NPDC-1, a Novel Regulator of Neuronal Proliferation, Is Degraded by the Ubiquitin/Proteasome System through a PEST Degradation Motif*‡

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Normal cell growth is characterized by intricate regulation of proliferation and differentiation. These processes intersect at the regulation of cell cycle regulatory proteins (for review, see Refs. 1 and 2). Proliferation promotes positive regulation of cell cycle proteins (e.g. cyclins and cyclin-dependent kinases (Cdk))1, whereas differentiation results in inhibition of these cell cycle proteins. As the cell cycle progresses through G1, S, G2, and M phase, regulatory events not only occur at the level of protein modification and expression but also at the level of gene transcription (3).

One target of cell cycle regulation is the transcription factor E2F-1. The E2F-1-DP-1 (4) transcription complex controls the gene expression of proliferating cell nuclear antigen (5), B-myb, cyclin A, and dihydrofolate reductase (6). DNA binding and transcription mediated by E2F-1 is regulated by the retinoblastoma tumor suppressor protein (pRb), and the ability of pRb to bind E2F-1 is regulated by the Cdk4/6-cyclin D active kinase complex (7), which can hyperphosphorylate pRb and release it from E2F-1. These events are exquisitely synchronized within the cell cycle and are regulated by the availability of activated kinase complex, which in turn is regulated by a variety of signaling cascades originating from external signaling events. Passage through the cell cycle is a highly regulated event, and a variety of safeguards have been incorporated into this process to prevent inadvertent entry. These include regulation of the expression of cell cycle mediators (e.g. cyclins and Cdkks), regulation of active kinase complexes (e.g. specific modulators of phosphorylation), and the expression of specific inhibitors of kinase complex activity. The latter of these is an emerging area of research and is of great interest in that it provides a potential avenue for tissue-specific regulation of cell cycle events.

Neuronal proliferation, differentiation, and control clone-1 (NPDC-1) gene encodes a 34-kDa protein that is primarily expressed in neural tissues. NPDC-1 has been shown to bind to E2F-1, a variety of cyclins, and to drive differentiation events in neuronal precursor cells (8). Binding of E2F-1 by NPDC-1 inhibits E2F-1 DNA binding and E2F-1-mediated transcription events (9). Related to this, NPDC-1 has been shown to inhibit [3H]thymidine incorporation in a number of cell lines and to decrease their ability to grow in soft agar (8). Cumulatively, these observations suggest that NPDC-1 interacts with cell cycle control proteins to regulate neuronal differentiation and, consequently, may function as a tumor suppressor protein in these cells. Although NPDC-1 is differentially expressed in neuronal tissues, very little is known about the mechanisms regulating its expression.

Expression of proteins regulating cell proliferation and differentiation can occur at the transcriptional, translational, and
post-translational level. Whereas Cdks are thought to be con-
stitutively expressed throughout the cell cycle, the levels of
cyclins and Cdk inhibitors have been shown to be regulated by
both transcriptional and post-transcriptional processes. The obser-
vation that many cyclins and Cdk inhibitors are unstable proteins has implicated regulated protein degradation as a
critical mechanism in cell cycle control (for review, see Ref. 10).

Intracellular levels of the cell cycle regulatory protein E2F-1,
an NPDC-1 binding partner, have also been shown to be regu-
lated by the ubiquitin/proteasome-targeted protein degrada-
tion pathway (11). Targeted proteolysis allows for the rapid
removal of cell cycle regulators and promotes irreversible tran-
sitions between cell cycle phases. Furthermore, the rapid
removal of positive regulators of cell cycle events prevents them from
interfering with the regulation of downstream cell cycle events.

NPDC-1 associations with cell cycle regulation and regulators of cell cycle progression suggest its expression may also be necessarily regulated at the post-translational level.

In previous studies we have identified NPDC-1 as a regula-
tor of transcriptional events mediated by retinoic acid (12). NPDC-1 was isolated from a yeast functional screen designed to
identify proteins that altered retinoid X receptor (RXR) homodimer-mediated transcription. This screen identified NPDC-1 as a co-regulator of RXR-mediated transcription, and as with E2F-1, the mechanism of inhibition appeared to be through altered retinoic acid receptor (RAR/RXR) DNA bind-
ing. Differentiation factors such as retinoic acid negatively
regulate cell cycle progression by altering cell cycle protein
function. Furthermore, retinoic acid has been shown to modu-
late the expression of cyclins and Cdk inhibitors (13). In the
case of D-type cyclins, retinoic acid alters cyclin availability by
increasing its degradation (14). These data suggest that, by
some yet to be resolved mechanism, retinoic acid signaling events can target the degradation of cell cycle regulators.

In this study we identified NPDC-1 as a protein that under-
goes rapid turnover. The degradation of NPDC-1 was shown to
be dependent upon the ubiquitin/proteasome pathway. Bioin-
formatics were used to identify a ubiquitin-targeted PEST se-
quence within the carboxyl terminus of NPDC-1. The PEST
sequence was shown to be necessary for NPDC-1 degradation and
was regulated by a phosphorylation event mediated by
extracellular signal-regulated (ERK) kinases. Functional impor-
tance of this pathway was suggested in that the removal of the
PEST sequence significantly increased NPDC-1-associated inhi-
bition of retinoic acid receptor-mediated transcription. These
data support the hypothesis that NPDC-1 normal cellular
function and potential role as a tumor suppressor is regulated at
the level of protein stability.

EXPERIMENTAL PROCEDURES

Plasmids and Constructs—The human NPDC-1 cDNA (12) sequence was subcloned into pEGFP-N1 vector (Clontech) to create a construct that would express hNPDC-1 protein tagged with GFP at its carboxyl terminus. A region of hNPDC-1 extending from the unique AgeI site through the carboxyl terminus was PCR-amplified out of a previously described pCMV-T7-hNPDC mammalian expression construct (15) using the following primers: forward, 5'-ggcgacctgctggctgccagcggagc-3', and reverse, 5'-ggcgacctgctggctgccagcggagc-3'. The reverse primer created an AgeI site at the 3' end of the hNPDC-1 PCR fragment, and this PCR product was subcloned into the AgeI site of pCMV-T7-hNPDC-1 vector between the ApaI and SmaI sites of hNPDC-1. This subcloning created a full-length hNPDC-1 terminated with an AgeI restriction site but no stop codon. The resultant plasmid, pCMV7/3
hNPDC-1 vector was subcloned into pCMV-T7-hNPDC-1 vector for expression.

Creation of a FLAG-tagged hNPDC-1 expression plasmid was acc-
complished by PCR amplification of the hNPDC-1 gene from the pCM-
V-T7-hNPDC-1 vector (forward primer, 5'-ccttttacgctgctgtcttcgtccttcgactg-
agcggagcgtgagc-3') and subcloning this PCR fragment back into a NotI-R
site of pCMV-T7-hNPDC-1 vector. The reverse primer incorpo-
ated a coding sequence for the FLAG epitope (underlined in oligonu-
cleotide), resulting in an expression construct (hNPDC-FLAG) in which
hNPDC-1 has a FLAG epitope fused at its carboxyl terminus. In addi-
tion, a rat NPDC-1 with a carboxyl-terminal FLAG tag was generated as
previously described (15), and it was observed that this construct could be used interchangeably with the human NPDC-1 construct.

The construction of pET-32a (Novagen) bacterial expression con-
structs for hNPDC-1 and hNPDC-1 mutants have been described else-
where (12). These constructs generate an hNPDC-protein tagged at its
amino terminus with thioredoxin (Trx), His6, and T-Tag (binds to T-
protein, Novagen). The hNPDC-D1-GFP deletion mutant was created
by subcloning the EcoRI-BamHI hNPDC-D1 fragment, isolated from the
pET-32a-hNPDC-D1 construct (12), into the EcoRI-BamHI sites of the
pEGFP-N1 vector. The hNPDC-D1 mutant contained amino acids
1–268 and is truncated at the start of hNPDC-1 predicted PEST sequence.

Tissue Culture, Western Blotting, and Microscopy—PC12 cells were
maintained in Dulbecco's modified Eagle's medium cell culture media supplemented with 10% horse serum, 5% fetal bovine serum, and 50 mg/ml gentamicin. Western blot analyses were performed essentially as previous described (16). Briefly, PC12 cells propagated in 100-mm tissue culture dishes (Costar) were harvested at 80% confluence and plated onto 60-mm dishes precoated with polylysine. Cells were treated as indicated in the figure legends and lysed in buffered RLB (50 m M Tris, pH 7.6, 150 mM NaCl, 20 mM MgCl2, 1% Nonidet P-40, and 10% glycerol). Buffer RLB was supplemented with a protease inhibitor mix (Calbiochem) or a phosphatase inhibitor mix (Calbiochem) where indicated. Fifty micrograms of protein were subjected to SDS-PAGE on either 10 or 12.5% gels. Gels were Western-blotted to nitrocellulose membranes and probed with indicated antibodies. GFP fusion proteins were detected using a monoclonal GFP antibody from Santa Cruz at a dilution of 1:200. FLAG-tagged hNPDC-1 protein was detected with the M2 anti-FLAG antibody from Sigma. Recombinant hNPDC-1 was identi-
fied by immunoblotting for its thioredoxin fusion partner using a
cyclins and Cdk inhibitors have been shown to be regulated by
extracellular signal-regulated (ERK) kinases. Functional impor-
tance of this pathway was suggested in that the removal of the
PEST sequence significantly increased NPDC-1-associated inhi-
bition of retinoic acid receptor-mediated transcription. These
data support the hypothesis that NPDC-1 normal cellular
function and potential role as a tumor suppressor is regulated at
the level of protein stability.
NPDC-1 Is Degraded by the Proteasome

NPDC-1 is degraded by the proteasome, as shown by various experiments. One experiment involved using Metabolic labeling to study in vivo phosphorylation of hNPDC-1 in PC12 cells. Cells were transfected with a pCMV-hNPDC-FLAG mammalian expression construct and grown in 80-mm plates coated with polylysine. After 24 h, cells were washed and starved for 60 min in phosphate-free Dulbecco modified Eagle's medium (Cellgro). Proteins were labeled by adding 0.5 mCi of [32P]orthophosphate (PerkinElmer Life Sciences) for 4 h. Cells were washed and harvested in RLB buffer containing phosphatase inhibitors (Calbiochem). hNPDC-1 was affinity-purified from duplicate samples with M2 anti-FLAG-agarose as described above. Both samples were subjected to SDS-PAGE separation. One gel was transferred to a nitrocellulose membrane for immunoblot analysis with M2-FLAG antibody. The second gel was dried and exposed to film for 2 h to detect radiolabeled proteins.

A series of kinase proteins was purchased from New England Biolabs. These included ERK, protein kinase A, glycogen synthase kinase 3, casein kinase I, and casein kinase II. Kinase labeling was performed as described in the manufacturer's protocol. Briefly, 5 μg of recombinant hNPDC-1 was added to a 1× kinase buffer mix (as provided by New England Biolabs) containing [γ-32P]ATP. Reactions were carried out under conditions of equal enzyme concentration for 1 h. Reactions were stopped with 2× SDS-PAGE sample buffer, subjected to SDS-PAGE separation, and analyzed by autoradiography. Similar experiments conducted for varying time points as indicated in figure legends were stopped with 2× SDS-PAGE sample buffer and analyzed as described above.

RESULTS

NPDC-1 Is Rapidly Degraded in PC12 Cells—To study hNPDC-1 function in vivo, several mammalian expression constructs were generated. All constructs were verified by DNA sequencing. PC12 cells transfected with an hNPDC-GFP construct were initially analyzed for GFP expression by fluorescence microscopy 48 h post-transfection. No cells were observed to express the hNPDC-GFP fusion (Fig. 1A). In contrast to the hNPDC-GFP fusion protein, the native GFP expressed from the pEGFP-N1 vector was easily observed, with a transfection efficiency of ~30%. No hNPDC-GFP was observed with increased exposure times, whereas GFP quickly became overexposed after 30 s (data not shown).

After sequencing the hNPDC-pEGFP clone a second time and confirming the integrity of the construct, we examined fusion protein expression by a more sensitive immunoblotting protocol (Fig. 1B). PC12 cells transfected with either the pEGFP control plasmid or the hNPDC-GFP plasmid were incubated for 48 h and collected in cell lysis buffer. Fifty micrograms of protein from each cell lysate was separated on a 12.5% SDS-PAGE gel and transferred to nitrocellulose membrane. A rabbit polyclonal antibody directed against GFP was used to probe the blot. As expected a protein migrating at the approximate molecular weight for GFP was easily detected. In fact, a robust band appeared at the shortest exposure time of 2 s.
contrast, a protein migrating at the theoretical molecular weight for the hNPDC-GFP fusion protein was not observed until the membrane had been exposed for 2 min. The hNPDC-GFP fusion protein detected at 2 min was observed to be severalfold less than the GFP control protein.

A plausible explanation for the above data would be that hNPDC-1 is a rapidly degraded protein in PC12 cells and never accumulates to significant levels. To explore this hypothesis, the expression of hNPDC-GFP was monitored in the presence of proteasome inhibitors.

### hNPDC-1 Levels Are Regulated by the Proteasome

Many cell cycle proteins (22) and signal transduction proteins are regulated by protein turnover (for review, see Ref. 23). An important protein degradation system is the ubiquitin/proteasome degradation machine (24). Proteins enter this degradation system by being targeted for destruction by the addition of multiple ubiquitin molecules. Once properly targeted, the protein to be degraded is delivered to the proteasome. The protein enters the proteasome and is cleaved into oligopeptides. The net result of proteasome degradation is an irreversible decreased function of the target protein.

It is well established that protease inhibitors directed toward the catalytic function of the proteasome can be used to monitor proteasome function (25). The inhibitor MG-132 has frequently been used to turn off the proteasome and allow targeted proteins to accumulate in the cell (19). PC12 cells growing in microscope slide culture chambers were transfected with mammalian expression vectors containing either the GFP control protein or hNPDC-GFP. After 24 h, cells were treated with 40 μM MG-132 where indicated. After an additional 24 h of incubation cells were washed with phosphate-buffered saline and fixed in 5% formaldehyde. Cell membranes were extracted with 0.1% TritonX-100 and stained with Texas Red-phalloidin. Cells were examined and photographed under conditions that would show all cells (phalloidin-stained; Fig. 2A, top panels) or transfected cells (cells having GFP fluorescence: Fig. 2A; bottom panels). PC12 cells transfected with pEFGP were easily identified by robust expression of the GFP control protein. In contrast, PC12 cells transfected with hNPDC-pEFGP failed to express GFP and could not be identified as transfected cells. However, PC12 cells treated with the proteasome inhibitor MG-132 displayed robust green cells indicative of GFP expression (Figs. 2A and 2B). Taken together these studies suggest that NPDC-1 is a rapidly degraded protein that is processed in a proteasome-dependent manner.

### The Expression of Endogenous NPDC-1 Is Regulated at the Post-translational Level

The above experiments using overexpressed and tagged versions of NPDC-1 provide strong experimental evidence for post-translational regulation of NPDC-1. To investigate the potential for endogenous post-translational regulation of NPDC-1, an NPDC-1-specific polyclonal antisera was generated to an internal peptide epitope (amino acids 53–70) of the human NPDC-1 protein. This antiserum specifically recognized an ~35-kDa protein, which would correspond...
with the predicted molecular mass of human NPDC-1. To evaluate post-translational regulation of NPDC-1, PC12 cells were treated with cycloheximide for 0–120 min and subsequently analyzed for their expression of endogenous NPDC-1 and a control protein calnexin (Fig. 3A). As seen in Fig. 3A, levels of NPDC-1 protein significantly dropped after 30 min of incubation in cycloheximide, whereas calnexin remained unchanged.

To further evaluate whether this reduction in endogenous NPDC-1 might be due to proteasomal activity, the same experiment was performed in the presence of the proteasomal inhibitor MG-132 (Fig. 3B). As seen in Fig. 3B, the protein levels of endogenous NPDC-1 remain unchanged after 2 h of treatment with cycloheximide in the presence of MG-132. These data cumulatively support the findings that endogenous human NPDC-1 protein is under stringent post-translational regulation through specific proteasomal targeting mechanisms.

NPDC Undergoes Ubiquitination in Vivo and in Vitro—The results above suggest that hNPDC-1 is degraded by the proteasome. An important component of proteasome-mediated degradation is the proper targeting of the protein to be degraded (for review, see Ref. 26). The targeting process is mediated by a complex enzyme system designated the ubiquitin conjugation complex. The ubiquitin conjugation complex attaches the 8-kDa protein ubiquitin to specific lysine residues within the target protein sequence. Subsequently, additional ubiquitin molecules are added to conserved lysine residues within ubiquitin. This process results in multiple ubiquitin chains being attached to the target protein. Therefore, it can be hypothesized that hNPDC-1 isolated from proliferating PC12 cells contains polyubiquitin chains.

To examine this possibility PC12 cells were transfected with mammalian expression hNPDC-FLAG constructs and grown in normal PC12 media. After 36 h, cells were washed once in complete medium and resuspended in PC12 media containing increasing amounts of MG-132 (Fig. 4). After an additional 4 h of incubation, cells were harvested in lysis buffer containing 40 μM MG-132 and 5 mM NEM (inhibits isopeptidases, which can remove Ub chains). Anti-FLAG-agarose was used to affinity-purify hNPDC-FLAG from clarified lysates. The anti-FLAG-bound proteins were resuspended in SDS-PAGE sample buffer and electro-

![Figure 2](image-url)
were normalized to the calnexin band intensities, and a -fold increase or decrease was calculated with ImageQuant software, the NPDC-1 band intensities were normalized to the calnexin band intensities, and the fluorescent signal was captured using a labeled secondary antibodies were used for identifying specific immuno-reacting bands, and the fluorescent signal was captured using a Typhoon scanner. The intensity of each NPDC-1 and calnexin band was calculated with ImageQuant software, the NPDC-1 band intensities were normalized to the calnexin band intensities, and a -fold increase or decrease was calculated with respect to 0 min.

were phosphorylated through a 12.5% SDS-PAGE gel. Proteins were transferred to a nitrocellulose membrane and probed with anti-FLAG antibody to verify the amount of hNPDC-1 present in the pellet. Subsequently, blots were stripped and reprobed with anti-ubiquitin. As seen in Fig. 4A, a high molecular weight smear, characteristic of polyubiquitination, increases in intensity with increased concentration of MG-132, demonstrating that hNPDC-1 is targeted by the ubiquitin/proteasomal pathway.

To confirm that the ubiquitin chains observed in Fig. 4A were directly attached to hNPDC-1 and not an artifact of an interacting protein, we carried out ubiquitination experiments with rabbit reticulocyte lysate. Rabbit reticulocyte lysate has been shown to contain all the necessary components of the ubiquitin/proteasome pathway (19). Recombinant hNPDC-1 was purified from E. coli and diluted into ubiquitin assay buffer containing GST-tagged ubiquitin. Increasing amounts of rabbit reticulocyte lysate were added as indicated. Fig. 4B shows that high molecular weight bands appear above hNPDC with increasing rabbit reticulocyte lysate. The sizes of these bands are approximately multiples of the 38-kDa molecular weight of the GST-ubiquitin fusion protein. Taken together, the above experiments support the hypothesis that hNPDC-1 is degraded in a manner that is dependent on the ubiquitin/proteasomal system.

**NPDC-1 Contains a PEST Motif That Directs Its Targeted Degradation**—Proteins that undergo rapid turnover contain sequence identifiers that target the protein for degradation. Therefore, bioinformatics was used to identify a degradation sequence in hNPDC-1. A BLAST search of the NCBI non-redundant data base failed to find common destruction motifs such as the destruction box found in cyclins (27); therefore, a PESTfind algorithm was employed to analyze the hNPDC-1 sequence for potential PEST amino acid sequences. A PEST motif is a polypeptide sequence enriched in proline, glutamate, serine, and threonine (28). These regions are hydrophilic, at least 12 amino acids long, and target proteins for rapid degradation. The PESTfind program revealed one possible PEST sequence located at the carboxyl terminus of hNPDC-1 (Fig. 5A).

An hNPDC-1 deletion mutant was created in which the carboxyl terminus of hNPDC was truncated just before the start of the PEST sequence (Fig. 5B). This mutant, termed hNPDC-D1, was subcloned into the pEGFP-N1 mammalian expression vector to create hNPDC-D1-GFP. The expression of hNPDC-D1-GFP was compared with full-length hNPDC-GFP by using visible GFP as a marker for expression. PC12 cells transfected with a GFP control vector, an hNPDC-GFP mammalian expression construct, or the hNPDC-D1-GFP mammalian expression construct were fixed and stained as described under “Experimental Procedures” and visualized using fluorescence microscopy (Fig. 5C). As seen previously, the hNPDC-GFP fusion protein was not observed in the absence of the proteasome.

![Fig. 3. Endogenous NPDC-1 is regulated at the post-translational level through targeting to the proteasome. PC12 cells were treated for the indicated times with 150 μM cycloheximide in the absence (A) and presence (B) of the proteasomal inhibitor MG-132. Cell lysates were fractionated on SDS-PAGE gels, Western-blotted to nitrocellulose membranes, and probed for the presence of NPDC-1 and calnexin using NPDC-1 and calnexin-specific antisera. Fluorescin-labeled secondary antibodies were used for identifying specific immunoreacting bands, and the fluorescent signal was captured using a Typhoon scanner. The intensity of each NPDC-1 and calnexin band was calculated with ImageQuant software, the NPDC-1 band intensities were normalized to the calnexin band intensities, and a -fold increase or decrease was calculated with respect to 0 min.](http://www.jbc.org/content/370/7/7468.full.pdf+html)

![Fig. 4. NPDC-1 is ubiquitinated. A, PC12 cells were transfected with an hNPDC-1 mammalian expression construct tagged at the carboxyl terminus with the FLAG epitope (hNPDC-FLAG). 48 h post-transfection cells were treated for 4 h with increasing concentrations of the proteasome inhibitor MG-132 (0, 1, 10, 20, 50, and 100 μM). Cells were harvested in lysis buffer containing 5 mM NEM to prevent ubiquitin chain degradation. hNPDC-FLAG was immunoprecipitated (IP) from 500 μg of cell lysate and subsequently split into two equal aliquots. One aliquot was immunoblotted (IB) and probed with anti-ubiquitin. To control for loading, the second aliquot was probed with an anti-FLAG-M2 antibody. B, bacterially expressed hNPDC-1 was analyzed as a substrate for direct ubiquitination by rabbit reticulocyte lysate. Each reaction contained 10 μg of Trx-tagged recombinant hNPDC-1, 300 μg/ml GST-ubiquitin, and increasing amounts (0, 1, 10, 25, and 50%) of rabbit reticulocyte lysate. Reactions were stopped by the addition of an equal amount of 2× SDS-PAGE sample buffer. Proteins were separated on SDS-PAGE gels, immunoblotted onto nitrocellulose membranes, and probed with the anti-thioredoxin antibody.](http://www.jbc.org/content/370/7/7468.full.pdf+html)
inhibitor MG-132. In contrast, hNPDC-D1-GFP expression was completely independent of MG-132 and produced a transfection efficiency equal to that of the GFP control (Fig. 5D).

To further validate the PEST sequence as a site of hNPDC-1 ubiquitination, in vitro ubiquitination assays were performed using the recombinant hNPDC-D1 mutant protein (Fig. 6). As seen in Fig. 6, incubation of hNPDC-1, hNPDC-D1, and ubiquitin proteins in the rabbit reticulocyte in vitro assay system resulted in strong ubiquitin-specific labeling of unmutated hNPDC-1 protein but very little, if any, labeling of the hNPDC-D1 mutant protein. The above analysis and experiments suggest that hNPDC-1 contains the PEST protein degradation signal, and hNPDC-1 degradation can be inhibited by removal of this sequence.

Removal of the NPDC-1 PEST Sequence Impacts Its Ability to Repress Retinoic Acid-mediated Transcription—We have previously shown that hNPDC-1 can bind the retinoic acid receptors RAR and RXR and repress transcription events mediated by these receptors (12). Mapping studies characterizing the binding of hNPDC-1 to RXR clearly demonstrate that the NPDC-D1 mutant lacking the PEST sequence retains strong binding to RXR (12). Therefore, it can be hypothesized that enhancement of NPDC-1 stability through deletion of the PEST sequence would facilitate NPDC-1 repression of retinoic acid-stimulated transcription events. To examine this possibility,
Fig. 7. The NPDC-D1 mutant is a potent inhibitor of retinoic acid stimulated transcription. HEK-293 cells were co-transfected with mammalian expression constructs for GFP, NPDC-GFP, or NPDC-D1-GFP, a luciferase reporter gene driven by a retinoic acid-inducible promoter and a mammalian expression construct for β-galactosidase. Transfected cells were incubated in the presence of 10−6 M all-trans retinoic acid (atRA). After 48 h cells were lysed and assayed for luciferase activity and β-galactosidase activity as described under “Experimental Procedures.” Average luciferase values were normalized to the constitutive β-galactosidase response. Values are expressed as percent inhibition, with the normalized luciferase response for the retinoic acid-stimulated GFP construct serving as the 100% induction parameter.

RAR-mediated in vitro transcription assays were performed in the presence and absence of mammalian expression constructs for hNPDC-1 and the hNPDC-D1 mutant. As shown in Fig. 7, the hNPDC-D1 mutant is a significantly better inhibitor of retinoic acid-induced transcription than full-length hNPDC-1. This result suggests that proteasomal degradation of hNPDC-1 could serve as a regulatory mechanism for retinoic acid-induced transcription.

NPDC-1 Is Phosphorylated by a Number of Kinases—Phosphorylation has been shown to be one of the main mechanisms for modulating the activities of proteins like NPDC-1 that have direct links to transcriptional regulation and cellular proliferation (for review, see Refs. 29 and 30). To evaluate the in vitro phosphorylation state of hNPDC-1, metabolic labeling studies were performed. PC12 cells were transfected with a mammalian expression construct for hNPDC-FLAG, incubated for 24 h, and then labeled in phosphate-free media with [32P]phosphate. Cells were harvested in lysis buffer containing phosphatase inhibitors, and 300 μg of protein was subjected to immunoprecipitation with anti-FLAG M2 monoclonal antibody. The resulting pellet was divided evenly, with half of the immunoprecipitate subjected to SDS-PAGE for autoradiography. The remaining half was subjected to immunoblotting to verify that hNPDC-FLAG was in fact precipitated (Fig. 8A). As seen here, an immunoprecipitable band of the approximate molecular weight for hNPDC-1 was labeled with [32P]phosphate.

We next attempted to identify the kinases that might be responsible for phosphorylation of hNPDC-1. A panel of kinases was purchased from New England Biolabs, and the kinases were tested for their ability to phosphorylate recombinant hNPDC-1 protein. Fig. 8B shows that ERK2, glycogen synthase kinase 3, cdc2, casein kinase I, and casein kinase II were able to transfer the [γ-32P]phosphate from ATP to hNPDC-1. In contrast, protein kinase A showed no activity toward the hNPDC-1 substrate.

Phosphorylation of hNPDC-1 by ERK2 Accelerates Ubiquitination—Numerous examples of kinase-regulated proteolytic events exist in the literature, most notably in the regulation of cyclin levels (30). Furthermore, phosphorylation has been shown to regulate the degradation of several PEST-containing proteins (31, 32). To assess the impact ERK kinase has on the regulation of NPDC-1 degradation, we evaluated the effects phosphorylation by ERK kinase might have on in vitro ubiquitination kinetics of hNPDC-1. Recombinant Trx-S-NPDC-1 protein was phosphorylated in vitro with ERK2 kinase for 1 h. The kinase-treated protein reaction was diluted 1:2 in ubiquitination buffer and was subjected to ubiquitination time course assays containing rabbit reticulocyte lysate and recombinant ubiquitin protein (Fig. 9). As seen in Fig. 9A, the ubiquitination of hNPDC-1 protein was increased after phosphorylation of hNPDC-1 by ERK2 kinase.
FIG. 9. Phosphorylation increases the degradation kinetics of NPDC. A, recombinant S-thioredoxin-tagged hNPDC-1 was phosphorylated for 1 h with 500 units of ERK kinase in a reaction containing cold ATP. The reaction was added to an equal volume of ubiquitination buffer containing 10% rabbit reticulocyte lysate and recombinant Ub. Reactions were stopped at the indicated time points with 2× SDS-PAGE sample buffer, electrophoresed on SDS-PAGE gels, and immunoblotted for NPDC-1 with the anti-thioredoxin antibody (Anti-Trx). B, HEK-293 cells were transfected with an hNPDC-FLAG mammalian expression construct. 24 h later cells were treated with the indicated amount of PD-98059. After an additional 24 h, cells were treated with fresh PD-98059 and 40 μM MG-132 for 4 h. Cells were lysed in the presence of MG-132 and NEM to preserve ubiquitin chains. FLAG-tagged hNPDC-1 was pulled-down with anti-FLAG M2 beads. The pellet was divided, and one-half was immunoblotted and probed for ubiquitin using anti-ubiquitin anti-sera, whereas the other half was analyzed for total hNPDC-1 by immunoblotting and probing with anti-FLAG M2 antibody.

(compare the relative amounts of ubiquitin conjugates in the ERK kinase-treated samples to their respective no kinase controls). These data suggest that the phosphorylation state of NPDC-1 is linked to its degradation.

The effect of ERK kinase on hNPDC-1 activity was further examined in vivo. HEK-293 cells were transfected with an hNPDC-FLAG mammalian expression construct and subsequently assayed for ubiquitination in the presence and absence of the mitogen-activated protein kinase inhibitor PD-98059 (Fig. 9B). Ubiquitinated hNPDC-1 was observed to decrease in a dose-dependent fashion when pretreated with increasing concentrations of PD-98059. These studies suggest that members of the mitogen-activated protein kinase family regulate hNPDC-1 in vivo.

DISCUSSION

NPDC-1 is a protein restricted in its tissue distribution and previously described as a regulator of neuronal differentiation (8). Although NPDC-1 has not been aligned with the pathology of any specific tumorigenesis, the in vivo and in vitro data supporting the NPDC-1 role in the suppression of cellular proliferation would suggest that this protein could function as a tumor suppressor (8, 9). NPDC-1 was originally described as being expressed exclusively in the neural tissue (8) but has recently been described to also be expressed in heart, lung, and pancreas (12, 33). Studies involving neural tissue demonstrate that NPDC-1 mRNA expression levels appear to be induced upon neuronal differentiation, with the highest levels expressed in cells that have exited the cell cycle (8, 34). Data presented here provide evidence for an alternate mechanism for regulating endogenous levels of NPDC-1 protein, that being post-translationally through the ubiquitin/proteasome system. Of importance here is the large body of evidence suggesting that the proteasomal degradation pathway is intimately linked to the regulation of cell proliferation (35) as well as carcinogenesis (14). These data place NPDC-1 in the middle of this developing story, and it can be hypothesized that signaling events that activate the ubiquitination/proteasomal degradation pathways in cells expressing NPDC-1 would also reduce the levels of this putative tumor suppressor.

The ability of a protein to be degraded by the proteasome is dependent on the small protein tag ubiquitin (for review, see Ref. 35). This 8-kDa protein is attached to lysine residues of target proteins via the action of ubiquitin ligases termed E1 (E1 is ubiquitin-activating enzyme), E2 (ubiquitin carrier protein), and E3 (ubiquitin-protein isopeptide ligase). The current research data suggest that the E3 ligase subunit targets the protein substrate (26). Studies presented here demonstrate that NPDC-1 is rapidly ubiquitinated when overexpressed in PC12 cells and can be ubiquitinated in the rabbit reticulocyte lysate in vitro ubiquitination system. These results suggest that there exists an E3 ubiquitin ligase or ligases that are responsible for targeting NPDC-1 for degradation. The observations that hNPDC-1 can be ubiquitinated using rabbit reticulocyte lysate would further suggest a possible regulatory pathway for NPDC-1 outside of neuronal tissues. Alternatively, NPDC-1 degradation could be mediated by a common E3 ligase that is present in both brain and rabbit reticulocyte lysate. Whatever the case, the ability to in vitro and in vivo ubiquitinate hNPDC-1 should provide a viable tool for the future identification of the E3 ligase(s) that interacts with hNPDC-1 (19).

Cellular proliferation is regulated by phosphorylation cascades that ultimately result in up-regulation of transcriptional events and progression through the cell cycle (1). One well characterized system is the Raf/MEK/ERK signal transduction pathway. This hierarchical kinase cascade culminates in ERK kinase phosphorylation of transcription factors whose activation are required for progression through the cell cycle (36). Data presented here suggest an additional role for ERK kinase in this pathway, that being targeting degradation of protein factors inhibitory to proliferation. Somewhat similar to what was observed here for NPDC-1, ERK kinase has also recently been shown to regulate the protein stability of the differentiation factor Maf (37). Activation of ERK kinase was observed to increase the degradation of the lens-specific transcription factor Maf, and mutant forms of Maf resistant to degradation were observed to promote differentiation of lens cells from neuronal precursors (37). These data suggest that ERK kinase, in addition to its role as a positive regulator of transcription events required for cellular proliferation, may also target the destruction of transcription-linked protein inhibitors of cellular proliferation.

NPDC-1 expression has previously been shown to modulate gene expression events mediated by both retinoid receptors (12) and E2F-1 (9). Retinoids, metabolites of vitamin A, are established molecular mediators of embryonic development of both brain (38–40) and lung (41). Previous reports demonstrate that NPDC-1 is expressed in both brain and lung and that NPDC-1...
can inhibit retinoic acid-mediated transcription (12). Recently it has been shown that during lung and neuronal differentiation, retinoic acid can induce proteasome-mediated protein degradation of several proteins, including cyclin D1 (42). Some what contradictory to these results are the published observations that retinoic acid can directly activate Raf kinase, which leads to increased ERK kinase activity (43, 44), a pathway that would seemingly drive dedifferentiation and proliferation. One potential explanation for convergence of these diametrically opposed signaling pathways is that it may be necessary to establish an environment within proliferating cells that ensures the cell is competent to respond to retinoic acid. Indeed, activation of ERK2 in HL-60 lung epithelial cells (43) and PC12 cells (45) increases the cell sensitivity to retinoic acid. The data presented here support a role for NPDC-1 in both neuronal and lung development through a signaling pathway, Raf/MEK/ERK, that is known to converge with retinoid receptor-mediated transcription events. Establishment of NPDC-1 in the above signal transduction pathways may have important implications for cancer research, particularly tumors resistant to retinoic acid treatment.

E2F-1 has been shown to be critical in promoting proliferation (46) and is often dysregulated in human cancers (47–49). NPDC-1 has previously been shown to also potently inhibit E2F-1-mediated transcriptional events (9). Furthermore, NPDC-1 can form a direct interaction with E2F-1 (9, 12). Here we show that NPDC-1 is degraded by the ubiquitin/proteasome system, that phosphorylation of hNPDC-1 by ERK kinase can serve as a mechanism for targeting this event, and that inhibition of this event strongly inhibits retinoic acid-stimulated transcription events. These data suggest that the stabilization of NPDC-1 increases inhibition of retinoic acid-mediated events, and by extension of this model it can be hypothesized that stabilization of NPDC-1 at the protein level would also similarly inhibit transcription events mediated by E2F-1.

In summary, the studies presented here indicate that NPDC-1 has for the first time been shown to be regulated by the ubiquitin/proteasome system. The degradation of NPDC-1 is targeted through a PEST degradation motif located in the NPDC-1 carboxyl terminus and is regulated via phosphorylation by ERK kinase. These data provide a novel mechanism for regulation of NPDC-1 at the level of protein expression. These new data together with previous studies that suggest NPDC-1 may function as a tumor suppressor protein may provide a new avenue for tissue-specific tumorigenesis only detectable through proteomic technologies.
NPDC-1, a Novel Regulator of Neuronal Proliferation, Is Degraded by the Ubiquitin/Proteasome System through a PEST Degradation Motif
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