EG-1 interacts with c-Src and activates its signaling pathway

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Abstract. EG-1 is significantly elevated in breast, colorectal, and prostate cancers. Overexpression of EG-1 stimulates cellular proliferation, and targeted inhibition blocks mouse xenograft tumor growth. To further clarify the function of EG-1, we investigated its role in c-Src activation. We observed that EG-1 overexpression results in activation of c-Src, but found no evidence that EG-1 is a direct Src substrate. EG-1 also binds to other members of the Src family. Furthermore, EG-1 shows interaction with multiple other SH3- and WW-containing molecules involved in various signaling pathways. These observations suggest that EG-1 may be involved in signaling pathways including c-Src activation.

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 c-Src, p53, c-myc, ras, Rb (retinoblastoma), BRCA-1 and -2 (breast cancer susceptibility genes), Her-2, cyclin D1, and PTEN (phosphatase and tensin homolog) (1).

We previously discovered a novel gene EG-1 that is induced in endothelial cells by tumor conditioned media (endothelial derived gene-1) (2). In addition to its expected presence in the endothelial cells of blood vessels, EG-1 expression is significantly elevated in cancer cells from several types of adenocarcinomas including breast, colorectal and prostate (3). We reported that overexpression of EG-1 stimulates cellular proliferation *in vitro* and increases xenograft size *in vivo* (4). In subsequent study, we have observed that EG-1 targeted inhibition significantly blocks human breast cancer cell proliferation *in vitro* and xenograft growth in mice (unpublished data). 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 non-catalytic and conserved domains (5). The presence of an N-terminal poly-proline region in the EG-1 sequence suggests that EG-1 might interact with SH3 (Src homology) and WW domains (6). This led us to screen EG-1 for binding to a large panel of SH3 and WW containing proteins, where we repeatedly observed an association between EG-1 and c-Src (cellular Rous Sarcoma viral oncogene).

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Materials and methods

Cell culture. Human embryonic kidney HEK-293 cells were purchased from American Tissue Type Culture Collection (ATCC, Rockville, MD). The cells were maintained in Dulbecco's minimal essential medium (DMEM, Invitrogen, Carlsbad, CA) with 10% heat-inactivated FCS (fetal calf serum), 100,000 units/l penicillin, and 100 mg/l streptomycin, at 37˚C in 5% CO_2.

Domain binding assay. Domain array assays were obtained from Panomics (Redwood City, CA). Four SH3-containing protein blots (TranSignal™ SH3) and two WW-containing protein blots (TranSignal WW) were used following the manufacturer's protocol. Briefly, histidine tagged EG-1 was overexpressed in HEK-293 cells by transient transfection. Then, EG-1 protein was affinity purified and incubated with SH3 or WW Domain Array membranes with gentle shaking for 2 h at room temperature. After incubation, the membranes were washed three times with wash buffer for 10 min each at room temperature. We then incubated the membrane with anti-histidine HRP conjugate for 1 h at room temperature. The membranes were washed three times again as above and visualized with HRP-based chemiluminescence detection. The blots were scanned and density was measured with Scion Image software (Scion Corp., Frederick, MD).

Transfection. We used the pcDNA3.1D/V5-His-TOPO vectors (Invitrogen) and pShuttle-IRES-hrGFP-1 (Stratagene, La Jolla, CA) vectors to carry the full-length human EG-1 gene, according to the manufacturer's instructions. Empty vectors were used as negative controls. Specifically, standard lipofectamine (Invitrogen) was utilized for obtaining transient transfectants according to the manufacturer's protocol. Expression of the EG-1 gene was confirmed by Western blot analyses. Twenty-four hours after transfection, cell lysates were harvested and subjected to IP (immunoprecipitation) or IB (immunoblotting).

Generation of antibodies. Polyclonal antibodies that recognize a specific peptide epitope on human EG-1 were generated by

Table I. EG-1 binds to SH3 (A) and WW (B) domains of many proteins.

A, Gene	Full name of gene	Fold
Tec	Tyrosine-protein kinase Tec	5.3
Yes1	Yamaguchi sarcoma virus oncogene homolog 1	4.8
c-Src	Cellular Rous Sarcoma viral oncogene homolog	4.5
OSF	Osteoclast stimulating factor 1	4.3
BTK	Bruton tyrosine kinase	3.9
SLK	Proto-oncogene tyrosine protein kinase FYN	3.9
ABL2	Abelson-related protein; Arg	3.8
PIG2	1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase γ 2	3.8
SNX9	Sorting nexin 9	3.4
NPH1	Juvenile nephronophthisis 1 protein	2.8
PSTPIP1	Proline-serine-threonine phosphatase interacting protein 1; CD2 tail-binding protein	2.7
DFKZp434D-D4	Similar to hypothetical protein; Domain no. 4	2.7
Stam	Signal transducing adaptor molecule	2.6
PI3ß	Phosphoinositide-3-kinase regulatory ß subunit	2.6
PI3α	Phosphatidylinositol 3-kinase regulatory α subunit	2.6
Tim	Rho guanine nucleotide exchange factor (GEF) 5	2.5
KIAA0418-D4	KIAA0418 gene product; likely ortholog of five SH3 domains; Domain no. 4	2.5
KIAA0790	KIAA0790 protein	2.5
PSD95	Presynaptic density protein 95	2.5
Hck	Hemopoietic cell kinase	2.5
NEBL	Nebulette	2.5

B, Gene	Full name of gene	Fold
MAGI-3-D1	Membrane-associated guanylate kinase-related MAGI-3, WW Domain no. 1	5.0
TAZ	Transcriptional co-activator with PDZ-binding motif (TAZ)	4.5
NEDD4L-D2	NEDD4-like ubiquitin ligase 3, WW Domain no. 2	4.4
MAGI-3-D2	Membrane-associated guanylate kinase-related MAGI-3, WW Domain no. 2	3.7
YAP1	Yes-associated protein 1, 65 kDa (YAP65)	3.2
NEDD4-D3	Ubiquitin-protein ligase Nedd-4, WW Domain no. 3	2.6

Quality Controlled Biochemicals (QCB, Hopkinton, MA). Pre-immune and immune sera were harvested. Polyclonal antibodies were also affinity purified. For Western analysis, the secondary antibody used was horseradish peroxidase-conjugated monoclonal anti-rabbit IgG (cat. A1949, gamma-chain specific, Sigma). Antibodies to c-Src and Yes were purchased from Cell Signaling (Beverly, MA).

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 min. The cell lysate was centrifuged at 12,000 x g for 10 min, 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 was 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% non-fat dry milk in TBST (Tris-buffered saline, 0.1% Tween-20), and then incubated with a 1:500 dilution of primary antibody for 2 h. The blots were then washed three times over 30 min in TBST, and incubated for 1 h with horseradish peroxidase-conjugated secondary antibody mouse anti-rabbit IgG (1:10,000), and then washed in TBS-Tween as before. The membranes were then developed using the Supersignal West Pico Chemiluminescent Western blotting detection system according to the manufacturer's instructions (Pierce, Arlington Heights, IL). Ponceau S staining was performed to demonstrate equal loading.

Immunoprecipitation. Cell lysates were pre-incubated solely with protein A/G Plus-Agarose (Santa Cruz Biotechnology, Santa Cruz, CA) at 4˚C for 1 h, then the mixture was centrifuged at 3,000 x g for 5 min to pellet these beads and any non-specific interacting proteins. One mg of supernatant protein was incubated with anti-c-Src or anti-Yes antibody and 30 μl

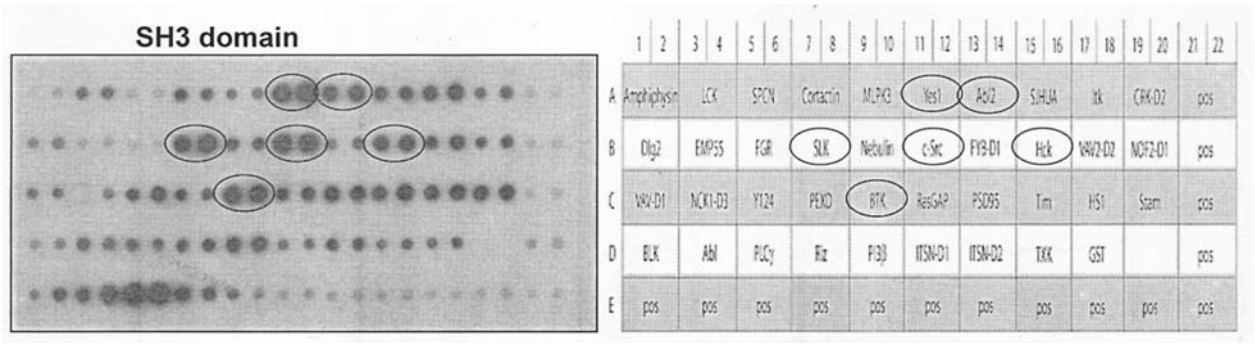

Figure 1. EG-1 binds to SH3 domains of many proteins. One representative of blots is shown.

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 or mouse IgG (Santa Cruz Biotechnology) and protein A/G- Plus-Agarose (for negative controls). After incubation, immunocomplexes were pelleted by centrifugation at 3,000 x g for 5 min at 4˚C. The pellets were then re-suspended 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 blotting.

In vitro kinase assay. EG-1 was synthesized by TNT® T7 Coupled Reticulocyte Lysate System (RLS, Promega, Madison, WI) following the manufacturer's protocol, or immunoprecipitated from EG-1 overexpressed HEK-293 cells (4). The EG-1 purified by immunoprecipitation was washed once in TBS to remove detergents. Each pellet was suspended in reaction buffer (5 μl 100 mM $MnCl_2$, 1.25 μl 1 M HEPES, pH 7.3, 0.25 μl 100 mM sodium orthovanadate). γ-^{32}P (10 μCi) was added and incubated at 25˚C for 45 min. To terminate the kinase reaction, we added 15 μl of Laemmli sample buffer. The samples were boiled for 5 min, then proteins were resolved by 10% SDS-PAGE gel. The gel was dried and exposed to X-ray film for varying lengths of time.

Results

EG-1 interacts with many SH3 and WW domain proteins. We screened 150 proteins with SH3 domains and 67 proteins with WW domains. To compare the relative interactions of these SH3 and WW domain proteins with recombinant EG-1, the blots were scanned and quantitated. Table IA lists those SH3 domain proteins that have at least 2.5-fold binding with EG-1 in comparison with negative control, which is measured by the interaction of EG-1 with GST (glutathione S-transferase). We repeatedly observed an association between EG-1 and c-Src (Fig. 1). Other than c-Src, the following exhibited the most binding with EG-1: Yes1 (Yamaguchi sarcoma virus oncogene homolog 1), Tec (tyrosine protein kinase Tec), OSF (osteoclast stimulating factor), BTK (Bruton tyrosine kinase), SLK (proto-oncogene tyrosine protein kinase FYN), ABL2 (Abelson-related protein), and PIG2 (1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase γ 2). For WW domains, EG-1 appears to interact with MAGI-3 (membrane-associated guanylate kinase inverted-3) and NEED4 (neural precursor cell expressed, developmentally down-regulated 4)-like ubiquitin ligase 3 (Table IB). The negative control is measured by the interaction of EG-1 with GST (glutathione S-transferase).

EG-1 interacts with c-Src and Yes. Several different polyclonal antibodies against various EG-1 peptide fragments were made. We found that peptides no. 34-53 (PGAPRPSSSTLVDELES SFE) reliably generated antibodies well suited for Western blot analysis. The sequence for this epitope is unique, as determined by searches in the Genbank database.

To study EG-1-src binding in living mammalian cells, we transiently transfected HEK-293 cells with EG-1 plasmid or with vector alone (Fig. 2A). Lane 1 (EV) shows that HEK-293 cells express EG-1 and c-Src. Transfection with EG-1 plasmid increases EG-1 without affecting the c-Src level (lane 2, panels 1 and 3). Immunoprecipitation of cell lysates with anti-Src antibody, followed by immunoblotting with anti-EG-1 antibody demonstrated that EG-1 interacts with c-Src (panel 5). Furthermore, EG-1 overexpression resulted in a higher proportion of c-Src proteins that are bound to EG-1. Similarly, we validated the interaction between EG-1 and Yes protein as seen in Fig. 2B. As with EG-1-Src complexes, we found that EG-1 interacts with endogenous Yes and that this binding is similarly enhanced in cells overexpressing EG-1.

EG-1 is not a substrate of c-Src. We next investigated whether EG-1 is a natural substrate of c-Src kinase. *in vitro* kinase assay showed that c-Src does not phosphorylate EG-1 (Fig. 3). The accuracy of the kinase assay system was ensured by introducing a natural c-Src substrate STAT (signal transducer and activator of transcription) (7) to the system (lane 5). STAT was phosphorylated, indicating that the system worked well. c-Src did not phosphorylate EG-1 synthesized by the Reticulocyte Lysate system (lane 2) nor EG-1 synthesized by HEK-293 cells following affinity purification (lane 4).

EG-1 overexpression is involved in the activation of c-Src. We investigated whether overexpression of EG-1 is related with the activation of c-Src. HEK-293 cells were transiently transfected with empty vector or EG1. EG-1 overexpression did not change the protein level of total c-Src (Fig. 4, panel 1). However, EG-1 overexpression is tightly related with an activated status of c-Src, as manifested by decreased levels of inactive non-p-Tyr419 c-Src (panel 3) and increased active

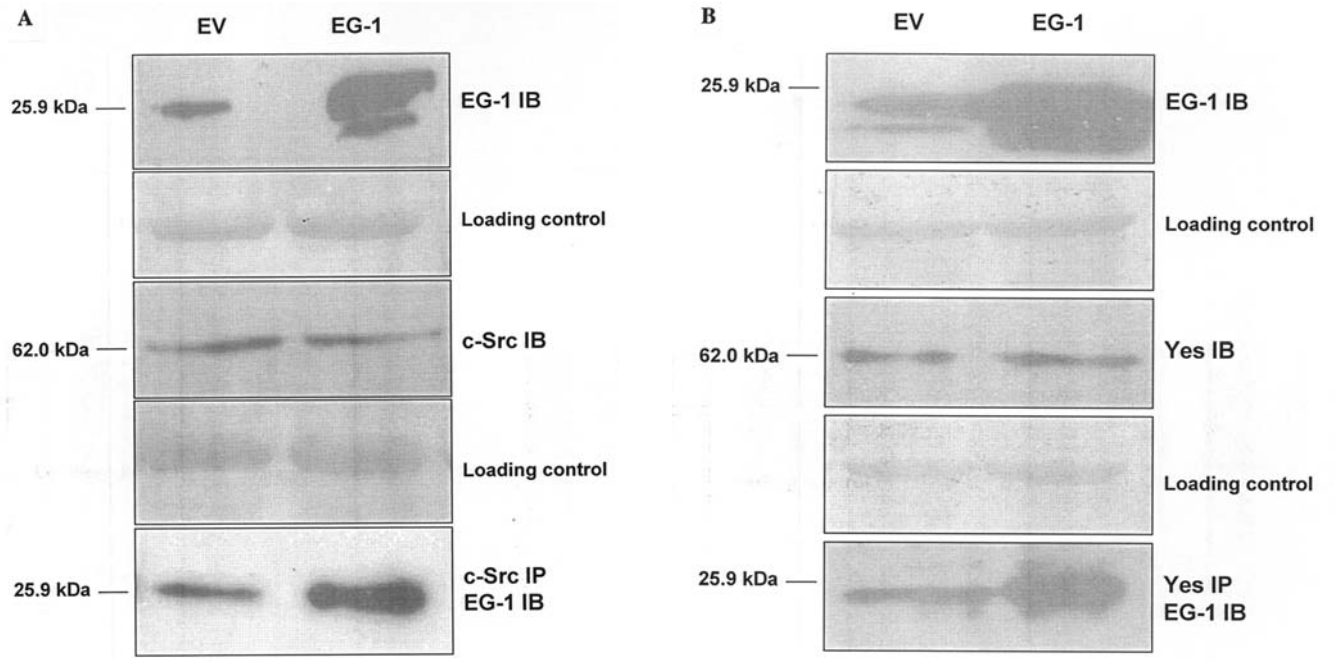

Figure 2. EG-1 binds c-Src (A) and Yes (B). HEK-293 cells were transiently transfected with empty vector (EV) or EG-1. Twenty-four hours after transfection, cell lysates were harvested and subjected to IP (immunoprecipitation) or IB (immunoblotting). Ponceau S staining was performed to demonstrate equal loading.

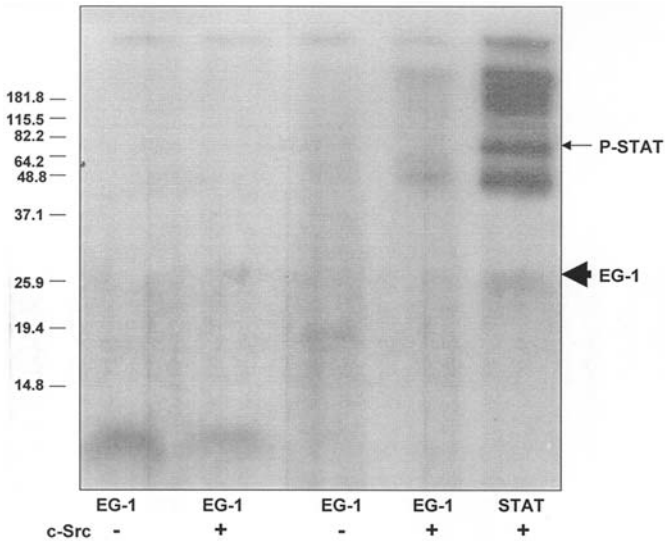

Figure 3. EG-1 is not a substrate of c-Src in *in vitro* kinase assay. Lane 1, EG-1 synthesized by RLS (Reticulocyte Lysate System) + [^{32}P]-ATP, without c-Src. Lane 2, EG-1 synthesized by RLS + [^{32}P]-ATP + active c-Src. Lane 3, EG-1 purified from HEK-293 cells + [^{32}P]-ATP, without c-Src. Lane 4, EG-1 purified from HEK-293 cells + [^{32}P]-ATP + active c-Src. Lane 5, natural c-Src substrate STAT + [^{32}P]-ATP + active c-Src. This lane serves as positive control of the kinase assay system.

non-p-Tyr530 c-Src (panel 9). EG-1 overexpression after transfection was confirmed by Western blotting (panel 7).

Discussion

In the present study, we observed that EG-1 overexpression results in c-Src activation. However, EG-1 was not a direct substrate of c-Src, nor did it increase c-Src expression. We also demonstrated that EG-1 binds to the Src family of protein tyrosine kinases c-Src and Yes, and possibly FYN and Hck (Hemopoietic cell kinase, Table IA). EG-1 overexpression was correlated with activation of c-Src. These observations collectively support the hypothesis that the novel gene EG-1 forms a protein-protein complex with c-Src, which then becomes catalytically active.

c-Src is a member of the Src family of cytoplasmic tyrosine kinases that regulate cell growth, differentiation, cell shape, migration and survival (8). c-Src has been reported to be overexpressed and to play a role in human carcinomas of the breast, colon, and others (9). Src family tyrosine kinases are often activated by receptor tyrosine kinases, such as EGF-R (epidermal growth factor receptor) or PDGF-R (platelet derived growth factor receptor) (10). The mechanisms of c-Src regulation are complex (11). The SH1 kinase domain contains the autophosphorylation site Tyr419 required for activation. The C terminus contains the Tyr530 which inactivates c-Src when phosphorylated, resulting in a closed configuration. Thus, in the cell, c-Src can be regulated by various kinases and phosphatases. Multiple receptor tyrosine kinases, G-protein-coupled receptors, FAK (focal adhesion kinase) and adhesion/cytoskeleton molecules can also participate (8). Other regulatory processes include ubiquitylation, mutations, and nitric oxide signaling (12). Certain SH3 binding agents, such as the HIV Nef protein, may also activate c-Src (13). Data derived from our study shows that EG-1 may be added as another c-Src activator.

As a result of EG-1 binding, c-Src presumably phosphorylates the MAPK pathway which has been shown to be crucial in promoting cellular proliferation and is situated downstream from c-Src (14). Previously, we had shown that the phosphorylated active forms of p44/42 MAP kinase, JNK (Jun-terminal kinase) and p38 kinase are elevated in EG-1

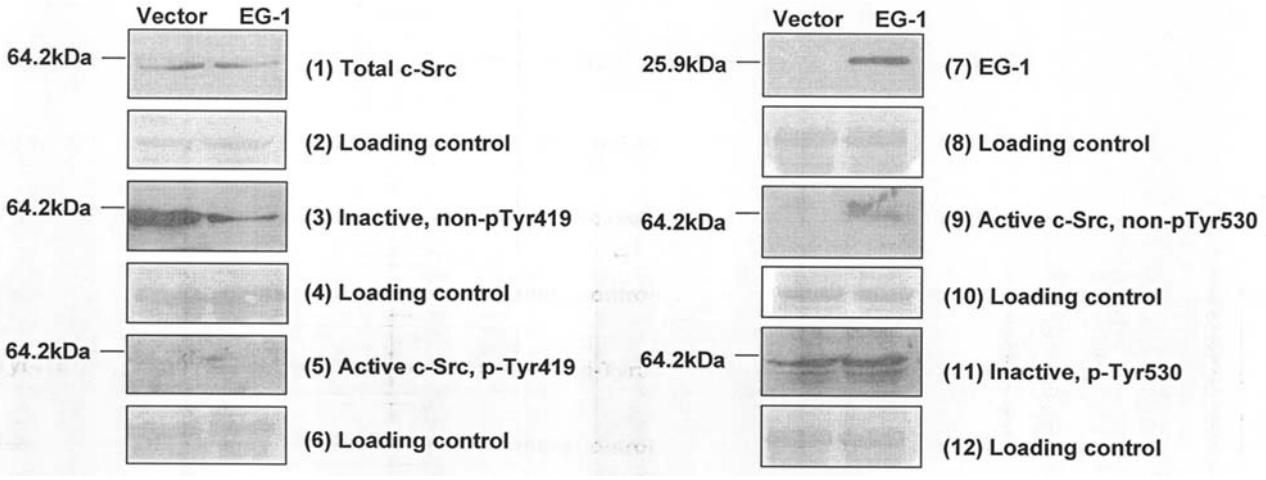

Figure 4. EG-1 activates c-Src. HEK-293 cells were transiently transfected with empty vector (EV) or EG-1. Whole cell lysates were collected and subjected to Western analysis. Ponceau S staining was performed to demonstrate equal loading.

transfected cells (4). During our work on EG-1, we became aware of two recent and unrelated publications on proteins with identical sequences to EG-1. In one publication, the Ramesh group from Harvard called this protein Magicin for Merlin and Grb2 interacting cytoskeletal protein (15). Magicin is described to associate with the actin cytoskeleton, and is proposed to have a role in receptor-mediator signaling at the cell surface. Via affinity binding, blot overlay and co-immunoprecipitation assays, the Ramesh group demonstrated that magicin binds directly to Grb2 (growth factor receptor bound 2 protein). Our SH3 panels contained one entry for each of the two SH3 binding domain fragments of Grb2, which revealed individual weak binding with EG-1 (data not shown). This is consistent with the group Ramesh' observation that full-length Grb2 displayed the strongest binding to EG-1/magicin. Our finding of EG-1's effects on the MAPK pathway may also be explained via its direct interaction with Grb2 (16). A second publication reported that the EG-1 protein product also functions as a member of the Mediator, a multi-protein transcriptional coactivator that is expressed ubiquitously in eukaryotes for induction of RNA polymerase II transcription by DNA binding transcription factors (17). As Med28, it is one subunit of the 'adaptor' that bridges RNA polymerase II with its DNA binding regulatory proteins and transduces both positive and negative signals (18). Anatomically, this is consistent with our previous report that EG-1 was present on immunohistochemical studies in the nucleus as well as in the cytoplasm (3).

In conclusion, it appears thus far that EG-1 is an important protein that has multiple interactions with crucial cellular pathways, such as Src, other tyrosine kinases (Tec, BTK), and the oncogene ABL (19). Overexpression of EG-1 induces key signaling pathways involved in cellular proliferation, an important component of the malignant phenotype. Our findings suggest that EG-1 may be an important target in the design of novel therapy against cancer.

Acknowledgments

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