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Elevation of Intracellular cAMP Inhibits Growth Factor-Mediated Matrix Metalloproteinase-9 Induction and Keratinocyte Migration

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ABSTRACT

Receptor tyrosine kinases are regulators of diverse cellular functions including cell growth, cell survival, differentiation, locomotion, and morphogenesis. Activation of the cAMP-dependent protein kinase A inhibits receptor tyrosine kinase-stimulated growth responses in a number of cell types. In this study, we investigated the consequences of elevated cAMP on growth factor-mediated keratinocyte migration and matrix metalloproteinase (MMP)-9 induction in a human keratinocyte cell line. We found that elevation of intracellular cAMP by forskolin abolishes epidermal growth factor (EGF)- or scatter factor/hepatocyte growth factor-dependent colony dispersion. Concentrations of forskolin that inhibit growth factor-induced motility also eliminate EGF- or scatter factor/hepatocyte growth factor-dependent induction of the 92-kDa gelatinase/MMP-9. In contrast to findings obtained in fibroblasts, elevated intracellular cAMP did not interfere with growth factor-dependent activation of the p42/44 extracellular signal-regulated kinases, indicating that cAMP-dependent inhibition of migration and MMP-9 induction does not occur through perturbation of the extracellular signal-regulated kinases/mitogen-activated protein kinase pathway. However, forskolin effectively inhibited EGF-dependent activation of c-Jun N-terminal kinase and p38, demonstrating that cAMP selectively interferes with a different subset of growth factor-induced mitogen-activated protein kinase signaling cascades than reported previously in fibroblasts. These findings illustrate that EGF concurrently activates multiple mitogen-activated protein kinase signaling cascades in keratinocytes and suggests that each pathway contributes to maximal EGF-dependent migration and proteinase induction.

Epithelial cell migration is an important aspect of wound healing, embryogenesis, and tumor metastasis (Lauffenburger, 1996; Hudson and McCawley, 1998). Activation of receptor tyrosine kinases such as the epidermal growth factor (EGF) receptor regulates many processes involved in cell migration including dissolution of cell-cell contacts, local proteolysis, and directional locomotion (Lauffenburger, 1996; Hudson and McCawley, 1998; Wells et al., 1998). EGF-dependent keratinocyte migration requires de novo gene expression (McCawley et al., 1998), and among the many genes regulated by receptor tyrosine kinases are members of the matrix metalloproteinase (MMP) family of extracellular matrix degrading proteinases (Boyd, 1996; McCawley et al., 1998). Recent findings illustrate that MMPs play important roles in a broad range of cellular functions (Shapiro, 1998), and we have shown previously that 92-kDa gelatinase/MMP-9 induction is important for EGF-dependent keratinocyte migration (McCawley et al., 1998).

The intracellular second-messenger cAMP has been shown to modulate receptor tyrosine kinase-dependent signaling pathways and subsequent biological responses. cAMP has been shown to have a direct impact on receptor tyrosine kinase signal transduction upstream of gene transcription. Activation of cAMP-dependent protein kinase [protein kinase A (PKA)] has been reported to phosphorylate Raf-1 and thereby interfere with the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) cascade in fibroblasts, resulting in inhibition of growth factor-induced DNA synthesis (Cook and McCormick, 1993; Sevetson et al., 1993; Wu et al., 1993; Huang et al., 1994). Thus, elevation of intracellular cAMP can result in rapid attenuation of growth factor-induced signal transduction and gene expression by disrupting the ERK/MAPK signaling cascade.

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ABBREVIATIONS: EGF, epidermal growth factor; MMP, matrix metalloproteinase; PKA, cAMP-dependent protein kinase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; SF/HGF, scatter factor/hepatocyte growth factor; JNK, c-Jun N-terminal kinase; GST, glutathione *S*-transferase; NGF, nerve growth factor; HOG, high-osmolarity glycerol; SCC, squamous cell carcinoma.

cAMP is additionally recognized as an independent regulator of many cellular processes that are also modulated by receptor tyrosine kinases. Elevation of cAMP levels governs a variety of cell functions such as cell proliferation, survival, and differentiation, either enhancing or inhibiting the response depending on cell type and context (Daniel et al., 1998). Furthermore, cAMP has been implicated in modulation of cell migration and metalloproteinase expression in certain cell types. Increased cAMP levels have been reported to inhibit fibroblast motility (O'Neill et al., 1985; Iwamoto et al., 1993). In addition, elevation of intracellular cAMP promotes collagen-dependent dissolution of cell-cell junctions, but inhibits migration of NBT-II rat bladder carcinoma cells (Morton and Tchao, 1994; Rodier et al., 1995) as well as collagen-directed migration of endothelial cells and colon carcinoma cells (Lampugnani et al., 1990; Ogasawara et al., 1997). In contrast, cAMP fosters Lewis lung carcinoma cell migration (Young et al., 1990, 1993) and dibutyryl cAMP has been reported to slightly enhance collagen-directed keratinocyte migration (Iwasaki et al., 1994).

Because findings on the role of cAMP in cell motility are contradictory and largely address extracellular matrix-driven migration, we evaluated the actions of elevated cAMP on growth factor-induced keratinocyte migration and MMP-9 induction. We found that elevation of cAMP antagonizes EGF-stimulated responses, but cAMP alone does not promote either colony dispersion or expression of MMP-9 in a human keratinocyte cell line [squamous cell carcinoma (SCC) 12F)]. We have reported previously that EGF and scatter factor/hepatocyte growth factor (SF/HGF) activate the p42/44 ERK, c-Jun N-terminal kinase (JNK), and p38 MAPK cascades and that sustained ERK activation is required for growth factor-stimulated MMP-9 induction and migratory responses (McCawley et al., 1999). Previous studies have established that the ERK, JNK, and p38 MAP kinases each contribute to MMP-9 gene expression (Boyd, 1996; Gum et al., 1997; Himelstein et al., 1997; Simon et al., 1998; McCawley et al., 1999), but it is currently unclear which MAPK pathway(s) are necessary for growth factor-stimulated MMP-9 production. Interestingly, cAMP did not inhibit growth factor-dependent activation of the p42/44 ERK/MAPKs; however, EGF-dependent stimulation of JNK and p38 MAPK cascades was disrupted. These findings demonstrate that cAMP inhibits a different subset of growth factor-induced MAPKs in keratinocytes than in fibroblasts and further identify human keratinocytes as one of a limited number of cell types in which cAMP-dependent inhibition of JNK activation has been detected (Hsueh and Lai, 1995; Rao and Runge, 1996; Shapiro et al., 1996; Li et al., 1997). Furthermore, cAMP-dependent inhibition of p38 represents a novel observation in mammalian cells, although genetic evidence in yeast supports interactions between these pathways (Varela et al., 1995; Marquez and Serrano, 1996; Siderius et al., 1997). Together with our previous work, the findings indicate that sustained ERK activation may be required but is not sufficient for growth factor-dependent keratinocyte migration and MMP-9 induction, and provides additional evidence supporting a role for other MAPK pathways in growth factor stimulation of these responses.

Materials and Methods

Cell Lines and Cell Culture. SCC 12F cells were originally derived from a tumor of the facial epidermis and were generously provided by Dr. William A. Toscano, Jr. (University of Minnesota, Minneapolis) and maintained and treated as described previously (McCawley et al., 1998, 1999). Murine EGF was obtained from Biomedical Technologies Inc. (Stoughton, MA), and SF/HGF was a generous gift from Genentech (South San Francisco, CA). Forskolin, 1,9-dideoxyforskolin, dibutyryl cAMP, theophylline, H89, and SB202190 were obtained from Calbiochem (La Jolla, CA) and dissolved in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide did not exceed 0.1% (v/v) in any experiment.

Measurements of Cell Motility. Evaluation of colony dispersion (cell scattering) was performed as described previously (McCawley et al., 1999). Briefly, cells were subcultured and maintained in growth medium until colonies of greater than 16 cells were established. Cultures were deprived of growth factors and serum for 24 h before treatment with or without ligand at the concentrations and times indicated in the figure legends. Colony dispersion or in vitro re-epithelialization was documented by photography. Photographs of cell cultures were taken using a Nikon N2000 camera mounted on a Nikon Diaphot-TMD inverted phase contrast microscope. Results shown are representative of at least three independent experiments.

Western Blot Analysis. Activated MAPK species were detected using phosphospecific phospho p44/42 MAPK (Thr-202/Tyr-204) monoclonal antibody (New England Biolabs, Beverly, MA) directed against the dually phosphorylated, active forms of the proteins according to the vendor's instructions and as described previously (McCawley et al., 1999). SCC 12F cells were serum-deprived for 24 h before stimulation with ligand at the concentrations and for the times indicated in the figure legends. Detection of total ERK as loading control was accomplished using a pan-ERK antibody (Transduction Laboratories, Lexington, KY).

Kinase Assays. JNK and p38 activity were measured by the immunocomplex assays described previously (McCawley et al., 1999). Briefly, SCC 12F cells were serum-deprived for 24 h before stimulation with ligand at the concentrations and for the times indicated in the figure legends. Cell lysate (100 μg) was incubated with either 5 μl of anti-JNK or 10 μl of anti-p38 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and 30 μl of either protein A-agarose (JNK) (Life Technologies, Gaithersburg, MD) or protein G-agarose (p38) (Sigma, St. Louis, MO) at 4°C for 1 to 2 h. The immunocomplexes were washed and incubated in kinase buffer at 30°C for 20 min with [γ-^{32}P]ATP (NEN Life Science Products, Boston, MA) and either glutathione *S*-transferase (GST)-c-Jun (JNK assay) or GST-ATF-2 (p38 assay). The kinase reactions were terminated by the addition of Laemmli sample buffer. The proteins were resolved using 10% SDS-polyacrylamide gel electrophoresis minigels. Substrate phosphorylation was detected by autoradiography and quantified using a Bio-Rad model GS-700 imaging densitometer (Bio-Rad Hercules, CA). pGEX-2T-c-Jun(1–79 amino acids) was the gift of Dr. Daniel Mueller (Department of Medicine, University of Minnesota). pGEX-3X-ATF-2 was the gift of Dr. Benoit Dérijard (Center de Biochimie, Nice, France). GST fusion proteins were expressed and purified as described previously (McCawley et al., 1999).

Zymogram Analysis. SCC 12F cells were serum-deprived for 24 h before growth factor treatment in fresh serum-free medium. Conditioned medium collected from control (untreated) and growth factor-treated cell cultures was analyzed for proteinase activity by substrate-gel zymography as described previously (McCawley et al., 1999). Briefly, equal amounts of total protein from experimental samples was fractionated on 10% SDS-polyacrylamide gels containing 0.1% gelatin. After electrophoresis, gels were washed with 2.5% Triton X-100 for 30 min at room temperature, then incubated with substrate buffer (50 mM Tris, 0.2 M NaCl, 5 mM $CaCl_2$, 0.02% Brij 35, pH 7.6) for 24 to 48 h at 37°C. Proteinase activity is visualized as clear areas in a Coomassie Blue-stained gel. Relative proteinase

activities were quantitated using a Kodak 440CF Image Station. Results shown are representative of a minimum of three independent experiments.

cAMP Accumulation Assay. [^{3}H]cAMP accumulation was measured in SCC 12F cells as described previously (Witt-Enderby and Dubocovich, 1996). Confluent SCC 12F cells plated in a 12-well culture dish were labeled with 2 μCi/ml [^{3}H]adenine in Dulbecco's modified Eagle's medium/Ham's F-12 for 6 h, then washed twice with 1× PBS. Cells were stimulated for 10 min with the indicated concentrations of forskolin or 10 nM EGF in the presence of 30 μM rolipram. The medium was aspirated, and the incubation was terminated by adding 5% trichloroacetic acid followed by incubation at 4°C for 16 h. [^{3}H]cAMP was isolated from [^{3}H]ATP using Dowex (Bio-Rad) and alumina columns (Sigma) and quantitated by liquid scintillation counting. Recovery was normalized by spiking the columns with a known amount of [^{14}C]cAMP (52.3 mCi/mmol; NEN Life Science Products).

Results

Forskolin Enhances cAMP Accumulation and Inhibits its EGF- and SF/HGF-Dependent Colony Dispersion. Increased cAMP levels have been reported to inhibit fibroblast motility; however, there are contradictory reports on the role of cAMP in epithelial cell migration (O'Neill et al., 1985; Lampugnani et al., 1990; Iwamoto et al., 1993; Morton and Tchao, 1994; Rodier et al., 1995; Ogasawara et al., 1997). Therefore, we evaluated SCC 12F cell migration in response to cAMP. First, we measured cAMP accumulation after exposure to EGF and the adenylyl cyclase activator forskolin. cAMP levels were measured as the amount of [^{3}H]cAMP formed in response to stimulation of SCC 12F cells labeled with [^{3}H]adenine. EGF stimulation did not alter cAMP levels; however, forskolin promoted a concentration-dependent accumulation of cAMP, with 100 μM forskolin increasing cellular cAMP approximately 70-fold over basal levels (Fig. 1).

The role of cAMP in receptor tyrosine kinase-dependent migration was assessed in a colony-dispersion assay. Forskolin did not promote a migratory response in SCC 12F cells at concentrations up to 100 μM (Fig. 2B). However, when SCC 12F cells were incubated with increasing concentrations of forskolin before growth factor stimulation, an inhibition of EGF- and SF/HGF-dependent colony dispersion was observed (Fig. 2). Partial inhibition of EGF-mediated scattering was detected at forskolin concentrations of 10 μM (Fig. 2F), and a near-complete inhibition occurred on pretreatment with 30 μM forskolin (Fig. 2E). No colony dispersion was detected in the presence of 100 μM forskolin (Fig. 2D). Similarly, 30 μM forskolin blocked SF/HGF-dependent colony dispersion (Fig. 2J). Treatment of cells with the negative control 1,9-dideoxyforskolin did not inhibit EGF-dependent colony dispersion (Fig. 2H). Comparable results were obtained using two other agents that elevate cAMP levels by different mechanisms; dibutyryl cAMP (a cAMP analog) and theophylline (a phosphodiesterase inhibitor), and in an in vitro re-epithelialization assay (data not shown).

Forskolin Inhibits EGF- and SF/HGF-Dependent MMP-9 Induction. Because we have shown previously that growth factor-dependent induction of MMP-9 is required for SCC 12F colony dispersion (McCawley et al., 1998), we wanted to determine whether increased intracellular cAMP accumulation inhibited receptor tyrosine kinase-regulated MMP-9 expression. Conditioned medium was collected from SCC 12F cells stimulated with EGF or SF/HGF in the presence or absence of increasing concentrations of forskolin and analyzed by gelatin zymography (Fig. 3). Forskolin concentrations of 100 μM modestly inhibited basal levels of MMP-9 (Fig. 3B). However, pretreatment of SCC 12F cells with forskolin resulted in a concentration-dependent inhibition of growth factor-stimulated MMP-9 induction (Fig. 3B). Near-complete inhibition of both EGF- and SF/HGF-dependent MMP-9 induction was observed at 100 μM forskolin (Fig. 3, A and B). Similarly, forskolin inhibited EGF-dependent induction of MMP-9 in normal human keratinocyte cultures (data not shown). In contrast, the negative control 1,9 dideoxy forskolin did not inhibit basal or EGF-stimulated MMP-9 induction (Fig. 3C). The findings illustrated in Figs. 2 and 3 demonstrate that forskolin alone does not stimulate keratinocyte migration or MMP-9 induction, but does effectively interfere with receptor tyrosine kinase-dependent responses. Forskolin did not alter EGF-dependent tyrosine phosphorylation (data not shown) as has been reported in fibroblasts (Barbier et al., 1999), suggesting that cAMP accumulation interferes with downstream signal transduction cascades.

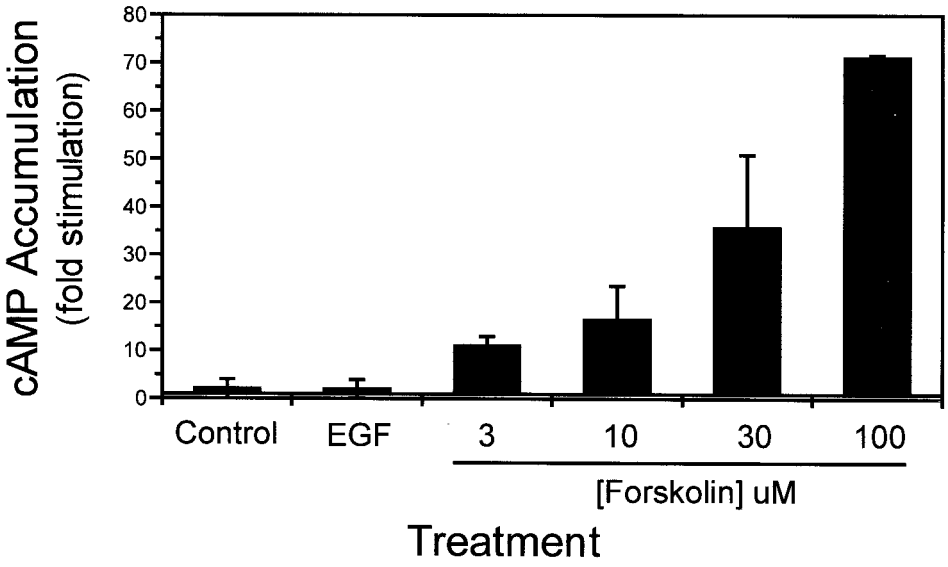

Fig. 1. Forskolin enhances cAMP accumulation in SCC 12F. [^{3}H]adenine-labeled cells were stimulated for 10 min with the indicated concentration of forskolin or EGF (10 nM). Control indicates cultures that received no treatment. [^{3}H]cAMP was isolated and quantitated as described in *Materials and Methods*. Results are from three independent experiments, and the values are expressed as fold stimulation relative to the control (untreated cells = 1.0) ± S.D.

Receptor Tyrosine Kinase-Stimulated ERK Activation Is Not Inhibited by Forskolin. Activation of PKA by cAMP has been reported to impinge on the ERK/MAPK signal transduction pathway (Cook and McCormick, 1993; Sevetson et al., 1993; Wu et al., 1993; Huang et al., 1994). We have shown that receptor tyrosine kinase-mediated keratinocyte migration and MMP-9 induction require sustained p42/44 ERK/MAPK activation (McCawley et al., 1999); therefore, inhibition of cellular responses by forskolin might be predicted to be attributable to interference with growth factor-dependent ERK activation. To test this possibility, SCC 12F cells were incubated with forskolin before growth factor stimulation, and ligand-dependent ERK activation was evaluated. As shown in Fig. 4, forskolin did not inhibit EGF-stimulated ERK activation at early or extended time points (Fig. 4A). Similarly, SF/HGF-dependent stimulation of ERK activation was insensitive to elevated intracellular cAMP levels (Fig. 4B). These results suggest that inhibition of receptor tyrosine kinase-dependent keratinocyte migration and MMP-9 induction by cAMP does not occur through perturbation of the ERK/MAPK pathway.

An additional role for PKA in modulation of ERK activity has been reported. In PC12 cells, maximal sustained activation of ERKs by nerve growth factor (NGF) was found to be dependent on PKA, and coincubation with the PKA inhibitor H89 significantly blocked ERK1 activation by NGF (Yao et al., 1998). Because sustained ERK activation is required for growth factor-dependent induction of MMP-9 in keratinocytes (McCawley et al., 1999), we examined the potential contribution of PKA to this response. As suggested by the absence of forskolin-dependent induction of colony dispersion or MMP-9 expression (Figs. 2 and 3), PKA activation does not appear to be required for sustained ERK activation in SCC 12F cells. Pretreatment with H89 at concentrations that effectively blocked NGF-stimulated ERK activation in PC12 cells (Yao et al., 1998) did not interfere with EGF-stimulated ERK activation at early or extended time points (Fig. 5A). Furthermore, H89 did not inhibit EGF-dependent MMP-9 induction (Fig. 5B) or SCC 12F colony dispersion (data not shown).

Inhibition of EGF-Dependent JNK and p38 Activation by Forskolin. There is evidence that the JNK and p38

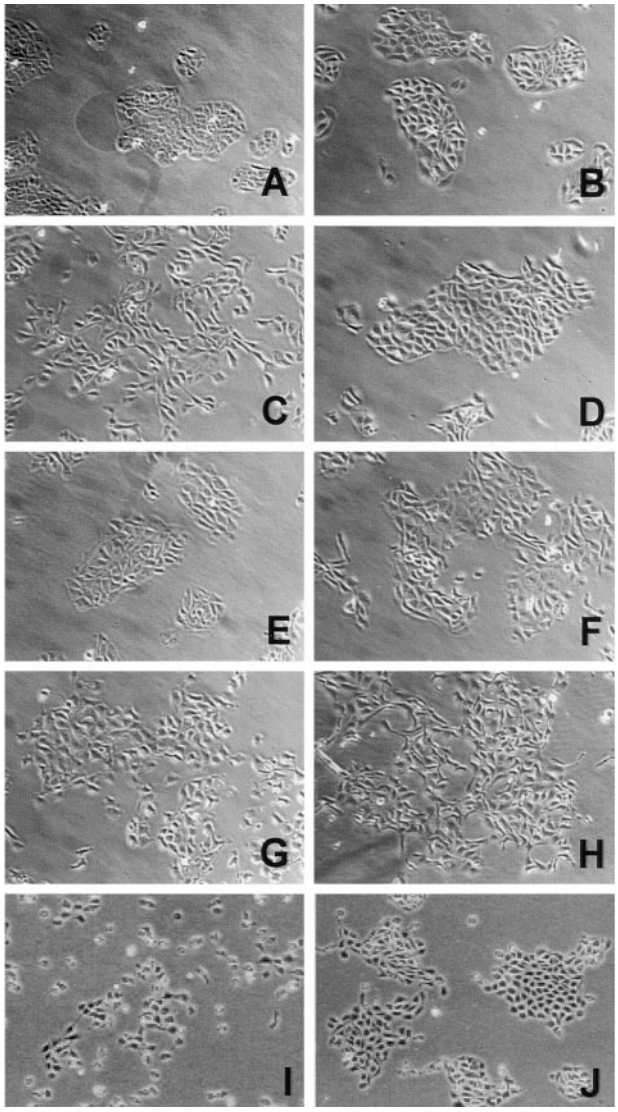

Fig. 2. Forskolin inhibits receptor tyrosine kinase-mediated migration. SCC 12F cells were grown as described under *Materials and Methods* and serum-deprived for 24 h before growth factor stimulation. Forskolin or 1,9-dideoxyforskolin was added 5 min before addition of EGF (10 nM) or SF/HGF (10 ng/ml). Colony dispersion was documented by photography 18 h after adding growth factor. A, no treatment; B, 100 μM forskolin; C, 10 nM EGF; D, 10 nM EGF + 100 μM forskolin; E, 10 nM EGF + 30 μM forskolin; F, 10 nM EGF + 10 μM forskolin; G, 10 nM EGF + 3 μM forskolin; H, 10 nM EGF + 50 μM 1,9-dideoxyforskolin; I, 10 ng/ml SF/HGF; J, 10 ng/ml SF/HGF + 30 μM forskolin. Results are representative of at least four independent experiments.

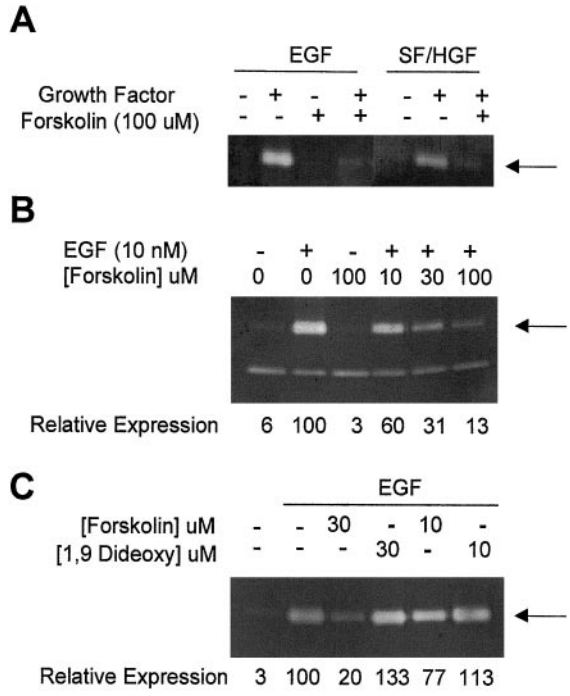

Fig. 3. Forskolin inhibits EGF-stimulated MMP-9 induction. Gelatin zymography was performed as described under *Materials and Methods* using conditioned media collected from cells treated with (+) or without (−) growth factor in the presence or absence of forskolin for 24 h. The arrow indicates MMP-9. A, cells were pretreated with 100 μM forskolin for 5 min before addition of EGF (10 nM) or SF/HGF (10 ng/ml). B, cells were pretreated with the indicated concentrations of forskolin for 5 min before addition of EGF (10 nM). MMP-9 production was quantitated using a Kodak 440CF Image Station and normalized to EGF as 100%. Results are representative of at least three independent experiments. C, cells were pretreated with the indicated concentrations of forskolin or 1,9-dideoxyforskolin (1,9 dideoxy) for 5 min before addition of EGF (10 nM). MMP-9 production was quantitated as described in B.

MAPK pathways are involved in MMP-9 gene expression (Boyd, 1996; Gum et al., 1997; Himelstein et al., 1997; Simon et al., 1998; McCawley et al., 1999). Disruption of the constitutively activated JNK signaling cascade inhibits high basal MMP-9 expression in UM-SCC-1 cells (Gum et al., 1997) and phorbol ester-stimulated MMP-9 induction is abolished by inhibitors of p38 (Simon et al., 1998). We have shown that EGF activates both JNK and p38 in SCC 12F cells (McCawley et al., 1999), and in a limited number of cell types, cAMP has been reported to inhibit JNK activation (Hsueh and Lai, 1995; Rao and Runge, 1996; Shapiro et al., 1996; Li et al., 1997). Therefore, we wanted to establish whether PKA-dependent pathways might interfere with growth factor-stimulated activation of the JNK and p38 MAPK cascades.

As illustrated in Fig. 6 and reported previously (McCawley et al., 1999), EGF stimulates transient activation of JNK (9.89- ± 1.67-fold in three independent experiments) in SCC 12F cells. Pretreatment of cells with 50 μM forskolin abolished EGF-dependent JNK activation (Fig. 6A) but did not substantially interfere with anisomycin-dependent JNK activation (Fig. 6B). Inhibition of JNK activation by PKA has been observed in T lymphocytes (Hsueh and Lai, 1995), smooth muscle cells (Rao and Runge, 1996; Shapiro et al., 1996), and rat liver epithelial cells (Li et al., 1997). Importantly, cAMP did not inhibit stress-dependent JNK activation in keratinocytes (Fig. 6B) or rat liver epithelial cells (Li et al., 1997), indicating that the inhibition is specific to particular stimuli.

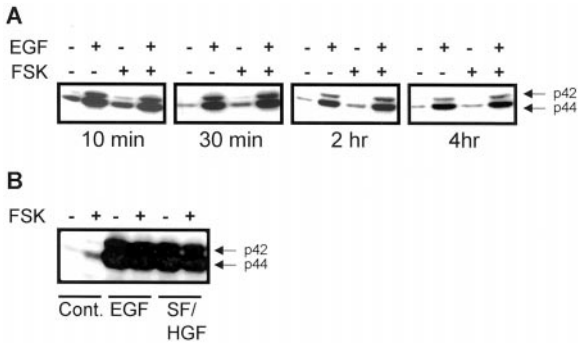

Fig. 4. Forskolin does not inhibit ERK activation by growth factors. SCC 12F cells were grown as described under *Materials and Methods* and serum-deprived for 24 h before treatment. A, cells were incubated in the presence or absence of 50 μM forskolin (FSK) for 15 min before EGF (10 nM) stimulation and incubated at 37°C for the indicated times. Whole cell lysates were collected and fractionated on 10% SDS-polyacrylamide gel electrophoresis, and the dually phosphorylated ERK proteins were detected by immunoblot analysis as described under *Materials and Methods*. B, cells were treated as described above and stimulated with either EGF (10 nM) or SF/HGF (10 ng/ml) or received no growth factor (Cont.) as indicated. Results are representative of at least three independent experiments.

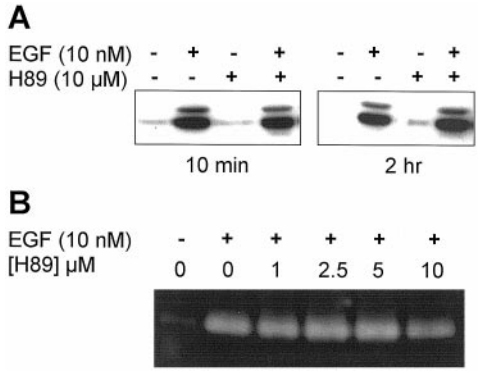

Fig. 5. PKA activity is not required for sustained activation of p42/44 ERKs or induction of MMP-9 by EGF. A, the dually phosphorylated ERKs were detected by immunoblot analysis as described under *Materials and Methods* and the legend to Fig. 4. Cells were treated without (−) or with (+) H89 (10 μM) for 10 min before addition of EGF (10 nM) for the indicated times. B, cells were pretreated with H89 at the indicated concentrations for 10 min before addition of EGF (10 nM) for 18 h. Conditioned medium was analyzed by gelatin zymography as described under *Materials and Methods*.

To further investigate the role of intracellular cAMP elevation in modulating MAPK signal transduction cascades, we examined EGF-dependent activation of p38. EGF stimulates a moderate (4.96- ± 1.84-fold in three independent experiments) and transient activation of p38 (McCawley et al., 1999) (Fig. 7A). This stimulation appears to make a contribution to MMP-9 expression and cell migration because inhibition of p38 activity by SB202190 results in a partial, but reproducible, decrease in the EGF-stimulated response (Fig. 7B) and disruption of SCC 12F colony dispersion (data not shown). SB202190 concentrations of up to 30 μM abolished EGF-dependent stimulation of p38 activation but did not inhibit JNK activity as measured in an immunocomplex assay (data not shown). Pretreatment of cells with 50 μM forskolin inhibited EGF-dependent activation of p38 (Fig. 7A), and as observed for JNK, forskolin did not substantially interfere with anisomycin-dependent p38 activation (data not shown). These findings (Figs. 5, 6, and 7), and those of others (Hsueh and Lai, 1995; Rao and Runge, 1996; Shapiro et al., 1996; Li et al., 1997), suggest that PKA interaction with specific MAPK cascades differs between fibroblasts and other cell types (Cook and McCormick, 1993; Sevetson et al., 1993; Wu et al., 1993; Huang et al., 1994; Hsueh and Lai, 1995; Rao and Runge, 1996; Shapiro et al., 1996; Li et al., 1997). Furthermore, the results illustrated in Figs. 6 and 7 suggest that although p38 activation contributes to EGF-induced MMP-9 expression, other pathways are involved in regulating MMP-9 expression.

Discussion

The results reported in this study illustrate interactions between growth factor- and cAMP-mediated signaling pathways with regard to keratinocyte migration and MMP-9 induction. Activation of the EGF receptor did not enhance cAMP accumulation (Fig. 1), consistent with reports that stimulation of cAMP in response to receptor tyrosine kinase activation is dependent on expression of adenylyl cyclase type 5, which is not detected in keratinocytes (Chen et al., 1995). Elevation of cAMP levels in the absence of growth factor treatment did not stimulate colony dispersion or MMP-9 expression; however, cAMP inhibited several EGF- and SF/HGF-dependent responses including migration, MMP-9 induction, and DNA synthesis (Figs. 2 and 3 and data not shown). A mechanism by which cAMP may interact with receptor tyrosine kinase-mediated signaling pathways is through modulation of MAPK cascades.

There is evidence that MMP-9 gene expression is regulated by the ERK, JNK, and p38 MAP kinases (Boyd, 1996; Gum et al., 1997; Himelstein et al., 1997; Simon et al., 1998; McCawley et al., 1999). We have shown previously that EGF-stimulated MMP-9 induction requires sustained ERK activation (McCawley et al., 1999). Our observation that forskolin inhibits EGF-

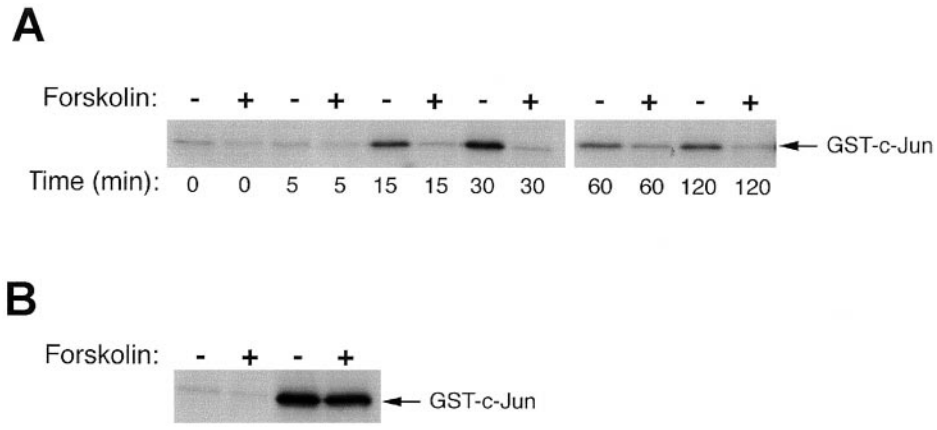

Fig. 6. Forskolin inhibits EGF-stimulated JNK activation. SCC 12F cells were grown as described under *Materials and Methods* and serum-deprived for 24 h before growth factor stimulation. SCC 12F were incubated in the presence (+) and absence (−) of 50 μM forskolin for 30 min before incubation with 20 nM EGF for the indicated times (A) or 190 nM anisomycin for 30 min (B). JNK activation was assayed as described under *Materials and Methods*. Data shown are representative of at least three independent experiments. Phosphorylation of GST-c-Jun was quantified using a Bio-Rad model GS-700 imaging densitometer, and kinase activation was normalized to untreated control cultures (control values = 1.0). Maximal stimulation of JNK activation by EGF at 30 min was 9.89 ± 1.67-fold in three independent experiments (average normalized kinase activation ± S.D.).

stimulated MMP-9 induction (Fig. 3), and reports that cAMP inhibits ERK activation in fibroblasts (Cook and McCormick, 1993; Wu et al., 1993; Mineo et al., 1996) lead us to investigate whether forskolin inhibits EGF-stimulated ERK activation. Surprisingly, elevation of intracellular cAMP did not inhibit ERK activation in SCC 12F keratinocytes (Fig. 4). These results indicate that although sustained ERK activation is required for EGF-stimulated MMP-9 induction, this signal is not sufficient and suggests that cAMP inhibits other signaling pathways that are important for MMP-9 induction. Accordingly, we found that elevated intracellular cAMP selectively inhibits EGF-dependent JNK and p38 activation in keratinocytes (Figs. 6 and 7). Importantly, this inhibition is associated with loss of growth factor-dependent MMP-9 induction and migratory response. Thus, JNK and p38 appear to be involved in but not sufficient for growth factor-stimulated MMP-9 gene expression. This is supported further by the observation that keratinocyte growth factor stimulates JNK and p38 activity in SCC 12F cells but does not induce MMP-9 or promote colony dispersion (McCawley et al., 1998, 1999). Additional evidence that multiple MAP kinase cascades are required for keratinocyte migration is provided by Zeigler et al. (1999), who reported that neither the ERK nor JNK pathways alone were sufficient for growth factor-induced migration of normal human keratinocytes.

The p38 MAPK pathway has been shown to be essential for phorbol ester-stimulated MMP-9 induction (Simon et al., 1998), and we find that p38 activation by EGF partially contributes to growth factor-regulated MMP-9 expression (Fig. 7B). SB202190 is a highly selective inhibitor of p38 (Lee et al., 1994) and did not disrupt EGF-dependent JNK activation in SCC 12F cells (data not shown). Forskolin, which inhibits both JNK and p38 activation (Figs. 6 and 7), disrupts EGF-stimulated responses more effectively than does SB202190, suggesting that JNK activation may be involved in growth factor-stimulated MMP-9 induction (Figs. 2 and 3). This suggestion is supported by the findings of others that JNK activation stimulates MMP-9 gene expression (Gum et al., 1997; Himelstein, 1997). Collectively, our findings demonstrate that EGF concurrently activates multiple MAPK signaling cascades and suggest that coordinate regulation of all three pathways is necessary for maximal EGF-dependent migration and proteinase induction.

Inhibition of JNK by cAMP has been observed in a limited number of model systems. Forskolin coordinately inhibited endothelin- or thrombin-induced ERK2 and JNK activation in airway smooth muscle cells (Shapiro et al., 1996). In contrast, cAMP weakly impaired Raf-1 activation and did not inhibit ERK activation in T lymphocytes; however, cAMP significantly disrupted JNK activation, thereby illustrating selective inhibition of one MAPK cascade (Hsueh and Lai, 1995). Similarly, in vascular smooth muscle cells, cAMP inhibited thrombin-induced JNK1 activation and c-Jun expression but did not compromise thrombin-induced ERK activation and c-Fos expression (Rao and Runge, 1996). PKA-dependent inhibition of JNK, but not ERK, activation was also detected in GN4 rat liver epithelial cells (Li et al., 1997). A calcium-dependent tyrosine kinase (CADTK/PYK2) pathway implicated in JNK activation has been proposed as a target for forskolin-dependent inhibition of JNK activation in rat liver epithelial cells (Li et al., 1997). Additionally, angiotensin II- or thapsigargin-stimulated, but not anisomycin-dependent, JNK activation was cAMP-sensitive, leading to the conclusion that there is divergence between the mechanisms of stress versus calcium-dependent stimulation of the JNK cascade (Li et al., 1997). Based on the difference in sensitivity to inhibition by elevated intracellular cAMP, our

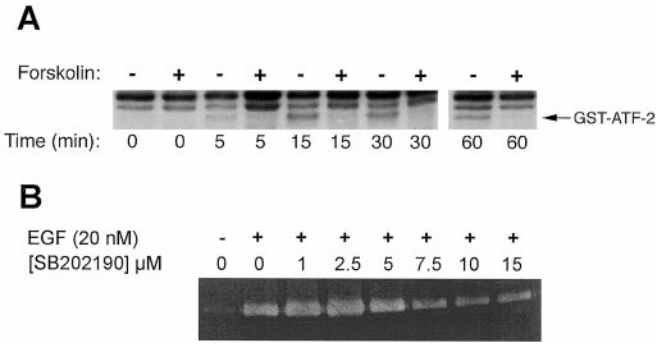

Fig. 7. Forskolin inhibits EGF-stimulated p38 activation. SCC 12F cells were grown as described under *Materials and Methods* and serum deprived for 24 h before growth factor stimulation. A, SCC 12F cells were incubated in the presence (+) and absence (−) of 50 μM forskolin for 30 min before incubation with 20 nM EGF for the indicated times. p38 activation was assayed as described under *Materials and Methods*. Concentrations of SB202190 up to 30 μM had no effect on either basal or EGF-stimulated JNK activation as determined by immunocomplex assay (data not shown). Data are representative of at least three independent experiments. B, SCC 12F cells were treated with 20 nM EGF in the absence or presence of the indicated concentrations of SB202190 for 24 h, and conditioned medium was analyzed by gelatin zymography as described under *Materials and Methods*. Phosphorylation of GST-ATF-2 was quantified as described in the legend to Fig. 6. Maximal stimulation of p38 activation by EGF at 30 min was 4.96 ± 1.84-fold in three independent experiments.

findings suggest that there is divergence between receptor tyrosine kinase-mediated and stress-induced activation of JNK and p38 in keratinocytes (Figs. 6 and 7).

Although examples of inhibitory interactions between PKA and JNK have been identified in mammalian cells, evidence for similar interactions between the PKA and p38 pathways have to date been documented only in yeast. p38 is the mammalian counterpart of the yeast high-osmolarity glycerol (HOG) MAPK. The *Saccharomyces cerevisiae HSP12* gene is induced by a HOG-dependent signaling pathway, and mutations that result in high PKA activity inhibit salt- or heat stress-induced *HSP12* gene expression, thereby indicating interactions between these signaling pathways (Varela et al., 1995; Siderius et al., 1997). Similarly, yeast *PMR21ENA1* gene induction is mediated by the HOG MAPK pathway, and PKA negatively modulates expression of this gene (Marquez and Serrano, 1996). Our findings extend those described in yeast to directly identify cAMP-dependent inhibition of tyrosine kinase-stimulated p38 activation in mammalian cells (Fig. 7).

Based on our results, disruption of sustained ERK activation (McCawley et al., 1999) or concurrent inhibition of JNK and p38 activity by cAMP can interfere with growth factor-mediated MMP-9 induction and migratory response in keratinocytes. These findings indicate that multiple MAPK pathways are required for maximal stimulation of keratinocyte migration and MMP-9 production in response to growth factors. Identification of the mechanisms that lead to differences in cAMP interactions with specific MAPK cascades in various cell types will be an important area for additional investigation.

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