Recent studies suggest a crucial role for protein kinase A (PKA) in the regulation of growth factor signaling. However, the effect of PKA on the transcription of growth factor-responsive genes has drawn less attention. Here we have investigated the signaling mechanisms involved in the activation of an activator protein-1 (AP-1)-driven, growth factor-specific enhancer element, fibroblast growth factor-inducible response element (FiRE). The activation was found to be mediated by three phorbol 12-O-tetradecanoylphorbol-13-acetate-response element-related DNA elements of FiRE, including motif 4 and two distinct elements of motif 5 (referred to as M5-1 and M5-2). All three elements were required for full FiRE activity. Stimulation of cells with fibroblast growth factor-2 (FGF-2) induced the binding of AP-1 to motif 4 and M5-2, whereas M5-1 did not show detectable binding. The FGF-2-induced FiRE activation appeared to require cooperation of the Ras/ERK and PKA pathways. Inhibition of either of the pathways abolished the binding of AP-1 complexes to motif 4 and motif 5 and the subsequent FiRE activation. By contrast, costimulation of cells with FGF-2 and the PKA activator 8-bromo-cyclic AMP increased the binding of AP-1 to FiRE and potentiated the level of transcriptional activity. The cooperative functional cooperation of these two pathways was confirmed by experiments with cell lines stably expressing 5-OH-4-pharmaceuticals-inducible oncogenic Raf-1 (ΔRaf-1;ERK(DD)). Noticeably, the induction systems showed variations with respect to regulation of AP-1-driven activation of FiRE. These differences were likely to originate from the ability of these two systems to induce the differential activation pattern of the Ras/ERK pathway.

Binding of growth factors to their transmembrane receptors induces signal transduction through intracellular signaling pathways. The main pathway transporting such signals to the nucleus is the Ras/ERK pathway, through which signals are transferred by sequential activation of specific adaptor proteins and protein kinases characteristic of this pathway. Recent studies (4–7) suggest that the cAMP-dependent protein kinase (protein kinase A (PKA)) has an important role in the regulation of mitogen-induced signaling via the Ras/ERK pathway. The main molecular mediators of such regulation are the Raf-1 and B-Raf serine/threonine kinases (8, 9). Depending on the cell line studied and the stimulus used, the effect of PKA can be either stimulatory or inhibitory (8, 10–12).

Although the link between growth factor signaling and PKA is becoming generally recognized, little is known of the role of PKA in the regulation of growth factor-induced transcription. To address this issue we made use of the recently discovered FGF-inducible response element (referred to as FiRE), which is an AP-1-driven far upstream enhancer element specifically activated by FGFs. This 170-bp element is located at −10 kb from the translation initiation site of the murine syndecan-1 gene. It is a likely candidate to mediate the growth factor-induced expression of syndecan-1 in vivo. In mouse fibroblast cells (NIH3T3) FGF-2 is the most potent activator of FiRE, whereas stimuli such as platelet-derived growth factor, EGF, or serum do not activate it. FiRE contains five DNA motifs (motifs 1–5) capable of binding specific transcription factors. Previous data indicate that motifs 1 and 2 are occupied both in FGF-2-stimulated and unstimulated cells, whereas motifs 3–5 are occupied in FGF-2-treated cells only. Motif 1 appears to bind a so far unidentified 46-kDa protein, whereas motif 2 binds USF-1. Following FGF stimulation, motif 3 displays binding to an AP-2-like protein, referred to as an FGF-inducible nuclear factor (FIN-1), whereas motifs 4 and 5 bind AP-1 complexes. Studies with various deletion mutants have shown that all other motifs except motif 2 are required for the full FGF-2 response of FiRE (13, 14).

We have studied the mechanisms of AP-1-mediated FiRE activation in FGF-2-stimulated cells. AP-1 is a sequence-specific transcription factor composed of members of the Jun (c-Jun, Jun-B, and Jun-D) and Fos (c-Fos, Fos-B, Fra-1, and Fra-2) protein families. Jun and Fos belong to the family of basic region leucine zipper proteins and bind DNA as Jun-Jun and Jun-Fos dimers (15, 16). The most avid binding is to the TRE (15, 17). The DNA binding specificity, affinity, and orientation of the AP-1 complex depend on the dimer composition, sequence of the binding site, and the surrounding sequence.

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1 The abbreviations used are: ERK, extracellular regulated kinase; FGF-2, fibroblast growth factor-2; PKA, protein kinase A; AP-1, activator protein-1; USF-1, upstream stimulatory factor-1; H89, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)IH-imidazole; FiRE, FGF-inducible response element; DMEM, Dulbecco’s modified medium; 4-OHT, 4-hydroxytamoxifen; TRE, phorbol 12-O-tetradecanoyl-13-acetate (TPA)-response element; EGF, epidermal growth factor; CAT, chloramphenicol acetyltransferase; CRE, cAMP-response element; CREB, cAMP-response element-binding protein.
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context (18–21). In naturally existing promoter and enhancer elements, AP-1-binding sites often deviate from the optimal recognition sequence. Such variation probably contributes to the differential functions of different Jun-Fos dimers with regard to various regulatory elements (16). All Fos and Jun genes are ubiquitously expressed early response genes. Their expression is rapidly induced in response to various stimuli, such as growth factors, cytokines, and cellular stress. Promoters and enhancers containing AP-1-binding elements regulate a large number of different genes. Interestingly, also the promoters of c-fos and c-jun genes contain AP-1-binding sites creating an autoregulatory loop to control their own expression (15, 22, 23). The activity of AP-1 complexes is regulated on several levels, including transcriptional and post-transcriptional mechanisms affecting the expression of AP-1 proteins and post-translational mechanisms such as phosphorylation altering the DNA-binding affinity and transactivation potential.

Our recent data suggests that PKA would have a crucial role in balancing the growth factor-induced signal transduction through the Ras/ERK pathway (24) and also in regulating the growth factor-activated transcription (25). In this paper, we have attempted to elucidate the mechanisms by which the level of transcriptional activity of FiRE is regulated. We demonstrate that the AP-1-mediated induction of FiRE activity in response to FGF-2 requires cooperation of the Ras/ERK and PKA signaling pathways. In addition, we show that although sustained activation of the Ras/ERK pathway leads to FiRE activation, the process still requires active PKA. These data suggest a crucial function of basal PKA activity for repression by signaling through the Ras/ERK pathway. Furthermore, based on the new data, an updated model of FiRE is presented.

EXPERIMENTAL PROCEDURES

Materials—The cell-permeable, PKA-specific inhibitor H89 (used at 10 μM), the non-degradable cAMP analogue 8-Br-cAMP (used at 500 μM), the MEK-1 inhibitor PD98059 (used at 20 μM), and the p38 pathway inhibitor SB203580 (used at 20 μM) were purchased from Calbiochem. Human recombinant FGF-2 (from PeproTech-Rocky Hill, NJ) and and was used at 10 ng/ml. 4-Hydroxytamoxifen (4-OHT, used at 100 nM) and the antibody against active ERK1/2 were from Sigma. Antibodies for Jun and Fos family members, anti-ERK2 (C-14), and anti-ERα antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies used in supershift assays recognizing all Jun family members were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies used in Western blot assays were c-Jun (H-79), and Fos family members were c-Jun/AP-1 (DXX) and c-Fos (KXX). Antibodies used in supershift assays recognizing all Jun family members were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies used in Western blot assays were c-Jun (H-79), JunB (N-17), JunD (329), c-Fos (K-25), FRA-1 (N-17), FRA-2 (Q-20), and FosB (H-75).

Cell Culture, Plasmids, Transfections, and CAT Assays—NIH3T3 mouse fibroblasts were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum to ~80% confluence. For treatment with FGF-2 or with 4-OHT, fetal calf serum was replaced with 1% carboxymethyl-Sephadex-eluted fetal calf serum (CMS) 24–48 h before stimulation. Other treatments were initiated 30 min prior to the start of FGF-2 or 4-OHT treatments. Construction of the p271FiRE-CAT reporter plasmid (13), PKAδ/ε expression plasmid MT-REV (26, 27), mutRaf-1 (pRSV Raf-1C) (28), mutMEK-1 (pMCL-HA-MKK1-R7TM) (29), and ΔRaf-1:ER[DD] (30) have been described previously. For transient transfections, NIH3T3 cells were plated at equal density on 6-well plates (Falcon) 2 days before transfections. Plasmid DNA was transfected into the cells by the calcium phosphate method (31). To monitor the transfection efficiency, 1 μg of a β-galactosidase-expressing plasmid (pSV-β-galactosidase, Promega) was cotransfected. Three parallel transfections were made for all assays. Following transfection, the media were changed to DMEM containing 1% carboxymethyl-Sephadex and FGF-2, or 4-OHT was added. After 24 h the cells were collected, and CAT assays were performed by the xylene extraction method, followed by measurement of CAT activities by liquid scintillation counting and β-galactosidase activities spectrophotometrically at 420 nm. Stable transfections of p271FiRE-CAT were made by simultaneous introduction of pBOS plasmid (Promega) and a 10-fold molar excess of the CAT reporter plasmid by the calcium phosphate method and selecting cells with 750 mg/ml G418. Several independent clones were pooled. The cDNA encoding ΔRaf-1:ER[DD] was cloned into the pCDN6 vector (Invitrogen), and transfected cells were selected on the basis of their resistance to Blasticidin (1 μg/ml). Individual clones were selected and tested for their responsiveness to 4-OHT.

Targeted Mutagenesis—Point mutations (null and TRE mutations) to the AP-1-binding motifs of FiRE were generated directly to the p271FiRE-CAT reporter plasmid by PCR using the Stratagene Quick-Change™ Site-directed mutagenesis kit, according to the manufacturer's instructions. Resultant mutant constructs were confirmed by sequencing and their binding capacity was studied by gel retardation assays. The following mutations were generated (the wild type binding sequences are underlined, the mutated binding sequences are double-underlined, and the mutated nucleotides are indicated in boldface): For null mutations: motif 4 to M4null, TGGGTC → AAAGCC; M5-1 to M5-1null, TGGGTC → AAAGCC; M5-2 to M5-2null, TGGGTC → AAAGCC. For TRE mutations: motif 4 to M4-TRE, TGGGTC → AAAGCC; M5-1 to M5-1-TRE, TGGGTC → AAAGCC; M5-2 to M5-2-TRE, TGGGTC → AAAGCC.

Nuclear Extracts and Gel Retardation Assays—For preparation of nuclear extracts, NIH3T3 cells were plated on 16-cm dishes, grown to ~70–80% confluence, serum-starved, and treated as indicated. Nuclear proteins were extracted as described previously (13, 14). For gel retardation assays, double-stranded oligonucleotides were end-labeled with γ-[32P]ATP (ICN Biomedicals) by T4 polynucleotide kinase (Promega). The following oligonucleotides were used (only the top strand is shown; the wild type binding sequences are underlined, the mutated binding sequences are double-underlined, and the mutated nucleotides are indicated in boldface). For wild type oligonucleotides: motif 2, 5′-TTGGCAGACACCTGGGAGGATG-3′; motif 4, 5′-GGCAACCTGGGAGGATG-3′; motif 5 (wt), 5′-GCTGACAGATGGTACATGACTGTGTTGAGG-3′; For null mutation oligonucleotides: M5-1null, 5′-GGCAACACATTGGGAGGATG-3′; M5-2null, 5′-GGCAGGATGGTACATGACTGTGTTGAGG-3′; M5-3null, 5′-GCTGACACATGGGAGGATG-3′; M5-4null, 5′-GCTGACACATGGGAGGATG-3′.

For TRE mutation oligonucleotides: M4-TRE, 5′-GGCAGGATGGTACATGACAC-CTGGGAGGATG-3′; M5-1-TRE, 5′-AGCACATGGTACATGACTGTGTTGAGG-3′; M5-2-TRE, 5′-TATGGTACATGACTGTGTTGAGG-3′; M5-3-TRE, 5′-TATGGTACATGACTGTGTTGAGG-3′. The conditions of the binding reactions and gel runs have been described previously (13, 14). For supershift assays, 1 μl (2 μg) of specific antibody was added to the reaction mixture 20 min before the labeled oligonucleotide.

Cell-free extracts—Cells were plated on 30-mm dishes, grown to ~80% confluence, serum-starved, and subjected to the indicated treatments. Subsequently, the cells were solubilized in 150 μl of Laemmli/SDS buffer and sonicated to shear the chromosomal DNA. The lysates were run on 12% SDS-PAGE and transferred to nitrocellulose membrane (Schleicher & Schuell). The membrane was incubated overnight at 4°C under a gentle rotation in 10 ml Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20, and 5% (v/v) non-fat dry milk containing the specific antibodies. The primary antibodies were diluted as follows: anti-active ERK1/2 1:10,000, anti-ERK2 1:4,000, and antibodies for specific Jun-Fos family members 1:1,000. The specific bands were detected using the ECL chemiluminescence detection method (Amersham Biosciences) by exposure on x-ray films. To study the loading of the samples, membranes were stripped with 0.1 μg/ml, pH 2.5 (3 times for 5 min), and washed briefly with 10 ml Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween 20, followed by blocking and immunodetection with anti-ERK antibodies.

RESULTS

M5-1, a Low Affinity TRE-like Element Required for FiRE Activation—FiRE have been shown to contain two Jun-Fos-binding motifs, motifs 4 and 5. Analysis of deletion mutants indicated that both motifs are required for the FGF-2-induced activation of FiRE (13). Mutations of motif 4 caused a 5-fold decrease in activation of FiRE (M5-1 and M5-2) which closely resemble TRE (TRE, 5′-GGCAACACATTGGGAGGATG-3′). To study the role of motif 4, M5-1 and M5-2 in growth factor-induced FiRE activation, they were mutated in order to prevent their binding of AP-1. To retain the overall FiRE structure as intact as possible, point mutations with minimal sequence alterations were made (see “Experimental Procedures”). NIH3T3 cells were stably transfected with a reporter construct containing the various
mutated forms of FiRE in front of the CAT reporter gene (p271FiREmut-CAT). The cells were serum-starved and treated overnight with FGF-2, followed by measurement of CAT activity. The null mutation of motif 4 (M4null) resulted in a complete loss of FiRE activity, whereas individual null mutations of M5-1 or M5-2 (M5-1null or M5-2null) both resulted in 70% reduction of activity, and the M5-2null mutation has a somewhat more potent inhibitory effect. When both M5-1 and M5-2 were mutated together (M5-1/M5-2null), activation of FiRE was completely lost (Fig. 1A). The results show that all putative AP-1-binding elements were required for FiRE activation by FGF-2 and suggest a tight cooperative function between all TRE-like sequences in motif 4 and motif 5.

Because both M5-1 and M5-2 seemed essential for FiRE activation, we next studied protein binding to these elements by gel retardation analyses with oligonucleotides containing intact and null mutated binding sites (for details see “Experimental Procedures”). Although the sequence of M5-1
differs from the consensus AP-1 sequence by only one nucleotide, it did not display detectable protein binding (M5-2null oligonucleotide), whereas intact M5-2 showed clear FGF-2-dependent binding of AP-1 complexes (M5-1null oligonucleotide) (Fig. 1B). A crucial role for M5-1 in FiRE activity was further demonstrated by mutating it to the classical M5-1-TRE, which showed clear FGF-2-dependent binding of AP-1 complexes (Fig. 1D). This mutation abolished FiRE activation almost completely, whereas the same mutation in the other putative AP-1-binding sites of FiRE did not have any inhibitory effect (Fig. 1C). Importantly, mutating M5-2 and motif 4 to the AP-1 consensus site did not alter the growth factor specificity of FiRE. FiRE retained its responsiveness to FGF-2 but still remained non-responsive to serum or EGF (data not shown). On the basis of these data, we propose an updated schematic model of FiRE (Fig. 1E).

**Ras/ERK Pathway Transports the FGF-2 Signal Required for FiRE Activity**—To study the role of the Ras/ERK pathway in FGF-2-induced activation of FiRE, 3T3-p271FiRE-CAT cells were serum-starved and treated overnight with FGF-2 alone or in combination with the MEK-1 inhibitor PD98059 or with the p38 pathway inhibitor SB203580, followed by determination of CAT activity. Inhibition of the Ras/ERK pathway significantly reduced FiRE activation, whereas inhibition of p38 pathway had no effect (Fig. 2A). Furthermore, we transiently transfected the kinase-inactive forms of Raf-1 (mutRaf-1) and MEK-1 (mutMEK-1) in wild type NIH3T3 cells together with the p271FiRE-CAT reporter plasmid, and we measured the
ability of FGF-2 to activate FiRE. Expression of either of the dominant inhibitory proteins significantly down-regulated FiRE activation (Fig. 2B), indicating that the FGF-2-elicited signal required for FiRE activity is transduced via the Ras/ERK pathway.

To investigate how the Ras/ERK pathway activation influenced the binding of AP-1 proteins to FiRE, serum-starved cells were pretreated for 30 min with PD98059 or SB203580 before a 5-h stimulation with FGF-2. Nuclear proteins were isolated, and gel retardation assays were performed with double-stranded oligonucleotides corresponding to the transcription factor-binding sites of FiRE. Inhibition of the Ras/ERK pathway with PD98059 had no effect on binding of USF-1 to motif 2, whereas the FGF-2-induced AP-1 binding to motif 4 was significantly reduced. Interestingly, pretreatment with PD98059 did not inhibit binding of AP-1 complex to motif 5, although the migration of the resultant protein complex appeared slightly altered. This may suggest changes in the composition or post-transitional modification of the proteins in the complex. Inhibition of the p38 pathway with SB203580 did not affect binding of transcription factors to any of the sites studied (Fig. 2C).

Sustained Activation of the Ras/ERK Pathway Activates FiRE—Growth factors activate several signaling systems besides the Ras/ERK pathway. Depending on the cell line, such activation may involve RalGDS, p38, PLC, Src, and phosphatidylinositol 3-kinase pathways (32–39). Therefore, to address specifically the role of the Ras/ERK pathway in the regulation of FiRE, we established NIH3T3 cell lines stably expressing a conditionally active form of oncogenic Raf-1 (H9004 Raf-1:ER[DD]) (30, 40, 41). The kinase activity of H9004 Raf-1:ER[DD] can be induced by 4-OHT, which elicits a rapid activation of the Ras/ERK pathway without affecting other signaling pathways (42, 43).

Introduction of 4-OHT to serum-starved 3T3-H9004 Raf-1:ER[DD] cells induced a rapid and sustained ERK phosphorylation, confirming the activity of the fusion protein (Fig. 3A). However, the cells required a prolonged 4-OHT stimulation to achieve an ERK phosphorylation level comparable with that produced by FGF-2 treatment. Importantly, 4-OHT-induced activation of the Ras/ERK pathway was not regulated by PKA (Fig. 3B), whereas pretreatment of the cells with H89 increased and prolonged the FGF-2-induced ERK phosphorylation, remaining clearly detectable as long as 5 h from the start of the induction. By contrast, when the cells were pretreated with the cAMP analogue 8-Br-cAMP to activate PKA, FGF-2-induced ERK phosphorylation was significantly decreased (Fig. 3C).

To study the ability of 4-OHT to activate FiRE, 3T3-H9004 Raf-1:
ER[DD] cells were transiently transfected with the p271FIRE-CAT reporter construct and treated with 4-OHT alone or in combination with PD98059 or SB203580. As shown in Fig. 4A, FIRE was activated by 4-OHT stimuli. The activation was abolished by PD98059, whereas SB203580 had no effect. These data suggest that sustained signaling through the Ras/ERK pathway activates FIRE. Furthermore, FIRE was also activated in wild type NIH3T3 cells that were cotransfected with p271FIRE-CAT and constitutively active MEK-1 (data not shown).

To investigate how 4-OHT regulated the binding of AP-1 complexes to FIRE nuclear extracts from differentially treated 3T3-ΔRaf1:ER[DD], cells were prepared, and gel retardation assays were performed. Treatment of cells with 4-OHT had no effect on protein binding to motif 2, whereas it induced binding of AP-1 complexes to motif 4. The binding was abolished by pretreatment with PD98059 but not with SB203580. Likewise, 4-OHT induced protein binding to motif 5, but in contrast to cells treated with FGF-2, the binding was abolished with PD98059 (Fig. 4B). These data suggest that sustained activation of the Ras/ERK pathway would be sufficient to induce binding of AP-1 complexes to FIRE and consequently induce the activation of FIRE.

**PKA Regulates Binding of AP-1 Complexes to FIRE**—To elucidate the role of PKA in AP-1-mediated activation of FIRE, serum-starved 3T3-p271FIRE-CAT cells were pretreated with the PKA inhibitor H89 or with the PKA activator 8-Br-cAMP followed by overnight FGF-2 stimulation. Inhibition of PKA completely abolished FIRE activation, whereas activation of PKA dramatically increased FIRE activity (Fig. 5A). We have demonstrated that the FGF-2-induced activation of FIRE can also be inhibited by expressing dominant negative PKA-RI-subunits (25). To investigate the role of PKA in the 4-OHT-induced activation of FIRE, 3T3-ΔRaf1:ER[DD] cells were transiently transfected with the p271FIRE-CAT reporter construct and treated with 4-OHT alone or in combination with chemicals modifying PKA activity. Inhibition of PKA with H89 dramatically decreased the FIRE activity, whereas activation of PKA had no effect (Fig. 5B). Inhibition of PKA down-regulated the FIRE activity also in wild type NIH3T3 cells that were transiently transfected with the p271FIRE-CAT reporter plasmid together with constitutively active MEK-1 (data not shown). These data conclusively show that PKA regulates transcriptional activity of FIRE in both cell models studied.
activity might direct other transcription factors such as members of the CREB and CREM families to bind FiRE. To demonstrate that after combined treatments with FGF-2 and 8-Br-cAMP motifs 4 and 5 still bound Fos-Jun complexes, supershift experiments were performed with antibodies recognizing all Jun or Fos family members (aJun and aFOS, respectively) (Fig. 6B). Both antibodies abolished binding to motifs 4 and 5, indicating that the elements indeed bound Fos-Jun complexes. Taken together, the data suggest that PKA influences the level of FiRE activity by regulating the amount of Fos-Jun complexes bound to FiRE.

To assess whether PKA would also regulate the binding of AP-1 in response to sustained activation of the Ras/ERK pathway the serum-starved 3T3-ΔRaf-1:ER[DD] cells were treated with 4-OHT alone or in combination with H89 or 8-Br-cAMP (12 h). Nuclear extracts were prepared, and gel retardation assays were performed. The treatments did not affect protein binding to motif 2. By contrast, inhibition of PKA reduced binding of AP-1 complexes to the motifs 4 and 5, whereas activation of PKA significantly increased protein binding to both motifs (Fig. 7A). To investigate whether combinatorial treatment with the PKA activator 8-Br-cAMP and 4-OHT increased binding of Jun-Fos complexes to motifs 4 and 5, supershift experiments with aJun and aFOS antibodies were performed. The antibodies abolished the binding (Fig. 7B) similarly to what was seen in FGF-2/8-Br-cAMP costimulated cells (Fig. 6B), indicating that both motifs bound Fos-Jun proteins. These results imply that PKA has a role in regulating the FiRE activation upon sustained signaling via the Ras/ERK pathway. Furthermore, the results suggest that the basal PKA activity may have a direct function in the regulation of growth factor-induced gene transcription.

Analysis of Expression of Fos and Jun Family Members in FGF-2- and 4-OHT-stimulated Cells—Data presented above demonstrate that activation of FiRE in response to FGF-2 or the sustained Ras/ERK pathway activity requires binding of AP-1 complexes to the motifs 4 and 5 and that this involves active PKA. Composition of the Fos-Jun dimers determines the transcriptional activity of the AP-1 complex. Because the abundance of different Fos and Jun family members directly determines the composition of the AP-1 complex (44), we studied the expression of individual Fos and Jun proteins in differentially treated cells by Western blotting (Fig. 8).

Expression of c-Fos, in response to FGF-2, was transient returning to the basal level 12 h after the start of the induction. Combined treatment with FGF-2 and 8-Br-cAMP increased the expression, the increase being most conspicuous at the 3- and 6-h time points. Interestingly, inhibition of PKA before FGF-2 induction did not affect the levels of c-Fos, indicating that PKA is not necessary for its FGF-2-induced expression. The expression of c-Fos was induced also by 4-OHT, and the expression appeared to be PKA-dependent. The c-Fos protein migrated as a broad band, suggesting differential phosphorylation of the protein (45). The expression of Fra-1 and Fra-2 has been shown to be delayed as compared with other Fos family members (46). In our studies, low levels of Fra-1 were detected after a 6-h FGF-2 induction. Fra-1 was absent when the activity of Ras/ERK pathway was blocked with PD98059. Although the fra-1 promoter does not contain PKA-responsive elements (47), the levels of Fra-1 protein were altered by modulation of PKA activity. Both FGF-2 and 8-Br-cAMP induced expression of Fra-2. These two stimuli in combination further increased Fra-2 expression, whereas inhibition of PKA did not affect Fra-2 levels.

Expression of Jun proteins showed that variations in PKA activity did not have significant effects on their FGF-2-induced expression, although promoter regions of junB and junD genes contain potential PKA-responsive elements (48). Inhibition of the Ras/ERK pathway down-regulated the expression of JunB but had very little effect on the expression of c-Jun and JunD. In 3T3-ΔRaf-1:ER[DD] cells, 4-OHT-induced prolonged activation of the Ras/ERK pathway activated the expression of c-Jun and JunB. In this system the expression of JunB was clearly regulated by PKA. JunD was present also in quiescent cells, and its levels were not affected by any of the treatments.

In summary, our data indicate that PKA is involved in the
regulation of the growth factor-induced expression of Fos family proteins, whereas its effect on the expression of Jun family proteins is less prominent. These findings suggest that the PKA-mediated regulation of FiRE may involve alterations in the expression of Fos family proteins and subsequent changes in the composition of AP-1 complexes bound to FiRE.

**DISCUSSION**

Recently we described the FGF-specific enhancer element referred as FiRE on the murine syndecan-1 gene, and we demonstrated that PKA was involved in regulating its transcriptional activation in response to growth factor stimuli (13, 25). Results presented in this paper elucidate the mechanisms controlling the AP-1-mediated activation of FiRE. The putative AP-1-binding sites of FiRE deviate from the optimal AP-1 recognition sequence, which is an imperfect palindrome containing two separate half-sites \(5'-\text{TGA(C/G)TCA}-3'\). Fos-Jun heterodimers can thus potentially bind to regulatory elements in two opposite orientations, both of which have been observed in Fos-Jun-TRE crystals (49). Alterations in the sequence flanking the AP-1 site or in the sequence of either of these half-sites may determine the preferred orientation in which heterodimeric Fos-Jun complexes bind to the specific element (20, 21). Furthermore, additional transcription factors binding to the adjacent sites and interacting with Fos-Jun complexes may define the orientation (16, 50). Because specific interactions between transcription factors and basal transcription machinery are crucial in transcriptional induction, it is evident that orientation of the transcription factor in relation to the promoter exerts a dramatic influence on activation capacity of defined transcription factor (51). The putative AP-1 sites of FiRE contain only one intact half-site, TGGCTCA in motif 5-1, TGACTGT in motif 5-2, and TGAGCCA in motif 4. Precise orientation of AP-1 complexes may therefore be required for FiRE activity. The sequence variations may also contribute to the differential functions of different Fos-Jun complexes bound to these elements. Furthermore, sites with somewhat weaker binding affinities of Fos-Jun complexes may also impose a requirement for interactions with other transcription factors.

Motif 5 contains two TRE-like sequence elements that were both required for FiRE activation (Fig. 1A). Both these elements have sequences with close resemblance to the consensus AP-1-binding site. Although M5-1 differed from consensus AP-1 site only by one nucleotide, it did not seem to bind AP-1 complex or any other protein (Fig. 1B). What could be the...
function of M5-1 in the regulation of FiRE? It may bind a specific factor that cannot be detected in gel retardation assays due to low binding affinity. Previously, Li and co-workers (52) described a corresponding low affinity AP-1 site regulating the expression of the *fas* gene in T-cells. It should be noted that binding of transcription factor to a short oligonucleotide might differ completely from binding to the intact regulatory element that may occur in combination with other factors. Protein binding to M5-1 site may thus require additional transcription factors, such as M5-2-bound AP-1. Moreover, M5-1 may serve a crucial function to orchestrate formation and maintenance of the transcriptionally active protein complex formed around the FiRE. This view may be supported by the notions that when the M5-1 site was mutated to the AP-1 consensus site, the FiRE activation was abolished, perhaps because the resultant high affinity binding of AP-1 prevented the entry of Fos-Jun complex to the adjacent M5-2 site and furthermore interfered with the ordered recruitment of other transcription factors to FiRE. Noticeably, the same mutation in other TRE-like elements of FiRE did not affect on FGF-2-induced FiRE activation. Because heterodimeric Fos-Jun complexes bind to the consensus AP-1 site as roughly equal mixtures of two distinct orientation isomers, it is possible that the M5-1 site and the factor(s) bound to it define the orientation of adjacent AP-1 complex bound to the TRE-mutated motif and facilitate the activation of FiRE in response to FGF-2.

The Ras/ERK pathway is the most important pathway transducing growth factor signals to the nucleus leading to phosphorylation of specific transcription factors and activation of target genes (3, 53). The strict control over the magnitude and duration of kinase activity is mandatory to induce physiologically acceptable level of gene activation. PKA have been shown have an effect on the Ras/ERK signaling by various different mechanisms (11, 54–59). Our data suggest that PKA would negatively regulate the FGF-2-induced Ras/ERK signaling in the cell line studied. We have shown that the inhibition of PKA activity prior to FGF-2 stimulus resulted in overactivation of the Ras/ERK signaling, whereas an increase of PKA activity prior to FGF-2 stimuli decreased the signal flow (Fig. 3C). This observation points to the model where PKA functions to balance the growth factor-induced signal transduction through the Ras/ERK pathway. NIH3T3 fibroblast cells display detectable PKA activity without any stimuli, and it can be anticipated that such basal activity do have a functional role. Basal PKA activity is likely to participate in regulating the growth factor-induced transcription as well as in regulating the magnitude of growth factor-induced Ras/ERK signaling. It is possible that the basal activity also prevents nonspecific signaling and reduces the meaningless signal flow by maintaining a threshold.
that must be overcome before signal transduction through the Ras/ERK pathway is activated. When this threshold is overcome, PKA could control the signal strength, thus providing a mechanism for limiting and fine-tuning the growth factor signaling.

We show that cooperation of PKA and Ras/ERK pathways is required for AP-1-mediated FiRE activation in response to FGF-2. Interestingly, active PKA was also required for FiRE activation with inducible Raf-1, and this suggests that even the basal PKA activity would have a significant role in regulating the AP-1-mediated transcription. What are the mechanisms by which these two pathways cooperatively regulate AP-1-mediated FiRE activation? Both of these signaling pathways regulate the expression of Fos-Jun proteins as well as the transcriptional activity of AP-1 complexes. Our data suggest that PKA activity would determine the amount of Fos-Jun complexes bound to the motifs 4 and 5 and consequently the activation level of FiRE. Inhibition of PKA activity prior to FGF-2 stimuli blocked binding of AP-1 complexes to these motifs and subsequently prevented activation of FiRE although the FGF-2-induced activity of the Ras/ERK pathway was clearly prolonged. Combinatorial induction with growth factor and PKA-activating agents led to synergistic binding of AP-1 to these motifs and an increase in FiRE activity, although the signaling via the Ras/ERK pathway was clearly more transient than in the cells treated with FGF-2 alone. Comparable synergistic transcriptional activation was recently shown for proencephalin gene in neuroblastoma cell line (60) and for urokinase-type plasminogen activator gene in mouse mammary carcinoma cells (61). PKA uses several parallel mechanisms to regulate the abundance and the transcriptional activity of AP-1 components. It controls their expression by the CREB/ATF family transcription factors, which recognize the CRE-like sequences in the promoter regions of junB, junD, c-fos, and fra-2 genes (48, 62). Furthermore, PKA regulates the transactivating capacity of AP-1 complexes by directly phosphorylating members of the Fos family (c-Fos, Fra-1, and Fra-2) and by regulating the nuclear translocation of c-Fos (63–66). The transcriptional activity of c-Jun may also be regulated by PKA, albeit without direct phosphorylation (67). Moreover, inhibitory protein-1 forms a complex with Fos and Jun proteins preventing their nuclear translocation and DNA binding. PKA has been shown to phosphorylate inhibitory protein-1 and restore the AP-1 activity (68, 69).

Transient activation of the Ras/ERK pathway induces short-term expression of c-Fos and Fos-B, whereas sustained activation leads to elevated expression of Fra1, Fra2, c-Jun, and JunB (70, 71). The duration of the Ras/ERK pathway activation may thus determine the availability of different Fos and Jun proteins and therefore influence AP-1-dependent gene expression, because the composition of AP-1 dimer determines its transcriptional activity (44, 72). Changes in the activation level of the Ras/ERK pathway may also have direct effect on transcription of AP-1-regulated genes, because the kinases regulated by this pathway control the transcriptional activity of AP-1 by phosphorylating c-Fos, FosB, and Fra-2, and JunD proteins (73–76). Furthermore, pathways other than PKA can drive the CRE-mediated regulation of fos and jun genes. Growth factor-induced activation of several signaling pathways including the Ras/ERK and the p38 pathways activate kinases phosphorylating CREB at Ser-133 that leads to CRE-mediated activation of PKA-responsive genes such as c-fos (35, 37, 77–80). These observations underline the tight regulatory relationship between these two systems in controlling the AP-1/CRE-mediated gene activation.

It is reasonable to assume that alterations in PKA activity upon FGF-2 stimulation would influence the Jun and Fos fami-
illy members forming the AP-1 complex binding to FiRE. Fra-2 is the only member of Fos family that was identified to bind motifs 4 and 5 in fibroblasts treated alone with FGF-2 or in combination with FGF-2 and 8-Br-cAMP. We were not able to identify the specific Jun proteins bound to these motifs, and thus it is possible that the AP-1-binding sites of FiRE do not discriminate between the Jun family members (data not shown). When interpreting this preliminary data one should be careful, because the gel retardation assay as well as the supershift assays do have their strict limitations when trying to identify the factors bound to specific elements.

The relationship between the Ras/ERK pathway and PKA in the regulation of FiRE activity was ascertained with cells expressing the \( \Delta \text{Raf-1:ER[DD]} \) fusion protein (3T3-\( \Delta \text{Raf-1:ER[DD]} \)). In these cells sustained activation of the Ras/ERK pathway was sufficient to induce activation of FiRE. However, the mode of FiRE activation differs from that seen in FGF-2-stimulated cells. First, in FGF-2-treated cells, inhibition of the Ras/ERK pathway with PD98059 had no effect on binding of AP-1 to motif 5, whereas in 4-OHT-stimulated cells the binding was abolished. Second, costimulation with FGF-2 and 8-Br-cAMP increased the activity of FiRE, whereas 8-Br-cAMP together with 4-OHT did not result in such a synergistic effect. FGF-2 elicits transient activation of the Ras/ERK pathway (Fig 3C), whereas activation of \( \Delta \text{Raf-1:ER[DD]} \) with 4-OHT leads to gradually increasing and sustained activation (Fig. 3A). Furthermore, FGF-2 is known to activate parallel pathways to the Ras/ERK, which are likely to have a profound effect on the reporter system studied and AP-1-mediated transcription in general. By contrast, 4-OHT presumably activates only the Ras/ERK pathway. However prolonged Ras/ERK pathway activation with 4-OHT (≥ 20 h) has been proposed to induce HB-EGF gene expression (43). In certain cell models the responses detected after prolonged induction with 4-OHT might thus involve the autocrine response to produced growth factors. Because in fibroblasts FiRE is not responsive to EGF, it is likely that responses detected were directly mediated by the Ras/ERK pathway activated by the oncogenic Raf-1. Finally, the role of PKA in these two systems was different. Whereas PKA regulated the FGF-2-induced activation of the Ras/ERK pathway (Fig. 3C), it did not have any effect on stimulation with \( \Delta \text{Raf-1:ER[DD]} \) fusion protein (Fig. 3B). It is probable that the differences detected between these induction systems in respect to FiRE activity corresponds to their ability to induce different expression levels of specific Fos and Jun proteins as well as different post-translational modifications on these AP-1 components and the other transcription factors able to bind FiRE. The data suggest that direct comparison between these two systems is impossible, and furthermore, using only one of these systems would be inadequate to draw the complete picture of AP-1-driven regulation of FiRE.

In conclusion, we demonstrate that AP-1-mediated FiRE activity in response to FGF-2 requires cooperation of Ras/ERK and PKA signaling pathways. In addition, we have shown that FiRE activation in response to sustained activation of the Ras/ERK pathway with oncogenic Raf-1 (\( \Delta \text{Raf-1:ER[DD]} \)) also requires PKA. These observations suggest crucial function of basal PKA activity for AP-1-mediated FiRE activation and underlines the important role of PKA in general regulating the cellular responses to the signals transported via the Ras/ERK pathway.

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2 J.-P. Pursiheimo, personal observations.
Cooperation of Protein Kinase A and Ras/ERK Signaling Pathways Is Required for AP-1-mediated Activation of Fibroblast Growth Factor-inducible Response Element (FiRE)

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