pin1-null mice, which display age-dependent motor and behavioral defects, tau pathologies, and neuronal loss (Liou *et al.*, 2003), results that suggest that Pin1 protects against neurodegeneration, but how? One suggestion is based on a model of neurodegeneration stating that stressed postmitotic neurons (e.g., exposed to β -amyloid peptides) are abnormally induced to enter the cell cycle, leading to the appearance of mitotic proteins (including p-T231-tau), resulting in neuronal apoptosis (Neve and McPhie, 2006). The protective role of Pin1 is to alter the phosphorylated conformation of mitotic proteins to enable dephosphorylation of tau at Thr 231 by exposing the site to PP2A phosphatase activity. The absence of Pin1 in null mice or the low soluble Pin1 levels seen in AD brains, prevents phosphatase action at the hyperphosphorylated tau sites (reviewed in Lu, 2004).

Like tau, neurofilaments contain many S/T-P phosphate acceptor sites that are targeted by several different kinases and phosphatases. In contrast to tau, however, NFs, particularly NF-H, are enriched with numerous KSP repeat motifs in the tail domain (43–100 depending on species), sites for proline-directed kinase phosphorylation. Phosphorylation of these tail domain sites is normally restricted to the axonal compartment as NF polymers are assembled and transported along the axon and results in extension of sidearms that interact with other NFs, NF-associated proteins, and microtubules to form a stable, structural lattice. We suggest that Pin1, distributed throughout the neuron, is directly involved in modulating NF phosphorylation to affect its distribution in the cell body and axon. By deregulating the system via induction of neurodegeneration (glutamate excitotoxicity), to evoke NF pathologies resembling those seen in ALS, we have been able to identify a role for Pin1 in the regulation of NF phosphorylation.

MATERIALS AND METHODS

All experiments were replicated a minimum of four times, and results shown are representative Western blots or images. All quantitative data were obtained through densitometric scanning, and results are expressed as $\pm$ SEM.

Antibodies

Pin1 antibodies were obtained from Cell Signaling Technologies (Beverly, MA) and Oncogene Research Products (Boston, MA) and used for Western blotting (1:1000) and immunohistochemistry (1:100), respectively. RT-97 antibody 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 dilution 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 (clone N52), and DAPI (Sigma-Aldrich, St. Louis, MO) were used at 1:10000 for Western blotting and 1:1000 for nuclear counterstaining, respectively.

Plasmids and Pin1-Small Interfering RNA

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. Transfection of wild-type and DN-Pin1 constructs were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Wild-type Pin1 was cloned into pGEX-5X-1 obtained from Amersham Biosciences (Piscataway, NJ) for glutathione S-transferase (GST) expression studies. Control nonsilencing and Pin1-small interfering RNA (siRNAs; silencing) were designed as follows. Control siRNA (nonsilencing) sense and antisense sequences were as follows: 5'-r(UUUUCCGACACGUCACGU)d(TT)-3' and 5'-r(ACGUGACACGUUCGGAGAA)d(TT)-3', respectively. Pin1 siRNA (silencing) sense and antisense sequences were as follows: 5'-r(GCUCAGGCCGAGU-GUACUA)dTdT-3' and 5'-r(UAGUACACUGCCGUGAGC)dTdT-3', respectively. 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 heated at 90°C for 1 min and then incubated at 37°C for 60 min before transfection. Final concentrations (40 nM) of siRNAs were transfected into embryonic day 18 (E18) primary cortical neurons (5 d in culture [DIC]) using Lipofectamine 2000 reagent according to the manufacturer's instructions. After 48 h the cells were either fixed for immunohistochemical analyses or lysed with lysis buffer for Western blot analyses.

Reagents

5-Hydroxy-1, 4-naphthoquinone (juglone) and L-glutamic acid (glutamate) were purchased from Sigma-Aldrich. Juglone was prepared as described (Chao *et al.*, 2001) and used at 30 μ M in dorsal root ganglion (DRG), and glutamate was used at 0.1 mM and 10 μ M for 6 h in cortical neurons and DRG neurons, respectively.

GST-Pin1 Protein Expression and Pulldown Assays

GST and GST-Pin1 were expressed in *Escherichia coli* and purified according to the manufacturer's instructions (Amersham Biosciences). Purified GST and GST-Pin (20 mg of each) were then used in GST-pulldown assays from rat brain lysates. Rat brain lysates, 10%, were prepared in immunoprecipitation (IP) lysis buffer containing 50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 5 mg/ml leupeptin, 2 mg/ml aprotinin, 5 mg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride (PMSF) described previously (Kesavapany *et al.*, 2004). GST and GST-Pin1 were incubated with the lysates overnight at 4°C, washed three times, and then separated by SDS-PAGE on 4–20% acrylamide gels. Gels were stained with Coomassie and destained, and differential bands were excised and sent for mass spectrometric identification. For Western blotting analyses, samples were transferred onto nitrocellulose and phosphorylated neurofilament-H (p-NF-H) was detected using RT-97 antibody.

Mass Spectroscopic Identification

Excised Coomassie-stained and destained gel slices were subjected to in-gel tryptic digestion, and then dried digests were processed as previously described (Jaffe *et al.*, 2004).

Co-IP

Rat brain was homogenized in ice-cold IP lysis buffer (see above) using 40 strokes of a Dounce homogenizer on ice. Homogenates were centrifuged for 30 min at 4°C at 14,000 rpm. Supernatants were precleared using protein A-Sepharose beads (Sigma), and 500 μ g of total protein was used in IP experiments using the polyclonal Pin1 antibody and immunoglobulin was captured using protein A-Sepharose beads overnight at 4°C. After three washes with IP lysis buffer, immunoprecipitates were heated in 2 $\times$ SDS sample buffer, separated by SDS-PAGE and transferred onto nitrocellulose membranes for Western blotting analyses. Phosphorylated NF-H was immu-

not detected using RT-97 antibody. Co-IPs of similarly prepared rat brain lysates using RT-97 antibody specific for phosphorylated NF-H were also carried out to detect Pin1 with the Pin1 antibody.

SDS-PAGE and Sample Preparation

Total homogenates, 10%, of spinal cord were made as described previously (Kesavapany *et al.*, 2004). Samples were heated for 10 min at 95°C and then aliquoted and frozen at -80°C. Western blots were prepared from equal volumes of total lysates. Soluble lysates were produced by using 40 strokes of a Dounce homogenizer in ice-cold IP lysis buffer containing 50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 5 mg/ml leupeptin, 2 mg/ml aprotinin, 5 mg/ml pepstatin, and 1 mM PMSF, incubated for 20 min on ice, and then centrifuged at 14,000 rpm for 20 min to obtain the soluble supernatant. Protein concentrations for soluble lysates were determined with the bicinchoninic acid assay system (Pierce, Rockford IL). Western blots were obtained from equal amounts of soluble lysates. All neuronal cell lysates were made by scraping the neurons directly into 2× SDS sample buffer and heating at 95°C for 10 min. Protein balancing for total homogenates and confirmation of protein loading was achieved through immunodetection of tubulin. Once cooled, samples were separated by SDS-PAGE and processed for Western blotting analyses. Signals were scanned and densitometrically measured using a GS800 scanner and its accompanying Quantity One program (Bio-Rad, Hercules, CA).

ALS and AD Tissue

Closely matched, age and postmortem time, control, and ALS-affected spinal cord sections and spinal cord tissues were obtained from the Robarts Research Institute Brain Bank (London ONT, Canada). Matched control and AD tissue were obtained from the Harvard Brain Tissue Resource Center. Samples that were matched in terms of region (Brodman layer), age, and postmortem time were used to produce total and soluble lysates (described earlier).

Immunohistochemical Staining of Spinal Cord Sections

Paraffin-embedded control and ALS-affected spinal cord sections were prepared for immunostaining through xylene treatment and gradual rehydration with 95–75% ethanol. Sections were blocked and then incubated with primary antibodies overnight at 4°C in 0.6% Triton X-100/3% bovine serum albumin (Sigma). Secondary antibodies were incubated for 1 h at room temperature in the dark, and slides were coverslipped using GelMount (Biomed, Foster City, CA). Images were captured with a 20× objective on a Zeiss LSM510 using LSM Image Software managed with Adobe Photoshop.

Primary rat DRG Cultures and Treatments

Pregnant female rats or mice were killed, and embryos were removed from the uterus at E15, as previously described (Olah *et al.*, 2001). DRG neurons were plated onto poly-lysine-coated glass coverslips and grown for 4 d before treatment ± juglone/glutamate. For sequential treatment, neurons were first pretreated with 30 μM juglone for 3 h and then with 10 μM glutamate for 6 h. Two homogenate preparations were prepared: a total homogenate where neurons were harvested directly into SDS sample buffer and heated before processing and a soluble homogenate (described above) to determine soluble levels of Pin1.

Primary Rat Cortical Neuron Cultures and Treatments

E16–18 rat cortical neuron cultures were produced as described previously (Kesavapany *et al.*, 2004). Typically, neurons were transfected after 3DIC, incubated for 5 h, and then treated with glutamate. Neurons were grown for 7 d before treatment + juglone and glutamate. For sequential treatment, neurons were first pretreated with 30 μM juglone for 3 h and then with 0.1 mM glutamate for 6 h. Two homogenate preparations were prepared: a total homogenate where neurons were harvested directly into SDS sample buffer and heated before processing and a soluble lysate to determine soluble levels of Pin1 prepared with IP lysis buffer described earlier.

Immunocytochemical Staining of Neuronal Cultures

Primary rat cortical and DRG neurons were plated on poly-lysine-coated glass coverslips and processed for immunocytochemistry as previously described (Kesavapany *et al.*, 2004). Coverslips were mounted using GelMount (Biomed, Foster City, CA). In situ cytotoxicity kits were obtained from Roche (Indianapolis, IN) and TUNEL (terminal deoxynucleotidyl transferase-mediated nick end labeling) staining was performed according to the manufacturer's instructions before immunocytochemistry. Images were captured with an oil immersion 63× objective on a Zeiss LSM510 using LSM Image Software managed with Adobe Photoshop (San Jose, CA).

Preparation of Molecular Models

Molecular models were prepared with the aid of DeepView (Guex and Peitsch, 1997). The NF-H tail domain has an amino acid composition characteristic of "intrinsically unstructured" protein (Fink, 2005). This was confirmed for the C-terminal 600 residues of human NF-H by applying the

IUPred algorithm via the website, <http://iupred.enzim.hu/> (Dosztanyi *et al.*, 2005). Because nearly all the proline-associated serines in this NF-H domain become phosphorylated in mature axons (Jaffe *et al.*, 1998a,b), these serines evidently become accessible to kinases during this processing. Thus it seems reasonable to model each unphosphorylated repeat unit as a loop centered on the S-P residues. Accordingly, in constructing Figure 8, the DeepView loop-building tool was applied to each repeat unit as an extended peptide, and then these were ligated and minimized. The *cis* transition of SP2 was modeled by rotating the S-P ω torsion by 180° while leaving the N-terminal residues stationary.

RESULTS

Pin1 Associates with the p-NF-H

We used GST pulldown assays to investigate whether Pin1 binds to p-NF-H. GST and GST-Pin1 were expressed and purified by immobilizing the protein on glutathione-Sepharose beads. The bound GST fusion proteins were then incubated with rat brain lysates overnight. The samples, after washing, were separated by SDS-PAGE, and gels were stained and destained. Bands present in GST-Pin1 pulldown but not in the GST lanes were excised, subjected to in gel tryptic digestion, and identified using tandem mass spectroscopy or MS-MS mass spectroscopy. Peptides from two different proteins were conclusively identified including tau and the band at 200 kDa (arrowed in Figure 1A) that was NF-H. Two peptides that belonged to NF-H were identified as TLDVKSPEAK and SPADKFPEK, where the serine in the first peptide was phosphorylated. Other bands on the gels were MAP1b (X1) and NF-M (X2). X3 was found to contain keratin, which is a common contaminating band found in these types of studies, and X4 was a degradation product of GST-Pin1. One band was identified as tau, but we chose to focus our efforts on NF-H because tau has been well characterized by other groups. NF-M studies are part of our future plans. To confirm this result, Pin1 was immunoprecipitated from rat brain lysates and p-NF-H was immunodetected using RT-97 antibody. Pin1 readily pulled down phosphorylated NF-H (Figure 1B). Pin1 coimmunoprecipitated with p-NF-H when p-NF-H was pulled down using RT-97 antibody (Figure 1C).

NF-H Phosphorylation Is Increased in AD Tissue

Although the majority of reports have concentrated on hyperphosphorylated tau as the major cytoskeletal pathology in Alzheimer's disease (reviewed in Stoothoff and Johnson, 2005), p-NF-H is also a constituent of aggregates in AD brain (Sternberger *et al.*, 1985; Cork *et al.*, 1986; Wang *et al.*, 2001). We wanted to compare the levels of NF-H phosphorylation in control and AD tissue. Closely matched control and AD homogenates were subjected to SDS-PAGE before immunodetection of p-NFH. We found a threefold increase in p-NF-H in AD samples compared with controls, but found no change in soluble Pin1 levels. Total levels of NF-H did not change (Figure 2).

NF-H Phosphorylation Is Increased in ALS without Any Change in Pin1 Levels

Because cytoskeletal proteins, especially NF-H, are reported to be aberrantly phosphorylated in ALS spinal cord and affected motor neurons (Straube-West *et al.*, 1996; Strong *et al.*, 2001; Rao and Nixon, 2003), we compared p-NF-H expression in ALS-affected spinal cord tissue with that in normal controls. Western blots of total homogenates from ALS spinal cords together with controls (closely matched as to age and postmortem time) were immunoassayed for p-NF-H using RT-97 antibody. Although soluble Pin1 levels can change during AD (Lu *et al.*, 1999), we found that soluble Pin1 levels were unchanged in these ALS samples compared

Figure 1. Pin1 binds to phosphorylated neurofilament-heavy chain (p-NF-H). (A) GST pull-down assays in rat brain lysates. Samples were separated by 10% SDS-PAGE, and gels were Coomassie stained. Protein bands corresponding to approximately 200 kDa were excised and identified by MS-MS mass spectrometry. This protein was identified as NF-H (arrowed). Other bands present in the gel are as follows: X1, MAP1b; X2, NF-M; X3, keratin; and X4, GST-Pin1 degradation product. Tau, GST-Pin1, and GST are also indicated. A representative gel from five experiments with identical results is shown. (B) A co-IP using anti-Pin1 antibody (Oncogene). Samples were separated by SDS-PAGE and subjected to Western blotting using the RT-97 antibody, which specifically detects p-NF-H. Arrowed band shows the species co-IP with Pin1. + and -, presence and absence of immunoprecipitating antibody, respectively. (C) Co-IP was done using RT-97 antibody to immunoprecipitate p-NF-H. Pin1 was detected in the immunoprecipitate. Arrowed bands show p-NF-H and Pin1 species. + and -, presence and absence of immunoprecipitating antibody, respectively.

with controls. Nevertheless, p-NF-H was elevated in all ALS samples compared with controls (Figure 3A) without any change in total NF levels.

p-NF-H and Pin1 Colocalize in Aggregates Found in ALS Spinal Cord

To determine whether Pin1 colocalizes with p-NF-H aggregates in ALS spinal cord, spinal cord sections were immunostained for Pin1 and for p-NF-H. p-NF-H and Pin1 staining were evident in all control neurons, and both were uniformly distributed throughout the sections (Figure 3B, g-i). By contrast, distinct differences in the staining pattern were observed in the ALS-affected sections (Figure 3B, j-l). Aggregates of p-NF-H were observed in ALS-affected sections and the uniform nature of Pin1 and p-NF-H staining was disrupted, with Pin1 colocalizing in the NF-H aggregates. Specificity for aggregate staining was confirmed by sections without primary antibody (Figure 3B, bottom panels).

Glutamate Toxicity in DRG Neurons Produces Accumulations of p-NF-H and Pin1

DRG neuronal cultures have been used as models for the neurotoxicity seen in ALS (Durham *et al.*, 1997). DRG neurons subjected to excitotoxic or oxidative stress exhibit increased kinase activity and accumulate p-NF-H in their cell bodies (Shea *et al.*, 2004). On glutamate treatment, we observed an approximate twofold increase in p-NF-H levels relative to untreated neurons. When DRG neurons were treated with juglone, a specific Pin1 inhibitor (Lee *et al.*, 2001), before glutamate exposure, NF-H phosphorylation remained at untreated levels. Pin1 levels were unchanged by glutamate treatment (Figure 4A). DRG neurons, identically treated, were also fixed and immunostained (Figure 4B, a-i). Though Pin1 was expressed in cell body and neurites in untreated DRG neurons, p-NF-H was confined to their processes (Figure 4B, a-c). Glutamate treatment caused a

Figure 2. NF-H phosphorylation is increased in Alzheimer's disease (AD) tissue. Top panels, total protein lysates from age/region-matched controls (CON) and AD brain were made and separated by SDS-PAGE. The presence of p-NF-H (top panel) and Pin1 (third panel) were immunodetected, and equal loading was confirmed by tubulin. The increase in p-NF-H was evident in AD samples without any change in soluble Pin1 levels. Quantitation is shown in the bar graph below expressed as $\pm$ SEM of densitometric measurements of p-NF-H and Pin1 signals normalized to tubulin measurements. Total NF levels were unchanged.

Figure 3. NF-H phosphorylation and aggregation are increased in ALS spinal cord tissues. (A) Total lysates of spinal cord tissues from control and ALS were separated by SDS-PAGE. The Western blot shows an increase in phosphorylated NF-H in ALS tissue compared with the controls (CON) without any significant change in Pin1 levels. Quantitation is shown in the bar graph at right, with tubulin showing equal loading. p-NF-H and Pin1 densities were normalized to tubulin densities (obtained through densitometric scanning) expressed as $\pm$ SEM. Total NF levels were unchanged. (B) Spinal cord sections from ALS patients and closely matched controls were immunostained for Pin1 (red) and p-NF-H using RT-97 (green). In the ventral region of gray matter the distribution of both Pin1 and p-NF-H were uniform in the area. (g–i) However, in the ventral horn of the white matter of ALS spinal cord, p-NF-H and Pin1 colocalized in aggregates (j–l). Controls without primary antibody did not show any staining. Scale bars, 20 μ m.

marked increase in cell body staining of p-NF-H, where it colocalized with Pin1 with a concomitant loss of Pin1 expression in neurites (Figure 4B, d–f). Juglone treatment before glutamate treatment, abolished the cell body accumulation of p-NF-H (Figure 4B, g–i). We used different glutamate treatment concentrations in the cortical and DRG neuron experiments because cortical neurons have a higher tolerance to glutamate exposure; we treated cortical neurons with 0.1 mM glutamate and DRG neurons, which are more sensitive to glutamate, with 10 μ M glutamate (Crawford *et al.*, 2000; Lee *et al.*, 2001).

Glutamate Toxicity Increases p-NF-H Accumulation in Cortical Neuron Perikarya

Seven-day-old cortical neuronal cultures were treated with or without glutamate for 6 h or pretreated for 3 h with 30 μ M juglone before glutamate treatment. The neurons were harvested for SDS-PAGE and Western blotting analyses (Figure 5A); identically treated neurons on coverslips were fixed and immunostained for p-NF-H and Pin1 (Figure 5B). There was a twofold increase in p-NF-H in cortical neurons treated with glutamate. This level was reduced nearly to that of controls when neurons were incubated with juglone before glutamate exposure. Pin1 levels were not significantly changed (Figure 5A, bar graph). There was a marked increase in p-NF-H staining in the neuronal cell body without any change in Pin1 staining; the two proteins colocalized in

this compartment (Figure 5B, e–h). The cell body accumulation of p-NF-H was reduced when neurons were incubated with juglone before glutamate exposure (Figure 5B, i and l), colocalization of Pin1 and p-NF-H was also reduced. Untreated neurons exhibited little or no staining of p-NF-H in the cell body, although Pin1 was predominantly localized in this area (Figure 5B, a–d). We also obtained identical results using another well-characterized p-NF-H antibody, SMI31 (data not shown).

Glutamate-mediated Increase in Phosphorylated NF-H Is Reduced by Dominant Negative Pin1 and Pin1-siRNA Transfection

To confirm that Pin1 activity was responsible for this effect, we used transfections of dominant negative Pin1 (DN-Pin1) as well as transfections of Pin1-siRNA. DN-Pin1 was constructed in the laboratory of Silvio Gutkind and has been well characterized in the laboratory (unpublished data). Five-day-old neurons were transfected with DN-Pin1, and after 5 h they were treated with glutamate for 6 h. DN-Pin1-transfected neurons were identified through its green fluorescent protein (GFP) tag, which showed that the transfected neuron exhibited decreased p-NF-H staining. Additionally, in transfected neurons, cell body accumulation of p-NF-H was reduced (Figure 6A). Identically transfected and treated neurons were harvested for SDS-PAGE analyses. Neurons treated with glutamate exhibited a twofold increase

Figure 4. Glutamate excitotoxicity induces p-NF-H accumulations in DRG neuronal cell bodies. (A) Five-day-old DRG neurons were nontreated, treated with 10 mM glutamate for 4 h, or pretreated with 30 μ M juglone then treated with 10 μ M glutamate for 4 h. Total lysates from the DRG samples were subjected to Western blotting to detect p-NF-H and Pin1. p-NF-H increased upon treatment with glutamate, which was reduced to nontreated levels when DRG neurons were pretreated with juglone before glutamate exposure. Tubulin expression served as loading control. Quantitation shown in bar graph at right as $\pm$ SEM of four separate experiments. (B) Five-day-old DRG neurons were nontreated (a–c), treated with 10 μ M glutamate for 6 h (d–f) or pretreated with 30 mM juglone and then treated with 10 μ M glutamate for 6 h (g–i). In nontreated DRG neurons, Pin1 was localized to the cell body, whereas p-NF-H was found mainly in the processes. On glutamate treatment there was an increase in p-NF-H and colocalization with Pin1 in the cell bodies. This was reduced when neurons were pretreated with juglone before glutamate exposure. Scale bar, 20 μ m.

in p-NF-H levels compared with nontreated neurons, and this was reduced back to “normal” levels when DN-Pin1 was overexpressed. Transfected DN-Pin1 migrated as a GFP fusion protein at approximately 50 kDa, whereas endogenous Pin1 migrated at approximately 18 kDa. Statistical analyses showed that this mediation of p-NF-H was significant (Figure 6B). Additionally, reduced phosphorylated NF-H levels were evident in Pin1-siRNA-transfected neurons according to Western analyses. Using immunocytochemistry, it was found that the extensive accumulations of p-NF-H in the neuronal perikarya were reduced in Pin1-siRNA-transfected neurons (Figure 6C). Identically transfected and treated neurons were harvested and p-NF-H and Pin1 were immunodetected. Glutamate treatment caused a robust increase in p-NF-H levels in control siRNA-transfected neurons, which was reduced when Pin1-siRNA was transfected, confirming the inhibitor as well as the DN-Pin1 data (Figure 6D). Note also that the Pin1 siRNA resulted in a 50% knockdown in the expression of Pin1 compared with the control siRNA.

Inhibition of Pin1 Reduces Glutamate-mediated Apoptosis

Glutamate can be neurotoxic and prolonged treatment can induce apoptosis (Lee *et al.*, 2000). The phosphorylation of

NF-H and its accumulation in the cell body has also been described as leading to neurotoxicity and apoptosis (Rao and Nixon, 2003). To determine whether phosphorylated NF-H accumulations in cell bodies correlate with neuronal apoptosis in our system and whether inhibition of endogenous Pin1 affects this, 7-d-old cortical neurons were untreated, treated with glutamate or pretreated with 30 μ M juglone before glutamate exposure. Approximately 10% of untreated cortical neurons were TUNEL positive; this increased to 40% upon glutamate treatment. Pretreatment with juglone before glutamate treatment reduced TUNEL-positive neurons to $\sim$ 15%, approaching numbers comparable to nontreated neurons (Figure 7A). Representative TUNEL images are shown, whereas quantitation in the bar graph represents TUNEL-positive counts from four separate experiments, where 10 independent fields were counted (Figure 7B). In glutamate-treated cortical and DRG neurons p-NF-H was also elevated with no changes in soluble or total Pin1 levels. We chose to use a 6-h treatment of glutamate, because this produced extensive accumulation of p-NF-H accompanied by neuronal death. When treatment times were extended, the percentage of TUNEL-positive neurons increased but neurons also began to detach from the coverslips (data not shown).

Figure 5. Glutamate-mediated increases in p-NF-H levels in cortical neurons is inhibited by juglone. (A) Seven-day-old primary cortical neurons were nontreated, treated with 0.1 mM glutamate for 6 h, or pretreated with 30 μ M of the Pin1 inhibitor juglone for 3 h and then treated with 0.1 mM glutamate for 6 h. Total lysates were subjected to Western blotting. p-NF-H increased in the glutamate (Glut)-treated samples and were reduced to nontreated (NT) levels when neurons were treated with juglone before glutamate exposure (Jug+Glut). Soluble Pin1 levels did not change. Bar graph on the right shows the results of four experiments expressed as $\pm$ SEM. (B) Neurons were nontreated (NT, a–d), glutamate treated (Glut, e–h), and pretreated with juglone before glutamate exposure (Jug+Glut, i–l), fixed and stained for p-NF-H using RT-97 antibody (red), Pin1 (green), and DAPI (blue). Nontreated neurons exhibited p-NF-H staining in the processes with little or no staining in the cell body, which increased upon glutamate treatment. Cell body p-NF-H staining was reduced when neurons were pretreated with the Pin1 inhibitor before glutamate treatment. Scale bar, 20 μ m.

DISCUSSION

Recent studies implicating Pin1 in tau hyperphosphorylation and paired helical filament (PHF) accumulation in neurodegeneration (Augustinack *et al.*, 2002a,b; Liou *et al.*, 2003) lend support to the hypothesis that Pin1 may also modulate NF phosphorylation in normal and pathological situations. NF-H/M contain multiple proline-directed Ser/Thr repeat residues in the C-terminal tail domain. Normally these only become phosphorylated in the axonal compartment. Pin1 specifically targets phosphorylated Ser/Thr-P sites and switches a kinase inaccessible *cis* conformation to an accessible *trans* configuration. This can affect structure and function of the target protein (Wulf *et al.*, 2004). Our data from excitotoxic-stressed neurons suggest that Pin1 regulates at least some of the phosphorylated KSP repeats in the NF-H carboxy-terminal tail domains. Neuronal stress (e.g., oxidative, excitotoxic) disrupts the relative activities of kinases and phosphatases that tightly regulate topographic phosphorylation. This disruption involves abnormal activation of proline-directed kinases such as Cdk5, Erk1/2, SAPK, and p38, and results in p-NF-H (and/or hyperphosphorylated tau) accumulations within perikarya, leading to cell death (Shea *et al.*, 1988, 2004; Davis *et al.*, 1995; Brownlee *et al.*, 2000). By examining the glutamate-induced neurodegeneration model, which evokes NF pathologies resembling those seen in ALS, we have identified a role for Pin1 in the regulation of NF phosphorylation. We show that p-NF-H is overexpressed in ALS spinal cord tissues and colocalizes with Pin1 in aggregates in ventral horn cells, similar to the Pin1 copurification with PHF tangles in AD (Lu *et al.*, 1999).

We find that p-NF-H is also overexpressed in AD brains and, as in ALS, soluble Pin1 expression is unchanged, in contrast to the decline of Pin1 reported in AD brain (Lu *et al.*, 1999) and hippocampus (Butterfield *et al.*, 2006; Sultana *et al.*, 2006). The reasons for these differences are not understood. Pin1 GST pulldown assays of rat brain lysates show that Pin1 associates with p-NF-H and that both proteins colocalize in axons of untreated DRG and cortical neurons, but not in cell bodies. However, glutamate-induced neurotoxicity produces overexpression of p-NF-H accompanied by increased p-NF-H in perikarya where it colocalizes with Pin1, a result rescued by Pin1 activity inhibition, either with juglone pretreatment, and/or with cotransfection with DN-Pin1 or Pin1 SiRNA. Moreover, these cells become apoptotic, possibly because of obstructed p-NF-H transport into axons (Shea *et al.*, 2004). All this occurs in the absence of any change in soluble Pin1 expression and suggests that Pin1 catalytic or binding activity is responsible. In contrast to Pin1 rescue of tau hyperphosphorylation by dephosphorylating a key site, Thr 231, thus preventing neurodegeneration (Davis *et al.*, 1995; Lu *et al.*, 2003; Galas *et al.*, 2006), Pin1 stimulates perikaryal phosphorylation of NF-H in stressed neurons. How can this difference be reconciled?

A model of its role in regulating tau phosphorylation in stressed neurons has been suggested. Glutamate stress, surprisingly, induces tau dephosphorylation in cortical neurons containing phosphorylated fetal tau, whereas p-tau abnormally accumulates in cell bodies (Davis *et al.*, 1995). Oxidative stress also induces tau dephosphorylation with specific phosphorylated epitopes exhibiting different levels of de-

Figure 6. Glutamate-mediated increase in phosphorylated NF-H is reduced by overexpression of DN-Pin1 and Pin1-siRNA. (A) Five-day-old cortical neurons were transfected with DN-Pin1 and after 5 h were treated with 0.1 mM glutamate (Glut). Neurons were immunostained and p-NF-H was detected using RT-97 (red) and DN Pin1 detected through expression of GFP. Only neurons transfected with DN Pin1 exhibited reduced phosphorylated NF-H in the cell body. Scale bar, 20 μ m. (B) Identical samples from A were harvested for lysates. Total cell lysates were made and separated by SDS-PAGE and then subjected to Western blotting. Phosphorylated NF-H (p-NF-H) was detected using RT-97. Pin1 was immunodetected using anti-Pin1 antibody. p-NF-H was reduced in the DN-Pin1-transfected sample. Transfected Pin1 (tPin1) migrated at approximately 50 kDa because it was a GFP fusion protein, compared with 18 kDa of endogenous Pin1 (ePin1). Equal loading was confirmed by detection of tubulin. The Western blot panels are representative of five independent experiments, and quantitation of p-NF-H is shown in the bar graph on the right, where densitometric measurements of RT-97 were normalized to tubulin measurements and expressed as $\pm$ SEM. (C) Five-day-old neurons were transfected with either control siRNA (a–d and e–h) or Pin1-siRNA (i–l) and then treated with 0.1 mM glutamate (Glut, 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. Only neurons transfected with Pin1-siRNA exhibited reduced p-NF-H in the cell body. Scale bar, 20 μ m. (D) Identical samples from C were harvested and separated by Western blotting for immunodetection of p-NF-H and Pin1. Signals were normalized to tubulin levels. Pin1-siRNA reduced glutamate-induced increases in p-NF-H.

phosphorylation (Galas *et al.*, 2006). Significantly, there is no change in Pin1 levels. Juglone, the Pin1 inhibitor, partially prevents dephosphorylation of the Thr 231 epitope, whereas in a Pin1 knockout, stress-induced dephosphorylation at Thr 231 is prevented without affecting dephosphorylation at other sites (Galas *et al.*, 2006). This suggests that Pin1 regulates tau phosphorylation by targeting dephosphorylation of a key binding site responding to unbalanced kinase:phosphatase activities. This may result from *cis-trans* isomerization and induced conformational changes that expose other phosphorylated tau sites to phosphatases. It is important to acknowledge that in addition to groups implicating Pin1 reductions with increased prevalence to AD and other dementias, Pin1 presence has also been found to be involved in disease and apoptotic processes. A recent report showed that Pin1 activity enhanced the formation of Lewy bodies, a pathological hallmark found in PD and overexpression of DN-Pin1 inhibited formation of these inclusions (Ryo *et al.*, 2006). Thus, Pin1 activity impacts target proteins with dis-

tinct results in different neurodegenerative diseases. Another recent report showed that Pin1 is involved in mitochondrial mediated apoptosis. The activation of c-Jun N-terminal kinase (JNK) signaling induced the disassociation of Pin1 from the neuron-specific JNK scaffold protein JIP3, which promotes the binding of Pin1 with phosphorylated BH3-only protein BIMEL. This stabilized BIMEL and activated the mitochondrial apoptotic machinery (Becker and Bonni, 2006). Whether this process is involved in neurodegeneration remains to be seen.

Because NF-H possesses numerous KSP repeat phosphate acceptor sites in the tail domain, which are prime targets of several proline-dependent kinases, Pin1 regulation of these sites may differ fundamentally from its regulation of the one important site in tau, Thr 231. Interpretation of our data suggests a novel model in which Pin1 is a key player in the topographic regulation of NF phosphorylation (Pant and Veeranna, 1995; Grant *et al.*, 2006). According to the model, NF monomers and oligomers within the cell body are tran-

Figure 7. Inhibition of Pin1 reduces glutamate mediated neuronal cell death. (A) Seven-day-old cortical neurons were nontreated (a), treated with 0.1 mM glutamate for 6 h (b), or pretreated with juglone then glutamate (c) and presence of apoptotic neurons examined by TUNEL-FITC staining. Nuclei were counterstained using DAPI. TUNEL-positive cells increased upon glutamate treatment and declined after neurons were treated with juglone before glutamate treatment. Scale bar, 20 μ m. (B) Quantitation is shown in the bar graph where five independent areas containing a minimum of 50 neurons were counted. Neuronal death increased nearly four times upon exposure to glutamate, and this was reduced nearly twofold when neurons were treated with juglone before glutamate treatment.

siently prevented from polymerizing into filaments by phosphorylation of specific NF head domain Ser residues by protein kinase A (PKA) and PKC (Sihag *et al.*, 1988; Hisanaga *et al.*, 1994). Phosphorylation of head domain sites in a head-to-tail tetramer (and higher) NF-subunit oligomers may interact with exposed tail domain KSP sites to block initiation of their phosphorylation. This is supported, in part, by the observation that PKA phosphorylation of NF-M head domain sites in rat cortical neurons inhibits phosphorylation of tail domain KSP sites (Zheng *et al.*, 2003). Hence, though Pin1 is present and presumably active in neuronal cell bodies (Hamdane *et al.*, 2006), phosphorylated S/T-P target sites are unavailable for Pin1 binding. Within the axon initial segment, however, as NF oligomers bind to microtubules (MTs) for transport into the axon, S/T phosphatases are activated, the head domain sites are dephosphorylated and NF polymerization begins. During axonal transport tail domain sites thus become accessible to proline-directed kinases and as phosphorylation proceeds, the tails extend, cross-linking adjacent NFs and interacting with microtubules. Pin1 now plays a major role within the axon, by inducing *cis-trans* isomerizations of critical p-S/T-P sites, driving the tail domains to extend and become stabilized. The details of Pin1 interactions with axonal pNF are described below; meanwhile, we shall focus on the destabilizing effect of stress on the processing of NFs within the cell body.

Glutamate excitotoxicity evokes an influx of Ca^{2+} that activates several proline-directed kinases. Cdk5, for example, is hyperactivated when calpain, a calcium-dependent protease, cleaves p35 to produce a more active and stable p25 (Lee *et al.*, 2000). Additionally, Erk1/2 and other stress-activated kinases such as SAP/JNK and p38 are also elevated in response to ischemic and excitotoxic stimuli (Zheng *et al.*, 2003). The kinase:phosphatase activity ratio in the cell body is altered to favor proline-directed kinases. Moreover, the influx of Ca^{2+} activates calcineurin, a phosphatase that may dephosphorylate NF head domain p-Ser sites and induce abnormal NF polymerization within the cell body. Phosphorylated tail domain multiple KSP repeats are exposed evoking Pin1 binding and promoting sustained, aberrant, perikaryal accumulation of p-NF-H. This, in turn,

inhibits axonal transport that leads to cell death. Inhibition of Pin1 activity with juglone, a dominant negative form of Pin1 or siRNA-mediated reduction of the levels of Pin1, prevent a sustained extension and phosphorylation of C-terminal tail domain KSP repeats and stops p-NF-H accumulation in the cell body.

An alternative mechanism may involve Pin1 directly. Because Pin1 activity can be switched off by PKA phosphorylation at Ser16 (Lu *et al.*, 2002), this regulation may control NF phosphorylation within neuronal perikarya during neurofilament protein synthesis. Some forms of AKAP, which can bind both PKA and phosphatases such as calcineurin, function to regulate their relative activities in relation to presynaptic AMPA receptor insertion (Houslay, 2006) and perhaps function similarly with regard to neurofilament phosphorylation. Thus "stress conditions," such as glutamate treatment, may turn off PKA in response to increased Ca^{2+} influx and aberrantly activate Pin1.

If there is a physiological role for Pin1 in the axonal compartment, it may incorporate these facts: 1) Pin1 only acts after phosphorylation at (S/T)-P repeat sites, 2) proline-directed kinases will not phosphorylate serines or threonines that are *cis*-bonded to proline residues, and 3) NF-H tail domains, which contain ~43/44 S/T-P repeat units, are not detectably phosphorylated in normal neuronal perikarya, and yet become extensively phosphorylated during axonal transport and assembly into neurofilaments. Figure 8 illustrates one way that Pin1 may participate in the normal process of NFH tail domain phosphorylation in axons. Although there is little information about the tertiary structure of this domain, it appears to be unstructured, (i.e., does not contain neither sheets nor helices; Fink, 2005). We assume, for purposes of discussion, that the unphosphorylated tail domain is globular, with only the most C-terminal repeats on its surface accessible to kinases, whereas more N-terminal repeat units are sterically sequestered within the core. However, the negative charges introduced by multiple proline-directed S/T-phosphorylations will cause the repeat units to unfold and allow kinases access to adjacent repeat units, except at certain sites where instead, phosphorylation induces *cis* isomerization of the p-S/T-P bond. We propose

Figure 8. Hypothetical mechanism for Pin1 regulation of NF-H phosphorylation. The three adjacent KSP repeat units used to illustrate this model are the human NF-H sequence 742–761. We arbitrarily diagram the kinase phosphorylation of NF tail domain repeats as normally starting at the most C-terminal repeat unit and proceeding toward their N-terminus. The more N-terminal repeat units are assumed to be sterically shielded from kinase access by burial within the tail domain until the adjacent C-terminal units are phosphorylated: (1) Tail domain phosphorylation occurs at kinase-accessible C-terminal repeats (yellow spheres); phosphorylation “unwinds” the outer repeat units and permits kinase access to additional repeat units. (2) In some cases, two or more phosphorylations will initiate a *trans* to *cis* isomerization of the last phosphorylated S-P bond. This causes a local conformation that does not expose further tail domain phosphorylation sites. However, if active Pin1 is available it will rapidly return the *cis* p-S/T-P, to *trans* and allow additional phosphorylation to proceed normally in axons. (3) However, if this occurs in perikarya, premature extension of side-arms may prevent neurofilament subunit transport out of perikarya and cause aggregation of p-NF-H subunits.

that in such cases and in the absence of active Pin1, the *cis* bond not only blocks kinase action, but also produces a local conformation that blocks access to new phosphorylation sites (Figure 8B, 3). When active Pin1 is available, however, its binding to the pS/T-P sites will regenerate the *trans* isomers, induce a conformational change, and expose adjacent S/T-P sites to initiate further kinase action. Because Pin1 can only act on phosphorylated S/T-P sites, one or more phosphorylated *cis* S/T-P bond must already be present so that Pin1 can isomerize it and cause further unraveling of the tail domain.

As shown here, Pin1 action on NF-H during neurotoxic conditions can result in aberrant phosphorylation and aggregation of neurofilaments in cultured neurons. This suggests that regulation of Pin1 binding to NF-H is necessary for normal posttranslational control of topographic NF-H tail domain phosphorylation within neuronal perikarya and the axonal compartment. Thus, inhibiting Pin1 activity or reducing its levels during neurotoxicity is an attractive target for therapeutic intervention to reduce the formation of

these aggregates in neuronal cell bodies. Pin1 antagonists, currently being sought in the treatment of cancer, may potentially be beneficial to suppress the neurotoxic accumulation of phosphorylated NF-H found in neurodegenerative disorders such as AD, PD, and ALS.

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