E1AF/PEA3 Activates the Rho/Rho-Associated Kinase Pathway to Increase the Malignancy Potential of Non–Small-Cell Lung Cancer Cells

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Abstract

E1AF/PEA3, an Ets family transcription factor, is frequently overexpressed in non–small-cell lung cancers (NSCLCs). Overexpression of E1AF increases motility and invasion of VMRC-LCD and NCI-H226 NSCLC cells, which lack endogenous E1AF expression, and the effect is synergistically increased by hepatocyte growth factor (HGF). The small GTPase Rho/Rho-associated kinase (ROCK) pathway is also involved in motility and invasion. To determine the role of the Rho/ROCK pathway in malignant phenotypes induced by E1AF, we analyzed VMRC-LCD cells transfected with an E1AF expression vector (LCD-E1AF cells) or with empty vector (LCD-vector cells). LCD-E1AF cells had more GTP-bound (active) Rho than LCD-vector cells and Rho activation was synergistically increased by HGF. The Rho activation by E1AF and HGF was also shown in NCI-H226 cells. Phosphorylation of myosin light chain (MLC), a downstream effector of ROCK signaling, was higher in LCD-E1AF cells than in LCD-vector cells, especially under HGF treatment. A specific ROCK inhibitor, Y27632, strongly suppressed MLC phosphorylation, cell motility, and invasion. In nude mice implanted s.c. and intrapulmonarily, LCD-E1AF cells made more local tumors than LCD-vector cells (six of six versus one of seven mice and four of seven versus one of seven mice, respectively). Three of the four mice with lung tumors from LCD-E1AF cells had lymph node metastases whereas the mouse with LCD-vector tumors did not. LCD-E1AF tumors showed higher MLC phosphorylation than LCD-vector tumors. These results suggest that E1AF activates the Rho/ROCK pathway in an HGF-enhanced manner and its activation is important in E1AF-induced motility and invasion as well as tumorigenesis and metastasis in NSCLC cells.

Introduction

Cell motility and invasion as well as tumorigenesis and metastasis characterize the malignancy potential of cancer. Members of the Ets-related oncprotein family are transcription factors with a highly conserved ETS domain; they play important roles in the regulation of gene expression during such biological processes as oncogenesis (1). One Ets-related family member, E1AF (2), a human homologue of mouse PEA3 (3), was isolated by its ability to bind adenovirus E1A enhancer elements. E1AF can up-regulate transcription from several subclasses of matrix metalloproteinase (MMP) genes in transient expression assays (4). Expression of E1AF confers an invasive phenotype on MCF7, a human breast cancer cell line (4). E1AF expression is correlated with the transcription of MMPs and the invasive phenotype in oral squamous cell carcinoma cell lines (5). We have previously reported that E1AF is frequently overexpressed in non–small-cell lung cancers (NSCLC) and its expression increases motility and invasion of NSCLC cell lines VMRC-LCD and NCI-H226, which normally lack E1AF expression (6). In addition, recent in vivo experiments show that E1AF increases the metastatic activity of fibrosarcoma cells through expression of membrane type 1 MMP (7) and that mammary oncogenesis induced by HER2/neu is strongly inhibited by blockade of E1AF (8). These observations suggest the E1AF has roles in various malignant phenotypes of cancer cells but the mechanisms remain to be discovered.

Rho, a well-known member of the p21 Ras superfamily of small GTPases, exhibits both GDP/GTP binding and GTPase activities. Rho serves as a molecular switch, regulating signal transduction from receptors in the membrane to various cellular events related to cell morphology (9), motility (10), cytoskeletal dynamics (11, 12), and tumor progression (13, 14; for reviews, see refs. 15–17). Rho activates Rho-associated kinase (ROCK), which then increases phosphorylation of myosin light chain (MLC; ref. 18). Phosphorylated MLC induces the contraction of myosin and subsequently organizes the actin stress fibers and focal adhesions. Activation of Rho/ROCK signaling is also known to stimulate the organization of actin stress fibers and to enhance the motility and invasion of rat hepatoma cells (19, 20), human glioma cells (21), and human ovarian cancer cells (22). Recently, studies using clinical specimens showed a relationship between the expression level of RhoC, an isoform of Rho, and tumor aggressiveness in breast cancer (23–25), pancreatic cancer (26), and NSCLCs (27). Furthermore, RhoC also enhances metastasis of melanoma cells (28) and lung cancer cells (29) in mouse model systems.

To determine whether the Rho/ROCK pathway is involved in E1AF-associated malignant phenotypes in NSCLCs, we examined E1AF- and vector-transfected VMRC-LCD NSCLC cells for Rho activity and its relation to motile and invasive activities. To increase the effects of E1AF, we used hepatocyte growth factor (HGF), a strong cell-scattering factor that is frequently overexpressed in various human cancers including NSCLCs. We have shown that HGF acts synergistically with E1AF to increase motile and invasive activities in VMRC-LCD cells (6). We treated the cells with a specific ROCK inhibitor, Y27632 (20), to determine whether activation of the Rho/ROCK pathway is required for...
the enhancement of motile and invasive activities by E1AF. Furthermore, we examined E1AF- and vector-transfected cells for tumorigenic and metastatic activities in vivo and their relationship with phosphorylation of MLC, a downstream effector of ROCK.

Materials and Methods

Cell lines and cell culture. VMRC-LCD and A549 NSCLC cell lines were obtained from the Health Science Research Resource Bank of Japan (Osaka, Japan). NCI-H226 and NCI-H520 NSCLC cell lines were kindly provided by Dr. H. Oie (Navy Medical Oncology Branch, National Cancer Institute, Bethesda, MD). All cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 0.03% glutamine at 37°C in an atmosphere of 5% CO₂. LCL-E1AF cells (VMRC-LCD cells transfected with the E1AF-expression vector pCMV-E1AF and pRSVneo), LCL-vector control cells (VMRC-LCD transfected with the empty vector pEV3S and pRSVneo), H226-E1AF cells (NCI-H226 cells transfected with the E1AF-expression vector pCMV-E1AF and pRSVneo), and H226-vector control cells (H226 cells transfected with the empty vector pEV3S and pRSVneo) have been described in our previous article (6). Although not stated in that article, they were pools of G418-resistant cells consisting of at least 100 independent clones, which could minimize possible clonal variations. The established cells had been stocked in aliquots in liquid nitrogen until experiments were started. Only cells in early passages were used for the experiments to avoid molecular changes during prolonged period of culture.

pLNTK-E1AF–infected SiHa cells were cultured in DMEM supplemented with 10% FBS and 0.03% glutamine at 37°C in an atmosphere of 5% CO₂ and used for positive control for Western blot analysis of E1AF protein (30).

Western blot analysis of E1AF. Western blot analyses of E1AF were done as previously described (30). Briefly, purified total protein (30 μg) from isolated subconfluent cells was separated by SDS-PAGE, transferred to nitrocellulose membranes, and reacted with an anti-E1AF monoclonal antibody. The primary antibodies were detected using an antirabbit antibody conjugated with horseradish peroxidase and visualized by the Amersham enhanced chemiluminescence (ECL) system (Amersham, Arlington Heights, IL). pLNTK-E1AF–infected SiHa cells were used for positive control of E1AF protein expression (30). The same lysates were used in Western blotting of actin as an internal control.

In vitro motility and invasion assays. Transwell cell culture chambers (pore size, 8 μm; Costar, Cambridge, MA) were used for the motility and invasion assays. For the motility assay, 1 × 10⁵ LCD-E1AF and LCD-vector cells were suspended in serum-free RPMI 1640 with 0.1% bovine serum albumin (BSA; Sigma Chemical, St. Louis, MO) and added to the upper chamber. The reverse side of the upper chamber filter was coated with 10 μg human cellular fibronectin (Biomedical Technologies, Stoughton, MA) as a chemoattractant. Serum-free RPMI 1640 with 0.1% BSA was added to the lower chamber. For the invasion assay, 2 × 10⁵ LCD-E1AF and LCD-vector cells were suspended in serum-free RPMI 1640 with 0.1% BSA and added to the upper chamber. The upper chamber filter was coated with 10 mg mouse Matrigel (Becton Dickinson Labware, Franklin Lakes, NJ) and the reverse side of the upper chamber filter was coated with 10 μg human cellular fibronectin as a chemoattractant. Serum-free RPMI 1640 with 0.1% BSA was added to the lower chamber. In both motility and invasion assays, a specific ROCK inhibitor, Y27632 (0, 1, 10, or 100 μM/L; Calbiochem, Darmstadt, Germany), was added to the upper and lower chambers and HGF (40 ng/mL; Toyobo, Osaka, Japan) was added to the lower chamber as indicated. These cells were incubated at 37°C in an atmosphere of 5% CO₂ for 24 hours for both motility and invasion assays.

Cell growth assay. Cell growth was measured using an assay based on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; non-radioactive proliferation assay, Promega, Madison, WI). Cells (1 × 10⁴) in 0.1 mL of RPMI 1640 with 10% FBS or 0.1% BSA, with or without 100 μg/mL Y27632, were seeded onto each well of 96-well plates and then incubated at 37°C in an atmosphere of 5% CO₂ for 1, 3, or 5 days. To each well was added 15 μL of Dye Solution (Promega) and then cells were incubated at 37°C for 4 hours. After 4 hours, 100 μL of Solubilization/Stop Mix solution (Promega) were added to each well. One hour later, the contents of the wells were mixed to yield a uniformly colored solution. The absorbance at 590 nm was recorded using a 96-well plate reader and the survival fraction was quantified.

Rho pull-down assay. The Rho pull-down assay was done as previously described (31). Briefly, cells (3.5 × 10⁶/mL) were plated and cultured under 10% serum conditions with or without HGF for 24 hours. After incubation, cells were washed twice with PBS and lysed in Laemmli’s SDS sample buffer (32). Cell lysates were clarified by centrifugation. For controls, LCD-vector cell lysate or H226-vector cell lysate containing 1.5 mg total protein was incubated at 30°C for 30 minutes with 100 μM/L GTPγS (Upstate Biotechnology, Lake Placid, NY) for the positive control or with 100 μM/L GDP (Upstate Biotechnology) for the negative control. Cell lysates containing 1.5 mg total protein were incubated with 30 μg RhoGDI-agarose beads (Upstate Biotechnology) at 4°C for 45 minutes. The beads were washed thrice with PBS. GTP-bound Rho proteins were detected by Western blotting using a polyclonal antibody against Rho (RhoA, RhoB, and RhoC; Upstate Biotechnology) overnight at 4°C. The primary antibodies were used as in (B) were done using H226-vector and H226-E1AF cells.

Figure 1. Rho activation by E1AF in NSCLC cells. A, Western blot analysis of E1AF protein of LCD-vector and LCD-E1AF cells, H226-vector and H226-E1AF cells, and A549 and NCI-H520 cells. P.C., positive control (pLNTK-E1AF–infected SiHa cells; ref. 30). B, Western blot analysis of intracellular levels of GTP-bound Rho and total Rho in LCD-vector and LCD-E1AF cells. For GTP-bound (active) Rho, total protein isolated from cells cultured for 24 hours, with or without HGF (40 ng/mL), was incubated with RhoGDI-agarose beads to bind active Rho. Then GTP-bound Rho was detected by Western blotting using an anti-Rho (RhoA, RhoB, and RhoC; Upstate Biotechnology) antibody.
were detected using anti-rabbit antibody conjugated with horseradish peroxidase and visualized with the Amersham ECL system. The same lysates were used in Western blotting of total Rho and actin as an internal control for the comparison of levels of GTP-bound Rho.

**Western blot analysis of myosin light chain.** LCD-E1AF and LCD-vector cells were cultured in RPMI 1640 with 10% FBS for 24 hours, then with or without 40 ng/mL HGF, and finally with 100 μmol/L Y27632 for 0, 1, 4, 8, 12, and 24 hours. Cells were washed twice with PBS and lysed in ice-cold lysis buffer [10 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 0.1% deoxycholate, 0.1% SDS, 100 μg/mL leupeptin, 100 μg/mL aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride]. Cell lysates containing 30 μg total protein were separated on SDS-polyacrylamide gels, transferred to nitrocellulose membrane, and then reacted with anti-MLC antibody from mice (Sigma Chemical) or anti–phosphorylated MLC antibody from rabbits (Cell Signaling, Beverly, MA). The primary antibodies were detected using antimouse or antirabbit antibody conjugated with horseradish peroxidase and visualized with the Amersham ECL system.

**Western blot analysis of mouse tumor homogenates.** Single s.c. tumor from a mouse implanted with LCD-vector cells or a mouse with LCD-E1AF cells, single lung tumor from a mouse with LCD-vector cells, a pool of lung tumors from five mice with LCD-E1AF cells, and a pool of mediastinal lymph node tumors from four mice with LCD-E1AF cells were homogenized and sonicated in 1.0 mL of ice-cold lysis buffer. Specimens were centrifuged at 900 g for 15 minutes and supernatants were filtered through sterile filters of 10 μm pore size (Toyo Roshi, Tokyo, Japan). Cell lysates containing 30 μg total protein were separated on SDS-polyacrylamide gels, transferred to nitrocellulose membrane, and then reacted with anti-MLC antibody from rabbits (Cell Signaling, Beverly, MA). The primary antibodies were detected using antimouse or antirabbit antibody conjugated with horseradish peroxidase and visualized with the Amersham ECL system.

**Mice.** Female BALB/c athymic nude mice, 4 to 6 weeks old, were purchased from Clea Japan, Inc. (Tokyo, Japan) and maintained in a specific pathogen-free environment throughout the experiment.

**Tumor implantation.** LCD-E1AF or LCD-vector cells (1 × 10^7) were injected i.v. into the tail vein or s.c. into the flank of 6- to 8-week-old nude mice. The mice injected s.c. were examined for localized tumor and for metastasis to mediastinal lymph node, liver, kidney, and spleen. The weight of each tumor was measured.

The implantation of LCD-E1AF and LCD-vector cells into the lung was done as previously described (33) with some modifications. Briefly, the left chest of each anesthetized mouse was incised (5 mm incision) just below the inferior border of the scapula, and 20 μL of suspension containing 1 × 10^7 VMRC-LCD cells and 20 μg of Matrigel were injected into the left lung parenchyma through the intercostal space. The skin incision was closed with 3-0 silk. Mice were sacrificed on days 14, 28, and 42 after tumor cell implantation. Tumors were examined in lung, mediastinal lymph node, liver, kidney, and spleen and were weighed.

**Histologic examination.** The sacrificed mice were examined and then the tumors in the lungs and mediastinal lymph nodes were removed and weighed. After careful macroscopic examination, tumors were fixed with 10% formalin, embedded in paraffin, cut into 4-μm sections, and then stained with H&E.

**Effect of Y27632 on VMRC-LCD cell growth.** Cells were cultured with or without 100 μmol/L Y27632 and with 10% FBS or 0.1% BSA. Cell growth was analyzed by MTT assay.

**Figure 2. In vitro motility and invasion of LCD-vector (open columns) and LCD-E1AF (closed columns) cells with various concentrations of Y27632. Cells were incubated for 24 hours with various concentrations of Y27632, with or without HGF treatment (40 ng/mL), and the number of migrated cells was counted at a magnification of ×200. A and B, motility assay (A) and invasion assay (B) without HGF; C and D, motility assay (C) and invasion assay (D) with HGF. Columns, mean; bars, SD.**

**Figure 3. Effect of Y27632 on VMRC-LCD cell growth. Cells were cultured with or without 100 μmol/L Y27632 and with 10% FBS or 0.1% BSA. Cell growth was analyzed by MTT assay. ○, LCD-vector in FBS without Y27632; △, LCD-vector in FBS with Y27632; ●, LCD-E1AF in FBS without Y27632; ▲, LCD-E1AF in FBS with Y27632; ○, LCD-vector in BSA without Y27632; △, LCD-vector in BSA with Y27632; ●, LCD-E1AF in BSA without Y27632; ▲, LCD-E1AF in BSA with Y27632. Points, mean; bars, SD.**

**Figure 2. In vitro motility and invasion of LCD-vector (open columns) and LCD-E1AF (closed columns) cells with various concentrations of Y27632. Cells were incubated for 24 hours with various concentrations of Y27632, with or without HGF treatment (40 ng/mL), and the number of migrated cells was counted at a magnification of ×200. A and B, motility assay (A) and invasion assay (B) without HGF; C and D, motility assay (C) and invasion assay (D) with HGF. Columns, mean; bars, SD.
mice and anti–phosphorylated MLC antibody from rabbits. The primary antibodies were detected using antimouse or antirabbit antibody conjugated with horseradish peroxidase and visualized with the Amersham ECL system.

**Statistical analysis.** Statistical differences in the means were examined by Student’s unpaired two-tailed \( t \) test.

**Results**

**E1AF activates Rho in VMRC-LCD cells.** We have previously reported frequent overexpression of E1AF in NSCLC cell lines, except for VMRC-LCD and NCI-H226 cells, using Northern blot analysis and generation of E1AF-expressing VMRC-LCD and NCI-H226 cells (6). Western blot analysis using an anti-E1AF monoclonal antibody confirmed the presence of E1AF protein expression in LCD-E1AF and H226-E1AF cells and the absence of endogenous E1AF protein in LCD-vector and H226 vector cells (Fig. 1A). Endogenous E1AF protein expression was detected in A549 and NCI-H520 cells whereas it was weaker than that in LCD-E1AF and H226-E1AF cells.

To determine the effect of E1AF expression on Rho activity in VMRC-LCD, we measured the intracellular levels of GTP-bound (active) Rho using a pull-down assay system. We compared the activity of Rho in LCD-E1AF cells and LCD-vector cells with and without HGF treatment. As shown in Fig. 1B, the level of the GTP-bound (active) Rho was higher in LCD-E1AF cells than in LCD-vector cells. HGF enhanced the level of active Rho strongly in LCD-E1AF cells but had little effect in LCD-vector cells. Total Rho protein levels were similar under each condition in each cell line (Fig. 1B), showing that the activation was not caused by an increase in Rho expression.

We did the same experiments using H226-E1AF and H226-vector cells (Fig. 1C) as in LCD-E1AF and LCD-vector cells. H226-E1AF cells also showed higher active Rho levels than H226-vector cells in a HGF-enhanced manner whereas total Rho protein was similar between the cells, suggesting that the activation of Rho protein by E1AF and HGF without an increase in its expression is not due to variation in NSCLC cell lines.

**A Rho-associated kinase inhibitor decreases cell motility and invasion.** We next determined the effects of Y27632, a small-molecule inhibitor of ROCK. As in our previous report (6), LCD-E1AF cells showed significantly more motile and invasive activities than LCD-vector cells (motility, \( P = 0.04 \); invasion, \( P = 0.04 \); Fig. 2A and B) especially under HGF treatment (motility, \( P = 0.009 \); invasion, \( P = 0.009 \); Fig. 2C and D). Y27632 dose-dependently inhibited motility and invasion in both cell lines and at high Y27632 concentration, these activities became similarly low (Fig. 2), indicating that Y27632 decreased the motile and invasive activities more in LCD-E1AF cells than in LCD-vector cells.

To assess whether Y27632 was toxic, MTT assays were done with or without 10% serum. As shown in Fig. 3, Y27632 at 100 \( \mu \)mol/L, the highest concentration used in Fig. 2, did not affect the growth of either VMRC-LCD cell line with or without serum.

**E1AF increases phosphorylation of myosin light chain whereas a Rho-associated kinase inhibitor decreases its phosphorylation.** To analyze the effect of E1AF on ROCK activity in VMRC-LCD cells, we measured the phosphorylation of MLC.

### Table 1. Tumorigenic and metastatic activities of LCD-vector and LCD-E1AF cells after s.c. and intrapulmonary implantation in nude mice

<table>
<thead>
<tr>
<th>S.c. implantation (day 56)</th>
<th>Intrapulmonary implantation (day 42)</th>
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<tr>
<td>Primary*</td>
<td>Metastasis*</td>
</tr>
<tr>
<td>LCD-vector</td>
<td>1/7 (0.23 ± 0.62 g)</td>
</tr>
<tr>
<td>LCD-E1AF</td>
<td>6/6 (4.73 ± 3.29 g)</td>
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*Data are shown as no. of mice with tumor/no. of total mice (tumor weight per mouse shown as mean ± SD).

\( P < 0.01, \) LCD-E1AF versus LCD-vector.

\( P < 0.05, \) LCD-E1AF versus LCD-vector.

Metastatic site of all three mouse was mediastinal lymph node.
a downstream effector of ROCK, using Western blot analysis. As shown in Fig. 4, phosphorylated MLC was more abundant in LCD-E1AF cells than in LCD-vector cells. HGF strongly enhanced the phosphorylation of MLC in LCD-E1AF cells but not in LCD-vector cells. Y27632 inhibited the phosphorylation of MLC induced by E1AF and HGF to an undetectable level.

**E1AF increases tumorigenic and metastatic activities in association with phosphorylated myosin light chain.** The tumorigenicity and metastasis potential of LCD-E1AF and LCD-vector cells was investigated by injection into nude mice. Eight weeks after i.v. injection of LCD-E1AF or LCD-vector cells, no tumor was observed in any organs (data not shown). After s.c. injection, all of six mice injected with LCD-E1AF cells and one of seven mice injected with LCD-vector cells had local tumors in flank subcutis 56 days after transplantation (Table 1). The s.c. tumors of LCD-E1AF cells were significantly heavier than those of LCD-vector (4.73 ± 3.29 versus 0.23 ± 0.62 g, \( P < 0.005 \)). No metastatic tumors were observed in any organs from either cell line. When intrapulmonarily injected, LCD-E1AF and LCD-vector cells formed local tumors in the lung in four of seven and one of seven nude mice, respectively, 42 days after transplantation (Table 1). Each tumor-bearing mouse had one lung tumor. Lung tumors of LCD-E1AF cells were significantly heavier than those of LCD-vector (66 ± 65 versus 9 ± 23 mg, \( P < 0.05 \)). Metastatic tumors from LCD-E1AF cells were observed in mediastinal lymph nodes in three of the four mice with lung tumors at 42 days (Fig. 5A). The number of mediastinal metastasis was three for one mouse at 42 days and one for the other three mice. The mean tumor weight was 71 ± 92 mg at 42 days after transplantation. Histopathologic examinations confirmed adenocarcinoma occupying the mediastinal lymph node (Fig. 5B). In contrast, there was no metastasis in mediastinal lymph nodes from the mouse with lung tumors of LCD-vector cells. There were no metastases from tumors of either cell line in the other organs.

To test whether Rho/ROCK signal is activated by E1AF in vivo, we examined the phosphorylation of MLC in tumors developed from LCD-E1AF and LCD-vector cells. By Western blot analysis (Fig. 5C), phosphorylated MLC was much more abundant in s.c., lung, and mediastinal lymph node tumors from LCD-E1AF cells than in s.c. and lung tumors from LCD-vector cells.

**Discussion**

This is the first study showing that E1AF overexpression results in the activation of the Rho/ROCK signaling pathway. HGF accentuated this activation strongly. The inhibition of cell migration and invasion by a ROCK inhibitor suggests that Rho/ROCK activation is necessary for cell migration and invasion induced by E1AF. Furthermore, we have shown an increase in tumorigenesis and metastasis caused by E1AF expression, which also results in phosphorylation of MLC, a downstream target of Rho/ROCK signaling.

We have previously shown that E1AF increases migration and invasion of cancer cells and that the increased invasion is associated with E1AF-induced expression of MMPs and urokinase plasminogen activator (uPA; refs. 2, 4–6). In this study, we have shown abundant GTP-bound Rho in E1AF-transfected cells, suggesting that another mechanism, the activation of Rho, may be involved in migration and invasion enhanced by E1AF. Extensive studies have shown that increased motility and invasion induced by Rho are mediated through its downstream molecules, ROCK and MLC (15–18); MLC phosphorylation was shown in E1AF-transfected cells in the present study. Furthermore, a specific ROCK inhibitor, Y27632, inhibited cell migration and motility and decreased phosphorylation of MLC, suggesting that activation of Rho/ROCK signaling is necessary for E1AF-induced migration and invasion.

The Rho/ROCK signaling pathway has been shown to be involved in motility and invasion of various cancer cell lines, such as rat
hepatoma cells (20), human glioma cells (21), and human ovarian cancer cells (22). Studies using clinical specimens showed a relationship between the expression level of RhoC, an isoform of Rho, and tumor aggressiveness in breast cancer (23–25) and pancreatic cancer (26). Recently, NSCLCs have also been shown to express RhoC mRNA and protein at higher levels than do nontumor tissues, and the RhoC expression level is correlated to vascular permeation (27). Taken together with the accumulating data showing association of Rho/ROCK signaling with migration and invasion, the present results suggest that the Rho/ROCK signaling has a central role in migration and invasion enhanced by E1AF in NSCLC cells.

E1AF has previously been correlated with altered expression of several genes, including uPA, MMP, and another Ets family gene, Ets-1 (2, 6). LCD-E1AF cells were found to overexpress Ets-1 and the kinetics of HGF-mediated activation of Ets-1 is better correlated with uPA level than with E1AF level (6). This may suggest that the biological changes in the cell line may be mediated by altered expression of Ets-1 or possibly other Ets family members. All of ~30 Ets family transcription factors bind GGAA/T core sequence with some specificity depending on flanking sequences, suggesting their redundant functions (34). To determine their involvement in the phenotypes of E1AF-overexpressing cells, experiments with small interfering RNA of Ets-1 and other Ets family members possibly overexpressed in the cells will be required.

To our knowledge, the relationship of not only E1AF but also of other Ets family members, including Ets-1, with the activation of Rho has not been previously reported. Although E1AF and Ets-1 are transcription factors, their expression did not increase total Rho protein, suggesting that the activation of Rho is not caused by their transcriptional regulation. Rho activity is regulated by guanine nucleotide exchange factors (GEF) and GTPase-activating proteins (GAP; ref. 35). Possible mechanisms for the Rho activation by E1AF include transcriptional regulation of GEFs and GAPs directly by E1AF or indirectly by potential mediators like Ets-1. A growing number of GEFs and GAPs for the Rho family have been identified, which by now include 69 Db1-related GEF proteins, the largest family of RhoGEP, and more than 30 RhoGAPs (36, 37). To determine whether the activation of Rho by E1AF involves such mechanisms, comprehensive analyses including microarray analysis should be investigated in the near future.

The detection of endogenous E1AF protein in A549 and NCI-H520 cells is consistent with our previous study showing the E1AF mRNA expression in these cells and suggests roles of the endogenous E1AF protein in NSCLC cell lines. Loss of function studies of E1AF in the E1AF-expressing cell lines would clarify the roles of E1AF in the observed phenotypes, including Rho signaling activation, especially in a physiologic context.

The tumorigenicity potential of E1AF is indicated by the observed increase in incidence and weight of tumors in the skin and lung of nude mice developed from E1AF-transfected cells compared with vector-transfected cells. Tumorigenesis by E1AF has been investigated for breast tumors. E1AF is overexpressed in the vast majority of human breast cancers and in nearly all HER2/Neu–positive tumors (38). Using MMTV-Neu transgenic mice, Shepherd et al. (8) showed that expression of a dominant-negative E1AF transgene under the control of the MMTV promoter in mammary epithelial cells dramatically delayed the onset of mammary tumors and reduced the number and size of such tumors in individual mice. On the other hand, Xing et al. (39) reported that E1AF suppresses HER2 promoter activity in human tumor-derived cell lines, dependent on an E1AF binding site in this promoter, and inhibits tumorigenesis of the cells. However, several articles have shown that E1AF can activate E1AF-responsive promoters, including the human HER2/neu promoter in various cell types (3, 8, 40–42). Bojovic and Hassell (43) noted that the expression of these same genes is inhibited when E1AF protein is expressed at very high levels and suggested that transcriptional squelching accounts for these observations. Thus, both our results and observations in breast cancer indicate the tumorigenic activity of E1AF but this activity may depend on various conditions including cell type.

The development of mediastinal lymph node metastases from lung tumors of E1AF-transfected cells, but not from the lung tumor of vector-transfected cells, suggests that E1AF may enhance metastasis of NSCLC cells. These observations are consistent with previous reports showing that E1AF increases in vivo metastatic activities of fibrosarcoma cells (7) and breast cancer cells (44). Highly phosphorylated MLC in the local and metastatic tumors developed from E1AF-transfected cells, suggesting an association between E1AF tumorigenic and metastatic activities and the increased activity of the Rho/ROCK pathway. Rho has been shown to have transforming activities (13, 14, 24) and may have some role in the observed tumorigenesis by E1AF. The association of Rho/ROCK signaling with metastasis is also consistent with recent reports showing that RhoC enhances metastasis of melanoma cells (28) and lung cancer cells (29) in association with migration and invasion.

In conclusion, these results suggest that E1AF induces activation of the Rho/ROCK pathway, which plays an important role for malignant phenotypes including motility and invasion as well as tumorigenesis and metastasis enhanced by E1AF in NSCLC cells. Inhibition of this pathway by molecules such as Y27632 may have therapeutic potential to control NSCLCs, most of which overexpress E1AF.

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References


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