Journal of Cellular Biochemistry 98:185–193 (2006)

Ndrg4 Enhances NGF-Induced ERK Activation Uncoupled With Elk-1 Activation

Shigeki Hongo,* Takuya Watanabe, Keiko Takahashi, and Akira Miyazaki

Department of Biochemistry, Showa University School of Medicine, Shinagawa-ku, Tokyo 142-8555, Japan

Abstract *Ndrg4* is expressed predominantly in the early postnatal rat brain and may be related to neural cell differentiation. PC12 cell lines stably expressing increased levels of Ndrg4 protein display enhanced NGF-induced phosphorylation of MEK and ERK. In contrast, the Ndrg4-C2-overexpressed PC12 cell lines showed attenuated NGF-promoted phosphorylation of Elk-1, which is a nuclear target of ERK. A reporter assay also indicated that Ndrg4-C2 suppresses Elk-1-mediated transcriptional activation and SRE reporter expression. The suppressive effect of Ndrg4-C2 on NGF-induced activation of Elk-1 was abolished by colchicine but not by cytochalasin D, suggesting that microtubules are involved in the reduced activation of Elk-1 by Ndrg4. Ndrg4 may play a role in supporting the activation of ERK and its target proteins needed for neuronal differentiation and in reducing the activation of Elk-1 implicated in cell growth. J. Cell. Biochem. 98: 185–193, 2006. © 2006 Wiley-Liss, Inc.

Key words: Ndrg4; ERK signaling pathway; Elk-1; serum response element; microtubules; NGF; PC12 cells

The central nervous system is formed by neural precursor cells after many developmental steps. A number of genes are considered to be expressed during restricted periods to advance the development at the corresponding stages. We identified a novel gene, *Ndrg4*, which is expressed predominantly in the early postnatal rat brain and was originally named *Bdm1* [Yamauchi et al., 1999]. The *Ndrg4* gene encodes six isoforms with molecular masses of 37–43 kDa [Maeda et al., 2004]. Ndrg4 mRNA expression was shown to increase with the differentiation of mouse embryonal carcinoma P19 cells and rat pheochromocytoma PC12 cells into neuron-like cells by retinoic acid and nerve growth factor (NGF), respectively [Yamauchi et al., 1999].

Human *NDRG4* is a member of the *NDRG* gene family that includes *NDRG1–3*. *NDRG1* expression is upregulated during differentiation of some carcinoma cell lines [van Belzen et al., 1997] and downregulated during cell transformation [van Belzen et al., 1997; Kurdistani et al., 1998; Guan et al., 2000]. NDRG1 is also upregulated in response to some cellular stresses caused by various chemicals [Kokame et al., 1996; Zhou et al., 1998]. Moreover, *NDRG1* was identified as a target of p53 [Kurdistani et al., 1998] and as a metastatic suppressor gene [Guan et al., 2000]. *NDRG2*, which is most closely related to *NDRG4*, as well as *NDRG1*, were demonstrated to reduce cell growth [Kurdistani et al., 1998; Guan et al., 2000; Deng et al., 2003]. NDRG1–3 are expressed ubiquitously in the organism [Zhou et al., 2001], whereas NDRG4 is present only in the brain and heart [Yamauchi et al., 1999; Zhou et al., 2001]. Recently, NDRG1/Rit42 protein was shown to associate with the microtubules in centrosomes and to participate in the spindle checkpoint [Kim et al., 2004]. However, the cellular functions of the NDRG family proteins, especially Ndrg4 protein, remain obscure.

Previously, we reported that Ndrg4 protein can modulate activating protein-1 (AP-1) activity and may regulate neurite outgrowth in NGF-treated PC12 cells [Ohki et al., 2002]. AP-1 is a group of transcription factors consisting of the Fos and Jun families [Angel and Karin, 1991]. Three types of enzymes, extracellular

Grant sponsor: Japan Society for the Promotion of Science; Grant number: 16590895.

*Correspondence to: Dr. Shigeki Hongo, Department of Biochemistry, Showa University School of Medicine, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan.
E-mail: shongo@med.showa-u.ac.jp

Received 26 August 2005; Accepted 10 November 2005

DOI 10.1002/jcb.20763

signal-regulated kinase (ERK), Fos regulating kinase (FRK), and Jun N-terminal kinase (JNK), can affect AP-1 activity by transcriptional and post-translational regulations [Karin, 1995]. The Ras–ERK pathway functions in cell proliferation and differentiation [Seger and Krebs, 1995] and is stimulated in response to NGF in PC12 cells [Segal and Greenberg, 1996]. The human *smap8* gene product, which corresponds to rat Ndrg4-B2, was suggested to play a role in PDGF-induced mitogenesis of a rat aortic smooth muscle cell line by amplification of the ERK signal [Nishimoto et al., 2003]. ERK1 and ERK2 (ERK1/2) translocate to the nucleus to phosphorylate and transcriptionally activate a subfamily of ETS domain-containing transcription factors known as ternary complex factors (TCFs) [Sheng et al., 1988; Brunet et al., 1999]. Elk-1, which is a member of the TCFs, associates with serum response factor (SRF) dimers to form a ternary transcription complex and binds to the serum response element (SRE) within the promoter of immediate early genes, such as the c-*fos* gene [Wasylyk et al., 1998]. However, c-Fos protein is reported to block neuronal differentiation of PC12 cells [Braselmann et al., 1992].

To study the molecular mechanism of the support of neuronal differentiation by Ndrg4, we examined phosphorylation of components of the ERK signaling cascade in PC12 cells stably transfected with Ndrg4-C2, and further determined the effects of Ndrg4 overexpression on activation of Elk-1. We found that Ndrg4 enhanced NGF-induced ERK activation, but rather attenuated Elk-1 activation uncoupled from ERK signaling in PC12 cells.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), Opti-MEM, fetal bovine serum, horse serum, Lipofectamine^{TM} 2000, and pcDNA3.1/myc-His vector were obtained from Invitrogen (Carlsbad, CA). NGF was purchased from Upstate (Lake Placid, NY). Antibodies to phospho-Raf (Ser338), phospho-MEK1/MEK2 (MEK1/2) (Ser217/221), ERK1/ERK2 (ERK1/2), phospho-ERK1/2 (Thr202/Tyr204), phospho-90 kDa ribosomal S6 kinase (p-p90RSK, Ser380), phospho-Elk-1 (Ser383), phospho-p38 MAP kinase (Thr180/Tyr182), and anti-rabbit IgG antibody conjugated to horseradish peroxidase were obtained from Cell Signaling Technology (Beverly, MA). Anti-mouse IgG antibody conjugated to horseradish peroxidase and Lumi-LightPLUS Western Blotting Substrate were obtained from Roche Diagnostics (Mannheim, Germany). Anti-HA monoclonal antibody (F-7) was from Santa Cruz Biotechnology (Santa Cruz, CA). The pSRE and pAP1 luciferase (Luc) reporter plasmids containing respective promoter element fused to the herpes simplex virus-thymidine kinase TATA-like promoter were obtained from BD Biosciences Clontech (Palo Alto, CA). PathDetect Elk1 trans-Reporting System was from Stratagene (La Jolla, CA). Dual-Luciferase Reporter Assay System and *Renilla* luciferase reporter vector (phRL-TK) were purchased from Promega (Madison, WI). All other chemicals were of the highest grade available.

Cell Culture

PC12 cells (RIKEN RCB0009) were cultured in DMEM supplemented with 5% fetal bovine serum (FBS) and 5% horse serum (HS) in a humidified atmosphere of air with 5% CO_2. Cells were seeded onto 3.5-cm dishes (6×10^5 cells/dish) or 6-cm dishes (1.2×10^6 cells/dish) for Western blotting analyses. After overnight culture, cells were induced to differentiate by addition of 50 ng/ml of mouse 2.5S NGF.

Construction of Expression Vectors for Ndrg4 Protein Isoforms

Expression vectors for production of hemagglutinin (HA)-tagged fusion Ndrg4 protein isoforms were generated. The plasmid pcDNA3.1/myc-His was digested with *Hind*III and *Pme*I and blunt-ended using Blunting high (Toyobo, Osaka, Japan). An oligonucleotide (gcggccgcc accatgTACCCATACGATGTTCCAGATTACG-CTgctagctatctcgag) containing the Kozak consensus sequence, HA-epitope sequence (upper case), and *Nhe*I and *Xho*I sites, was annealed with its complement and inserted into the blunt-ended vector to generate the HA expression vector, pcDNA–HA. Complementary DNAs for Ndrg4-A1, Ndrg4-A2, Ndrg4-C1, and Ndrg4-C2 were produced by reverse transcription-polymerase chain reaction (RT-PCR) [forward primer for Ndrg4-A1 and Ndrg4-A2: GACGCACTAGTCCGGAGTGCTGGGATGGG-GAACATGAC; forward primer for Ndrg4-C1 and Ndrg4-C2: GACGCACTAGTAAGGTGC-TGGGACACAGGCTCCAACTG; reverse primer:

GAACGCGTCGACGAGGACTTCAGCAGGAC-ACCTCCATG] and subcloned into the pGEM-T-Easy vector. The cloned cDNAs were ascertained to be the respective Ndrg4 isoform cDNAs by DNA sequencing. These cDNAs were cut out with *Sal*I and *Spe*I and then inserted into *Nhe*I- and *Xho*I-treated pcDNA–HA to construct expression vectors for production of HA-Ndrg4 fusion proteins (pcDNA–HA–Ndrg4-A1, -A2, -C1, -C2). The nucleotide sequences around the junction of ligation were determined again by sequencing to confirm in-frame ligation.

Generation of PC12 Cell Clones Stably Transfected With Ndrg4-C2

Transfection of PC12 cells was carried out using Lipofectamine 2000 according to the manufacturer's instructions. Cell lines stably expressing Ndrg4-C2 were obtained after selection for growth in the presence of G418 (800 μg/ml) and characterized for HA–Ndrg4-C2 expression by Western blotting analysis.

Western Blotting Analysis

Cells were washed with Dulbecco's phosphate buffered saline (PBS), and scraped into 62.5 mM Tris-HCl buffer (pH 6.8) containing 1% sodium dodecyl sulfate (SDS), 1 mM sodium orthovanadate, 10 mM NaF, and Complete^{TM} protease inhibitor cocktail. The scraped lysates were homogenized by passing through 23-gauge needles to shear the DNA, followed by determination of protein using the BCA protein assay reagent (Pierce, Rockford, IL). Before loading, SDS, 2-mercaptoethanol, and bromophenol blue were added to the lysates to concentrations of 2%, 5%, and 0.005%, respectively, and heated at 95°C for 5 min. Equal amounts of total cellular protein per sample (15–30 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene fluoride membranes. Proteins were analyzed with specific antibodies and either horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibody. Specific proteins were detected with Lumi-LightPLUS substrate and exposure to X-ray film. The bands on the X-ray film were scanned and their densities were measured using Scion Image.

Promoter *cis*-Reporter Assay

PC12 cells were seeded onto 24-well plates (1.2×10^5 cells/well) and maintained in DMEM supplemented with 5% FBS and 5% HS and without antibiotics for 24 h. PC12 cells were transfected with an expression vector for Ndrg4-C2 protein (pcDNA–HA–Ndrg4-C2, 200 ng) or an empty vector (pcDNA–HA, 200 ng) together with the reporter construct for SRE or AP-1 (200 ng) using Lipofectamine 2000. *Renilla* luciferase reporter vector (phRL-TK, 3 ng) was co-transfected as an internal control. Twenty-four hour after transfection, cells were incubated in medium containing 0.5% FBS and 0.5% HS for a further 20 h followed by stimulation with 50 ng/ ml NGF for 8 h. Cells were lysed and the Dual luciferase assay was performed. Data were normalized by the activity of *Renilla* luciferase reporter vector.

PathDetect Trans-Reporter Assay

The Elk-1-mediated transcriptional activity was assayed using a PathDetect kit. PC12 cells were seeded and maintained as described above. The cells were transfected with 200 ng of pFR-Luc, 10 ng of pFA2-Elk1, and 5 ng of phRL-TK (*Renilla* luciferase control vector), along with the indicated amount of pcDNA–HA–Ndrg4 or empty vector pcDNA–HA. Cells were then treated as above to calculate luciferase activity.

RESULTS

Ndrg4 Enhances NGF Phosphorylation of MAPK/ERK Pathway Components and Represses NGF Phosphorylation of Elk-1 in PC12 cells

The Ras–ERK pathway is known to be the major cellular signaling pathway that induces neuronal differentiation of PC12 cells in response to NGF [Segal and Greenberg, 1996]. To analyze the molecular mechanism underlying the enhanced NGF-induced PC12 cell differentiation by Ndrg4 [Ohki et al., 2002], we examined the phosphorylation of ERK1 and -2 (ERK1/2) and their nuclear target Elk-1 in a stably Ndrg4-C2-transfected PC12 cell line (PC12/Ndrg4-C2). Among the six Ndrg4 protein isoforms [Maeda et al., 2004], we first examined Ndrg4-C2, as Ndrg4-C2 mRNA is expressed at only modest levels in the developing brain [Maeda et al., 2004] and is considered to be appropriate to study the effects of transfected Ndrg4 on the action of endogenous signaling molecules. Figure 1A shows that endogenous ERK1 and ERK2 were phosphorylated more strongly after 15 min, 60 min, and 120 min of NGF treatment in PC12/Ndrg4-C2 cells than in

the parent PC12 cells. Though expression of ERK1/2 was shown to be decreased in PC12 and PC12/Ndrg4-C2 cells after 15 min of NGF treatment, the levels of ERK1/2 in the two cell lines were similar. Figure 1B shows 1.4-fold total phosphorylation of ERK1 and ERK2 in PC12/Ndrg4-C2 cells after 15 min of NGF treatment. In contrast, phosphorylation of Elk-1 was weaker in PC12/Ndrg4-C2 cells than in control PC12 cells at 15 min, 60 min, and 120 min of NGF treatment (Fig. 1A,C). Phosphorylated Elk-1 level showed little increase by NGF in PC12/Ndrg4-C2 cells, although the original level at time 0 in PC12/Ndrg4-C2 cells was higher than that in PC12 cells (Fig. 1C). Thus, PC12/Ndrg4-C2 cells sustained a lower level of phosphorylation of Elk-1 after NGF treatment.

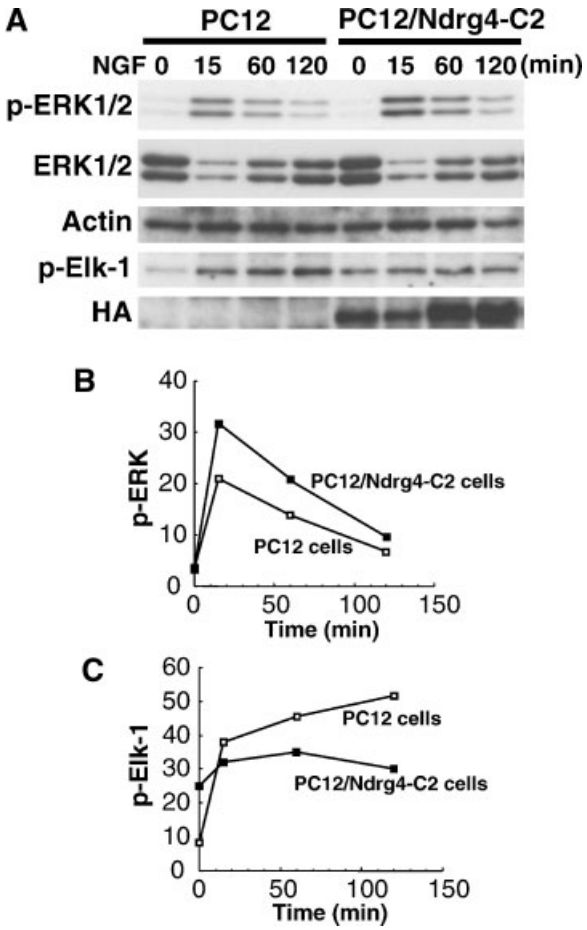

Fig. 1. Time-dependent enhanced phosphorylation of ERK1/2 and suppressed phosphorylation of Elk-1 in stably Ndrg4-C2-transfected PC12 cells. **A**: Parent PC12 cells and a PC12 cell clone stably transfected with Ndrg4-C2 were incubated in medium containing 0.1% fetal bovine serum and 0.1% horse serum for 20 h and treated with NGF for the indicated times. The cell lysates were prepared and used to assay phospho-ERK1/2 and phospho-Elk-1 by Western blotting. The membrane used for the analysis of phosphorylated ERK1/2 was re-probed with anti-ERK1/2 antibody. The same membrane was also re-probed with anti-actin antibody to confirm loading of an equal amount of protein in each lane. **B**, **C**: The densities of bands on the blots were determined using Scion Image and are expressed in arbitrary units.

Previously, we obtained PC12 cell clones expressing various levels of Ndrg4 protein during establishment of antisense RNA-expressing cell clones [Ohki et al., 2002]. To rule out phenotypic variation among subclones, we examined phosphorylation of ERK and Elk-1 in these cell clones after treatment with NGF. Endogenous Ndrg4 protein level was not changed when the cell clones were treated with NGF for 15 min. Before NGF treatment, the levels of ERK phosphorylation were very low and difference in the basal level could not be detected among the cell clones (data not shown). Phosphorylation of ERK1/2 was found to be upregulated in a PC12 cell clone containing a high level of Ndrg4 protein [PC12/AS2-q(H) cells] as compared to control cells (mock and parent PC12 cells) by NGF treatment for 15 min (Fig. 2). Phosphorylation of 90-kDa ribosomal S6 kinase (p90RSK), which is a cytoplasmic substrate for ERK, was also shown to be enhanced in PC12/AS2-q(H) cells. By comparison, phosphorylation of ERK1/2 was weak in cell clones containing low levels of Ndrg4

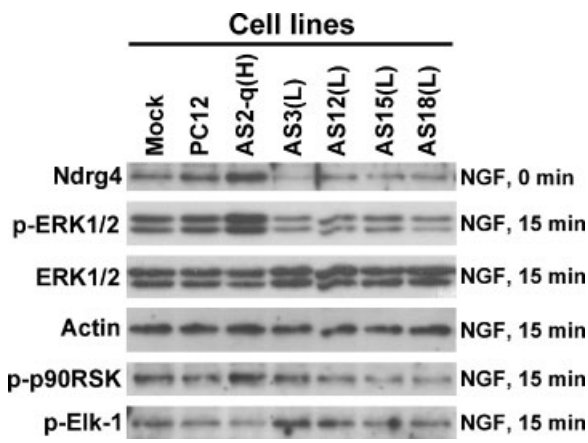

Fig. 2. NGF-induced phosphorylation of ERK pathway components and Elk-1 in PC12 cell subclones. PC12 cell clones stably expressing higher (H) and lower (L) levels of Ndrg4 protein than parent PC12 cells or mock transfected controls were treated with NGF (50 ng/ml) for 15 min and examined for phosphorylation of endogenous ERK1/2, p90RSK, and Elk-1 by Western blotting analyses. The blots were stripped and re-probed with anti-ERK antibody to compare the amounts of ERK loaded. The **top panel** shows expression of Ndrg4 protein before stimulation of the cell clones with NGF.

protein [AS3(L), AS12(L), AS15(L), and AS18(L) cells]. In this way, correlation between a level of cellular Ndrg4 protein and strength of NGF-dependent phosphorylation of ERK1/2 was verified. In contrast, phosphorylation of Elk-1 was confirmed to be repressed in PC12/AS2-q(H) cells (Fig. 2).

Phosphorylation of Other Signaling Molecules After Overexpression of Ndrg4

Next, we examined from what stage the components of the NGF-dependent ERK signal cascade are stimulated by Ndrg4. NGF-induced phosphorylation of MEK1/2 (MAP kinase or ERK kinase 1 and 2), upstream regulators of ERK, was enhanced in stably Ndrg4-C2-transfected PC12 cells during exposure to NGF for 120 min (Fig. 3). However, no enhancement of Raf phosphorylation was observed (Fig. 3). Therefore, it is unlikely that Ndrg4-C2 enhances ERK phosphorylation through Raf-1 activation, which is elicited by other factors, such as phosphatidylinositol 3-kinase [Sun et al., 2000].

The p38 MAP kinase, a subfamily of the MAP kinase superfamily, has been reported to be activated by treatment with NGF in PC12 cells [Morooka and Nishida, 1998] and p38 is known to activate Elk-1 [Wasylyk et al., 1998]. JNK is hardly activated by NGF in PC12 cells [Xing et al., 1998]. The reduction of Elk-1 phosphorylation by overexpressed Ndrg4 may be due to diminished p38 activity. Therefore, phosphorylation of p38 was compared between the parent PC12 cells and PC12/Ndrg4-C2 cells. As shown in Figure 3 (lower panel), no decrease was observed in phosphorylation of p38 in PC12/Ndrg4-C2 cells, suggesting that the attenuation of Elk-1 phosphorylation by Ndrg4-C2 transfection is not due to decrease of p38 activity.

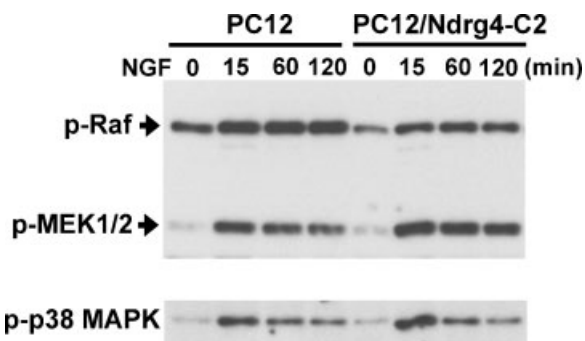

Fig. 3. Time courses of phosphorylation of various signaling molecules in a PC12 cell clone overexpressing Ndrg4 protein. Parent PC12 cells and PC12 cells stably transfected with Ndrg4-C2 were incubated in medium containing 0.1% fetal bovine serum and 0.1% horse serum for 20 h and treated with NGF (50 ng/ml) for the indicated times. The cell lysates were prepared and used to assay phospho-Raf, phospho-MEK1/2, and phospho-p38 by Western blotting. Phospho-Raf and phospho-MEK1/2 were determined on the same blot.

Ndrg4 Suppresses NGF-Induced Transcription From the SRE

We studied NGF-induced transcriptional activity from the SRE in Ndrg4-C2-transfected PC12 cells, as phosphorylated Elk-1 is recruited to the SRF dimer and the SRE to form the ternary transcription complex. Transcriptional activity of AP-1, which can be regulated by ERK [Karin, 1995], was also examined. We used the reporter vector, in which a specific response element is located upstream of the luciferase reporter gene. Figure 4 shows that NGF-induced SRE activation was repressed to 68% by co-expression of Ndrg4-C2 as compared to that obtained with the empty plasmid. Under the same condition, NGF-induced AP-1 reporter expression was enhanced 2.6-fold by co-expression of Ndrg4-C2.

Ndrg4 Suppresses NGF-Induced Activation of Elk-1

To determine whether Ndrg4 protein affects the NGF-induced activation of Elk-1 in PC12 cells, the PathDetect trans-reporting system

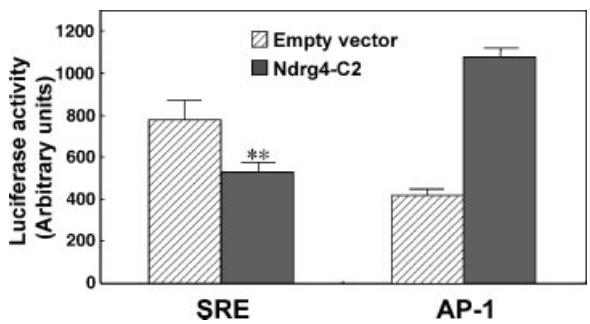

Fig. 4. Ndrg4-C2 suppresses NGF-induced transcription from the SRE. PC12 cells were transfected with an empty vector (200 ng) or an expression vector for Ndrg4-C2 protein (200 ng) together with luciferase vectors under the control of AP-1 and SRE (200 ng). *Renilla* luciferase reporter vector (phRL-TK, 3 ng) was co-transfected as an internal control. Twenty-four hours after transfection, cells were incubated in medium containing 0.5% fetal bovine serum and 0.5% horse serum for a further 20 h followed by stimulation with 50 ng/ml NGF for 8 h. Cells were lysed and the Dual luciferase assay was performed. Data were normalized by the activity of *Renilla* luciferase reporter vector and expressed in arbitrary units. Data are from triplicate assays in two independent experiments and are expressed as mean ± SD. Statistical analyses were based on total observation. $^{**}P < 0.001$ versus empty vector (Paired *t*-test).

was used. In this system, the fusion protein consisting of the activation domain of transcription activator and the yeast GAL4 DNA binding domain is tested. Luciferase expression is activated when the transcription activation domain of the fusion protein is phosphorylated and the fusion protein binds to the promoter of the luciferase reporter gene containing GAL4 binding sites. We co-transfected PC12 cells with the expression vector pcDNA–HA–Ndrg4-C2 encoding Ndrg4-C2 cDNA and the Elk-1 reporter system, followed by NGF treatment for 8 h. Expression of Ndrg4-C2 was shown to attenuate the NGF-induced Elk-1 reporter activity in an Ndrg4-C2 dose-dependent manner as compared to controls transfected with the empty plasmid (pcDNA–HA) (Fig. 5). A dose of 200 ng of pcDNA–HA–Ndrg4-C2 elicited a reduction in Elk-1 reporter activity to 70% as compared to that obtained with the same dose of the control plasmid.

We then compared the ability to reduce NGF-induced Elk-1 reporter activity among Ndrg4 protein isoforms. Ndrg4-A1, Ndrg4-A2, and Ndrg4-C1 at a dose of 200 ng reduced the Elk-1-mediated luciferase activity to 70% of that in controls in almost the same manner as Ndrg4-C2 and no significant differences were observed in the reducing ability among the Ndrg4 protein isoforms (data not shown).

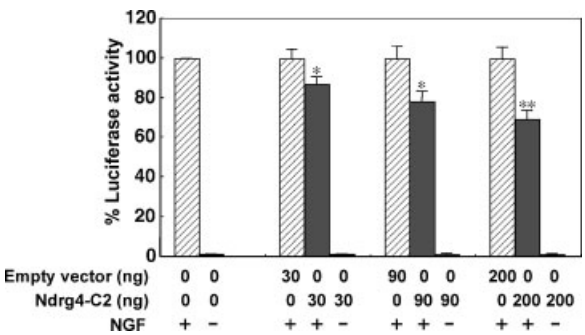

Fig. 5. Ndrg4 suppresses Elk-1-mediated transcriptional activation. The Elk-1 luciferase activity assay was performed using the PathDetect in vivo signal transduction pathway trans-reporting system. Trans-reporting constructs, including pFA2-Elk1, and pFR-Luc plasmids, and increasing amounts of Ndrg4 expression vector (pcDNA–HA–Ndrg4-C2) (solid bars) or empty vector (pcDNA–HA) (hatched bars) were transiently transfected into PC-12 cells. *Renilla* luciferase reporter vector (phRL-TK) was co-transfected as an internal control. Twenty-four hours after transfection, cells were incubated in medium containing 0.5% fetal bovine serum and 0.5% horse serum for a further 20 h followed by stimulation with (+) or without (−) 50 ng/ml NGF for 8 h. Cells were lysed and the Elk-1 luciferase activity was measured using the Dual-Luciferase Reporter Assay System. Data were normalized by the activity of *Renilla* luciferase reporter vector and expressed as percentages relative to controls transfected with an equal amount of empty vector (pcDNA–HA). Data are from triplicate assays in three independent experiments and are expressed as means ± SD. Statistical analyses were based on total observations. $*P < 0.01$ versus empty vector; $**P < 0.001$ versus empty vector (Paired *t*-test).

Suppressive Effect of Ndrg4 on Activation of Elk-1 Is Abolished by a Microtubule-Disrupting Agent

We studied the effects of cytoskeleton-disrupting agents on the Ndrg4-induced attenuation of Elk-1 activation, because a part of ERK in the cells has been reported to be present on microtubules [Reszka et al., 1995; Morishima-Kawashima and Kosik, 1996] and the cytoskeleton can regulate the nuclear entry of ERK [Smith et al., 2004], which activates Elk-1. PC12 cells were preincubated for 30 min with colchicine, a microtubule-disrupting agent, before stimulating cells with NGF for 8 h. Figure 6 (left) shows that colchicine at 1 μM and 10 μM abolished the Ndrg4-C2-induced suppression of Elk-1 activation, and 10 μM colchicine elicited a slight enhancement in activation of Elk-1. Another agent that disrupts actin microfilaments, cytochalasin D, did not prevent the Ndrg4-C2-dependent suppression of Elk-1 activation (Fig. 6, right).

DISCUSSION

Here, we described the functional relevance of Ndrg4 protein to Ras–ERK pathway signaling, which is important in the neuronal differentiation of PC12 cells by NGF. We found that Ndrg4 protein positively regulates the NGF-dependent intracellular ERK pathway by enhancing the phosphorylation of signaling components, MEK1/2 and ERK1/2. In contrast, Ndrg4 protein repressed the NGF-induced phosphorylation and activation of Elk-1, which is a nuclear target for ERK. Thus, the enhanced phosphorylation of ERK by Ndrg4 overexpression was not associated with augmented phosphorylation/activation of Elk-1. Elk-1 is a member of the Ets family of transcription factors, which have been implicated in cell proliferation [Buchwalter et al., 2004]. Ndrg4 possibly regulates expressions of the genes that contain the SRE in their promoters, such as c-*fos* [Meyer et al., 1993], *egr-1* [Rupprecht et al., 1993], and *jun B* [Perez-Albuerne et al., 1993]. Previously, we reported that Ndrg4-overexpressing PC12 cell clones showed increased differentiation scores

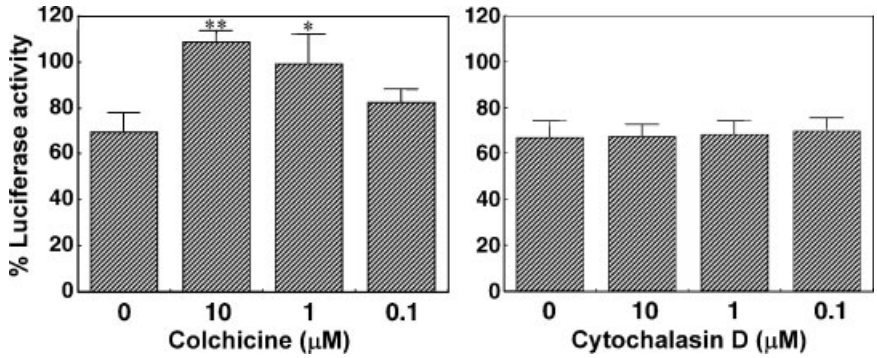

Fig. 6. The suppressive effect of Ndrg4 on Elk-1-mediated transcriptional activation is abolished by a microtubule-disrupting agent. Trans-reporting constructs, including pFA2-Elk1 and pFR-Luc plasmids, and the Ndrg4 expression vector pcDNA–HA–Ndrg4-C2 (200 ng) or empty vector pcDNA–HA (200 ng) were transiently transfected into PC12 cells. Control empty vector was used at each concentration of test agent. After maintaining the cells as described for Figure 5, cells were preincubated with the indicated concentrations of colchicine (**left**) or cytochalasin D (**right**) for 30 min and treated with NGF for 8 h. Colchicine is a microtubule-disrupting agent and cytochalasin D is an actin microfilament-destabilizing agent. Elk-1 luciferase activity was measured using the cell lysate and Dual-Luciferase Reporter Assay System. Data are from triplicate assays in two independent experiments and are expressed as means ± SD. Statistical analyses were based on total observations. $*P < 0.01$ versus colchicine (0 μM); $**P < 0.001$ versus colchicine (0 μM) (Paired *t*-test).

in response to NGF [Ohki et al., 2002]. Ndrg4 may support the ERK-mediated phosphorylation of proteins required for neuronal differentiation and suppress the proliferative effect of Ras.

The mechanism by which Ndrg4 suppresses Elk-1 activity could be inferred from the data presented here and our results provide clues to further our understanding of the cellular function of Ndrg4. The abolishment of the Ndrg4-dependent reduction of Elk-1 transcriptional activity by colchicine suggests that Ndrg4 influences Elk-1 activation via the interaction of Ndrg4 with microtubules in the cytoplasm. Smith et al. [2004] described a similar phenomenon; in retinoic acid-induced endoderm differentiation of embryonic stem and carcinoma cells, nuclear entry of activated MAP kinase is restricted to suppress the activation of Elk-1 and an intact cytoskeleton is required for this effect. Uncoupling between ERK and Elk-1 activation has been reported, for example, in the expression of KSR [Sugimoto et al., 1998], Gab2 [Zhao et al., 1999], and Dab2 [Smith et al., 2001]. Recently, it was reported that NDRG1/Rit42 protein, the gene product of another member of the NDRG family, is a microtubule-associated protein and participates in the spindle checkpoint, localizing in the centrosomes [Kim et al., 2004]. A part of MAPK/ERK was shown to associate with microtubules in neural cells [Reszka et al., 1995] and other cell types [Morishima-Kawashima and Kosik, 1996; Willard and Crouch, 2001]. It is worthwhile examining whether Ndrg4 associates with the microtubules or ERK signaling components and restricts the translocation of ERK to the nucleus. Similarly, the mechanism by which Ndrg4 enhances the activation of ERK pathway members may be to due to interactions between Ndrg4 protein and ERK pathway components via microtubules or their associated proteins.

Ndrg4-C2 attenuated NGF-induced transactivation activity of Elk-1 and *cis*-acting SRE activity. To the contrary, Ndrg4-C2 enhanced NGF-induced AP-1-luciferase activity. We also reported that Ndrg4 overexpression elevated DNA binding activity of AP-1 as determined by gel shift assay [Ohki et al., 2002]. The ability of Ndrg4 protein to reduce the NGF-induced activation of Elk-1 indicated by the results of the present study appear contradictory to the increased AP-1 activity, as Elk-1 is a transcriptional activator for c-*fos* gene and c-Fos can compose AP-1. The reason for this inconsistency is not known at present. One possible explanation is that the AP-1 components may have been altered quantitatively or qualitatively by Ndrg4 expression. In accordance with this idea, we obtained the result that NGF-stimulated phosphorylation of c-Jun was enhanced by overexpression of Ndrg4 (data not shown).

In summary, the results of the present study indicate that Ndrg4 enhances NGF-induced

ERK signaling components and attenuates NGF-induced Elk-1 activation for transcription in PC12 cells, probably supporting neuronal differentiation.

ACKNOWLEDGMENTS

This work was supported in part by Grant-in-Aid for Scientific Research (C) (16590895 to A. M.) from Japan Society for the Promotion of Science and the High-Technology Research Center Project from the Ministry of Education, Culture, Sports, Science and Technology.

REFERENCES

Angel P, Karin M. 1991. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. Biochim Biophys Acta 1072:129–157.

Braselmann S, Bergers G, Wrighton C, Graninger P, Superti-Furga G, Busslinger M. 1992. Identification of Fos target genes by the use of selective induction systems. J Cell Sci Suppl 16:97–109.

Brunet A, Roux D, Lenormand P, Dowd S, Keyse S, Pouyssegur J. 1999. Nuclear translocation of p42/p44 mitogen-activated protein kinase is required for growth factor-induced gene expression and cell cycle entry. EMBO J 18:664–674.

Buchwalter G, Gross C, Wasylyk B. 2004. Ets ternary complex transcription factors. Gene 324:1–14.

Deng Y, Yao L, Chau L, Ng SS, Peng Y, Liu X, Au WS, Wang J, Li F, Ji S, Han H, Nie X, Li Q, Kung HF, Leung SY, Lin MC. 2003. N-Myc downstream-regulated gene 2 *(NDRG2)* inhibits glioblastoma cell proliferation. Int J Cancer 106:342–347.

Guan RJ, Ford HL, Fu Y, Li Y, Shaw LM, Pardee AB. 2000. *Drg-1* as a differentiation-related, putative metastatic suppressor gene in human colon cancer. Cancer Res 60: 749–755.

Karin M. 1995. The regulation of AP-1 activity by mitogen-activated protein kinases. J Biol Chem 270:16483–16486.

Kim KT, Ongusaha PP, Hong YK, Kurdistani SK, Nakamura M, Lu KP, Lee SW. 2004. Function of Drg/Rit42 in p53-dependent mitotic spindle checkpoint. J Biol Chem 279:38597–38602.

Kokame K, Kato H, Miyata T. 1996. Homocysteine-respondent genes in vascular endothelial cells identified by differential display analysis: GRP78/BiP and novel genes. J Biol Chem 271:29659–29665.

Kurdistani SK, Arizti P, Reimer CL, Sugrue MM, Aaronson SA, Lee SW. 1998. Inhibition of tumor cell growth by *RTP/rit42* and its responsiveness to p53 and DNA damage. Cancer Res 58:4439–4444.

Maeda A, Hongo S, Miyazaki A. 2004. Genomic organization, expression, and comparative analysis of noncoding region of the rat *Ndrg4* gene. Gene 324:149–158.

Meyer DJ, Stephenson EW, Johnson L, Cochran BH, Schwartz J. 1993. The serum response element can mediate induction of c-fos by growth hormone. Proc Natl Acad Sci USA 90:6721–6725.

Morishima-Kawashima M, Kosik KS. 1996. The pool of MAP kinase associated with microtubules is small but constitutively active. Mol Biol Cell 7:893–905.

Morooka T, Nishida E. 1998. Requirement of p38 mitogen-activated protein kinase for neuronal differentiation in PC12 cells. J Biol Chem 273:24285–24288.

Nishimoto S, Tawara J, Toyoda H, Kitamura K, Komurasaki T. 2003. A novel homocysteine-responsive gene, *smap8*, modulates mitogenesis in rat vascular smooth muscle cells. Eur J Biochem 270:2521–2531.

Ohki T, Hongo S, Nakada N, Maeda A, Takeda M. 2002. Inhibition of neurite outgrowth by reduced level of NDRG4 protein in antisense transfected PC12 cells. Brain Res Dev Brain Res 135:55–63.

Perez-Albuerne ED, Schatteman G, Sanders LK, Nathans D. 1993. Transcriptional regulatory elements downstream of the JunB gene. Proc Natl Acad Sci USA 90:11960–11964.

Reszka AA, Seger R, Diltz CD, Krebs EG, Fischer EH. 1995. Association of mitogen-activated protein kinase with the microtubule cytoskeleton. Proc Natl Acad Sci USA 92:8881–8885.

Rupprecht HD, Sukhatme VP, Lacy J, Sterzel RB, Coleman DL. 1993. PDGF-induced Egr-1 expression in rat mesangial cells is mediated through upstream serum response elements. Am J Physiol 265:F351–F360.

Segal RA, Greenberg ME. 1996. Intracellular signaling pathways activated by neurotrophic factors. Annu Rev Neurosci 19:463–489.

Seger R, Krebs EG. 1995. The MAPK signaling cascade. FASEB J 9:726–735.

Sheng M, Dougan ST, McFadden G, Greenberg ME. 1988. Calcium and growth factor pathways of c-*fos* transcriptional activation require distinct upstream regulatory sequences. Mol Cell Biol 8:2787–2796.

Smith ER, Capo-chichi CD, He J, Smedberg JL, Yang DH, Prowse AH, Godwin AK, Hamilton TC, Xu XX. 2001. Disabled-2 mediates c-Fos suppression and the cell growth regulatory activity of retinoic acid in embryonic carcinoma cells. J Biol Chem 276:47303–47310.

Smith ER, Smedberg JL, Rula ME, Xu XX. 2004. Regulation of Ras-MAPK pathway mitogenic activity by restricting nuclear entry of activated MAPK in endoderm differentiation of embryonic carcinoma and stem cells. J Cell Biol 164:689–699.

Sugimoto T, Stewart S, Han M, Guan KL. 1998. The kinase suppressor of Ras (KSR) modulates growth factor and Ras signaling by uncoupling Elk-1 phosphorylation from MAP kinase activation. EMBO J 17:1717–1727.

Sun H, King AJ, Diaz HB, Marshall MS. 2000. Regulation of the protein kinase Raf-1 by oncogenic Ras through phosphatidylinositol 3-kinase, Cdc42/Rac and Pak. Curr Biol 10:281–284.

van Belzen N, Dinjens WNM, Diesveld MPG, Groen NA, van der Made ACJ, Nozawa Y, Vlietstra R, Trapman J, Bosman FT. 1997. A novel gene which is up-regulated during colon epithelial cell differentiation and down-regulated in colorectal neoplasms. Lab Invest 77:85–92.

Wasylyk B, Hagman J, Gutierrez-Hartmann A. 1998. Ets transcription factors: Nuclear effectors of the Ras-MAP-kinase signaling pathway. Trends Biochem Sci 23:213–216.

Willard FS, Crouch MF. 2001. MEK, ERK, and p90RSK are present on mitotic tubulin in Swiss 3T3 cells: A role for the MAP kinase pathway in regulating mitotic exit. Cell Signal 13:653–664.

Xing J, Kornhauser JM, Xia Z, Thiele EA, Greenberg ME. 1998. Nerve growth factor activates extracellular signal-regulated kinase and p38 mitogen-activated protein kinase pathways to stimulate CREB serine 133 phosphorylation. Mol Cell Biol 18:1946–1955.

Yamauchi Y, Hongo S, Ohashi T, Shioda S, Zhou C, Nakai Y, Nishinaka N, Takahashi R, Takeda F, Takeda M. 1999. Molecular cloning and characterization of a novel developmentally regulated gene, *Bdm1*, showing predominant expression in postnatal rat brain. Brain Res Mol Brain Res 68:149–158.

Zhao C, Yu DH, Shen R, Feng GS. 1999. Gab2, a new pleckstrin homology domain-containing adapter protein, acts to uncouple signaling from ERK kinase to Elk-1. J Biol Chem 274:19649–19654.

Zhou D, Salnikow K, Costa M. 1998. *Cap43*, a novel gene specifically induced by Ni^{2+} compounds. Cancer Res 58:2182–2189.

Zhou RH, Kokame K, Tsukamoto Y, Yutani C, Kato H, Miyata T. 2001. Characterization of the human NDRG gene family: A newly identified member, NDRG4, is specifically expressed in brain and heart. Genomics 73:86–97.