PEA-15 Inhibits Tumor Cell Invasion by Binding to Extracellular Signal-Regulated Kinase 1/2

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Abstract
Phosphoprotein enriched in astrocytes of 15 kDa (PEA-15) binds to extracellular signal-regulated kinase 1 and 2 (ERK1/2) mitogen-activated protein (MAP) kinases to alter ERK1/2 cellular localization and target preferences and binds to adaptors in the extrinsic cell death pathway to block apoptosis. Here, we report that PEA-15 protein expression is inversely correlated with the invasive behavior of breast cancer in an immunohistochemical analysis of a breast cancer progression tissue microarray. Short hairpin RNA–mediated inhibition of PEA-15 expression increased the invasion of PEA-15–expressing tumor cells in vitro, suggesting a causative role for PEA-15 in the inhibition of invasion. This causative role was confirmed by the finding that the enforced expression of PEA-15 in invasive tumor cells reduced invasion. The effect of PEA-15 on tumor invasion is mediated by its interaction with ERK1/2 as shown by the following: (a) PEA-15 mutants that fail to bind ERK1/2 did not inhibit invasion; (b) overexpression of ERK1 or activated MAP/ERK kinase (MEK) reversed the inhibitory effect of PEA-15; (c) when an inhibitor of ERK1/2 activation reduced invasion, PEA-15 expression did not significantly reduce invasion further. Furthermore, we find that the effect of PEA-15 on invasion seems to relate to the nuclear localization of activated ERK1/2. PEA-15 inhibits invasion by keeping ERK out of the nucleus, as a PEA-15 mutant that cannot prevent ERK nuclear localization was not able to inhibit invasion. In addition, membrane-localized ERK1, which sequesters endogenous ERK1 to prevent its nuclear localization, also inhibited invasion. These results reveal that PEA-15 regulates cancer cell invasion via its ability to bind ERK1/2 and indicate that nuclear entry of ERK1/2 is important in tumor behavior. [Cancer Res 2007;67(4);1536–44]

Introduction
Extracellular signal-regulated kinases (ERK1/2) control diverse cellular functions, such as proliferation, survival, and migration (1–5). ERK1/2 is regulated, in part, by its subcellular localization (6), which can be controlled by phosphoprotein enriched in astrocytes of 15 kDa (PEA-15), a small, death effector domain (DED)–containing protein (7). PEA-15 has at least two distinct functions within the cell: that of regulating ERK1/2 localization by sequestering them in the cytoplasm (8, 9), and of blocking apoptosis by interfering with the assembly of the death-induced signaling complex (reviewed in ref. 10). Increased ERK1/2 activity is found in a majority of breast cancers; downstream of activated Ras and growth factor receptors, ERK1/2 is a major effector kinase in proprogression signaling pathways (11, 12). Alteration of ERK1/2 signaling is therefore an important facet of tumor behavior.

PEA-15 was first identified as an astrocytic protein (13), but has subsequently been identified in a variety of human and mouse tissues (14, 15). It is a regulator of apoptosis, competitively inhibiting the binding of DED-containing proteins to initiator caspases (16). In addition, PEA-15 regulates the activity of integrin adhesion receptors through its interaction with ERK1/2. Active H-Ras leads to inactivation of integrins via an ERK1/2-dependent mechanism. PEA-15 reverses integrin suppression by binding ERK1/2 and redirecting it away from the plasma membrane (9, 17). Because PEA-15 also redirects ERK1/2 activity away from the nucleus, it reduces ERK1/2-dependent transcription (7). Cells can regulate the effects of PEA-15 by phosphorylation at Ser104 and Ser116 (15). Phosphorylation of PEA-15 at these sites by protein kinase C (PKC) and CamKII or Akt, respectively, prevents the binding of ERK1/2 and promotes the binding of the Fas-associated protein with death domain (FADD; ref. 18).

Regulation of apoptosis and ERK1/2 localization and function by PEA-15 suggests that the level of PEA-15 expression could influence cell survival and proliferation, two key determinants of carcinogenesis (19). However, studies in model systems and a few patients have yielded conflicting data. The adenovirus protein E1A reduces tumorigenicity and proliferation of ovarian cancer cells via increased PEA-15 expression (20). In contrast, PEA-15 mRNA levels are increased in metastatic squamous cell carcinoma versus nonmetastatic transformed cells (21), and mRNA analysis suggests that increased mRNA levels are also seen in breast cancer versus normal breast tissue (22). Overexpression of PEA-15 in a transgenic mouse model increases susceptibility to chemically induced skin cancers (23). In addition, increased expression of PEA-15 protein in a cohort of 20 human breast cancers was linked to resistance to chemotherapy (24). Here, we have examined the expression of PEA-15 protein in a panel of 252 breast cancer and normal mammary tissue samples and find that PEA-15 protein is expressed in normal mammary epithelial cells, and its expression is decreased in pathologically invasive cancers, suggesting an inverse relationship between PEA-15 expression and tumor invasion. This relationship was confirmed by observing that PEA-15 expression inhibits cancer cell invasion in vitro, and that short hairpin RNA (shRNA)–mediated reduction of PEA-15 expression increases tumor cell invasion. Finally, we report that the ability of PEA-15 to regulate invasion depends on its ability to bind to ERK1/2, thereby preventing their access to nuclear targets.
Materials and Methods

Cell culture. Chinese hamster ovary (CHO) cells, glioblastoma A172 cells, and HeLa cells were obtained from the American Type Culture Collection (Manassas, VA). Hs757T cells were a generous gift from Dr. Barbara Mueller (The La Jolla Institute for Molecular Medicine, La Jolla, CA). CHO, A172, and HeLa cells were cultured in DMEM with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% L-glutamin, and 1% nonessential amino acids (all from Invitrogen, Carlsbad, CA). Hs757T cells were cultured in a similar medium, with the addition of 10 μg/mL insulin.

DNA constructs and transfection. Most PEA-15 and ERK cDNA expression constructs used in this work have been described previously (9). The PEA-15 mutant L123R was initially described by Hill et al. (25). ERK1 K257E CAAX was constructed using Quikchange Site–directed mutagenesis expression constructs used in this work have been described previously (9).

RNA interference. Plasmid-based siRNA directed against PEA-15 and a control siRNA targeting the nonspecific region of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were constructed in pSilencer-16 (Ambion, Austin, TX) and were a gift from Dr. Joseph Ramos (University of Hawai‘i-Manoa, Honolulu, HI).

CHO and HeLa cells were transfected using Lipofectamine Plus, and glioblastoma A172 cells were transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer's recommended protocols. DNA concentrations were kept constant within experiments. Media were replaced 3 and 6 h posttransfection, respectively. Hs757T cells were transfected using an Amaxa nucleofector device, cell line solution V, program T-30 (Amaza GmbH, Cologne, Germany). All cells were used 24 h posttransfection. For HeLa cell invasion assays, pEGFP-C1 was cotransfected with the designated plasmids at a 1:10 ratio of pEGFP-C1 to construct of interest. For glioblastoma A172 cell invasion assays, green fluorescent protein (GFP) was not cotransfected.

Antibodies, Western blotting, and immunohistochemistry. Rabbit polyclonal anti–PEA-15 4513 was initially described by Krueger et al. (15). Mouse antihegminatinii (HA) tag and were used by DNA constructs and transfection.

Western blotting was performed on samples from the indicated cell lines. Membranes were blocked in 5% nonfat dry milk in Tris-buffered saline (TBS), and primary antibodies were applied overnight at 4°C. The primary antibodies used were as follows: pCHA-MEK2 222/226D (MEK DD) was provided by Dr. M. Weber (University of Virginia, Charlottesville, VA). The pEGFP-C1 vector was obtained from BD-Clontech (Palo Alto, CA). A plasmid-based siRNA directed against PEA-15 and a control siRNA were a gift from Dr. B. Felding-Habermann (The Scripps Research Institute, La Jolla, CA). Anti-MMP-9 and anti-MMP-2 from EMD Biosciences (San Diego, CA) were a gift from Dr. B. Felding-Habermann (The Scripps Research Institute, La Jolla, CA). For MMP blotting, cells transfected with vector alone or pCDNA3 HA-PEA-15 were lysed in 500 μL M2 lysis buffer [20 mM Tris (pH 7.6), 0.5% NP40, 250 mM NaCl, 2.5 mM EDTA, 3 mM MgCl2, 0.02% Protease inhibitor cocktail (Roche, Indianapolis, IN)]. Protein concentration was determined by bicinecinonic acid (BCA) assay (Pierce Biotechnology, Rockford, IL). Fifty micrograms of total cell lysate were resolved on 4%–20% polyacrylamide gels (Invitrogen) in 3%–8% SDS-PAGE buffer. Nitrocellulose membranes were blotted for MMP expression using the above MMP antibodies at a concentration of 1 μg/mL. Antibodies and antigens were analyzed by Western blot hybridization. Immunohistochemistry was done as described by Krueger et al. (15). Tissue array slides were obtained from the Cooperative Breast Cancer Tissue Resource (BCBCTR, Rockville, MD) fully mounted. The slides were kept at 4°C before and after staining. Tissue array analysis. The tissue array (TMA case set 2) was constructed in four replicate blocks. We obtained one section from each replicate; three were used for experimental staining and one was used for immunoglobulin G (IgG) control. The IgG control slide was used to estimate background staining, which was negligible. Each of the experimental slides was scored for average staining intensity by two investigators, to whom the pathologic status of the samples was unknown. These investigators scored staining intensity as 0, no staining; 1, low staining intensity; 2, medium staining intensity; or 3, high staining intensity. The investigators also scored the percentage of epithelial positive for staining, but because these scores were statistically uniform, only staining intensity scores are presented here. Cores that were missing or did not contain any epithelial cells were not scored. Before data analysis, the scores were compared between investigators and were highly comparable. Average staining intensity was then compared with the pathologic information provided for the samples by the CBCTR. This includes age at diagnosis, tumor-node-metastasis (TNM) stage, pathologic case type, race, number of nodes positive, and the size of invasive cancer when available. Statistically significant correlation was found between case type, as well as TNM stage (Fig. 1). There was no association between PEA-15 staining intensity and the age at diagnosis or race (data not shown).

Information about estrogen and progesterone receptor expression, HER2 status, and menopausal status was not available for this array.

Statistical analysis. The average staining intensities between case types and TNM stages were compared using one-way ANOVA, which yielded F = 5.34; df, 4; P = 0.001 for case type and F = 7.15; df, 622; P < 0.001 for TNM stage, indicating that the differences between means reached statistical significance. Post hoc testing using the Tukey-Kramer multiple comparison test showed no significant difference between the normal and ductal carcinoma in situ (DCIS) groups, but significant differences between normal (or DCIS) and all other groups (node negative, node positive, and metastasis). In parallel, no difference was seen between the normal and stage 0 (tumor in situ), but significant differences were seen between these groups and stages I to IV. No statistical difference was seen between stage IIA and stage I, IIA, or IV. We used likelihood ratio χ2 statistics, commonly denoted G2, to test for the homogeneity of staining intensity frequencies among the normal, DCIS, node negative, node positive, and metastasis subgroups (ref. 26, section 3.3.2, p. 48–49). (Staining intensities could be classified as 0, 1, 2, or 3; hence, overall frequencies from the five subgroups could be conveniently summarized in a 5 × 5 contingency table, the rows corresponding to the subgroups and the columns corresponding to the staining intensity categories.) To plot the distribution of staining intensities, the 0 and 1 score groups were combined to yield low, medium, and high groupings. The number of cores scoring in each grouping is represented as a ratio to the total number of cores of that case type. All calculations were done using NCSS statistical software (NCSS, Kaysville, UT).

Invasion assay. Hs757T cells invade in response to a gradient of 20% serum on BD BioCoat Matrigel invasion chambers (BD Biosciences, Bedford, MA). The stimulus was also sufficient for glioblastoma A172 cells. HeLa cells invade in response to growth factor gradients (27). Baseline invasion rates of ~10% were observed in response to epidermal growth factor (EGF, Peprotech, Rocky Hill, NJ). The basic invasion assay was done as follows: cells were transfected with the relevant plasmids and allowed to recover. At 24 h posttransfection, cells were trypsinized, pelleted, and resuspended in 0.5% serum-containing media (starvation media). Approximately 2.5 × 105 cells were plated in a single well of a six-well invasion chamber plate in starvation media. A total volume of 2 mL starvation media was placed in the bottom chamber, to which 20% FBS or 10 ng/mL EGF was added. The cells were allowed to invade overnight, ~16 to 18 h. Invading and noninvading cells were then collected from the top and bottom chambers using trypsin and placed in separate fluorescence-activated cell sorting (FACS) tubes containing 10% FBS DMEM. The cells were pelleted and resuspended in exactly 200 μL of 10% DMEM. For each condition, the upper and lower chamber cell populations were analyzed by FACS analysis for GFP expression. For Fig. 4C, the mitogen-activated protein (MAP)/ERK kinase (MEK) inhibitor PD98059 (Invitrogen) was added as necessary to the upper chamber at a final concentration of 50 μM. Relative invasion is given as the ratio of the percentage of GFP-expressing cells in the lower and upper chambers. This controls for transfection efficiency between conditions and provides a direct measure of invasion in transfected cells only.

In invasion assays using glioblastoma A172 cells, we were not able to cotransfect GFP due to off-target effects on invasion. In this case, FACS analysis was used to measure invasion of the total cell population.


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Therefore, to control for cell number, the upper and lower cell populations were resuspended in the same volume and subjected to flow cytometry for the same amount of time at a constant flow rate. Invasion was calculated as the ratio of total cell number in the lower versus the upper chambers and expressed as a percentage of vector control.

At the time cells were plated into the invasion chambers, the remainder of the transfected cells was lysed for Western blotting of protein expression using 200 μL M2 lysis buffer. Protein concentration was determined by BCA assay. Twenty micrograms of total cell lysate were resolved on 4% to 20% polyacrylamide gels in SDS-PAGE buffer. Nitrocellulose membranes were blotted for PEA-15, HA, ERK, and GAPDH at dilutions listed above.

Results

Expression of PEA-15 is reduced in invasive breast tumors. PEA-15 inhibits certain ERK1/2-mediated functions and some forms of apoptosis, suggesting that its expression could modulate the behavior of tumors. Indeed, expression of this protein has been linked to both increased and decreased tumorigenesis in therapeutic model systems (20, 24). To explore a possible role of PEA-15 in altering tumor behavior, we used immunohistochemistry to examine PEA-15 expression in human mammary cancers of known invasive and metastatic types. We affinity purified a previously described (15) polyclonal anti-PEA-15 antibody and found that it reacted with a single band of molecular weight ~15 kDa upon immunoblotting of CHO cells and glioblastoma A172 cells. A band of increased intensity and slightly higher molecular weight was seen in HA-PEA-15–transfected CHO cells, and no band was seen in lysate from astrocytes derived from PEA-15 knock-out mice (ref. 28; Supplemental Fig. S1A).

![Figure 1. PEA-15 expression is reduced in invasive tumors. A, tissue array tumor cores were stained with affinity-purified anti-PEA-15 4513. A control tissue array slide was stained with normal rabbit IgG at an equivalent concentration, and all slides were counterstained with Mayer’s hematoxylin to detect nuclei. Images are representative of respective case type: normal breast, 10× magnification; normal breast, 40× magnification; DCIS, 10×; rabbit IgG staining of DCIS, 10×; node negative invasive adenocarcinoma, 10×; metastasis of invasive adenocarcinoma, 10×. B, the average staining intensity of each case type shows a significant inverse correlation between PEA-15 expression and invasive pathology. ANOVA and post hoc multivariate analysis indicated a significant difference (P < 0.001) between the normal/DCIS and node-negative, node-positive, and metastasis groups. No significant difference was found between normal and DCIS or between the invasive case types. Columns, mean staining intensity; bars, SE. C, the average staining intensity correlates with the onset of invasion through the basement membrane, as indicated by the TNM stage. Statistical analysis indicated no difference between the normal and stage 0 groups, nor between stages I, IIA, IIB, IIIA, and IV. However, a statistically significant difference was observed between the normal/DCIS and node-negative, node-positive, and metastasis groups. No significant difference was found between normal and DCIS or between the invasive case types. Columns, means; bars, SE. D, the distribution of staining intensity scores shows the change in staining pattern between normal/DCIS and invasive case types. Scores were grouped as follows: 0–1, low; 2, medium; 3, high. Data represent the ratio of the number of samples in each score grouping (low, medium, high) to the total number of samples.](image-url)
In immunohistochemistry, this antibody very strongly stained PEA-15-overexpressing transfected CHO cells (arrowheads); PEA-15–expressing A172 glioblastoma cells showed more general but less intense staining, whereas untransfected CHO cells exhibited minimal staining. Specificity of staining was confirmed by its absence from astrocytes derived from PEA-15 knock-out mice (Supplemental Fig. S1B) and lack of reactivity of the cells with control rabbit IgG (data not shown). Thus, the antibody specifically reacted with PEA-15 in both immunoblotting and immunohistochemistry.

Having confirmed the specificity of this affinity-purified antibody, we used it to stain a tissue array consisting of 288 cores taken from 252 breast cancer and normal mammary tissue samples and 36 nonbreast tissues and cell lines, obtained from the CBCTR.3 Three replicate arrays were stained with anti-PEA-15, and each replicate was scored for staining intensity independently by two blinded investigators. One replicate array was stained with normal rabbit IgG as an additional specificity control. In all mammary-derived tissues, the intensity of PEA-15 staining was homogenous throughout the entire epithelial cell population of each core (Fig. 1A). Therefore, scoring of the percent of cells stained ranged between 75% and 100% and was not incorporated into the PEA-15 staining scores presented in Fig. 1B–D. The intensity of PEA-15 staining was measured as the average staining intensity of the epithelial cells in each sample. The average staining intensity was recorded as 0, no staining; 1, light staining; 2, medium staining; and 3, intense staining, as per widely used pathologic scoring systems (29–31). PEA-15 staining was absent from stromal fibroblasts, adipocytes, and myocytes associated with epithelial ducts and acini (Fig. 1A). Furthermore, staining was uniformly absent in normal rabbit IgG controls (Fig. 1A).

Normal mammary epithelium (Fig. 1A) exhibited intense staining, as did DCIS. In contrast, in invasive tumors, PEA-15 expression was reduced or absent. Invasion is defined here as penetration of tumor cells beyond the basement membrane. Cells might also invade into the vasculature and thereby reach regional lymph nodes and distant sites, but this is not required for a tumor to be defined as invasive (32). The degree of reduction in PEA-15 expression seemed similar in invasive primary tumors (with or without lymph node involvement) and distant metastases (Fig. 1A). Quantification of the average PEA-15 staining intensity (Fig. 1B) confirmed that PEA-15 expression was reduced in invasive carcinoma. Staining intensities were significantly greater in the normal/DCIS subgroups compared with the invasive node-negative/node-positive/metastasis subgroups (Fig. 1B). ANOVA analysis of the mean staining intensity of each case type indicated a significant difference between the means. Additional post hoc analysis indicated no significant difference between normal and DCIS groups (Tukey-Kramer $P > 0.1$), but significant differences between normal and node negative ($P < 0.005$), node positive ($P < 0.005$), and metastases ($P < 0.001$). No significant differences were seen between node-negative, node-positive, and metastasis groups. Similar $P$ values were returned by pairwise comparison with DCIS.

Similar correlations were seen when the staining intensities were analyzed by TNM stage, a pathologic grading system based on tumor size, invasion of tumor cells into adjacent lymph nodes, and

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3 http://cbctr.nci.nih.gov
the presence of metastases (Fig. 1C). Between normal and stage 0, \( P > 0.1 \); between normal and stage I, \( P < 0.05 \); stage IIA, \( P < 0.005 \); stage IIB, \( P < 0.01 \); stage IIIA, \( P < 0.001 \); and stage IV, \( P < 0.002 \). Comparable \( P \) values were returned by pairwise comparison between stage 0 and stages I, IIA, IIB, IIIA, and IV.

The distribution of staining intensity scores for each case type (Fig. 1D) further shows the dramatic change in PEA-15 expression between noninvasive and invasive breast tumors. We used \( \chi^2 \) analysis to study the differences in the proportions of staining scores (33) and found that staining intensities were similar in the normal and DCIS subgroups: \( G^2 = 0.875 \), degrees of freedom \( (df) = 2 \), \( P = 0.65 \). Similarly, staining intensities were similar in the node-negative, node-positive, and metastasis subgroups: \( G^2 = 4.003 \), \( df = 6 \), \( P = 0.69 \). However, staining intensity frequencies varied dramatically between the normal/DCIS and the invasive node-negative/node-positive metastasis categories: \( G^2 = 42.72 \), \( df = 12 \), \( P < 10^{-5} \). Thus, the loss of PEA-15 expression was a marker of the transition between noninvasive (DCIS) and invasive mammary epithelial tumors.

Alterations in PEA-15 expression affect tumor cell invasion. As noted above, the loss of PEA-15 expression was correlated with increased invasiveness in human breast cancers, as indicated by the ability of the tumor to invade through the basement membrane into the surrounding stroma (32). We used an \textit{in vitro} assay that models local basement membrane invasion to test for a potential causal role for PEA-15 in altered invasiveness. The human mammary carcinoma cell line, Hs578T, was originally isolated from an invasive carcinoma and has shown high \textit{in vitro} activity in Boyden chamber invasion assays (34–36). This cell line did not express PEA-15 and was highly invasive in a Matrigel invasion assay. Hs578T cells transfected with a cDNA encoding PEA-15 (Fig. 2B) exhibited \~{}40\% to 50\% reduction in invasion compared with vector-transfected control cells (Fig. 2A). Thus, increasing PEA-15 levels can inhibit the invasion of a breast cancer cell line.

Having shown that reexpressing PEA-15 can inhibit invasion, we asked whether reducing endogenous PEA-15 expression could increase invasion. Human glioblastoma A172 is a poorly invasive tumor cell line that expresses high levels of PEA-15 (Supplemental Fig. S1A). shRNA-mediated reduction of PEA-15 expression doubled the invasiveness of the A172 cells (Fig. 2C) and reduced endogenous PEA-15 expression by 60\% to 80\% (Fig. 2D). shRNA transfection did not reduce the expression of another gene product, GAPDH (Fig. 2D), and a scrambled shRNA had no effect on PEA-15 expression or invasion (data not shown). Transfection with a cDNA-encoding hamster PEA-15, which is not susceptible to the shRNA, reduced the invasion of the shRNA-transfected cells to levels lower than those in the parent cells. This further reduction in invasion is most likely due to PEA-15 overexpression, because transfection with PEA-15 alone also reduced invasion to this level (Fig. 2C and D).

Mutations that block the PEA-15 interaction with ERK1/2 prevent inhibition of tumor cell invasion. The foregoing experiments showed that PEA-15 expression could regulate invasion in breast cancer and glioblastoma. To examine the mechanism of the PEA-15 effect, we extended the analysis to HeLa cells, a widely studied, moderately invasive, human cervical cancer cell line. HeLa cells do not express PEA-15 and are easily transfectable using cationic lipid transfection reagents, thus facilitating study of the regulation of invasion by PEA-15. PEA-15 interacts with several signaling pathways that could modulate invasion; for example, PEA-15 binds both ERK1/2 and RSK2 and blocks their nuclear accumulation and phosphorylation of nuclear substrates (7, 37). In addition, PEA-15 can bind to DED-containing proteins to inhibit certain forms of apoptosis (10) and can bind to phospholipase D and increase its expression (38). We previously reported that mutations in the PEA-15 DED (PEA-15 D74A) and in the COOH-terminal tail (PEA-15 L123R) can inhibit binding to ERK1/2 (9, 17). Each of these mutations reduced the ability of PEA-15 to inhibit the invasion of HeLa cells (Fig. 3A), although they were well expressed (Fig. 3B). The D74A mutation does not impair interaction with RSK2, and L123R binds to phospholipase D (37, 38), suggesting that neither interaction with RSK2 nor phospholipase D is sufficient for suppression of invasion. Furthermore, the interaction of PEA-15 with the DED proteins (i.e., FADD) is promoted by PEA-15 phosphorylation at Ser\textsuperscript{104} and Ser\textsuperscript{116} (18); in contrast, ERK1/2 binding is inhibited by phosphorylation at these sites. Phosphorylation-mimicking Asp mutations at these positions block ERK1/2 binding (15), and PEA-15 bearing these mutations was well expressed but failed to inhibit invasion (Fig. 3A and B). Thus, all of the mutations that perturb the capacity of PEA-15 to bind to ERK1/2 block its ability to inhibit tumor invasion. However, we identified mutations that prevent inhibition of invasion, yet preserve binding to each of the other known partners of PEA-15 (RSK2, phospholipase D, and FADD). Hence, the interaction of PEA-15 with ERK1/2 seems to be essential for inhibition of invasion.

If PEA-15 binding to ERK1/2 is required for inhibition of invasion, then overexpression of ERK1 should overcome the effects of increased PEA-15 expression. When ERK1 was cotransfected with PEA-15, there was no inhibition of HeLa invasion; indeed, invasion was slightly stimulated (Fig. 4A). Furthermore, expression of an activated form of MEK (MEK DD) also rescued the effect of PEA-15 on invasion (Fig. 4B). We then asked whether expression of
PEA-15 was sufficient to block ERK-mediated tumor cell invasion. As seen in Figs. 2 and 3, PEA-15 blocked invasion of both HeLa and Hs578T ~50% to 60%. Treatment of HeLa cells with the MEK inhibitor, PD98059, also inhibited invasion by ~50%. Expression of PEA-15 concurrent with PD98059 treatment did not significantly reduce invasion further (Fig. 4C). Western blotting confirmed the reduction in ERK1/2 activity following PD98059 treatment (Fig. 4C, bottom right). Thus, based on the effects of PEA-15 mutations and of ERK1 overexpression, we conclude that PEA-15 blocks invasion by binding to ERK1/2.

**PEA-15 inhibits invasion by preventing nuclear localization of ERK1/2.** ERK1/2 can regulate invasion by phosphorylating nuclear substrates, thus increasing the transcription of migration-promoting genes (39). However, PEA-15 can also affect transcription-independent activities of ERK1/2, such as suppression of integrin activation (9), and recent studies showed that changes in integrin activation can regulate tumor invasion (40–42). PEA-15 I15A, does not interfere with PEA-15 binding to ERK, but does inhibit the ability of PEA-15 to prevent nuclear localization of ERK1/2 (7). Expression of PEA-15 I15A did not inhibit invasion, suggesting that the nuclear export of ERK1/2 is required for the effect of PEA-15 on tumor cell invasion (Fig. 5A).

Tethering ERK1 to the plasma membrane by the addition of a COOH-terminal H-ras farnesylation sequence (ERK1-CAAX) inhibits the nuclear translocation and transcriptional activities of endogenous ERK1/2 (6) by sequestering endogenous ERK1 at the plasma membrane. Expression of ERK1-CAAX in HeLa cells inhibited invasion, suggesting that preventing nuclear entry of ERK is sufficient to block invasion (Fig. 5A). Transfection of PEA-15 slightly reduced the invasion of ERK1-CAAX–expressing cells; however, there was some residual invasion relative to cells transfected with PEA-15 alone. We suspected that the inability of PEA-15 to further block invasion was due to competition between the membrane-tethered ERK1-CAAX and endogenous ERK1/2 for PEA-15. To test this idea, we used ERK-1 K257E, an ERK mutant that fails to bind PEA-15 (9). ERK-1 K257E-CAAX inhibited invasion, presumably due to the sequestration of endogenous ERK at the plasma membrane.

![Graphs of invasion assays](image-url)
plasma membrane. However, PEA-15 transfection suppressed the invasion of ERK1 K257E-CAAX–expressing cells to levels comparable to those transfected only with PEA-15. PEA-15 failed to reduce the invasion of cells expressing untethered ERK1 K257E, presumably because PEA-15 cannot bind and prevent the entry of ERK1 K257E into the nucleus (Fig. 5C and D). Thus, mutations that inhibit the capacity of PEA-15 to prevent nuclear entry of ERK1/2 also prevent PEA-15 from inhibiting tumor cell invasion. Conversely, sequestering ERK1 in the cytoplasm blocks invasion. Hence, we ascribe the anti-invasive activity of PEA-15 to its capacity to bind ERK1/2 and prevent phosphorylation of their nuclear substrates.

As mentioned above, ERK1/2 phosphorylates nuclear targets, increasing the transcription of many proinvasion genes (39). Of these, perhaps the best characterized are members of the MMP family. The above data suggest that the expression of PEA-15 would modify the expression of ERK1/2-dependent genes. As an initial test of this hypothesis, we transfected Hs578T and HeLa cells with PEA-15 and examined MMP expression. All MMPs tested, including MMP-1, MMP-2, MMP-9, MMP-13, and MMP-3, exhibited decreased expression in cells transfected with PEA-15, whereas the housekeeping protein, GAPDH, remained unaffected (Fig. 6). Thus, PEA-15 is able to alter expression of ERK1/2-dependent promigratory genes.

Discussion

The DED protein, PEA-15, binds to numerous cytoplasmic targets, thus regulating multiple cellular functions potentially involved in the pathogenesis of cancer. Here, we have examined the relationship of PEA-15 expression to the behavior of a human cancer. Immunohistochemistry of mammary tissue arrays revealed that PEA-15 is expressed in mammary epithelium and in carcinoma in situ; loss of PEA-15 expression was correlated with invasive behavior in more advanced tumors. In vitro experiments established a causative role for PEA-15 in the inhibition of invasion. PEA-15 sequesters ERK1/2 in the cytoplasm and inhibited invasion by preventing the nuclear accumulation of ERK1/2 and subsequent expression of proinvasion proteins. These results reveal that PEA-15 regulates cancer cell invasion and tumor progression via its ability to bind ERK1/2 and indicate that nuclear entry of ERK1/2 is important in tumor behavior.

PEA-15 protein is present in normal breast epithelium, and loss of PEA-15 expression was correlated with invasive behavior of breast tumors. These results contrast with those of Stassi et al., who reported that PEA-15 was overexpressed in 16:20 human breast cancers. In their study, high PEA-15 expression correlated with high Akt activity and poor patient prognosis (24). Our studies examined 12 times as many unselected mammary tissue samples as Stassi et al. and found that PEA-15 expression was inversely correlated with invasiveness. However, it is noteworthy that we did observe high PEA-15 expression in ~20% of invasive cancers. Phosphorylation of PEA-15 by Akt and PKC prevents ERK1/2 binding and promotes the antiapoptotic activity of PEA-15 (15, 18). Therefore, the high Akt activity observed by Stassi et al. may explain the inconsistency between the two studies. There is already substantial PEA-15 phosphorylation in normal breast epithelium (15), and we

Figure 5. PEA-15 inhibits invasion by sequestering ERK1/2 in the cytoplasm. A, expression of hamster PEA-15 I15A, which binds ERK1/2 but does not block their nuclear accumulation, does not inhibit invasion. Furthermore, transfection of ERK1-CAAX (which would draw active ERK1 to the membrane) inhibits invasion, and additional expression of PEA-15 does little to increase this inhibition. Columns, means; bars, SE; n = 3. *, P < 0.01 as compared with vector control. B, representative expression of PEA-15 and ERK1 constructs in cells used for invasion assay; anti-ERK (top), anti-PEA-15 (middle), anti-GAPDH (bottom). C, an ERK1 mutant that does not bind PEA-15 rescues invasion (K257E). However, addition of a membrane targeting sequence (CAAX) to this PEA-15–insensitive ERK renders it no longer able to reverse PEA-15–mediated inhibition of invasion. Columns, means; bars, SE; n = 3. *, P < 0.01 as compared with vector control. D, representative expression of PEA-15 and ERK1 constructs in cells used for invasion assay; antihemagglutinin (top) and anti-GAPDH (bottom).
noma, and other cancers (Unigene, Bethesda, MD), and our studies show that expression of PEA-15 inhibits tumor invasion.

Figure 6. PEA-15 reduces expression of MMPs. Expression of MMP-1, MMP-2, MMP-9, MMP-13, and MMP-3 is reduced in Hs578T (A) and HeLa (B) cells expressing PEA-15. Lysates from PEA-15 or vector-transfected cells were probed for the listed MMPs and GAPDH. The blots were quantified by densitometry analysis using the Odyssey IR imaging system from Licor. Columns, percentage of expression in vector-transfected cells; bars, SD. Hs578T: n = 3 for MMP-2, MMP-9, MMP-13, and GAPDH, n = 1 for MMP-1 and MMP-3; HeLa; n = 3 for MMP-1, MMP-2, MMP-9, MMP-13, and GAPDH, n = 1 for MMP-3.

The findings presented here suggest that PEA-15 is a regulator of the behavior of mammmary epithelial cancers. ERK1/2 kinases play a critical role in the capacity of Ras and its downstream targets, Raf1 and MEK1, to increase both proliferation and invasion of tumor cells (30). PEA-15 also inhibits the in vitro migration of astrocytes, possibly by changing the expression of an isoform of PKC-α kinase (51). In our studies, we found that the effects of PEA-15 on invasion could be explained by its interactions with ERK1/2. PEA-15 expression correlated with reduced breast cancer invasiveness, and enforced expression of PEA-15 inhibited the invasion of cancer cell lines in vitro. Furthermore, using mutants of PEA-15 and/or ERK, we showed that PEA-15 blocks invasion by binding to ERK1/2 kinases. Previous studies focused on how specific enzymatic activities can regulate the activation of ERK1/2 (3); the present work shows that the biological consequences of ERK1/2 signaling accounts for the effects of PEA-15 on tumor cell invasion, and that this effect is likely to be mediated by blocking the nuclear localization of ERK1/2 and the resulting transcriptional activity.

The wide expression of PEA-15 suggests that it may regulate the behavior of many types of tumors. PEA-15 is highly expressed in astrocytes (13); however, PEA-15 mRNA is found in many epithelial and nonepithelial tissues, and we found staining for PEA-15 protein in control tissues on the tissue array, including those in normal appendix, prostate, kidney, and endometrium (data not shown), where PEA-15 was seen in multiple cell types. PEA-15 mRNA is found in glioblastoma, breast adenocarcinoma, melanoma, and other cancers (Unigene, Bethesda, MD), and our studies showed its capacity to regulate the invasive behavior of tumor cell lines derived from both the ectoderm (brain and breast) and mesoderm (cervix). Therefore, it is reasonable to suggest that the effects of PEA-15 on breast, cervical, and glial cancer cell behavior could occur in other tumor cells; further study will be required to define the breadth of the impact of PEA-15 in cancer. This study is not sufficient to conclude that PEA-15 is an independent factor related to tumor aggressiveness. However, our findings that PEA-15 inversely correlates with the invasion of breast cancer in vivo, and that expression of PEA-15 suppresses invasion of breast tumor cells, suggest that the level of PEA-15 expression may contribute to the biological behaviors of breast cancer.

In this work, several lines of evidence show that the effects of PEA-15 on tumor cell invasion was mediated by its interaction with ERK1/2 and suggest that this interaction prevents the nuclear localization of ERK1/2. A mutational analysis of PEA-15 showed that those mutations that perturbed ERK1/2 binding interfered with the ability of PEA-15 to block invasion, and we could complement the effect of PEA-15 on invasion through overexpression of ERK or constitutively active MEK. Tethering ERK1 to the plasma membrane by means of a prenylated membrane-targeting sequence (ERK1-CAAX) sequesters ERK1 in the cytoplasm (6). We found that ERK1-CAAX suppressed invasion, suggesting that sequestration of ERK1 can block invasion. Notably, ERK1-CAAX also reduces integrin activation (9), and recent studies (41, 42) suggest that this could also block invasion. This explanation seems unlikely to account for the effects of ERK1-CAAX and PEA-15 on invasion because the transfection of cells with PEA-15 increases integrin affinity while inhibiting invasion. Finally, the anti-invasive activity of PEA-15 was blocked by a mutation that prevents its export from the nucleus. ERK1/2 fosters increased transcription of genes such as MMPs (43, 44) and urokinase plasminogen activator and its receptor (45). ERK1/2 can also promote invasiveness by reducing the transcription of tight junction proteins (46) and metastasis suppressor genes (47). In addition, extranuclear activities of ERK1/2, including the activation of cell motility machinery (48) and disruption of focal adhesions (49), may also contribute to increased invasiveness. Expression of multiple MMPs, which are known to be regulated by the ERK-sensitive activator protein-1 promoter, was reduced in cells expressing PEA-15. Therefore, we conclude that interference with ERK1/2 signaling accounts for the effects of PEA-15 on tumor cell invasion, and that this effect is likely to be mediated by blocking the nuclear localization of ERK1/2 and the resulting transcriptional activity.

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The findings presented here suggest that PEA-15 is a regulator of the behavior of mammary epithelial cancers. ERK1/2 kinases play a critical role in the capacity of Ras and its downstream targets, Raf1 and MEK1, to increase both proliferation and invasion of tumor cells (30). PEA-15 also inhibits the in vitro migration of astrocytes, possibly by changing the expression of an isoform of PKC-α kinase (51). In our studies, we found that the effects of PEA-15 on invasion could be explained by its interactions with ERK1/2. PEA-15 expression correlated with reduced breast cancer invasiveness, and enforced expression of PEA-15 inhibited the invasion of cancer cell lines in vitro. Furthermore, using mutants of PEA-15 and/or ERK, we showed that PEA-15 blocks invasion by binding to ERK1/2 kinases. Previous studies focused on how specific enzymatic activities can regulate the activation of ERK1/2 (3); the present work shows that the biological consequences of ERK1/2 signaling in tumor cells can be redirected by cellular expression of endogenous ERK1/2 modulators such as PEA-15. Because of the pivotal role of ERK1/2
signaling and apoptosis in many cancers, our findings suggest that the enforced expression of proteins such as PEA-15 may offer a means to change tumor cell behavior. Similarly, agents that increase PEA-15 expression, such as adenovirus EIA (20), or those that modulate its phosphorylation may also provide new therapeutic options.
PEA-15 Inhibits Tumor Cell Invasion by Binding to Extracellular Signal-Regulated Kinase 1/2
