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A new ERK2 binding protein, Naf1, attenuates the EGF/ERK2 nuclear signaling

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Abstract

Extracellular signal regulated kinase1/2 (ERK1/2), an important factor in signal transduction, controls cell growth, differentiation, and death. To elucidate the details of the mechanism of ERK1/2 signaling in human cells, we isolated Nef-associated factor 1α (Naf1α) by a yeast two-hybrid system, which bound to human ERK2. The binding was confirmed by a pull-down assay in vitro and immunoprecipitation in vivo. Upon EGF treatment, Naf1α was phosphorylated by the EGF/MEK/ERK2 signal transduction pathway. To identify the role of Naf1α in the ERK2 signaling, Naf1α-expressing Saos-2 cells were analyzed for ERK2 nuclear translocation and activation of its downstream target. Overexpression of Naf1α suppressed ERK2 entering into the nucleus and inhibited the ERK2-dependent Elk1-driven *luciferase* transcription, suggesting Naf1α to be an attenuator of activated ERK2 signaling.

Keywords: ERK2; MAPK; Signal transduction; Nuclear transport; Nef-associated factor; Yeast two-hybrid system

The mammalian mitogen-activated protein (MAP) kinase pathway contributes to the control of cell growth, differentiation, and tumorigenesis in response to various stimuli such as growth factors, mitogens, hormones, and stresses. Three distinct MAPKs, extracellular signal regulated kinase1/2 (ERK1/2, p42–p44 MAPK), p38 MAPK, and c-Jun N-terminal kinase (JNK), have been identified to be involved in this signal pathway [1,2]. Epidermal growth factor (EGF) and other growth factors transmit signals to ERK1/2 sequentially through the cell surface and intracellular cytoplasmic signaling molecules of receptor tyrosine kinase, ras, raf, and MAPK-ERK kinase (MEK1/2). MEK1/2 activates ERK1/2 by phosphorylating threonine and tyrosine in a TEY sequence within the kinase domain [3–5]. The activated ERK1/2 in turn phosphorylates cytoplasmic protein substrates such as p90 RSK [6], MAP kinase interacting kinase1 (Mnk1) [7], and other cytoskeletal proteins [8], or upon translocation to the nucleus, a set of transcription factors such as Elk-1 [9], Ets [10], and others is activated. Such phosphorylated ERK1/2 substrates are mostly transcription factors, which activate the transcription of genes needed for cell responses with different stimuli.

ERK1/2 activities are primarily regulated at two levels, one at phosphorylation, and the other at translocation from the cytoplasm to the nucleus. The former is controlled by the balance of kinase and phosphatase activities. Two phosphatases, MAP kinase phosphatases (MKPs) with dual specificities for tyrosine and serine/threonine [11–13] and protein tyrosine phosphatases

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(PTP-SL) with a speciality for threonine [14], have been reported. When ERK1/2 is activated, the phosphorylation promotes ERK1/2 to dimerize and then ERK1/2 is rapidly translocated from the cytoplasm to the nucleus [15]. However, in some cases, ERK1/2 can still be detected in the cytoplasm. Cytoplasmic retention of the ERK1/2 is controlled by cytoplasmic anchor proteins such as MEK1/2 [16], β-arrestin [17,18], or by the dephosphorylation with MKP3 [11–13] and PTP-SL [14]. However, the involvement of other factors that modulate ERK1/2 signaling pathways has yet to be explored.

In the present study, we searched for a protein that interacted with ERK2 using a yeast two-hybrid system. As a result, we isolated a gene corresponding to Naf1α, one of the isoforms of HIV Nef-associated factor 1 (Naf1) or HIV virion-associated matrix–interacting protein (VAN) [19,20]. It has been reported that Naf1 is conserved among vertebrates and expressed rather ubiquitously in various human tissues. We found for the first time that Naf1α was phosphorylated by the EGF/MEK/ERK2 signal pathway, but in turn modulated ERK2 signaling by trapping ERK2 in the cytoplasm and reducing ERK2 nuclear signals.

Materials and methods

Yeast two-hybrid system. Interactive cloning experiments, including relevant controls, were performed as described previously with minor modifications [21,22]. In brief, human ERK2 cDNA encoding codons from 9 to 360 was fused to the C-terminus of the GAL4-DNA-binding domain at the *Eco*RI and *Xho*I sites of the pGBDU vector. The pGBDU-ERK2 yeast bait plasmid was used to screen a human liver cDNA library constructed in pAD-GAL4-2.1 (Stratagene, La Jolla, CA, USA) for interacting proteins. Transformants were first selected for leucine, uracil, and histidine prototropies. The positive colonies were then replica-plated onto synthetic complete medium minus uracil, histidine, leucine, and adenine (SC − Ura, − His, − Leu, − Ade). The plasmids were recovered from the growing clones, co-transformed again with pGBDU-ERK2 into PJ69-4A, and selected for their ability to activate transcription from the reporters. The plasmid DNAs from positive clones were transformed into *Escherichia coil* DH5α and the inserts were verified by sequencing.

Plasmid constructions. Construction of the full-length human Naf1α cDNA in the pcDNA3.1-His A vector which contained Xpress epitope (Invitrogen, Carlsbad, CA, USA) was described previously [19]. For the expression of Naf1α-C, the *Eco*RI–*Xho*I fragment was excised from the isolated cDNA clone and ligated at the relevant sites of p3XFLAG-CMV-10 (Sigma, St. Louis, MO, USA) to make the N-terminally FLAG-tagged fusion protein. The N-terminal GST-tagged fusion protein of ERK2 was constructed by ligation of human ERK2 cDNA (codons 9–360) in the frame to the C-terminal end of glutathione-*S*-transferase (GST) using the pGEX-4T-1 vector with *Eco*RI and *Xho*I. GFP-ERK2 was also made by inserting the same human ERK2 cDNA into the C-terminal end of the GFP with *Eco*RI and *Xho*I. The sequences of all the constructed expression plasmids were verified as having the correct reading frame by sequencing.

Cell culture and isolation of cell clones expressing Naf1α. Saos-2 cells were cultured with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37 °C in an atmosphere of 5% CO_2 as described previously [23].

Saos-2 cells were plated at 1×10^6 cells/100 mm dish, and the next day, the cells were transfected with 0.5 μg of either pcDNA-His-Naf1α, p3XFLAG-CMV-10-Naf1α-C, or their empty vectors by a calcium phosphate precipitation method [24]. After 48 h of transfection, 500 μg/ml neomycin derivative, G418 (Sigma Chemicals), was added and cultured for two weeks by replacing with fresh medium containing G418 every 5 days. The cells in the single colonies that appeared were trypsinized and grown as individual clones. The transfectants (Naf1α/Saos-2 or Naf1α-C/Saos-2) were selected for the highest levels of protein expression of Naf1α and Naf1α-C, respectively, by Western blotting or immunostaining of cells. Saos-2 cell clones carrying the empty vectors (pcDNA3.1-HisA/Saos-2 or p3XFLAG-CMV-10/Saos-2) were also cloned.

Immunological reagents. Anti-ERK2 (monoclonal D-2 and polyclonal K-23), anti-p53 (DO-1), and anti-GST (monoclonal 12) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-FLAG M1 monoclonal and anti-FLAG BioM2 monoclonal antibodies were from Sigma Chemicals. Phospho-ERK1/2 monoclonal antibody (Thr202/Tyr204), phospho-AKT (Thr308) polyclonal antibody, and phospho-p70S6K1(Thr421/Ser424) polycolnal antibody were from Cell Signaling Technology (Beverly, MA, USA). Anti-Xpress monoclonal antibody was from Invitrogen (Carlsbad, CA, USA).

Conjugated secondary antibodies were from Santa Cruz Biotechnology: horseradish peroxidase (HRP)-conjugated anti-rabbit IgG, HRP-conjugated anti-mouse antibody, and streptavidin-peroxidase-conjugated-anti-mouse antibody. Alexa Fluor 488 goat anti-mouse IgG was from Molecular Probes (Eugene, OR, USA). Protein G–Sepharose and ECL reagents were from Amersham Pharmacia Biotechnology (Tokyo, Japan).

Phosphorylation of Naf1α by EGF and drug treatment. The cells were cultured for 24 h in serum-free DMEM and treated with EGF (50 ng/ml) 10 min before the harvest. LY294002 (50 μM, PI3K inhibitor, ALEXIS, San Diego, CA, USA) or U0126 (50 μM, MEK inhibitor, Cell Signaling) was added 1 h or 1.5 h, respectively, before the cell harvest. The cells were lysed with NP40 buffer (20 mM Hepes, pH 7.4, 5 mM EDTA, 150 mM NaCl, 0.1% NP40, 50 mM NaF, 1 mM glycerophosphate, and 5 mM $Na_4P_2O_7$) supplemented with a tablet of Complete protease inhibitor cocktail (Roche Diagnostics, Boehringer–Mannheim, Germany) per 50 ml, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM Na_3VO_4. Protein concentrations were determined using a Bio-Rad protein assay DC kit (Bio-Rad, Hercules, CA, USA).

In vitro binding assay using glutathione–S-transferase (GST)-fused proteins. E. coli BL21 carrying the GST-ERK2 plasmid was grown in Luria–Bertani medium containing 50 μg/ml ampicillin and induced with 0.5 mM isopropyl-β-thiogalactopyranoside (IPTG) at 26 °C overnight. The induced bacteria were lysed as described previously [25]. The bacterial lysates were incubated for 1 h at room temperature with glutathione–Sepharose beads (Amersham Pharmacia Biotechnology) in binding buffer as recommended by the supplier and then centrifuged for 5 min to remove the supernatants. The Saos-2 cell lysates were incubated with 5 μl glutathione beads conjugated with GST fusion ERK2 protein at 4 °C for 3 h and the beads were washed three times with phosphate-buffered saline (PBS). Proteins were eluted with 1× sample buffer, subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE), and immunoblotted with suitable antibodies.

Immunoprecipitation. Antibodies were conjugated with protein G beads as described previously [25] and then incubated with cell lysates overnight at 4 °C. The beads were pelleted by centrifugation and washed three times with lysis buffer before eluting the proteins by boiling with 1× sample buffer.

Western blotting. This was performed as described previously [23].

Protein phosphatase treatment. Immunoprecipitates of Naf1α were treated with λ protein phosphatase (New England Biolabs, Beverly, MA, USA) in 50 μl λ protein phosphatase reaction mixture containing 20 mM Tris–HCl (pH 7.6), 2 mM $MnCl_2$, 5 mM DTT, and 400 U

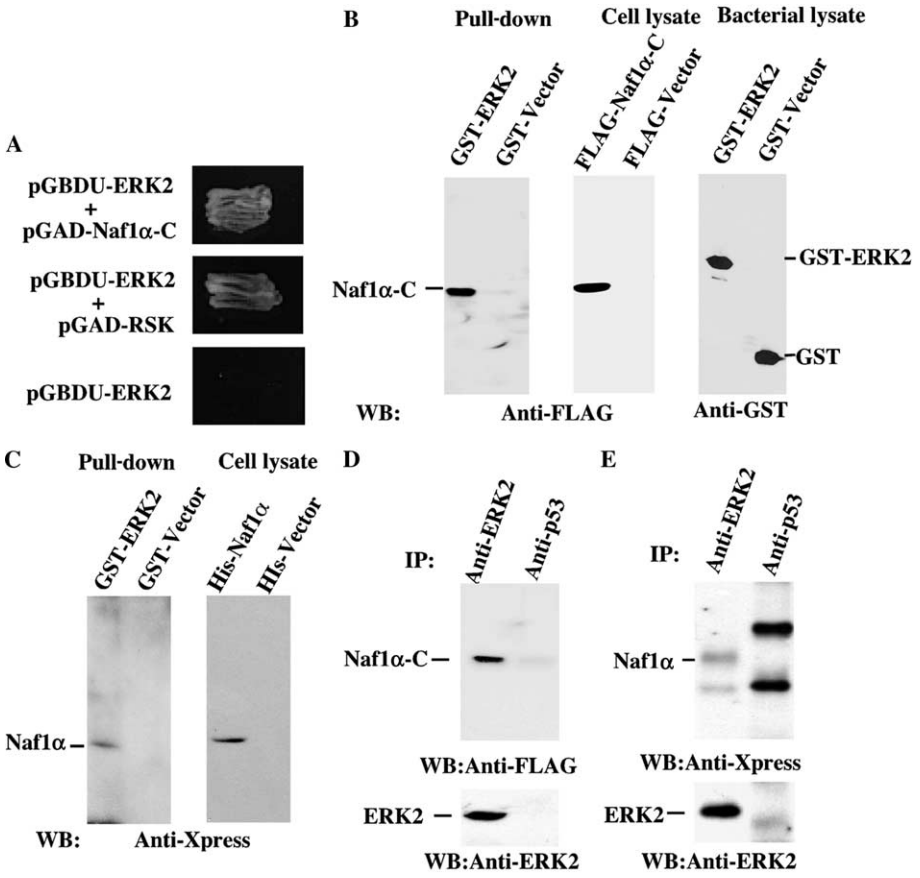

Fig. 1. Interaction of Naf1α and ERK2. (A) Association of Naf1α-C with human ERK2 in the yeast two-hybrid system. Yeast PJ69-4A was co-transfected with pGBDU-ERK2 as a bait and pGAD-Naf1α-C (upper panel) or pGAD-RSK2 (middle panel). The transformed yeasts were selected on SC-Ura–His–Leu–Ade. (B) Pull-down assay of Naf1α-C. GST-ERK2 fusion protein or GST protein from the vector (right panel) was mixed with cell extract of FLAG-Naf1α-C/Saos-2 cells (middle panel), and the complex formed was collected on glutathione–Sepharose beads. The beads were washed and the protein that remained associated with the beads was separated by SDS–PAGE and Western blotted. (C) Pull-down assay of Naf1α. GST-ERK2 protein used in (B) was mixed with protein extract of His-Naf1α/Saos-2 cells (right panel). The complex formed was pulled down and detected as described in (B). (D) Immunoprecipitation of FLAG-Naf1α-C and ERK2. Anti-ERK2-conjugated protein G–Sepharose beads were mixed with cell extracts of Naf1α-C/Saos-2 cells. The beads were washed, and proteins on the beads were analyzed by Western blotting using anti FLAG-BioM2. (E) Immunoprecipitation of His-tagged Naf1α with ERK2. Immunoprecipitation was performed as described in (D). Anti-p53 antibody-conjugated Sepharose beads were used as negative control.

phosphatase, as recommended by the supplier. The controls contained the same ingredients except for the phosphatase.

Immunofluorescence microscopy. Naf1α/Saos-2 cells were grown on cover glasses and transfected with the GFP-ERK2 plasmid by a calcium phosphate precipitation method [24]. After 24 h, the cells were treated with EGF or U0126 as described above and then fixed with ice-cold methanol/acetone (1:1, v/v) for 3 min. After fixation, the cells were blocked with 5% dried non-fat milk in TBS-Ca (Tris-buffered saline containing 1 mM $CaCl_2$, pH 7.6) for 1 h. After washing, the cells were stained with anti-Xpress antibody for 1 h at room temperature, incubated with Alex-fluor-conjugated anti-mouse IgG for 45 min at room temperature, and then washed with TBS-Ca buffer. The nuclei were stained with DAPI as described previously [26]. The cells were observed under a fluorescent microscope (Olympus BX50).

Luciferase reporter assays. ERK2-dependent transcription was measured using the Elk-1 driven luciferase reporter system (PathDetect Elk-1 *trans*-Reporting System, Strategene, La Jolla, CA, USA). In brief, Naf1α/Saos-2 and pcDNA 3.1-HisA/Saos-2 cells were transfected with pFA2-Elk1 (50 ng/plate) or pFC2-dbd (50 ng/plate) as control, along with pFR-Luc (1 μg/plate), and pRL-SV40 (10 ng/plate). One day after transfection, the cells were serum-starved for 24 h and then incubated in the complete medium with or without EGF (50 ng/ml) for 5 h before preparing the cell lysates. The luciferase activities were determined by a Dual Luciferase Report Assay System (Promega, Madison, WI, USA). Equal aliquots of cell lysates were assayed alternatively for firefly and *Renilla* luciferase activities. Firefly activities were normalized to the *Renilla* luciferase activities.

Results

Interaction of Naf1α with human ERK2

A human cDNA library linked to a Gal4-DNA activation domain was used to screen against human ERK2 cDNA linked to a GAL4-DNA-binding domain as a bait. Four clones out of approximately 2×10^6 transformants were found to contain portions of the Naf1α open reading frame (ORF) after sequencing and BLAST searching, in addition to four clones of

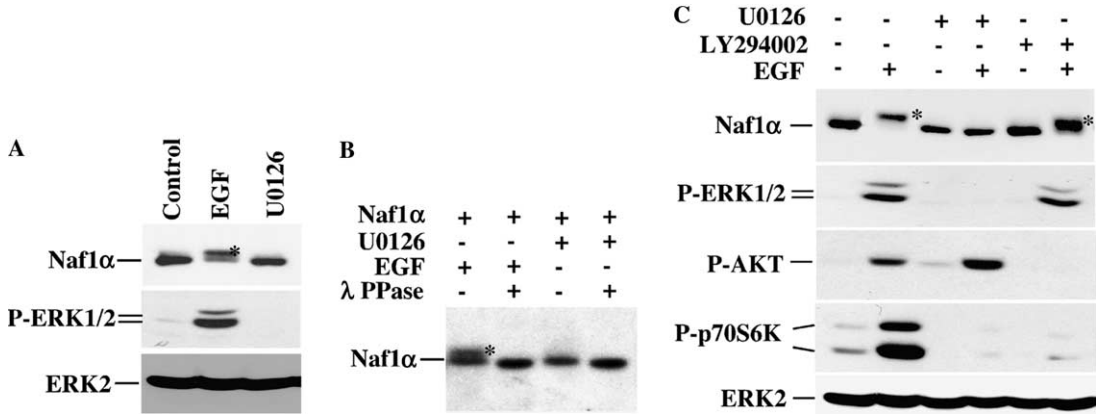

Fig. 2. Naf1α is phosphorylated through the EGF/MEK/ERK2 pathway. Naf1α/Saos-2 cells were serum starved. (A) Cells were treated with MEK inhibitor, U0126 (50 μM) or without any treatment as a control, or stimulated with EGF (50 ng /ml). (B) Naf1α/Saos-2 cell extracts after various treatments in (A) were immunoprecipitated with anti-ERK2 antibody. The immunoprecipitates were treated with λ protein phosphatases. (C) Cells were pretreated with 50 μM U0126, 50 μM LY294002 before being treated with EGF as described in Materials and methods. The same amount of cell lysates in (A) and (C), or the immunoprecipitates in (B) were separated by 6% SDS–PAGE, and then analyzed by Western blotting using the anti-Xpress antibody for His-Naf1α, anti-phospho(P)-ERK1/2, anti-phospho(P)-AKT, anti-phospho (P)-p70S6K, or anti-ERK2 antibody. The slowly migrating band of Naf1α is marked as "*."

p90RSK2, which was reported to bind to ERK2 (Fig. 1A). Three of the clones designated as Naf1α-C encoded amino acid residues from 310 to 636 of the C-terminal half and the remaining one those from 231 to 636.

The interaction of Naf1α-C with ERK2 was further confirmed by pull-down assay. As shown in Fig. 1B, GST-ERK2 was able to pull-down 3XFLAG-tagged Naf1α-C expressed in Saos-2 cells. The pull-down assay also showed that GST-ERK2 was able to pull down His-tagged full-length Naf1α in Saos-2 cells (Fig. 1C). Immunoprecipitation further confirmed the binding between endogenous ERK2 and exogenously expressed Naf1α in Saos-2 cells. The cell lysates from the Naf1α/Saos-2 or Naf1α-C/Saos-2 cells were incubated with the anti-ERK2 antibody protein-conjugated G–Sepharose beads. As shown in Fig. 1D, Naf1α-C was immunoprecipitated with anti-ERK2 antibody while it was not with anti-p53-antibody used as a negative control. Full-length His-tagged Naf1α protein expressed in Saos-2 cells was also found in the immunoprecipitated complex with ERK2 (Fig. 1E). These results confirmed that Naf1α can bind to ERK2 in human cells in its native condition.

Naf1α is phosphorylated by EGF/MEK/ERK2

We studied whether Naf1α could be phosphorylated through the EGF/MEK/ERK signal transduction pathway since it bound to ERK2. As shown in Fig. 2A, when Naf1α/Saos-2 cells were treated with EGF, a new band of Naf1α with a slower mobility appeared. However, this band was not significantly detected in the control or U0126 (MEK inhibitor)-treated cells. The results suggest that this slower migrating band may be a phosphorylated form of Naf1α, because of its binding to ERK2. To confirm this, the Naf1α protein was treated with λ protein phosphatase. As shown in Fig. 2B, the slower migrating band disappeared, suggesting that the mobility shift was due to phosphorylation.

The search for consensus ERK phosphorylation sites (S/TP or PXS/TP, [1]) showed 10 sites in Naf1α but none for consensus MEK phosphorylation site (TEY).

The EGF signal is transduced not only to ERK1/2, but also to PI3K [27,28]. We examined the inhibitory effect of Naf1α phosphorylation by a PI3K inhibitor, LY294002. As shown in Fig. 2C, LY294002 did not block the appearance of the slower migrating band of Naf1α, induced by EGF, while U0126 did. However, LY294002 effectively inhibited the EGF-induced phosphorylation of AKT and p70 S6K1, as AKT and p70 S6K1 are downstream effectors of PI3K [29,30]. These results, taken together, suggest that Naf1α in Saos-2 was phosphorylated primarily through the EGF/MEK/ERK pathway, but not through the PI3K pathway.

ERK1/2 nuclear signal transduction is blocked by Naf1α

As Naf1α has been reported to contain both nuclear localization and nuclear export signals by which it can shuttle between the cytoplasm and the nucleus [20], we examined whether Naf1α was modified in its cellular localization after the phosphorylation. As shown in Fig. 3, Naf1α was primarily localized in the cytoplasm in both EGF-treated and U0126-treated Naf1α/Saos-2 cells. Since Naf1α was present in the cytoplasm and ERK1/2 was reported to be in the cytoplasm before activation with growth factors, we studied whether the Naf1α can block the activated ERK2 entering into the nucleus. GFP-ERK2 was transiently expressed in Naf1α/Saos-2 cells and parental Saos-2 cells, which were

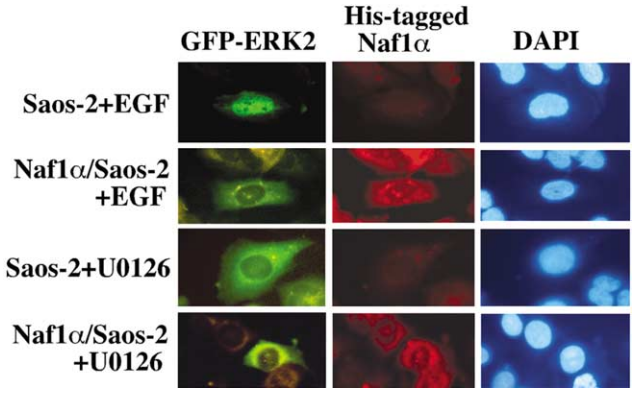

Fig. 3. Localization of GFP-ERK2 in Naf1α/Saos-2 cells. Saos-2 cells (first and third rows) or Naf1α /Saos-2 cells (second and fourth rows) were transfected with the GFP-ERK2 plasmid DNA. After 4 h of transfection, cells were serum starved for 24 h and then treated with 50 ng/ml of EGF for 10 min (the upper two rows) or 50 μM of U0126 for 1.5 h (the lower two rows). Cells were fixed and stained as described in Materials and methods, and visualized using a conventional fluorescent microscope (Olympus BX50-FLA).

then treated with EGF or U0126. GFP-ERK2 in Naf1α/Saos-2 cells was localized in the cytoplasm when treated with either EGF or U0126, respectively (Fig. 3, 2nd and 4th rows). However, in EGF-treated parental Saos-2 cells, GFP-ERK2 was localized in the nucleus, while U0126 treatment blocked the nuclear accumulation of GFP-ERK2 (Fig. 3, first and third rows). These results suggested that Naf1α blocked the GFP-ERK1/2 translocation into the nucleus.

In the nucleus, it has been reported that activated ERK1/2 can further activate transcription factors such as Elk-1 by phosphorylation [9]. We questioned whether or not Naf1α can inhibit ERK2-dependent Elk1-driven luciferase activity. The Elk-1 *trans*-reporting system was employed in this study. As shown in Fig. 4A, EGF treatment of the pcDNA3.1-HisA/Saos-2 cells increased the pFR-luc activity by nearly 70-fold compared with the untreated cells. However, in the Naf1α/Saos-2 cells, the increase was only about 5-fold by EGF treatment, suggesting that Naf1α reduced the ERK2-dependent Elk1 transactivation. To further confirm this result, Saos-2 cells were transfected with increasing amounts of His-Naf1α plasmid DNA together with pFA2-Elk1 and pFR-luc plasmid DNAs. As shown in Fig. 4B, the Elk1-dependent luciferase activities decreased gradually with the increasing amounts of Naf1α plasmid DNA, suggesting that the expression of Naf1α can inhibit the ERK2-dependent activation of Elk1. Taken together, these results suggested that Naf1α suppressed the ERK2 nuclear signaling.

Discussion

In this study, we found that ERK2 bound to Naf1α and that Naf1α in turn blocked the ERK2 nuclear signaling. It has been reported that the critical step in the EGF/ERK signaling cascade is the change in cellular localization of phosphorylated ERK1/2 from the cytoplasm to the nucleus, which results in the activation of transcription factors of Elk-1 and others [1]. However, in the Naf1α-overexpressing Saos-2 cells, both translocation to the nucleus and activation of Elk-1 were blocked by Naf1α which was detected in the cytoplasm, when compared with the parental Saos-2 cells. Further, we showed that the suppression of Elk-1 activation was dependent on the Naf1α-expression levels (Fig. 4). Thus, Naf1α may play a role as a cytoplasmic anchor protein and reduce ERK2 nuclear signaling.

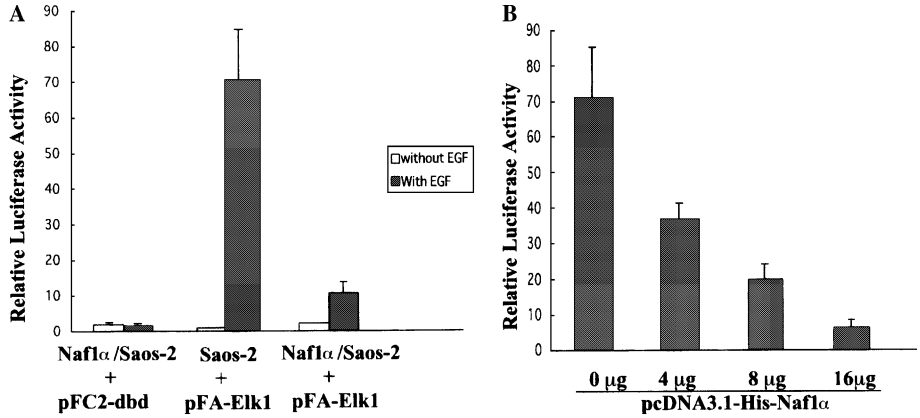

Fig. 4. Effect of Naf1α overexpression induced ERK2-dependent activation of Elk1-dependent transcription. (A) Naf1α/Saos-2 or pcDNA-His/Saos-2 cells were transfected with 1 μg pFR-Luc and 50 ng pFA2-Elk-1 or pFC2-dbd as a negative control together with 10 ng pRL-SV40 as described in Materials and methods. (B) Saos-2 cells were transfected with increasing amounts of pcDNA-HisA-Naf1α plasmid DNA with 1 μg pFR-Luc, 50 ng pFA2-Elk-1, and 10 ng pRL-SV40. After serum starvation, cells were treated with or without EGF for 5 h and harvested for the dual luciferase assay. The luciferase activities were expressed as the means ± SD from two independent experiments.

The blockade of ERK-nuclear transport by Naf1α was similar to that by β-arrestin or MEK1/2. However, β-arrestin is a scaffold protein that traps ERK2 in the endosomes but at the same time enhances the ERK2 activity [18]. MEK1/2 traps ERK2 in the cytoplasm with its nuclear export signal (NES), but, once phosphorylated, ERK2 is immediately dissociated from the MEK complex and translocated into the nucleus [16]. Contrary to these factors, Naf1α is phosphorylated by the EGF/MEK/ERK2 pathway. The cytosolic localization of Naf1α was not changed, even when the cells were stimulated by the EGF treatment. Whether the phosphorylation of Naf1α is attributed to the blockade of ERK2 nuclear transport has yet to be elucidated. Whatever the blocking mechanism of ERK2 translocation to the nucleus may be, Naf1α could be a new regulator, so that ERK2 signals may be attenuated by Naf1α.

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