EGF and Î²1 Integrin Convergently Regulate Migration of A431 Carcinoma

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EGF and β1 Integrin Convergently Regulate Migration of A431 Carcinoma Cell through MAP Kinase Activation

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[1]*Abbreviations used in this paper*: COL, collagen; EGF, epidermal growth factor; EGFR, EGF receptor; MAPK, mitogen-activated protein kinase

Running title: MAP Kinase in Cell Migration

ABSTRACT

We found that the convergently the EGF-induced signal and the collagen-induced signal activate MAPK, which induces migration. We examined the signaling mechanisms of EGF-induced cell migration on collagen using the A431 carcinoma cell. EGF (10 ng/ml) induced migration on collagen, but inhibited proliferation. Using a MAPK cascade inhibitor, PD98059, it was shown that EGF-induced migration on collagen was mediated by MAPK whereas EGF-induced migration on fibronectin and vitronectin were not. PD98059 also showed that activation of MAPK induced by EGF enhanced the adhesiveness of A431 cells to collagen. By western blotting analysis, the kinetics of MAPK phosphorylation induced by EGF and collagen was examined separately, and convergently. First of all, EGF without collagen caused transient mitogen-activated protein kinase (MAPK) phosphorylation. Collagen without EGF caused MAPK to be immediately and transiently dephosphorylated, and rephosphorylated followed by sustained hyperphosphorylation. EGF together with collagen caused an immediate, and sustained, hyperphosphorylation. These facts suggest that the transient MAPK dephosphorylation induced by collagen is required for migration in order to maintain an appropriate level of sustained phosphorylation. Furthermore, we found that adhesion of A431 cells to collagen was blocked by the anti-β1 integrin antibody or by the mixed antibodies composed of anti-α1, -α2, and -α3 antibodies, indicating that collagen-induced MAPK phosphorylation was mediated through α1β1, α2β1 and α3β1 integrins.

Key words: Cell migration, EGF receptor, integrin, MAP kinase

INTRODUCTION

Cell migration is a crucial phase in cancer invasion that requires convergent signals from motogenic cytokines and adhesion receptors such as integrins serving as the transmembrane link. Epidermal growth factor (EGF) elicits migration in a wide variety of cells [1-6]. EGF potentiates the signal-transduction pathway for cell proliferation, which differs from the pathway for migration [1, 7]. Mitogen-activated protein kinases (MAPK) are rapidly activated in cells stimulated by a variety of mitogens or motogens including EGF, which delivers essential signals via Ras to a protein kinase network involving the Raf-1 kinase, MEK, and MAPK [8, 9]. It has therefore been proposed that these kinases are arranged in a linear cascade, in which Raf-1 phosphorylates and activates MEK, which in turn phosphorylates and activates MAPK. MAPK carries the signal to cytoplasmic kinases in the motogenic pathway [4], or to the nucleus, where it phosphorylates transcription factors capable of mediating immediate early gene induction in mitogenic and motogenic pathway [10].

Human squamous cell carcinomas frequently display an increase in the expression of the EGF receptor (EGFR), first demonstrated for A431 cells [11]. Increased EGFR expression renders the proliferation of A431 cells less dependent on an exogenous source of EGF [12]. It also further enhances the EGF-induced motogenic responses of squamous cell carcinoma cell lines compared with human epidermal keratinocytes [5, 6], and may thus contribute to the invasiveness of squamous cell carcinoma cells. However, cell motility and invasiveness are still dependent on high doses of EGF [6, 13-15], although the same level of EGF stimulation inhibits proliferation of A431 cells [11, 15, 16]. The early response of A431 cells to EGF induces rapid alterations in the organization of the actin microfilament system that result in extensive ruffling, lamellipodia formation, and cortical actin polymerization [13, 14, 17, 18]. These processes precede and accompany cell migration.

Integrins, along with structural linking proteins, lead to the formation of membrane-associated signaling complexes with tyrosine kinases, serine/threonine kinases and the corresponding substrates in the focal contacts [10, 19, 20, 21]. Ligation and clustering of integrins cause activation of focal adhesion kinase following second messenger Grb2, and the Sos to Ras-independent MAPK cascade [10, 19, 20], or the adapter protein Shc associated with Grb2 independent of the focal adhesion kinase-activated MAPK cascade [21]. The integrins which induce MAPK activation are limited [21-23]. Since the signaling pathways leading to cell migration are different among integrin species, a number of studies have focused on the divergence of signaling [3, 4, 24, 25]. However, a significant degree of multiplicity exists among the integrins in terms of ligand specificity and most cells simultaneously express several different integrins [26]. This fact makes it difficult to determine the precise role of specific integrins in migration. The limited integrin species and EGF share the role of MAPK activation [27]. There is some integration of both the signals from integrin receptors and the signals from growth factor receptors in order to organize the cytoskeleton [6, 13, 27, 28].

In this paper, we show that EGF- and β1- integrin convergently activate MAPK which lead to an appropriate level of sustained MAPK activation for migration of A431 cells on collagen.

MATERIALS AND METHODS

Reagents and adhesive ligands

EGF (Genzyme, Cambridge, MA) was used. Cycloheximide, actinomycin D and 3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO). PD98059 was purchased from New England BioLabs (Beverly, MA). Collagen (COL) type I was purified from human placentas [29]. Fibronectin and vitronectin were purified from human plasma [30, 31].

Cell lines

A human esophageal squamous carcinoma cell line, A431, and a human squamous carcinoma cell line, HSC-3, obtained from the Human Science Research Resource Bank (Osaka, Japan) were used. The cells of subconfluent cultures, maintained in Eagle's minimum essential medium (MEM, Flow Laboratories, Irvine, Scotland) containing 10% fetal bovine serum (Gibco BRL., Grand Island, NY) were harvested with 0.25% trypsin, and then the trypsin was inactivated by mixing with 0.25% of a soybean trypsin inhibitor (Sigma) in MEM. The cells were washed with serum-free MEM containing 0.5% heat-denatured bovine serum albumin (BSA/MEM) (Sigma), maintained in suspension for 30 mins at 4 ° C, and were used for all the experiments in the present study.

Adhesion assay

Polystyrene, 96-well plates were coated with 10 μg/ml COL in phosphate-buffered saline (PBS, pH 7.4) overnight at 4 ° C. The wells were then blocked with BSA/MEM overnight at 4 ° C. Then, 50,000 cells in BSA/MEM, in quadruplicate determinations, were loaded onto ligands and incubated in a CO_2 incubator for 20 mins. Non-adherent cells were removed by gentle washing and the remaining adherent cells were quantified with a MTT assay [32]. To inhibit adhesion, anti- α1 (polyclonal, Chemicon, LaJolla, CA), -α2 (P1E6, DAKO, Carpenteria, CA), -α3 (P1B5, DAKO), -β1 (4-145, Sumitomo Electric, Tokyo, Japan), and -β3 antibodies (VNR5, Takara, Tokyo, Japan) were used.

Migration assay

A cell migration assay was performed in a 24-well Transwell^{TM} chamber containing a polycarbonate membrane with 8 μm-pores (Costar, Cambridge, MA). The undersurface of the membrane was coated with 10 μg/ml of human plasma fibronectin or human placental COL in PBS at 4 ° C overnight. Cells were then treated with EGF for 30 mins at 4 ° C.

After washing via centrifugation, the resuspended cells (50,000 cells) in BSA/MEM, in quadruplicate determinations, were loaded onto a membrane and incubated for 18 to 24 hours. Cells, which had migrated onto the surface of the substrate-coated membrane, were fixed and stained with 2% crystal violet in 2% ethanol. After washing cells with distilled water, dye-stained cells were eluted with acetic acid. The optical density of the eluate at 595 or 620 nm was measured. The background optical density was evaluated on BSA-coated membranes and subtracted from all data points.

Immunoblotting of ERK1/2 and phosphorylated ERK1/2

Cell samples were lysed by adding an SDS sample buffer containing 62.5 m*M* of a Tris-HCl buffer (pH 6.8), 2% SDS, 10% glycerol, mercaptoethanol, and 0.1% bromophenol blue. Then, samples were heated to 95 ° C for 5 mins, separated by 10% SDS-PAGE, transferred to a polyviniliden fluoride membrane, and blocked in 5% nonfat dry milk in Tris-buffered saline (pH 7.6). The anti-ERK1/2 antibodies (New England BioLabs) and anti-phospho-ERK1/2 (Thr^{202}/Tyr^{204}) antibodies (New England BioLabs) used as primary antibodies were detected using a chemiluminescence system (New England BioLabs). The blotting images were scanned with a computer-associated scanner and the densities of the bands were analyzed with NIH image.

Protein assay

We used samples which had the same protein amount. To determine the protein amount, a protein assay dye (Bio-Rad, Hercules, CA) was used. Samples in the cell lysis buffer or SDS-PAGE buffer had unnegligible concentrations of detergents and reducing agents, and then we used a diluted solution at 1:25.

RESULTS

EGF Potentiates Migratory activity of A431 Cells on COL, but Inhibits Proliferating Activity on COL

In the migration assay, A431 cells migrated in small numbers both on COL, fibronectin, and vitronectin (Fig. 1, 2), but did not migrate on BSA. EGF markedly enhanced migration in a dose-dependent manner at concentrations between 1 and 10 ng/ml both on COL (Fig. 1A), fibronectin and vitronectin. Migratory activity culminated at a concentration of 10 ng/ml EGF.

A431 cells are well known to overexpress EGFR [11]. Several authors [11, 33, 34] have reported that high doses of EGF inhibited proliferation of cells overexpressing EGFR, while low doses of EGF stimulated proliferation. Furthermore, cell motility and proliferation are generally mediated through different pathways [1], whereas they sometimes use the same MAPK pathway [4, 5]. To test the effect of EGF on proliferation at a concentration that enhanced cell migration on COL, A431 cells were treated with 10 ng/ml of EGF and cultured on COL, and their numbers were counted. After 1, 3 and 5 days, cells proliferated on COL without EGF, but did not proliferate when treated with 10 ng/ml of EGF (Fig. 1B). Therefore, we used 10 ng/ml of EGF as an appropriate concentration, which induced cell migration but not proliferation, in the following experiments to examine the signal transduction pathway for cell migration.

Migration on COL is MAPK-dependent, but Migration on Vitronectin and Fibronectin are not

The MAPK cascade has been reported to be involved in the migration-directed signal transduction system [2, 5, 10], but the activation of MAPK induced by adhesion to the matrix, which was required for cell migration on COL, is integrin species-dependent [21, 22]. A specific inhibitor of the activation of the MAPK kinase, PD98059 [35], reacted with A431 cells during migration on various ligands. PD98059 inhibited EGF-induced migration on COL in a dose-dependent manner (Fig. 2A). In contrast, PD98059 did not inhibit migration on either fibronectin, or vitronectin (Fig. 2B, C), indicating that EGF-induced migration on collagen was mediated by MAPK whereas EGF-induced migration on fibronectin and

vitronectin were not.

A431 Cells Adhere to COL Using Integrins α1β1, α2β1, and α3β1

Activation of MAPK is integrin-species dependent [21-23]. Therefore, we tried to determine which integrins induced MAPK activation. First, integrin subunits expressed in A431 cells were screened using a highly sensitive method: reverse transcription-PCR using mRNA purified from A431 cells. A431 cells expressed α1, α2, α3, α4, α5, α6, αv, β1, β3, and β5 (data not shown). Putatively, the integrins that A431cells use for adhesion to COL are α1β1, α2β1, and α3β1. Next, we attempted to determine the integrin species actually used for adhesion to COL. Specific antibodies to integrins were reacted with A431 cells for 30 min before they were loaded onto COL. The results of adhesion assays showed that the anti-β1 antibody inhibited adhesion of A431 cells to COL, although the anti-β3 antibody did not. The anti-α1 antibodies alone did not inhibit adhesion to COL, nor did the anti-α2 or -α3 antibodies alone either. Attempts to use two different antibodies mixed, anti-α1 and anti-α2, anti-α2 and anti-α3, and anti-α3 and anti-α1, failed to inhibit adhesion. Then, these three antibodies were mixed and an adhesion assay was done, resulting in inhibition of adhesion (Fig. 3). These findings indicate that the integrins α1, α2 and α3 are utilized for adhesion of A431 cells to COL, and that one, two or possibly all three of them induced the signal to MAPK.

MAPK-mediated Signaling Directs Adhesion and Spreading on Collagen

Recently, it has been demonstrated that adhesion and spreading of cells on different ligands is dependent on MAPK activity (29, 36). Adhesion assays were performed using A431 cells that were treated with 10 ng/ml EGF for 30 min at 37 ° C and were washed before plating. Since all the cells treated, or untreated, with EGF were bound to collagen 60 min after loading onto collagen, we examined early adhesion within 20 mins. EGF enhanced adhesiveness to collagen within 20 min (Fig. 4A). When cells were pretreated with 100 μM

PD98059 in addition to EGF, the original adhesiveness was restored (Fig 4A). Cells not treated with EGF were well-spread and large, or elongated with cytoplasmic extensions on collagen at 90 mins. Cells treated with EGF were also spread on collagen, but cell shapes were rather small, and round to polygonal. PD98059 blocked the change of cell shape induced by EGF (Fig. 4B). Thus, we infer that the effect of EGF on adhesiveness and spreading were mediated by MAPK.

Migration of A431 Cells on Collagen Requires de novo Protein Synthesis

It has been reported that *de novo* protein synthesis is required for EGF-induced PKC-mediated cell migration on vitronectin [3], but is not required for MAPK-mediated cell migration on COL [4]. Therefore, we attempted to determine whether or not the ensuing signal from MAPK involved in cell migration could direct gene expression. A gene expression inhibitor, actinomycin D, and a protein synthesis inhibitor, cycloheximide, were tested. Cells were treated with one of the inhibitors, prior to treatment with EGF. After washing, the inhibitors were readded and the cells were allowed to migrate. As indicated in Figs. 5A and 5B, treatment of A431 cells with 5 μ*M* cycloheximide or 50 ng/ml of actinomycin D affected EGF-induced migration on COL, fibronectin and vitronectin. As a negative control, HSC-3 cells were used. Treatment with the same concentration of cycloheximide did not affect EGF-induced migration of HSC-3 cells on COL (Fig. 5C). These results suggest that MAPK-mediated cell migration requires *de novo* protein synthesis in A431 cells.

EGF Activates ERK1/2 Transiently in A Dose-dependent Manner

Since A431 cell migration was both EGF- and COL-dependent, the EGF-induced and COL-induced activation of MAPK were examined separately, and their synergy was also examined. First, we examined the kinetics of EGF-induced MAPK activation. Cells in suspension were treated with EGF at 10 ng/ml, and the phosphorylated ERK1/2 kinase and

ERK1/2 of the cell lysates were detected by immunoblotting. The band densities of ERK1/2 were unchanged over a 60 min time course (Fig. 6A). As has been shown in A431 cells and other cells overexpressing EGFR [15, 37], the band densities of phosphorylated ERK1/2 reflecting MAPK activity elevated within 15 mins, and returned to the control level after 60 mins (Fig. 6A, B). The effect of EGF on the phosphorylation of ERK 1/2 was dose-dependent after 15 mins (Fig. 6C), coinciding with the fact that EGF-induced migration was dose-dependent. Furthermore, to detect whether PD98059 which inhibited the migration of A431 cells on collagen is directed to MAPK phosphorylation, PD98059 was reacted with cells prior to treatment with EGF. EGF-induced phosphorylation of ERK 1/2 at 15 mins was inhibited by PD98059 (Fig. 6D).

Adhesion of Cells to COL Decreases MAPK Activity Transiently and Maintains Mild Activation

Second, to detect activation of MAPK by COL, A431 cells were loaded on COL or vitronectin without EGF stimulation (data not shown). A431 cells adhered to COL partly after 15 mins, and almost all cells were adhered to COL within 30 mins. Cells did not completely adhere to vitronectin after 30 mins, as has been shown in other cells [32]. Since non-adherent cells are unlikely to induce signals to the inside of the cells, we discarded the non-adherent cells by repeated gentle washing. The protein concentrations were determined using a protein assay dye, and immunoblotting was applied to identical amounts of protein samples. Since ligand binding induces persistent activation of MAPK [27, 28], the kinetics of MAPK activities were examined over a long time interval. While adhesion of A431 cells to COL did not alter the amount of ERK1/2 up to 180 mins, phosphorylated ERK1/2 rose to 150% from 60 to 180 mins (Fig. 7A). On the other hand, phosphorylated ERK1/2 decreased after 30 mins (Fig. 7A). Thus, the phosphorylated ERK1/2 decreased rapidly at 15 mins, and it began to return to the control level at 30 mins, followed by further elevation (Fig. 7A).

Phosphorylated ERK1/2 after adhesion to vitronectin remained unchanged (Fig. 7B). These results support the idea that cell migration on COL is MAPK-dependent, while cell migration on vitronectin is MAPK-independent. However, while it has been reported that elevation of MAPK activity is an early response [10, 20]; a transient decrease in MAPK activity has never been reported. Although the significance of hypophosphorylation of MAPK is unknown, to learn whether this phenomenon is a general response, another cell line, HSC-3, which also shows MAPK-dependent cell migration on COL, was analyzed. The kinetics of HSC-3 were similar to those of A431 cells (Fig. 7C).

EGF and Adhesion to COL Synergize to Maintain Certain Levels of Sustained MAPK Activation

To examine the kinetics of MAPK synergistic activation by both EGF and adhesion to COL, immediately after addition of EGF to the cell suspension, the cells were loaded onto COL. Surprisingly, the kinetics of the two stimuli together resulted in a simple pattern: gradual activation and a subsequent sustained activation increasing to 200% over 4 hours (Fig. 7D), in contrast to the complex pattern induced by only adhesion to COL. We thereby suggest that the transient down-regulation of MAPK activity induced by adhesion to COL in synergy with the transient up-regulation of MAPK activity induced by EGF plays an important role in maintaining an appropriate level of MAPK activation leading to cell migration.

DISCUSSION

In the present study, we showed 10 ng/ml EGF inhibited proliferation on collagen but caused maximal-migration. It is known that high concentration of EGF inhibits proliferation and low concentration of EGF enhances proliferation, and the phenomenon has been clearly related to overexpression of EGFR [11, 33]. While it is known that signals induced by EGF for proliferation and migration are different [1, 7], they are hardly separable because the same

stimulus causes different cell responses through the same pathway, the MAPK. Since EGF-induced proliferation inhibition is mediated by MAPK-independent protein kinase C signaling in A431 cells [16], the A431 cells and migration assay system we used was a good model to analyze phenomena relating to cell motility. In the present study, we showed that EGF-induced cell migration on COL was MAPK-dependent.

We found that the targets of MAPK signaling involved in cell migration were cell adhesion to matrix and the following cell spreading. We showed that this signaling was not from COL-induced MAPK activation, but from EGF-induced MAPK activation, since PD98059 was washed out before the cells were loaded onto collagen. The EGF-induced MAPK signaling enhanced cell adhesion to collagen. Similarly, increased integrin-ligand affinity has been shown to result in an increase in cell migration [27, 39]. In addition to the enhancement of adhesion, EGF-induced MAPK activation decreased cell spreading. Cell spreading with focal adhesion is required for integrin-induced MAP kinase [28] and cell migration [4, 25]. Conversely, detachment from substratum and a decrease in spreading are also required for cell migration. Previous reports have also demonstrated that EGF induced disassembly of focal adhesions and a decrease in spreading [40].

There seem to be other targets of MAPK affecting cell migration. One of them is the myosin light chain kinase [4, 41], which we did not examine in the present study. We also found *de novo* protein synthesis is required for migration of A431 cells. Many other cells reported do not require *de novo* protein synthesis for migration [4, 24]. In contrast, FG carcinoma cells require transcription for αvβ5-dependent migration 3]. It has also been shown that A431 cells require *de novo* protein synthesis, which is transcribed by a transcription factor, AP-1, for EGF-induced migration [42]. Thus, we conclude that protein synthesis required for migration seems to be cell-dependent rather than a signaling-dependent event.

Migration-directed signaling induced by EGF seems to be less affected by overexpression of EGFR, but may influence the migration-directed signal from β1-integrins, which induced a transient decrease in MAPK phosphorylation. It has been reported that there are signaling pathways connecting EGFR and integrins; the signal from EGFR is directed to integrins and that from integrins to EGFR [43, 44]. Thus, integrin-mediated signaling may be affected to some extent by EGFR.

The transient decrease in MAPK phosphorylation induced by COL that we demonstrated seems to play an important role in cell migration since the signals synergized with the signals mediated by EGF resulted in rapid and sustained MAPK phosphorylation. A transient activation induced by EGF, but not a decrease in MAPK phosphorylation induced by COL, has been reported, but this transient activation was not sufficient for cell migration [8, 27]. In fact, transient activation was observed after treatment with EGF, and the level was dose-dependent and closely related to dose-dependent migration. Interestingly, stimulation by EGF and the COL matrix evoked a gradual and sustained phosphorylation of MAP kinase resulting in cell migration. Thus, an appropriate level of MAPK activation is at least necessary for migration, and the COL-mediated signaling appears to regulate MAPK so as to maintain the appropriate level of activity.

We found that cell migration on COL was MAPK-dependent, but migration on vitronectin and fibronectin was not MAPK-dependent. These facts further confirm the idea that EGF-induced MAPK activation alone is insufficient, and that the additional signals induced by integrins are required. For example, Klemke *et al.* [2] showed that αvβ5–directed cell migration on vitronectin was mediated by protein kinase C. Likewise, α2β1-directed cell migration on COL is regulated by MAPK [4], and β1- and β3-mediated migration is triggered through a distinct signaling mechanism [25]. These observations suggest that several distinct integrins activate MAPK. In fact, several recent reports

revealed a part of the integrin repertoire, which induced activation of MAPK. Integrin-mediated signaling upregulates Raf-1, MEK, and MAPK by Grb2 binding to focal adhesion kinase or Shc following activation of Src and Sos [19-21]. The association with Shc defines a class of integrins: α1β1, α5β1, αvβ3 [21], and α6β4 [23] which cause tyrosine phosphorylation. α2β1, α3β1, α6β1 and β2 integrins do not induce this event [21]. Another study has claimed that α2β1 induced MAPK activation [22]. We showed that α1β1, α2β1 and α3β1 were expressed and used for adhesion to COL in A431 cells. Since α3β1 is not involved in MAPK activation [21] and α2β1 is controversial [21, 22], COL-induced MAPK activation in A431 cells may well be mediated by α1β1 and/or α2β1.

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FIGURE LEGENDS

Fig. 1. EGF at a concentration of 10 ng/ml induces A431 cell migration maximally on COL, but inhibits cell proliferation on COL. (A) Cell migration was determined using a modified Boyden chamber with an 8 μm porous membrane coated with 10 μg/ml of COL solution. Cells were allowed to traverse the membrane for 16-20 hours in the presence of various concentrations of EGF (2 to 10 ng/ml) and no EGF (NT). The cells were then stained with crystal violet. The optical density of the eluate with acetic acid was measured at 620 nm. Each bar represents the mean±SE of four replicate wells. (B) Cells were cultured in 35 mm dishes coated with 10 mg/ml of COL solution at a starting population of $5X10^5$ cells per dish. After 1, 3 and 5 days, cells were trypsinized and numbers of cells were counted directly using a hemocytometer. A431 cells were cultured on COL without EGF (closed circles), or treated with 10 ng/ml EGF (open circles). Each bar represents the mean±SE of three replicate dishes.

Fig. 2. EGF-induced migration on COL is inhibited by a MEK inhibitor. Cells were pretreated with various concentrations of PD98059 (0 to 100 μ*M*) for 30 mins. Then, EGF (10 ng/ml) was added and it was incubated for 30 mins, and washed out. PD98059 was readded and cells were allowed to migrate for 16-20 hours on COL (A), fibronectin (B) or vitronectin (C). As a control, cells without any treatment (NT) were used. Each bar represents the mean±SE of four replicate wells.

Fig. 3. Inhibition of adhesion using anti-integrin antibodies. A431 cells were reacted with anti-β1, -β3, -α1, -α2, or -α3 antibodies. Mixed antibodies, anti-α1 and -α2, anti-α2 and -α3, anti-α3 and -α1, anti-α1, -α2 and -α3, were also reacted to cells. Then, cells were loaded on COL for 20 min. Non-adherent cells were thoroughly washed out, and adherent cells were measured with a MTT assay. Each bar represents the mean±SD of four replicate wells.

Fig. 4. Effect of EGF on adhesion and spreading on collagen. (A) Adhesion assay on collagen. A431 cells were pretreated with PD98059 (0 or 100 μM). Then, EGF (10 ng/ml) was added and it was incubated for 30 mins, and washed out. Same concentrations of PD98059 was readded to the cells. Cells were allowed to adhere for 10-20 mins on COL . As a control, cells without any treatment (NT) were used. Cells adhered were quantified with a MTT assay. Each bar represents the mean±SD of four replicate wells. (B) Cell spreading assay. Cells treated with EGF or PD98059 in similar conditions to (A) were loaded on collagen and incubated for 90 mins.

Fig. 5. EGF-induced migration on COL is inhibited by protein synthesis inhibitors. A431 cells (A, B) and HSC-3 cells (C) were pretreated with various concentrations of cycloheximide (0 to 5 μM) (A, C) or actinomycin D (0 to 50 ng/ml) (B) for 30 mins. Then, EGF (10 ng/ml) was added and it was incubated for 30 mins, and washed out. Cycloheximide or actinomycin D was readded and a migration assay was done on COL. As a control, cells without any treatment (NT) were used. Each bar represents the mean±SE of four replicate wells.

Fig. 6. Effect of EGF on MAPK phosphorylation. (A, B) A431 cells were reacted with 10 ng/ml of EGF and maintained in suspension for 15, 30, 45 and 60 mins. The cells were lysed in detergent, and subjected to SDS-PAGE and immunoblotting with anti-ERK1/2 antibodies (top of A) or anti-phosphorylated ERK1/2 antibodies (bottom of A). Densities of phosphorylated ERK1/2 measured by a densitometer are plotted in a line chart (B). (C) Cells were pretreated with 100 μM PD98059 for 30 min before reacting with 10 ng/ml EGF for 15 min. (D) Cells were reacted with EGF at various concentrations (2 to 10 ng/ml) for 15 or 30 mins, and subjected to immunoblotting with anti-phosphorylated ERK1/2 antibodies.

Fig. 7. Effect of adhesion to COL on MAPK phosphorylation. A431 cells were loaded on COL (A) or vitronectin (B). After washing, cells were lysed in detergent and subjected to

SDS-PAGE and immunoblotting with anti-ERK 1/2 antibodies (top and middle of A, B) or anti-ERK1/2 antibodies (bottom of A). The densities of the bands in the middle of A were measured and plotted in a line chart. (C) Similarly, HSC-3 cells were loaded on COL, and subjected to immunoblotting with anti-phosphorylated ERK1/2 antibodies. (D) A431 cells were treated with EGF, loaded immediately on COL, and incubated for various time intervals (15 to 240 mins). The cell lysates were subjected to immunoblotting with anti-phosphorylated ERK1/2. The protein concentrations of the cell lysates adhered to COL were measured and equal amounts of proteins were applied to SDS-PAGE.

Fig. 1.

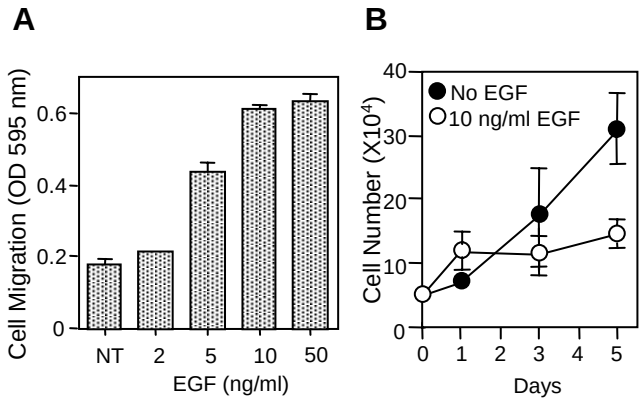


Fig. 2.

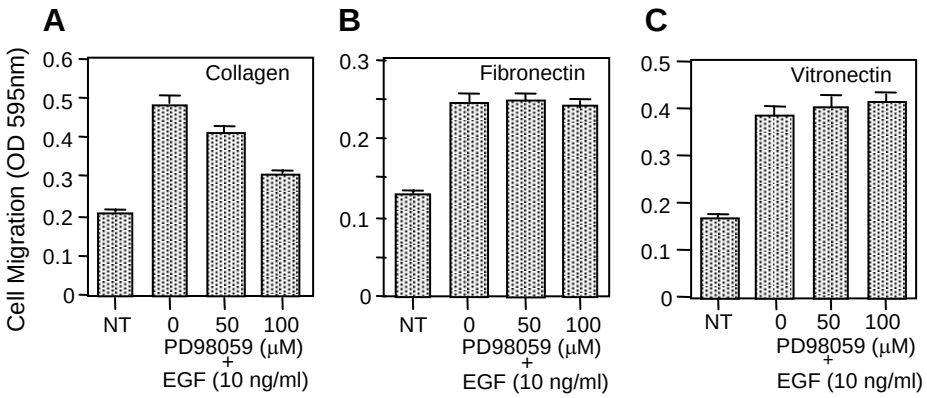


Fig. 3.

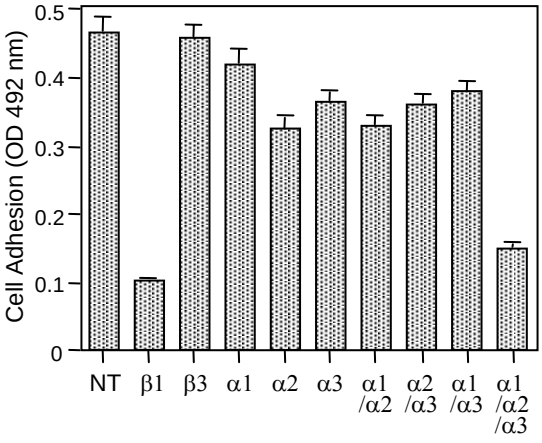


Fig. 4.

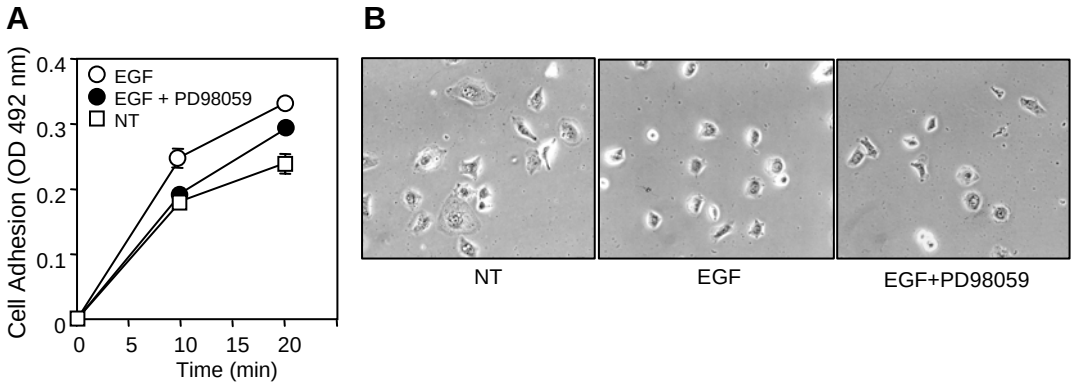


Fig. 5.

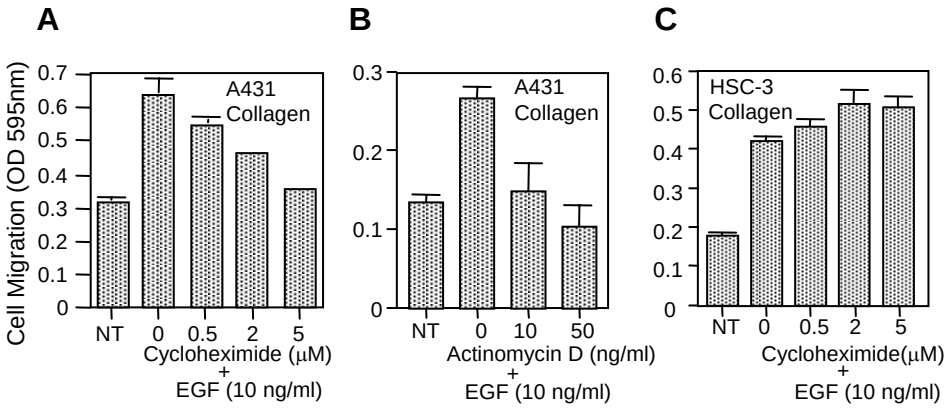


Fig. 6.

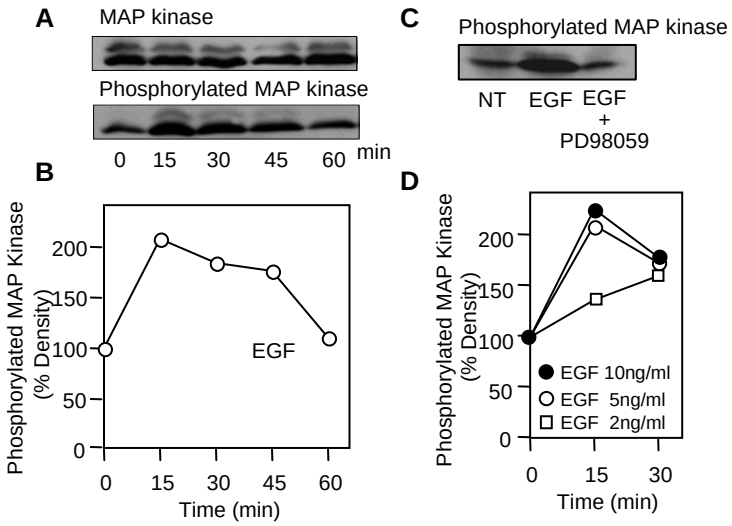
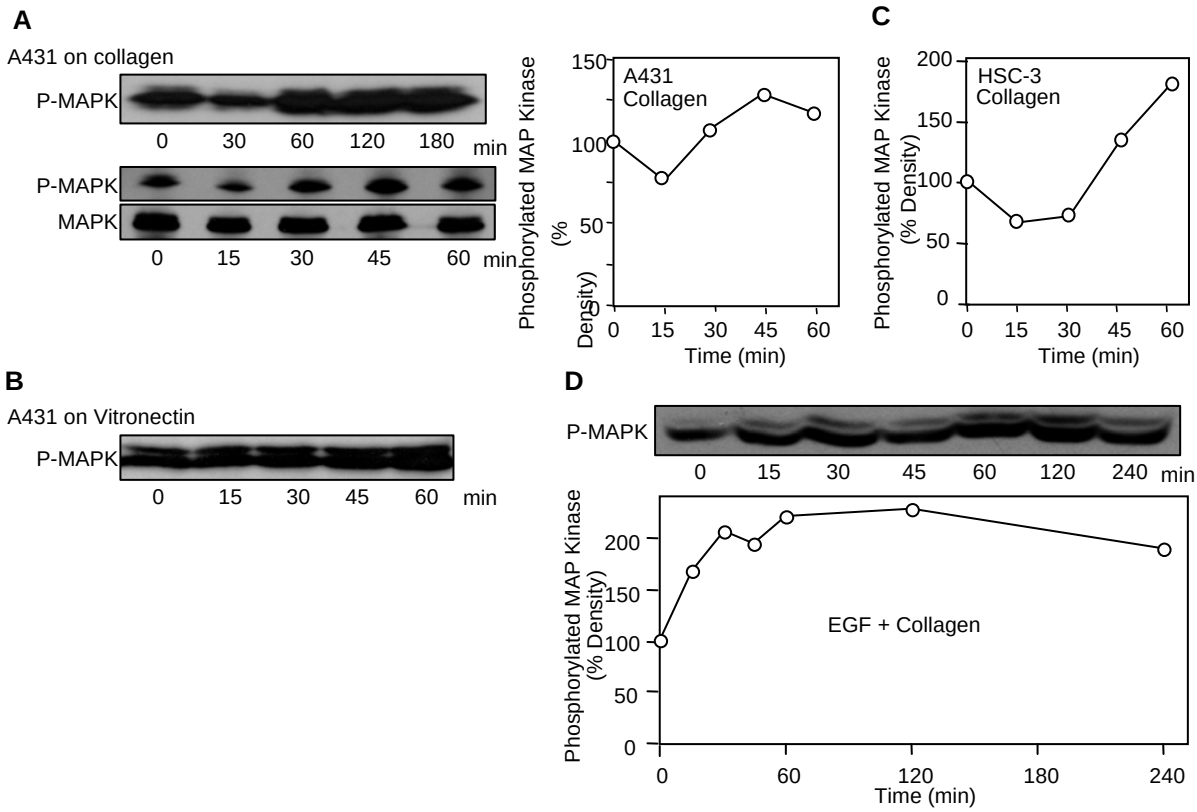


Fig. 7.


A
A431 on collagen
P-MAPK
0 30 60 120 180 min
P-MAPK
MAPK
0 15 30 45 60 min
Phosphorylated MAP Kinase (% Density)
A431 Collagen
Time (min)
C
HSC-3 Collagen
Phosphorylated MAP Kinase (% Density)
Time (min)
B
A431 on Vitronectin
P-MAPK
0 15 30 45 60 min
D
P-MAPK
0 15 30 45 60 120 240 min
Phosphorylated MAP Kinase (% Density)
EGF + Collagen
Time (min)