The Transcription Factor AP-1 Is Required for EGF-induced Activation of Rho-like GTPases, Cytoskeletal Rearrangements, Motility, and In Vitro Invasion of A431 Cells

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Abstract. Human squamous cell carcinomas (SCC) frequently express elevated levels of epidermal growth factor receptor (EGFR). EGFR overexpression in SCC-derived cell lines correlates with their ability to invade in an in vitro invasion assay in response to EGF, whereas benign epidermal cells, which express low levels of EGFR, do not invade. EGF-induced invasion of SCC-derived A431 cells is inhibited by sustained expression of the dominant negative mutant of c-Jun, TAM67, suggesting a role for the transcription factor AP-1 (activator protein-1) in regulating invasion. Significantly, we establish that sustained TAM67 expression inhibits growth factor–induced cell motility and the reorganization of the cytoskeleton and cell-shape changes essential for this process: TAM67 expression inhibits EGF-induced membrane ruffling, lamellipodia formation, cortical actin polymerization and cell rounding. Introduction of a dominant negative mutant of Rac and of the Rho inhibitor C3 transferase into A431 cells indicates that EGF-induced membrane ruffling and lamellipodia formation are regulated by Rac, whereas EGF-induced cortical actin polymerization and cell rounding are controlled by Rho. Constitutively activated mutants of Rac or Rho introduced into A431 or A431 cells expressing TAM67 (TA cells) induce equivalent actin cytoskeletal rearrangements, suggesting that the effector pathways downstream of Rac and Rho required for these responses are unimpaired by sustained TAM67 expression. However, EGF-induced translocation of Rac to the cell membrane, which is associated with its activation, is defective in TA cells. Our data establish a novel link between AP-1 activity and EGFR activation of Rac and Rho, which in turn mediate the actin cytoskeletal rearrangements required for cell motility and invasion.

Key words: AP-1 • Rho-like GTPases • invasion • motility • EGFR

Invasion, the movement of cells through cellular and extracellular matrix barriers into neighboring tissue, distinguishes malignant from benign tumors. Although a simple pathological distinction, invasion itself is a complex process entailing alterations in cell–cell and cell–extra-

1. Abbreviations used in this paper: AP-1, activator protein-1; CAT, chloramphenicol acetyl transferase; Col, collagenase; ECM, extracellular matrix; EGFR, epidermal growth factor receptor; GAP, GTPase-activating proteins; GDI, guanine nucleotide dissociation inhibitors; GEF, guanine nucleotide exchange factors; HEK, human epidermal keratinocytes; HGF/SF, hepatocyte growth factor/scatter factor; HPV, human papilloma virus; JNK, c-Jun NH2-terminal kinase; SRF, serum response factor; SCC, squamous cell carcinomas.
ically transformed fibroblasts regulates the expression of such a multigenic invasion program (Hennigan et al., 1994; Lamb et al., 1997a).

AP-1 encompasses a complex family of transcription factors composed primarily of heterodimers between Fos and Jun family proteins (reviewed in Karin et al., 1997). The prototypes of each family, c-fos and c-jun, were first identified in their transduced forms as retroviral oncogenes, highlighting the important role of AP-1 in transformation. Subsequently, it has been shown that AP-1 activity is required for complete transformation by a wide variety of oncogenes (Lloyd et al., 1991; Rapp et al., 1994; Suzuki et al., 1994; Johnson et al., 1996; Kralova et al., 1998). AP-1 is activated by numerous signal transduction pathways. Through regulating target gene expression, AP-1 couples short-term signals elicited by extracellular stimuli, such as growth factors, to long term adaptive changes in cell phenotype (Curran, 1988). Initial studies in fibroblasts identified AP-1 as an essential regulator of cell proliferation (Holt et al., 1986). However, regulating proliferation is not the sole contribution of AP-1 to malignant transformation.

A governing role for AP-1 in the regulation of cell shape, motility, and invasion has also emerged. In fos-transformed fibroblasts there is gross cytoskeletal reorganization, resulting in a diminution of actin stress fibers and focal contacts, and altered motility (Miller et al., 1984; Hennigan et al., 1994; Lamb et al., 1997a,b). Many of the genes whose expression is either enhanced or repressed in fos-transformed fibroblasts, such as Mts-1, CD44, stromelysin 1, ezrin, and urokinase-type plasminogen activator (Hennigan et al., 1994; Miao and Curran, 1994; Jooss and Muller, 1995; Lamb et al., 1997a,b), are clearly implicated in cytoskeletal organization, cell motility, or remodelling of the ECM. Through the inactivation of AP-1 components and ablation of individual AP-1 target gene products, we have demonstrated that AP-1 activity is an essential requirement for morphological transformation and in vitro invasion of growth factor-treated and v-fos–transformed fibroblasts (Lamb et al., 1997a,b).

In epidermal cells, a critical role for AP-1 during the conversion of benign papillomas, induced by Ha-ras, into invasive SCC has been demonstrated using the mouse skin multistage model of carcinogenesis (Greenhalgh and Yuspa, 1988; Domann et al., 1994a; Saez et al., 1995). Other studies have indicated that the tumorigenicity (Domann et al., 1994b) and in vitro invasiveness (Dong et al., 1997) of mouse SCC can be suppressed by sustained expression of a dominant-negative deletion mutant of c-Jun, TAM67, which inhibits AP-1-mediated transcription (Brown et al., 1993; Domann et al., 1994b; Dong et al., 1997) without affecting proliferation (Domann et al., 1994b; Dong et al., 1997). In human epidermal cells immortalized by expression of human papilloma virus (HPV) 18, coexpression of v-fos promotes progression to a malignant phenotype (Pei et al., 1993). Such cells have elevated levels of AP-1 activity, and expression of TAM67 suppresses their anchorage independent growth concomitant with loss of AP-1 transactivation (Li et al., 1998). The results of the above studies are consistent with a role for AP-1 in the progression of benign epidermal cells to invasive SCC. However, the mechanism by which AP-1 controls a complex process such as invasion and therefore its precise role in malignant progression are unknown.

In contrast to the mouse skin carcinogenesis model, human SCCs rarely possess mutationally activated RAS genes (Clark et al., 1993). Instead they display an increase in the expression of the EGFR (Hendler and Ozanne, 1984; Stanton et al., 1994), first demonstrated for A431 cells (Fabricant et al., 1977). Increased EGFR expression has diverse consequences: acting through an autocrine mechanism, it renders the proliferation of A431 cells less dependent upon an exogenous source of EGF (Fan et al., 1994). It also enhances EGF-induced motogenic responses of SCC-derived cell lines compared with human epidermal keratinocytes (HEKs; McCawley et al., 1997), and may thus contribute to the invasiveness of SCC cells.

The early responses of A431 cells to EGF stimulation have been well documented. EGF induces rapid alterations in the organization of the actin microfilament system that result in extensive membrane ruffling, lamellipodia formation, cortical actin polymerization, and cell rounding (Chinkers et al., 1979; Schlessinger and Geiger, 1981; Rijken et al., 1991; Peppelenbosch et al., 1993). These processes precede and accompany cell movement (reviewed in Lauffenburger and Horwitz, 1996 and Mitchison and Cramer, 1996). Actin cytoskeletal rearrangements are regulated by members of the Rho-like GTPase family in all cell types surveyed, including epithelial cells (reviewed in van Aelst and D’Souza-Schorey, 1997 and Hall, 1998). In epithelial MDCK cells Rac is required for membrane ruffling and lamellipodia formation during hepatocyte growth factor/scatter factor (HGF/SF)-induced scattering (Ridley et al., 1995). Further, Rac and Rho proteins have been implicated in the invasion of carcinoma cells (Keely et al., 1997; Shaw et al., 1997; Yoshioka et al., 1998). The involvement of Rac and Rho in the motility and invasion of epithelial cells is consistent with findings in fibroblasts and T-lymphoma cells showing a requirement for these molecules in ras-induced transformation (Qiu et al., 1995a,b), as well as in invasion and growth factor-induced chemotaxis (Michiels et al., 1995; Anand-Apte et al., 1997). These studies in combination implicate Rho-like GTPase-induced cytoskeletal reorganization in the process of invasion.

In this study, we demonstrate that EGF can induce the in vitro invasion of SCC-derived cells but not benign epidermal keratinocytes. We establish that AP-1 is required for this response, since sustained expression of TAM67 in A431 cells blocks EGF-dependent in vitro invasion. Significantly, we find that TAM67 expression inhibits EGF-induced cell motility and in particular the cytoskeletal rearrangements and cell shape changes essential for this process. This occurs because EGF can no longer activate the small Rho-like GTPases Rac and Rho in TAM67-expressing cells, establishing a novel link between EGF signaling, AP-1 activity, and Rho-like GTPases.

Materials and Methods

Materials

Human recombinant EGF was obtained from R&D Systems (Abingdon, UK). TRITC- and FITC-labeled phalloidin and propidium iodide were purchased from Sigma Chemical Co. (Poole, UK), whereas FITC- and
TRITC-labeled anti-mouse immunoglobulin G (IgG) were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Antibodies used for immunoblotting, with the exception of the EGFR Western blot, were fractionated by SDS-PAGE and transferred electrophoretically to polyvinylidene difluoride membranes (Immobilon P; Millipore Corp., Billerica, MA) and processed as TIFF images. Several similar fields of immunoblots were developed and described before (Hennigan et al., 1994). Identical wounding assays were performed with monolayers of NA and TA cells grown on glass coverslips, in which case cells were fixed and stained for F-actin, as below, 24 h after wounding.

AP-1 Transactivation Assay

AP-1 activity in NA and TA cells was assayed using a reporter plasmid (–73 Col-CAT) as previously described (Havasentine et al., 1992). This plasmid encodes for the chloramphenicol acetyl transferase (CAT) gene under the control of an AP-1 responsive element present within the collageneis (Col) gene promoter. CAT activity is normalized against activity arising from the –63 Col-CAT reporter plasmid, which lacks the AP-1 binding site. 3 × 10⁶ cells were seeded in 60-mm plates and transfected with 4 µg of either –73 Col-CAT or –63 Col-CAT using lipofectAMINE. After transfection was completed, cells were incubated for 24 h in either serum-containing medium or serum-free medium supplemented with 10 ng/ml EGF. After this time, CAT activity in cell extracts containing equal amounts of protein was determined by standard techniques (Gorman et al., 1982).

In Vitro Invasion Assay

An inverse invasion assay in serum-free conditions was performed and quantitated as previously described (Hennigan et al., 1994; Lamb et al., 1997a), using growth factor depleted Matrigel-coated polycarbonate chambers (Transwell™ 8-µm pore size filters). In brief, in this assay cells were first allowed to attach to the underside of a Transwell™, and then chemoattracted across the 8-µm pore size filter and into the Matrigel below. This assay was repeated. Cells above the filter were stained with propidium iodide and nuclei viewed in regular 10-µm confocal z-sections from the bottom of the filter visualized using a MRC 600 confocal illumination unit (Bio-Rad Laboratories, Hercules, CA) attached to a Nikon Diaphot inverted microscope. Images of labeled cell nuclei at each height were processed as tagged-image file formats (TIFF) images, and pixels quantitated using a computer program developed and described before (Hennigan et al., 1994).

Cells at and above 20 µm from the bottom of the filter (i.e., in the Matrigel layer) have no cellular contact with the filter and are thus considered to have invaded the matrix (data not shown). Percentage chemotaxis is the ratio of positive pixels at and above the top of the filter to the total combined number of pixels above and below the filter, whereas percent invasion is the ratio of pixels at and above 20 µm from the bottom of the filter to the total number of pixels above and below the filter. For addition of EGF to the assay, human recombinant EGF at a concentration of 10 ng/ml was added above the Matrigel layer in serum-free DME. Transwells™ were placed in the wells of conventional 24-well tissue culture plates containing serum-free DME.

Western Blot Analysis

For all Western blots, cells were washed twice in cold PBS. With the exception of the EGFR Western blot, cells were then lysed for 30 min on ice in 50 mM Hepes, pH 7.0, 250 mM NaCl, 0.5% Nonidet P-40, 5 mM EDTA, 50 mM NaF, 200 µM sodium orthovanadate, 2 mM benzamidine, 50 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 10 µg/ml soybean trypsin inhibitor. For the EGFR Western blot, cells were lysed in 50 mM Hepes, pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 100 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 50 mM β-glycerophosphate, 2 mM PMSF, 10 µg/ml aprotinin, and 10 µg/ml leupeptin. Lysates were centrifuged for 15 min at 14,000 rpm at 4°C. Protein concentrations were determined by bichoronic acid assay (Sigma Chemical Co., St. Louis, MO). 50–80 µg of extracted protein were fractionated by SDS-PAGE and transferred electrophoretically to polyvinylidene difluoride membranes (Immobilon P; Millipore Corp., Bedford, MA). Immunoblots were blocked and probed with antibodies against TAM67, EGFR, active MAPK, ERK2, c-Met, phospho-tyrosine, Rac, and RhoA according to protocols supplied by the manufacturers. The Western blots were developed with the enhanced chemiluminescence Western blotting system (Nycoderm Amersham, Buckinghamshire, UK). Blots were then visualized by exposure to Kodak X-Omat film for various times.

Scattering Assays

The stimulatory effect of EGF on cell motility was measured for A431, NA, and TA clones. Cells were seeded at a density of 2 × 10⁴ cells/well on 6-well plates. Cells were allowed to attach and when they had formed small colonies were transferred to serum-free medium for ~2 d. Colonies were then treated with EGF (2, 5, or 10 ng/ml) for 2 d. Colonies were photographed before and two days after the addition of growth factor using phase-contrast optics.

Wound-healing Assays

A431, NA, or TA cells were plated in 60-mm tissue culture dishes at 2 × 10⁶ cells/plate, cultured overnight in complete medium, and then a confluent monolayer was formed transferred to serum-free medium for a minimum of 2 d. Before digital time-lapse imaging, cells were then lysed for 30 min on ice with 50 mM Hepes-buffered serum-free DME and cells filmed using an MRC 600 Nikon Diaphot confocal laser microscope at 37°C for 5 min before and 45 min after the addition of EGF. Phase signals at 30-s intervals were collected with a COMOS program (Bio-Rad Laboratories) and processed as TIFF images. Several similar fields of A431, NA, and TA cells were filmed with identical results.

Microinjection

Cells for microinjection were seeded on glass coverslips, left to attach overnight in complete medium, and then transferred to serum-free medium for two days before microinjection. C3 transferase was microinjected...
In the cytoplasm of NA13 cells at a concentration of 200 μg/ml, together with FITC-Dextran as an inert marker protein to identify injected cells. C3 transfected at this concentration inhibited serum induced stress fiber formation in 208F fibroblasts (data not shown). C3 was microinjected over a 10-min period and then cells were returned to the incubator for a further 15 min before treating them with EGF for either 5 or 15 min before fixation. The myc-tagged constructs pEXV14RhoA, pEXV12Rac1 encoding constitutively active forms of RhoA and Rac1, respectively (Qui et al., 1995), were microinjected into the nucleus of NA13 and TA37 cells. 12–18 h later cells were fixed and double stained for the myc-epitope and actin filaments using an anti-myc mAb (9E10) and phalloidin, respectively. NA13 cells were similarly microinjected with a plasmid, pEXVN17Rac1, encoding a dominant-negative mutant form of Rac1 tagged with the myc-epitope (Qui et al., 1995b). Microinjected cells left to express the plasmid 12–18 h were treated with EGF for either 5 or 15 min and then fixed and stained as above. Microinjection was performed using a microscope (Axiovert 135m; Zeiss, Inc., Thornwood, NY) attached to an Eppendorf Microinjection Unit (Microjector model 5242, Micromanipulator model 5170, and Heat Controller model 3700). During microinjection cells were maintained at 37°C and before microinjection medium was replaced with Heps-buffered serum-free DME. Microinjected plasmids C3 were diluted in microinjection buffer (5 mM potassium glutamate/150 mM KCl).

**Immunofluorescence**

For immunofluorescence cells were fixed in 4% formaldehyde for 15 min at room temperature and washed several times in PBS containing 0.1% Triton X-100. For visualization of filamentous actin, fixed cells were stained with 500 ng/ml phalloidin for 10 min at room temperature in PBS containing 0.1% Triton X-100. For myc-epitope staining of the microinjected cells blocking of nonspecific antibody binding was performed with PBS containing 0.1% Tween-20 and 5% skimmed milk (Marvel) for 45 min at room temperature. Blocked slides were incubated for 1 h at room temperature in blocking buffer with 1:20 dilution of anti-myc antibody (9E10), washed and then incubated for 45 min at room temperature in fresh blocking buffer containing anti-mouse IgG. Stained slides were washed and mounted (Vectashield; Vector Laboratories, Inc., Burlingame, CA). For visualization of endogenous Rac, cells were blocked in BSA and stained according to the protocol supplied by the manufacturer. Control staining with an isotype matched primary antibody indicated that Rac immunocytochemical staining was specific for the antibody used (data not shown). All slides were viewed on the laser confocal microscope. Fluorescence images were obtained with conventional FITC and TRITC excitation settings on the laser confocal microscope described above and processed as TIFF images.

Images perpendicular to the substratum (y-z projections) of the cells were constructed, using laser scan confocal microscopy on phalloidin stained cells, allowing visualization of cell height.

**Results**

**SCC-derived Cell Lines Invade in Response to EGF**

SCC-derived cell lines express elevated levels of EGFR compared with normal human keratinocytes in culture (Hendler and Ozanne, 1984; Stanton et al., 1994). We used a quantitative in vitro invasion assay (Hennigan et al., 1994; Lamb et al., 1997a) to test whether EGFR overexpression correlates with increased invasion potential. For our studies we used a number of well characterized SCC-derived cell lines (Edington et al., 1995; Malliri et al., 1996) for which the levels of EGFR expression are known (Stanton et al., 1994). We also included normal HEKs, HPV16 E6 and E7 immortalized HEKs (TFK104), and benign immortal keratinocytes (HaCaT) to determine whether invasiveness is an exclusive property of malignant epithelial cells. Unlike the SCC-derived cell lines, none of the benign keratinocyte cell lines overexpress EGFR (Stanton et al., 1994). Further, none of the benign epidermal cells invaded in this assay, even in the presence of EGF (Fig. 1 a). In contrast, whereas none of the SCC-derived cell lines tested invaded spontaneously, they all invaded after EGF treatment (Fig. 1 a). A431 cells are an example of the SCC-derived cell lines shown in Fig. 1 a. Without EGF no A431 cells migrated to the top of the filter; on addition of EGF cells migrated to a distance of 50 μm from the bottom of the filter (Fig. 1 b). Invasion of A431 cells was dependent upon the concentration of EGF added to the top of the Matrigel and was maximal for 10 ng/ml (data not shown). Invasion was abrogated both by anti-EGFR antibodies demonstrated to inhibit EGFR signaling (Modjtahedi et al., 1993) and by CGP52411 (a gift from Dr. N. Lydon, Ciba Geigy Pharmaceuticals, Basel, Switzerland) that inhibits EGFR tyrosine kinase activity (Buchdunger et al., 1994; data not shown). We conclude that invasion of SCC-derived cells lines correlates with EGFR overexpression and is dependent on EGFR signaling.

![Figure 1](https://jobs.rupress.org/jcb.rupress.org/...
Invasion of A431 Cells Is Inhibited by Expression of the Dominant Negative Mutant of c-Jun, TAM67

Previously, we have demonstrated that invasion of v-fos-transformed or EGF-treated fibroblasts requires functional AP-1 (Lamb et al., 1997a). To determine if AP-1 activity is required for EGF-dependent in vitro invasion of human SCC-derived cell lines, we generated A431 transfectants constitutively expressing the c-Jun deletion mutant, TAM67. A431 cells were selected because their response to EGF treatment is well characterized and because they demonstrate a marked invasion response to EGF. TAM67 has been clearly demonstrated to nullify AP-1 transcriptional activity (Brown et al., 1993; Domann et al., 1994b; Dong et al., 1997; Li et al., 1998). G418-resistant A431 subclones were obtained and tested by Western blot analysis for expression of TAM67. Three A431 TAM67-expressing clones, TA5, TA36, and TA37, and two nonexpressing G418-resistant clones, NA13 and NA15, were used most extensively in subsequent experiments (Fig. 2a). Sustained TAM67 expression was found to inhibit basal and EGF-induced AP-1 transcriptional transactivation using a collagenase promoter-CAT reporter construct (Fig. 2b). As was previously demonstrated for malignant mouse epidermal cell lines (Domann et al., 1994b; Dong et al., 1997), introduction of TAM67 did not alter the growth rates of expressing clones (data not shown).

The in vitro invasion assay was used to determine whether sustained expression of TAM67 altered the ability of A431 cells to respond to EGF. 14 TA clones and 12 NA clones were examined for their invasive response to EGF treatment (10 ng/ml). Whereas all the NA clones retained their invasive response to EGF, none of the TA clones invaded the Matrigel in response to EGF. Quantitation of the invasion of two representative NA clones and three representative TA clones is shown in Fig. 2c. Confocal images of cell nuclei from the invasion assay are also shown for one NA and one TA clone in Fig. 2d. It was evident from the analysis of the in vitro invasion assay that chemotaxis itself of TA cells was impaired, as cells failed to migrate even to the top of the micropore filter (Fig. 2c).

All the SCC-derived cell lines that invade in vitro in response to EGF, including A431 cells, have high levels of EGFR (Stanton et al., 1994). We tested whether the impaired invasiveness of TA cells resulted from decreased EGFR expression and/or signaling activity. Quantitation of the level of EGFR expression for A431 and NA and TA clones by Western blotting (Fig. 3a) or by ligand binding assays (data not shown) revealed that the level of EGFR expression was equivalent in TA, NA, and A431 cells. Furthermore, EGF-induced EGFR autophosphorylation was equivalent in A431, NA, and TA cells, as determined by Western blot analysis using an anti-phosphotyrosine antibody (Fig. 3b), and by an EGFR in vitro kinase assay (data not shown). The ability of EGF to stimulate phosphorylation of MAPK was also equivalent in TA, NA, and A431 cells (Fig. 3c). Thus, TAM67 expression inhibits chemotaxis and invasion of A431 cells without directly interfering with EGFR expression, ligand activation of its tyrosine kinase activity, or its ability to activate MAPK.

TAM67 Inhibits EGF- and HGF/SF-stimulated Colony Scattering as Well as Wound Closure

The greatly reduced chemotactic response of TA cells to EGF stimulation suggested that they might display impaired motility in response to EGF. A431, NA, and TA cells normally grow as packed colonies. Colonies of A431 cells respond to HGF/SF ligation of its receptor, c-Met, by...
scattering, which requires the disruption of cell–cell junctions and an increase in cell motility (Tajima et al., 1992). Like the EGFR, c-Met is a ligand-stimulated protein tyrosine kinase. We found that EGF, in addition to HGF/SF, induced scattering in A431 cells, as had previously been demonstrated for SCC-derived cells HSC-1, which also overexpress EGFR (Fujii et al., 1996). To assess whether inhibition of AP-1 activity directly inhibits motility of A431 cells, we tested both EGF and HGF/SF for colony scattering activity on A431 cells, two NA clones, and three TA clones. EGF efficiently induced scattering of A431 and NA colonies within 48 h, but did not induce scattering of TA colonies (Fig. 4a). Treatment of A431 or NA cells with the EGFR inhibitor CGP52411 inhibited EGF-stimulated colony scattering (data not shown). Similar results were obtained with HGF/SF treatment even though A431, NA, and TA cells all expressed equivalent levels of c-Met (data not shown). These results suggest that TAM67 inhibits growth factor-stimulated cell–cell disruption and/or cell motility.

Monolayers of cells respond to wounding by migrating into the wound to effect closure. Closure of wounds of ~0.75 mm in monolayers of A431 or NA cells occurred within 48 h (Fig. 4b) by a process that was independent of mitomycin C treatment (at a concentration 0.15 μg/ml that inhibits DNA synthesis by 90%) and therefore of cell proliferation (data not shown). Wounding itself was a potent stimulus for cell migration. In contrast to A431 and NA cells, wounded monolayers of TA cells did not migrate significantly into the wound, failing to close it over a 48-h period (Fig. 4b).

We noted that in EGF-induced colony scattering, scattered cells appeared rounded with pronounced lamellipo-
dia (Fig. 4, a and insets). Time-lapse digital microscopy of EGF-treated A431 cells revealed that the rounded cells were motile and extended lamellipodia in the direction of movement (data not shown). In the wound healing assays, cells that had moved into the wound were also rounded. Rounded cells displayed an increase in F-actin that was polarized in its distribution with a higher concentration towards the wound (Fig. 4c). In contrast, TA cells, which did not move in response to EGF or wounding, did not round (Fig. 4c). These results suggest that cell rounding is part of the motogenic response of A431 cells.

**TAM67 Inhibits EGF-stimulated Rearrangements of the Actin Cytoskeleton and Cell Rounding/Contraction**

EGFR activation leads to rapid and pronounced membrane ruffling in A431 cells, which is followed by significant cortical actin polymerization (Chinkers, 1979; Schlessinger and Geiger, 1981; Rijken et al., 1991; Peppelenbosch et al., 1993). No increase in actin stress fibers, a common consequence of growth factor stimulation of fibroblasts and other epithelial cells (van Aelst and D’Souza Schorey, 1997; Hall 1998), has been noted for A431 cells. Pretreatment of A431 or NA cells with the EGFR inhibitor CGP52411 prevented EGF-induced actin cytoskeletal rearrangements and cell rounding (data not shown) indicating that EGF-stimulated EGFR tyrosine kinase activity was required to initiate these rapid changes.

We investigated whether EGFR activation results in actin cytoskeletal rearrangements in TA cells, culminating in cell rounding. Serum-starved NA and TA cells displayed low levels of F-actin as judged by phalloidin staining (Fig. 5, a and b). EGF treatment of NA cells resulted in membrane ruffles (Fig. 5c) and lamellipodial extensions (Fig. 5c, insert) within 5 min, which was followed by a dramatic increase in polymerized cortical actin by 15 min (Fig. 5e). No increase in actin stress fibers was detected at 5 or 15 min after EGF treatment (Fig. 5c and e). When TA cells were treated with EGF, membrane ruffling, lamellipodia formation, and cortical actin polymerization were all greatly reduced (Fig. 5, d and f). The cortical actin polymerization occurring in NA cells 15 min after EGF treatment was associated with an increase in cell height. The bottom panels of Fig. 5, a, b, e, and f show confocal microscopy optical sections made perpendicular to the substratum through the cells at the position of the colony indicated by the arrow. As judged by comparing these bottom panels, 15 min of EGF treatment resulted in a significant increase in height as they contract and become rounded. Pretreatment of A431 or NA cells with the EGFR inhibitor CGP52411 prevented EGF-induced actin cytoskeletal rearrangements and cell rounding (data not shown) indicating that EGF-stimulated EGFR tyrosine kinase activity was required to initiate these rapid changes.
increase in cell height in NA cells, which was much reduced in TA cells. The presence of membrane ruffles, lamellipodia, and polymerized cortical actin was tested in A431 cells and clones NA13, NA15, TA5, TA36, and TA37 with similar results as shown in Fig. 5 for clones NA13 and TA37. 40 min after treatment with EGF, the great majority of A431 and NA cells became rounded/contracted, whereas TA cells did not (Fig. 6). EGF-induced rounding was tested in eight NA and eight TA clones with results similar to those shown in Fig. 6 for A431 cells and clones NA13, TA37, and TA36. We conclude that sustained expression of TAM67 inhibits EGF-induced cytoskeletal rearrangements and cell rounding.

Rac and Rho Mediate EGF-induced Cytoskeletal Rearrangements in A431 Cells. EGF Fails to Activate Rho-like GTPases in TA Cells

To study the involvement of Rho-like GTPases in EGF-induced cytoskeletal changes, Rac1 was selectively inhibited by microinjection of NA13 cells with a plasmid encoding a myc-epitope-tagged dominant negative mutant of Rac1 (DN-Rac) and Rho by microinjection of Clostridium botulinum exoenzyme C3 transferase. After an overnight period, following microinjection of DN-Rac, or 15 min after the microinjection of C3 transferase, cells were treated with EGF for 5 or 15 min and stained for F-actin with phalloidin. Expression of DN-Rac inhibited membrane ruffles and lamellipodia formation (Fig. 7, a and b), indicating that Rac activation is required for the formation of these membrane protrusions in EGF-treated NA13 cells. No accumulation of polymerized cortical actin nor cell rounding was detected in the C3 transferase-microinjected NA13 cells after 15 min of EGF treatment (Fig. 7, c and d), although membrane ruffles were observable after 5 min EGF treatment (data not shown), indicating that C3 transferase was not inhibiting Rac activity. This observation demonstrates that Rho activity is required for EGF-induced cortical actin polymerization and cell rounding. Furthermore, treatment of NA13 cells microinjected with DN-Rac with EGF for 15 min blocked cortical actin polymerization (Fig. 7, c and d). This suggests that Rac activation is upstream of Rho activation in the EGF-dependent pathway regulating cortical actin polymerization in A431 cells.

The inability of EGF to induce membrane ruffles, lamellipodia, and cortical F-actin in TA cells suggests that EGFR signaling to the actin cytoskeleton is compromised in these cells. This could be due to a failure to activate Rac
and Rho, the absence or functional inactivity of certain downstream effectors of Rac and Rho, or a combination of both. To determine whether the downstream effectors of Rac and Rho responsible for the EGF-induced cytoskeletal rearrangements are present and functional in TA cells, plasmids directing the expression of myc-epitope-tagged activated forms of Rac1 and RhoA (RacV12 and RhoV14) were introduced by microinjection into NA13 and TA37 cells. Expression of RacV12 resulted in the appearance of F-actin at the site of cell to cell contacts, where RacV12 also accumulated (Fig. 8, a–d), as has also been reported for MDCK cells (Ridley et al., 1995; Hordijk et al., 1997; Takaishi et al., 1997). Expression of RhoV14 resulted in an increase in F-actin around the cell cortex and rounded morphology resembling that which occurred after EGF treatment (Fig. 8, e–h). It also induced a low but detectable increase in actin stress fibers, which did not occur after EGF treatment (data not shown). These results indicate that the main cytoskeletal rearrangement in A431 cells mediated by Rho is the polymerization of cortical actin rather than the formation of stress fibers. Most significantly, however, actin structures formed in both NA13 cells and TA37 cells after microinjection of either RacV12 or RhoV14 were equivalent (compare Fig. 8 a with c and e with g). These results indicate that the downstream effectors of Rac and Rho required for the specific EGF-induced actin cytoskeletal and morphological changes are functional in TA cells, which suggests that EGF activation of Rac and Rho is compromised in TA cells.

No assay is presently available to directly measure the activation of Rho. However, in mouse dermal fibroblasts Rac has been shown to be recruited from the cytoplasm to the plasma membrane upon growth factor stimulation concomitant with its activation (Azuma et al., 1998). In serum-deprived NA13 and TA37 cells Rac as detected by immunofluorescence was not found associated with the plasma membrane (Fig. 9 A, a and b). Upon EGF stimulation though Rac was detected in the plasma membrane of NA13 but not TA37 cells (compare Fig. 9 A, c and e with d and f), indicating that EGF stimulation does not activate Rac in TA cells.

One explanation for the absence of actin cytoskeleton rearrangements after EGF treatment in TA cells could be due to decreased expression of Rac and Rho in TA cells. This possibility was excluded by the demonstration that the levels of Rac and Rho protein in extracts from A431, NA13, NA15, TA36, and TA37 cells, as determined by Western blot analysis using antibodies specific for Rac and Rho, were equivalent (Fig. 9 B).

Discussion

Through investigating the regulation of human SCC invasion, we have made several interesting findings that establish a novel link between EGF signaling, AP-1, and the regulation of cell motility. First, in vitro invasion of SCC-derived cells is dependent upon EGF stimulation, and this response to EGF does not occur in benign epidermal cells. Second, expression of the c-Jun deletion mutant, TAM67, inhibits EGF-induced cytoskeletal rearrangements necessary for membrane ruffling, lamellipodia formation, cell rounding, and ultimately motility and invasion. Third, TAM67 expression inhibits EGF activation of Rho-like GTPases, the critical regulators of these events.

We have shown that invasion in response to EGF distinguishes SCC-derived cells from benign epidermal cells, including those that are immortal. An obvious distinction between the malignant and benign epidermal cells is their level of expression of EGFR, which suggests that overexpression of growth factor receptors could be an important step in the emergence of the invasive phenotype. This is supported by the finding that whereas colon-adenoma-derived cells do not invade in vitro in response to EGF, malignant variants of these cells that overexpress the EGFR do (Brunton et al., 1997). However, EGFR overexpression may not be sufficient to render benign cells responsive to EGF-induced invasion, since loss of invasion suppressor genes also appears to be critical for the invasive phenotype of cancers (Liotta et al., 1991; Birchmeier et al., 1995).

EGF-induced in vitro invasion of the SCC-derived cell
line A431 is dependent upon AP-1 activity since sustained expression of TAM67 in these cells inhibited this response, concomitant with suppression of AP-1 transactivation. In this study, we establish that a major role of AP-1 in facilitating invasion of carcinoma cells is its ability to couple growth factor signaling to cell motility. We demonstrate that TAM67-mediated inhibition of AP-1 results in both impaired chemotaxis and scattering of A431 cells in response to EGF.

The early responses of A431 cells to EGF, which include formation of membrane ruffles and lamellipodia, cortical actin polymerization, and cell body retraction (rounding), are all processes assumed to occur repeatedly during the long-term response of cell migration (Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996). Sustained TAM67 expression inhibited not only the long-term motile response of A431 cells to EGF, but also these early responses. We contend that the failure of EGF to induce cytoskeletal and morphological changes in TA cells is in large part responsible for their impaired motile response. Further, our findings imply that continued expression of one or more AP-1 target genes is required in order to transduce an EGF signal into a motogenic response. This hypothesis is at least consistent with the findings of Ridley et al. (1995) who also demonstrated a requirement for de novo gene expression during HGF/SF-induced motility of MDCK cells.

In this study, we establish that EGF-induced actin cytoskeletal rearrangements and morphological changes in A431 cells are dependent upon activation of Rac and Rho, as documented for many other cell types (van Aelst and D’Souza-Schorey, 1997; Hall, 1998). Whereas Rac is required for EGF-induced membrane ruffling and lamellipodia formation in A431 cells, Rho regulates cortical actin polymerization and cell rounding. Further, our findings that (a) dominant-negative Rac1 inhibits cortical actin polymerization and cell rounding, as well as lamellipodia formation and membrane ruffling; (b) C3 transferase inhibition of Rho inhibits only EGF induced cortical actin polymerization and cell rounding; and (c) activated RhoA by itself is sufficient to induce cortical actin polymerization only are consistent with the proposed hierarchy suggested for Swiss 3T3 cells, where growth factor stimulation of Rho-mediated events are dependent upon Rac activation (Nobes and Hall, 1995). However, unlike the situation in fibroblasts (Nobes and Hall, 1995), activated Rac1 by itself appears insufficient to activate Rho in A431 cells.

In fibroblasts and epithelial MDCK cells, Rho-mediated actin polymerization results in an increase in stress fibers (Ridley and Hall, 1992; Ridley et al., 1995). However, in A431 cells expression of RhoV14 or activation of Rho by EGF stimulation results largely in cortical actin polymerization and cell rounding, but not stress fiber formation. A similar response to Rho activation occurs in neuroblastoma-derived cells (Kranenburg et al., 1997; van Leeuwen et al., 1997). This suggests that the type of actin reorganization mediated by Rho in response to growth factor signaling is cell type and perhaps stimulus specific.

As already alluded to, we believe that Rho-mediated rounding observed after EGF treatment of A431 cells is an important component of their motogenic response. We have noted in both EGF-induced cell scattering and wound healing experiments that the motile cells are rounded. In the wound healing experiments the leading edge cells round up, break away from the monolayer, and migrate into the wound. During EGF-induced scattering cell rounding precedes and is maintained during movement. Time-lapse digital microscopy reveals that the round motile cells extend lamellipodia in the direction of movement. This mode of movement is not restricted to A431 cells and reflects a more common form of epidermal cell motility, since normal HEK cells stimulated to move also migrate as round cells (Owens, D., unpublished data).

From the absence of actin cytoskeletal rearrangements in EGF-treated TA cells, coupled with loss of motility and invasiveness, we postulated that expression of TAM67 might be compromising signaling pathways involving Rac and Rho. In principle, this block could occur either upstream of Rac and Rho preventing their activation, at the level of expression of Rac and Rho, or downstream of Rac and Rho by reducing the activity of effector molecules, or a combination of all of these. Our results are most consistent with the first explanation. Rac activation is associated with membrane translocation (Azuma et al., 1998), and whereas EGF induces the translocation of Rac to the plasma membrane in NA cells, it does not in TA cells. No significant alterations in the level of expression of Rac or Rho were detected in TA cells. Furthermore, expression of constitutively active forms of Rac1 and RhoA induced indistinguishable cytoskeletal effects in TA and NA cells, indicating that at least a subset of the effector pathways downstream of Rac and Rho are intact in both cell types.

However, constitutively active forms of Rac and Rho alone did not induce A431 or NA cells to move within a 48-h time period (data not shown), indicating that neither molecule in the absence of EGF signaling is sufficient to induce cell motility. Expression of dominant negative Rac within these cells prevented EGF-stimulated cytoskeletal rearrangements, cell rounding, and motility (Fig. 5 and data not shown), indicating that Rac activation is required for all these responses. To the extent that motility is required for invasion, we contend that Rac would also be required for invasion.

On the basis of the above analysis, we anticipate that possible candidates for AP-1 target genes required for EGF-induced motogenic responses include the multiple known upstream regulators of Rho-like GTPase activity (van Aelst and D’Souza-Schorey, 1997; Hall, 1998), and potentially other as yet unidentified regulators. Two aspects appear to be involved in growth factor regulation of Rho-like GTPase activity: one is the recruitment of these molecules from the cytosol to the membrane, the other is guanine nucleotide exchange. However, the precise sequence of these events has yet to be determined. A number of guanine nucleotide exchange factors (GEFs) have been identified that catalyze guanine nucleotide exchange. GTPase-activating proteins (GAPs) serve to inhibit Rho-like GTPases by accelerating the conversion of GTP-bound forms to their inactive GDP-bound forms. Guanine nucleotide dissociation inhibitors (GDI) sequester Rho-like GTPases in the cytosol and also inhibit GEFs from stimulating the exchange of GDP for GTP. Members of the ERM family of proteins, which include ezrin, radixin, and moesin, can function as activators of Rho-like GTP-
GTPases have been reported to activate the c-Jun NH2-like GTPases act upstream of signaling cascades that stimulate the expression of other AP-1–regulated genes required for cell motility and invasion, but not in TA cells. These genes could include proteases that degrade ECM (Hennigan et al., 1994; Miao and Curran, 1994; Tremble et al., 1995), as well as proteins that mediate cell–cell adhesion (Reichmann et al., 1992; Fialka et al., 1996).

It has been demonstrated in mammalian cells that Rho-like GTPases act upstream of signaling cascades that stimulate expression and activation of AP-1 components, as well as functioning to alter cell shape and motility. Rho-like GTPases have been reported to activate the c-Jun NH2-terminal kinase (JNK; Coso et al., 1995; Minden et al., 1995; Teramoto et al., 1996) and are also required for stimulation of the serum response factor (SRF; Hill et al., 1995). Our results suggest the possibility of a positive feedback connection between AP-1 function and activation of Rho-like GTPases that could serve to amplify a motogenic and invasion stimulus.

Dorsal closure during Drosophila embryogenesis provides another instance where AP-1 and Rho-like GTPases function coordinately to regulate cytoskeletal changes and movement of epithelial cells (reviewed in Martin-Blanco, 1997). During dorsal closure, Rac in the leading most migrating cells activates AP-1 via the JNK signaling pathway, whereas AP-1 directs the expression of genes such as decapentaplegic, Dpp (a TGF-β homologue). Dpp in turn elicits its cytoskeletal changes in more laterally positioned epithelial cells. Cell shape changes in leading edge cells, whereas dependent upon AP-1 activity, are independent of Dpp signaling (Riesgo-Escovar and Hafen, 1997a). In more lateral epithelial cells, Dpp signaling still appears to require Dfos function (Riesgo-Escovar and Hafen, 1997b). This has lead to the suggestion that certain cytoskeletal elements or their regulators are direct effectors of AP-1 signaling during dorsal closure. Based on our results, we suggest that this connection also exists in vertebrate epithelial cells.

In conclusion, we propose that sustained expression of TAM67 functions as an inhibitor of a multigenic invasion program through suppressing the activity of AP-1. A subset of AP-1 target genes appear to provide molecular bridges between growth factor stimulation and activation of Rac and Rho, which in turn regulate morphological and motile responses (Fig. 10). In this respect, AP-1 target genes could include upstream activators of Rac and Rho (Fig. 10, X), but equally might include inhibitors of these molecules (Fig. 10, Y). The effect of TAM67 on downstream signaling from the EGFR appears specific to the Rho-like GTPase cascade, since the ability of EGF to activate MAPK is unimpaired in TA cells (Fig. 10). Our findings strengthen the hypothesis that AP-1 activity is essential for the invasion of malignant human epithelial cells.

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