The Novel Gene EG-1 Stimulates Cellular Proliferation

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Abstract

We recently discovered a novel gene and named it endothelial-derived gene 1 (EG-1). Previously, we have shown that the expression of EG-1 is significantly elevated in the epithelial cells of breast cancer, colorectal cancer, and prostate cancer. Here, we report that EG-1 can stimulate cellular proliferation. Transfection experiments which overexpressed the full-length EG-1 gene in human embryonic kidney HEK-293 cells or human breast cancer cell lines resulted in significantly increased in vitro proliferation, in comparison with transfection with empty vectors. On the other hand, small interfering RNA cotransfection resulted in inhibition of proliferation. S.c. xenograft assays were carried out in a severe combined immunodeficient mouse model. We found that injection of high EG-1 expressing HEK-293 clones resulted in significantly larger tumors, in comparison with clones carrying the empty vectors. To further clarify the function of this gene, we investigated its interaction with Src and members of the mitogen-activated protein kinase (MAPK) family. Immunoprecipitation with anti-Src antibody, followed by immunoblotting with anti–EG-1 antibody, showed an association between these two molecules. Overexpression of EG-1 was correlated with activation of the following kinases: extracellular signal-regulated kinases 1 and 2, c-jun-NH2-kinase, and p38. These observations collectively support the hypothesis that the novel gene EG-1 is a positive stimulator of cellular proliferation, and may possibly be involved in signaling pathways involving Src and MAPK activation. (Cancer Res 2005; 65(14): 6159-66)

Introduction

Cancer is a major cause of morbidity and the second leading cause of death in the American population. Several major oncogenes and tumor suppressor genes have been identified to contribute to the neoplastic transformation of epithelial cells. These include src, p53, c-myc, ras, Rb (retinoblastoma), BRCA-1 and BRCA-2 (breast cancer susceptibility genes), Her-2, cyclin D1, and PTEN (phosphatase and tensin homologue; ref. 1). Other alterations in the cell, such as DNA methylation, contribute to the overall genetic instability, and abnormal maintenance of telomeres results in replicative immortality (2).

Another important biological phenomenon in the tumorigenic and metastatic phenotype involves the process of angiogenesis. Three decades of experimental evidence has shown that the growth and metastasis of solid tumors is dependent on their ability to initiate and sustain new capillary growth (i.e., angiogenesis; ref. 3). Multiple clinical observations in human cancer have added support to the hypothesis that tumors are angiogenesis dependent. The number of vessels in a tumor specimen correlates with the disease stage and can add prognostic value independent of other routinely used markers (4). Furthermore, the levels of various angiogenic factors in bodily fluids have been shown to correlate with prognosis in cancer patients (5–7). Many agents have been developed to inhibit tumor angiogenesis, and there have been reports of some encouraging results (8, 9).

Several researchers, including our laboratory, have investigated the difference between molecules of the proliferating tumor endothelium and those of the normal quiescent endothelium (10, 11). To closely mimic a tumor environment, we have attempted to identify endothelial gene products expressed in response to a mixture of growth factors found in tumor-conditioned media. Toward this goal, we used a subtraction hybridization method called suppression subtractive hybridization (12). In human umbilical vein endothelial cell populations exposed to conditioned media from human cancer cells (13) for 4 hours, we have isolated multiple clones (14, 15). One of these differentially expressed genes is endothelial-derived gene 1 (EG-1; ref. 16). In addition to its expected presence in blood vessels, EG-1 expression is significantly elevated in the epithelial cells of several cancers including breast, colorectal, and prostate cancer (17). In this article, we present in vitro and in vivo data which suggest that EG-1 stimulates cellular proliferation and may be involved in signaling pathways involving Src and mitogen-activated protein (MAP) kinase (MAPK) activation.

Materials and Methods

Cell culture. Human embryonic kidney HEK-293 cells and the human breast cancer cell lines MCF-7 and MDA-MB-231 were purchased from American Tissue Type Culture Collection (Rockville, MD). The cells were maintained in RPMI (Invitrogen, Carlsbad, CA) with 10% heat-inactivated FCS, 100,000 units/L penicillin, and 100 mg/L streptomycin at 37°C in 5% CO2.

Proliferation assay. The cells were plated onto 48-well culture plates at 10,000 cells/well and incubated at 37°C in 5% CO2 for 48 hours in DMEM with 10% FCS. On the third day, 1 μCi of [methy13H]thymidine (Amersham, Piscataway, NJ) was added to each well. Approximately 15 hours later, the plates were washed with PBS. The cells were fixed with trichloroacetic acid, washed with ethyl alcohol, and lysed with sodium hydroxide. After adding glacial acetic acid, the radioactivity of the cell lysates was counted in scintillation solution (Searl/Verse, Fisher, Pittsburgh, PA). The in vitro assays were done in triplicates. Certain experiments were carried out with MAPK inhibitors (PD98059, SB203580, and U0126) purchased from Calbiochem (La Jolla, CA). The cells were treated with 10 μmol/L of one of the above inhibitors for 24 hours before harvest.

Transfection. We used the pcDNA3.1D/V5-His-TOPO vectors (Invitrogen) to carry the full-length human EG-1 gene according to the instructions of the manufacturer. Empty vectors were used as negative controls. Specifically, standard calcium-phosphate DNA coprecipitation was used for

Note: M. Lu and L. Zhang contributed equally to the work.

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obtaining stable transfectants. Individual clones were selected for Geneticin (Invitrogen) resistance over a period of several weeks. Expression of the EG-1 gene by individual clones was confirmed by Northern and Western blot analyses.

For transient transfection, we used the pcDNA3.1/D/V5-His-TOPO and pShuttle-IREs-hrGF-1 (Strategene, La Jolla, CA) vectors to carry the full-length EG-1 gene. Empty vectors were used as negative controls. Liposomal reagents were used to transfect the pcDNA3.1/D/V5-His-TOPO vectors into cells using the MBS Mammalian Transfection Kit according to the protocol of the manufacturer (Strategene).

**Small interfering RNA.** EG-1 expression knockdown was achieved by transfecting a lentivirus vector expressing a small interfering RNA (siRNA) against EG-1, cis-linked with a green fluorescent protein expression cassette, into HEK-293 cells or breast cancer cells. The pcSCEG plasmid (U6-shRNA-EG1-CMV-GFP) was constructed by ligating the BamHI/EcoRI digests of pCSG and the U6-shRNA-EG1 PCR product. The U6-shRNA-EG1 PCR was done using a hU6-containing plasmid at an annealing temperature of 60°C with the primers 5'-GGGGGATCCCAAGTTCCGGGGAGGAAGGGCCTATTCCC-3' for siRNA1, 5'-GGGGGATCCCAAGTTCCGGGGAGGAAGGGCCTATTCCC-3' for siRNA2, 5'-GGGGGATCCCAAGTTCCGGGGAGGAAGGGCCTATTCCC-3' for siRNA3, and 5'-GGGGGATCCCAAGTTCCGGGGAGGAAGGGCCTATTCCC-3' for siRNA4. Each siRNA was transfected into HEK-293 cells using the MBS Mammalian Transfection Kit according to the protocol of the manufacturer (Strategene).

**Western blot analysis.** Cell pellets were lysed in preheated 0.025 mol/L Tris (pH 7.4), 0.001 mol/L EDTA, and 0.3% SDS, and then boiled for 5 minutes. The cell lysate was centrifuged at 12,000 × g for 10 minutes, and the supernatant was saved. Protein concentration was measured by the Bradford assay (Bio-Rad, Hercules, CA). For Western blot analysis, ~40 μg of protein were separated by a 12% SDS-PAGE gel, and transferred to a nitrocellulose membrane by electrophoretic blotting. The membrane was blocked overnight (4°C) with 5% nonfat dry milk in TBS-0.1% Tween 20, and then incubated with a 1:500 dilution of EG-1 antisera for 2 hours. The blots were then washed thrice over 30 minutes in TBS-Tween 20, and incubated for 1 hour with horseradish peroxidase–conjugated secondary antibody goat anti-rabbit IgG (1:10,000), and then washed in TBS-Tween 20 as before. The membranes were then developed using the Supersignal Western Chemiluminescent Western blotting detection system according to the instructions of the manufacturer (Pierce, Arlington Heights, IL).

**Immunoprecipitation.** Cells lysates were preincubated solely with protein A/G Plus-Agarose (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C for 1 hour, and the mixture was centrifuged at 3,000 × g for 5 minutes to pellet these beads and any nonspecific interacting proteins. One milligram of supernatant protein was incubated with anti–EG-1 antibody and 30 μl of protein A/G Plus-Agarose overnight at 4°C under agitation, and 1 mg of proteins from the same source was incubated with normal rabbit IgG (Santa Cruz Biotechnology) and protein A/G Plus-Agarose (for negative controls). After incubation, immunocomplexes were pelleted by centrifugation at 3,000 × g for 5 minutes at 4°C. The pellets were then resuspended and washed three additional times with immunoprecipitation buffer to remove nonspecific interactions. Laemmli loading buffer was then added to the beads. After boiling, the proteins were separated by 12% SDS-PAGE and analyzed by Western blot.

**Size exclusion chromatography-mass spectrometry.** Anti-FLAG M2 affinity gel (Sigma) was used to purify recombinant FLAG-tagged EG-1 peptide from transiently transfected HEK-293 cells, following the protocol of the manufacturer. Briefly, cell lysates were collected and loaded onto the column under gravity flow. The column was washed and then eluted with seven 1-mL aliquots of 0.1 mol/L glycine-HCl at pH 3.5 into vials containing 25 μL of 1 mol/L Tris (pH 8.0). The vial with the highest concentration of the EG-1 protein (vial 2) was subjected to mass spectrometry (MS).

The protocol of Whitelegge et al. (18) was used to separate the EG-1 fraction by size exclusion chromatography before analysis in a mass spectrometer (size exclusion chromatography-MS). Approximately 10 μg of EG-1 suspended in 0.1 mol/L glycine-HCl at pH 7.0 were dried down in vacuo (SpeedVac) and then resuspended in 100 μL of 90% formic acid immediately before size exclusion chromatography-MS. The size exclusion chromatography was done using a mobile phase of CHCl3/methanol/1% aqueous formic acid (4:1, v/v/v) and a Super SW 2000 column (4.6 × 300 mm, Tosoh Bioscience, Montgomeryville, PA) at 250 μL/min and 40°C. Before delivery to the electrospray-ionization source, the column effluent was monitored with a UV detector (280 nm). Electrospray ionization MS was done using a triple quadrupole instrument (API III, Applied Biosystems) tuned and calibrated as described (19). Data were processed using MacSpec 3.3, Hypermass, and BioMultiview 1.3.1 software (Applied Biosystems).

**Microfluidic chromatography with tandem mass spectrometry.** After the EG-1 peptides were eluted from the FLAG column, we confirmed the presence and purity of EG-1 by Western blotting. Then the sample was subjected to 12% SDS-PAGE, and the gel was stained by SYPRO Ruby Protein Gel Stain (Molecular Probes, Eugene, OR) following the manual from the manufacturer. Subsequently, the EG-1 bands were excised and dehydrated in acetone for 3 minutes, and dried completely by SpeedVac. DTT (10 mmol/L) dissolved in 100 mmol/L NH4HCO3 was added to the sample, which was then incubated for 1 hour at 56°C. The liquid was removed, and 55 mmol/L iodoacetamide dissolved in 100 mmol/L NH4HCO3 was added for 45 minutes at room temperature in the dark. The sample was washed in 100 mmol/L NH4HCO3 for 10 minutes, dehydrated in acetonitrile for 30 minutes, followed by swelling in
100 mmol/L NH₄HCO₃ for 30 minutes, dehydrated again in acetonitrile for 30 minutes, and finally dried completely by SpeedVac. Then, the sample was digested in trypsin solution (50 mmol/L NH₄HCO₃, 5 mmol/L CaCl₂, 12.5 ng/µL trypsin) on ice for 45 minutes. The liquid was then removed, and the sample incubated in the same solution without trypsin at 37°C overnight. On the following day, the sample was washed with 20 mmol/L NH₄HCO₃ then the extraction of the EG-1 peptides was carried out with 5% formic acid and 50% acetonitrile for 20 minutes, and repeated twice. The sample was extracted once with acetonitrile for 20 minutes. All the postigestion extractions were pooled together and dried down by SpeedVac.

Samples were analyzed by microliquid chromatography with tandem mass spectrometry (micro-LC-MS/MS) with data-dependent acquisition (LCQ-DECA, ThermoFinnigan, San Jose, CA) after dissolution in 5 µL of 70% acetic acid (v/v). A reverse-phase column (200 µm × 10 cm; PLRP/S, 5 µm, 300 Å; Michrom Biosciences, San Jose, CA) was equilibrated for 10 minutes at 1.5 µL/min with 95% A, 5% B (A, 0.1% formic acid in water; B, 0.1% formic acid in acetonitrile) before sample injection. A linear gradient was initiated 10 minutes after sample injection ramping to 60% A, 40% B after 50 minutes and 20% A, 80% B after 65 minutes. Column eluent was directed to a coated glass electrospray emitter (TaperTip, TT150-50-50-CE-5, New Objective) at 3.3 kV for ionization without nebulizer gas. The mass spectrometer was operated in “triple-play” mode with a survey scan (m/z 400-1,500), data-dependent zoom scan and MS/MS. Individual sequencing experiments were matched to a custom protein sequence database using Sequest software (ThermoFinnigan).

Statistical analysis. Descriptive statistics, such as mean and SE, were used to summarize the results. The ANOVA test was done for comparison among the various groups followed by the Bonferroni posttest. The Student’s t test was used for comparison between only two groups. Statistical significance is defined by P < 0.05.

Results

The EG-1 gene and peptide. A BLASTN search for sequence homology done in the GenBank database revealed that EG-1 has no significant homology to any gene with a known function. The homology of the human EG-1 peptide to its mouse counterpart is 94.9%, and 95.5% to its rat counterpart. The homology between mouse and rat EG-1 is 98.9%. A Profile Scan search revealed a proline-rich region in the NH₂ terminus (Fig. 1A and B). Of interest, we have found similar sequences to EG-1 in several insects, but these seem to lack the NH₂-terminal polyproline region.

Western blot analysis showed that the transfected full-length EG-1/FLAG exists primarily as a 22 kDa protein, with possible degradation peptide products (Fig. 1C and D). It should be noted...
that our transfectant EG-1 product contains at the NH₂ terminus 15 additional amino acids containing the FLAG tag (MDYKDDDDKNSAGSNMAAPLGGMFSGQPPQAPPGLPGQASLLQAAPGAPRPSSSTLV-

...sequence. These siRNAs were cotransfected with a full-length cDNA of EG-1 into HEK-293 cells. The proliferation results are as follows: 34,847 ± 2,060 cpm in vector-transfected cells; 26,892 ± 801 cpm in cells transfected with siRNA1; 25,785 ± 970 cpm in cells transfected with siRNA2; and 12,548 ± 12 cpm in cells transfected with siRNA3 (Fig. 2C). ANOVA analysis shows that the inhibitory effect exerted by siRNA3 is significant in comparison with that by vector alone (P < 0.001). Whole-cell lysates were collected 24 hours after transfection, subjected to SDS-PAGE, then immunoblotted with anti-EG-1 antibody. This analysis showed minimal levels of EG-1 peptide in cells cotransfected with siRNA3 (Fig. 2D).

Overexpression of EG-1 stimulates breast cancer cell proliferation. We transiently transfected a full-length cDNA of EG-1 carrying a FLAG tag into the human breast cancer cell lines MCF-7 and MDA-MB-231. Subsequent experiments showed that the EG-1–transfected MCF-7 cells have increased proliferation (9,613 ± 694 cpm) in comparison with the ones transfected with empty vectors (7,213 ± 252 cpm). Cotransfection with EG-1 and siRNA3 abrogated the increased proliferation (5,947 ± 264 cpm, P = 0.0002; Fig. 3A). The same phenomenon was observed with MDA-MB-231 cells transfected with empty vector (34,651 ± 3,756 cpm), with EG-1 (61,605 ± 2,288 cpm), and with EG-1 and siRNA3 combination (34,197 ± 429 cpm, P < 0.0001; Fig. 3C). Figure 3B and D shows the corresponding levels of EG-1 peptide in these cells, as shown with Western blot analysis of cell lysates.

EG-1 overexpression increases the tumorigenicity of mouse xenografts. We next examined the effect of EG-1 on the tumorigenicity of permanently transfected and wild-type HEK-293 cells in SCID mice. One group of mice was injected s.c. in the

<table>
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<th>Table 1. EG-1 peptides identified by MS/MS</th>
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<tr>
<td><strong>Sequence</strong></td>
</tr>
<tr>
<td>K.KPAPDIPQGLAYLEQASANIPALKPT.</td>
</tr>
<tr>
<td>K.PAPDIPQGLAYLEQASANIPALKPT.</td>
</tr>
<tr>
<td>K.RLIQSLVQKPEVQIK.E</td>
</tr>
<tr>
<td>R.LQSLVQKPEVIKEDVSEL.R.N</td>
</tr>
<tr>
<td>K.KPAPDIPQGLAYLEQASANIPALKPT.</td>
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<tr>
<td>K.RLIQSLVQKPEVQIK.E</td>
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<tr>
<td>K.RLIQSLVQKPEVQIK.E</td>
</tr>
<tr>
<td>R.LQSLVQKPEVQIKEDVSEL.R.N</td>
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<td>R.LQSLVQKPEVQIK.E</td>
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<td>R.QTECFFLQK.R</td>
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<td>K.EVSELNELQ.R.K</td>
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<tr>
<td>R.LQSLVQKPEVQIK.E</td>
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<tr>
<td>K.FLDIA.RQ</td>
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*aSequence of EG-1 peptide matched to tandem mass spectrum.

¹Numbered according to complete FLAG-tagged sequence; MDYKDDDDKNSAGSNMAAPLGGMFSGQPPQAPPGLPGQASLLQAAPGAPRPSSSTLV-DELELESSFEACFASLVQSQYDYNQ1ETQGVQ1QKFLDIAQRQTECFFLQKRQLSVQKPEVQIKEDVSELRELQLKDVALVQKHTKLRHWWQ-VQLEDNVQHKPKAPDIPQGLAYLEQASANIPALKPT.

²For the peptides highlighted in bold, a cross correlation coefficient (XC) of >2.55 for a 1+ charge state, 3.39 for a 2+ charge state, and 3.78 for a 3+ charge state or greater is considered a highly significant match. Lower scoring matches are included because they are tryptic peptides identified without specifying this option in the search.

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flank with wild-type HEK-293 cells, a second group with a stably transfected empty vector clone, and the third group with one EG-1–overexpressing clone. There were four mice per group. At day 30, the xenograft tumor sizes are as follows: 1,204 mm$^3$ in the wild-type group, 899 mm$^3$ in the empty vector group, and 1,956 mm$^3$ in the EG-1 vector group (Fig. 4A). ANOVA analysis shows that the stimulatory effect exerted by EG-1 transfection in the xenografts is significant in comparison with that by vector alone ($P < 0.01$) as well as with that by wild-type ($P < 0.05$). This phenomenon was similarly observed in other xenograft experiments using other stable clones generated in our laboratory (Fig. 4C; $P < 0.0001$).

To examine the expression of the EG-1 product in mouse tumors, multiple xenograft samples were analyzed by immunohistochemistry using the anti–EG-1 antibody. The histologic slides were reviewed by a Board-certified pathologist (J.Y.R). Figure 4B shows a representative slide prepared from a vector-transfected tumor, and a slide from an EG-1–transfected tumor. The EG-1–transfected xenografts seem to express a higher amount of the EG-1 peptide, in comparison with those transfected with empty vector.

**EG-1 overexpression influences certain kinase pathways.** Western blot analysis of cell lysates showed that the phosphorylated p44/42 MAP kinase level is elevated in EG-1–transfected HEK-293 cells in comparison with those transfected with empty vector.

![Figure 2](image-url)

**Figure 2.** A, proliferation of HEK-293 cells. Lane 1, wild-type; lane 2, permanent clone of HEK-293 cells transfected with empty vector; lane 3, permanent clone of HEK-293 cells transfected with EG-1. Columns, mean expressed in counts per minute; bars, SE. $P < 0.001$. B, one milligram of the above cell lysate proteins was immunoprecipitated with anti–EG-1 antibody overnight, then immunoblotted with the same antibody. C, proliferation of HEK-293 cells transfected with EG-1. Lane 1, cotransfected with empty vector; lane 2, cotransfected with siRNA1; lane 3, cotransfected with siRNA2; lane 4, cotransfected with siRNA3. Columns, mean expressed in counts per minute; bars, SE. $P < 0.001$. D, Western blot analysis of EG-1 expression of the above cell lysates.

![Figure 3](image-url)

**Figure 3.** Proliferation of MCF-7 (A) and MDA-MB-231 (C) human breast cancer cells. Lane 1, cells transfected with empty vector; lane 2, cells transfected with EG-1; lane 3, cells transfected with both EG-1 and siRNA3. Columns, mean expressed in counts per minute; bars, SE. $P < 0.001$. Western blot analysis of EG-1 expression of the above cell lysates compared with purified EG-1 peptide positive control. B, MCF-7; D, MDA-MB-231.
The phosphorylated forms of the JNK (Fig. 5B) and the p38 kinase (Fig. 5C) are similarly increased in EG-1–overexpressing cells. Of note, we also observed similar changes in phospho-JNK levels in MCF-7 cell experiments, and in p44/42 MAPK levels in MDA-MB-231 cell experiments (data not shown). We further investigated the proliferation of EG-1–transfected HEK-293 cells when treated with the following MAPK inhibitors: PD98059 (inhibits MAPK), SB203580 (inhibits p38), and U0126 (inhibits p44/42 MAPK).

Figure 5. Western blot analysis of kinase expression in HEK-293 cell lysates. Equivalent amounts of protein were loaded per lane. Lane 1, permanent clone of HEK-293 cells transfected with empty vector; lane 2, permanent clone of HEK-293 cells transfected with EG-1. Expression of phosphorylated versus nonphosphorylated p44/42 MAP kinase (A), JNK (B), and p38 kinase (C).

D, MAPK inhibitors block the increase of proliferation by EG-1 overexpression in HEK-293 cells. Columns, mean expressed in counts per minute; bars, SE. Cells were wild type (WT), transfected with empty vector (EV), or transfected with EG-1 (NT). P = 0.015. In the group with EG-1 transfection, cells were either not treated (NT), or treated with vehicle (DMSO) or MAPK inhibitors (PD98059, SB203580, and U0126; 10 μmol/L). P < 0.05.
EG-1 overexpression in HEK-293 cells (Fig. 5; MAP/extracellular signal-regulated kinase (ERK) kinase 1 and 2). Equivalent amounts of cell lysate proteins were immunoprecipitated with an anti-Src antibody (lanes 1 and 2). Lane 3, negative control, in which normal mouse antibody was used to immunoprecipitate an equivalent amount of the same cell lysate of transiently EG-1–transfected HEK-293. Lane 4, positive control, in which an equivalent amount of the same cell lysate was immunoprecipitated with anti-EG-1 antibody. Subsequently, all lanes were blotted with anti-EG-1 antibody.

Western blot analysis of EG-1 expression in HEK-293 cell lysates. Figure 6.

to c-Src (Fig. 6). The anti–EG-1 antibody, and discovered that EG-1 does indeed bind to c-Src (Fig. 6), transfect HEK-293 cells with either empty vector or with EG-1 plasmid, and prepared cell lysates. We then immunoprecipitated c-Src with anti-c-Src antibody, followed by Western blotting using the anti–EG-1 antibody, and discovered that EG-1 does indeed bind to c-Src (Fig. 6).

Discussion

We report here that the novel gene EG-1 can stimulate cellular proliferation. Overexpression of EG-1 in transfected cells resulted in a marked increase of in vitro proliferation. On the other hand, siRNA cotransfection resulted in inhibition of proliferation. In vivo, mouse xenograft models showed that EG-1 transfecteds had a growth advantage as evidenced by the formation of larger s.c. tumors. Because cellular proliferation is an important component of the malignant phenotype, the current observations are consistent with our previous reports on the expression profile of this novel gene. We have shown in our first publication that the expression of EG-1 is significantly elevated in cancerous compared with benign epithelial cells, as seen in Northern blot analyses (16). Subsequent immunohistochemical studies of several human pathologic specimens confirmed our hypothesis that EG-1 is associated with the malignant phenotype of the common epithelial-derived cancers of the breast, colon, and prostate (17).

Our present observations are unique in part because EG-1 is a novel gene with completely unknown function, which was discovered in our laboratory 2 years ago. The fact that this gene is highly conserved in mammals suggests that it may serve an important and fundamental role in cellular biology. The EG-1 protein product is unique because we could not find any homology of its tryptic peptides with any known proteins in the database. Thus, the major finding is that a unique and novel gene is a positive stimulator of cellular proliferation. To investigate possible mechanisms for the stimulatory activity of EG-1, we analyzed the well-known MAPK family kinase signaling pathway which has been shown to be crucial in promoting cellular proliferation (20). We used the stable clones that overexpress EG-1, and compared their MAPK activity with that expressed by stable clones carrying only empty vectors. By Western blot analysis, we found that EG-1 overexpression was correlated with activation of the following MAP kinases: ERK-1 and -2, JNK, and p38. Further work is needed to study the network involved in this interaction, and to determine whether the MAPK pathway really plays a role in EG-1–associated cellular proliferation.

A key to the understanding of cellular signal transduction pathways is to determine whether certain proteins of interest interact with one another. Protein-protein interactions are often mediated by noncatalytic and conserved domains (21). To further clarify the function of EG-1, we analyzed the sequence of this highly conserved gene. EG-1 has two tyrosines at positions 68 and 163. The presence of an NH2-terminal polyproline region suggests that EG-1 may interact with Src homology 3 domains. This led us to screen more than 100 proteins with Src homology 3 domains by domain array assays, in which we repeatedly observed an association between EG-1 and c-Src (data not shown). Immunoprecipitation with anti-Src antibody, followed by immunoblotting with anti–EG-1 antibody, showed an interaction between these two molecules. c-Src is a member of the Src family of cytoplasmic tyrosine kinases that regulate cell growth, differentiation, cell shape, migration, and survival (22). c-Src has been reported to be overexpressed and to play a role in human carcinomas of the breast, colon, and others (23). Src family tyrosine kinases are often activated by receptor tyrosine kinases, such as epidermal growth factor receptor or platelet-derived growth factor receptor (24). Further work is needed to elucidate the effects of the observed association between Src and EG-1.

The above observations collectively support the hypothesis that the novel gene EG-1 is a positive stimulator of cellular proliferation. Because cellular proliferation is an important component of the malignant phenotype, our present findings suggest that EG-1 may be an important target in the design of novel cancer therapies. In addition, we have now shown that EG-1 overexpression is potentially associated with the well-known MAP kinase family signaling pathway, which could underline the mechanism of the function of EG-1. Finally, we have observed that EG-1 forms protein-protein complexes with the Src tyrosine kinase, which is critical in the regulation of cell growth, differentiation, migration, and survival. The cross-talk between MAPK family and c-Src has been reported by several groups (25, 26). When c-Src is catalytically active, it is presumed to phosphorylate epidermal growth factor...
receptor (EGFR) monomers on tyrosine residues leading to EGFR transactivation. Tyrosine-phosphorylated EGFR forms complex with adapter proteins, including growth factor receptor binding protein 2-Sos, which activate Ras and the ERK pathway (27).

We think that these results will form the basis for further studies of this interesting gene, and the possible translation of the discovery of this molecule into potential use in cancer diagnosis and/or treatment.

References
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