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Induction of Id-1 by FGF-2 Physiology Involves Activity of EGR-1 and Sensitizes Neuroblastoma Cells to Cell Death

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Abstract

Inhibitor of differentiation-1 (Id-1) is a member of helix–loop–helix (HLH) family of proteins that regulate gene transcription through their inhibitory binding to basic-HLH transcription factors. Similarly to other members of this family, Id-1 is involved in the repression of cell differentiation and activation of cell growth. The dual function of Id-1, inhibition of differentiation, and stimulation of cell proliferation, might be interdependent, as cell differentiation is generally coupled with the exit from the cell cycle. Fibroblast growth factor-2 (FGF-2) has been reported to play multiple roles in different biological processes during development of the central nervous system (CNS). In addition, FGF-2 has been described to induce “neuronal-like” differentiation and trigger apoptosis in neuroblastoma SK-N-MC cells. Although regulation of Id-1 protein by several mitogenic factors is well-established, little is known about the role of FGF-2 in the regulation of Id-1. Using human neuroblastoma cell line, SK-N-MC, we found that treatment of these cells with FGF-2 resulted in early induction of both Id-1 mRNA and protein. The induction occurs within 1 h from FGF-2 treatment and is mediated by ERK1/2 pathway, which in turn stimulates expression of the early growth response-1 (Egr-1) transcription factor. We also demonstrate direct interaction of Egr-1 with Id-1 promoter in vitro and in cell culture. Finally, inhibition of Id-1 expression results in G_2/M accumulation of FGF-2-treated cells and delayed cell death.

Fibroblast growth factor-2 (FGF-2) or basic fibroblast growth factor (bFGF) is a member of the family of heparin-binding growth factors, which comprises up to 23 growth factors (Nishimura et al., 1999; Grothe and Nikkhah, 2001; Eswarakumar et al., 2005), and binds tyrosine kinase receptors FGFR1-4 (Sahni et al., 1999). The pleiotropic action of FGF-2 influences many processes during embryonic development, and supports malignant growth (Basilico and Moscatelli, 1992; Grose and Dickson, 2005). These broad effects are particularly marked in the embryonic central nervous system (CNS), where FGF-2 is

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extensively expressed, and regulates neural proliferation, differentiation, and survival (Ledoux et al., 1992; Baird, 1994; Creancier et al., 2000). Similarly, FGF-2 has been shown to induce neural differentiation of multipotent brain stem cells (Palmer et al., 1999) and neuronal progenitors cell lines (Gage et al., 1995; Raballo et al., 2000). Inhibition of cellular growth by FGF-2 has been demonstrated in peripheral primitive neuroectodermal tumor-derived SK-N-MC cells, in which it causes a delay in cell cycle progression through the G_2 phase arrest (Smits et al., 2000). In the same cell line FGF-2 can also induce differentiation and apoptosis (Kim et al., 2004; Russo et al., 2004; Ma et al., 2008).

Inhibitor of differentiation-1 (Id-1) belongs to a group of helix–loop–helix (HLH) proteins that act as dominant-negative factors, forming homo- and heterodimers with other basic-HLH transcription factors (Benezra et al., 1990; Ristow, 1996). This group of proteins, namely inhibitors of DNA binding (Id-1 to Id-4), lacks the basic residues adjacent to the HLH domain, which binds to DNA sequences that contain the canonical “E box” recognition sequence (Massari and Murre, 2000), and thus formed Id-bHLH heterodimers are unable to bind to DNA. Functionally, Id-1 has been shown to stimulate cell proliferation, inhibit cell differentiation, and stimulate angiogenesis (Lister et al., 1995; Lyden et al., 1999; Ling et al., 2005). Increased expression of Id-1 has been found in many types of human cancers including cervical, prostate, and breast cancers (Ouyang et al., 2002; Schindl et al., 2003; Schoppmann et al., 2003; Wong et al., 2004). Conversely, down-regulation of Id-1 expression is thought to be required for cellular differentiation in a variety of cell types, including neural cells (Jogi et al., 2002; Du and Yip, 2010).

In this study, we show transient up-regulation of Id-1 expression in neuroblastoma SK-N-MC cells treated with FGF-2. This induction is mediated through the activation of the MAP kinases members ERK1/2. We also show that the zinc-finger transcription factor early growth response-1 (Egr-1) is the ERKs downstream effector that binds directly to the Id-1 promoter, leading to its transcriptional activation. Inhibition of Id-1 expression by shRNA strategies sensitizes SK-N-MC cells to FGF-2-mediated G_2/M accumulation and cell cycle arrest delaying the pro-apoptotic effects of FGF-2.

Materials and Methods

Cell cultures

Human neuroepithelioma SK-N-MC cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA). When required, cells were treated with FGF-2 (Invitrogen) at a concentration of 10, 20, or 40 ng/ml.

Inhibitors and antibodies

U0126, SB239063, SP600125, and LY24002 were purchased from EMD Biosciences (Darmstadt, Germany). [α-^{32}P]ATP and [γ-^{32}P]ATP were obtained from PerkinElmer Life Sciences (Waltham, MA). Fugene 6 was from Roche (Basel, Switzerland). Human recombinant FGF-2 was purchased from Invitrogen. Id-1 (C-20) and Egr-1 (588) antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Polyclonal anti-p42/p44 MAP kinases (137F5) and polyclonal anti-phospho-p42/p44 (Thr202/Tyr204) were from Cell Signaling Technology (Danvers, MA). Anti-Grb2 was from Transduction Laboratories (BD Biosciences, San Jose, CA).

Mutational analysis of Id-1 promoter

Seven 5′-deletion mutants of the Id-1 promoter were generated by PCR amplification from the full-length Id-1 sequence and subcloned into the pGL3 reporter vector.

Site-directed mutagenesis was performed using a plasmid-containing the Id-1 promoter sequence −986/+95 bp (pId-1 F5) as template for the PCR using the Quick-Change II site-directed mutagenesis kit (Strategene, La Jolla, CA) according to the supplier's protocol. The primers were: Egr-1/Id-1 Fw: mut, 5′-GGCGAC**CGCCCATGC**GGCGCCAGC-3′, and reverse. Egr-1-binding site is in bold and the altered nucleotides are underlined.

Transfection and luciferase assays

SK-N-MC cells were transfected using Fugene 6 (Roche). Cells were plated at a density of 5×10^4 cells per well in a 24 wells plate 48 h prior to transfection. Co-transfection was performed according to the manufacturer's protocol using 450 ng of reporter plasmid and 120 ng of Renilla luciferase pRL-TK control plasmid (Promega, Madison, WI) for 24 h. Following this period, the transfection mixture was removed and replaced with media with or without 40 ng/ml FGF-2 for 24 h. When required, cells were pretreated for 30 min with 15 μM of UO126, 10 μM of SB239063, 25 μM of SP600215, or 50 μM of LY24002 prior to FGF-2 induction. Cell extracts were subsequently prepared and assayed using the Dual Luciferase kit (Promega) as per the manufacturer's instructions. Luciferase activities were normalized with Renilla values. Data are presented as the fold activation of the FGF-2 treated versus untreated samples. Results shown represent the mean of at least three independent experiments.

Western blots

Cell protein extracts were prepared by centrifugation and lysis of the cell pellet in the appropriate volume of RIPA buffer (50 mM Tris pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM sodium orthovanadate, phosphatase inhibitors, and protease inhibitor cocktails). Thirty to 50 μg of whole cell lysate were separated on a 4–15% SDS–PAGE (Bio-Rad, Hercules, CA) and transferred to nitrocellulose membrane, followed by immunoreaction with the indicated antibodies.

Northern blots

Total RNA from SK-N-MC cells (6.5×10^5 cells in 100 mm^2 diameter dishes) was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA (15 μg) was resolved on formaldehyde-containing 1.2% agarose gel and transferred to Hybond-N nylon membranes (GE Healthcare) and cross linked by ultraviolet irradiation. A 410 bp DNA fragment corresponding to the Id-1-coding region was radiolabeled with [α-^{32}P] dCTP and used as a probe. The filter was pre-hybridized for 1 h at 42°C in UltraHyb solution (Ambion, Austin, TX) and hybridized for 16 h at 42°C with the specific probe (10^6 cpm/ml). The blot was then rinsed twice for 5 min with 2 × SSC 0.1% SDS at 42°C and washed twice with 0.2× SSC, 0.1% SDS at 42°C for 20 min. The filter was exposed at −80°C to a Fuji XAR-5 film with Kodak intensifying screen. To verify RNA loading, the filter was rehybridized with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA probe.

Chromatin Immunoprecipitation assay

ChiP assay was performed with a kit (Upstate Biotechnology, Lake Placid, NY) according to the manufacturer's instructions. 1×10^6 SK-N-MC cells were cross-linked in a 1% formaldehyde solution for 10 min at 37°C. Cells were then lysed in 200 μl of SDS buffer and sonicated to obtain DNA fragments of 500 bp average length. After centrifugation, the

cleared supernatants were diluted 10-fold with ChIP buffer and incubated with 2 μg of α-Egr-1 or normal rabbit serum (NRS) at 4°C. Immune complexes were precipitated, washed, and eluted as recommended. DNA–protein cross-links were reversed by heating at 65°C for 4 h, and the DNA fragments were purified and dissolved in 20 μl of water. Two microliters of each sample were used as a template for PCR amplification. Id-1 oligonucleotide sequences for the PCR primers were: Id-1_ChIP Fw: 5′-CGCAAGAAACGCATTCCCAG-3′ and Id-1_ChIP Rev: 5′-AGCGGCTCAG ACCGTTAGAC-3′.

This primer set encompasses the Id-1 promoter segment from nucleotide −1,092 to −905, to generate a 187 bp amplification product which includes the Egr-1-binding site. PCR was performed at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s for 25 cycles. DNA samples were analyzed by electrophoresis on 1.2% agarose gels.

Electrophoretic mobility shift assay

Nuclear extracts were prepared as previously described (Andrews and Faller, 1991). Electrophoretic mobility shift assays (EMSAs) were performed by incubating 10 μg of nuclear extracts with [γ-^{32}P]-end-labeled double-stranded oligonucleotide probes (5.4×10^4 cpm) in binding buffer containing 10 mM Tris (pH 7.5), 75 mM KCl, 10% glycerol, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 0.25 mM DTT, and 1.0 μg poly(dI – dC) at 4°C for 20 min. Super shifts were performed by adding 2.0 μg antibody to the extract 30 min prior to incubation with probe. Protein/DNA complexes were resolved on 6% polyacrylamide gel run in 0.5× TBE, at 170 V at room temperature. The primers used for mutagenesis were also used for EMSA.

Generation of Id-1 shRNA lentivirus

The third generation-based ViraPower Lentivirus Expression System (Invitrogen) was used to generate lentiviruses encoding Id-1 or non-targeting (NT) shRNAs (Sigma Mission shRNA). Lentiviruses were produced by co-transfection of 293FT cells with four plasmids in the ratio of 1:4 (5 μg each) using calcium phosphate transfection kit (Promega). The medium was replaced with fresh medium after 8 h. After an additional 40 h the medium containing the viral particles was harvested and subjected to centrifugation, followed by filtration through a 0.45 μm membrane and frozen at −80°C.

Lentiviral transductions of SK-N-MC

SK-N-MC were plated at a density of 1×10^5 cells per well in a six wells plate 48 h prior to infection with lentiviruses encoding Id-1 shRNA, or NT shRNA. In a pilot experiment, we determined the amount of virus that was non-toxic to the cells by diluting the initial stock as follows: undiluted, 1:2, 1:4, 1:8, 1:16, and 1:32. Cells were incubated with the virus for 16 h followed by a change of medium. Efficient reduction of Id-1 expression was evaluated after 24, 48, and 72 h by Western blot.

Cell survival assay

SK-N-MC were plated at a density of 5×10^4 in 12-well plates 48 h prior to infection with lentiviruses encoding Id-1 shRNA, NT shRNA at a 1:4 dilution with complete medium. Twenty-four hours post-infection the medium was replaced with complete medium in the absence or presence of 40 ng/ml FGF-2 and incubated for an additional 1, 24, and 48 h. Cells were collected in 1 ml of DMEM medium and viable cells were counted with GuavaEasy Cyte system by using Guava CytoSoft ViaCount program according to the manufacturer's recommendations (Guava Technologies, Hayward, CA).

Cell cycle analysis

SK-N-MC cells were infected as previous described. Aliquots of cells, 1×10^5–10^6/ml, were fixed in 70% ethanol for 30 min at 4°C. Cells were centrifuged at 1,600 rpm and the resulting pellets were resuspended in 200 μl of Guava Cell Cycle Reagent solution (Guava Technologies). Cell cycle distribution was analyzed with GuavaEasy Cyte system by using Guava CytoSoft cell cycle program according to the manufacturer's recommendations.

Statistical analysis

Data are presented as mean ±SEM. Comparison between two experimental groups was done by the Student's *t*-test.

Results

FGF-2 up-regulates Id-1 expression

In the first set of experiments, we have investigated the ability of FGF-2 to modulate expression of Id-1 in human SK-N-MC cells. Figure 1A (upper part) shows a time course experiment in which Id-1 protein levels were detected in SK-N-MC cells grown in the presence of serum and after treatment with FGF-2 (40 ng/ml) for 15 min, 30 min, 1, 2, 4, 6, 12, and 24 h. Increased levels of Id-1 were detected 30 min after FGF-2 treatment, further increasing in the next 2 h, and slowly decreased to the basal levels within 12 h. Quantitatively, more than 40% increase in the levels of Id-1 were observed already 15 min after treatment with FGF-2, with a peak of sevenfold increase 2 h post-treatment, followed by a gradual reduction to basal levels in the next 10–20 h (Fig. 1B). The ability of FGF-2 to induce up-regulation of Id-1 expression was confirmed by Northern blot analysis of Id-1 mRNA in SK-N-MC cells under the same experimental conditions. Figure 1C shows low levels of Id-1 mRNA in cells grown in the presence of serum and up to 30 min following FGF-2 treatment. Levels of mRNA robustly increased 1 h after FGF-2 treatment, and decreased rapidly to basal levels thereafter. Quantitatively, a twofold increase of Id-1 mRNA expression was detected 1 h after FGF-2 treatment as illustrated in Figure 1D. Northern blot to detect levels of GAPDH was used to monitor RNA loading conditions and to normalize Id-1 densitometry. Transcriptional regulation of Id-1 expression by FGF-2 was additionally confirmed by functional assay in which a reporter plasmid containing the full-length Id-1 promoter was transfected into SK-N-MC cells grown in the presence or absence of FGF-2. The results in Figure 1E show more than twofold increase in the Id-1 promoter activity after FGF-2 treatment, when compared to untreated cells.

Altogether, these data show that FGF-2 stimulates transient transcription and expression of Id-1 on both mRNA and protein levels in SK-N-MC cells.

FGF-2 activates Id-1 promoter through the ERK1/2 pathway

Previous reports have shown that FGF-2 treatment of SK-N-MC cells can result in a prolonged induction of ERK1/2 (Gentilella et al., 2008; Ma et al., 2008). FGF-2 has been reported to activate the PI3-K pathway in hippocampal neural progenitors (Peltier et al., 2007) and, to a lesser extent the p38 and JNK pathways (Gentilella et al., 2008; Ma et al., 2008). Therefore, in the next series of experiments we have investigated the contribution of the ERK1/2, PI3-K, p38, and JNK pathways in the up-regulation of Id-1. SK-N-MC cells were pre-treated with the MEK1 inhibitor UO126 (15 μM), the p38 MAPK inhibitor SB239063 (10 μM), the JNK inhibitor SP600215 (25 μM), and the PI3-K inhibitor LY24002 (50 μM), following by 1 h treatment with 40 ng/ml of FGF-2. Western blot in Figure 2A illustrates that among all the inhibitors used, only the ERK1/2 inhibitor UO126 (Fig. 2A, lane 3) significantly prevented the induction of Id-1 after 1 h treatment with FGF-2

(Fig. 2A, lane 2), while no major effect on Id-1 induction was observed for p38 MAPK, JNK, or PI3-K inhibitors (Fig. 2A, lanes 4–6).

Promoter functional assay further confirmed that the activation of transcription of the full-length human Id-1 promoter sequence induced by FGF-2 was reduced in SK-N-MC cells pre-treated with UO126 (Fig. 2B; $P < 0.05$). Pre-treatment of SK-N-MC cells with the p38 inhibitor SB230963 had no effect on the activation of Id-1 promoter. Treatment with the JNK inhibitor SP600215 resulted in a higher basal level of Id-1 promoter activation, which further increased with the addition of FGF-2. Those changes, however, were not statistically significant compared to the control without inhibitor. LY24002 had only a partial effect on the FGF-2-mediated activation of Id-1 promoter.

The efficacy of UO126 in inhibiting Id-1 transcription was further confirmed by Western blot analysis (Fig. 2C). Similarly to the results depicted in Figure 1A, up-regulation of Id-1 protein was observed between 30 min and 4 h after FGF-2 treatment. FGF-2 treatment results in a marked and sustained phosphorylation of ERK1/2, which also paralleled with increased expression of Egr-1. Pre-treatment of cells with UO126 prior to induction with FGF-2 prevented up-regulation of Id-1 and, as expected, efficiently inhibited ERK1/2 phosphorylation and Egr-1 expression.

To investigate which region in the Id-1 promoter was involved in the FGF-2-mediated activation of transcription, we have generated a series of reporter constructs containing the full-length human Id-1 promoter sequence and seven 5′ deletion mutants (Fig. 3A), and we have tested their responsiveness to FGF-2 treatment in SK-N-MC cells. Figure 3B shows that treatment with FGF-2 enhanced the activity of Id-1 promoter full-length (F1). Luciferase activity was not affected by removal of the Id-1 promoter sequences spanning nucleotides from −2,114 to −986 and the deletion mutants F2, F3, F4, and F5 behaved essentially as the full-length. Conversely, removal of sequences beyond the −986 nucleotide drastically reduced Id-1 promoter activity both at basal level and after FGF-2 induction (F6; $P < 0.001$). Examination of the Id-1 promoter sequence spanning the region between −986 and −904 using Transfac algorithms (www.biobase-international.com) revealed the presence of overlapping Egr-1/SP-1/YY1 element-binding sites (−959 to −976) and one putative CREB-binding site at position −922 from the transcription start site.

Up-regulation of Id-1 expression involves activity of Egr-1

Based on the results obtained using various inhibitors (Fig. 2) and previous studies showing Egr-1-dependent transcription of Id-1 (Tournay and Benezra, 1996), we next investigated the possible interaction of Egr-1 with the Id-1 promoter sequence spanning the region comprised between −986 and −904 using chromatin immunoprecipation (Chipitsyna et al., 2004) assay (Fig. 4A). Cell lysates obtained from SK-NM-C cells untreated or treated with FGF-2 in the absence or presence of the MEK inhibitor UO126 were immunoprecipited using an Egr-1 antibody or NRS as a negative control. PCR amplification of Id-1 promoter containing the putative Egr-1-binding site was detected in SK-N-MC cells treated with FGF-2 for 1 h (Fig. 4A, lane 2), but not in the sample obtained from untreated cells (Fig. 4A, lane 1). As expected, significantly lower amounts of PCR product were observed in cells pre-treated with UO126 prior to stimulation with FGF-2 (Fig. 4A, lane 3) compared to cells untreated with the inhibitor (lane 2). The specificity of Egr-1 binding to the Id-1 promoter (Fig. 4A, lanes 4–6), equal loading and efficiency of DNA amplification (lanes 7–9) were evaluated by a Chromatin Immuno-Precipitation (Chipitsyna et al., 2004) assay performed with NRS and from the input DNA, respectively. Next, to confirm in vitro interaction between Egr-1 protein and its putative-binding sequences we performed EMSA and super shift analysis using nuclear extracts from SK-N-MC cells under various conditions. Sequence analysis of Id-1 promoter revealed that the predicted Egr-1-binding site differs one

nucleotide from the canonic Egr-1-binding motif (Egr-1-binding motif 5′-GCGGGGGCG-3′, Egr-1/Id-1-binding site 5′-GCGCGG GCG-3′). Therefore, in this assay we have utilized the canonical Egr-1-binding motif as a positive control (Fig. 4B). A DNA–Egr-1 complex (asterisk) was detected in nuclear extracts obtained from cells treated for 1 h with FGF-2 when incubated with a DNA probe containing the canonical (Fig. 4B, lane 2) or the Egr-1-binding motif predicted in silico (Fig. 4B, lane 5). The difference in the intensity of the DNA/Egr-1 complex, Egr-1 consensus sequence versus Egr-1 putative sequence (lanes 2 and 5, respectively), might be due to the difference in the nucleotide sequence. Nonetheless, pretreatment of the cell cultures with UO126 followed by FGF-2 stimulation showed a strong reduction of DNA–protein complexes (Fig. 4B, lanes 3 and 6), further corroborating the FGF-2-mediated Egr-1/Id-1 pathway. In addition, mutation of two additional nucleotides in the putative Egr-1-binding site on the Id-1 promoter sequence further inhibited the complex formation (lane 14), confirming the specificity of the binding. Super shift assay in which the nuclear extracts were incubated with an antibody to Egr-1, prevented the formation of the DNA/Egr-1 complex (lanes 7–9), while no changes were observed when nuclear protein (treated under same condition) were incubated with NRS (lane 11).

Finally, we have used the Egr-1-binding site mutant described in Figure 4B to confirm the role of Egr-1-binding domain in the activation of Id-1 promoter activity upon treatment with FGF-2. As shown in Figure 4C, a point mutation in the Egr-1-binding site of Id-1 promoter slightly decreased the level of activation of Id-1 transcript ($P < 0.05$), confirming a potential role of the Egr-1-binding site in the up-regulation of Id-1 upon treatment with FGF-2.

ShRNA-mediated inhibition of Id-1 expression promotes G_2/M accumulation in FGF-2-treated SK-N-MC cells

To investigate the role of FGF-2-induced transient expression of Id-1 we used lentiviral shRNA strategy targeting Id-1. Figure 5A shows down-regulation of Id-1 expression in SK-N-MC cells upon lentiviral transduction with shId-1 and control NT shNT. As expected, treatment of the cells with FGF-2 failed to up-regulate Id-1 expression levels in cells transduced with shId-1 when compared to controls, shNT, and untransfected. In terms of morphological changes associated with the induction of differentiation by FGF-2, no changes in the number and length of neuronal-like processes were observed in shId-1 transduced cells compared to controls (data not shown). In terms of cell survival, no particular differences were observed in the first 24 h in cells with down-regulated levels of Id-1 compared to controls (Fig. 5B). However, the number of viable cells grown in complete medium for 48 h was significantly lower in cells transduced with shId-1 compared to controls (Fig. 5B, compare ShNT with ShId-1; $P < 0.05$). Addition of FGF-2 triggered massive cell death in the control shNT cells (ShNT + FGF-2) at 48 h, while in the same conditions shId-1 cells survived much better (ShId-1 + FGF-2; $P < 0.05$).

We further determined the cell cycle distribution of shId-1 transduced cells and controls with or without FGF-2. Average results from three independent experiments are shown in Figure 5C. No difference is observed in shId-1 and control shNT at time zero (T0). Twenty-four hours after stimulation with FGF-2 about 15% of shNT control cells shifts from G_0/G_1 to G_2/M ($P < 0.05$). The effect of FGF-2 in cells with down-regulated levels of Id-1 resulted in 23% reduction in the population of cells in G_0/G_1 and 33% increase in the number of cells in G_2/M. Comparison between FGF-treated control and shId-1 transduced cells highlights an increase of 28% in cells in G_2/M when Id-1 is down-regulated ($P < 0.001$).

Altogether, our results show that inhibition of Id-1 expression via shRNA improves cell survival which is associated with G_2/M arrest, and addiction of FGF-2 in the absence of Id-1 expression further increases cell accumulation in G_2/M.

Discussion

The family of Id proteins is known to participate in proliferation and differentiation in a variety of cell types including neural cells (Benezra et al., 1990; Nagata and Todokoro, 1994; Lyden et al., 1999). Regulation of Id-1 expression can be induced after a mitogenic stimulation (Nagata and Todokoro, 1994; Bergonzini et al., 2004) and it is required for the progression through the S phase of cell cycle (Zebedee and Hara, 2001). Although FGF-2 generally functions as a mitogenic factor (Grose and Dickson, 2005), it may also induce growth inhibition, as demonstrated in Ewing's Sarcoma family of Tumors (ESFT) cells (van Puijenbroek et al., 1997). In neuroblastoma SK-N-MC cells, the FGF-2-mediated growth arrest leads to differentiation (Kim et al., 2004) and eventually to apoptosis (Sturla et al., 2000; Kim et al., 2004; Williamson et al., 2004) by a mechanism that involves accumulation of phospho-ERK1/2 in the cytoplasm (Ma et al., 2008). Similarly to other growth factors, we found that FGF-2 treatment results in a transient expression of Id-1 prior to its down-regulation and cellular differentiation. While down-regulation of Id-1 has been shown to be required for the differentiation process (Deed et al., 1994), the role of this protein in the early stages of cell differentiation, when its expression actually increases, is less clear.

In this study, we have used the human neuroblastoma cell line SK-N-MC as a model for neuronal differentiation and investigated the regulation of Id-1 by FGF-2. We have shown that treatment of SK-N-MC cells with FGF-2 induced up-regulation of Id-1 at the transcriptional level, which in turn resulted in increased expression of Id-1 protein levels as well (Fig. 1). The subsequent decrease of Id-1 protein to the basal levels (Fig. 1) is in line with previous findings showing that the differentiation program promoted by various growth factors induces a burst of Id-1 expression first, and is followed by its down-regulation (Hara et al., 1994; Bergonzini et al., 2004). Since previous studies have shown various effects of FGF-2 depending on its concentration (Russo et al., 1999; Kim et al., 2004; Russo et al., 2004; Ma et al., 2008; Higgins et al., 2009), we determined whether different amounts of FGF-2 would result in increased Id-1 expression. We found that Id-1 levels are increased regardless of the concentration of FGF-2 utilized to treat SK-N-MC cells (data not shown). Relative to differentiation and cell death, no differences were observed among the cells treated with different amounts of FGF-2 (data not shown), suggesting that, at least in this system, FGF-2 induces comparable effects at the tested concentrations.

Similar to other growth factor receptors FGF-2 activation also triggers phosphorylation of Akt, ERK1/2, and CREB through the activation of PI3-K/Akt, MEK, JNK, and p38 MAPK pathways, respectively (Kim et al., 2004; Johnson-Farley et al., 2007; Peltier et al., 2007; Ditlevsen et al., 2008; Gentilella et al., 2008). We have further investigated the signaling pathways involved in FGF-2-mediated Id-1 up-regulation and found that only the specific ERK1/2 inhibitor UO126 significantly repressed the up-regulation of Id-1 at transcriptional and protein levels. In addition, treatment of the cells with UO126 partially prevented FGF-2-induced cell death (data not shown). Although a subtle reduction of Id-1 messenger levels and transcriptional activity was observed in cells treated with p38 MAPK and JNK inhibitors, respectively, this reduction did not affect Id-1 protein expression levels (Fig. 2). In terms of morphological changes, no differences were observed in cells treated with FGF-2 in the presence of the p38 or the JNK inhibitors; while cells treated with UO126 showed no signs of neuronal differentiation (data not shown). In agreement with the well-established role of Id-1 in mitogenic gene signaling cascades (Norton, 2000), we have identified the zinc-finger transcriptional factor Egr-1 as possible candidate of the up-regulation of Id-1. In line with previous studies using different cell lines stimulated with various factors (Tournay and Benezra, 1996; Jorda et al., 2007; Subbaramaiah et al., 2008), we also found a correlation between increased levels of Egr-1 and up-regulation of Id-1 protein after treatment with FGF-2 (Fig. 2).

To investigate the regulatory factors involved in the FGF-2-mediated induction of Id-1, we have performed a mutational analysis of the Id-1 promoter. Our results showed more than twofold increase in the transcriptional activity of Id-1 full-length upon stimulation with FGF-2. Deletion of the Id-1 promoter region containing Egr-1, Sp1, YY1, and CREB-binding site (Fig. 3B mutant F5) determined about twofold reduction of FGF-2-induced Id-1 transcriptional activity. Although the lack of Egr-1, Sp1, YY1, and CREB-binding site did not completely abolish the FGF-2-mediated induction of Id-1 transcriptional activity (Fig. 3B mutant F5), removal of the DNA sequences between −986 and −904 drastically decreased the basal level of Id-1 transcription (data not shown). Of interest, in a similar study Villano and White (2006) have reported that the region containing Egr-1, Sp1, YY1, and CREB-binding sites of Id-1 promoter plays an important role in the all-trans retinoic acid (at-RA)-induced expression of Id-1 in human keratinocytes.

To corroborate our results, we showed that the up-regulation of Egr-1 reflected an increased binding of Egr-1 protein to its putative-binding site on the Id-1 promoter (Fig. 4A,B). The role of Egr-1 in regulating Id-1 promoter was determined by gel shift and reporter assays using inhibitors and promoter mutation analysis (Fig. 4B,C). Single or double mutations in the Egr-1-binding site of the Id-1 promoter resulted in a consistent but not complete lack of the induction of Id-1, suggesting that additional elements might be involved in the FGF-2-mediated up-regulation of Id-1. Differently from previous reports in which ERK-mediated up-regulation of Id-1 contributes to cell invasiveness (Subbaramaiah et al., 2008), the FGF-2/ERK induced up-regulation of Id-1 might be involved in cell death.

In terms of cellular survival, loss of Id-1 partially protected cells from FGF-2-mediated cell death, suggesting that the pro-apoptotic effects of FGF-2 might be, at least in part, mediated by increased levels of Id-1 (Fig. 5B).

Different from the classic mechanism of growth inhibition in which the cells arrest in G_1 phase, FGF-2 appear to induce growth inhibition as a consequence of a delay in G_2 progression in SK-N-MC cells (Smits et al., 2000). In line with previous observations from Smits et al. (2000), our data also showed an increased number of cells in G_2/M after FGF-2 treatment (Fig. 5), and this trend was enhanced after down-regulation of Id-1 by shRNA. This result, however, is in contrast with previous work from Higgins et al. (2009) showing that FGF-2 produces cell cycle arrest in G_0/G_1 phase in SK-N-MC cells. Of interest, our data and the work from Smits et al. were both obtained in cells grown in presence of serum, while the experiments by Higgins et al. were performed in the absence of serum. As also suggested by Higgins et al. the different experimental settings may account for the discrepancies in the cell cycle.

Conclusion

Inhibition of Id-1 expression appears to be a common event following induction of cellular differentiation mediated by various factors (Norton et al., 1998; Zebedee and Hara, 2001; Ruzinova and Benezra, 2003; Bergonzini et al., 2004). However, less clear is the fact that down-regulation of Id-1 is preceded by its transient up-regulation (usually in the first 3 h of treatment). Taking advantage of the dual effects of FGF-2 on cell death and differentiation of SK-N-MC cells, in the present study we attempted to determine a possible role for Id-1 transient up-regulation as a contributing factor to FGF-2-induced cell death. Further investigation is however required to fully understand the molecular mechanism of such event.

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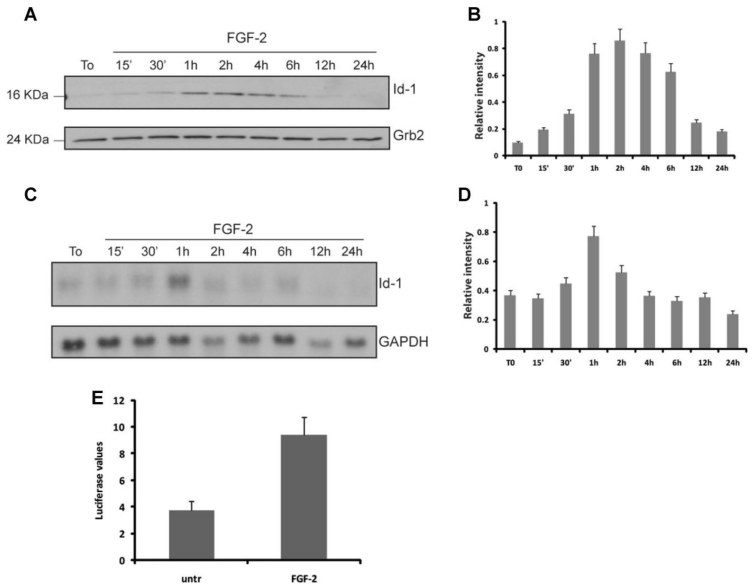


Fig. 1.

Regulation of Id-1 expression by FGF-2 in SK-N-MC cells. A: Western blot analysis of Id-1 from SK-N-MC cells induced with FGF-2 (40 ng/ml).Upper part shows expression of Id-1 at the indicated time points after FGF-2 stimulation. Anti-Grb2 was used as loading control (lowerpart). B: Densitometry analysis of Id-1 protein levels from part A after normalization for Grb2. C: Northern blot analysis of Id-1 mRNA at indicated time points. GAPDH was used as an internal control for the integrity of RNA and RNA loading. D: Densitometry analysis of Id-1 mRNA in the samples treated with or without FGF-2 (40 ng/ml) after normalization for GAPDH. E: Luciferase assay to monitor full length (2,114 bp) Id-1 promoter activity in SK-N-MC cells in the absence or presence of FGF-2 (40 ng/ml). Luciferase values were normalized by renilla values. Results are representative of three independent experiments with standard deviation.

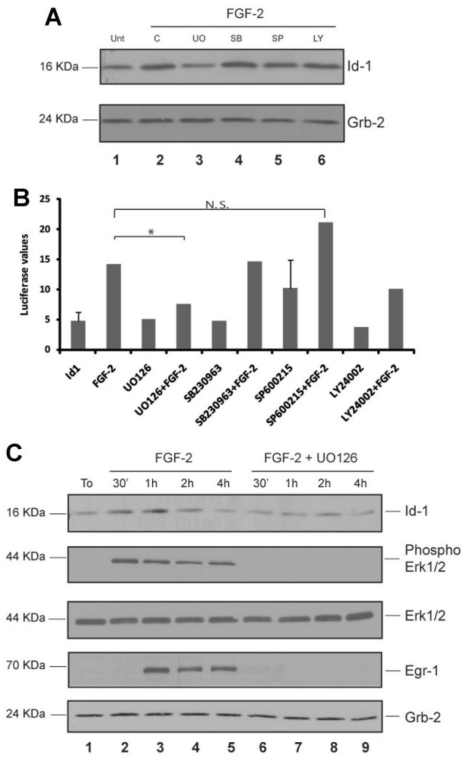


Fig. 2.

MEK pathway contributes to regulation of Id-1 expression. A: Western blot analysis to detect Id-1 (upper part) in SK-N-MC cells treated with FGF-2 (40 ng/ml) in the absence (c, lane 2) or presence of UO126 (15 μM, lane 3), SB239063 (10 μM, lane 4), SP600215 (25 mM, lane 5), and LY24002 (50 μM, lane 6) after 1 h treatment with FGF-2. Anti-Grb2 antibody (lower part) was used as loading control.

B: Luciferase assay to monitor full-length (2,114 bp) Id-1 promoter activity in SK-N-MC cells after treatment with FGF-2 (40 ng/ml) in the presence or absence of UO126 (15 μM), SB239063 (10 μM), SP600215 (25 μM), and LY24002 (50 μM). Fold activation of luciferase values was determined from three independent experiments after normalization with renilla activity. The asterisk represents $P < 0.05$. N.S., not statistically significant. C: Western blot analysis to detect Id-1 in SK-N-MC cells treated with FGF-2 (40 ng/ml) in the absence (lanes 2–5) or presence of UO126 (15 μM; lanes 6–9) at the indicated time points. Immunoblot was performed using anti-Id-1 (first part), phosphorylated ERK1/2 (second part), total ERK1/2 (third part), and anti-Egr-1 (fourth part) antibodies. Anti-Grb2 was used as loading control.

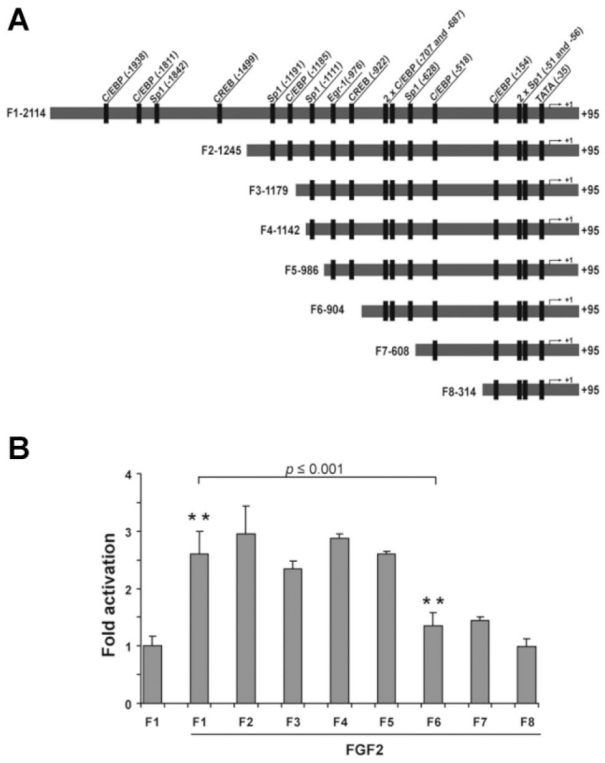


Fig. 3.

FGF-2 induces Id-1 promoter activity. A: Diagram showing the full-length Id-1 promoter sequence spanning from −2,114 to +95 bp, and the various 5′ deletion mutants, all cloned into the pGL3 reporter plasmid. B: FGF-2-induced transcriptional activation of deletion mutants of Id-1 promoter. Fold activation of mutated Id-1 promoters were calculated from FGF-2-induced transcriptional activities over transcriptional activities of the same mutant in the absence of FGF-2. Luciferase values were normalized by renilla values. Results are representative of three independent experiments with standard deviation. The double asterisk represents $P < 0.001$.

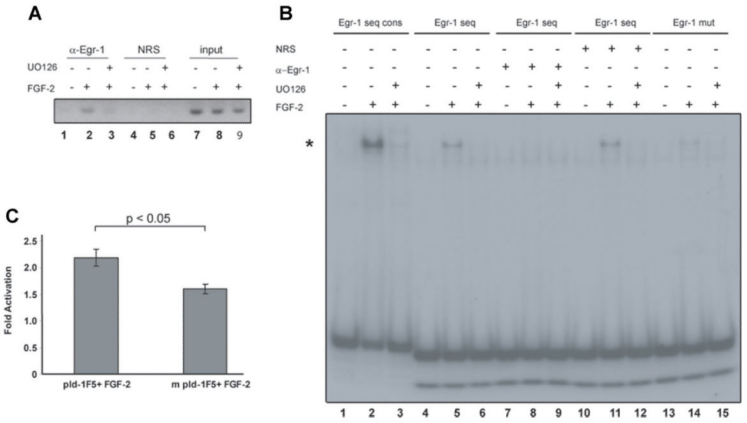
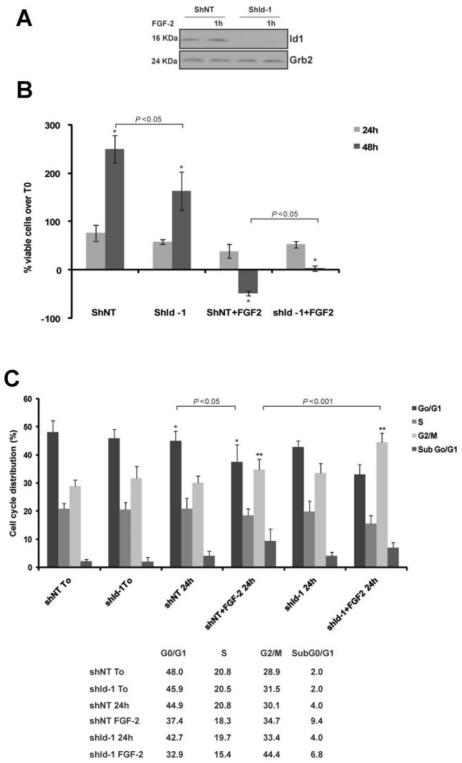


Fig. 4.

Egr-1 interacts with Id-1 promoter after FGF-2 induction. A: Chromatin Immunoprecipitation assay. SK-N-MC cells were treated with FGF-2 in the presence or absence of UO126. Chromatin-bound Egr-1 was immunoprecipitated using a polyclonal antibody against Egr-1 (lanes 1–3) or NRS (lanes 4–6), followed by PCR amplification using Id-1-specific primers and analyzed by agarose gels. Inputs genomic DNA was used as positive control (lanes 7–9). B: Electrophoretic mobility shift assay (EMSA) was performed using nuclear extracts from SK-N-MC treated for 1 h with FGF-2 (40 ng/ml) in the absence (lanes 2, 5,7, 11, 14) or presence UO126 (15 μM; lanes 3, 6,9, 12,15). Samples were incubate with putative Egr-1-binding site sequences (lanes 1–3), Egr-1-binding site sequences on Id-1 promoter (lanes 4–6) or a mutant with two mutations in the Egr-1-binding site (lanes 13–15). Supershift assays were performed using a polyclonal rabbit antibody against Egr-1 (lanes 7–9) or NRS (lanes 10–12). Asterisk shows Egr-1/DNA complex. Experiments shown in (A)and (B) are representative of three independent experiments. C: Transcriptional activity of pId-1 F5 (−986/+95) deletion mutant and its variant obtained by a mutagenesis of Egr-1-binding site (−976/−968). Fold activation of luciferase values was determined from three independent experiments after normalization with renilla activity. The asterisk represents $P < 0.05$.



	G0/G1	S	G2/M	SubG0/G1
shNT To	48.0	20.8	28.9	2.0
shId-1 To	45.9	20.5	31.5	2.0
shNT 24h	44.9	20.8	30.1	4.0
shNT FGF-2	37.4	18.3	34.7	9.4
shId-1 24h	42.7	19.7	33.4	4.0
shId-1 FGF-2	32.9	15.4	44.4	6.8

Fig. 5.

Effects of ShId-1 on cell survival and cell cycle. A: Western blot showing down-regulation of Id-1 expression in SK-N-MC cells transduced with ShId-1 lentiviral vector compared to control ShNT. B: Diagram showing percentage of viable cells cultured in the absence or presence of FGF-2 for 24 and 48 h. The asterisk represents $P < 0.05$. C: Cell cycle distribution analysis (diagram and table) of ShNT or ShId-1-transduced cells at time zero and stimulated with FGF-2 for 24 h. The experiments were repeated three times each in duplicate. Asterisk represents $P < 0.05$; double asterisk indicates $P < 0.001$.