The Transcriptional Corepressor NAB2 Inhibits NGF-induced Differentiation of PC12 Cells

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Abstract. The PC12 pheochromocytoma cell line responds to NGF by undergoing growth arrest and proceeding to differentiate toward a neuronal phenotype. Among the early genetic events triggered by NGF in PC12 cells are the rapid activation of the zinc finger transcription factor Egr1/NGFI-A, and a slightly delayed induction of NAB2, a corepressor that inhibits Egr1 transcriptional activity. We found that stably transfected PC12 cells expressing high levels of NAB2 do not differentiate, but rather continue to proliferate in response to NGF. Inhibition of PC12 differentiation by NAB2 overexpression was confirmed using two additional experimental approaches, transient transfection, and adenoviral infection. Early events in the NGF signaling cascade, such as activation of MAP kinase and induction of immediate-early genes, were unaltered in the NAB2-overexpressing PC12 cell lines. However, induction of delayed NGF response genes such as TGF-β1 and MMP-3 was inhibited. Furthermore, NAB2 overexpression led to downregulation of $p21^{WAF1}$, a molecule previously shown to play a pivotal role in the ability of PC12 cells to undergo growth arrest and commit to differentiation in response to NGF. Cotransfection with $p21^{WAF1}$ restored the ability of NAB2-overexpressing PC12 cells to differentiate in response to NGF.

Key words: Egr1 • NAB2 • $p21^{WAF1}$ • corepressor • differentiation

MANY complex cellular processes such as differentiation, proliferation, and apoptosis are initiated by extracellular stimuli, which in turn activate intracellular signaling cascades. These signaling pathways lead to changes in gene expression, which allow the cell to respond or adapt appropriately to environmental cues. Much interest has focused on early downstream nuclear targets of various signaling pathways such as immediate-early genes. In particular, those encoding transcription factors, such as Fos, Jun, and Egr family members are thought to provide a vital link between early cytoplasmic events and long-term alterations of gene expression.

The PC12 pheochromocytoma cell line responds to NGF by extending neurites and developing many other properties similar to those of sympathetic neurons (Greene and Tischler, 1976; Allen and Heinrich, 1987). PC12 cells have served as a model system to study a variety of problems related to neuronal differentiation and function. Egr1 (also called NGFI-A, Zif268, Krox24) is a member of a family of zinc finger transactivators which also includes Egr2/Krox20, Egr3 and Egr4/NGFI-C (for review see Gashler and Sukhatme, 1995) that was originally identified as an immediate-early gene dramatically induced by NGF in PC12 cells (Milbrandt, 1987). As a transcription factor, it may play a critical role in the regulation of genes that are induced at later times and whose products are crucial for establishing the differentiated phenotype.

Members of the Egr family of transactivators bind to a DNA consensus element, 5′-GCG(G/T)GGGCG-3′, which is found in the promoters of many genes (Christy and Nathans, 1989; Lemaire et al., 1990; Swirnoff and Milbrandt, 1995). These putative target genes are involved in a variety of processes that include proliferation, apoptosis and differentiation. Examination of mice deficient in members of the Egr family has revealed crucial roles for these transcription factors in the development and function of both the nervous and reproductive systems; the phenotypes of these mice include female infertility (Lee et al., 1996) and defects in myelination and hindbrain development (Swiatek and Gridley, 1993; Schneider-Maunoury et al., 1993; Topilko et al., 1994). In addition, analyses of

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mice deficient in various combinations of Egr proteins suggest that the Egr family is also critical for development of the central nervous system (Tourtellotte, W., and Milbrandt, J., unpublished observations).

Many transcription factors are influenced by their interaction with other proteins that modulate their transcriptional activity. A subclass of these cofactors, called corepressors, repress the activity of promoters to which they are recruited. One of the most well-studied corepressors is the retinoblastoma gene product, pRb, which controls progression through the cell cycle by binding to E2F and repressing transcription of several genes involved in DNA replication (Weinberg, 1995). Recently, a family of novel transcriptional corepressors consisting of NGFI-A binding protein 1 (NAB1)[1] and NAB2 was identified (Russo et al., 1995; Svaren et al., 1996). NAB proteins bind to a conserved R1 domain found in several members of the Egr family immediately upstream of the DNA binding domain. Egr1, Egr2, and Egr3 all possess this motif, whereas Egr4 lacks an R1 domain and is not repressible by NAB proteins. NAB binding to Egr proteins is mediated by a domain, NAB-conserved domain 1 (NCD1), which is strongly conserved in both NAB1, NAB2, as well as *Caenorhabditis elegans* and *Drosophila* NAB homologues (Svaren et al., 1996; Clements, M., and J. Milbrandt, unpublished data). NAB proteins do not interfere with DNA binding by Egr1, but rather actively repress transcriptional activity of promoters to which they are tethered (Swirnoff et al., 1998).

While Egr1 is rapidly induced by NGF in PC12 cells, the related Egr2 and Egr3 proteins are not expressed in these cells (Joseph et al., 1988; Patwardhan et al., 1991). NAB1 is constitutively expressed at a low level in PC12 cells, whereas NAB2 is induced by NGF, but with kinetics that are slightly delayed relative to the peak of Egr1 expression (Svaren et al., 1996). Egr4 is expressed at low levels in PC12 cells after NGF stimulation (Crosby et al., 1991), but it does not contain an R1 domain. This expression data therefore suggests that Egr1 and NAB2 are the relevant members of their families in regulating expression of Egr target genes in PC12 cells.

In this study, we have investigated the physiologic function of the Egr1/NAB2 transcription factor complex in PC12 cell differentiation. We generated PC12 cell lines that constitutively overexpress NAB2, and found that they fail to differentiate in response to NGF. Wild-type cells respond to NGF by withdrawing from the cell cycle before differentiation, whereas NAB2-overexpressing clones continued to divide. Similar effects on differentiation were observed when NAB2 was introduced using transient transfection or by infection with a NAB2-expressing adenovirus. Early events in the NGF signaling cascade, including activation of mitogen-activated protein (MAP) kinase (MAPK) and induction of immediate-early genes, were unaltered in NAB2-overexpressing cells. However, delayed response genes, such as TGF-β1 and MMP-3 (stromelysin/transin), were not induced in response to NGF in these NAB2 overexpressing cell lines. Furthermore, constitutive overexpression of NAB2 led to a dramatic downregulation of the cyclin-dependent kinase inhibitor (cdi) $p21^{WAF1}$ (also known as Cip1, Sdi1), a molecule previously shown to play a pivotal role in the ability of PC12 cells to withdraw from the cell cycle and differentiate in response to NGF (Yan and Ziff, 1995; Billon, 1996; Erhardt and Pittman, 1998). Consistent with these findings, transient cotransfection of an expression plasmid for $p21^{WAF1}$ restored differentiative competence to NAB2-transfected cells. These data suggest that NAB2 overexpression represses Egr1 activity and prevents downstream events that are required for PC12 differentiation. At least one of the important downstream events inhibited by NAB2 overexpression is activation of $p21^{WAF1}$, an event that is required for PC12 cells to exit the cell cycle and initiate differentiation (Mark and Storm, 1997).

Materials and Methods

Cell Culture and Transfections

Rat pheochromocytoma PC12 cells were cultured in DME supplemented with 10% FBS and 5% horse serum in 12% CO_2. Cells were induced to differentiate by adding NGF to a final concentration of 50 ng/ml. Differentiation was assayed by the appearance of neurite extensions greater than two cell body diameters in length. Transient transfections of PC12 cells were carried out using lipofectamine (GIBCO-BRL, Gaithersburg, MD) according to the manufacturer's recommended protocol. Stably transfected cell lines were selected by growth in medium containing 350 μg/ml G418. After 8 wk of selection, individual colonies were picked for expansion and further analysis. All of the stably transfected lines were maintained in G418 selection.

LacZ Reporter Gene Expression

PC12 cells were cotransfected with a cytomegalovirus (CMV)-driven lacZ reporter gene along with various other expression constructs in a 1:20 ratio using lipofectamine (GIBCO-BRL). Cultures were treated with NGF (50 ng/ml) for 3 d beginning 48 h after transfection. LacZ reporter gene expression was detected as described (Yao and Cooper, 1995).

Immunofluorescent Staining

Wild-type PC12 cells and NAB2-overexpressing PC12 cell lines were plated on gelatin-coated glass chamber slides and allowed to grow for 48 h. The cells were then incubated with or without NGF for 1 h, gently washed with PBS, and fixed with 4% PFA in PBS for 10 min. Cells were washed three times with PBS, permeabilized in 0.5% Triton X-100 in PBS for 10 min, rinsed with PBS, and then blocked with 5% FCS in PBS for 1 h. The cells were incubated with anti-NAB2 mAb 1C4 (Kirsch et al., 1996) at 1:200 dilution in 2% FCS, 0.2% Triton X-100 in PBS at 4°C for 16 h. After three washes of 5 min each in PBS, cells were incubated in goat anti–mouse Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA) at 1:500 dilution in 2% FCS PBS for 1 h at 25°C. After three washes with PBS for 5 min each, slides were mounted for fluorescence microscopy.

DNA Constructs

The CMV expression vector with a neomycin selectable marker, pCB6 (Brewer, 1994), was used to express human NAB2 (pCMVNAB2) (Svaren et al., 1996), Egr1/NGFI-A (Russo et al., 1993), NAB1 (Swirnoff et al., 1998), Egr4/NGFI-C, and fusions of either native or mutant (I293F) Egr1 R1 domain (amino acids 269–304) with the Gal4 DNA-binding domain (Russo et al., 1993), and the LacZ reporter gene (Svaren et al., 1996). The human $p21^{WAF1}$ (pCMV-p21) construct was purchased from American Type Culture Collection (Rockville, MD).

Recombinant Adenoviral Constructs and Infection

Adenoviral recombinants were prepared essentially as described (Ehrengruber et al., 1998). For AdGFP (green fluorescent protein) NAB2, the

1. *Abbreviations used in this paper*: cdi, cyclin-dependent kinase; CMV, cytomegalovirus; GFP, green fluorescent protein; MAP and MAPK, mitogen-activated protein and MAP kinase; NAB, NGFI-A binding protein; NCD, NAB-conserved domain.

full-length cDNA for human NAB2 (Svaren et al., 1996) was subcloned into the pAC adenoviral transfer plasmid and, by homologous recombination, inserted into the E1 region of adenovirus Ad5PacIGFP as described (Ehrengruber et al., 1998). The virus Ad5PacIGFP was prepared by ligation of an expression cassette, CMV GFP SV40pA, into the PacI site (in the E3 deletion) of AdPacI as described (Ehrengruber et al., 1998). The particular GFP used is a codon optimized cDNA with an attached mitochondrial tag prepared in the laboratory of Dr. B. Wold (Caltech, Pasadena, CA). The control virus, Ad5SJS2GFP, is deleted in E1 and contains the same GFP as a single transgene in the PacI site (Ehrengruber et al., 1998). The functionality of the NAB2 cDNA in the recombinant virus was checked by measuring repression of NGFI-A activity in CHO cells that had been cotransfected with NGFI-A cDNA and a luciferase reporter construct containing two NGFI-A–binding sites (Russo et al., 1993).

PC12 cells grown in six-well plates were infected at a viral titer of 10^9 plaque forming units/ml in DME with 5% FBS for 4 h. Thereafter, cells were washed three times with the same medium and then cultured in normal PC12 medium. 2 d after infection, cells were treated with NGF for 5 d, then examined and photographed using fluorescence microscopy.

Protein Extraction and Immunoblot Analyses

PC12 cells were dislodged by gentle shaking in PBS and pelleted by centrifugation at 4°C at 1,000 rpm for 5 min. After two washes with ice-cold PBS, cell pellets were resuspended in lysis buffer (50 mM Hepes, pH 7.9, 150 mM KCl, 10 mM $MgCl_2$, 0.1% NP-40, with protease inhibitors leupeptin, pepstatin A, PMSF, and aprotinin). Cell extracts were sonicated and debris was removed by centrifugation at 15,000 rpm at 4°C for 15 min. Total protein was loaded onto 10% or 15% SDS–polyacrylamide gels (Laemmli, 1970) and transferred to nitrocellulose. The filters were immunoblotted as described (Svaren et al., 1996). In some experiments, blots were stripped by incubating in 1.0% Tween-20, 0.1% SDS, and 0.2 M glycine, pH 2.2, for 1 h at 25°C, and then re-probed with other antibodies to ensure equal loading of samples.

Antibodies

Phospho-specific p44/42 MAPK (Tyr204) and p44/42 MAPK antibody (New England Biolabs Inc., Beverly, MA) were used at a 1:1,000 dilution to probe immunoblots. Antibodies against $p21^{WAF1}$, cyclin D1, and $p27^{Kip1}$ (Santa Cruz Biotechnology, Santa Cruz, CA) were used at 1 μg/ml. The NAB2 mAb 1C4 (Kirsch et al., 1996) was a gift from J. Johnson (University of Munich, Munich, Germany).

RNA Analysis

PC12 cells were treated with or without NGF for the indicated length of time. RNA was isolated using Trizol reagent (Molecular Research Center Inc., Cincinnati, OH) according to the manufacturer's instructions. RNA blot analysis was performed using 10 μg total RNA per lane as previously described (Svaren et al., 1996). The Egr1 probe was a 1,400-bp NcoI–MscI fragment; the TGF-β1 probe was the full-length rat TGF-β1 cDNA (985 bp); the MMP-3 (stromelysin/transin) probe was a BamHI–SalI fragment (nucleotides 1,408–1,703) from the rat gene.

Results

Overexpression of NAB2 in PC12 Cells

The expression of the Egr1 transactivator is rapidly, but transiently, induced as PC12 cells are stimulated to differentiate in response to NGF. The corepressor NAB2, which inhibits Egr1 activity, is also transiently induced by NGF in these cells, although induction of NAB2 is slightly delayed relative to that of Egr1 (Svaren et al., 1996). To investigate the consequences of altering Egr1 activity on PC12 cell physiology, we attempted to generate PC12 clones that overexpress Egr1 but were unable to do so, presumably because Egr1 represses growth either by promoting apoptosis or by direct inhibition of proliferation (Muthukkumar et al., 1995; Liu et al., 1996). However, we were successful in generating PC12 cell lines that constitutively overexpress its corepressor, NAB2. Immunoblot analysis with a NAB2 mAb (Kirsch et al., 1996) showed that in wild-type PC12 cells, the level of NAB2 is dramatically increased within 1 h after NGF treatment. In contrast, PC12 cell lines stably transfected with the CMV-NAB2 expression vector (N2-8, N2-61, N2-63, N2-64) express high levels of NAB2 in the absence of NGF (Fig. 1 *A*). NAB2 migrates as a doublet, reflecting different levels of phosphorylation (Sevetson, B., and J. Milbrandt, unpublished data). Immunocytochemical analysis with the anti-NAB2 mAb revealed that NAB2-overexpressing cell lines exhibit intense nuclear staining without NGF stimulation. There is also a marked increase in nuclear staining 1 h after NGF addition, reflecting induced expression of the endogenous NAB2 gene (Fig. 1 *B*).

NAB2 Overexpression Blocks NGF-induced Differentiation of PC12 Cells

In the absence of NGF, the NAB2-overexpressing line contains a higher percentage of cells that develop small ex-

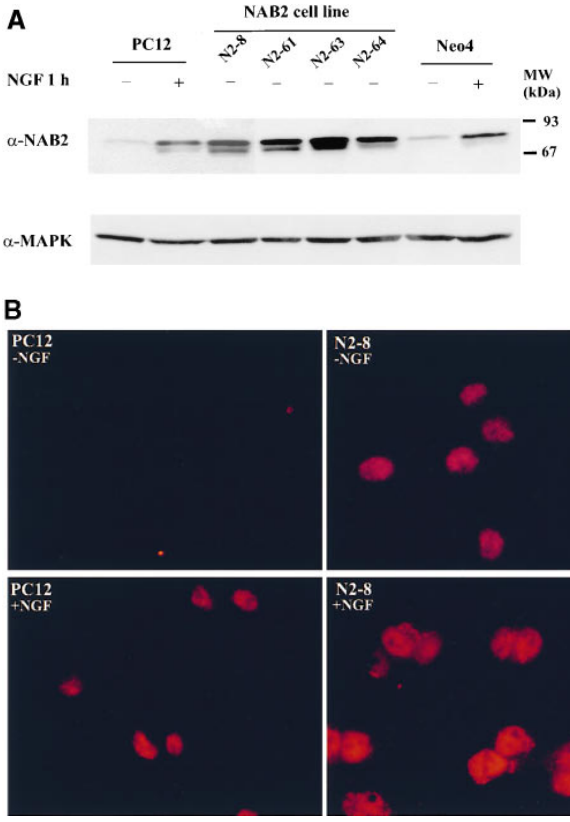


Figure 1. Overexpression of NAB2 in PC12 cell stable transfectants. (*A*) Total protein was isolated from wild-type PC12 cells, cell lines stably transfected with NAB2 (N2-8, N2-61, N2-63, N2-64), and a cell line transfected with non-recombinant vector (*Neo4*) grown in the presence or absence of NGF for 1 h. Protein lysates were electrophoresed on an SDS–polyacrylamide gel, blotted onto nitrocellulose, and then probed with an mAb directed against NAB2. To ensure equal loading of samples, the blot was stripped and re-probed with a p44/42 MAPK antibody. (*B*) Wild-type PC12 cells and a NAB2 stably transfected cell line, N2-8, were grown on chamber slides. The cells were either treated with NGF for 1 h or left untreated. Immunocytochemical analysis was performed using an mAb directed against NAB2.

tensions from the cell surface, as compared with wild-type PC12 cells. However, the most dramatic difference is observed after addition of NGF. After growing in the presence of NGF for several days, wild-type PC12 cells undergo a series of dramatic phenotypic changes and acquire properties characteristic of sympathetic neurons (Allen and Heinrich, 1987). The cell bodies of differentiated PC12 cells enlarge and become flatter, and each cell generally extends several long neurites. In contrast, clonal PC12 lines overexpressing NAB2 fail to differentiate after exposure to NGF for 6 d (Fig. 2). NGF treatment of cells overexpressing NAB2 causes them to enlarge slightly and develop only very short, stubby projections from the cell surface. The average number of neurites per NAB2-overexpressing cell is 3.1 ± 1.1 (as compared with 3.5 ± 1.2 for wild-type PC12 cells), and the average length of the neurite is 38 ± 13 μm (compared with 223 ± 177 μm for wild-type PC12 cells).

To determine whether NAB2 overexpression affects proliferation of PC12 cells, equivalent numbers of cells were plated and cell counts were performed at various times for ≤10 d in the presence of NGF. NAB2-overexpressing cell lines divide at a rate that is similar to wild-type PC12 cells in the absence of NGF (data not shown). However, whereas wild-type PC12 cells cease proliferation 3 to 4 d after exposure to NGF, the growth rate of NAB2-overexpressing cells was not altered by NGF (Fig. 3). These results suggest that NAB2 overexpression prevents cell cycle arrest, a prerequisite for NGF-induced differentiation (Mark and Storm, 1997).

To confirm that the results of NAB2 overexpression in stable cell lines was not due to the clonal selection process, we examined the ability of NAB2 to block NGF-induced PC12 differentiation in transient transfection experiments. PC12 cells were cotransfected with NAB2 and a CMV-driven lacZ reporter gene (to identify transfected cells). 2 d after transfection, cells were treated with NGF for 3 d. Approximately 70% of PC12 cells cotransfected with lacZ and NAB2 failed to develop neurites after 3 d of NGF treatment. In contrast, only 11% of cells cotransfected with lacZ and the non-recombinant control vector failed to develop neurites (Fig. 4, *A* and *B*).

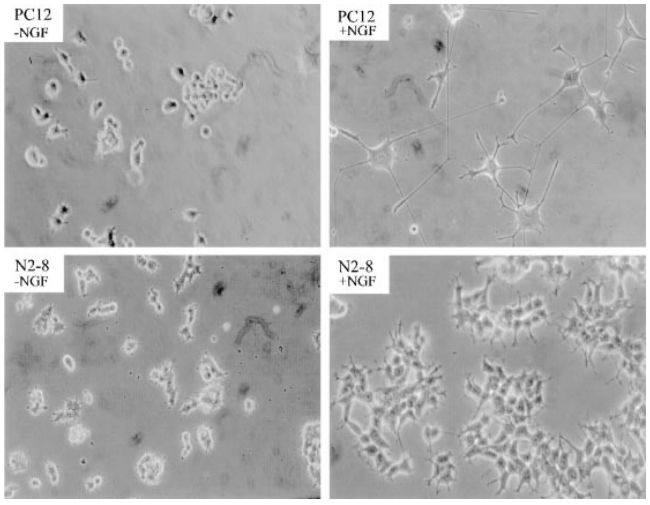


Figure 2. NAB2 overexpression blocks NGF-mediated differentiation of PC12 cells. Wild-type PC12 cells and PC12 cells overexpressing NAB2 (*N2-8*) were grown in the absence or presence of NGF for 6 d. The morphology of the cells was examined using phase-contrast microscopy. Note the contrast between the long, slender neurites emanating from wild-type cells and the short, stubby projections emanating from NAB2-overexpressing cells.

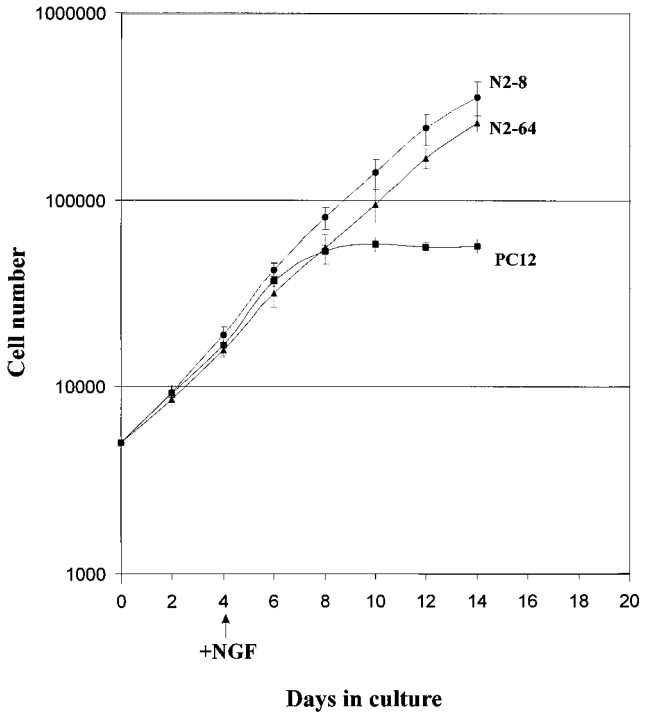
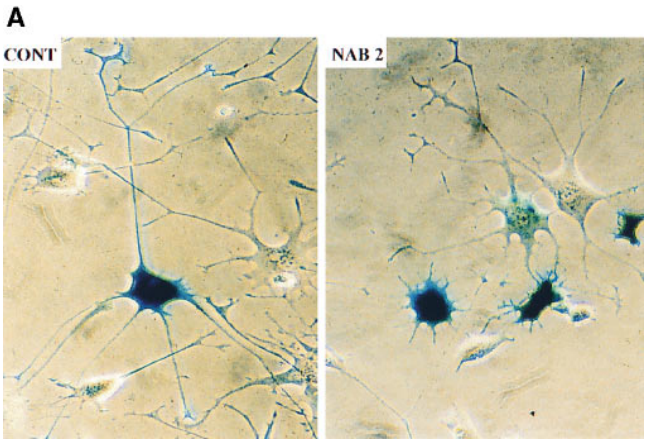
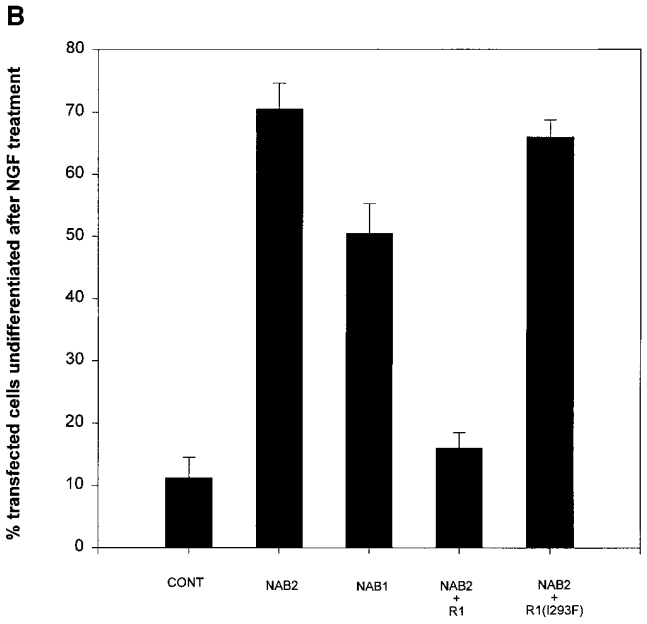


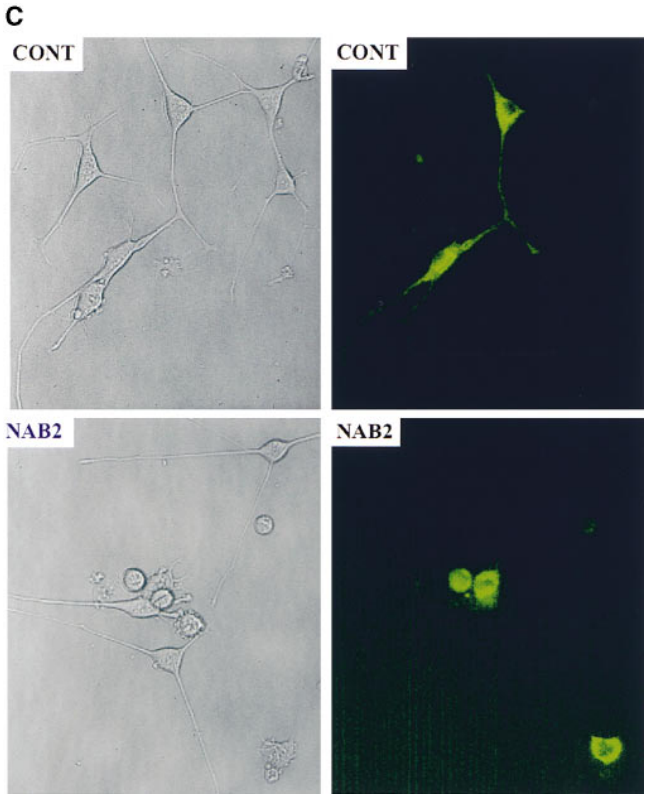
Figure 3. NAB2-overexpressing cells do not undergo growth arrest in response to NGF. Wild-type PC12 cells and the NAB2 stably transfected cell lines, N2-8 and N2-64, were plated at 5,000 cells/well in a 24-well plate. NGF was added to each culture at day 4. Medium was changed every other day and cell counts were performed on four wells from each cell line at the indicated time points. Error bars indicate the standard deviation.

Although NAB1 is not normally induced by NGF in PC12 cells, it is highly homologous to NAB2 and behaves very similarly to NAB2 in transcriptional repression assays (Russo et al., 1995; Swirnoff et al., 1998). Therefore, we also tested whether NAB1 would have the same effect as NAB2 on PC12 cell differentiation in transient cotransfection experiments. In these experiments, 50% of lacZ-positive cells did not develop neurites after treatment with NGF for 3 d. NAB1 appears to be slightly less effective than NAB2 in inhibiting PC12 cell differentiation, which may reflect an intrinsic difference between them or may be due to the lower level of NAB1 expression attained with this expression construct (Sevetson, B., and J. Milbrandt, unpublished data).

In our examination of NAB2 inhibition on NGF-mediated differentiation of PC12 cells, we also used NAB2 recombinant adenoviruses to overexpress NAB2. PC12 cells were infected with adenovirus AdGFP NAB2, which carries two independent expression cassettes for NAB2 and a GFP reporter gene. Infected cells were identified based on their GFP fluorescence and analysis of these infected cultures showed that most fluorescent (infected) cells did not differentiate after 5 d of exposure to NGF (Fig. 4 *C*). By







contrast, non-fluorescent (uninfected) cells in the same culture exhibited very long neurites indicative of the differentiated state. In addition, PC12 cells infected with the control adenovirus (containing only the GFP reporter gene) clearly differentiated after NGF treatment. These results demonstrate that adenoviral infection itself does not block PC12 cell differentiation and further support our finding that NAB2 overexpression inhibits NGF-mediated PC12 cell differentiation.

Overexpression of NAB2 in PC12 Cells Does Not Affect NGF Signal Transduction

Some of the earliest responses of PC12 cells to NGF include dimerization and autophosphorylation of the Trk A receptor and sustained activation of Ras-dependent MAPK (Szeberenyi, 1996). To determine whether the signal transduction pathway itself might be blocked by NAB2 overexpression, we first examined MAPK activation in NAB2-overexpressing cell lines after exposure to NGF. A Western blot with a phospho-specific MAP kinase antibody showed that the sustained activation of MAPK normally observed in NGF-treated PC12 cells is unaltered in the NAB2-overexpressing lines (Fig. 5 *A*). We also examined the NGF activation of the immediate-early gene Egr1 in these cells and found that Egr1 mRNA levels were elevated with the same time course in wild-type PC12 cells and those overexpressing NAB2 (Fig. 5 *B*). Together these data indicate that NAB2 interferes with NGF-induced PC12 cell differentiation at a step downstream of signal transduction and immediate-early gene induction.

Overexpression of NAB2 Blocks NGF-mediated Induction of TGF-β1 and MMP-3

In contrast to the immediate-early gene Egr1, there are a number of genes whose induction by NGF is dependent on de novo protein synthesis. NGF activates these genes, which include TGF-β1 and MMP-3 (stromelysin, transin), with a more extended time course. Furthermore, TGF-β1 has been shown to be regulated by Egr1 (Kim et al., 1994;

Figure 4. NAB2 inhibition of differentiation is prevented by the Egr1 R1 domain. (*A*) PC12 cells were cotransfected with a lacZ reporter plasmid at a 1:20 ratio with either control vector or pCMVNAB2. 2 d after transfection, cells were exposed to NGF for 3 d, and stained for lacZ gene expression. Photographs are representative fields of lacZ-positive cells transfected with either non-recombinant (*CONT*) vector or pCMVNAB2 as indicated. (*B*) PC12 cells were cotransfected with a lacZ reporter plasmid and either NAB2, NAB1, NAB2 + Egr1 R1 domain, NAB2 + mutant Egr1 R1 (I293F) or non-recombinant (*CONT*) vector as indicated. The histogram shows the percentage of lacZ-positive cells that remain undifferentiated after NGF treatment for 3 d beginning 2 d after transfection. For each condition, >100 lacZ-positive cells were counted. Cells with neurites longer than two cell body diameters were scored as morphologically differentiated. The data is derived from four independent transfections. Error bars indicate the standard deviation. (*C*) PC12 cells were infected with recombinant adenovirus AdGFP (*CONT*, *top*) or AdGFP NAB2 (*bottom*). NGF was added 2 d after infection and cells were maintained for an additional 5 d. The cells were visualized by phase-contrast (*left*) or fluorescence (*right*) microscopy. The green fluorescent (GFP-positive) cells are those that have been infected with adenovirus.

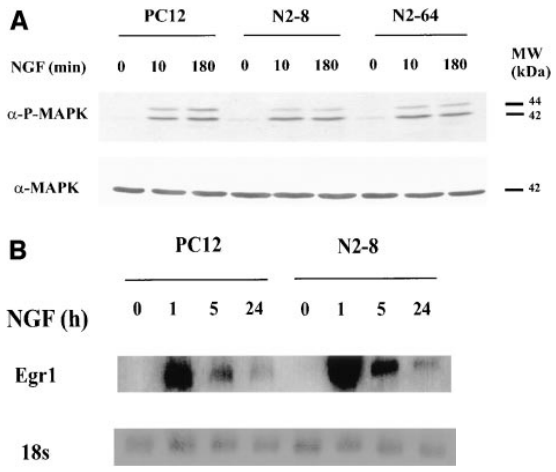


Figure 5. NGF-mediated MAP kinase activation and immediate-early gene induction are intact in NAB2-overexpressing PC12 cells. (*A*) Total protein was collected from wild-type PC12 cells and two NAB2 stably transfected cell lines, N2-8 and N2-64, which had been treated with NGF for the indicated times. The samples were blotted with an antibody against phosphorylated MAP kinase (*α-P-MAPK*). The blots were stripped and re-probed with anti–MAP kinase antibody (*α-MAPK*) to show that equal amounts of protein were loaded in each lane. (*B*) RNA was isolated from wild-type PC12 cells and a NAB2 stably transfected cell line (*N2-8*) after stimulation with NGF for the indicated length of time. RNA blot analysis was performed using 10-μg samples of total RNA per lane and a ^{32}P-labeled Egr1 probe. The 18S ribosomal RNA band visualized with ethidium bromide is shown below to demonstrate equal loading.

Liu et al., 1996), and MMP-3 has multiple Egr1 consensus binding elements in its promoter region (Yee et al., 1997). To determine whether the expression of these genes was altered by NAB2 overexpression, we stimulated wild-type and NAB2-overexpressing PC12 cells with NGF and compared the induction of these genes by RNA blot analysis (Fig. 6). In accord with previous reports, both of these genes were induced in wild-type PC12 cells, but their induction was abolished in NAB2-overexpressing cells. These results suggest that NAB2 overexpression is inhibiting Egr1-mediated gene activation.

Egr1 R1 Domain Prevents NAB2-mediated Inhibition of PC12 Cell Differentiation

To determine if NAB2-mediated inhibition of PC12 differentiation occurred through its interaction with proteins containing an R1 domain (presumably Egr1), we attempted to reverse this inhibition with the isolated R1 domain of Egr1, which acts as a potent competitor of NAB repression (Russo et al., 1995). PC12 cells were transiently cotransfected with NAB2 as well as the R1 domain of Egr1 fused to the Gal4 DBD (which provides a nuclear localization signal). The R1 domain of Egr1 dramatically reverses the inhibitory effect of NAB2 on NGF-induced PC12 cell differentiation (Fig. 4 *B*). Only 15% of cells that are cotransfected with the R1 domain and NAB2 fail to differentiate after NGF treatment, a percentage that is very similar to that observed with either control vector or R1 domain alone (11% or 10.5%, respectively). In contrast, transfection with a construct expressing a mutant R1 domain (I293F) that prevents interaction with NAB proteins (Russo et al., 1995; Svaren et al., 1996) failed to reverse the inhibitory effects of NAB2 on PC12 differentiation (68%). This result suggests that NAB2 overexpression blocks PC12 cell differentiation because of its interaction with R1 domain bearing proteins such as Egr1, which subsequently influence gene expression and regulate cellular processes.

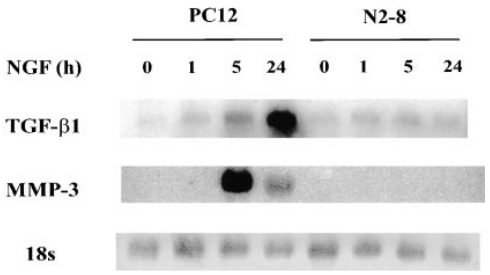


Figure 6. Overexpression of NAB2 blocks the induction of NGF-responsive late genes TGF-β1 and MMP-3. RNA was isolated from wild-type PC12 cells and a NAB2 stably transfected cell line (N2-8) after stimulation with NGF for the indicated length of time. RNA blot analysis was performed using 10-μg samples of total RNA per lane and a ^{32}P-labeled TGF-β1 or MMP-3 probe as indicated. The 18S ribosomal RNA band visualized with ethidium bromide is shown below to demonstrate equal loading.

NAB2 Overexpression Decreases Level of p21^{WAF1} Expression

Because NAB2 overexpression in PC12 cells appears to prevent them from undergoing growth arrest in response to NGF (Fig. 3), we examined the expression profiles of several proteins known to play important roles in cell cycle arrest and subsequent differentiation of NGF-treated PC12 cells. One such molecule is the cyclin-dependent kinase inhibitor, p21^{WAF1}. Levels of p21^{WAF1} are increased within 24 h after NGF treatment and continue to increase for up to 1 wk (Yan and Ziff, 1995), (data not shown). This induction of p21^{WAF1} correlates with an increase in the relative amount of hypophosphorylated (i.e., active) pRb (Yan and Ziff, 1995).

Immunoblot analyses of p21^{WAF1} levels before and after NGF treatment in wild-type PC12 cells clearly showed an increase after 48 h. However, in NAB2-overexpressing cell lines, p21^{WAF1} levels were dramatically downregulated (Fig. 7). Not only the induced level of p21^{WAF1} expression, but also the basal level (in the absence of NGF) was strongly decreased in NAB2-overexpressing cell lines. This effect on p21^{WAF1} levels was specific, as the levels of another cdi, p27^{Kip1}, and cyclin D1 were unaffected.

Expression of p21^{WAF1} Restores the Ability to Differentiate to NAB2-overexpressing Cells

To address the functional significance of p21^{WAF1} downregulation, PC12 cells were transiently transfected with NAB2 in the absence or presence of p21^{WAF1}. As previously observed, transfection of NAB2 alone resulted in dramatic inhibition of NGF-mediated differentiation (71% undifferentiated cells), however cotransfection of a p21^{WAF1} expression construct along with NAB2 largely restored the ability to differentiate in response to NGF (24% undifferentiated cells) (Fig. 8). In comparison, cotransfec-

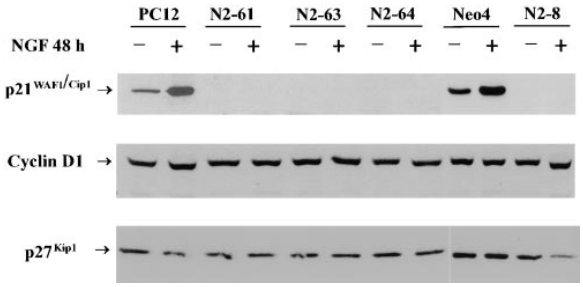


Figure 7. NAB2 overexpression results in decreased levels of $p21^{WAF1}$ protein. Wild-type PC12 cells, NAB2-overexpressing cell lines (N2-61, N2-63, N2-64, and N2-8), and a cell line transfected with non-recombinant vector (*Neo4*) were incubated in the absence or presence of NGF for 48 h. Protein lysates were electrophoresed on a SDS–polyacrylamide gel, blotted onto nitrocellulose, and then probed with an anti-$p21^{WAF1}$ antibody (*top panel*). The same blot was stripped and re-probed with antibodies against cyclin D1 (*middle panel*) or $p27^{Kip1}$ (*bottom panel*).

tion of Egr4 (which lacks an R1 domain) along with NAB2 was not able to restore the ability to differentiate in response to NGF (70% undifferentiated cells). Transfection with non-recombinant or $p21^{WAF1}$ vector alone resulted in a minimal number of undifferentiated cells (10% and 9.8%, respectively). It has been shown that $p21^{WAF1}$ induction by itself does not lead to PC12 cell differentiation in the absence of NGF (Yan and Ziff, 1995). These results clearly suggest that $p21^{WAF1}$ prevents NAB2-mediated inhibition of PC12 cell differentiation, consistent with previous work indicating that NGF-induced expression of $p21^{WAF1}$ is critical for exit from the cell cycle and subsequent differentiation of PC12 cells (Yan and Ziff, 1995; Billon, 1996; Erhardt and Pittman, 1998).

Discussion

To investigate the physiological role of NAB2 repression on Egr1 transcriptional activity, we developed PC12 cell lines that stably and constitutively overexpress NAB2. In contrast to wild-type PC12 cells, NAB2-overexpressing PC12 cells neither arrest their growth nor extend long neurites after NGF treatment. Analysis of multiple clonal lines indicates that NAB2-mediated inhibition of differentiation correlates with the level of NAB2 expression in each clone. The NAB2-mediated inhibition of NGF-induced differentiation was independently confirmed by transient transfection experiments and by infection with NAB2 recombinant adenoviruses.

Transfection of the isolated Egr1 R1 domain has previously been shown to reverse the repressive effects of NAB proteins in a dose-dependent manner (Russo et al., 1995). Indeed, this was one of the original observations that suggested the existence of a corepressor of Egr1 (Russo et al., 1993). NAB2 could not block differentiation of PC12 cells in the presence of this R1 domain competitor, suggesting that the ability of NAB2 to inhibit differentiation is dependent upon its interaction with Egr1 and/or other proteins containing a domain similar to the R1 domain. Furthermore, this result also demonstrates that effects of NAB2 on PC12 differentiation are specific, as they require an interaction through the NAB NCD1 domain.

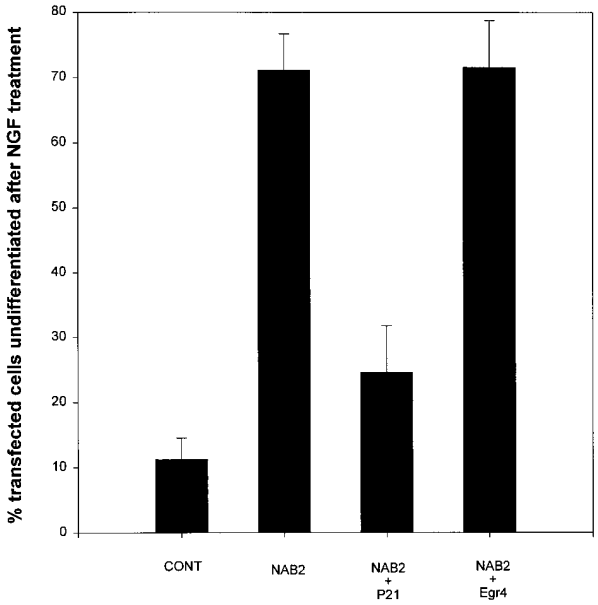


Figure 8. $p21^{WAF1}$ prevents the inhibition of differentiation mediated by NAB2 overexpression. PC12 cells were cotransfected with a lacZ reporter plasmid and either NAB2, NAB2 + $p21^{WAF1}$, NAB2 + Egr4, or non-recombinant vector, as indicated. The histogram shows percentages of lacZ-positive cells that remain undifferentiated after treatment with NGF for 3 d beginning 2 d after transfection. For each condition, >100 lacZ-positive cells were counted. Cells with neurites longer than two cell body diameters were scored as morphologically differentiated. The data is derived from four independent transfections. Error bars indicate the standard deviation.

Sustained MAPK activation is clearly required for NGF-induced differentiation of PC12 cells (Cowley et al., 1994). Our analysis of various steps in the signal transduction cascade revealed that MAPK activation was not affected by NAB2 overexpression. Similarly, the induction of immediate-early genes, exemplified by Egr1, was also unaffected. In contrast, the induction of delayed response genes such as TGF-β1 and MMP-3 was clearly inhibited. This inhibition may be important for the lack of neurite outgrowth in these cells. Although these cells do not extend normal neurites, short, stubby projections can be observed on these cells after NGF treatment. The character of these projections may be secondary to the loss of MMP-3, as this extracellular matrix metalloprotease has been shown to be involved in axonal growth in vivo, including a role in growth cone invasiveness (Nordstrom et al., 1995). The function of TGF-β1 in PC12 cell differentiation is unclear, but it is a bona fide Egr1 target gene (Kim et al., 1994; Liu et al., 1996) that is clearly induced in response to NGF, and is, of course, a potent influence on cell physiology. These results, indicating that the effects of NAB2 are exerted after immediate-early gene induction, are consistent with its normal pattern of expression (induced after Egr1) and its role as a corepressor of Egr1.

The inability of NAB2-overexpressing cells to arrest growth suggested a possible cell cycle regulatory defect. Indeed, examination of the levels of cdk inhibitors revealed that $p21^{WAF1}$ levels are significantly lower in cells that stably overexpress NAB2. This effect was specific as

expression levels of the related cdi, $p27^{Kip1}$, and of cyclin D1 were unaffected by NAB2 overexpression. The precise mechanism through which NAB2 overexpression leads to lowered levels of $p21^{WAF1}$ remains to be elucidated; however, this downregulation is likely to result directly in an inability of these cells to differentiate, as induction of $p21^{WAF1}$ has been shown to play an important role in NGF-mediated differentiation of PC12 cells (Yan and Ziff, 1995; Billon, 1996; Erhardt and Pittman, 1998). This conclusion is supported by experiments demonstrating that coexpression of $p21^{WAF1}$ prevents the inhibition of differentiation mediated by NAB2 overexpression. It will be interesting to determine whether NAB2 overexpression in other cell types leads to decreased $p21^{WAF1}$ levels and subsequent alterations in cell growth.

In summary, we have found that overexpression of NAB2, a corepressor of the NGF-induced immediate-early protein Egr1, blocks NGF-mediated differentiation of PC12 cells. Although it has been almost a decade since the identification of Egr1 as an immediate early gene in NGF-mediated PC12 cell differentiation (Milbrandt, 1987), our data provides some of the first evidence that Egr1 activity is required for PC12 cell differentiation. Furthermore, our data suggest that NAB2 regulates Egr activity in such a way as to provide the correct amplitude and/or temporal regulation of Egr target gene expression. Such a role for NAB2 is consistent with its slightly delayed induction by NGF treatment relative to that of Egr1 (Svaren et al., 1996). The very existence of such a "brake" on Egr activity further highlights the importance of Egr activity in regulating differentiation pathways. Because altering the Egr1/NAB2 ratio has profound effects in this model of neuronal differentiation, it seems likely that these factors may also influence differentiation and growth in other systems as well.

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