Inhibition of Anchorage-independent Growth of Transformed NIH3T3 Cells by Epithelial Protein Lost in Neoplasm (EPLIN) Requires Localization of EPLIN to Actin Cytoskeleton

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Epithelial protein lost in neoplasm (EPLIN) is a cytoskeleton-associated protein characterized by the presence of a single centrally located *lin-11*, *isl-1*, and *mec-3* (LIM) domain. We have reported previously that EPLIN is down-regulated in transformed cells. In this study, we have investigated whether ectopic expression of EPLIN affects transformation. In untransformed NIH3T3 cells, retroviral-mediated transduction of EPLIN did not alter the cell morphology or growth. NIH3T3 cells expressing EPLIN, however, failed to form colonies when transformed by the activated Cdc42 or the chimeric nuclear oncogene EWS/Fli-1. This suppression of anchorage-independent growth was not universal because EPLIN failed to inhibit the colony formation of Ras-transformed cells. Interestingly, the localization of EPLIN to the actin cytoskeleton was maintained in the EWS/Fli-1- or Cdc42-transformed cells, but not in Ras-transformed cells where it was distributed heterogeneously in the cytoplasm. Using truncated EPLIN constructs, we demonstrated that the NH₂-terminal region of EPLIN is necessary for both the localization of EPLIN to the actin cytoskeleton and suppression of anchorage-independent growth of EWS/Fli-1-transformed cells. The LIM domain or the COOH-terminal region of EPLIN could be deleted without affecting its cytoskeletal localization or ability to suppress anchorage-dependent growth. Our study indicates EPLIN may function in growth control by associating with and regulating the actin cytoskeleton.

INTRODUCTION

The cell–cell and cell–matrix interaction, required for the maintenance of organized epithelia, is dependent on cytoskeleton (Drubin and Nelson, 1996). Cytoskeleton plays a pivotal role in many cellular processes, including cell polarity, adhesion, movement, membrane trafficking, cytokinesis, and distribution of receptors and signaling molecules (Stossel, 1993; Fishkind and Wang, 1995; Gumbiner, 1996; Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996). Structurally, cytoskeleton is composed of actin fibers, intermediate filaments, and microtubules. In addition, vari-

ous proteins associated with the cytoskeleton provide specialized functions, such as linking the cytoskeleton to the sites of cell–cell and cell–matrix interaction or to different cytoskeletal filament networks (Clark and Brugge, 1995; Fuchs and Yang, 1999). Various studies have indicated that the cytoskeleton may play a critical role in cellular transformation (Gluck *et al.*, 1993; Braverman *et al.*, 1996; Pawlak and Helfman, 2001). Proteins involved in the regulation of cell–matrix interaction and the organization of the actin stress fibers can function as either oncogenes or tumor suppressors (Hunter, 1997; Van Aelst and D'Souza-Schorey, 1997). In addition, activation of receptor tyrosine kinases or Ras, an event frequently associated with oncogenesis, induces a profound and rapid rearrangement of the actin cytoskeleton (Rodriguez-Viciano *et al.*, 1997; Hall, 1998), suggesting a critical link between the cytoskeleton and transformation.

Epithelial protein lost in neoplasm (EPLIN) was initially identified as a gene that is transcriptionally down-regulated in oral cancer cells (Chang *et al.*, 1998). Subsequent studies

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Abbreviations used: aa, amino acid; EPLIN, epithelial protein lost in neoplasm; LIM, *lin-11*, *isl-1*, and *mec-3* domain; PBS, phosphate-buffered saline.

have shown that EPLIN expression is either lost or down-regulated in a number of human epithelial cancer cells (Maul and Chang, 1999). There are two known isoforms of EPLIN (α and β), generated by alternative promoter usage from a single copy gene located on chromosome 12 (Chen *et al.*, 2000). These two isoforms differ only at the NH₂ terminus, where the presence of additional 160 amino acids (aa) distinguishes the longer EPLIN- β (759 aa) from the shorter EPLIN- α (600 aa). Both isoforms are found along the actin stress fibers and focal adhesion plaques, suggesting their role in the regulation of cell shape or cell-matrix interaction. The expression of EPLIN isoforms in tissues is highly variable (Maul *et al.*, 2001), indicating these two isoforms may have a similar, but nonredundant function. The amino acid sequence of EPLIN is unique, except for a single centrally located 54 aa *lin-11*, *isl-1*, and *mec-3* (LIM) domain capable of forming two closely spaced zinc-binding subdomains. Many LIM proteins participate in cell differentiation, as components of transcription machinery (Dawid *et al.*, 1998). Another class of LIM proteins includes cytoskeletal proteins such as zyxin (Schmeichel and Beckerle, 1997) and paxillin/hic-5/leupaxin (Turner, 2000), which are found at the site of cell-matrix adhesion, and limatin/LIMAB1 (Kim *et al.*, 1997) and DbLIM (Prassler *et al.*, 1998), which associate with the actin cytoskeleton. Although the functions of many of these LIM proteins are still under investigation, genetic analysis indicates that UNC-115, a *Caenorhabditis elegans* ortholog of aBLIM/limatin, functions as a cytoskeletal linker protein that acts in axon guidance (Lundquist *et al.*, 1998). For cysteine-rich proteins, CRP3/MLP knockout mice develop cardiomyopathy characterized by marked disruption in the architecture of myofibrils (Arber *et al.*, 1997).

Other than the subcellular localization to the cytoskeleton, the function of EPLIN is not known. In many cancer-derived or transformed cell lines, the expression of EPLIN is significantly down-regulated, suggesting that the loss of EPLIN may contribute to the transformed phenotype. To address this question, we have examined whether the expression of EPLIN can reverse or alter the growth phenotype or the morphology of NIH3T3 cells transformed by the activated Ras (RasV12), the activated Cdc42 (Cdc42V12), or the chimeric nuclear oncogene EWS/Fli-1.

MATERIALS AND METHODS

Construction of EPLIN Expression Plasmids

The translated region of EPLIN was amplified with the high-fidelity *Pfu* polymerase (Stratagene, La Jolla, CA), by using the following primers: for EPLIN- α (EPLIN- α ATG and EPLIN-TGA); EPLIN- β (EPLIN- β ATG and EPLIN-TGA); EPLIN- α Δ C (EPLIN- α ATG and EPLIN Δ C 3'); EPLIN- β Δ C (EPLIN- β ATG and EPLIN Δ C 3'); and EPLIN Δ N (EPLIN Δ N 5' and EPLIN-TGA). Polymerase chain reaction-amplified fragments were digested with *Bam*HI and *Sal*I and cloned directionally into the corresponding sites of the pCMV5-Flag vector. EPLIN- α Δ LIM was constructed from by amplifying the NH₂-terminal half (primers: EPLIN- α ATG and EPLIN Δ LIM 3') and COOH-terminal half (EPLIN Δ LIM 5' and EPLIN-TGA) of EPLIN individually. Amplified fragments were then digested with *Bam*HI, *Asp*718, and *Sal*I, and cloned into the *Bam*HI- and *Sal*I-digested pCMV5-Flag vector. The primer sequences were as follows: EPLIN- α ATG (GCAGGATCCAAAATGGAATTTGTCTAGGAG); EPLIN- β ATG (GCAGGATCCAAGATGGAATCATCTCCATTAA-TAG); EPLIN-TGA (CTTGCGGCCGCTCGACTCACTCTTCATC

CTCATCTCTC); EPLIN Δ C 3' (CCGTCGACTCATTATCATAGTTGC-CCTTAGATT); EPLIN Δ N 5' (CCGGATCCAAGAAGTTTCAGGCA-CCTGCAAG); EPLIN Δ LIM 3' (CAGGTACCTGAACTTCTTCAT-TGCTTTG); and EPLIN Δ LIM 5' (CCGGTACCTCACTTCAATCA-ACTCTTTAA). The restriction enzyme recognition sites used in cloning are italicized. The coding region of EPLIN, including the NH₂-terminal flag epitope, was excised from the pCMV5-Flag backbone and cloned into the SR α -MSV-tkneo vector. Nucleotide sequence of EPLIN constructs used in this study was verified by DNA sequencing.

Retroviral Transduction

Retroviral constructs encoding RasV12, Cdc42V12, and EWS/Fli-1 were gifts of J. Collicelli, O. Witte, and C. Denny (University of California, Los Angeles, CA). NIH3T3 cells were maintained in DMEM supplemented with 5% newborn calf serum (Hyclone Laboratories, Logan, UT), penicillin, and streptomycin. The EPLIN constructs in the SR α -MSV-tkneo vector and the ψ (-) packaging plasmid were cotransfected into 293T cells by a calcium phosphate precipitation method. Sixty hours after the transfection, the conditioned media containing the packaged viruses were collected and used as retroviral stock. Sixteen hours before infection, ~250,000 NIH3T3 cells were seeded on 100-mm plates. Retroviral infection was carried out using 2 ml of conditioned media in the presence of polybrene. After retroviral infection, polyclonal populations of transduced cells were selected in the presence of 500 μ g/ml G418 for neomycin resistance.

Growth of Cells in Semisolid Media

Transformation was assessed by colony formation in soft agar. Cells (5000/60-mm plate) were embedded in soft agar in the presence of 20% newborn calf serum, as described by Lugo and Witte (1989). After 14–18 d of growth, the colony counts were enumerated from photographs captured with an IS-1000 Digital Imaging System by using Alpha Imager 2000 software. Each experiment was carried out in triplicate.

Immunoblot Analysis

Protein lysates were prepared by boiling tissue culture cells in 0.2% SDS in 25 mM Tris-HCl, pH 7.5 and 1 mM EDTA. Total cellular protein (15–30 μ g) was fractionated by SDS-PAGE and transferred to nitrocellulose membrane for immunoblotting with anti-EPLIN antibodies (Maul and Chang, 1999), M2 (anti-flag) monoclonal antibody (mAb; Sigma-Aldrich, St. Louis, MO), anti-Ras (Transduction Laboratories, Lexington, KY), or anti-Cdc42 (Santa Cruz Biotechnology, Santa Cruz, CA).

In Situ Immunofluorescence

U2-OS cells were cultured in DMEM supplemented with 10% fetal calf serum on glass coverslips that had been coated with fibronectin (Sigma-Aldrich) (10 μ g/ml in phosphate-buffered saline [PBS]). Exponentially growing cells were transfected with the EPLIN expression plasmids by using LipofectAMINE (Invitrogen, Carlsbad, CA). Twenty-four hours after the transfection, the glass coverslips were fixed in 3% formaldehyde (Ladd Research Industries, Burlington, VT) in PBS for 10 min and permeabilized in 0.2% Triton X-100 in PBS for 5 min. The glass coverslips were then incubated in a blocking buffer (0.1% Tween 20 and 10% goat serum in PBS) for 30–45 min. Endogenous and transfected EPLIN mutants were visualized by staining with anti-EPLIN antibody (1:250 dilution) (Maul and Chang, 1999) and the M2 mAb (2 μ g/ml), respectively, followed by fluorescein isothiocyanate-conjugated goat anti-rabbit or anti-mouse IgG (Molecular Probes, Eugene, OR). The filamentous actin fibers were stained with rhodamine-conjugated phalloidin (Molecular Probes). All incubations were carried out at room temperature. The coverslips were mounted with ProLong (Molecular Probes) and viewed under a Nikon Diaphot microscope equipped with fluorescent light sources. Similarly, polyclonal population of transduced

Figure 1. Overexpression of EPLIN in NIH3T3 cells. (A) Expression of EPLIN in NIH3T3 cells transduced with EPLIN- α or - β (lanes 1 and 2), or empty virus (lane 3), human BeWo choriocarcinoma cells (lane 4), U2-OS osteosarcoma cells (lane 5), and HeLa cells (lane 6) was examined in an immunoblot analysis. Equal amounts ($\sim 15 \mu\text{g}$) of total cell lysates were fractionated by SDS-PAGE. The levels of EPLIN expression in the transduced NIH3T3 cells were comparable with the levels of endogenous EPLIN in BeWo, U2-OS, or HeLa cells. (B) Light microscopy photographs ($320\times$ magnification) of NIH3T3 cells transduced with SR α , EPLIN- α , and EPLIN- β are shown.

NIH3T3 cells were plated on fibronectin-coated glass coverslips and stained the same way. The RasV12-infected NIH3T3 cells were viewed under a confocal microscope.

RESULTS

EPLIN Does Not Affect Morphology or Growth of Untransformed Cells

To study the role of EPLIN in oncogenic transformation, we subcloned both α and β isoforms of EPLIN in the retroviral vector SR α -MSV-tkneo (SR α). NIH3T3 cells were infected with retroviruses and selected with G418 to obtain a polyclonal population of stably transduced cells. Equal amounts of cell lysates prepared from the transduced cells were analyzed by immunoblot with anti-EPLIN antisera. At baseline, NIH3T3 cells express low levels of EPLIN- α and - β (Figure 1A). In the transduced cells, the level of EPLIN was considerably higher and was comparable to the endogenous levels of EPLIN in U2-OS osteosarcoma or BeWo choriocarcinoma cells. In a previous study using U2-OS cells engineered to overexpress EPLIN under the control of a tetracycline inducible promoter, we had reported that EPLIN- β can be growth inhibitory (Maul and Chang, 1999). At the physiological range of EPLIN expression achieved by retroviral infection, the transduced cells were indistinguishable from

Figure 2. Growth rate and saturation density of transformed NIH3T3 cells are not affected by the presence of EPLIN. Growth rate (A–D) and saturation density (E) of the RasV12-, Cdc42V12-, and EWS/Fli-1-expressing NIH3T3 cells were measured. A polyclonal population of stably transduced cells was plated at 250,000 cells/well in six-well plates and cultured in the presence of 5% newborn calf serum. At indicated time points, cell counts were determined. The saturation cell density was measured on day 7. Data shown are representative of two to three independent experiments.

the control cells in cell morphology and growth characteristics (Figures 1B and 2).

EPLIN Can Suppress Anchorage-independent Growth of NIH3T3 Cells Transformed by Cdc42V12 or EWS/Fli-1, but Not by RasV12

To study the effect of EPLIN on transformation, polyclonal population of NIH3T3 cells expressing either EPLIN- α or - β was infected with a retrovirus encoding RasV12, Cdc42V12, EWS/Fli-1, or the control SR α vector. The prior infection with EPLIN-encoding retrovirus did not alter the efficiency

of the second round of infection, as evidenced by the appearance of characteristic morphological changes when RasV12 or EWS/Fli-1 was transduced (Figure 3E). RasV12, Cdc42V12, and EWS/Fli-1 were chosen in this study because the mode of transformation by these three oncogenes is thought to be distinct from one another. Ras is known to activate a variety of different mitogenic pathways, including the phosphatidylinositol 3-kinase, mitogen-activated protein kinases, and nuclear factor- κ B (Vojtek and Der, 1998). Cdc42, a member of the Rho family of small GTPases, is involved in the activation of c-Jun NH₂-terminal kinase and modulation of cytoskeleton (Hall, 1998). EWS/Fli-1, a fusion protein found in childhood Ewing's sarcoma or peripheral neuroendocrine tumors, is believed to function as an aberrant transcription factor to alter expression of a number of target genes, leading to the transformed phenotype (Denny, 1996).

Although the growth rate and the saturation density varied depending on which oncogene was expressed in the cell, they were not significantly altered by the presence of EPLIN (Figure 2). The effect of EPLIN, however, was more apparent when the growth of transformed cells was measured in a soft agar assay. The number of colonies in soft agar was reduced by ~80% in the Cdc42V12 transformed cells when either EPLIN- α or - β was expressed (Figure 3). Similarly, EPLIN suppressed anchorage-independent growth of EWS/Fli-1-transformed cells. The ability of EPLIN to suppress anchorage-independent growth of transformed cells was not universal, because the colony formation of RasV12-transformed cells was not significantly altered by the presence of EPLIN- α or - β . The levels of EPLIN- α or - β in the transformed cells used in these experiments were equivalent (Figure 3D). RasV12- and EWS/Fli-1-transduced cells adopted a distinct morphology (e.g., round and refractile for RasV12; small with long cytoplasmic processes for EWS/Fli-1). These morphological features were not affected by EPLIN expression (Figure 3E).

Because oncogenic transformation frequently disrupts the cytoskeleton, we next examined whether subcellular localization of EPLIN is altered in transformed cells. As expected, the transduced EPLIN was distributed along the actin cytoskeleton in the control NIH3T3 cells (Figure 4, A and B). NIH3T3 cells expressing Cdc42V12 and EPLIN were morphologically similar to the control cells, except for a slightly more prominent actin stress fibers (Figure 4C). In these cells, EPLIN was colocalized to the actin filaments in a pattern essentially identical to that seen in the control cells (Figure 4D). EWS/Fli-1-expressing cells were round and had prominent cortical actin fibers at the periphery of the cell (Figure 4E). Although the cell shape and actin cytoskeleton were considerably different in EWS/Fli-1-expressing cells, EPLIN still colocalized to the actin fibers present at the periphery of the cell (Figure 4F). RasV12-transformed cells were spindle shaped and showed diminished staining for actin cytoskeleton (Figure 4G). In these cells, EPLIN was distributed heterogeneously throughout the cell, rather than localizing to the actin filaments (Figure 4H). Furthermore, the EPLIN staining in RasV12-transformed cells was in a speckled pattern along the plasma membrane, suggesting that EPLIN may be associated with membrane protrusions. These findings suggested that the subcellular localization of EPLIN to the actin filaments can be altered by oncogenic transforma-

tion and raised a possibility that the inability of EPLIN to suppress colony formation of RasV12-transformed cells may rest on this altered subcellular localization.

EPLIN Increases Actin Fibers in EWS/Fli-1-transformed NIH3T3 Cells

In both Cdc42V12 and EWS/Fli-1-expressing cells, the subcellular localization of EPLIN to the actin cytoskeleton was not disturbed. However, unlike the Cdc42V12-expressing cells, which maintained the characteristic fibroblast-like morphology, the EWS/Fli-1-expressing cells were round and lacked the actin stress fibers. We next examined the morphology of the EWS/Fli-1-transformed cells in more detail, focusing on the actin cytoskeleton. NIH3T3 cells displayed well-organized meshwork of stress fibers (Figure 5A). The appearance of actin stress fibers was not altered by EPLIN at physiological range of expression (Figure 4; our unpublished data). In the EWS/Fli-1-transduced cells, the actin filaments were organized mostly as cortical actin, with a near complete absence of the actin stress fibers (Figure 5B). In these cells, the presence of either EPLIN- α or - β induced a formation of densely packed actin filaments in the periphery of the cell (Figure 5, C and D). The increase in actin filaments, although atypical in appearance, indicates that EPLIN may induce the formation or, alternatively, enhance the stability of actin filaments.

NH₂-terminal Region of EPLIN Is Required to Inhibit Anchorage-independent Growth of EWS/Fli-1-transformed Cells

To identify the EPLIN domain required for the suppression of anchorage-independent growth, we generated EPLIN truncation mutants. Using the centrally located LIM domain as a reference point, we constructed mutants lacking the LIM domain or either side of it in the SR α -MSV-tkneo vector (Figure 6A). These truncated proteins were modified at the NH₂ terminus with a flag-epitope for detection. An immunoblot analysis with the M2 anti-flag mAb showed that each EPLIN truncation mutant could be stably transduced into NIH3T3 cells (Figure 6B). None of the EPLIN truncation mutants affected the cell morphology or the growth characteristics (our unpublished data). In soft agar assays, the constructs lacking the LIM domain (EPLIN- $\alpha\Delta$ LIM and EPLIN- $\beta\Delta$ LIM) or the COOH-terminal region (EPLIN- $\alpha\Delta$ C and EPLIN- $\beta\Delta$ C) suppressed anchorage-independent growth to a similar extent as the wild-type EPLIN proteins (Figure 6C). EWS/Fli-1-transformed cells coexpressing EPLIN Δ N continued to form colonies in soft agar, although the colony numbers were somewhat diminished. These studies allowed us to conclude that the LIM domain or the COOH-terminal 305 aa of EPLIN is not required for the suppression of colony formation.

Localization of EPLIN to Cytoskeleton Correlates with Its Ability to Suppress Anchorage-independent Growth

We next investigated whether any of the truncation affected the subcellular localization of EPLIN. In NIH3T3 cells, the endogenous EPLIN is expressed at an insufficient level to properly determine its localization by in situ immunofluo-

Figure 3. Presence of EPLIN inhibited anchorage-independent growth of NIH3T3 cells expressing Cdc42V12 or EWS/Fli-1, but not RasV12. Soft agar assays were carried out using 5000 cells/60-mm well in the presence of 20% newborn calf serum. The colony formation is presented as percentage relative to the number of colonies in the absence of EPLIN. The absolute number of colonies in a typical experiment is shown above the bar. (A) NIH3T3 cells transduced with RasV12. (B) NIH3T3 cells transduced with Cdc42V12. (C) NIH3T3 cells transduced with EWS/Fli-1. In each panel, the cells were also transduced an empty virus (SR α), EPLIN- α , or EPLIN- β . (D) Top, expression levels of EPLIN- α and - β in the control SR α , RasV12-, Cdc42V12-, and EWS/Fli-1-transformed NIH3T3 cells are shown. Bottom, expression levels of RasV12 and Cdc42V12 in the control SR α , EPLIN- α , and - β -transduced NIH3T3 cells are shown. For immunoblot 15 μ g of total cell lysates was fractionated by SDS-PAGE and probed with anti-flag (for detection of EPLIN), anti-Ras, or anti-Cdc42 antibodies. (E) Light microscopy photographs (160 $\times$ magnification) of EPLIN- α and - β cells transduced with RasV12, Cdc42V12, and EWS/Fli-1 are shown.

Figure 4. Subcellular localization of EPLIN in transformed cells. NIH3T3 cells expressing EPLIN- α were plated on fibronectin-coated glass coverslips and then analyzed by in situ immunofluorescence by using rhodamine-phalloidin and anti-EPLIN antibodies to visualize actin filaments (A, C, E, and G) and EPLIN (B, D, F, and H), respectively. (A and B) NIH3T3 cells transduced with EPLIN- α and a control empty virus (SR α). (C and D) NIH3T3 cells transduced with EPLIN- α and Cdc42V12. (E and F) NIH3T3 cells transduced with EPLIN- α and EWS/Fli-1. (G and H) NIH3T3 cells transduced with EPLIN- α and RasV12.

rescence. Therefore, we used U2-OS cells, which expresses sufficiently high level of endogenous EPLIN to allow a comparison of the subcellular localization of transfected EPLIN mutants to the endogenous protein. In situ immunofluorescence with anti-EPLIN antisera showed that the endogenous EPLIN in U2-OS cells is distributed predominantly along the actin stress fibers (Figure 7A). The transiently expressed wild-type EPLIN- α and - β , which can be distinguished from the endogenous EPLIN by the NH₂-terminal flag-epitope, was also found along the actin stress fibers (Figure 7, B and C). This pattern of subcellular localization was not affected by the deletion of the COOH-terminal region or the LIM domain (Figure 7, D and E). Interestingly, EPLIN Δ N, which did not suppress the anchorage-independent growth of EWS/Fli-1-transformed cells, stained heterogeneously in the cell without localizing to the actin stress fibers, indicating that the NH₂-terminal 220 aa of EPLIN- α contains the actin cytoskeleton-targeting sequences (Figure 7F). The transduced wild-type EPLIN (Figure 4, A and B) and its

Figure 5. EPLIN increases the actin fibers in EWS/Fli-1-transformed cells. NIH3T3 cells were plated on fibronectin-coated coverslips and analyzed by in situ immunofluorescence by using rhodamine-phalloidin to visualize the actin filaments. (A) NIH3T3 transduced with a control empty virus (SR α). (B) EWS/Fli-1-transduced NIH3T3 cells. (C) EWS/Fli-1- and EPLIN- α -transduced NIH3T3 cells. (D) EWS/Fli-1- and EPLIN- β -transduced NIH3T3 cells.

mutants, except for EPLIN Δ N, displayed an essentially identical pattern of localization to the actin filaments in NIH3T3 cells (our unpublished data). The correlation between the localization of EPLIN to the actin filaments and its ability to suppress anchorage-independent growth suggest that proper subcellular localization is important for the function of EPLIN.

DISCUSSION

In the current study, we used the NIH3T3 cells as a model system to study the role of EPLIN in transformation. Our main conclusion is that ectopic expression of EPLIN can suppress anchorage-independent growth of transformed NIH3T3 cells. Furthermore, our study indicates that the localization of EPLIN to the actin cytoskeleton may be important for its ability to suppress anchorage-independent growth. In RasV12-transformed cells, which continued to form colonies in soft agar in the presence of EPLIN, EPLIN was distributed heterogeneously in the cell rather than localizing to the actin cytoskeleton. This altered distribution of EPLIN could represent a nonspecific consequence of transformation, which frequently alters cell morphology and cytoskeleton. However, the finding that EPLIN continues to be associated with actin cytoskeleton in either the Cdc42V12- or EWS/Fli-1-transformed cells argues against such interpretation and indicates that the subcellular localization of EPLIN can be altered only by certain oncogenic stimuli, including those brought about by RasV12. The importance of proper subcellular distribution of EPLIN is also suggested in the finding that EPLIN Δ N, which failed to colocalize to the actin cytoskeleton, also failed to suppress anchorage-independent growth of EWS/Fli-1-transformed cells. Last,

Figure 6. NH₂-terminal region of EPLIN is required for the suppression of anchorage-independent growth of the EWS/Flt-1-transformed cells. (A) Extent of deletion in each truncation mutants is diagrammed. (B) Expression of truncated EPLIN in the EWS/Flt-1-transformed cells was determined by an immunoblot using the M2 anti-flag mAb. The bands corresponding to the truncated EPLIN are marked by *. The M2 anti-flag mAb also recognizes EWS/Flt-1 (indicated by an open arrowhead), which has the flag-epitope at the COOH terminus. (C) Growth of transduced cells expressing both EPLIN and EWS/Flt-1 in soft agar was determined as described in EXPERIMENTAL PROCEDURES.

in EWS/Flt-1-transformed cells, the presence of EPLIN led to an increase in the amount of actin filaments, indicating that EPLIN may be involved in the maintaining the integrity of actin cytoskeleton in the cell.

Most untransformed cells require cell adhesion for proliferation and undergo either growth arrest or apoptosis when deprived of cell adhesion. In the absence of adhesion-dependent stimulation, the response of mitogen-activated protein

Figure 7. NH₂ terminal region of EPLIN is required for its localization to the actin cytoskeleton. U2-OS cells cultured on fibronectin-coated glass coverslips were transfected with the control SRα (A), EPLIN-α (B), EPLIN-β (C), EPLIN-αΔC (D), EPLIN-αΔLIM (E), or EPLINΔN (F) plasmid. Twenty-four hours after the transfection, the cells were fixed in formaldehyde and stained for actin filaments by using rhodamine-phalloidin (right) or for EPLIN by using anti-EPLIN antibody (A) or the M2 anti-flag mAb (B-F). M2 anti-flag mAb only recognizes the transfected EPLIN proteins, which are modified with a flag-epitope at the NH₂ terminus.

kinases to the growth factor is diminished (Lin *et al.*, 1997; Renshaw *et al.*, 1997). The growth in soft agar, which reflects the loss of this dependence on cell adhesion, is frequently viewed as a reliable criterion for neoplastic transformation (Schwartz, 1997). How EPLIN, a cytoskeleton-associated protein, can suppress the anchorage independence of NIH3T3 cells transformed by Cdc42 or EWS/Fli-1 is not known. Cdc42 mediates the formation of actin microspikes and filopodia in response to bradykinin or other external agonist by binding N-WASp or related proteins to promote Arp2/3-dependent actin polymerization (Nobes and Hall, 1995; Rohatgi *et al.*, 1999). Cdc42 has also been implicated in the activation of serum response factor (Hill *et al.*, 1995), c-Jun NH₂-terminal kinase and its downstream target c-Jun (Coso *et al.*, 1995; Minden *et al.*, 1995), the ternary complex factor protein Elk-1 (Whitmarsh *et al.*, 1997), and p38 mitogen-activated protein kinase (Zhang *et al.*, 1995). EWS/Fli-1 is a chimeric transcription factor resulting from a fusion of the NH₂ terminus of the EWS gene, which encodes a protein related to the human TATA box protein-associated factor hTAFII68 (Bertolotti *et al.*, 1996), to the COOH terminus of ets transcription factors (Denny, 1996). In Cdc42V12-, EWS/Fli-1-, or RasV12-transformed NIH3T3 cells, the expression of EPLIN did not alter the levels of extracellular signal-regulated kinase1/2 phosphorylation or the reduction of extracellular signal-regulated kinase1/2 phosphorylation seen upon removing anchorage-dependent stimulation (our unpublished data). Therefore, we do not believe that EPLIN influences this mitogen-activated kinase pathway.

Stable expression of activated forms of Cdc42 is known to oppose the activities of RhoA (Sanders *et al.*, 1999), leading to a reduction in stress fibers (Qiu *et al.*, 1997). EWS/Fli-1 also affects cell morphology, leading to a reduction in actin stress fibers. Loss of cytoskeletal architecture is thought to be a contributing factor, rather than a consequence, of neoplastic transformation (Janmey and Chaponnier, 1995; Ben-Ze'ev, 1997; Pawlak and Helfman, 2001). Expression of several actin-binding proteins, including tropomyosin, vinculin, α -actinin, and gelsolin has shown to be down-regulated in many transformed cells (Kaneko *et al.*, 1995). Restoring these proteins can reverse the malignant phenotype in several different experimental models of transformation (Gluck *et al.*, 1993; Braverman *et al.*, 1996; Kwon *et al.*, 1997). In the NIH3T3 cells expressing EPLIN, the introduction of Cdc42V12 did not diminish the actin stress fibers (Figure 3C). In addition, the EWS/Fli-1-transformed cells displayed increased amount of actin filaments when EPLIN was present. Last, the EPLIN Δ N mutant lacking the NH₂-terminal 220 aa failed to localize to the cytoskeleton or suppress colony formation. These observations suggest that the primary role of EPLIN may involve maintaining the integrity of actin cytoskeleton in the cell, which, through a yet-to-be-defined mechanism, restores the anchorage dependence of the transformed cells.

EPLIN overexpression did not affect the colony formation of RasV12-transformed cells. Ras activates a variety of mitogenic pathways (Vojtek and Der, 1998). One possibility is that Ras simply is a more potent oncogene than either Cdc42 or EWS/Fli-1, and can overcome the ability of EPLIN to reinforce the actin stress fibers. Equally plausible is the possibility that the activated Ras prevents or alters the localization of EPLIN to the stress fibers. We currently favor

the latter possibility based on observation that EPLIN failed to colocalize with the actin stress fibers and was found in a speckled pattern distributed throughout the cytoplasm in RasV12-transformed cells. In addition, the extent to which EPLIN colocalizes to the actin stress fibers is variable in different cell lines (Maul and Chang, 1999) (our unpublished data), which suggests that the subcellular localization of EPLIN can vary depending on the cellular background and may be regulated by cytosolic signaling events. Further studies on the regulation and sequence requirement for the cytoskeletal localization of EPLIN are needed to clarify whether the failure of EPLIN to inhibit anchorage-independent growth of RasV12-transformed cells is a direct consequence of its failure to localize to the actin cytoskeleton.

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REFERENCES

- Arber, S., Hunter, J.J., Ross, J., Jr., Hongo, M., Sansig, G., Borg, J., Perriard, J.C., Chien, K.R., and Caroni, P. (1997). MLP-deficient mice exhibit a disruption of cardiac cytoarchitectural organization, dilated cardiomyopathy, and heart failure. *Cell* 88, 393–403.
- Ben-Ze'ev, A. (1997). Cytoskeletal and adhesion proteins as tumor suppressors. *Curr. Opin. Cell Biol.* 9, 99–108.
- Bertolotti, A., Lutz, Y., Heard, D.J., Chambon, P., and Tora, L. (1996). hTAF(II)68, a novel RNA/ssDNA-binding protein with homology to the pro-oncoproteins TLS/FUS and EWS is associated with both TFIID and RNA polymerase II. *EMBO J.* 15, 5022–5031.
- Braverman, R.H., Cooper, H.L., Lee, H.S., and Prasad, G.L. (1996). Anti-oncogenic effects of tropomyosin: isoform specificity and importance of protein coding sequences. *Oncogene* 13, 537–545.
- Chang, D.D., Park, N.H., Denny, C.T., Nelson, S.F., and Pe, M. (1998). Characterization of transformation related genes in oral cancer cells. *Oncogene* 16, 1921–1930.
- Chen, S., Maul, R.S., Kim, H.R., and Chang, D.D. (2000). Characterization of the human EPLIN (epithelial protein lost in neoplasm) gene reveals distinct promoters for the two EPLIN isoforms. *Gene* 248, 69–76.
- Clark, E.A., and Brugge, J.S. (1995). Integrins and signal transduction pathways: the road taken. *Science* 268, 233–239.
- Coso, O.A., Chiariello, M., Yu, J.C., Teramoto, H., Crespo, P., Xu, N., Miki, T., and Gutkind, J.S. (1995). The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. *Cell* 81, 1137–1146.
- Dawid, I.B., Breen, J.J., and Toyama, R. (1998). LIM domains: multiple roles as adapters and functional modifiers in protein interactions. *Trends Genet* 14, 156–162.
- Denny, C.T. (1996). Gene rearrangements in Ewing's sarcoma. *Cancer Invest* 14, 83–88.
- Drubin, D.G., and Nelson, W.J. (1996). Origins of cell polarity. *Cell* 84, 335–344.

- Fishkind, D.J., and Wang, Y.L. (1995). New horizons for cytokinesis. *Curr. Opin. Cell Biol.* 7, 23–31.
- Fuchs, E., and Yang, Y. (1999). Crossroads on cytoskeletal highways. *Cell* 98, 547–550.
- Gluck, U., Kwiatkowski, D.J., and Ben-Ze'ev, A. (1993). Suppression of tumorigenicity in simian virus 40-transformed 3T3 cells transfected with alpha-actinin cDNA. *Proc. Natl. Acad. Sci. USA* 90, 383–387.
- Gumbiner, B.M. (1996). Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell* 84, 345–357.
- Hall, A. (1998). Rho GTPases and the actin cytoskeleton. *Science* 279, 509–514.
- Hill, C.S., Wynne, J., and Treisman, R. (1995). The Rho family GTPases RhoA, Rac1, and CDC42Hs regulate transcriptional activation by SRF. *Cell* 81, 1159–1170.
- Hunter, T. (1997). Oncoprotein networks. *Cell* 88, 333–346.
- Janmey, P.A., and Chaponnier, C. (1995). Medical aspects of the actin cytoskeleton. *Curr. Opin. Cell Biol.* 7, 111–117.
- Kaneko, S., Satoh, Y., Ikemura, K., Konishi, T., Ohji, T., Karasaki, Y., Higashi, K., and Gotoh, S. (1995). Alterations of expression of the cytoskeleton after immortalization of human fibroblasts. *Cell Struct. Funct.* 20, 107–115.
- Kim, A.C., Peters, L.L., Knoll, J.H., Van Huffel, C., Ciciotte, S.L., Kleyn, P.W., and Chishti, A.H. (1997). Limatin (LIMAB1), an actin-binding LIM protein, maps to mouse chromosome 19 and human chromosome 10q25, a region frequently deleted in human cancers. *Genomics* 46, 291–293.
- Kwon, H.J., Yoshida, M., Nagaoka, R., Obinata, T., Beppu, T., and Horinouchi, S. (1997). Suppression of morphological transformation by radicicol is accompanied by enhanced gelsolin expression. *Oncogene* 15, 2625–2631.
- Lauffenburger, D.A., and Horwitz, A.F. (1996). Cell migration: a physically integrated molecular process. *Cell* 84, 359–369.
- Lin, T.H., Chen, Q., Howe, A., and Juliano, R.L. (1997). Cell anchorage permits efficient signal transduction between ras and its downstream kinases. *J. Biol. Chem.* 272, 8849–8852.
- Lugo, T.G., and Witte, O.N. (1989). The BCR-ABL oncogene transforms Rat-1 cells and cooperates with v-myc. *Mol. Cell Biol.* 9, 1263–1270.
- Lundquist, E.A., Herman, R.K., Shaw, J.E., and Bargmann, C.I. (1998). UNC-115, a conserved protein with predicted LIM and actin-binding domains, mediates axon guidance in *C. elegans*. *Neuron* 21, 385–392.
- Maul, R.S., and Chang, D.D. (1999). EPLIN, epithelial protein lost in neoplasm. *Oncogene* 18, 7838–41.
- Maul, R.S., Sachi Gerbin, C., and Chang, D.D. (2001). Characterization of mouse epithelial protein lost in neoplasm (EPLIN) and comparison of mammalian and zebrafish EPLIN. *Gene* 262, 155–160.
- Minden, A., Lin, A., Claret, F.X., Abo, A., and Karin, M. (1995). Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. *Cell* 81, 1147–1157.
- Mitchison, T.J., and Cramer, L.P. (1996). Actin-based cell motility and cell locomotion. *Cell* 84, 371–379.
- Nobes, C.D., and Hall, A. (1995). Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* 81, 53–62.
- Pawlak, G., and Helfman, D.M. (2001). Cytoskeletal changes in cell transformation and tumorigenesis. *Curr. Opin. Genet. Dev.* 11, 41–47.
- Prassler, J., Murr, A., Stocker, S., Faix, J., Murphy, J., and Marriott, G. (1998). DdLIM is a cytoskeleton-associated protein involved in the protrusion of lamellipodia in *Dictyostelium*. *Mol. Biol. Cell* 9, 545–559.
- Qiu, R.G., Abo, A., McCormick, F., and Symons, M. (1997). Cdc42 regulates anchorage-independent growth and is necessary for Ras transformation. *Mol. Cell Biol.* 17, 3449–3458.
- Renshaw, M.W., Ren, X.D., and Schwartz, M.A. (1997). Growth factor activation of MAP kinase requires cell adhesion. *EMBO J.* 16, 5592–5599.
- Rodriguez-Viciana, P., Warne, P.H., Khwaja, A., Marte, B.M., Papin, D., Das, P., Waterfield, M.D., Ridley, A., and Downward, J. (1997). Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras. *Cell* 89, 457–467.
- Rohatgi, R., Ma, L., Miki, H., Lopez, M., Kirchhausen, T., Takenawa, T., and Kirschner, M.W. (1999). The interaction between N-WASP and the Arp2/3 complex links Cdc42-dependent signals to actin assembly. *Cell* 97, 221–231.
- Sanders, L.C., Matsumura, F., Bokoch, G.M., and de Lanerolle, P. (1999). Inhibition of myosin light chain kinase by p21-activated kinase. *Science* 283, 2083–2085.
- Schmeichel, K.L., and Beckerle, M.C. (1997). Molecular dissection of a LIM domain. *Mol. Biol. Cell* 8, 219–230.
- Schwartz, M.A. (1997). Integrins, oncogenes, and anchorage independence. *J. Cell Biol.* 139, 575–578.
- Stossel, T.P. (1993). On the crawling of animal cells. *Science* 260, 1086–1094.
- Turner, C.E. (2000). Paxillin and focal adhesion signaling. *Nat. Cell Biol.* 2, E231–E236.
- Van Aelst, L., and D'Souza-Schorey, C. (1997). Rho GTPases and signaling networks. *Genes Dev.* 11, 2295–2322.
- Vojtek, A.B., and Der, C.J. (1998). Increasing complexity of the Ras signaling pathway. *J. Biol. Chem.* 273, 19925–19928.
- Whitmarsh, A.J., Yang, S.H., Su, M.S., Sharrocks, A.D., and Davis, R.J. (1997). Role of p38 and JNK mitogen-activated protein kinases in the activation of ternary complex factors. *Mol. Cell Biol.* 17, 2360–2371.
- Zhang, S., Han, J., Sells, M.A., Chernoff, J., Knaus, U.G., Ulevitch, R.J., and Bokoch, G.M. (1995). Rho family GTPases regulate p38 mitogen-activated protein kinase through the downstream mediator Pak1. *J. Biol. Chem.* 270, 23934–23936.