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Essential Role of Mitogen-Activated Protein Kinase Pathway and c-Jun Induction in Epidermal Growth Factor-Induced Gene Expression of Human 12-Lipoxygenase

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ABSTRACT

The role of mitogen-activated protein kinase signaling and the transcription factor c-Jun in epidermal growth factor (EGF)-induced expression of 12-lipoxygenase in human epidermoid carcinoma A431 cells was studied. EGF increased the activation of extracellular signal-regulated kinase (ERK) and c-Jun amino terminal kinase (JNK) in a time-dependent manner. Treatment of the cells with an mitogen-activated protein kinase kinase inhibitor, PD098059 (30 μM), inhibited the EGF- and pSV2ras-induced expression of 12-lipoxygenase mRNA. Transfection of the cells with Ras, ERK2, Rac, JNK dominant negative mutants pMMrasDN, K52R ERK2, RacN17, and mJNK all inhibited the EGF-induced promoter activation of the 12-lipoxygenase gene. EGF induced the expression of c-Jun and the activity of transcription factor activator protein 1 in cells, and these effects were blocked by the treatment with K52R ERK2 and mJNK. Overexpression of c-Jun increased the expression of 12-lipoxygenase mRNA and enzyme activity. Furthermore, the Sp1-binding sites in the promoter region of the 12-lipoxygenase gene were requisite for c-Jun response, which was similar to that previously observed in EGF response. The results indicate that the EGF-induced expression of 12-lipoxygenase in A431 cells was mediated through the Ras-ERK and Ras-Rac-JNK signal pathways. Subsequent induction of c-Jun led by ERK and JNK activation was essential for this EGF response.

Arachidonate 12-lipoxygenase (arachidonate:oxygen 12-oxidoreductase; EC1.13.11.31) in the platelet was the first mammalian lipoxygenase discovered (Hamberg and Samuelsson, 1974). It catalyzes the transformation of arachidonic acid into 12(*S*)-hydroperoxyeicosatetraenoic acid. It is subsequently converted to 12(*S*)-hydroxyeicosatetraenoic acid [12(*S*)-HETE] by a glutathione-dependent peroxidase (Chang et al., 1982). In addition to human platelet 12-lipoxygenase, a second 12-lipoxygenase isozyme was found in porcine leukocyte (Yoshimoto et al., 1982). These two 12-lipoxygenases differ in substrate specificity and are expressed from different genes (Yamamoto, 1992). The human platelet-type 12-lipoxygenase was also found in human erythroleukemia cells (Funk et al., 1990; Izumi et al., 1990), epidermal cells (Takahashi et al., 1993), and epidermoid carcinoma cells (Chang et al., 1993).

The biological activities of 12(*S*)-HETE are less studied than the metabolites formed by 5-lipoxygenase catalysis. However, 12(*S*)-HETE plays a significant role in the pathogenesis of some epidermal and epithelial inflammation. A markedly elevated 12(*S*)-HETE level was found in psoriatic plague, whereas the level of prostaglandins E_2 and $F_{2\alpha}$ were only minimally elevated (Hammarstrom et al., 1975). In guinea pig skin, unequivocal growth promotion in addition to the inflammatory reaction was observed upon 12(*S*)-HETE activation (Chan et al., 1985). As a result, high concentration of 12(*S*)-HETE may contribute to the inflammatory changes and the abnormal epidermal hyperproliferation in the development of a psoriatic plaque. In the psoriatic lesions, overexpression of epidermal growth factor (EGF) receptors (Nanney et al., 1986), transforming growth factor-α (Elder et al., 1989), and platelet-type 12-lipoxygenase (Hussain et al., 1994) has been reported. Human epidermoid carcinoma A431 cells overexpresse the EGF receptors (Haigler et al., 1978). It is therefore a suitable cell model to study the EGF response in epidermal cells. EGF induced the expression of human 12-lipoxygenase in A431 cells (Chang et al., 1992). Enhancement of EGF-induced expression of 12-lipoxygenase is caused by transcriptional activation (Liu et al., 1997b). In the anal-

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ABBREVIATIONS: 12(*S*)-HETE, 12(*S*)-hydroxyeicosatetraenoic acid; EGF, epidermal growth factor; Sp1, simian virus 40 promoter factor 1; MEK, mitogen-activated protein kinase kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEKK, mitogen-activated protein kinase kinase kinase; JNK, c-Jun amino-terminal kinase; AP1, activator protein 1; kb, kilobase(s); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SV40, simian virus 40.

ysis of promoter activation, the two simian virus 40 promoter factor 1 (Sp1)-binding sites residing at −158 to −150 bp and −123 to −114 bp were required for EGF response (Liu et al., 1997a).

One of the early events in the EGF signaling pathway involves the coupling of EGF to receptor tyrosine kinase, which causes Ras activation by binding adapter protein Grb2 and the exchange protein Sos. Ras subsequently leads to the activation of Raf-1. Raf-1 phosphorylates and activates mitogen-activated protein kinase kinase (MEK), which, in turn, phosphorylates and activates p^{42}/p^{44} mitogen-activated protein kinases (MAPK) (Moodie et al., 1993), also named as extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2). Ras can also activate Rac and Rho, members of the Rho family of small GTPases (Ridley et al., 1992). Rac in turn activates a protein kinase cascade that leads to the activation of c-Jun amino-terminal kinase (JNK). This cascade includes MEK kinase (MEKK), which phosphorylates JNK kinase, which then phosphorylates and activates JNK (Derijard et al., 1995). We previously found that overexpression of Ha-ras in A431 cells induced the transcriptional stimulation of human 12-lipoxygenase promoter in a manner similar to that of EGF (Chen et al., 1997). In this study, the role of Ras and its downstream ERK and JNK signaling in EGF-induced expression of 12-lipoxygenase was reported. After the activation of the Ras-ERK and Ras-Rac-JNK signal pathways, the functional role of c-Jun induction, relaying the ERK and JNK signaling in EGF response was also illustrated. Evidence obtained from this study directly shows that Ras-ERK and Ras-Rac-JNK signaling, followed by the activation of c-Jun induction, was essential for mediating the EGF-induced gene expression of 12-lipoxygenase.

Experimental Procedures

Materials. Mouse EGF (natural, culture grade) was purchased from Collaborative Research (Bedford, MA). [α-^{32}P]dCTP (3000 Ci/mmol), [γ-^{32}P]ATP (5000 Ci/mmol), [1-^{14}C]arachidonic acid (56.3 mCi/mmol), the multiprime DNA labeling system, and nylon membrane (Hybond-N) were purchased from Amersham (Bucks, UK). PD098059 was from Calbiochem (La Jolla, CA). *o*-Nitrophenyl-β-galactopyranoside was from Sigma. The luciferase assay system and activator protein 1 (AP1) (*c-jun*) oligonucleotides were from Promega (Madison, WI). Oligo(dT)-latex was from Takara (Otsu, Shiga, Japan). Qiagen-tip 100 was from Qiagen (Hilden, Germany). β-Galactosidase plasmid driven by cytomegalovirus (pCMVβ) was from Clontech (Palo Alto, CA). The AP1 *cis*-reporting system containing the pAP1-Luc reporter plasmid was purchased from Stratagene (La Jolla, CA). Monoclonal antibodies against c-Jun and ERK2 were obtained from Transduction Laboratories (Lexington, KY). Rabbit polyclonal antibodies against c-Fos were purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal antibodies directed against the phosphorylated form of Thr202/Tyr204 ERK1/2 and Thr183/Tyr185 JNK were purchased from New England Biolabs (Beverly, MA). Antibody against JNK1 was from Santa Cruz Biotechnology (Santa Cruz, CA). Lipofectamine, Dulbecco's modified Eagle's medium, and Opti-MEM medium were obtained from Life Technologies (Grand Island, NY). Fetal bovine serum was from HyClone Laboratories (Logan, UT). All other reagents used were of the highest purity obtainable.

Cell Culture and EGF Treatment. Human epidermoid carcinoma A431 cells were grown at 37°C under 5% CO_2 in 10-cm plastic dishes containing 10 ml of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 U/ml penicillin. In this series of experiments, cells were treated with 50 ng/ml EGF in culture medium supplemented with 10% fetal bovine serum, unless stated otherwise.

Preparation of Microsomal Fraction. Cells in a 6-cm Petri dish were washed twice with PBS and scraped with a Teflon sheet in 50 mM Tris · HCl, pH 7.4. They were then sonicated with a Heat System-Ultrasonics Model W-375 sonicator (Farmingdale, NY). The homogenate was centrifuged at 9,000*g* for 20 min, and the resulting supernatant was centrifuged at 105,000*g* for 1 h in a Bechman L8-80 M ultracentrifuge (Palo Alto, CA). The resulting pellet was resuspended in 0.5 ml of 50 mM Tris · HCl, pH 7.4, and was designated as the microsomal fraction. All of these procedures were performed at 4°C. The protein content of microsomes was determined with bovine serum albumin (fraction V) as a standard (Lowry et al., 1951).

Assay of Microsomal 12-Lipoxygenase Activity. The assay mixture contained 8.5 μM [1-^{14}C]arachidonic acid (0.1 μCi) and the enzyme protein in microsomes in a final volume of 0.2 ml. The reaction was allowed to take place at 37°C for 20 min. The reaction mixture was acidified to pH 3.0 with 1 N HCl, extracted with 2 ml of ethyl acetate, and applied to thin-layer chromatography plates. The plates were developed in the organic phase of a solvent of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (11:5:2:10, v/v). Formation of [1-^{14}C]12(*S*)-HETE was determined by a system 2000 Imaging Scanner (Bioscan, Washington, DC).

Preparation of Nuclear Extracts. Cells from eight dishes (8×10^7 cells) were washed twice with PBS and scraped in 6 ml of PBS. Cells were collected by centrifuging at 400*g* for 10 min, resuspended in 10 volumes of buffer A (300 mM sucrose, 10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, and 0.1 mM EDTA) and homogenized by 20 strokes with a Dounce homogenizer A pestle (Wheaton, Millville, NJ). Buffer A and all buffers described below contained 0.5 mM phenylmethylsulfonyl fluoride, 1 mM orthovanadate, 2 μg/ml pepstatin A, and 2 μg/ml leupeptin. Nuclei were pelleted by centrifugation at 400*g* for 10 min. Pellets were resuspended in 10 volumes of buffer B [10 mM HEPES, pH 7.9, 400 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EGTA, and 5% glycerol (v/v)] and were homogenized by 20 strokes with a B pestle. The suspension was stirred for 1 h at 4°C and then centrifuged at 16,000*g* for 60 min in a microcentrifuge. Supernatants were collected and dialyzed for 16 h against 50 volumes of buffer C [20 mM HEPES, pH 7.9, 0.1 mM EDTA, 75 mM NaCl, and 20% glycerol (v/v)]. Dialysates were centrifuged at 7,500*g* for 10 min and the supernatants were stored at −70°C until use.

Western Blotting. An analytical 10% SDS-polyacrylamide slab gel electrophoresis was performed. The cell nuclear extracts or lysates (30 μg of protein of each) prepared from control and EGF-treated cells were analyzed. For immunoblotting, proteins in the SDS gels were transferred to a polyvinylidene difluoride membrane by an Electroblot apparatus. Mouse monoclonal antibodies against human c-Jun and c-Fos or rabbit polyclonal phospho-ERK1/2 and phospho-JNK were employed as the primary antibodies. Immunoblot analysis was carried out with mouse IgG antibody coupled to horseradish peroxidase. An enhanced chemiluminescence kit (Amersham) was used for detection. The density of the immunoblots was determined by an image analysis system installed with a software BIO-ID (Vilber Lourmat, France).

Gel-Shift Assay. AP1 (c-jun) oligonucleotides, 5′-CGCTTGATGAGTCAGCCGGAA-3′ were end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (Sambrook et al., 1989). The binding reaction was performed in a 15-μl reaction mixture containing 0.2 μg of poly(dI-dC).poly(dI-dC), 20 mM HEPES, pH 7.9, 0.1 mM KCl, 2 mM $MgCl_2$, 15 mM NaCl, 0.2 mM EDTA, 5 mM dithiothreitol, 10% (v/v) glycerol, 2% (w/v) polyvinyl alcohol, 6 μg of the cell nuclear extracts, and the radiolabeled probe (4×10^4 c.p.m.). The mixtures were incubated at room temperature for 30 min and loaded on a 4% (w/v) polyacrylamide gel. Electrophoresis was performed at a constant 300 V for 1 h. The gel was dried and autoradiographed.

RNA Preparation. Cells from four 10-cm petri dishes were harvested in 6 ml of solution D composed of 4 M guanidine thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosinate, and 0.1 M 2-mer-

captoethanol. Total cellular RNA was isolated using an acid guanidine thiocyanate-phenol-chloroform method. The RNA content was determined on 1% agarose gel that was stained with 1 μg/ml ethidium bromide. mRNA from total RNA was purified by using oligo(dT)-latex as described previously (Chang et al., 1993).

RNA Blot Analysis. For RNA separation, 20 μg of total RNA or 2 μg of mRNA were separated by electrophoresis on 1% agarose-glyoxal gel and transferred to a nylon membrane (Sambrook et al., 1989). The equivalency of samples was verified by the intensity of ethidium bromide staining of the 28 S and 18 S rRNA bands. The *Hin*dIII-*Bam*HI fragment [2.3 kilobases (kb)] of human platelet 12-lipoxygenase cDNA, the *Bam*HI fragment of c-Jun cDNA (2.3 kb), the *Eco*RI-*Xho*I fragment of c-Fos cDNA (2.0 kb), and the *Pst*I fragment of GAPDH cDNA (1.25 kb) were used as probes for the identification of 12-lipoxygenase mRNA, c-jun mRNA, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, respectively, in cells. Probes were labeled with [α-^{32}P]dCTP by using a multiprime DNA labeling system, and hybridization with the ^{32}P-labeled probes was performed with a rapid hybridization system (Amersham). The nylon membranes were washed three times at room temperature in 2× standard saline/phosphate/EDTA buffer (300 mM NaCl, 20 mM NaH_2PO_4, and 2 mM EDTA) containing 0.1% SDS. Each wash was carried out for 15 min. Autoradiography was then performed. The intensity of the hybridized band was determined by Fujix Bio-imaging Analyzer BAS1000 (Fuji Photo Film Co., Tokyo, Japan).

Construction of Luciferase Reporter Vector. The human 12-lipoxygenase promoter regions of various lengths were prepared either by restriction enzyme digestion of the genomic clone for the preparation of pXLO-1 or by the PCR amplification method for the preparation of pXLO-7-1, 8, and 8D, as described previously (Liu et al., 1997a). The mutants at Sp1 site (SPM) were constructed by the site-directed mutagenesis method as described previously (Liu et al., 1997a). All the DNA fragments were ligated into a luciferase plasmid pXP-1. Simian virus 40 (SV40) early promoter was obtained from pGL2-control vector (Promega) digested with *Bgl*II and *Hin*dIII, and ligated with pXP-1 to form vector SV40-LUC. All the plasmids for transfection was purified by the use of Qiagen-tip 100.

Transfection of Cells with Plasmids. The lipofection method was performed with lipofectamine according to the manufacturer's instruction with a slight modification. A431 cells were replated 36 h before transfection at a density of 3×10^5 cells in 2 ml of fresh culture medium in a 3.5-cm plastic dish. For use in transfection, 12.5 μl of lipofectamine was incubated with 0.5 μg of pXLO luciferase plasmid, 0.2 μg of β-galactosidase plasmid, or indicated plasmids as described in the figure legends in 1 ml of Opti-MEM medium for 30 min at room temperature. Cells were transfected by changing the medium with 1 ml of Opti-MEM medium containing the plasmids and lipofectamine, and then incubated at 37°C in a humidified atmosphere of 5% CO_2 for 24 h. After the change of Opti-MEM medium to 2 ml of fresh culture medium, cells were incubated for additional 48 h, unless stated otherwise.

Luciferase and β-Galactosidase Assays. The luciferase activity was measured by the luciferase assay system. The 2.5×10^6 transfected cells were washed with PBS and lysed in 150 μl of luciferase lysis reagent. After a 15-min incubation at room temperature, the lysate was centrifuged at 7200*g* for 15 s, and the supernatant solution was used as the cell lysate. Luciferase assay substrates in 100 μl were mixed with 30 μl of the cell lysate, and then the luciferase activity was measured by a Berthold Lumat LB 9501 luminometer. For the β-galactosidase assay, 30 μl of the cell lysate were mixed with 234 μl of the reaction buffer (0.1 M sodium phosphate, pH 7.5, 10 mM KCl, 1 mM $MgCl_2$, 0.1% (v/v) Triton X-100, and 5 mM β-mercaptoethanol), and the mixture was kept at 37°C for 10 min. The reaction was started by the additional of 66 μl of *o*-nitrophenyl-β-galactopyranoside solution (4 mg/ml in 0.1 M sodium phosphate at pH 7.5), and continued for 2 h at 37°C. The reaction was stopped by the addition of 150 μl of 1 M Na_2CO_3, and the absorbance at 420 nm was measured by a Hitachi U-3210 spectrophotometer. A 28% increase in the expression of pCMVβ-galactosidase was observed in cells treated with 50 ng/ml EGF. Transfection of cells with 0.5 μg of pRSVjun slightly altered the expression of β-galactosidase by a 47% increase. These effects in cells were of a negligible level if compared with those on the expression of luciferase activity induced by EGF and c-Jun overexpression. Therefore, the luciferase activities of cells in each culture dishes were normalized to their respective β-galactosidase activities in this series of experiments.

Results

Activation of ERK and JNK by EGF. EGF has been shown to activate the ERK and JNK pathways in several cell lines (Pai and Tarnawski, 1998; Pomerance et al., 1998). The effect of EGF on ERK and JNK activities in A431 cells was therefore studied. EGF stimulated the ERK and JNK activities in a time-dependent manner (Fig. 1), but not p38 MAPK (data not shown). Activation of ERK and JNK could initially be observed in cells treated with EGF for 0.5 min, and the maximum response was observed in cells treated with EGF for 5 min.

Effect of PD098059 on EGF- and Ha-ras-Induced Expression of 12-Lipoxygenase. To determine whether EGF-induced expression of 12-lipoxygenase was mediated by ERK activation, PD098059, an inhibitor of MEK, was used. Pretreatment of cells with 30 μM PD098059 significantly inhibited the EGF-induced 12-lipoxygenase mRNA expression (Fig. 2A).With the aid of a luciferase reporter, we have previously reported that overexpression of Ha-ras enhances the activity of 12-lipoxygenase promoter (Chen et al., 1997). The action of Ha-ras on the expression of 12-lipoxygenase mRNA

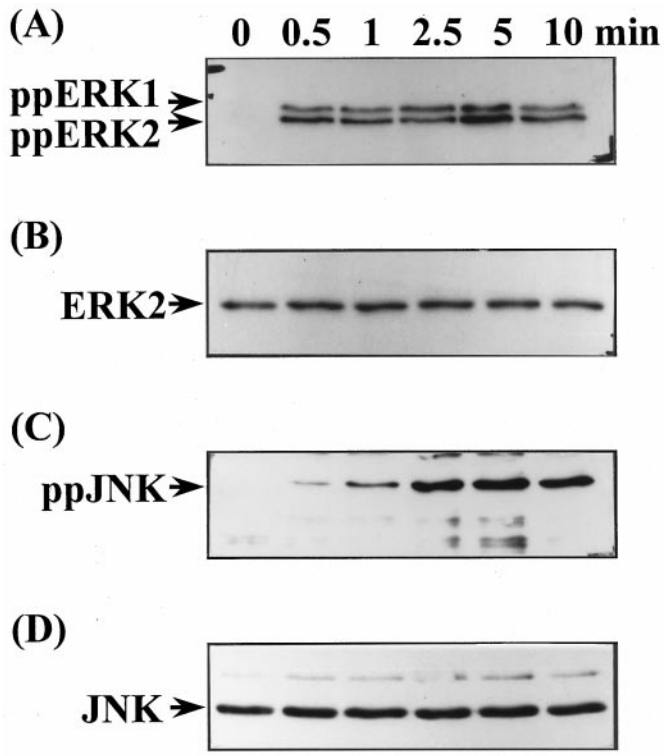

Fig. 1. Time-dependent effect of EGF on ERK and JNK activities. Confluent cells were starved for 24 h in serum-free culture medium before EGF treatment, and then treated with EGF in culture medium without serum. Whole-cell lysates were prepared and subjected to Western blotting using antibodies specific for phosphorylated form of ERK1/2 (A) and JNK (C) and those against ERK2 (B) and JNK (D).

was then studied. Vector pSV2ras was used in this series of experiments. Overexpression of Ha-ras in A431 cells enhanced the expression of 12-lipoxygenase mRNA in a time-dependent manner. Transfection of cells with 1.0 μg of vector pSV2ras for 24, 36, 60, and 72 h resulted in 51, 81, 100, and 110% increases in 12-lipoxygenase mRNA expression, respectively. On the other hand, the Ha-ras-induced expression of 12-lipoxygenase mRNA was completely inhibited by the treatment of cells with 30 μM PD098059 (Fig. 2B).

Effect of Dominant Negative Mutants of Ras, ERK, Rac, and JNK on EGF-Induced Promoter Activation of 12-Lipoxygenase. Expression vector of Ras-dominant negative mutant pMMrasDN (Feig and Cooper, 1988) was used to determine whether EGF-induced expression of 12-lipoxygenase was mediated by Ras signaling. Cells were transfected with a luciferase reporter gene and a dominant negative vector pMMrasDN for 68 h, followed by EGF stimulation. As shown in Fig. 3A, transfection of pMMrasDN in cells dose-dependently inhibited EGF-induced promoter activation of 12-lipoxygenase. Transfection of cells with ERK, Rac, and JNK dominant negative mutants K52R ERK2, RacN17 and mJNK, respectively, also inhibited the EGF-induced promoter activation of 12-lipoxygenase (Fig. 3A). Transfection of cells with dominant negative mutant K52R ERK induced a more complete inhibition of EGF-induced promoter activation than that with either dominant negative mutants mJNK or RacN17. Furthermore, overexpression of MEKK significantly stimulated the 12-lipoxygenase-reporter activity in a dose-dependent manner (Fig. 3B). These results indicate that the Ras-ERK and Ras-Rac-JNK signal pathways were essential for the EGF-induced expression of 12-lipoxygenase, and Ras-ERK pathway played a more significant role than Ras-Rac-JNK pathway in this EGF response.

Activation of c-Jun Expression by EGF. When MAPK is activated, it is translocated to nucleus (Khokhlatchev et al., 1998). Therefore activation of transcription factors such

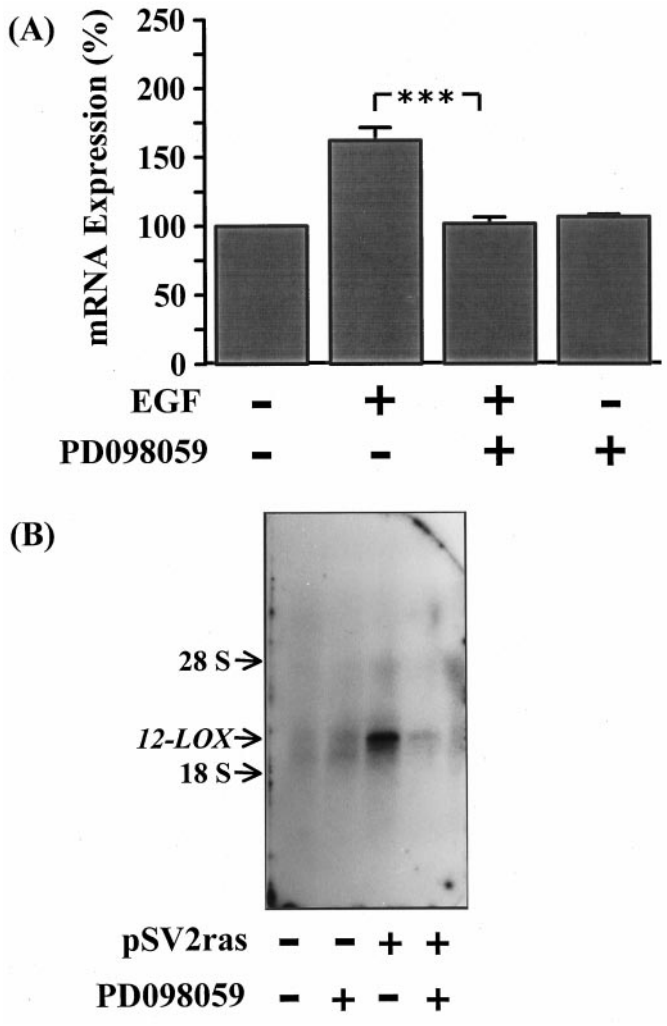

Fig. 2. Effect of PD098059 on EGF- and Ha-ras-induced MAPK activation and 12-lipoxygenase mRNA expression. A, confluent cells were treated with 30 μM PD098059 for 30 min followed by EGF treatment for 30 min. The medium was then switched to EGF-free culture medium containing 30 μM PD098059 for up to 18 h in the assay of 12-lipoxygenase mRNA expression. The intensity ratio of control cells was defined as 100%, and the relative intensity ratio of EGF-treated cells to inhibitor-treated cells in each experiment was calculated. Values for mRNA expression are means ± S.E.M. of three experiments. Data were analyzed statistically by Student's t test. B, cells were transiently transfected with 1 μg of pSV2ras for 68 h in the presence or absence of 30 μM PD098059 and the expression of 12-lipoxygenase mRNA was analyzed.

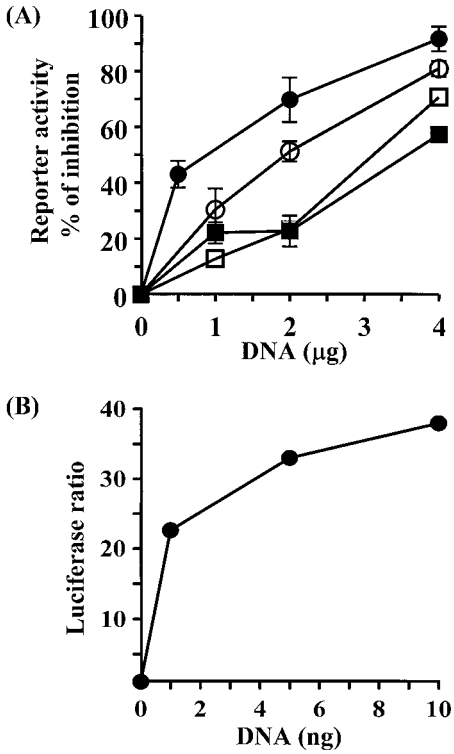

Fig. 3. Effect of dominant negative mutants of Ras, ERK2, Rac and JNK on EGF-induced promoter activation of 12-lipoxygenase gene. A, cells were cotransfected with 0.5 μg of pXLO-7-1 luciferase plasmid, 0.2 μg of β-galactosidase plasmid and dominant negative vectors pMMrasDN (○), K52R ERK2 (●), RacN17 (□), and mJNK (■) by the lipofection method. pBluescript KRSPA was used as a vector to adjust to the same amount of plasmids in each experiment. After the change of Opti-MEM medium to 2 ml of fresh culture medium, cells were incubated for an additional 24 h and then treated with 50 ng/ml EGF. After 30 min of EGF treatment, the medium was removed and then the cells were further cultured in fresh medium up to 18 h. The expression of luciferase activity and β-galactosidase activity was determined. B, a mixture of 0.5 μg of pXLO-7-1 luciferase plasmid, 0.2 μg of β-galactosidase plasmid, and pFC-MEKK plasmid was transfected into cells by the lipofection method. The luciferase expression ratio of pFC-MEKK-transfected and control cells is indicated. Values shown in A are means ± S.E.M. from at least three separate experiments done in triplicate in each experiment. Each value shown in B is from one experiment, and three determinations in control and pFC-MEKK-treated cells were performed.

as c-Fos and c-Jun may be induced in this signal pathway (Pulverer et al., 1991; Deng and Karin, 1994). Induction of c-Fos and c-Jun by EGF in A431 cells was then studied. EGF induced the expression of both c-jun mRNA and protein in a time-dependent manner (Fig. 4A). The maximum induction of mRNA and protein was observed in cells treated with EGF for 0.5 h and 1 h, respectively, and the maximum induction of c-Jun protein sustained at least up to 6 h after EGF treatment (Fig. 4B). EGF also induced the expression of c-fos mRNA (Fig. 4C). In comparison with the stability of c-jun mRNA, c-fos mRNA was degraded more quickly. Most of c-fos mRNA was degraded in 1 h after EGF treatment, but more than half of c-jun mRNA was still intact in the same time. In contrast to the long-term expression of c-Jun protein, the maximum induction of c-Fos protein was also observed at 1 h after EGF treatment, but the induction then declined and almost disappeared in cells treated with EGF for 6 h (Fig. 4C). To confirm that c-Jun induction is the consequence of MAPK signaling, the effect of the dominant negative vectors of ERK and JNK on the EGF-induced expression of c-Jun was then studied. As shown in Fig. 5, overexpression of the dominant negative mutant K52R ERK2 and mJNK in cells inhibited the EGF-induced expression of c-jun mRNA in a dose-dependent manner.

Activation of AP1 by EGF. c-Jun is thought to confer transcriptional enhancement through AP1 elements (Karin, 1995), including c-jun promoter activation (Angel et al., 1988). The activity of AP1 is regulated by several mechanisms, including alteration in the expression of specific AP1 components, which may affect dimer formation and consequent affinity, as well as protein phosphorylation (Karin, 1995). To investigate whether EGF induces AP1 activity, electrophoretic gel mobility shift assay and the reporter activity assay of pAP1-luciferase reporter were performed. As indicated in Fig. 6A, when nuclear extracts from EGF-treated cells were allowed to react with a DNA probe, band retardation was observed. Formation of the retarded band was blocked by the addition of unlabeled AP1 oligonucleotides. Incubation of nuclear extract with the specific antibodies against c-Fos and c-Jun significantly abolished the formation of the retarded band. Presence of both c-Jun and c-Fos antibodies abolished the formation of the retarded band synergistically, indicating that both c-Fos and c-Jun were the components of AP1 interacting with its promoter binding site. Moreover, EGF induced the binding activity of AP1 in cell nuclear extract to its promoter-binding site in a time-dependent manner (Fig. 6B). The induction was observed in cells treated with EGF for 5 min and reached the maximum at 1 h after EGF treatment. The functional assay for AP1 activation in cells was then performed with a pAP1-luciferase reporter. As indicated in Fig. 7A, EGF induced the expression of AP1-driven reporter activity in a time-dependent manner. Induction of the reporter expression in cells was not observed

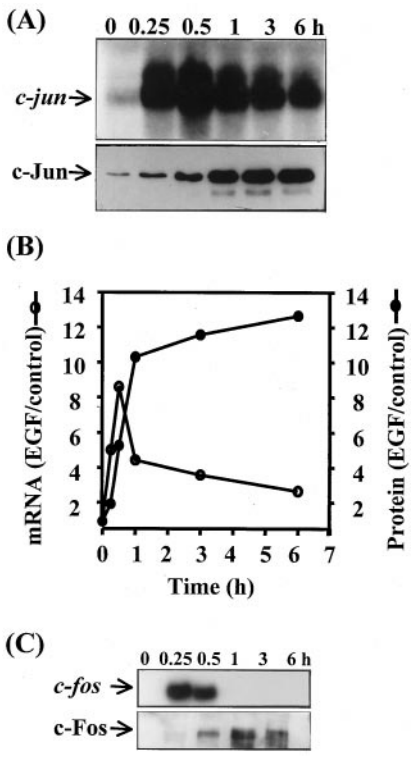

Fig. 4. Effect of EGF on the expression of c-Jun and c-Fos. Confluent cells were starved for 24 h in serum-free culture medium before EGF treatment, and then treated with EGF in culture medium without containing serum for a different time period as indicated. A, Northern blot analysis of mRNA and immunoblot analysis of c-Jun expression in nuclear extracts were performed. B, a kinetic change in the expression of c-jun mRNA and protein in cells treated with EGF is indicated. C, Northern blot analysis of mRNA and immunoblot analysis of c-Fos expression in nuclear extracts were performed.

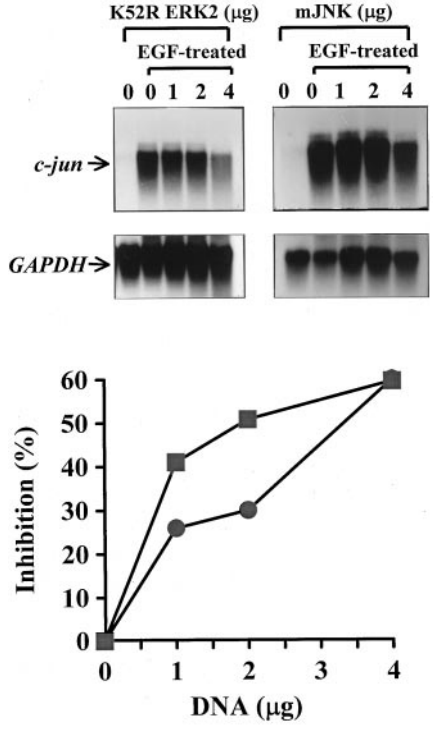

Fig. 5. Effect of ERK and JNK dominant negative mutants on EGF-induced expression of c-jun mRNA. A, cells were transfected with 1, 2, and 4 μg of ERK and JNK dominant negative forms K52R ERK2 and mJNK as described in Fig. 3. After incubation in serum-free medium for 12 h, cells were treated with EGF for 30 min. mRNA was purified by oligo(dT)-latex, and the expression of c-jun and GAPDH was analyzed. B, the intensity of the hybridized band was determined, and the mRNA ratio of c-Jun to GAPDH in each sample was obtained. The mRNA ratio of control cells was defined as 1, and the relative ratio of EGF- and dominant negative mutants-treated groups in each experiment was calculated. The percentage of K52R ERK2- (●) and JNK-induced (■) inhibition of c-Jun expression was determined.

until 40 min after EGF treatment. The time lag in the reporter assay compared with AP1 activation in gel shift assay might be because of the time needed for luciferase protein biosynthesis. To study the mediation of Ras signaling in EGF-induced AP1 activation, cells were transfected with the dominant negative mutants of ERK and JNK. As shown in Fig. 7B, overexpression of the dominant negative mutants K52R ERK2 and mJNK dose-dependently inhibited EGF-induced AP1 reporter activity. These results indicate that EGF activated the cellular AP1 activity through ERK and JNK signal pathways, which led to the increase in the expression of c-Jun.

Stimulation of 12-Lipoxygenase Expression by c-Jun Overexpression. The effect of transient transfection with an expression vector of c-Jun on the expression of 12-lipoxygenase mRNA and enzymic activity was studied. Overexpression of c-Jun induced the expression of 12-lipoxygenase mRNA in a dose-dependent manner (Fig. 8A) and also the expression of 12-lipoxygenase activity (Fig. 8B). Luciferase reporter vectors bearing various lengths of 5′-flanking regions of 12-lipoxygenase gene were then used to study the promoter activation induced by the overexpression of c-Jun. The results are summarized in Fig. 9. The transcription activities of luciferase-bearing vectors pXLO-1 (−951 bp) and pXLO-7-1 (−224 bp) were stimulated by overexpression of c-Jun. A 14- to 18-fold increase in activities was observed by comparing the luciferase activity in c-Jun-transfected cells with that of the control cells. An apparent decrease in the stimulatory response of c-Jun-transfection was observed in vectors bearing a promoter with a deletion from −224 (pXLO-7-1) to −100 bp (pXLO-8), indicating that a promoter region ranging from −224 to −100 bp was important for the c-Jun-stimulated response of 12-lipoxygenase expression. This observation was further confirmed with vectors with the 3′-deletion. A 15-fold stimulation of c-Jun response was also observed in pXLO-8D with a deletion of −1 to −83 bp. To identify the potential role of Sp1 binding sites of the promoter region in c-Jun response, the luciferase reporter vectors bearing the promoter with mutations at −123 to −114 bp (SPM6), at both −158 to −150 bp and −123 to −114 bp (SPM8), and at all three Sp1 binding sequences (SPM7) were used. A 65% decrease in c-Jun response was observed in SPM6, whereas the response was nearly abolished in vectors SPM7 and SPM8 (Fig. 9). These results indicate that the downstream and middle Sp1 sites residing at −123 to −114 bp and −158 to −150 bp played an important role in c-Jun-induced transcription of the human 12-lipoxygenase gene, as previously reported in EGF response (Liu et al., 1997a). To further examine the functional role of Sp1 in c-Jun induction, an SV40 early promoter that contains six Sp1-binding sites located in the 21-bp repeat region (Dynan and Tjian, 1983) was used. Plasmids SV40-LUC bearing the SV40 early promoter-driven luciferase gene and pRSVjun were transiently co-transfected into A431 cells. c-Jun overexpression induced a 6-fold increase in the luciferase expression driven by SV40 early promoter. These results indicated that the Sp1-binding sites in the promoter region of 12-lipoxygenase and SV40 play an important role in the gene expression of EGF and

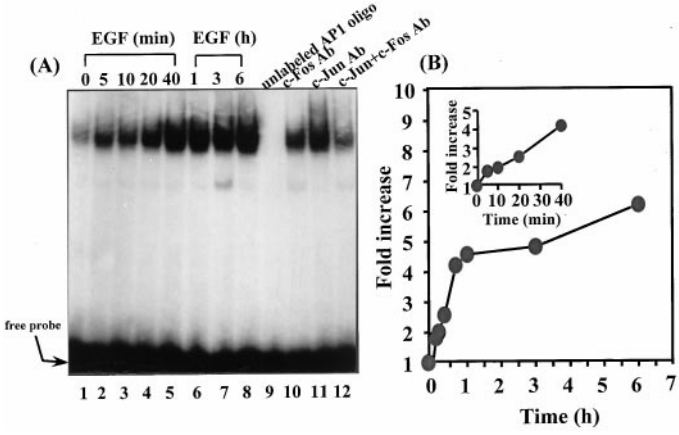

Fig. 6. Activation of AP1 activity by EGF. Confluent cells were starved for 24 h in serum-free culture medium before EGF treatment, and then treated with EGF in culture medium without serum. A, electrophoretic mobility shift assay was performed with ^{32}P-labeled AP1 (c-jun) oligonucleotides as described under *Experimental Procedures*. Nuclear extracts were prepared from starved cells treated with EGF for 5 min to 6 h (lanes 2–8). Competition assays with 1.75 pmol of unlabeled AP1 oligonucleotides (lane 9), 0.1 μg of c-Fos antibody (lane 10), 0.25 μg of c-Jun antibody (lane 11), and both c-Fos and c-Jun antibodies (lane 12) were performed by using nuclear extracts of cells treated with EGF for 6 h. B, the intensity of the binding activity of AP1 (lanes 1–8 as shown in A) was analyzed. The kinetic effect of EGF treatment on the binding activity of AP1 is indicated. The insert plot shows the increase of the binding activity of AP1 in the early stage of EGF-treated condition.

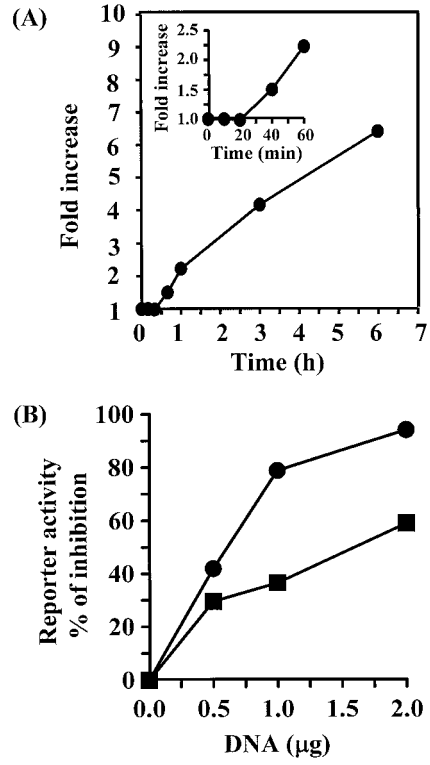

Fig. 7. Effect of ERK and mJNK dominant negative mutants on EGF-induced expression of AP1-driven reporter activity. A, cells were transfected with 0.2 μg of the pAP1-Luc plasmid and 0.2 μg of β-galactosidase by the lipofection method. After the change of Opti-MEM medium to 2 ml of fresh culture medium, cells were incubated for an additional 24 h and then starved for 12 h before treatment with EGF for 10 min to 6 h in serum-free culture medium. Expression of luciferase activity and β-galactosidase activity was determined. The insert plot shows the increase of luciferase activity in the early stage of EGF-treated condition. B, cells were cotransfected with 0.2 μg of pAP1-Luc, 0.2 μg of β-galactosidase, and a different amount (0.5~2 μg) of K52R ERK2 (●) or mJNK (■), and then incubated as described in A. The expression of luciferase activity and β-galactosidase activity of cells treated with 50 ng/ml EGF for 6 h were determined. Values are means ± S.E.M. of three determinations.

c-Jun responses. A slight induction of the luciferase reporter activity driven by 12-lipoxygenase gene promoter was also observed in cells transfected with an expression vector pSV-fos; however, the effect of c-Fos was only one eighth of that of c-Jun overexpression (data not shown).

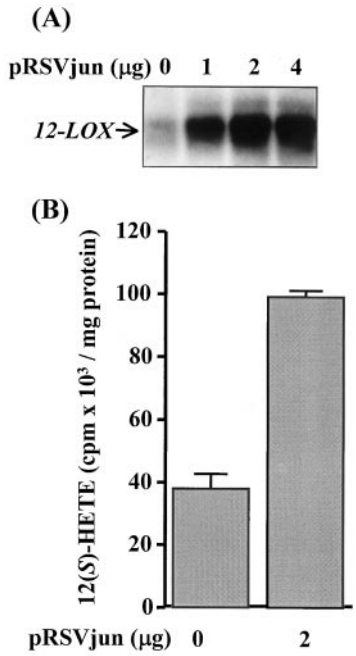

Fig. 8. Stimulation of 12-lipoxygenase expression by c-Jun overexpression. Cells were transfected with pRSVjun by the lipofection method. Expression of 12-lipoxygenase mRNA (A) and activity (B) of cells transfected with the expression vector for 68 h was determined. Values of enzyme activity indicated in B are means ± S.E.M. of three determinations.

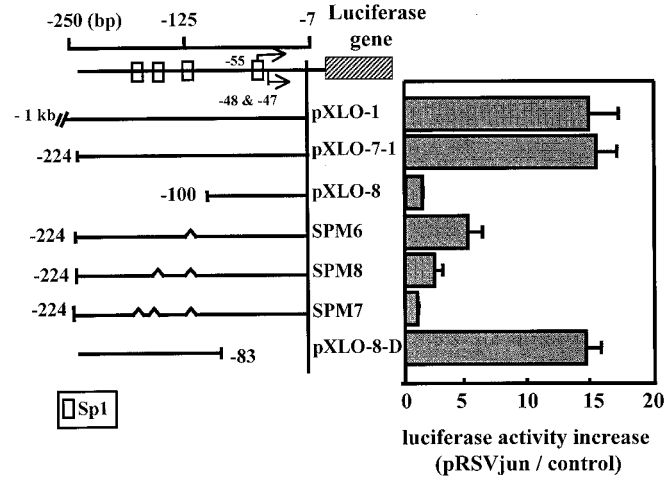

Fig. 9. Effect of c-Jun overexpression on the promoter activity of 12-lipoxygenase. Cells were transfected with 0.5 μg of luciferase plasmid-bearing 12-lipoxygenase gene promoter, 0.2 μg of β-galactosidase, and 0.5 μg of pRSVjun by the lipofection method. After the change of Opti-MEM medium to 2 ml of fresh culture medium, cells were incubated for an additional 48 h. The luciferase and β-galactosidase activities were then determined. Transcription initiation sites at −55G, −48G and −47A in A431 cells are also indicated as arrow marker. The expression ratio of pRSVjun-treated cells to control cells is indicated. Each value was from three separate experiments, and three determinations in control and pRSVjun-treated cells were performed in each experiment. Values are means ± S.E.M.

Discussion

In this study, the role of the ERK and JNK activation induced by Ras signaling, followed by the induction of c-Jun biosynthesis in EGF-induced expression of human arachidonate 12-lipoxygenase in A431 cells, was analyzed. We reported previously that the activation of human 12-lipoxygenase transcription by Ha-ras overexpression was similar to that induced by EGF (Chen et al., 1997). The two Sp1 binding sequences residing at −158 to −150 bp and −123 to −114 bp in 12-lipoxygenase gene promoter were requisite for both EGF and Ras responses (Liu et al., 1997a). These results indicate that the Ras signaling pathway may play a very significant role in EGF-induced expression of 12-lipoxygenase. In the present study, we provided new evidence indicating that Ras-ERK and Ras-Rac-JNK signal pathways are directly involved in EGF-induced expression of 12-lipoxygenase. First, the EGF-induced 12-lipoxygenase mRNA expression was inhibited by PD098059, a MEK inhibitor (Fig. 2A), indicating that MEK-ERK activation was essential for EGF-induced expression of 12-lipoxygenase mRNA. Secondly, overexpression of pSV2ras in A431 cells induced the expression of 12-lipoxygenase mRNA in a time-dependent fashion, which correlated well with its effect on the enhancement of the 12-lipoxygenase promoter activity reported previously (Chen et al., 1997). PD098059 inhibits the pSV2ras-induced expression of 12-lipoxygenase mRNA (Fig. 2B), indicating that ERK activation was crucial for the activation of 12-lipoxygenase mRNA expression induced by the overexpression of Ha-ras. Finally, inhibition of EGF-induced expression of 12-lipoxygenase by the overexpression of dominant negative expression vectors of Ras, ERK, Rac and JNK (Fig. 3) pointed to the direct mediation of Ras-ERK and Ras-Rac-JNK signaling in EGF-induced activation of 12-lipoxygenase gene.

Another important finding from this study is the demonstration of a pivotal role of c-Jun induction after the ERK and JNK activation in EGF-induced expression of 12-lipoxygenase. Activation of ERK is thought to activate numerous transcription-related proteins, such as p62TCF, c-Myc and AP1 (Karin and Hunter, 1995). Earlier studies show that an AP1-like element in the c-jun promoter mediates a positive autoregulation of the *c-jun* gene in HeLa TK$^-$ cells (Angel et al., 1988), and EGF induces the c-jun promoter through a Ras-to-Rac-MEKK pathway in HeLa cells (Clarke et al., 1998). The protein kinase MEKK phosphorylates JNK kinase, followed by the phosphorylation and activation of JNK (Derijard et al., 1995). JNK phosphorylates and activates several transcription factors, including c-Jun (May et al., 1998). Gel shift assay in this study revealed that the AP1 activated by EGF treatment in A431 cells was at least in part composed of a Jun-Fos heterodimer (Fig. 6A). Therefore, any changes in either c-Jun or c-Fos, which alters dimer formation, may affect the AP1 activity. Phosphorylation of AP1 is a key event to switch on AP1 activity. ERK1/2 phosphorylate c-Fos (Deng and Karin, 1994). The phosphorylation of the carboxyl-terminus of c-Fos is required for activation of an AP1 site specific for Jun-Fos heterodimers (McBride and Nemer, 1998). Phosphorylation of c-Jun can be caused by ERK activation (Pulverer et al., 1991) or by JNK activation (May et al., 1998). Two pieces of evidence were provided in this study to indicate that AP1 activation was essential for EGF-induced expres-

sion of the *c-jun* gene in A431 cells. First, the binding capacity of AP1 of nuclear extract to DNA probe of AP1 consensus element was induced before the expression of c-jun mRNA (Fig. 4 and Fig. 6B). Secondly, the reporter activity of AP1-driven luciferase vector in A431 cells was increased by EGF treatment in a time-dependent manner (Fig. 7A). Inhibition of EGF-induced reporter activity of AP1-driven luciferase vectors (Fig. 7B) and that of EGF-induced expression of c-jun mRNA (Fig. 5) were another two pieces of evidence indicating that the induction of c-Jun expression by EGF in A431 cells was at least in part mediated through ERK and JNK activation.

In the treatment of A431 cells with EGF, the expression of both of c-Jun and c-Fos was induced, but the induction of c-Jun was more significant than that of c-Fos protein. The maximum induction of c-Jun protein was observed in cells treated with EGF for 1 h, and then sustained for at least up to 6 h after EGF treatment (Fig. 4B). In contrast to the long-term expression of c-Jun protein, the maximum induction of c-Fos protein was also observed at 1 h after EGF treatment, but the induction then declined and almost disappeared in cells treated with EGF for 6 h (Fig. 4C). Although a slight induction of the luciferase reporter activity of 12-lipoxygenase promoter was observed in cells overexpressing c-Fos, it was only one eighth of the response of c-Jun overexpression. Therefore, the induction of c-Jun protein might contribute more than c-Fos protein in the increase of AP1 activity induced by EGF treatment in the response of 12-lipoxygenase induction. Overexpression of c-Jun in A431 cells activated the promoter activity of 12-lipoxygenase gene in the same fashion as EGF, as reported previously (Liu et al., 1997a). The promoter region ranging from −224 to −100 bp was important for the c-Jun response as for EGF response. With the aid of site-directed mutagenesis, two Sp1 sequences residing at −158 to −150 bp and −123 to −114 bp were identified as critical elements for c-Jun response (Fig. 9) as for EGF response. Similar requirement for the Sp1 consensus sequences for promoter activation was observed in the stimulation of 12-lipoxygenase in A431 cells treated with EGF and c-Jun overexpression. Therefore, induction of c-Jun biosynthesis in cells was an essential step in EGF-induced expression of 12-lipoxygenase. The maximum induction of c-Jun protein was observed in cells treated with EGF for 1 h (Fig. 4B), and the induction of 12-lipoxygenase mRNA and promoter activation was initially observed in cells treated with EGF for 9 h (Chang et al., 1993; Liu et al., 1997b). A lag period of 8 h is present between the expression of c-Jun protein and 12-lipoxygenase mRNA. Neither known AP1 binding sequence in the promoter region responsive to EGF nor apparent binding between the transcription factor AP1 and EGF-responsive promoter DNA is observed (Liu et al., 1997a). Although two Sp1 binding sites residing at −158 to −150 bp and −123 to −114 bp are essential in the mediation of EGF induction of 12-lipoxygenase gene, the EGF response is not essential because of the increase in Sp1 biosynthesis. No change of the binding between nuclear Sp1 proteins and promoter DNA was observed in control and EGF-treated cells (Liu et al., 1997a). Therefore, induction of Sp1 protein biosynthesis triggered by the transcription factor AP1 in EGF-induced activation of 12-lipoxygenase gene seems unlikely. Growth factor induction without significant change in nuclear factor binding has also been observed for Sp1 binding to the EGF response element in the human gastrin promoter (Merchant et al., 1995). We previously reported that not only the basal expression of 12-lipoxygenase but also EGF induction was regulated by Sp1-binding sites in the promoter region (Liu et al., 1997a). In addition, we recently found that the expression of Sp1, not Sp3, stimulated the activities of 12-lipoxygenase promoter and SV40 early promoter in *Drosophila melanogaster* Schneider SL2 cells with deficiency of Sp1 (Chen et al., 1999). These results suggest that Sp1 was required for the transcription of 12-lipoxygenase gene as a component of transcription factors, and interaction of Sp1 with other transcription factors may be necessary for EGF-induced activation of 12-lipoxygenase gene transcription. The mechanisms by which AP1 activation induced the expression of 12-lipoxygenase gene is still unclear. One possibility is the cooperative interaction of AP1 with Sp1 and other transcription factors. The cooperative complex formed between the nuclear factor of activated T cells and AP1 on the interleukin-2 enhancer has been reported (Wolfe et al., 1997).

In summary, we found that activation of Ras-ERK and Ras-Rac-JNK signaling, followed by the induction of c-Jun biosynthesis, was essential for EGF-induced gene expression of 12-lipoxygenase. The proposed pathway is described as follows. Induction of Ras by EGF is mediated by EGF receptor binding to Grb2 and Sos, a guanine nucleotide exchange factor for Ras (Chardin et al., 1993). Activation of Ras stimulates Raf, which then activates MEK, followed by the activation of ERK. Ras can also activate JNK through MEKK protein kinase cascade. EGF efficiently activates Ras-ERK and Ras-Rac-JNK pathway and then induces the activation of AP1. Biosynthesis of c-Jun is then induced, which enhances AP1 activity by AP1 formation of homodimers or heterodimers of c-Jun and c-Fos. Enhancement of AP1 may directly or indirectly interact with other unknown factors to regulate the function of Sp1. This activated-Sp1 complex may then trigger the expression of 12-lipoxygenase gene. This is the first report indicating that MAPK activation through Ras signaling, followed by induction of c-Jun, was essential for EGF-induced activation of 12-lipoxygenase in A431 cells. The present study, together with our previous reports (Chang et al., 1993; Liu et al., 1997a) delineated in part the mechanisms by which EGF mediates the transcriptional activation of 12-lipoxygenase in epidermal cells and linked the overexpression of EGF receptors (Nanney et al., 1986) and transforming growth factor α (Elder et al., 1989) to that of 12-lipoxygenase (Hussain et al., 1994) in psoriatic lesions.

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