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ERM Is Expressed by Alveolar Epithelial Cells in Adult Mouse Lung and Regulates Caveolin-1 Transcription in Mouse Lung Epithelial Cell Lines

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Abstract We previously identified an Ets cis-element in the mouse caveolin-1 promoter that is selectively activated in lung epithelial (E10), but not lung endothelial murine lung endothelial cell line (MFLM-4), cell lines and therefore appears important for differential, cell-specific caveolin-1 transcription. In the present study, we demonstrate that immunostaining of adult mouse lung detects the ETS protein Ets-related molecule (ERM PEA3) in distal lung epithelium in alveolar type I and II cells, but not in bronchial epithelium or lung endothelial cells. We tested ERM and polyomavirus enhancer activator 3 (PEA3) for their ability to increase endogenous caveolin-1 transcripts and to activate caveolin-1 promoter fragments containing the −865 Ets cis-element. Chromatin immunoprecipitation (ChIP) assays show that both ERM and PEA3 bind to the caveolin-1 promoter in murine E10, but not MFLM-4, cells. Normalized luciferase activities show that only ERM activates the caveolin-1 promoter in E10 cells, but neither protein enhances promoter activity in MFLM-4 cells. Mutation of the Ets site blocks ERM-mediated promoter activation in E10 cells. Furthermore, overexpression of ERM increases the cellular content of caveolin-1 mRNA and protein, in E10, but not MFLM-4, cells. The effects of PEA3 on the cellular content of endogenous caveolin-1 expression are variable. These results demonstrate that ERM is involved in caveolin-1 regulation in a murine lung epithelial, but not lung endothelial cell line. We conclude that transcriptional regulation of caveolin-1 differs markedly between lung epithelial and endothelial cell lines, perhaps explaining why the onset of caveolin-1 expression differs in epithelial and endothelial cells during lung development. J. Cell. Biochem. 102: 13–27, 2007.

Key words: caveolin-1; transcriptional regulation; alveolar type I cells; ETS proteins; ERM; PEA3

We previously predicted that the caveolin-1 promoter might be differentially regulated in lung epithelial and endothelial cells because, during lung development, the onset of caveolin-1 expression in these two cell populations in the peripheral lung differs markedly [Ramirez et al., 2002]. We recently reported the identification of a cis-element (located between −844 and −865 bp of the proximal mouse caveolin-1 promoter) containing the core Ets family consensus sequence, 5′GGAA/T-3′, that strongly enhances caveolin-1 gene expression in a murine lung epithelial cell line (E10), but has no significant effects on caveolin-1 transcription in a murine lung endothelial cell line (MFLM-4) [Kathuria et al., 2004]. We showed by gel shift analyses that, although three ETS family members, ETS-1, PEA3, and ERM, are expressed in both cell lines, they recognize and bind in vitro to the Ets site in the epithelial, but not in the endothelial, cell line [Kathuria et al., 2004]. In the present study, we localized the expression pattern of selected ETS proteins in the distal mouse lung. We then tested ERM and PEA3 for their ability to increase endogenous caveolin-1 transcripts and to activate caveolin-1 promoter fragments containing the identified −865 Ets cis-element.

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Identifying the ETS transcription factors responsible for transactivating the caveolin-1 promoter is complex because they comprise a very large family of proteins of more than 25 members in both mice and humans [Sharrocks, 2001; Galang et al., 2004; Hollenhorst et al., 2004]. In addition several detailed studies have shown that all tissues and cell types examined express at least 16 different ETS proteins simultaneously, albeit in different abundance [Hollenhorst et al., 2004].

Despite a high degree of overlapping expression of Ets genes within a single cell type, there is compelling evidence for cell-specific and promoter-specific utilization of ETS proteins. Using the promoter of the MHC class I-like glycoprotein CD1d1, Geng et al. [2005] shows that ELF-I enhances expression in B cells, but not other cell types, while in macrophages PU.1 inhibits transcription of the same promoter by binding to the same Ets cis-element. Likewise chromatin immunoprecipitation assays of Jurkat and HCT116 cells, both of which express ETS-1, ETS-2, and ELK1, show differential activation when tested with two different promoters. The EGR-1 (early growth response −1 gene) promoter binds only ELK-1 in both cell types, while the CDC2L2 (cell division control 2 like 1 gene) promoter binds ETS 1 and ETS2 in both cell types [reviewed by Hollenhorst et al., 2004]. These kinds of studies show that, while identification of the ETS proteins expressed by cells of interest is important, this information alone provides little insight into which proteins are functionally important for activation of a specific promoter.

A second level of complexity relates to understanding how specific ETS proteins function selectively from within a mixture of many such proteins in a single cell. A number of factors are known to contribute to the regulatory specificity of individual family members including protein abundance, post-translational modifications including phosphorylation, acetylation, and sumoylation, modifications by signaling molecules, interactions with partner proteins, and others [Chen et al., 2003; Hollenhorst et al., 2004; Kopp et al., 2004; Geng et al., 2005; Macauley et al., 2006]. Additional regulation is through autoinhibition, a process by which cis-acting inhibitory modules negatively regulate DNA binding through intramolecular interactions [Sharrocks, 2001]. Recent data suggest that some of these regulatory mechanisms may be quickly and dynamically regulated, as shown by changes in protein phosphorylation that confer graded activation and inactivation, correlating with graded DNA binding affinity [Pufall et al., 2005].

Our studies are focused on understanding caveolin-1 regulation in the lung, a major tissue site of expression of this protein. In lung, caveolin-1 is expressed by type I alveolar epithelial cells, fibroblasts, and endothelial cells which collectively are the major cell types of the gas exchange region of the lung. While little is known about its specific functions in the peripheral lung, many functions have been ascribed to caveolin-1 including negative regulation of molecular signaling proteins (e.g., Src tyrosine kinases), inactivation of certain enzymes (e.g., eNOS), entry of viruses, and others [Marjomaki et al., 2002; Richards et al., 2002; Peters et al., 2003; Sanchez-San Martin et al., 2004; Williams and Lisanti, 2005].

Targeted deletions of caveolin-1 lead to pulmonary hypertension and hyperproliferative lung cells [Drab et al., 2001; Razani and Lisanti, 2001; Zhao et al., 2002]. Furthermore, it was recently shown that siRNA-induced caveolin-1 knockdown in mice results in increased lung vascular permeability [Miyawaki-Shimizu et al., 2006]. These kinds of observations provide a strong impetus for exploring how caveolin-1 expression is regulated in lung cells and whether regulation is cell-type specific.

We have previously shown that the lung cell lines of interest express several ETS proteins, including ERM and PEA3 mRNA and protein [Kathuria et al., 2004]. PEA3 and ERM, members of the PEA3 subgroup of highly similar ETS proteins, appear to be involved in organogenesis, particularly of branching organs, and cancer development and progression [Sharrocks, 2001; Hollenhorst et al., 2004; 20–21]. Both are expressed in epithelium of the early lung bud. During branching morphogenesis, ERM expression remains epithelial while PEA3 is expressed in both epithelium and mesenchyme. In newborn mice, expression of both genes is primarily mesenchymal [Chotteau-Lelievre et al., 1997; Liu et al., 2003].

Northern analyses indicate that ERM, but not PEA3, is expressed in adult mouse lung [Chotteau-Lelievre et al., 1997; Liu et al., 2003]. Calculation of mRNA abundance by QRT-PCR indicates that ERM is present at high levels in adult human lung and that low levels of PEA3

are also detectable (~1 message copy/cell) [Hollenhorst et al., 2004]. Since the lung is composed of more than 40 different cell types there is the possibility that one or a few particular cell type(s) express relatively high levels of PEA3. Alveolar epithelial cells seem likely candidates since PEA3 is expressed in the distal epithelium during lung development and is highly expressed in lung adenocarcinomas, which are epithelial derivatives.

It has recently been shown by in situ hybridization that ERM is increasingly restricted to the distal lung epithelium during mouse lung development [Lin et al., 2006]. Furthermore, ERM, through its interactions with TTF-1 (Nkx2.1), is involved in regulation of surfactant protein-C (SP-C), a protein restricted to alveolar type II epithelial cells [Lin et al., 2006]. In the present study we have demonstrated by immunohistochemistry, that in the adult mouse, ERM is expressed in both alveolar type I and II cells, but not in bronchial epithelium or lung endothelial cells. Based largely on their expression patterns in murine lung, we hypothesized that PEA3 and ERM were likely candidates to activate caveolin-1 transcription in distal lung epithelial cells, but would perhaps have little or no effect in MFLM-4 cells.

To test this, we first performed chromatin immunoprecipitation (ChIP) binding assays which showed that both ERM and PEA3 bind the caveolin-1 promoter (either directly or indirectly) at or near the −865 Ets cis-element in vivo in E10 but not MFLM-4 cells. We then transfected the two lung cell lines with CMV-driven expression constructs for ERM and PEA3 and measured the effects on caveolin-1 expression in two ways. First, we cotransfected caveolin-1 promoter-luciferase constructs containing either the wild-type Ets site (−865 Luc) or mutated Ets site (−865 M Luc) and measured luciferase activity and, second, we assessed the effects of increased PEA3 or ERM expression on cellular content of caveolin-1 mRNA and protein, measured by Northern analyses and immunoblotting, as representative of activation of the endogenous caveolin-1 promoter.

We report here that the effects of PEA3 and ERM are cell-type dependent. ERM activates the caveolin-1 promoter in E10 cells, but neither protein increases transcriptional activity in MFLM-4 cells. Overexpression of ERM also increases the cellular content of both caveolin-1 mRNA and protein, likely due to increased activation of the endogenous promoter, in E10, but not MFLM-4 cells. The effects of PEA3 on the cellular content of endogenous caveolin-1 mRNA and protein were variable in the E10 cells. Taken together, these results demonstrate that ERM enhances caveolin-1 transcription in a murine lung type I epithelial cell line, but not in a MFLM. Understanding the details of this differential regulation is likely to be important, as PEA3, ERM, and caveolin-1 expression are known to be altered in lung tumor pathogenesis, progression, and metastasis.

MATERIALS AND METHODS

Immunohistochemistry

Lungs were fixed by intratracheal instillation of freshly prepared 4% paraformaldehyde/0.1% glutaraldehyde (Ladd) in 0.1 M phosphate buffer, pH 7.4. Tissues were stored overnight at 4°C and processed into paraffin using standard methods. Endogenous peroxidases were quenched with 3% H_2O_2 in MeOH for 15 min at room temperature, and sections were blocked with CAS-Block (Zymed) for 1 h at room temperature. Sections were incubated with primary antibody in PBS for 16 h at 4°C. For ERM, the tissue sections were incubated with goat polyclonal anti-ERM antibody (1:4,000–1:7,000 dilution) (catalog number sc-22807, Santa Cruz Biotechnology, CA.) Antibody binding was detected using the Vectastain Elite ABC kit as directed with diaminobenzidine as the chromagenic substrate. Control slides lacking primary antibody were included in all procedures. Immunostaining with an isotype-matched irrelevant antibody (goat polyclonal anti-FGF-10 antibody) was performed to test specificity of binding. Sections were counterstained with methyl green or left unstained and photographed in a Leitz Aristopan microscope using Improvision software.

Culture and Characterization of the Cell Lines

The mouse cell lines used for these studies are E10, an adult lung epithelial cell line, that expresses 13 mRNAs shown to be specific for the type I cell (versus type II cell) in murine lung [Kathuria et al., unpublished data], [provided by Dr. A Malkinson (University of Colorado, Denver, CO) and Dr. Randy Ruch (Medical College of Ohio)]; and MFLM-4, a fetal lung

endothelial cell line, [provided by Dr. Ann Akeson (Children's Hospital, Cincinnati, OH)]. E10 and MFLM-4 cells were cultured as described previously [Kathuria et al., 2004].

Chromatin Immunoprecipitation Assays

E10 and MFLM-4 cells (1×107) were fixed with 1% formaldehyde in DMEM media for 10 min at RT with gentle shaking. Cells were collected, centrifuged at 4,000 rpm for 10 min, washed twice with cold PBS, and resuspended in 300 μl of lysis buffer containing 1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.1, 1 mM PMSF, 1 mM pepstatin A, and 1 mM aprotinin. Samples were sonicated on ice in a sonicator (Fisher Scientific) to ~500 bps using a cup horn attachment [Power 5, 5 cycles (each cycle 5 min) of 25 s on, 5 s off]. Debris was removed by centrifugation at 13,000 rpm for 10 min at 4°C. 50 μl of the supernatant containing the DNA fragments were de-crosslinked at 65°C overnight and run on a 1% agarose gel to assess fragment size.

Input DNA (5% of total) was reserved for baseline measurements. The remainder was diluted to 2 ml in buffer containing 0.01% SDS, 1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1, and 150 mM NaCl, pre-cleared with salmon sperm DNA/protein A agarose beads (30 μl) and normal mouse IgG (1 μl) for 2 h at 4°C with rotation. Supernatants were incubated with normal mouse IgG (2 μg) (Santa Cruz, CA) or antibodies (2 μg) against ERM (rabbit polyclonal IgG, Santa Cruz #sc-22807X), or PEA3 (mouse monoclonal IgG, Santa Cruz #sc-113X), overnight at 4°C with rotation. Immune complexes were collected with salmon sperm DNA/protein A agarose beads (30 μl) for 2 h at 4°C, washed three times in dilution buffer (above) followed by one wash with dilution buffer containing 500 mM NaCl. The pellets were resuspended in 150 μl ChIP assay elution buffer (1% SDS, 0.1 M $NaHCO_3$), rotated at RT for 15 min, then centrifuged with collection of supernatants. Cross-links were reversed in all samples (including diluted inputs) by heating samples overnight at 65°C. Proteins were degraded with 40 μg proteinase K at 37°C for 30 min. DNA was extracted with phenol/ chloroform, precipitated in EtOH, and resuspended in 50 μl of H_2O. DNA fragments were analyzed by PCR for caveolin-1 fragments spanning the −865 ETS site (196 bp amplicon) and for β-actin (control for non-specific binding). Primers for caveolin-1 were as follows: 5′-ggccagcttttgaaactgat-3′ and 5′-ttctgcctggagattaaacactc-3′. Primers for β-actin were as follows: 5′-gcttctttgcagcctccttcgttg-3′ and 5′-tttgcacatgccggagccgttgt-3′.

DNA Constructs

Expression vectors for murine PEA3 (pCANmyc/PEA3) and ERM (pCANmyc/ERM) were gifts of Dr. John Hassell (McMaster University, Canada) and have been described previously [Bojovic and Hassell, 2001; Shepherd et al., 2001]. Briefly, the pCANmyc1 expression vector contains a CMV-promoter upstream of a c-Myc epitope. When cDNAs downstream of this epitope are expressed, fusion proteins bearing the Myc epitope are synthesized. pCANmyc/PEA3 contains full length mouse PEA3 cDNA cloned into pCANmyc1. pCANmyc/ERM contains full length mouse ERM cDNA cloned into pCANmyc1. Sequences were verified to ensure that no mutations were introduced.

To test whether the different cell lines could produce similar amounts of PEA3 and ERM from the expression vectors, within each transient transfection experiment two wells were independently analyzed to determine the abundance of exogenous ERM and PEA3 expression by immunoblot analysis using an anti-9E10 c-Myc monoclonal antibody (Zymed Laboratories, CA, 1:1,000). The cell lines were transfected with the appropriate c-Myc tagged effector plasmid (pCANmyc/PEA3, pCANmyc/ERM) or CMV-control; cell lysates were immunoprecipitated for c-Myc protein using the ProFound Mammalian c-Myc Tag IP/Co-IP kit (Pierce Biotechnology, IL) according to the manufacturer's protocol. For analysis of c-Myc protein, PVDF membranes were blocked in 1X TBST containing 10% dry milk (1 h, RT) exposed overnight at 4°C to anti-9E10 c-Myc monoclonal antibody and then to goat anti-mouse secondary antibody (1:10,000, 1 h, RT). Binding of labeled HRP-secondary antibodies was detected with Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). This assay therefore only detects ERM and PEA3 expression that is c-Myc tagged (i.e., exogenous ERM or PEA3).

−865 bp fragment of the 5′ caveolin-1 promoter (−865 Luc) was generated from murine genomic DNA by PCR cloning using the published mouse caveolin-1 promoter sequence (GenBank accession number AF124227), as previously described [Kathuria et al., 2004].

Briefly, purified fragment was ligated into the promoterless luciferase expression vector, pGL3-basic vector (Promega), and confirmed by sequence analysis. Constructs containing a mutated Ets cis-element between −865 and −844 (−865M Luc) were generated by PCR using −865 Luc as the template. The forward primers were oligonucleotides for the mutated Ets site (−865M: agaggatgt → cgcgtaagt). The reverse primer was a 30-mer oligonucleotide complementary to the 3′ wildtype sequence. After sequence verification, computer algorithms (Match-Public, Alibaba2) were used to ensure that no other known enhancer/repressor sites had been created in the mutant constructs. All constructs were digested with Nhe-1 and Sac-1 prior to insertion into the pGL3-basic vector. Both caveolin-1 constructs contain +62 bps of the untranslated region.

Transfection and Reporter Assay Activity

Constructs (−865 Luc or −865 M Luc and Renilla luciferase control plasmid) were transiently cotransfected with c-Myc tagged expression vectors encoding CMV-PEA3 (pCANmyc/PEA3), CMV-ERM (pCANmyc/ERM), or CMV-control into the cell lines using Lipofectamine transfection reagent (Invitrogen, CA) and Plus Reagent (Invitrogen, CA). Optimal transfection efficiency for the E10 cell line was achieved using 0.8×10^5 cells per 35-mm dish, 3 μg of total plasmid DNA (2 μg reporter constructs and 1 μg expression vectors), 5 μl DNA of transfection reagent, and 20 μl Plus Reagent. For the MFLM-4 cell line, 0.9×10^5 cells per 35-mm dish, 1.5 μg total plasmid DNA (1 μg of reporter constructs, 0.5 μg expression vectors), 2.5 μl DNA of transfection reagent, and 10 μl Plus Reagent were used for optimal transfection efficiency.

Cells were first grown to 60–80% confluency. The transfection mixture containing 125 μl of serum-free medium and the Lipofectamine Reagent were pre-incubated for 15 min. Plasmid DNA and Plus Reagent were incubated for 15 min to pre-complex DNA. The two mixtures were combined and further incubated for 15 min. The standard culture medium was replaced with 1 ml of fresh serum-free medium. The transfection mixture was added dropwise to the tissue culture dish, and after 3 h (37°C in 5% CO_2), an equal volume of serum-containing medium was added. The cells were incubated using standard growth conditions (37°C in 5% CO_2) for 48 h, harvested, washed three times with PBS, lysed, and analyzed for both luciferase activities with the Dual Luciferase Reporter assay kit (Promega) according to the manufacturer's instructions.

Luminescence was detected in a Berthold Lumat LB 9501 (Berthold, Nashua, NH) luminometer; promoter driven firefly luciferase activity was normalized to Renilla luciferase activity. The fold stimulation for luciferase was calculated as normalized luciferase activity obtained in cells expressing ETS family members divided by the luciferase activity of samples originating from vector-transfected control cells. Data are expressed as the mean of at least three experiments (duplicate samples) ± S.E. Luciferase activities are presented relative to the level of expression of a promoterless construct, pGL3. Data were analyzed by *t*-test with differences $P \leq 0.05$ considered significant.

Northern Blots

To determine if overexpressing PEA3 or ERM upregulated endogenous caveolin-1 mRNA expression, total RNA from cells transfected with the pCANmyc expression vectors or CMV-control alone was isolated using TRIZOL reagent (Life Technologies). Northern blots were prepared by the glyoxal/DMSO denaturation method. Total RNA (10 μg for E10 cells and 20 μg for MFLM-4 cells) was electrophoresed on 1.5% agarose gels, blotted, hybridized, and washed by standard methods [Cao et al., 2003]. [32P]-labeled probes [caveolin-1, 838 bp coding sequence (human)] were prepared by the random hexamer primer method. After the first exposure, blots were stripped and re-probed for β-actin to normalize for loading. Film exposure times were selected to ensure that signals were in a linear range. Densitometry (ImageQuant; Molecular Dynamics) of auto-radiograms was used for the semi-quantification of endogenous caveolin-1 gene expression. Images selected for publication are the most representative blots.

Western Blots

To determine if overexpressing PEA3 or ERM upregulated endogenous caveolin-1 protein expression, total protein from cells transfected with the pCANmyc expression vectors or CMV-control alone was isolated and immunoblotted for caveolin-1. Briefly, cell monolayers were washed with ice-cold phosphate buffered saline, and scraped with 500 μl PBS. The cells were

concentrated by centrifugation and resuspended in lysis buffer with inhibitors and then incubated with rotation (30 min, 4°C). The lysate was centrifuged for 2 min at 13,000 rpm at 4°C in a microcentrifuge and the supernatant (30 μg) was used for immunoblot analysis.

For analysis of caveolin-1 protein, PVDF membranes were blocked in 1X TBST containing 5% dry milk (1 h, RT) exposed overnight at 4°C to anti-caveolin-1 monoclonal antibody (BD Sciences, #610406, 1:1,000), and then to goat anti-mouse secondary antibody (1:10,000, 1 h, RT). Binding of labeled horseradish peroxidase-secondary antibodies was detected with Super-Signal West Pico Chemiluminescent Substrate (Pierce). For densitometry of autoradiograms, film exposure times were selected to ensure that signals were in a linear range. Images selected for publication are the most representative blots.

RESULTS

In the Adult Mouse Lung, ERM is Expressed in Both Alveolar Type I and II Cells, but not in Bronchial Epithelial and Lung Endothelial Cells

To determine the cell type(s) in the distal lung that express ERM, we performed immunohistochemistry on adult mouse lung tissue. We show expression of ERM in the cytoplasm of alveolar type I and II cells, but not in small airway epithelium or lung endothelial cells (Fig. 1). We do not detect PEA3 in normal adult

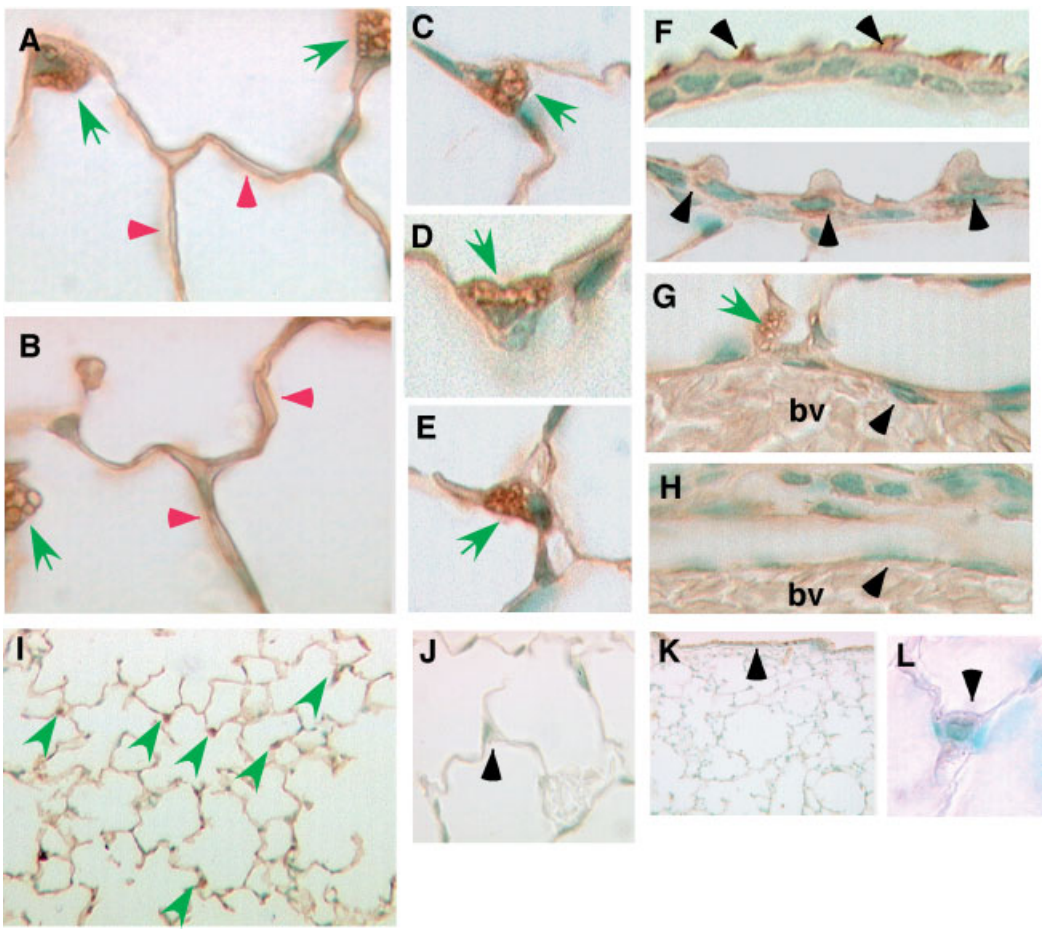

Fig. 1. Immunostaining of ERM in adult mouse lung. (**A-B**) Both epithelial type I (red arrows) and type II (green arrows) cells express ERM. (**B**) Continuous staining of the cells lining the air spaces indicates that epithelial type I cells express ERM in the cytoplasm. Staining of type I cells is seen on both surfaces of an alveolar septum, as would be expected. (**C-E**) At higher magnification, it is clear that type II cells are positive (green arrow). **(F)** Airway epithelial cells do not express ERM. There is non-specific staining in the cilia of bronchial epithelial cells. (**G-H**) Lung endothelial cells lining the blood vessels (bv) do not express ERM. (**I**) At lower magnification, type II cells are positive (green arrow). **(J)** Control sections using secondary antibody alone show no staining in the distal lung. (**K-L**) There is no staining with a non-specific isotype matched irrelevant antibody (goat polyclonal anti-FGF-10) except for non-specific staining in the cilia of bronchial epithelial cells (black arrow in (**K**)).

lung tissue by RT-PCR [Kathuria et al., 2004] or by immunohistochemistry (data not shown).

Staining of alveolar type I and II cells is detected in the adult lung (Fig. 1A–B). There is continuous staining of the alveolar walls in a pattern that matches the expression of the type I cell marker T1α, indicating that type I cells express ERM. Continuous staining on both sides of the alveolar septae is seen, consistent with the known location of type I cells. There does not appear to be nuclear staining of either alveolar type I or II cells. Higher magnification shows that type II cells express ERM (Fig. 1C–E). Sections of the adult lung show that blood vessels and airway epithelial cells do not stain for ERM (Fig. 1F–H). There is non-specific staining of cilia in the bronchial epithelial cells. Lower magnification shows that type II cells express ERM (Fig. 1I). Type I cells cannot be resolved at this magnification although there is a low level of signal in the thin alveolar septae, each of which is composed of two type I cells. Control sections using secondary antibody alone show no staining in the distal lung (Fig. 1J). There is no staining with a non-specific isotype matched control antibody (goat polyclonal anti-FGF-10) except for non-specific staining in the cilia of bronchial epithelial cells. (Fig. 1K–L).

ERM and PEA3 Bind to the Caveolin-1 Promoter In Vivo in E10 but not MFLM-4 Cells by ChIP Assays

To determine whether ERM and PEA3 bind to the caveolin-1 promoter in vivo, we performed ChIP experiments in E10 and MFLM-4 cells. After cross-linking the proteins bound to DNA followed by sonication, cell extracts were immunoprecipitated using IgG and antibodies against ERM or PEA3. After precipitation, samples were analyzed by PCR for the caveolin-1 promoter region spanning the −865 Ets site. Similar to our in vitro binding assays [Kathuria et al., 2004], these data clearly show that in vivo, PEA3 and ERM bind to the caveolin-1 promoter at or near the −865 Ets site in the E10, but not MFLM-4 cells. For β-actin, there is no difference between IgG and the specific antibodies (Fig. 2). Due to the variable sizes of promoter DNA fragments that are generated when DNA is sheared in ChIP assays, these experiments can only determine that ERM and PEA3 bind the promoter somewhere within the region of average size (0.5 and 1 kb) obtained by shearing chromatin. However, we previously showed by gel shift analyses that nuclear extracts from both lung cell lines contain proteins that can bind specifically to the ETS binding domain [Kathuria et al., 2004]. Furthermore, EMSA studies using a 32P-labeled probe with a mutated ETS site (agaggatgt → cgcgtaagt) did not form nuclear complexes in either cell line [Kathuria et al., 2004]. These combined ChIP and gel shift data strongly suggest that in vivo, PEA3 and ERM bind to the −865 Ets site in the caveolin-1 promoter.

Both E10 and MFLM-4 Cells Transfected With pCANmyc/PEA3 or pCANmyc/ERM Produce Exogenous PEA3 and ERM

Since the pCANmyc1 expression vectors contain a CMV-promoter upstream of a c-Myc epitope, when cDNAs downstream of this epitope are expressed, fusion proteins bearing this c-Myc epitope are synthesized. To ensure that constructs were able to drive PEA3 or ERM protein expression, cells transfected with pCANmyc/PEA3, pCANmyc/ERM, or CMV-control were harvested, immunoprecipitated for c-Myc, and then immunoblotted for c-Myc.

Immunoblots (Fig. 3A) only detect ERM and PEA3 expression that has been driven by the CMV-promoter. When E10 and MFLM-4 cells were transfected with pCANmyc/PEA3 or pCANmyc/ERM, both cell lines were able to produce exogenous PEA3 and ERM. Figure 3A shows immunoreactive bands detected at 67 kD and 82 kD in cells transfected with pCANmyc/PEA3 and pCANmyc/ERM, respectively. We observe no bands in cells transfected with CMV-control.

ERM Transactivates Caveolin-1 Promoter Activity in E10 Cells

Cotransfection of E10 cells with caveolin-1 promoter-luciferase constructs containing the wild-type Ets site (−865 Luc) and pCANmyc/ERM increases luciferase activity ∼fourfold (n = 3). Cotransfection of E10 cells with −865 Luc and pCANmyc/PEA3 did not significantly increase luciferase activity. Cotransfecting the caveolin-1 promoter-luciferase construct containing the mutated Ets site (−865M Luc) and either pCANmyc/ERM or pCANmyc/ PEA3 did not increase luciferase activity [Fig. 3B(1)].

Neither PEA3 nor ERM transactivate the caveolin-1 promoter in MFLM-4 cells. In

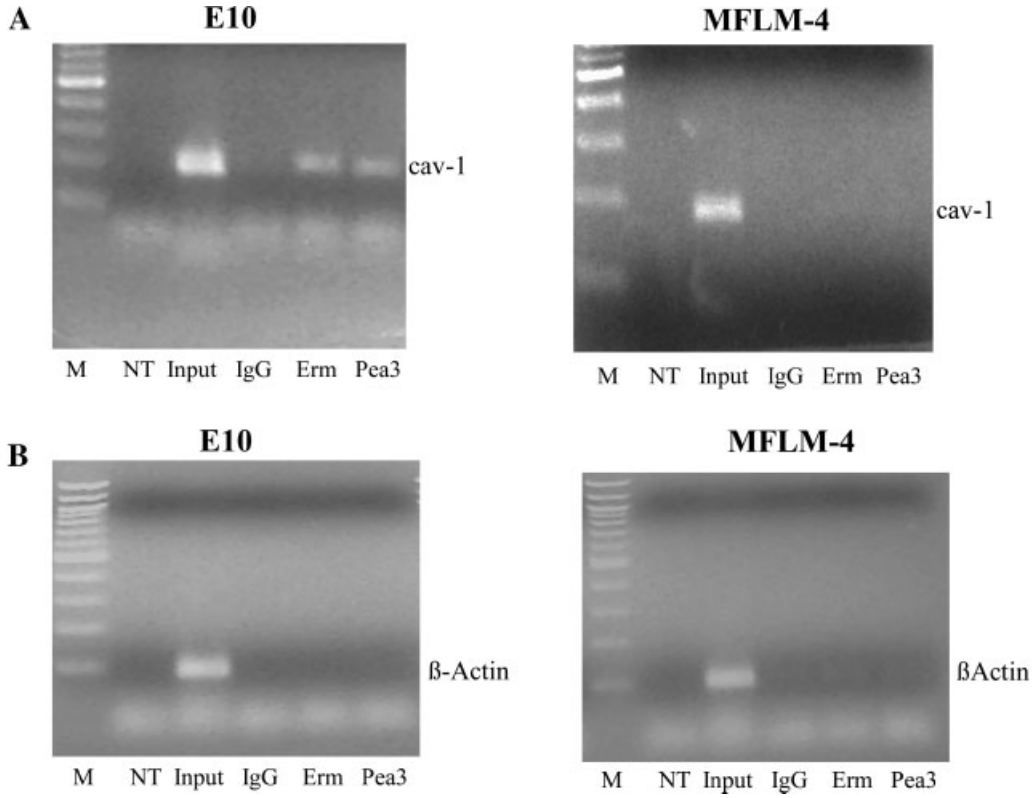

Fig. 2. Representative chromatin immunoprecipitation assay using IgG control or antibodies against ERM and PEA3. (**A**) After immunoprecipitation, samples were analyzed by PCR for caveolin-1 fragments spanning the ETS site (196 bp amplicon). These data show binding of ERM and PEA3 to a region at or near the −865 Ets site in the caveolin-1 promoter in E10, but not MFLM-4, cells. (**B**) Immunoprecipitated samples were analyzed by PCR for β-actin as a control. These data show no difference between IgG and the specific antibodies. M, marker; NT, no template.

MFLM-4 cells, neither pCANmyc/PEA3 nor pCANmyc/ERM cotransfected with −865 Luc or −865 M Luc increases luciferase activity compared with control vector [Fig. 3B(2)].

Overexpressing ERM Increases Endogenous Caveolin-1 mRNA in E10 Cells, but not MFLM-4 Cells

We next examined the effect of overexpressing PEA3 and ERM on endogenous caveolin-1 mRNA expression (n = 3). Figure 4 depicts the increased levels of the caveolin-1 transcript in cells transiently overexpressing ERM compared to control in E10 cells [Fig. 4A(1–3)]. In MFLM-4 cells, overexpression of ERM results in no change in caveolin-1 mRNA expression compared to CMV-control [Fig. 4B(1–3)]. Densitometry of these data normalized to β-actin control values (n = 3) shows that in E10 cells, transient overexpression of ERM induces a 1.4-fold increase in caveolin-1 mRNA compared to CMV-control [Fig. 4A(3)]. In E10 cells, the effect of transient overexpression of PEA3 on endogenous caveolin-1 mRNA is variable. In two independent transient transfection experiments [Fig. 4A(1,2)] overexpressing PEA3 either increased or decreased endogenous caveolin-1 transcription. Similar analysis by densitometry (n = 3) shows that overall, transient overexpression of PEA3 does not result in a statistically significant change in endogenous caveolin-1 mRNA expression compared to CMV-control in E10 cells [Fig. 4A(3)]. In MFLM-4 cells, overexpression of PEA3 results in no change in caveolin-1 mRNA expression compared to CMV-control [Fig. 4B(1–3)].

Overexpressing ERM Increases Endogenous Caveolin-1 Protein Expression in E10 Cells, but not MFLM-4 Cells

Figure 5 depicts the effects of overexpressing PEA3 and ERM on endogenous caveolin-1

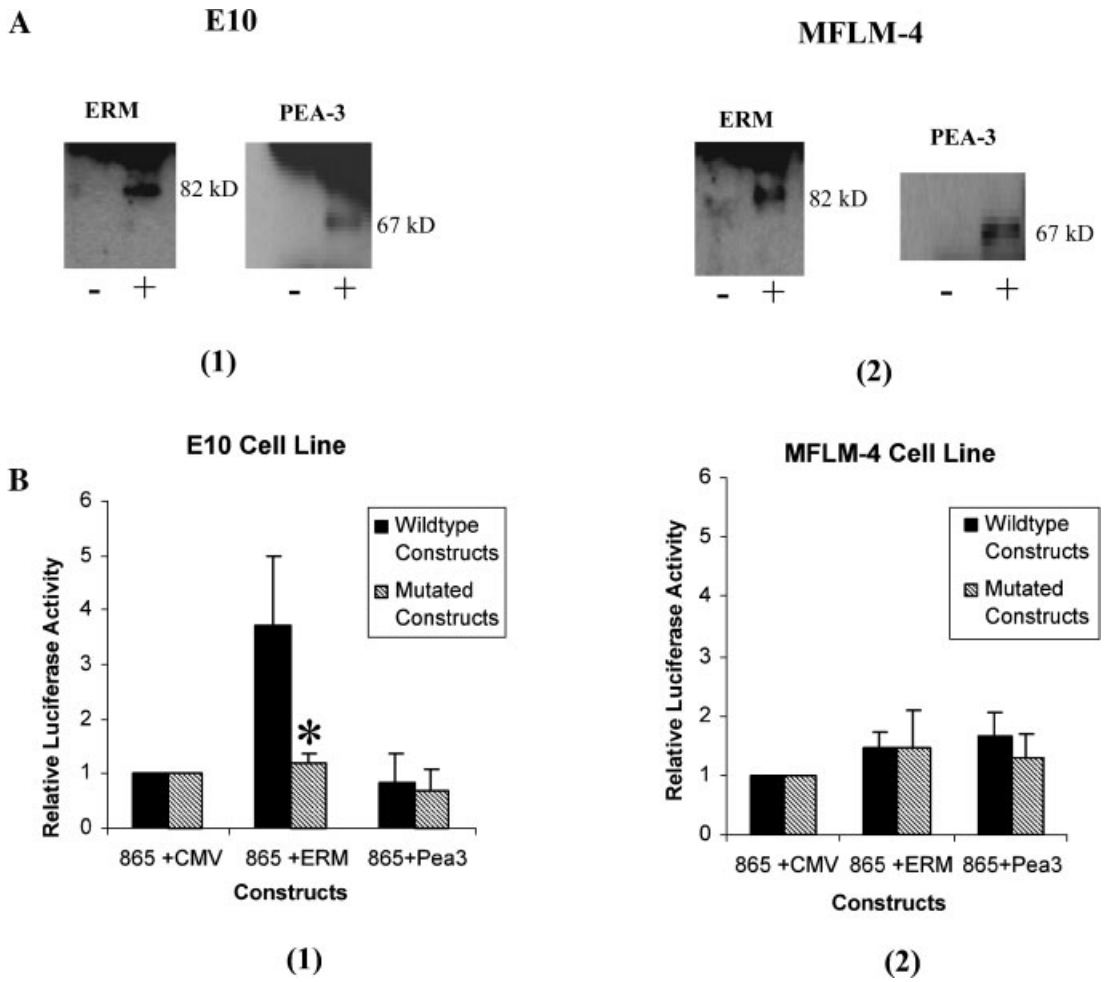

Fig. 3. (**A**) Representative Western blot analysis for PEA3 and ERM expression in transfected E10 and MFLM-4 cells. 50 μg of total protein/lane from cells transfected with pCANmyc/PEA3, pCANmyc/ERM, or CMV-control were immunoprecipitated for c-Myc, and then immunoblotted for c-Myc. When E10 and MFLM-4 cells were transfected with pCANmyc/PEA3 or pCANmyc/ERM, both cell lines were able to produce exogenous PEA3 and ERM. Immunoreactive bands are detected at 67 kD and 82 kD for cells transfected with pCANmyc/PEA3 and pCANmyc/ERM respectively, but not with CMV-control. (−) cells transfected with CMV-control; (+) cells transfected with pCANmyc/PEA3 or pCANmyc/ERM. (**B**) Cotransfection and reporter assay activity: After confirming the cell lines were capable of producing exogenous PEA3 and ERM, the indicated luciferase reporter constructs [865 Luc (black bars) or 865M Luc (hatched bars)] were transiently cotransfected with CMV-control (CMV), pCANmyc/ERM (ERM), or pCANmyc/PEA3 (PEA3) into E10 or MFLM-4 cell lines. (**1**) Normalized luciferase activity shows that −865 Luc promoter and ERM, but not PEA3, increases expression ~fourfold in E10 cells compared with control vector. (**2**) MFLM-4 cells cotransfected with −865 Luc or −865M Luc and PEA3 or ERM did not increase luciferase activity compared with control vector. (**1–2**) Coexpressing either ERM or PEA3 and −865M Luc did not increase promoter activity in any cell line compared with control vector. The fold stimulation for luciferase was calculated as normalized luciferase activity obtained in cells expressing ETS family members divided by the luciferase activity of samples originating from vector-transfected control cells. Data are expressed as the mean of at least three experiments (duplicate samples) ± S.D. Luciferase activities are presented relative to the level of expression of a promoterless construct, pGL3. Data were analyzed by Student's *t*-test with differences $P \leq 0.05$ considered significant (*).

protein expression (n = 3). We observe increased endogenous caveolin-1 protein expression in cells transiently overexpressing ERM in E10 cells [Fig. 5A(1–3)], but not MFLM-4 cells [Fig. 5B(1–3)]. Densitometry of these data normalized to β-actin control values (n = 3) shows that in E10 cells, transient overexpression of ERM induces a 2.5-fold increase in caveolin-1 protein compared to CMV-control [Fig. 5A(3)]. In E10 cells, the effect of transient overexpression of PEA3 on endogenous caveolin-1 protein is variable. Figure 5A(1,2) shows that in two independent transient transfection experiments overexpressing PEA3 can either

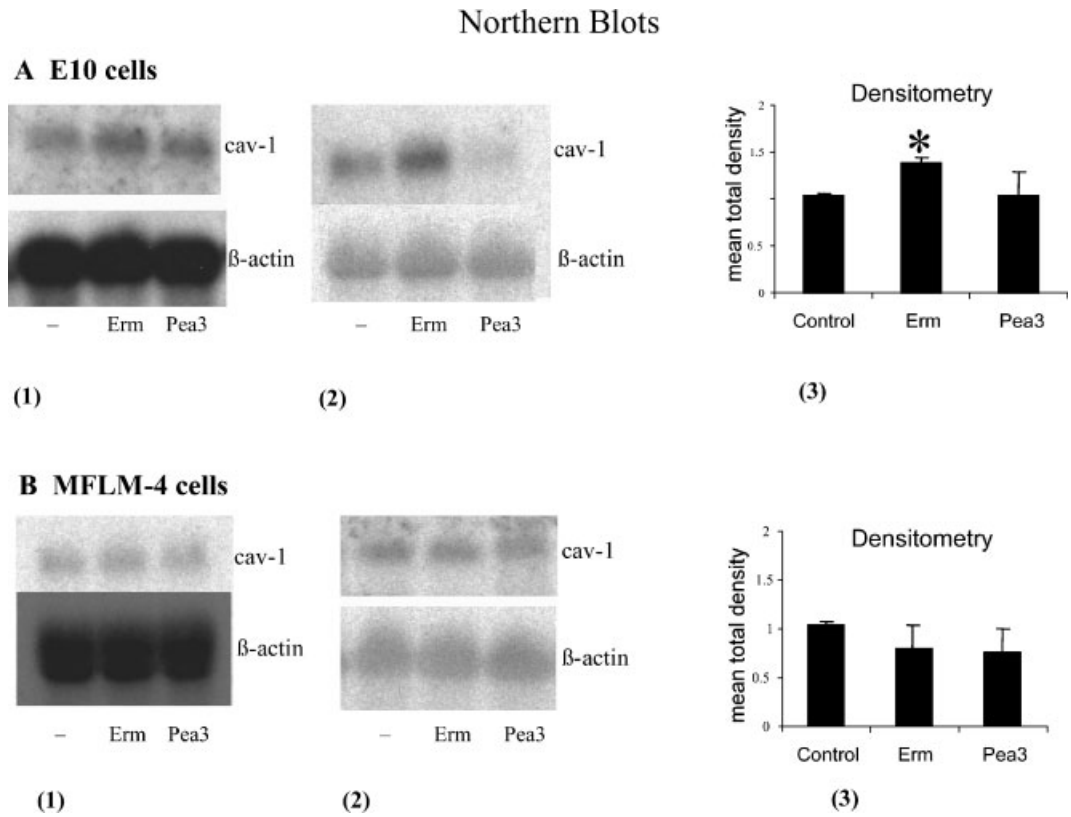

Fig. 4. Northern blot analysis for endogenous caveolin-1 expression in cells overexpressing PEA3 and ERM. (**A**1–2). Northern blot analysis of 10 μg RNA from E10 cells transfected with control CMV (–), pCANmyc/ERM (ERM), or pCANmyc/PEA3 (PEA3). (**B**1–2) Northern blots of 20 μg RNA from MFLM-4 cells transfected with control CMV (-), pCANmyc/ERM (ERM), or pCANmyc/PEA3 (PEA3). (**A**3 and **B**3) Densitometry of these data normalized to β-actin control values (n = 3) shows that in E10 cells, but not in MFLM-4 cells, transient overexpression with ERM induces a 1.4-fold increase in caveolin-1 mRNA expression compared to control CMV. (*) indicates $P < 0.05$.

increase or decrease endogenous caveolin-1 transcription. Densitometry of these data normalized to β-actin control values (n = 3) shows that in E10 cells, overall, transient overexpression of PEA3 does not increase endogenous caveolin-1 protein expression compared to CMV-control. In MFLM-4 cells, there is no change in caveolin-1 protein expression with overexpression of PEA3 [5B(1–3)].

DISCUSSION

We report herein our findings on the role of ETS proteins in the transcriptional regulation of caveolin-1 using murine lung cell lines as a model for our studies. We previously identified that an ETS cis-element differentially regulates transcription of the caveolin-1 gene in murine lung type I epithelial and endothelial cell lines [Kathuria et al., 2004]. The ETS protein family is large, complex, and widely expressed as described earlier [Sharrocks, 2001]. All cell types tested (23 tissues and cell lines) express at least 16 of the known 27 ETS family members and we have studied only six (ETS-1, PEA3, ERM, PDEF, ELF-3, ESE-3) selected because they are known to be expressed in the peripheral lung. Of these, only Ets-1, PEA3, and ERM were expressed (both mRNA and protein) in both cell lines representative of peripheral lung cells that express caveolin-1 [Kathuria et al., 2004].

We believe our data are the first to localize ERM protein expression in the adult lung. We show by immunohistochemistry studies of adult mouse lung that ERM, a member of the PEA3 subgroup of ETS transcription factors, is expressed in the cytoplasm of alveolar type I and II cells, but not airway epithelial cells or lung endothelial cells. It has recently been reported that ERM mRNA is increasingly restricted to the distal epithelium as lung development progresses, and is expressed only in type II cells in the adult lung by in situ

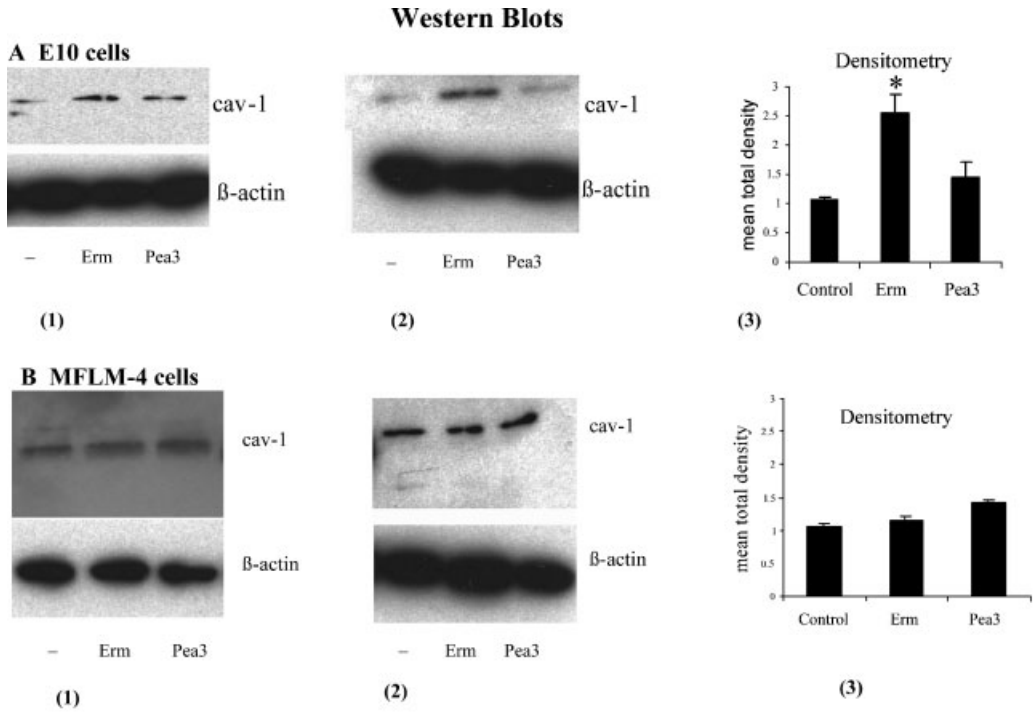

Fig. 5. Western blot analysis for endogenous caveolin-1 expression in cells overexpressing PEA3 and ERM. (**A** and **B**) Analysis of 30 μg of total protein/lane from cells transfected with CMV-control (–), pCANmyc/ERM (ERM), or pCANmyc/PEA3 (PEA3) shows increased levels of the 21 KD caveolin-1 protein in E10 cells, but not in MFLM-4 cells. (**A**3 and **B**3) Densitometry of these data normalized to β-actin control values shows that in E10, but not MFLM-4 cells, transient overexpression of ERM induces a 2.5-fold increase in endogenous caveolin-1 protein expression compared to CMV-control. (*) indicates $P < 0.05$.

hybridization [Lin et al., 2006]. Although our immunohistochemical findings support that in the adult mouse lung ERM is expressed in type II cells, we also demonstrate that ERM protein is expressed in alveolar type I cells. One plausible explanation for this discrepancy between the reported in situ results and our immunohistochemical findings of ERM expression is that, because type I cells are extremely flat (<0.2 μm thick), most areas of type I cytoplasm cannot be visualized in the light microscope. The usual in situ image of clustered autoradiographic grains within positive cells will therefore not be detectable [Rishi et al., 1995].

We did not detect ERM protein in mouse lung endothelial cells; yet, MFLM-4 cells, derived from lung mesenchyme at E14.5, express ERM. Since it has been previously reported that in late development, ERM is expressed in the mesenchyme, we believe that ERM expression in MFLM-4 cells indicates their origin from fetal lung mesenchyme. Similar to other reports, we did not detect PEA3 in normal adult lung tissue by RT-PCR [Kathuria et al., 2004] or by immunohistochemistry (data not shown).

In this report, we demonstrate that ERM binds and activates the caveolin-1 promoter in a cell-specific manner in alveolar type I (E10), but not lung endothelial (MFLM-4), cell lines, findings consistent with the expression patterns of ERM in alveolar type I and endothelial cells. ERM activation of caveolin-1 in E10 cells appears to be mediated by an Ets consensus cis-element at −865 to −844, a conclusion supported by our data showing in vitro binding of ERM to the caveolin-1 promoter in E10, but not MFLM-4 cells; mutation of two nucleotides within the −865 cis-element blocks ERM-induced transcription. Consistent with our findings, overexpression of ERM increases intracellular caveolin-1 mRNA and protein concentrations in E10 cells. Although we cannot yet rule out the possibility that this response is due to changes in mRNA stability, autoinhibition, or an indirect activation of the caveolin-1 promoter via other Ets-responsive genes, a simple explanation is that increased ERM also

directly activates the endogenous caveolin-1 promoter. These in vitro findings raise the possibility that caveolin-1 is regulated by ETS proteins in lung epithelial cells and that different types of peripheral lung cells such as fibroblasts and endothelial cells will utilize different family members, a testable conclusion that we view as likely. To further elucidate the role of ERM in caveolin-1 regulation, future studies will focus on siRNA knockdown of ERM in the cell lines.

We observe that overexpression of PEA3 or ERM at levels that were similar in both cell lines does not activate the caveolin-1 promoter in MFLM-4 cells, even though these cells express these endogenous transcription factors and caveolin-1. Future studies will focus on identifying the molecular mechanisms that account for the differential regulation of caveolin-1 by ETS proteins in the E10 and MFLM-4 cell lines. We speculate that the MFLM-4 cells express an inhibitory ETS and/or other protein or do not express a key co-factor (p300/CBP, ACTR, Sp100, and others) required for activation via ERM or PEA3. It was recently reported, for example, that KLF11-mediated repression can antagonize Sp1/SREBP-induced transcriptional activation of caveolin-1 in endothelial, but not fibroblast, cells [Cao et al., 2005]. Alternatively, in MFLM-4 cells, other transcription factor families may activate the caveolin-1 promoter. In skin fibroblasts, for example, Sp1, p53, E2F/DP-1, and SRE specific enhancers regulate the caveolin-1 promoter, [Bist et al., 1997; Engelman et al., 1999; Fielding et al., 1999], whereas in A14 and HEK293T cell lines, forkhead Box O (FOXO) regulates caveolin-1 [van den Heuvel et al., 2005].

Our data also do not rule out the possibility that other members of the ETS protein family can influence caveolin-1 transcription in the lung and elsewhere and, in fact, we think that this is probable. In E10 cells, our ChIP data show that PEA3 binds to the caveolin-1 promoter, and transient transfection experiments show variable responses of the endogenous caveolin-1 gene and protein to increases in PEA3 expression. Although not statistically significant, in one set of experiments there is increased caveolin-1 mRNA and protein in response to PEA3, a finding that differs from the activation of the −865 promoter-Luc construct in these cells that responds only to ERM.

These findings raise the interesting possibility that there may be additional Ets cis-elements elsewhere in the caveolin-1 promoter that we have not yet explored; both upstream and intronic regulatory elements are possibilities. In another promoter, the TβR-II (type II TGFβ receptor) promoter, two Ets sites are required for a synergistic response to ELF3, ETS-1, and PEA3 [Kopp et al., 2004].

It has also been shown that PEA3 activation of an Ets site produces an increase in osteopontin transcription synergistic with β-catenin-Lef-1 and c-Jun activation via their respective cognate cis-elements [El-Tanani et al., 2004]. Synergism is maximal when the two response elements are close to each other. PEA3 can also enhance transcription without binding to the Ets cis-element as shown by its interaction with USF-1 resulting in upregulation of bax [Firlej et al., 2005]. More recently it has been demonstrated that, although ERM by itself has little effect on the surfactant protein C (SPC) promoter, it significantly enhances TTF-1 mediated SPC transcription [Lin et al., 2006]. Interactions similar to these may contribute to differential activation and/or repression of the endogenous caveolin-1 promoter by ETS proteins in different lung cell types.

There are three models available to study transcriptional regulation in type I cells. E10, an adult lung epithelial cell line is a spontaneously immortalized cell line that expresses at least 13 type I cell markers including T1α and AQP-5, but not type II cell markers [Kathuria et al., unpublished data; Cao et al., 2003]. Limitations of the E10 cells are those inherent to using spontaneously immortalized cell lines. A second model available to study regulation in type I cells are primary type I cells isolated from normal adult lung. Isolation of type I cells from rat lungs by affinity methods using the anti-T1α antibody was recently reported [Chen et al., 2004], but to our knowledge, this isolation procedure has not yet been performed in the mouse. A third model is purified type II cells maintained in culture transdifferentiated to type I cells [Corti et al., 1996; Dobbs et al., 1998; Borok et al., 2002; Johnson et al., 2002)]. One possible limitation in studying transdifferentiated type II cells in culture, however, is that the biological relevance of promoter studies in cells whose differentiation characteristics are changing over time is unclear [Gonzalez et al., 2005]. While individually each of these models

has its own limitations, used together a comprehensive understanding can be gained on how ETS proteins regulate caveolin-1 in alveolar type I epithelial cells.

Although the E10 cell line is an acceptable model for studying regulation of type I cells, these cells are a spontaneously immortalized cell line, and the proliferative regulation of these cells is not normal. Since PEA3 is expressed in lung epithelial cells during development, and has been shown to be highly overexpressed in primary lung adenocarcinomas, an alternative explanation for PEA3 binding to the caveolin-1 promoter and the increased caveolin-1 mRNA and protein in response to PEA3 in the E10 cells (1 of 3 experiments) is that PEA3 may potentially regulate caveolin-1 in rapidly dividing or immortalized cells.

We believe that these new data on caveolin-1 transcriptional regulation may relate to gene expression patterns in lung cancers. There is clear evidence that expression of caveolin-1 and ETS proteins is altered in cancers. A recent report identified caveolin-1 as a direct target of EWS/Fli-1 (chimeric transcription factor encoded by gene fusion between EWS and the ETS gene Fli-1), and a key determinant of tumorigenicity in Ewing's sarcoma [Tirado et al., 2006]. In gene microarray studies of early lung adenocarcinomas, caveolin-1 mRNA is markedly downregulated [Powell et al., 2003]. Similar findings in other types of tumors have led to the proposal that caveolin may be a tumor suppressor gene, which is consistent with observations showing that caveolin-1 overexpression can inhibit cellular proliferation by mediating cell cycle arrest in G0/G1 [Williams and Lisanti, 2005]. However, several studies also show that caveolin-1 expression is markedly upregulated in late, poorly differentiated tumors becoming or already metastasized [Li et al., 2001; Ho et al., 2002].

Although the underlying changes in tumor cell behavior that accompany the dynamic changes in caveolin-1 expression are not known, these observations suggest the possibility that caveolin-1 protein may participate in some way in tumor cell detachment and seeding into new tissues, or in the signaling related to these events. The idea of a protein being either tumor suppressing or promoting, depending on tumor stage, has been demonstrated for other proteins including TGF-β, bcl-2, and Cox-2 [Shinoura et al., 1999; Trifan et al., 1999; Benson, 2004].

Likewise there are many examples showing that dysregulation of ETS protein expression is associated with carcinogenesis. Northern analyses show that PEA3 (E1af) mRNA is highly overexpressed in lung adenocarcinomas, likely leading to enhanced motility and invasiveness [Hiroumi et al., 2001]. This is consistent with some of the known targets of PEA3 that include matrix metalloproteinases −1, −3, and −9 and matrilysin that are involved in tumor cell invasiveness [Horiuchi et al., 2003]. ERM expression is an independent adverse prognostic factor for overall breast cancer survival [Chotteau-Lelievre et al., 2004]. We believe this to be the first study showing that caveolin-1 is a target for the PEA3 group of ETS transcription factors. This work demonstrates that under these experimental conditions, the cellular utilization of ETS proteins is cell-type specific. Since caveolin-1 may have cell-type specific tumor-modulating functions, understanding its regulation by ETS proteins in various cell types has important implications, particularly in the biology of lung and other cancers.

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