

Epidermal-growth-factor-induced proliferation of astrocytes requires Egr transcription factors

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Summary

Stimulation of astrocytes with epidermal growth factor (EGF) induced proliferation and triggered the biosynthesis of the transcription factor Egr-1, involving the activation of the extracellular signal-regulated protein kinase (ERK) signaling pathway. No differences in the proliferation rate of astrocytes prepared from wild-type or Egr-1-deficient mice were detected. However, expression of a dominant-negative mutant of Egr-1 that interfered with DNA-binding of all Egr proteins prevented EGF-induced proliferation of astrocytes. Site-directed mutagenesis of two crucial cysteine residues within the zinc finger DNA-binding domain revealed that DNA-binding of the Egr-1 mutant was essential to inhibit proliferation of EGF-stimulated astrocytes. Expression of NAB2 (a negative co-regulator of Egr-1, Egr-2 and Egr-3) or a dominant-negative mutant of Elk-1 (a key regulator of Egr-1 biosynthesis) abolished EGF-induced proliferation of astrocytes. Chromatin

immunoprecipitation experiments showed that Egr-1, Egr-2 and Egr-3 bound to the gene expressing basic fibroblast growth factor (bFGF) in EGF-stimulated astrocytes. Egr-2 and Egr-3 also interacted with the *bFGF* gene in EGF-stimulated astrocytes prepared from Egr-1-deficient mice, indicating that loss of Egr-1 is compensated by other Egr proteins. Together, these data show that Egr transcription factors are essential for conversion of the mitogenic signal of EGF into a proliferative response.

Supplementary material available online at
<http://jcs.biologists.org/cgi/content/full/122/18/3340/DC1>

Key words: Astrocyte, ERK, Egr-1, Elk-1, Proliferation, Basic fibroblast growth factor

Introduction

In the nervous system, a key feature of astrocyte reactivity is their proliferative response. Many types of insults to the central nervous system induce reactive gliosis, an astrocyte response that includes an increase in cell number, an extension of cellular processes, and an upregulation of glial fibrillary acidic protein (GFAP). Tissue culture studies showed that mitogens such as epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) stimulate DNA synthesis and proliferation in primary astrocytes and glioma cells (Kaufmann and Thiel, 2001; Riboni et al., 2001). Mitogenic stimulation of astrocytes with EGF and other mitogens leads to an activation of extracellular signal-regulated protein kinase (ERK, also known as MAPK) (Tournier et al., 1994; Kaufmann et al., 2001; Wang et al., 2002). The phosphorylated form of ERK translocates to the nucleus and induces a change in the genetic expression pattern of the cells, which is required for the proliferative response. One of the transcription factors that is synthesized as a result of ERK activation is the zinc finger protein Egr-1 (Thiel and Cibelli, 2002; Rössler et al., 2006). Originally, the gene expressing Egr-1 was discovered in a search for genes induced by growth factors (Lim et al., 1987; Christy et al., 1988; Lemaire et al., 1988; Sukhatme et al., 1988). Egr-1 preferentially binds to the GC-rich sequence 5'-GCGG/TGGGCG-3' (Christy and Nathans, 1989; Cao et al., 1993; Nakagama et al., 1995), with its DNA-binding domain comprised of three zinc finger motifs. The N-terminus of Egr-1 functions as an extended transcriptional activation domain (Thiel et al., 2000). In addition, an inhibitory domain between the activation domain and the DNA-binding domain has been identified that functions as

a binding site for two transcriptional co-repressors, NAB1 and NAB2. Both NAB1 and NAB2 block the biological activity of Egr-1 (Russo et al., 1993; Svaren et al., 1996; Thiel et al., 2000). The fact that Egr-1 and NAB2 expression is induced by the same stimuli, although on different time scales, suggests that expression of NAB2 functions as a negative feedback loop to reduce Egr-1 activity (Svaren et al., 1996).

A previous study using established human glioma cell lines revealed that EGF stimulation rapidly activated ERK, induced the biosynthesis of Egr-1, and triggered cell growth (Kaufmann and Thiel, 2001). In this study, it was concluded that Egr-1 may be an important 'late' component of the EGF-initiated signaling cascades and may function as a 'third messenger' connecting growth-factor stimulation with changes in gene transcription. Thus, induction of Egr-1 expression may be an integral part of the mitogenic pathway and continues the mitogenic signaling cascade via stimulation of the synthesis of growth factors or growth factor receptors. However, the proposed role for Egr-1 in controlling cell growth is largely based on the correlation between mitogenic responses and the induction of Egr-1 biosynthesis by mitogens. In addition, there are reports to the contrary, attributing to Egr-1 a growth inhibitory function in tumor cells, including gliomas (Huang et al., 1995; Calogero et al., 2001; Calogero et al., 2004).

The objective of this study was to show whether a causality exists between proliferation of astrocytes and the presence of Egr-1 transcriptional activity. The results show that Egr proteins are required to connect mitogenic stimulation with enhanced proliferation. In Egr-1-deficient mice, other Egr proteins compensate

for the loss of Egr-1. Moreover, we show that nuclear ERK and ternary complex factors are essential for stimulating proliferation in EGF-treated astrocytes.

Results

EGF stimulation of astrocytes triggers phosphorylation of ERK
Stimulation of the EGF receptor (a receptor tyrosine kinase) by its cognate ligand EGF activates the ERK signaling cascade. Using a phospho-specific antibody we showed that ERK2 is phosphorylated in astrocytes as a result of EGF stimulation (Fig. 1A). The activation of ERK by EGF was transient. Within 15 minutes of stimulation, phospho-ERK could be detected. ERK was mostly dephosphorylated and inactivated 45 minutes after stimulation.

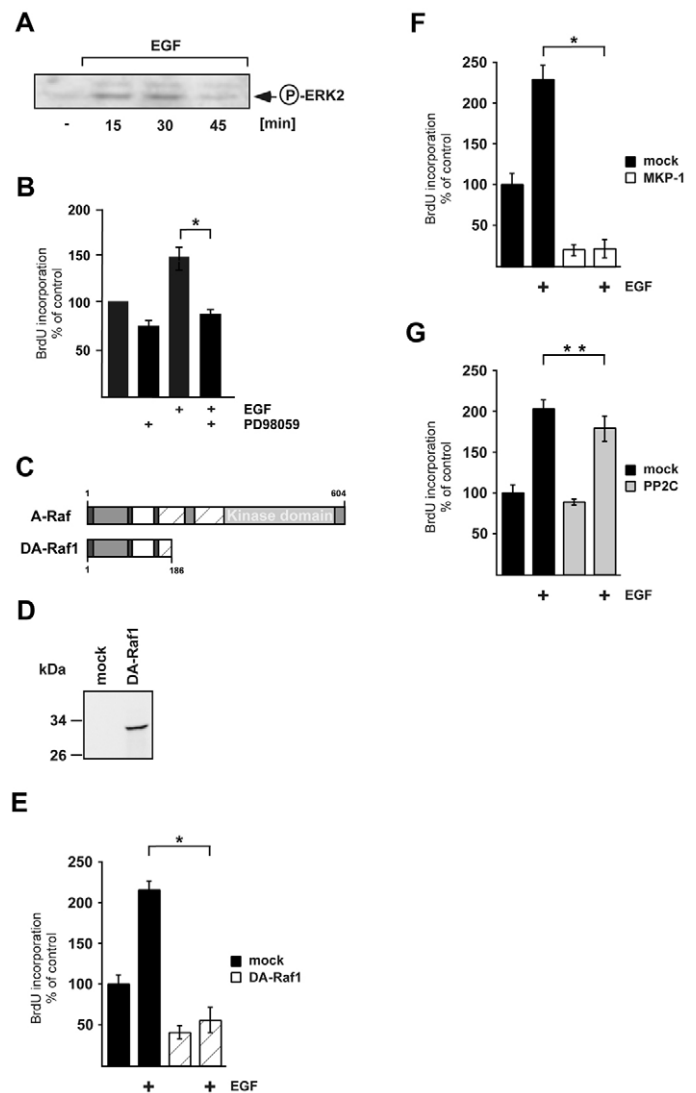
EGF-induced proliferation of astrocytes depends on the ERK signaling pathway

To evaluate astrocyte proliferation, we measured the incorporation of 5-bromo-2'-deoxyuridine (BrdU) into DNA as an indicator of DNA synthesis. Astrocytes were serum-starved for 24 hours and then treated with EGF for 24 hours. Fig. 1B shows that EGF stimulation of astrocytes induced a significant increase in BrdU incorporation (as estimated by an immunoassay) compared with unstimulated control cells, indicating that EGF functions as a mitogen for these cells. To elucidate the role of ERK in EGF-induced cell proliferation, we pre-incubated primary astrocytes with PD98059, a compound that inhibits the phosphorylation of the mitogen-activated protein kinase kinase (MAPK kinase, also known as MEK), before stimulation of the cells with EGF. Fig. 1B shows that PD98059 efficiently blocked the incorporation of BrdU into the DNA of EGF-treated astrocytes. Likewise, expression of DA-Raf1, a splicing form of A-Raf that functions as an antagonist of

the Ras-Raf-ERK signaling pathway, abrogated EGF-induced proliferation of astrocytes following stimulation with EGF (Fig. 1C-E). The phosphorylated and activated ERK translocates into the nucleus and changes the transcriptional program by phosphorylating transcriptional regulatory proteins. In the nucleus, MKP-1 is part of a negative feedback loop leading to the inactivation of ERK via dephosphorylation. Having shown that ERK activation is a key step in the mitogenic signaling cascade leading to proliferation of astrocytes, we tested whether overexpression of MKP-1 counteracts the EGF-induced proliferation of astrocytes. Fig. 1F reveals that expression of MKP-1 completely blocked the proliferative response in EGF-treated astrocytes. Moreover, the incorporation of BrdU into the DNA was even reduced in comparison to unstimulated cells, suggesting that MKP-1 dephosphorylated residual phosphorylated ERK in the nucleus. As a negative control, we expressed the protein phosphatase 2C (PP2C) in astrocytes. Fig. 1G shows that PP2C did not play any role in the mitogenic signaling cascade induced by EGF.

EGF stimulation of astrocytes induces the biosynthesis of Egr-1
Activation of the ERK signaling cascade is the major stimulus for induction of the biosynthesis of Egr-1 in many cellular systems (Thiel

Fig. 1. EGF stimulation triggers proliferation of astrocytes via activation of the ERK signaling pathway. (A) Phosphorylation of ERK2 in EGF-stimulated astrocytes. The cells were serum-starved for 24 hours and then treated with EGF (10 ng/ml) for the indicated periods. Whole-cell extracts were prepared and subjected to western blot analysis. The blot was incubated with an affinity-purified rabbit antibody directed against the phosphorylated active form of ERK2 (phospho-p42). (B) EGF induces proliferation of primary astrocytes. Cells were seeded in 96-well plates at a cell density of 1×10^4 cells per well in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS and incubated overnight. The serum concentration was lowered to 0% and the cells were incubated for another 24 hours. The effect of PD98059 on the proliferation of astrocytes was investigated in cells that had been pre-incubated for 1 hour with PD98059 (50 μ M) before stimulation. Cells were stimulated with EGF (10 ng/ml) for 24 hours and induction of DNA synthesis was measured by the incorporation of BrdU into the DNA. Incorporation of BrdU into the DNA was detected by immunoassay. Values shown are the mean \pm s.e.m. of quadruplicate samples from three experiments. *, values statistically significantly different from controls ($P < 0.0001$). (C) Modular structure of A-Raf and the DA-Raf1. (D) Expression of DA-Raf1 in lentiviral-infected astrocytes was detected using an antibody against the N-terminal myc-tag. Molecular-mass markers in kDa are shown on the left. (E) Astrocytes were infected with recombinant lentivirus encoding DA-Raf1. The transgene was expressed under the control of the human ubiquitin-C promoter. As a control astrocytes were infected with lentiviral stocks prepared with the lentiviral transfer vector pFUW (mock). Cells were treated with vehicle or EGF (denoted +) and induction of DNA synthesis was measured by the incorporation of BrdU into the DNA. *, values statistically significantly different from controls ($P < 0.0001$). (F,G) Overexpression of MKP-1, but not PP2C, blocks proliferation of astrocytes following EGF stimulation. Astrocytes were infected with a recombinant lentivirus encoding either MKP-1 (F) or PP2C (G). Mock-infected cells were analyzed as a control. Induction of DNA synthesis was measured by the incorporation of BrdU into the DNA. *, values statistically significantly different from controls ($P < 0.0001$); **, values statistically not significantly different from controls ($P > 0.05$). Graphs show means \pm s.e.m. of four samples from three experiments.



and Cibelli, 2002; Rössler et al., 2006). Primary astrocytes were serum-starved for 24 hours and then stimulated with EGF. The cells were harvested, nuclear extracts prepared and Egr-1 expression analyzed by immunoblotting. Egr-1 immunoreactivity was almost undetectable in unstimulated cells. EGF stimulation significantly increased its levels (Fig. 2A) with peak expression of Egr-1 occurring after 2 hours of stimulation. Preincubation of the cells with PD98059 blocked the biosynthesis of Egr-1 in EGF-stimulated astrocytes (Fig. 2B), indicating that ERK activation is a key event in the signaling cascades that connect EGF receptor stimulation with Egr-1 gene transcription. Thus, a correlation between EGF-induced proliferation and the biosynthesis of Egr-1 is observed in astrocytes.

Activation of chromatin-embedded Egr-1 promoter/luciferase reporter genes in EGF-stimulated astrocytes

The 5'-flanking region of the gene expressing Egr-1 contains five serum response elements (SREs) encompassing the consensus sequence CC[A/T]₆GG, also known as the CA₆G box. These motifs

are responsible for the induction of Egr-1 gene transcription by various extracellular signaling molecules (Thiel and Cibelli, 2002; Rössler et al., 2006). The SREs occur in two clusters in the Egr-1 promoter, a distal 5' cluster of three SREs and a proximal 3' cluster of two SREs. In addition, multiple binding sites for ternary complex factors (TCFs) are adjacent to the CA₆G boxes having the TCF consensus core sequence GGAA/T (Bauer et al., 2005).

To test the involvement of the proximal and distal SRE clusters of the Egr-1 promoter in mediating the responsiveness of the Egr-1 gene to EGF, we inserted two Egr-1 promoter/luciferase reporter genes into the chromatin of astrocytes using lentiviral gene transfer. The transfer vectors pFWEgr-1.1luc and pFWEgr-1.2luc, used to generate recombinant lentiviruses, encode Egr-1 promoter/luciferase reporter genes that contained 239 or 490 nucleotides of the human *EGR-1* gene 5'-upstream region, respectively, together with 235 nucleotides of the 5'-nontranslated region. The transfer vector pFWEgr-1SREluc encodes the luciferase reporter gene under the control of the proximal SREs #1 and #2 of the Egr-1 promoter. Fig. 2C shows a schematic representation of the integrated proviruses encoding Egr-1 promoter/luciferase reporter genes. Astrocytes were infected with recombinant lentiviruses and stimulated with vehicle or EGF. The addition of EGF significantly induced reporter gene transcription controlled by either 490 or 239 nucleotides of the human *EGR-1* promoter (Fig. 2D). The fact that the reporter genes #1 and #2 directed almost similar luciferase expression as a result of EGF stimulation indicates that the proximal, and not the distal SRE cluster, was important for the upregulation of Egr-1 transcription in EGF-stimulated cells.

This conclusion was confirmed in an experiment in which we directly measured the impact of the proximal SREs of the Egr-1 promoter on EGF-induced reporter gene transcription. Astrocytes were infected with a lentivirus encoding the luciferase gene under the control of the two proximal SREs of the Egr-1 promoter upstream of a minimal promoter (Fig. 2D). The transcriptional activity measured was no different to that of a reporter gene controlled by Egr-1 promoter sequences from -239 to +235. Thus, the most proximal SREs of the Egr-1 promoter are sufficient to transduce EGF signaling to the Egr-1 gene.

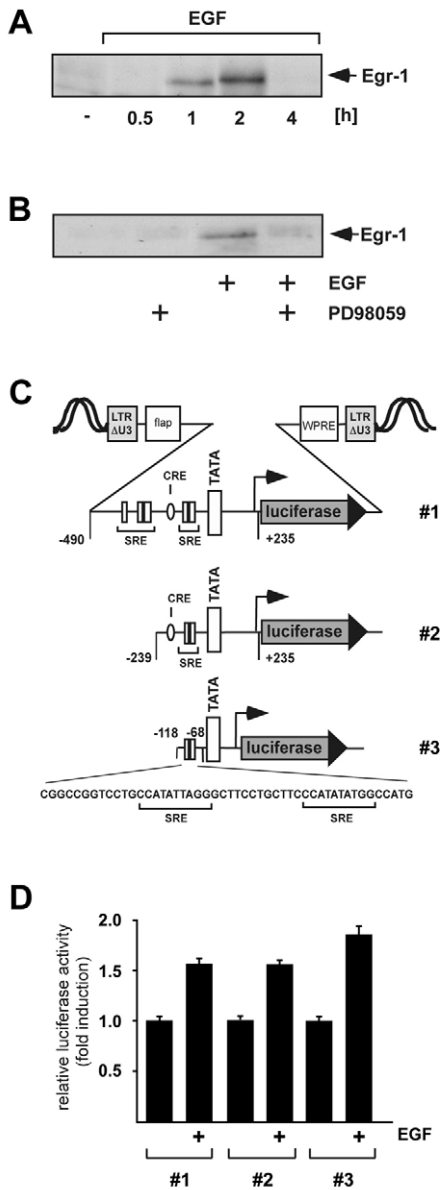


Fig. 2. Transcriptional upregulation of Egr-1 promoter/luciferase reporter genes and induction of Egr-1 biosynthesis in EGF-stimulated astrocytes. (A) Primary astrocytes were serum-starved for 24 hours and then treated with EGF (10 ng/ml) for the indicated periods. Nuclear extracts were prepared and subjected to western blot analysis. The blot was incubated with an antibody directed against Egr-1. (B) The effect of the MAPK kinase inhibitor PD98059 on the biosynthesis of Egr-1 was investigated in astrocytes that had been pre-incubated for 1 hour with PD98059 (50 μM) before stimulation with EGF (10 ng/ml). Nuclear extracts were prepared and subjected to western blot analysis. The blot was incubated with an antibody directed against Egr-1. (C) Schematic representation of integrated proviruses encoding Egr-1 promoter/luciferase reporter genes. The transfer vectors pFWEgr1.2luc (#1) and pFWEgr-1.1luc (#2) contain the sequences from -490 to +235 or from -239 to +235 derived from the human *EGR-1* gene. The transfer vector pFWEgr-1SREluc (#3) contains the two proximal SREs #1 and #2 of the Egr-1 promoter upstream of a minimal promoter. The important genetic elements within the Egr-1 regulatory region are depicted, including five SREs, and a CRE. The U3 region of the 5' LTR (long terminal repeat) of the transfer vector is deleted. The woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) and the HIV flap element are indicated. (D) Primary astrocytes were infected with recombinant lentiviruses prepared with the transfer vectors pFWEgr-1.1luc, pFWEgr-1.2luc or pFWEgr-1SREluc. The infected cells were treated with vehicle or EGF (denoted +) for 24 hours. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration. Graph shows means ± s.e.m. of four samples from three experiments.

Suppression of TCF activity prevents proliferation of astrocytes following EGF stimulation

Given the importance of SREs within the Egr-1 promoter, we next assessed the impact of TCF activation on the regulation of proliferation of astrocytes. To overcome the problem associated with redundancy of functions between the TCFs, we expressed a dominant-negative mutant of the TCF Elk-1, termed REST/Elk-1ΔC (Fig. 3A). This mutant retains the DNA-binding and SRF interaction domains, but lacks the C-terminal activation domain of Elk-1. REST/Elk-1ΔC additionally contains the N-terminal repression domain of the transcriptional repressor REST (Thiel et al., 1998), a FLAG epitope for immunological detection and a nuclear localization signal (NLS). Nuclear proteins of mock-infected astrocytes or astrocytes infected with a REST/Elk-1ΔC encoding lentivirus were fractionated by SDS-PAGE. The fusion protein was identified by western blot analysis using antibodies targeting the FLAG epitope (Fig. 3B). Fig. 3C shows that expression of REST/Elk-1ΔC prevented the proliferation of astrocytes that had been stimulated with EGF. We conclude that TCF activation is essential for conversion of mitogenic stimulation by EGF into a proliferative response.

Suppression of ATF2 activity did not interfere with the mitogenic signaling cascade induced by EGF in astrocytes

In addition to five SREs, the Egr-1 promoter contains a cyclic AMP response element (CRE) encompassing the sequence 5'-TCACGTCA-3'. It had been suggested that the CRE may control

Egr-1 gene transcription via activating transcription factor 2 (ATF2) activation by p38 stress-activated protein kinases (Rolli et al., 1999). To assess the role of ATF2 in the regulation of proliferation in astrocytes, we expressed a dominant-negative mutant of ATF2 termed ATF2ΔN that lacked the N-terminal regulatory region and the transcriptional activation domain. The modular structure of this mutant is depicted in Fig. 3D. The biological activity of this mutant has been demonstrated (Steinmüller and Thiel, 2003; Mayer et al., 2008). Fig. 3E shows that the dominant-negative mutant ATF2ΔN was synthesized in astrocytes following infection with recombinant lentiviruses. Fig. 3F reveals that expression of ATF2ΔN did not impair the proliferative response of astrocytes after stimulation with EGF.

Astrocytes derived from wild-type or Egr-1-deficient mice show a similar proliferation response to EGF

So far, we have shown that Raf, ERK and TCF activation are essential for the mitogenic responses in astrocytes. ERK and TCF activation are additionally required in many cellular systems for induction of the biosynthesis of Egr-1 (Thiel and Cibelli, 2002; Bauer et al., 2005). Thus, we can correlate the mitogenic responses and the induction of Egr-1 biosynthesis by mitogens. We prepared astrocytes from wild-type and Egr-1-deficient mice in order to investigate whether a causality exists between proliferation of astrocytes and the presence of Egr-1 transcriptional activity. Fig. 4A reveals that there were no differences between the EGF-induced growth rates of astrocytes derived from either wild-type or Egr-1-deficient mice.

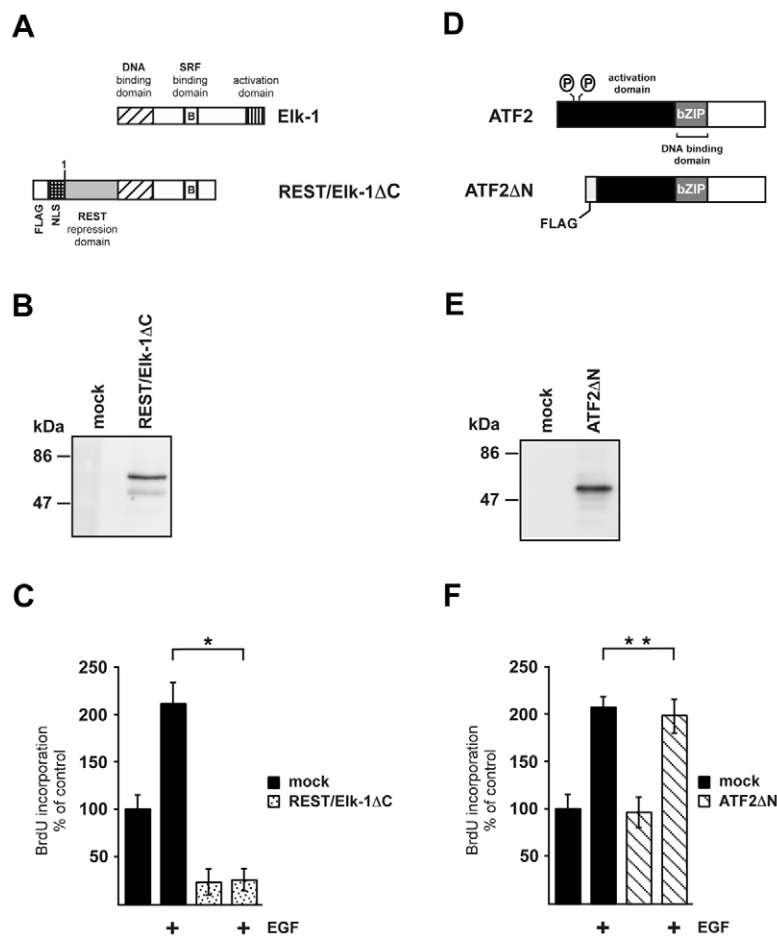


Fig. 3. Role of TCF Elk-1 and ATF2 in EGF-induced proliferation of astrocytes. (A) Schematic representation of wild-type Elk-1 and the dominant-negative mutant REST/Elk-1ΔC. The DNA-binding domain is located on the N-terminus. The transcriptional activation domain is located on the C-terminus. A regulatory domain lies within this transcriptional activation domain encompassing the key phospho-acceptor sites S383 and S389. Elk-1 binds with its B-domain to SRF, allowing the formation of the ternary Elk-1-SRF complex. The B-domain also couples the C-terminal phosphorylation of Elk-1 with enhanced DNA binding via the ETS domain. The dominant-negative mutant REST/Elk-1ΔC lacks the phosphorylation-regulated activation domain, but retains the DNA- and SRF-binding domains. The truncated Elk-1 is expressed as a fusion protein together with a transcriptional repression domain derived from the transcriptional repressor REST. (B) Western blot analysis of astrocytes either mock-infected or infected with a recombinant lentivirus encoding REST/Elk-1ΔC. Western blots were probed with an antibody against the FLAG tag. (C) Astrocytes were infected with a recombinant lentivirus encoding REST/Elk-1ΔC and treated with vehicle or EGF (denoted +). As a control, mock-infected cells were analyzed. Induction of DNA synthesis was measured by the incorporation of BrdU into the DNA. *, values statistically significantly different from controls ($P < 0.0001$). (D) Modular structure of ATF2 and the dominant-negative form ATF2ΔN. The dominant-negative mutant retains the basic region leucine zipper domain (bZIP) responsible for dimerization and DNA binding, but lacks the N-terminal transcriptional activation domain. (E) Western blot analysis of astrocytes infected with a lentivirus encoding ATF2ΔN. As a control, mock-infected cells were analyzed. Western blots were probed with the antibody directed against the FLAG epitope. Molecular-mass markers in kDa are shown on the left. (F) Astrocytes were infected with a recombinant lentivirus encoding ATF2ΔN and stimulated with EGF as indicated. As a control, mock-infected cells were analyzed. Induction of DNA synthesis was measured by the incorporation of BrdU into the DNA. **, values statistically not significantly different from controls ($P > 0.05$). Graphs show means \pm s.e.m. of four samples from three experiments.

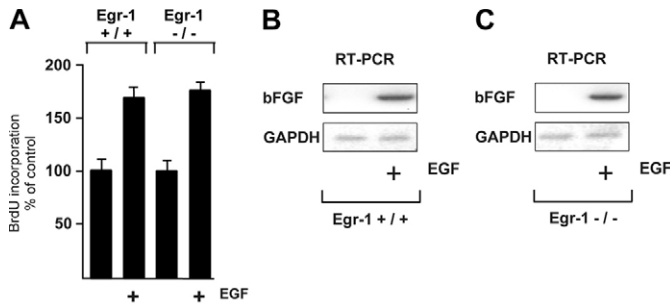


Fig. 4. Proliferation and bFGF synthesis in EGF-stimulated astrocytes derived from wild-type and Egr-1-deficient mice. (A) EGF induces proliferation of primary astrocytes derived from wild-type and Egr-1 knockout mice. Cells were stimulated with EGF (10 ng/ml), and induction of DNA synthesis was measured as described in the legend to Fig. 1. The incorporation of BrdU into the DNA was detected by immunoassay. Graphs show means \pm s.e.m. of four samples from three experiments. (B,C) Upregulation of *bFGF* mRNA concentrations in astrocytes prepared from wild-type (B) or Egr-1-deficient mice (C) as a result of EGF stimulation. Astrocytes were treated with vehicle or EGF (denoted +) for 2 hours. Total RNA was isolated, and the cDNA analyzed by RT-PCR using primers to detect *bFGF* mRNA.

EGF stimulation induces the expression of bFGF in astrocytes derived from wild-type or Egr-1-deficient mice. bFGF is a mitogen for astrocytes, and elevated bFGF levels have been detected in human glioma cells (Morrison, 1991; Holland and Varmus, 1998; Riboni et al., 2001). Interestingly, the human *bFGF* gene is transactivated by Egr-1 (Wang et al., 1997; Biesiada et al., 1998). In the murine *bFGF* gene, an Egr-1 binding motif was detected in the 5'-untranslated region (supplementary material Fig. S1). Thus, Egr-1 may regulate growth of astrocytes via transactivation of the gene encoding bFGF and the subsequent synthesis of bFGF. We therefore assessed the bFGF levels in EGF-stimulated astrocytes derived from wild-type and Egr-1 knockout mice. Fig. 4B,C shows that EGF treatment induced the expression of bFGF, regardless of the presence or absence of Egr-1 in the cells.

Expression of DA-Raf1, MKP-1 or REST/Elk-1 Δ C impairs proliferation of EGF-stimulated astrocytes prepared from Egr-1-deficient mice

The experiments performed with astrocytes prepared from Egr-1-deficient mice suggests that either Egr-1 plays no role in the control of astrocyte proliferation, or that other members of the Egr family compensate for the loss of Egr-1. Before addressing this problem, we confirmed the importance of Raf, nuclear ERK and TCF activation in this system. We prepared astrocytes from Egr-1-deficient mice and infected the cells with lentiviruses encoding either DA-Raf1 (Fig. 5A), MKP-1 (Fig. 5B), or REST/Elk-1 Δ C (Fig. 5D). The results show that proliferation of astrocytes was completely blocked after stimulation with EGF. Likewise, expression of PP2C or ATF2 Δ N did not change the proliferative response of EGF-treated astrocytes (Fig. 5C,E). These data indicate that activation of ERK, translocation of ERK into the nucleus, and subsequent activation of TCF are key events in the regulation of astrocyte proliferation.

Expression of a dominant-negative mutant of Egr-1 blocks the proliferation of astrocytes after EGF stimulation

To directly address the problem of compensation between the members of the Egr family, we expressed a dominant-negative mutant of Egr-1, Egr-1/Zn, that encodes the zinc finger DNA-binding domain of Egr-1 (Fig. 6A). In comparison with Egr-1, the

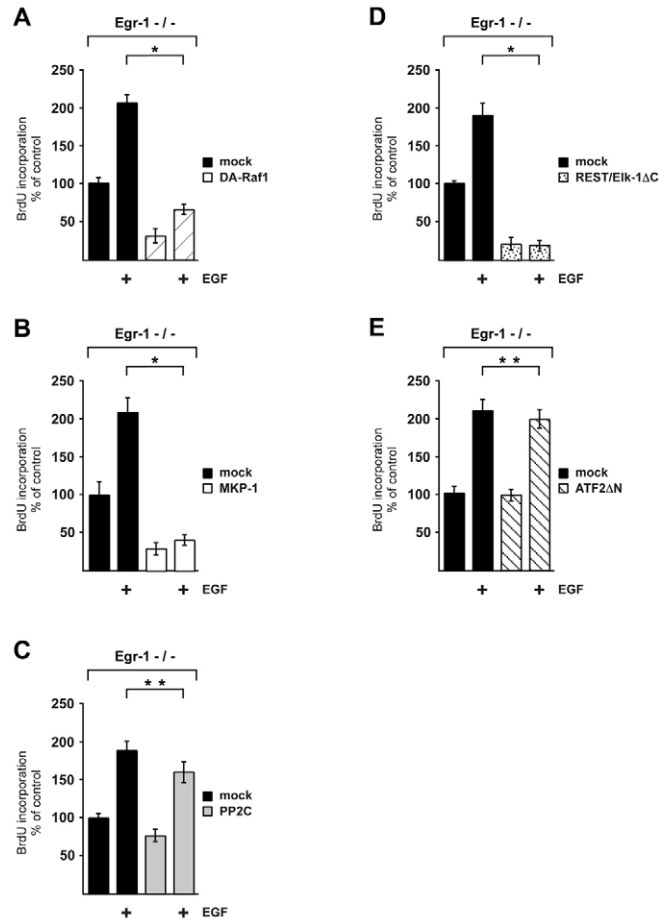


Fig. 5. Expression of DA-Raf1, MKP-1 or a dominant-negative mutant of Elk-1 prevents EGF-induced proliferation of Egr-1-deficient astrocytes. Astrocytes were infected with a recombinant lentivirus encoding either DA-Raf1 (A), MKP-1 (B), PP2C (C), REST/Elk-1 Δ C (D) or ATF2 Δ N (E). As a control, mock-infected cells were analyzed. Cells were treated with vehicle or EGF (denoted +). Induction of DNA synthesis was measured by the incorporation of BrdU into the DNA. *, values statistically significantly different from controls ($P < 0.0001$); **, values statistically not significantly different from controls ($P > 0.05$). Graphs show means \pm s.e.m. of four samples from three experiments.

mutant lacks the N-terminal transcriptional activation domain and the binding site for the transcriptional corepressors NAB1 and NAB2. Egr-1/Zn additionally contains a FLAG epitope for immunological detection and an NLS. Nuclear proteins of mock-infected astrocytes or astrocytes infected with an Egr-1/Zn-encoding lentivirus were fractionated by SDS-PAGE. The protein was identified by western blot analysis using antibodies targeting the FLAG epitope. Fig. 6B shows that the mutant protein was synthesized as expected. Fig. 6C,D reveal that expression of Egr-1/Zn blocked the proliferation of EGF-stimulated astrocytes, derived either from wild-type (Fig. 6C) or Egr-1 knockout mice (Fig. 6D). These data indicate that the Egr proteins control proliferation of astrocytes following stimulation of the cells with EGF.

DNA-binding activity of the Egr-1/Zn mutant is essential to block proliferation of EGF-stimulated astrocytes

The dominant-negative Egr-1/Zn mutant inhibits DNA binding of wild-type Egr proteins by blocking the cognate GC-rich binding sites for DNA binding. Thus, an impairment of DNA binding should

reduce or abolish the biological activity of the mutant. Therefore, we mutated crucial cysteine residues at positions 368 and 396 of zinc fingers 2 and 3, respectively, to serine residues (Fig. 7A). Similar mutations, introduced at the zinc finger domain of Sp1, abolished DNA binding (Lee et al., 2005). Electrophoretic mobility shift assays showed that the mutated Egr-1/Zn protein did not bind

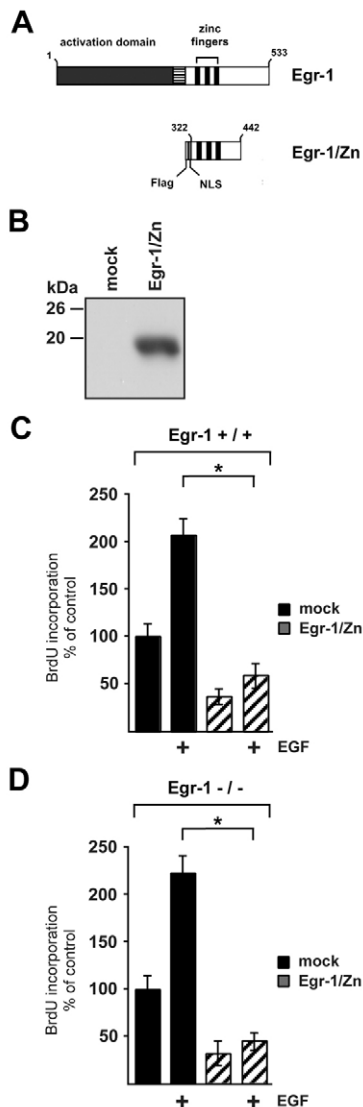


Fig. 6. Expression of a dominant-negative mutant of Egr-1 prevents EGF-induced proliferation of astrocytes. (A) Schematic representation of the modular structure of Egr-1 and Egr-1/Zn. The N-terminal activation domain and the zinc finger DNA-binding domain of Egr-1 are shown. The Egr-1 mutant Egr-1/Zn retains the zinc finger domain responsible for DNA binding, but lacks the transcriptional activation domain and the binding domain for NAB1 and NAB2. Egr-1/Zn also contains a triple FLAG tag and a nuclear localization signal (NLS) in its N-terminus. (B) Western blot analysis of astrocytes either mock-infected or infected with a recombinant lentivirus encoding Egr-1/Zn. Western blots were probed with an antibody against the FLAG tag. Molecular-mass markers in kDa are shown on the left. (C,D) Astrocytes prepared from wild-type (C) or Egr-1-deficient mice (D) were either mock-infected or infected with a recombinant lentivirus encoding Egr-1/Zn. Cells were treated with vehicle or 10 ng/ml EGF (denoted +) as indicated. Induction of DNA synthesis was measured by the incorporation of BrdU into the DNA. *, values statistically significant differently from controls ($P < 0.0001$). Graphs show means \pm s.e.m. of four samples from three experiments.

anymore to cognate Egr-1 binding motifs, derived from either the Egr-1 or the synapsin I promoter (Fig. 7B). Egr-1/Zn and the mutated version of Egr-1/Zn were synthesized in astrocytes following infection with recombinant lentiviruses (Fig. 7C). An analysis of EGF-induced proliferation revealed that these mutations of the DNA-binding domain reduced or abolished the activity of Egr-1/Zn to suppress the mitogenic signaling cascade in astrocytes (Fig. 7D,E). These data clearly show that transcriptional activation of Egr target genes is essential to couple EGF stimulation with enhanced proliferation of the cells.

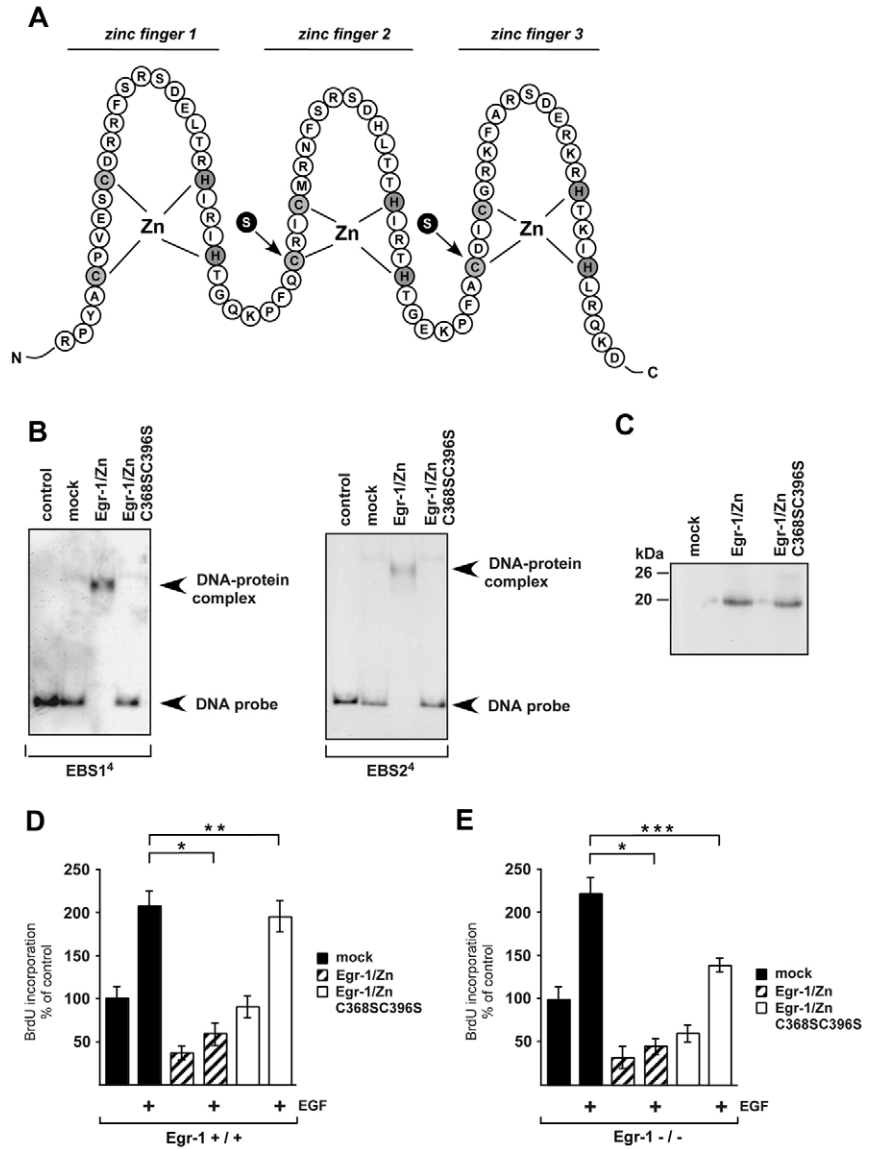
Expression of the negative cofactor NAB2 blocks the proliferation of astrocytes following EGF stimulation

To confirm the previous results, we expressed a FLAG-tagged NAB2 protein in astrocytes via lentiviral gene transfer. NAB2 is a transcriptional co-repressor that does not bind to DNA, but interacts with Egr-1, Egr-2 and Egr-3, leading to impairment of the transcriptional activity of the Egr transcription factors. We analyzed nuclear proteins derived from mock-infected astrocytes or astrocytes infected with a FLAG-NAB2-encoding lentivirus by SDS-PAGE. The protein was identified by western blot analysis using antibodies targeting the FLAG epitope. Fig. 8A shows that FLAG-NAB2 was synthesized as expected. Fig. 8B,C show that expression of NAB2 blocked the proliferation of EGF-stimulated astrocytes, derived either from wild-type (Fig. 8B) or Egr-1 knockout mice (Fig. 8C). We conclude that Egr proteins are essential for transforming the mitogenic signal into a proliferative response. Moreover, Egr target genes need to be activated for continuation of the mitogenic signaling cascade.

Egr-1, Egr-2 and Egr-3 bind under physiological conditions to the gene expressing bFGF in EGF-stimulated astrocytes

The previous results suggest that other Egr proteins compensate for the loss of Egr-1 in Egr-1-deficient mice. To test this assumption we analyzed the stimulus-induced binding of Egr proteins to the gene expressing bFGF. Fig. 9A shows a schematic representation of the 5'-upstream region of the murine *bFGF* gene, including the Egr-1 binding site in the 5'-untranslated region and the location of the primers used for PCR. The *bFGF* gene is in an open configuration in astrocytes, as shown by the fact that it is embedded into a nucleosomal context with histone H3 molecules carrying trimethylated lysine 4 (Fig. 9B), which is a marker of actively transcribed genes. Next, cross-linked and sheared chromatin, prepared from unstimulated astrocytes and astrocytes stimulated with EGF, was immunoprecipitated with antibodies directed against either Egr-1, Egr-2 or Egr-3. Fig. 9C (upper panel) shows that Egr-1, Egr-2 and Egr-3 bound to the regulatory region of the *bFGF* gene, but only when the cells had been stimulated with EGF. No binding of Egr proteins was detected to a distal region of the *bFGF* gene (Fig. 9C, lower panel). Naturally, no Egr-1 binding to the *bFGF* gene could be detected in astrocytes prepared from Egr-1-deficient mice. However, binding of Egr-2 and Egr-3 to the *bFGF* gene was similar to that in astrocytes prepared from wild-type mice (Fig. 9D).

The results obtained in expression experiments involving Egr-1/Zn and Egr-1/ZnC368SC396S suggested that Egr-1/Zn binds to Egr target genes and competes with wild-type Egr proteins for DNA binding. Chromatin immunoprecipitation (ChIP) experiments confirmed that Egr-1/Zn bound to the *bFGF* gene under physiological conditions (Fig. 9E, upper panel). In addition, ChIP experiments were performed with astrocytes that had been infected with a lentivirus encoding Egr-1/Zn and stimulated with EGF. Fig.



9E (lower panel) shows that expression of Egr-1/Zn prevented binding of Egr-1, Egr-2 and Egr-3 to the *bFGF* gene. Thus, the Egr-1 mutant binds to the 5'-untranslated region of the *bFGF* gene and inhibits DNA binding of wild-type Egr proteins.

Discussion

Since the discovery of the Egr-1 gene as an 'early growth response gene' (Sukhatme et al., 1988), research has been directed towards a function of Egr-1 in growth and proliferation. In fact, induction of Egr-1 gene transcription was monitored in many cell types in response to mitogens, and a direct role of Egr-1 in controlling proliferation has been proposed for astrocytes, glioma cells, glomerular mesangial cells, keratinocytes and T-cells (Perez-Castillo et al., 1993; Biesiada et al., 1996; Hofer et al., 1996; Kaufmann and Thiel, 2002; Rössler and Thiel, 2004). Egr-1 biosynthesis is strongly stimulated by activation of ERK (Kaufmann et al., 2001), the protein kinase that is activated by mitogens. The fact that genes encoding growth factors such as insulin-like growth factor II, platelet-derived growth factors A and B, and transforming growth factor β 1 have been identified as target genes of Egr-1 (Khachigian

et al., 1995; Svaren et al., 2000) suggests that Egr-1 continues the mitogenic signaling cascade via stimulation of growth factor synthesis. However, it is necessary to emphasize that the proposed role for Egr-1 in controlling cell growth is largely based on the correlation between mitogenic responses and the induction of Egr-1 biosynthesis by mitogens. The use of cells derived from Egr-1-deficient mice has helped to demonstrate a causal relationship between Egr-1 expression and cellular growth in a few cases. Delayed hepatocellular mitotic progression and impaired liver regeneration, as well as impairment of tumor progression of the prostate, has been observed (Abdulkadir et al., 2001; Liao et al., 2004). The objective of this study was to show whether a causality exists between proliferation of astrocytes and the presence of Egr-1 transcriptional activity.

The analysis of astrocytes derived from wild-type and Egr-1-deficient mice revealed that there were no differences in the proliferation of the cells following EGF stimulation. We hypothesized that other members of the Egr family, which all bind to a similar GC-rich target sequence, compensated for the loss of Egr-1. To overcome the problem associated with redundancy of

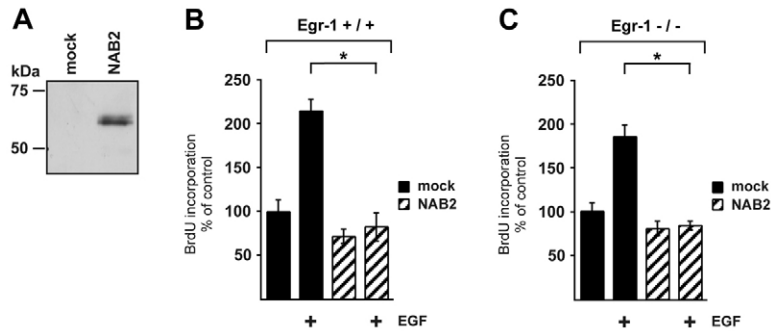
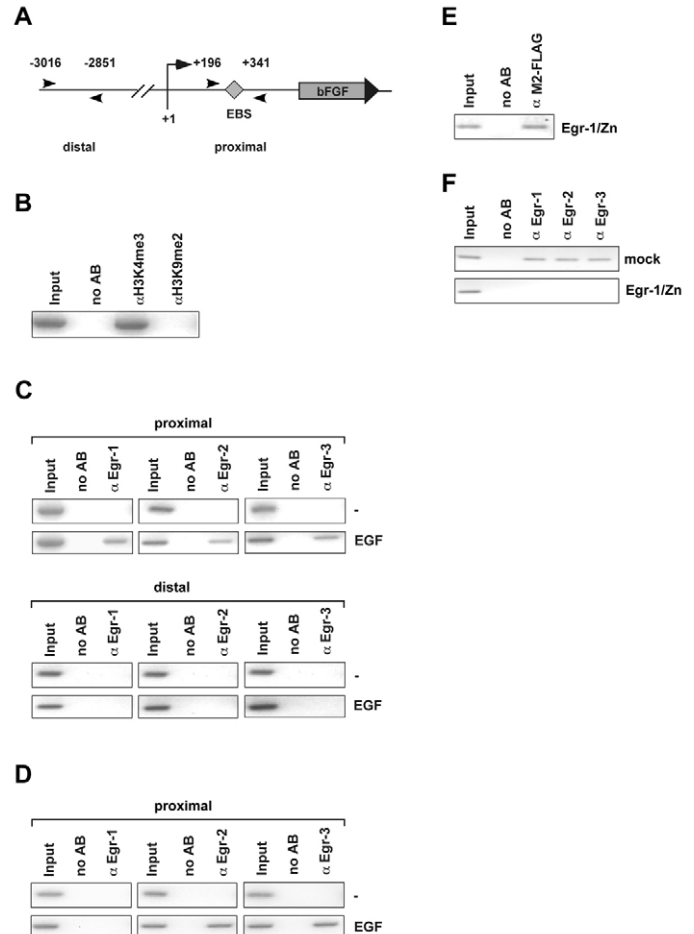


Fig. 8. Expression of NAB2 prevents EGF-induced proliferation of astrocytes. (A) Western blot analysis of astrocytes either mock-infected or infected with a recombinant lentivirus encoding FLAG-tagged NAB2. Western blots were probed with an antibody against the FLAG tag. Molecular-mass markers in kDa are shown on the left. (B,C) Astrocytes prepared from wild-type (B) or Egr-1-deficient mice (C) were either mock-infected or infected with a recombinant lentivirus encoding NAB2. Cells were treated with vehicle or 10 ng/ml EGF (denoted +) as indicated. Induction of DNA synthesis was measured by the incorporation of BrdU into the DNA. *, values statistically significantly different from controls ($P < 0.0001$). Graphs show means \pm s.e.m. of four samples from three experiments.

functions between the Egr proteins, we chose a dominant-negative approach. Expression of a dominant-negative mutant of Egr-1 that blocked the cognate binding sites of all Egr proteins abrogated proliferation of astrocytes following EGF stimulation, indicating that Egr activity is required to transform a mitogenic signal into a proliferative response. Furthermore, we showed that DNA binding of the Egr-1/Zn mutant is essential for interference with the mitogenic signaling in astrocytes, indicating that transcriptional

upregulation of Egr target genes is essential for connecting EGF stimulation with proliferation of the cells. Likewise, proliferation of astrocytes was prevented in cells overexpressing NAB2. This cofactor blocks transcription mediated by Egr-1, Egr-2 and Egr-3, all of which contain binding sites for NAB2 (O'Donovan et al., 1999). Together, these data establish a mechanistic link between Egr activity and proliferation of astrocytes and indicate that loss of Egr-1 in astrocytes prepared from Egr-1 knockout mice is

Fig. 9. ChIP experiments reveal binding of Egr-1, Egr-2 and Egr-3 to the *bFGF* gene in EGF-stimulated astrocytes. (A) Schematic representation of the *bFGF* gene. The start site of transcription is shown as well as the binding site for Egr proteins in the 5'-untranslated region. In addition, the locations of the fragments amplified by RT-PCR are shown. (B) Epigenetic modification of the *bFGF* gene in astrocytes. ChIPs were performed with anti-dimethylated H3K9 or anti-trimethylated H3K4 antibodies. Immunoprecipitated chromatin fragments were amplified with primers encompassing the Egr-1 binding site within the regulatory regions of the *bFGF* gene. As a negative control, no primary antibody was added (no AB). An aliquot of the total chromatin was also examined by RT-PCR (Input). (C) Binding of Egr-1, Egr-2 and Egr-3 to the regulatory region of the *bFGF* gene under physiological conditions. Cross-linked and sheared chromatin prepared from unstimulated astrocytes (denoted -) and astrocytes stimulated with EGF (10 ng/ml) was immunoprecipitated with antibodies directed against Egr-1, Egr-2 or Egr-3. Immunoprecipitated chromatin fragments were amplified with primers encompassing the Egr-1 binding site within the regulatory regions of the *bFGF* gene (proximal). For comparison, a distal genomic fragment was amplified as well (distal). As a negative control, no primary antibody was added (no AB). An aliquot of the total chromatin was also examined by PCR (Input). (D) Binding of Egr-2, and Egr-3 to the regulatory region of the *bFGF* gene in astrocytes prepared from Egr-1-deficient mice. Cross-linked and sheared chromatin prepared from unstimulated astrocytes (denoted -) and astrocytes stimulated with EGF (10 ng/ml) was immunoprecipitated with antibodies directed against Egr-1, Egr-2 or Egr-3. Immunoprecipitated chromatin fragments were amplified with primers encompassing the Egr-1 binding site within the regulatory region of the *bFGF* gene. As a negative control, no primary antibody was added (no AB). An aliquot of the total chromatin was also examined by PCR (Input). (E) The Egr-1 mutant Egr-1/Zn binds to the regulatory region of the *bFGF* gene. Astrocytes were infected with a recombinant lentivirus encoding Egr-1/Zn. ChIP was performed with M2-agarose to selectively precipitate the FLAG-tagged Egr-1/Zn mutant. Immunoprecipitated chromatin fragments were amplified with primers encompassing the Egr-1 binding site within the regulatory region of the *bFGF* gene. As a negative control, no primary antibody was added (no AB). An aliquot of the total chromatin was also examined by RT-PCR (Input). (F) ChIP was performed with chromatin isolated from astrocytes that had been mock-infected or infected with a lentivirus encoding Egr-1/Zn. The cells had been stimulated with EGF (10 ng/ml, 2 hours). Cross-linked and sheared chromatin was immunoprecipitated with antibodies directed against either Egr-1, Egr-2 or Egr-3. As a negative control, no primary antibody was added (no AB). An aliquot of the total chromatin was also examined by PCR (Input).



compensated by other Egr proteins. This view was corroborated by ChIP experiments. In Egr-1-deficient astrocytes, Egr-2 and Egr-3 still bound to the *bFGF* gene when the cells had been stimulated with EGF, thus explaining the increased levels of *bFGF* mRNA following EGF stimulation in the absence of Egr-1 expression. We conclude that the proliferation of astrocytes following stimulation with EGF is regulated by Egr transcription factors. The *bFGF* gene was analyzed as a known Egr-1 target gene and as a mitogen for astrocytes (supplementary material Fig. S2). Pharmacological inhibition of the bFGF receptor revealed that bFGF is not an essential intermediate for EGF-induced cell proliferation (supplementary material Fig. S2). Thus, the identification of Egr target genes encoding growth regulatory proteins will be the aim of future studies.

The analysis of Egr-1 promoter/luciferase reporter genes revealed that the most proximal SREs of the Egr-1 promoter are sufficient for transduction of EGF signaling to the Egr-1 gene. Transcriptional activation of Egr-1 is often preceded by an activation of Elk-1, a TCF. Phosphorylation of Elk-1 connects the ERK signaling cascade with SRE-mediated transcription. Elk-1 and other TCFs contact DNA and the serum response factor (SRF) to exhibit biological activity. Genetic inactivation of Elk-1 or other TCFs in transgenic mice revealed minimal changes of the phenotype (Ayadi et al., 2001; Cesari et al., 2004; Costello et al., 2004), suggesting that functional redundancy may exist. Therefore, we have assessed the necessity of TCF activation for EGF-induced proliferation of astrocytes by using a dominant-negative version of Elk-1 in loss-of-function experiments. Due to the binding to DNA and SRF, the Elk-1 mutant REST/Elk-1ΔC most probably also inhibits the activity of two other TCFs, SAP-1 and SAP-2. Expression of this Elk-1 mutant revealed that TCF activation is essential for the connection of mitogen stimulation and proliferation of astrocytes. This observation is in accordance with a previous study showing that TCF activation is required for proliferation of fibroblasts (Vickers et al., 2004).

Finally, we have shown that ERK activation is a key step in the mitogenic signaling cascade leading to proliferation of astrocytes. Expression of DA-Raf1, an antagonist of the Ras-Raf-ERK signaling pathway, or incubation of the cells with the compound PD98059 interfered with the mitogenic signaling cascade. Similarly, overexpression of MAPK phosphatase 1 (MKP-1; an enzyme that catalyzes the dephosphorylation and inactivation of ERK in the nucleus) blocked cell growth in EGF-stimulated astrocytes, indicating that ERK needs to translocate to the nucleus in order to stimulate cell proliferation. Thus, MKP-1 functions as a nuclear shut-off device that interrupts the mitogenic signaling cascade in the nucleus.

Materials and Methods

Mice

Transgenic mice containing an inactivated Egr-1 gene have been described (Topilko et al., 1997). These mice have an insertion of a LacZ-neo cassette between the promoter and the coding region that inactivates the Egr-1 gene. Moreover, an additional frame-shift mutation has been introduced at the beginning of the DNA-binding region. Homozygous *Egr-1*^{-/-} mice were produced by mating of heterozygous *Egr-1*^{+/-} mice.

Preparation and culture of primary astrocytes

Primary astrocyte cultures were prepared from the forebrain of 1- to 2-day-old neonatal mice. Excised brains were placed in buffer containing 137 mM NaCl, 5.4 mM KCl, 197 μM Na₂HPO₄, 35 μM KH₂PO₄, 5 mM glucose, and 58 mM saccharose (pH 6.5) on ice. Olfactory lobes and cerebellum were removed. The cerebral hemispheres were separated, and meninges and blood vessels removed. The cortices were cut into pieces and incubated in buffer containing 0.5% trypsin at 37°C for 10 minutes. The cell suspension was transferred to basal Eagle's medium (BME) (Sigma #B9638)

containing 10% fetal calf serum (FCS). Further dissection of the tissue was obtained by aspiration of the suspension through a narrowed Pasteur pipette. Astrocytes were used for the experiments after 7-10 days in culture.

Proliferation assays

Before stimulation, cells were seeded in 96-well plates at a cell density of 1 × 10⁴ cells per well and incubated for 24 hours. Cells were incubated for 24 hours in medium without serum before stimulation. Stimulation with EGF (Biochrom, Berlin, Germany, #W1325.950.500; used at a concentration of 10 ng/ml) and bFGF (Biochrom, #W1370950050; used at a concentration of 10 ng/ml) was performed as indicated. The MAPK kinase inhibitor PD98059 was purchased from Axxora (Lörrach, Germany, #385-023), dissolved in DMSO and used at a concentration of 50 μM. The bFGF receptor inhibitor PD173074 (Skaper et al., 2000) was purchased from Calbiochem (Bad Soden, Germany, #341607), dissolved in DMSO and used at a concentration of 10 nM. Induction of DNA synthesis was measured by the incorporation of the pyrimidine analog BrdU instead of thymidine into the DNA of proliferating cells using the Cell Proliferation ELISA kit from Roche Diagnostics (Mannheim, Germany, #1647229). The assay was performed according to the instruction manual with minor modifications. The labeling time with BrdU was 4 hours and incubation with the anti-BrdU peroxidase-conjugated antibody was for 90 minutes. Peroxidase activity was determined spectrophotometrically as described in the instruction manual. Each experiment was performed at least four times and the means ± s.e.m. (Student's *t*-test) are depicted.

Lentiviral gene transfer

The lentiviral transfer vectors pFUW-REST/Elk-1ΔC, pFUWATF2ΔN, pFUW-MKP-1, pFUW-mycPP2C, and pFUWEgr-1/Zn have been described elsewhere (Stefano et al., 2006; Bauer et al., 2007; Mayer et al., 2008). Site-directed mutagenesis was performed with the Quick-change kit from Stratagene (La Jolla, CA, #210518), using plasmid pFUWEgr-1/Zn as template and the oligonucleotides 5'-GCCAGAAG-CCCTTCCAGTCTCGAATCTGCATG-3', 5'-CATGCAGATTCGAGACTGGAA-GGGCTTCTGGC-3', 5'-GCGAGAAGCCTTTGGCCTCTGACATTTGTGGGAG-3', and 5'-CTCCACAAATGTCAGAGGCAAAAGGCTTCTCGC-3' as primers. To construct a FLAG-tagged NAB2 expression vector, we cloned an Ecl136II/XmnI-fragment, derived from plasmid pCMV-SPORT6.1-mNAB2 (accession #BC045138, obtained from the RZPD, Deutsches Ressourcenzentrum für Genomforschung) into the filled-in EcoRI site of plasmid p3XFLAG-CMV-7.1 (Sigma), generating plasmid pCMV-FLAGmNAB2. This plasmid was cut with Ecl136II and EcoRV. The fragment was inserted into the HpaI site of plasmid pFUW (Lois et al., 2002), generating the lentiviral transfer vector pFUWmNAB2. The lentiviral transfer vector pFUW-mycDA-Raf1 was generated via cloning of a filled-in XbaI-fragment, derived from plasmid pEF-BOS/Myc-DA-Raf1 (Yokoyama et al., 2007), into plasmid pFUW. The lentiviral transfer vectors pFWEgr-1.1luc and pFWEgr-1.2luc, encoding the luciferase reporter gene under the control of 239 or 490 nucleotides of the human *EGR-1* 5'-flanking region, have been described elsewhere (Rössler et al., 2008). The transfer vector pFWEgr-1SREluc encodes the luciferase reporter gene under the control of the proximal SREs #1 and #2 of the Egr-1 promoter. A minimal promoter was inserted downstream of the SREs consisting of a TATA box derived from the HIV long terminal repeat and an initiator element from the adenovirus major late promoter. The regulatory region of this transcription unit was derived from plasmid pEgr-1SREluc (Bauer et al., 2005). The viral particles were produced as previously described (Stefano et al., 2006) by triple transfection of 293T/17 cells with the *gag-pol-rev* packaging plasmid, the *env* plasmid encoding VSV-glycoprotein and the transfer vector.

Electrophoretic mobility shift assay

Binding was measured using the electrophoretic mobility shift assay (EMSA). Assays were carried out for 15 minutes at room temperature in a 20-μl reaction containing 42 mM HEPES (pH 7.9), 40 mM KCl, 2.7 mM MgCl₂, 0.13 mM ZnCl₂, 0.09 mM Na₂EDTA, 1.16 mM dithiothreitol, 5.3 mM spermidine, 44 mg/ml double-stranded poly(dI-dC), 130 mg/ml bovine serum albumin, 8.7% glycerol and double-stranded DNA probe. To generate the probe, we isolated fragments from plasmids pEBS1⁴CAT and pEBS2⁴CAT, containing four copies of an Egr-1-binding site derived either from the Egr-1 or the synapsin I gene, using SalI and XhoI restriction enzymes. The isolated fragments were incubated with the Klenow fragment of *Escherichia coli* DNA polymerase I in the presence of [^αP³²]dCTP. Nuclear extracts prepared from 293T cells that had been mock-transfected or transfected with plasmids pFUWEgr-1/Zn or pFUWEgr-1/ZnC368SC396S were added to the reaction mix, and the incubation was continued for 15 minutes. Free DNA and DNA-protein complexes were resolved by electrophoresis at 4°C on a 4% polyacrylamide gel (29:1 acrylamide:bisacrylamide) in 0.25 × TBE (1 × TBE is 50 mM Tris, 50 mM boric acid, 1 mM Na₂EDTA). The gel was dried and exposed to X-ray film.

Western blots

Nuclear extracts were prepared as described (Kaufmann and Thiel, 2002). Nuclear proteins (20 μg) were separated by SDS-PAGE and the blots were incubated with antibodies directed against Egr-1 (Santa Cruz Biotechnology, Heidelberg, Germany, #sc-189). To detect phospho-ERK, 20 μg of proteins derived from whole-cell extract preparations were separated on SDS-PAGE and transferred to nitrocellulose

membranes. Blots were probed with an antibody directed against the phosphorylated form of ERK2 (Promega, Mannheim, Germany, #V8031). Immunoreactive bands were detected using the ECL Plus system (Amersham, Braunschweig, Germany).

Reverse transcription polymerase chain reaction

Reverse transcription polymerase chain reaction (RT-PCR) to amplify *bFGF* mRNA was performed as previously described (Bauer et al., 2007) using the primers 5'-GGAAACAGAGGCAGGATGAA-3' and 5'-GAATAAGGGTTGCCAGACA-3'.

Chromatin immunoprecipitation

ChIP experiments were performed as described elsewhere (Rössler et al., 2008). To amplify the proximal region of the murine *bFGF* promoter, the primers 5'-GCCTAGCGGGACAGATTCTT-3' and 5'-GAGGGAGCCCTTGAGTGTA-3' were used. To amplify a distal region of the murine *bFGF* 5'-upstream region, the primers 5'-CACTAAGCCACCACACATGG-3' and 5'-TTGGTCCCCCTCAT-AATCTG-3' were used. The antibodies used for chromatin immunoprecipitation were anti-dimethyl H3K9 (Abcam, Cambridge, UK, #ab7312), anti-trimethyl H3K4 (Abcam, #ab8580), anti-Egr-1 (Santa Cruz Biotechnology, #sc-189), anti-Egr-2 (Santa Cruz Biotechnology, #sc-20690), and anti-Egr-3 (Santa Cruz Biotechnology, #sc-191). To detect binding of the Egr-1 mutant Egr-1/Zn to DNA, M2-agarose (Sigma, #A2220) was used, which interacts with the FLAG epitope of the mutant as described (Hohl and Thiel, 2005).

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