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Transcription of the Caveolin-1 Gene Is Differentially Regulated in Lung Type I Epithelial and Endothelial Cell Lines

A ROLE FOR ETS PROTEINS IN EPITHELIAL CELL EXPRESSION*

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In the lung, caveolin-1 is expressed in both type I alveolar epithelial and endothelial cells where it is hypothesized to modulate molecular signaling activities and progression of tumorigenesis. Developmentally, caveolin-1α is expressed in fetal lung endothelial, but not epithelial, cells; in adult lung, both cell types express caveolin-1α. To test the hypothesis that caveolin-1 transcription is differentially regulated in type I and endothelial cells, we characterized the proximal promoter of the mouse caveolin-1 gene in lung cell lines to identify factors that control its cell-specific expression. We show that caveolin-1 expression is regulated by an Ets *cis*-element in a lung epithelial cell line, but not a lung endothelial cell line, and that three ETS family members, ETS-1, PEA3, and ERM, recognize and bind the Ets site in the epithelial cell line. Based on these findings, we have identified the Ets *cis*-element as a region that accounts for differential transcriptional regulation of caveolin-1 in lung epithelial and endothelial cells.

The caveolin-1 promoter of several species has been cloned and sequenced, yet little is known about the protein transcription factors and *cis*-elements that regulate its transcription. In NIH 3T3 cells, caveolin promoter constructs containing 750 bp or 3 kb of upstream sequence show similar promoter activity (1), suggesting that in this cell line most of the regulatory regions are contained within the first 750 bp of the caveolin-1 promoter. In normal human skin fibroblasts, the caveolin-1 promoter is regulated by Sp1, p53, E2F/DP-1, and serum-response element-specific enhancers (2), whereas in vascular smooth muscle cells increases in free cholesterol stimulate caveolin-1 transcription by an SREBP-1-dependent mechanism (3).

The regulation of expression of caveolin-1 in the lung is especially interesting both because it is developmentally regulated and because two extensive cell populations expressing high levels of caveolin-1, *i.e.* alveolar epithelial type I cells and alveolar capillary endothelial cells, reside within a short distance from each other and therefore share many environmental influences (4). Furthermore, targeted deletions of caveolin-1 in mice show mainly a pulmonary phenotype, pulmonary hypertension as well as hyperproliferative and fibrotic lung tissue (5–7). Consistent with this hyperproliferative phenotype, embryonic fibroblasts derived from the null animals appear to have an increased rate of proliferation, which is partially reversed by re-expressing caveolin-1 protein from a viral expression vector (5). In intact animals loss of caveolin-1 leads to lung abnormalities at 2–4 months, about the same postnatal time point that caveolin-1α is first detectable by immunohistochemistry in type I cells in normal animals (4). The pulmonary phenotype appears to contribute to the shortened life span of the null animals although there are uncertainties about the actual cause of early death (8).

Caveolin-1, the main structural protein of caveolae, is a 21–24-kDa integral membrane protein that appears to organize specialized membrane domains into vesicles. Caveolae and/or caveolin-1 are proposed to be involved in tumorigenesis and in modulating signaling events, cholesterol trafficking, and chemokine activities (9–13). Caveolae have also been shown to provide a pathway for endocytosis of cellular prion proteins and some viruses including simian virus 40 and echovirus (14–17). In general, caveolin-1 modulates signal transduction by acting as a scaffolding protein that, by binding to signaling proteins, negatively regulates their activity.

Caveolin-1 is expressed in many cells including adipocytes, endothelial cells, fibroblasts, and type I alveolar epithelial cells. Two caveolin-1 isoforms have been identified. The α and β isoforms of caveolin-1 protein are identical except that the α isoform has an additional 31 amino acids at the amino terminus (18, 19). It is unclear whether the two isoforms have different functions. In mice the α and β isoforms are produced from different RNAs (full-length and 5′-variant) that differ in the first exon and in 620 bp of the 3′-untranslated region (19). *In vitro* transcription studies show that full-length caveolin-1 mRNA lacking the 5′-untranslated region can produce either the α or β isoform. Truncation of the 5′-untranslated region to 22 bp mostly produces the α isoform, whereas the 5′-variant, which lacks exon-1, produces only the β isoform (19).

Immunohistochemistry studies of embryonic, fetal, and adult mouse lung using an antibody that recognizes the caveolin-1α isoform show that caveolin-1α expression is present in developing endothelial cells but is not detectable in epithelial cells at the same developmental time points, suggesting differential regulation between the two cell types (4). No caveolin-1α expression is detectable in epithelial cells before birth. In the adult lung, caveolin-1α can be detected in both type I epithelial cells and endothelial cells but rarely in type II cells (4). Most interesting, in primary culture, alveolar type II cells growing on a plastic substratum acquire an alveolar type I phenotype as evidenced by an increase in many type I cell-specific proteins including caveolin-1. In these cells, caveolin-1 expression coincides with the

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TABLE I
Primer sequences

Gene	Primers/probes	Product size
		bp
Cav-1	5′-ACGATGTCTGGGGGCAAATAC 5′-CTTGACCACGTCGTCGTTGAG Probe catctacaagcccaacaacaaggcca	198
Cav-1β	5′-TAGCAAAAGTTGTAGCGCCAG 5′-CTTGACCACGTCGTCGTTGAG Probe catctacaagcccaacaacaaggcca	293
Ets-1	5′-AGCCGACTCTCACCATCATC-3′ 5′-GGATGCAGCGTCTGATAGGA-3′	542
Pea3	5′-GCTCATTTCATTGCTTGGAC-3′ 5′-GATTTGGCCTGCCTCCACTG-3′	580
Erm	5′-GGGAAATCTCGATCAGAGGACTG-3′ 5′-GGCTTCCTATCGTAGGCACAATAG-3′	338
Pdef	5′-GGCCTTCCAGGAGCTGGGCG-3′ 5′-GGACCTTGGGTTCTGGGATATCAG-3′	600
Elf-3	5′-TTCCCTGTGTTGCTGTAGAGAGG-3′ 5′-GTCTCATTTGCAGTCCATGTTGG-3′	192
Ese-3	5′-CCTGGACACCAACCAGCTAGATGC-3′ 5′-CCTGAAGATGCCTTCCGAACGGTC-3′	524

formation of caveolae and a down-regulation of SP-C (20, 21). To date, there are no studies reporting the identification of *cis*-elements or transcription factors important for caveolin-1 transcription in lung epithelial and endothelial cells.

To begin to understand the molecular mechanisms that regulate caveolin-1 transcription, we characterized the proximal promoter of the mouse caveolin-1 gene in lung cell lines to identify factors that control cell-specific expression, and we now demonstrate that 1.3-kb promoter of caveolin-1 contains such regulatory elements. Deletion, gel retardation, and mutation analyses in cell lines show that caveolin-1 expression is enhanced by a 20-bp region (−844 to −865 bp) in the lung epithelial cell line but not the lung endothelial cell line. An Ets-binding site is located within this region. We show here that three ETS family members, ETS-1, PEA3, and ERM, recognize and bind the Ets site in the epithelial cell line but not in the endothelial cell line. These studies have identified the Ets *cis*-element as a region that accounts for differential expression between cell lines representative of alveolar type I cells and lung endothelial cells.

EXPERIMENTAL PROCEDURES

Culture and Characterization of the Cell Lines—The murine cell lines used for promoter studies are E10, an adult lung epithelial cell line that expresses type I cell genes, 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). Selection of these two cell lines is consistent with our observations during lung development that caveolin-1α is expressed early in endothelial cells, whereas in epithelial cells it is first expressed postnatally (4).

E10 is a spontaneously immortalized cell line that expresses type I cell markers such as T1α and AQP-5, as shown previously, but not type II cell markers (22, 23). MFLM-4 is an SV40 large T antigen immortalized cell line that expresses the endothelial cell markers von Willebrand factor, vascular endothelial growth factor receptor, and angiopoietin-1 as shown earlier by RT-PCR[1] (24) and confirmed in our lab (data not shown). E10 cells were maintained in CMRL 1066 medium (Invitrogen), 10% fetal bovine serum (Invitrogen), 0.5 mM glutamine (Invitrogen), 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfate (Invitrogen). MFLM-4 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen), 10% fetal bovine serum (Invitrogen), 2 mM glutamine (Invitrogen), 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfate (Invitrogen).

Purification of RNA, QRT-PCR, and RT-PCR—Total RNA was isolated from newborn mouse lung or cell lines with TRIzol reagent using the manufacturer's protocol (Invitrogen) and treated with DNase using DNA-free kit (Ambion, Texas, CA). Isolated RNA (1 μg) was reverse-transcribed using avian myeloblastosis virus-reverse transcriptase (Promega, Madison, WI) following the manufacturer's protocol in a final volume of 25 μl.

The two cell lines were analyzed for caveolin-1α (full-length) and -β (5′-variant) expression by quantitative real time PCR (QRT-PCR) in an ABI Prism 7000 sequence detector (Applied Biosystems, Foster City, CA). Reverse transcriptions were diluted 1:32. Primers and probe sequences for caveolin-1 were designed using PrimerExpress software (Applied Biosystems) and are shown in Table I. Reactions were performed in 50 μl using Taqman Universal Master Mix (Applied Biosystems) and amplified under the same conditions for both cell lines. Reverse transcription products from three E10 and three MFLM-4 samples were analyzed in triplicate. The relative amount of RNA for caveolin-1α and -β was obtained using calibration curves performed for each isoform using normal adult lung RNA. Data were analyzed statistically using Statview software.

Expression of *Ets-1, Pea-3, Erm, Pdef, Elf-3,* and *Ese-3* in E10 and MFLM-4 cell lines was determined by semi-quantitative reverse transcription-PCR. The PCR was performed with 1 μl of the reverse transcription reaction and the Advantage-HF 2 polymerase mixture from Clontech (Palo Alto, CA) (94 °C, 30 s; 68 °C, 3 min; 35 cycles) for all genes. Primer sets and product sizes for each gene are shown in Table I.

Caveolin-1 Promoter Constructs—1.3 kb of the 5′ caveolin-1 promoter was generated from murine genomic DNA by PCR cloning. By using the published mouse caveolin-1 promoter sequence (GenBank™ accession number AF124227), 30-mer primer oligonucleotides were designed with adapters for Nhe-1 and Sac-1 and were used to amplify the caveolin-1 promoter sequence. The PCR was performed with 0.5 μg of mouse genomic DNA and Advantage-HF 2 polymerase mix (94 °C, 30 s; 68 °C, 3 min; 35 cycles) (Clontech). Fragments were digested with restriction enzymes Nhe-1 and Sac-1 and purified with QIAquick kit (Qiagen, Valencia, CA). The purified fragment was ligated into the promoterless luciferase expression vector, pGL3-basic vector (Promega), and confirmed by sequence analysis. By using similar methods, eight deletion promoter constructs containing −172, −269, −462, −800, −826, −844, and −865 bp and −1.1 kb 5′ from the transcription initiation site were generated and confirmed by sequence analysis. Constructs containing mutated Ets and forkhead sites were generated by PCR using −865 luciferase as the template. The forward primers were mutated oligonucleotides for the Ets site (865M, agaggatgt→cgcgtaagt) and each of the two-forkhead sites (844M, gtgtttaat→gtgtctcat; 826M, aatacaca→actacaca). The reverse primer was a 30-mer oligonucleotide complementary to the 3′ wild type sequence. After sequence verification, computer algorithms 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. All caveolin-1 constructs contain +62 bp of the untranslated region.

Transfection and Reporter Assay Activity—Constructs (Cav-1 promoter-luciferase and *Renilla* luciferase control plasmid) were transiently cotransfected into the cell lines using GeneJammer transfection reagent (Stratagene, La Jolla, CA). Optimal transfection efficiency for

[1] The abbreviations used are: RT-PCR, reverse transcription-PCR; EMSA, electrophoretic mobility shift assay; RT, room temperature; QRT-PCR, quantitative real time-PCR.

the E10 cell line was achieved using 0.8×10^5 cells per 35-mm dish, 2 μg of various reporter constructs, and 12 μl of DNA of transfection reagent. For the MFLM-4 cell line, 0.9×10^5 cells per 35-mm dish, 1 μg of reporter constructs, and 6 μl of DNA of transfection reagent was used. Cells were first grown to 60–80% confluency. The transfection mixture containing 100 μl of serum-free medium and the transfection reagent were preincubated for 10 min. Plasmid DNA was added to the mixture and further incubated for 10 min. The standard culture medium was replaced with 900 μl of fresh serum-containing 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 phosphate-buffered saline, 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; firefly luciferase activity was normalized to *Renilla* luciferase activity. Data are expressed as the mean of at least three experiments (duplicate samples) ± S.E. The SV40 promoter-luciferase construct was used as a positive control to show the maximum expression level in the two cell lines. 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 of $p \leq 0.05$ considered significant.

Nuclear Protein Extract Preparation—Nuclear proteins were isolated by using a mini-extraction procedure (25, 26). All procedures were performed with cold reagents and on ice. Briefly, confluent cells from 2 to 3 10-cm diameter plates were washed twice with 10 ml of phosphate-buffered saline, harvested with 1 ml of phosphate-buffered saline, and pelleted at 14,000 rpm for 1 min. The pelleted cells were resuspended in 20–40 μl of buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 0.2% (v/v) Nonidet P-40, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin) and lysed on ice with gentle vortexing for 5 min. The nuclei were pelleted at 3000 rpm for 5 min prior to protein extraction. Nuclear proteins were dissociated from native DNA-binding sites by the addition of buffer B (20 mM HEPES (pH 7.9), 420 mM NaCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 25% (v/v) glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin) and incubation with gentle vortexing for 10 min. The samples were centrifuged at 14,000 rpm for 10 min, and the supernatant containing nuclear proteins was collected and used for EMSA experiments and Western blot assays. Protein concentrations were determined by a modified Bradford method (Bio-Rad) using bovine serum albumin as a standard.

Immunodepletion of ETS Factors in Nuclear Extracts—Primary antibodies (4 μl) against either ETS-1 (rabbit polyclonal IgG, catalog number sc-350X, Santa Cruz Biotechnology, Santa Cruz, CA), PEA3 (mouse monoclonal IgG, catalog number sc-113X, Santa Cruz Biotechnology), or ERM (goat polyclonal IgG, catalog number sc-1955X, Santa Cruz Biotechnology) and RIPA buffer containing inhibitors (above) were incubated with 50 μg of nuclear protein extract (2 h, 4 °C). 30 μl of pre-washed A-G beads (Santa Cruz Biotechnology) were added (2 h, 4 °C) followed by centrifugation at 14,000 rpm (1 min, 4 °C) and collection of the supernatant. For the second depletion, the supernatants were incubated with 4 μl of antibody and with RIPA buffer containing the above inhibitors (2 h at 4 °C); 30 μl of pre-washed A-G beads (Santa Cruz Biotechnology) were added (2 h at 4 °C) and centrifuged at 14,000 rpm (1 min, 4 °C). For ETS-1 and PEA3, nuclear extract recovered from the second cycle of depletion was analyzed by immunoblot assay. For ERM, nuclear extract recovered from the second cycle of depletion was used for immunoprecipitation followed by Western blotting.

Immunoprecipitation—E10 and MFLM-4 nuclear protein extracts (50 μg) and ERM-immunodepleted nuclear protein were incubated with 4 μl of primary antibodies against ERM and RIPA buffer containing 2 μg/ml aprotinin and 0.5 mM phenylmethylsulfonyl fluoride (overnight, 4 °C) followed by 30 μl of pre-washed A-G beads (2 h, 4 °C). The samples were centrifuged at $14,000 \times g$ (1 min, 4 °C), and the precipitates were washed three times with RIPA buffer containing the above inhibitors. Samples were collected for immunoblot analysis.

Synthetic Oligonucleotides—Complementary oligonucleotides spanning the Ets-binding site (agaggatgt) with 4 bases of protruding 5′ ends purified by PAGE were purchased from Invitrogen. Annealing was performed at oligonucleotide concentrations of 10 μM in 50 μl of annealing buffer (10 mM Tris (pH 7.5), 10 mM $MgCl_2$, and 50 mM NaCl). Mixtures were heated to 95 °C for 5 min and gradually cooled to room temperature. A 1:10 dilution of this mixture was made in TE buffer (10 mM Tris (pH 8.0), 1 mM EDTA) and used as unlabeled competitor DNA in EMSA experiments. For use as the EMSA probe, annealed oligonucleotides (20 pmol) were labeled using 3 μl of [α-^{32}P]dATP and DNA polymerase large Klenow fragment (New England Biolabs, Beverly, MA). Probes were purified on Nuc Trap columns (Stratagene) and recovered in a final volume of 100 μl of STE at ~40,000 cpm/μl. Similar complementary oligonucleotides spanning a mutated ETS-binding site (agaggatgt→cgcgtaagt) were synthesized, annealed, labeled, and purified as above.

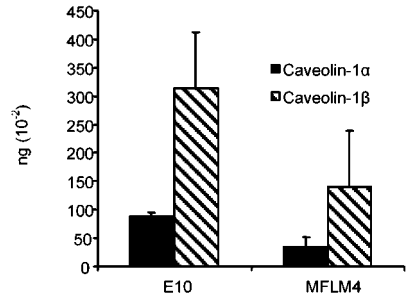


FIG. 1. **QRT-PCR analysis of caveolin-1 mRNA from E10 and MFLM-4 cell lines.** Both cell lines express the α (*black bars*) and β isoforms (*hatched bars*) of caveolin-1 mRNA. The relative amounts of each isoform were calculated using calibration curves obtained with mRNA from normal adult mouse lung. Data are expressed as the mean of three assays in triplicate ± S.E.

EMSA—Nuclear protein extracts (10 μg) or ETS-1, PEA3, and ERM-immunodepleted nuclear extracts (10 μg), 1 μg of poly(dI-dC) in 10 μl of buffer (50 mM Tris (pH 7.5), 50 mM NaCl, 2 mM EDTA, 2 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride) were incubated with labeled oligonucleotide (~20 fmol) at RT for 20 min. For competition experiments, unlabeled oligonucleotides were incubated with nuclear proteins for 10 min at RT prior to the addition of labeled oligonucleotides. For mutation experiments, mutated labeled oligonucleotide was added and then incubated for 20 min at RT. For supershift experiments, mixtures were incubated with 4 μl of ETS-1, PEA3, or ERM antibody at RT for 20 min followed by the addition of probe. For multiple antibody incubations, 2–3 μl of each antibody was used. Polyacrylamide gels (5%) were dried under vacuum and exposed at −70 °C for 3–5 days.

Western Blots—Western blot analysis was performed with nuclear proteins from E10 and MFLM-4 cell lines for ETS-1, PEA3, and ERM expression. For analysis of ETS-1 and ETS-1-immunodepleted protein, polyvinylidene difluoride membranes were blocked in 1× TBST containing 10% dry milk (1 h, RT) and exposed overnight at 4 °C to anti-ETS-1 (above, 1:1000) and then to goat anti-rabbit secondary antibody (1:10,000, 1 h, RT). For analysis of PEA3 and PEA3-immunodepleted protein, polyvinylidene difluoride membranes were incubated in 1× Tris-buffered saline containing 5% dry milk (1 h, RT) and exposed overnight at 4 °C to anti-PEA3 (above, 1:500) followed by horse anti-mouse secondary antibody (1:10,000, 1 h, RT). For analysis of ERM and ERM-immunodepleted protein, polyvinylidene difluoride membranes were blocked in 1× TBST containing 10% dry milk (1 h, RT) and exposed overnight at 4 °C to anti-ERM (above, 1:1000) and then to anti-goat 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).

RESULTS

E10 and MFLM-4 Cell Lines Express Endogenous Caveolin-1α and -β Isoforms—The E10 and MFLM-4 cell lines were analyzed for caveolin-1α and -β mRNA expression by QRT-PCR. These results show that both cell lines express endogenous caveolin-1α and -β mRNAs (Fig. 1) and that E10 and MFLM-4 cell lines have ~4 times higher expression of the 5′-variant mRNA (β isoform) compared with the full-length mRNA (α isoform). Although this pattern of mRNA expression is similar to that in both normal newborn and adult total lung as seen by semi-quantitative RT-PCR (data not shown), the two cell lines have not yet been analyzed for caveolin-1α and -β protein expression. These lung cell lines are therefore good models for studying the regulation of caveolin-1 because they express the endogenous caveolin-1 gene and, as shown previously (22–24), express the appropriate cell markers for differentiated epithelial and endothelial cells from lung.

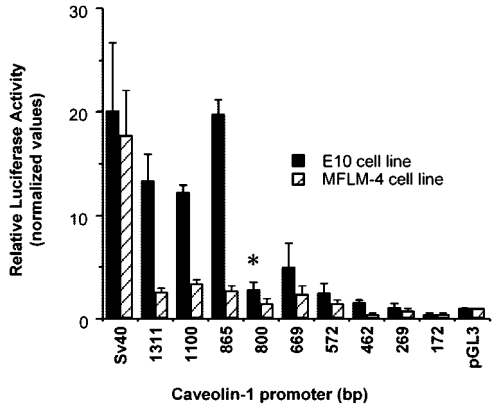


FIG. 2. **Deletion studies of the caveolin-1 promoter.** The indicated luciferase reporter constructs were transiently transfected into E10 (*black bars*) and MFLM-4 (*hatched bars*) cell lines. Deletion analysis shows that the −800 to −865-bp promoter increases expression ~8-fold in the epithelial but not the endothelial cell line. Luciferase reporter activity is normalized to activity of a cotransfected *Renilla* luciferase reporter. Normalized luciferase activity is shown relative to the promoterless pGL3 plasmid in each cell line. Activity from an SV40 promoter-luciferase construct used as a control shows that maximum expression levels for each cell line are approximately equal in this system. Data are expressed as the mean of three transfections with duplicate assays ± S.E. *, $p \leq 0.05$.

Deletion and Mutation Analyses Reveal the 1.3-kb Caveolin-1 Promoter Is Sufficient to Confer Specificity between Epithelial and Endothelial Cell Lines and That an ETS Consensus Site Is a Putative Enhancer for Caveolin-1—A 1.3-kb caveolin-1 promoter-luciferase construct was transiently transfected into the E10 and MFLM-4 cell lines. Fig. 2 compares luciferase activity of the caveolin-1 promoter between the two cell lines. In E10 cells, the expression of the 1.3-kb promoter construct (−1311-bp luciferase) is about 13-fold over that from a promoterless control construct (0-bp luciferase). In MFLM-4 cells, the expression of the 1.3-kb construct is about 2.5-fold higher than the promoterless control vector. Results ($n = 4$) show that the −1.3-kb caveolin-1 promoter fragment drives expression of luciferase activity in the lung epithelial cell line about 5-fold higher than in the endothelial cell line. The SV40 promoter-luciferase construct was used to show the maximum expression level in the two cell lines. Both E10 and MFLM-4 express nearly identical amounts of luciferase indicating that the two cell lines can produce and store approximately equal concentrations of luciferase protein under these conditions.

Ten deletion constructs were used for transient expression studies in the E10 and MFLM-4 cells ($n = 3$) to define regions that regulate promoter activity. Deletion analysis shows that expression from the caveolin −800 to −865-bp promoter increases expression 8-fold in the epithelial cell line but not endothelial cell line (Fig. 2). This preferential activation indicates the presence of enhancer elements important for differential regulation between endothelial and type I epithelial cell lines.

By using bioinformatic tools and programs including MatchPublic (www.gene-regulation.com/cgi-bin/pub/programs/match/match.cgi), AliBaba2 (wwwiti.cs.uni-magdeburg.de/~grabe/alibaba2), and Transcription Element Search software (bioinformer.ebi.ac.uk: 80/newsletter/archives/2/tess.html) that utilize Transcription Factor Database (TransFac) to search DNA sequences for the presence of transcription factor-binding elements, three potential *cis*-elements known to be important for lung epithelial cell gene regulation were identified in the caveolin-1 promoter region from −800 to −865 bp. These include an Ets transcription factor-binding site (−844 to −865 bp) and two winged-helix/forkhead consensus binding sites (−844 to −826 bp and −826 to −800 bp respectively). The Ets family of transcription factors is of particular interest because certain members, similar to caveolin-1, are expressed in the lung and are involved in tumorigenesis (27, 28). In addition, several members of the forkhead family group of transcription factors expressed in the lung are thought to be involved in developmental gene regulation, including hepatic nuclear-3/forkhead (HNF3/Fkh) which regulates transcription of lung-specific genes expressed by Clara and type II epithelial cells (29, 30).

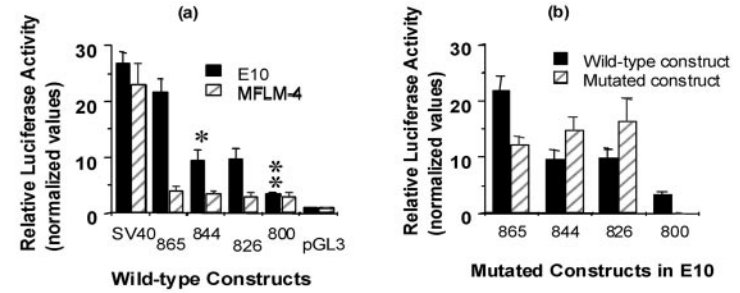
Fig. 3*a* shows that deleting the caveolin-1 promoter from −865 to −844 bp decreases expression in E10 cells from ~22- to ~10-fold over background, suggesting the Ets *cis*-element is a potential regulatory element in this region. The same deletion also decreases expression in MFLM-4 cells (from ~4- to ~3-fold over background), but the decrease is not statistically significant. A promoter deletion from −844 to −826 did not change expression levels in either cell line. This is in contrast to a −826 to −800 deletion that decreases expression from ~10- to ~3-fold in the E10 cell line but does not change expression levels in the MFLM-4 cell line, identifying the forkhead consensus site as a putative stimulatory region in the epithelial cell line.

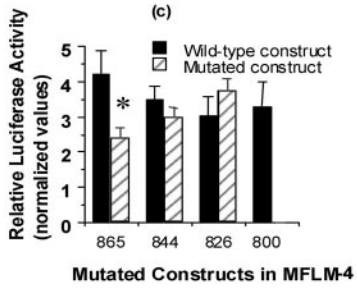
To determine whether these sites are transcriptionally active, constructs containing mutations of the Ets and forkhead-binding sites were prepared and assayed for luciferase activity in the two cell lines. Mutation of the Ets-binding site ($n = 3$) results in a statistically significant reduction in luciferase activity for both the E10 (~22- to ~12-fold) and the MFLM-4 (~4- to ~2.5-fold) cell lines (Fig. 3, *b* and *c*). Furthermore, gel shift analyses (described below in detail and in Fig. 4) show that extracts from both lung cell lines contain proteins that can bind specifically to the Ets binding domain. Because the caveolin-1 promoter −844 to −865 (the region of the Ets-binding site) has 6-fold higher activity in the E10 compared with MFLM-4 cells, it is likely that the Ets site is much more important for epithelial cell regulation. These data do not rule out the possibility, however, that the Ets site may be a weak activator in the MFLM-4 cell line.

Mutation of the forkhead sites did not decrease luciferase expression in either cell line suggesting that these elements are not active binding sites in the cell lines tested (Fig. 3, *b* and *c*). Although no other putative *cis*-elements are identified in the region −800 to −826 by using bioinformatic tools, it is possible that other sites yet unstudied may be important.

E10 and MFLM-4 Transcription Factors Interact with the −844 to −865-bp Region—Gel shift assays were performed to test for differential binding of epithelial and endothelial nuclear proteins to the DNA elements containing the Ets-binding site. EMSA analysis shows that the oligonucleotide containing the Ets site binds nuclear protein equally from both the E10 and MFLM-4 cell lines (Fig. 4*a*). Competition assays performed with 100–400-fold excess specific unlabeled oligonucleotide blocks complex formation with the labeled oligonucleotide in both cell types, indicating that binding is specific (Fig. 4*a*). Furthermore, the mutated oligonucleotide fails to form a complex with proteins from either cell type (Fig. 4*b*). The gel shift analyses therefore show that extracts from both lung cell lines contain proteins that can bind specifically to the Ets binding domain, suggesting that the Ets *cis*-element is a potential enhancer for caveolin-1.

E10 and MFLM-4 Cell Lines Express Ets-1, Pea3, and Erm—Six ETS family transcription factors were selected as candidates potentially involved in caveolin-1 regulation because they are either known to be expressed in lung or epithelial cells or to be involved in lung tumorigenesis (27, 28, 31). The genes se-





(d)

Wild type 865 (ETS site)	agaggatgt
Mutated 865	cgcgtaagt
Wild type 844 (Forkhead)	gtgtttaat
Mutated 844	gtgtctcat
Wild type 826 (Forkhead)	aatacaca
Mutated 826	actacaca

FIG. 3. **Deletion and mutation analyses of the caveolin-1 promoter.** *a*, normalized luciferase activity shows that promoter fragments −844 to −865 and −800 to −826 increase expression in E10 cells but not in MFLM-4 cells. Luciferase activity is expressed relative to pGL3. Mean values ± S.D.; $n = 3$. * and **, $p < 0.05$. *b*, normalized transcriptional activity in E10 cells is significantly decreased from the −865 promoter fragment mutated (*hatched bars*) as shown in *d* in the Ets site compared with the wild type construct (*black bars*). Mutation of either forkhead site as shown in *d* does not result in a statistically significant decrease in expression. Data are expressed as the mean of three transfections with duplicate assays ± S.E. *, $p \leq 0.05$. *c*, in MFLM-4 cells, mutation (*hatched bars*) of the −865 Ets site results in a small but statistically significant decrease in expression compared with wild type (*black bars*). Expression from fragments mutated in the forkhead element fragments at −844 and −826 (*hatched bars*) is not different from the wild type (*black bars*) sequence. Luciferase activity is normalized for *Renilla* luciferase activity. Means ± S.D.; $n = 3$. *, $p \leq 0.05$. *d*, sequences of mutated probes at −865, −844, and −826 are shown compared with wild type with substituted bases shown in *boldface*.

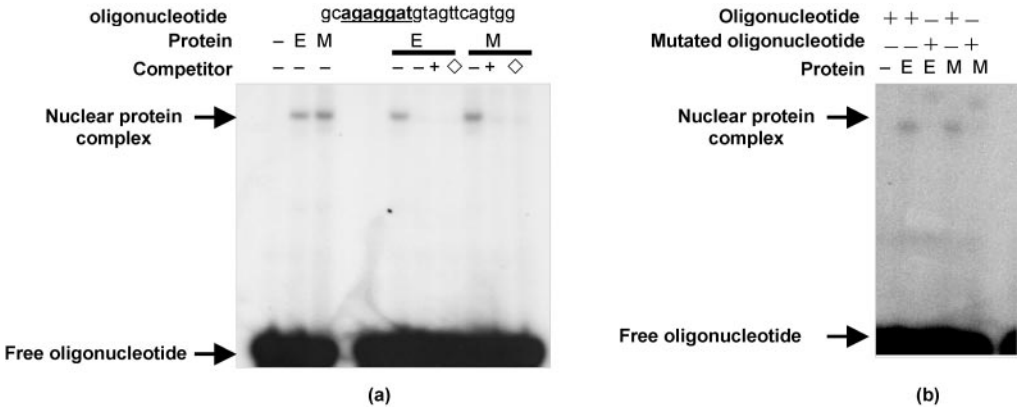


FIG. 4. **Nuclear proteins from E10 and MFLM-4 cell lines bind equally to the −844 to −865-bp promoter containing the Ets site.** *a*, EMSAs were performed using ^{32}P-labeled oligonucleotide (from bp −844 to −865). Competition assays with 100- (+) and 400-fold (◇) excess of unlabeled oligonucleotide block binding of E10 and MFLM-4 nuclear extracts (10 μg) with the labeled oligonucleotide. *b*, EMSA studies using a ^{32}P-labeled probe with a mutated Ets site (agaggatgt→**c**g**c**g**t**a**a**gt) did not form nuclear complexes in either cell line. *E*, E10; *M,* MFLM-4.

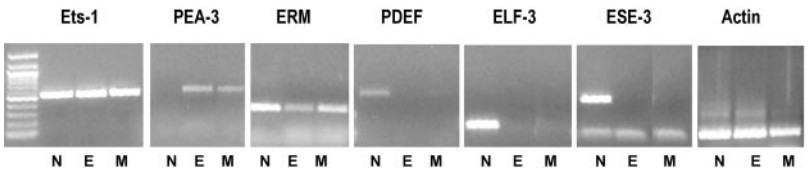


FIG. 5. **RT-PCR analysis of selected ETS family mRNAs in E10 and MFLM-4 cells.** Gel analysis of PCR products shows that both cell lines express *Ets-1, Pea3*, and *Erm* mRNAs and that neither expresses *Pdef, Elf-3*, nor *Ese-3*. With the exception of *Pea3*, all mRNAs can be detected in control mRNA from normal newborn mouse lung. Integrity of mRNAs is demonstrated by amplification of β-actin. *N*, newborn lung; *E*, E10 cells; *M*, MFLM-4 cells.

lected were *Ets-1, Pea3, Erm, Pdef, Elf-3,* and *Ese-3.* RT-PCR was used to determine whether E10 and MFLM-4 cell lines express these transcription factors. Fig. 5 shows the amplified products from mouse lung (*N*), E10 cells (*E*), and MFLM-4 cells (*M*). β-Actin shows equal amplification indicating that the RNA is intact. *Pdef, Elf-3*, and *Ese-3* are detectable in whole lung RNA, but not in either cell line. *Ets-1* and *Erm* are expressed in whole lung and in both cell lines. *Pea3* is not detectable in total lung but is expressed in both cell lines.

Immunoblot Analysis of ETS-1, PEA3, and ERM—E10 and

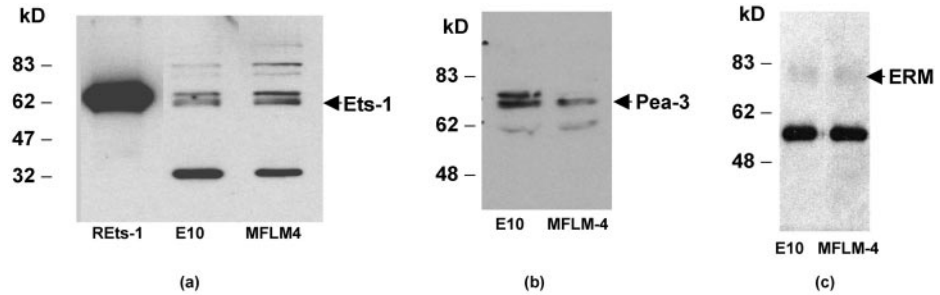


FIG. 6. **Western blot analysis for ETS-1, PEA3, and ERM in E10 and MFLM-4 nuclear proteins.** *a*, analysis of 10 μg of nuclear protein/lane using a polyclonal anti-ETS-1 antibody. Expression levels of ETS-1 protein detected as an immunoreactive doublet at the same molecular weight as positive control (*RETS-1*) show no differences in protein abundance between the two cell types. An unidentified nonspecific band at 32 kDa is seen in both cell lines. *RETS-1,* recombinant ETS-1 protein. *b*, analysis of 30 μg of nuclear protein/lane using a monoclonal anti-PEA3 antibody. A doublet at 72 kDa is detected in the E10 cell line. In contrast, a single 72-kDa immunoreactive band is observed in the MFLM-4 cell line. Based on published data, the higher molecular weight band detected in E10 cells likely represents a phosphorylated product (32, 33). *c*, 50 μg/lane of nuclear protein immunoprecipitated for ERM was analyzed by immunoblot assay using a polyclonal anti-ERM antibody. Expression levels of ERM protein show no differences in protein abundance between the two cell types. The bands detected at 55 kDa in both cell lines likely represent IgG heavy chains. *E*, E10 cells; *M,* MFLM-4 cells.

MFLM-4 nuclear extracts were analyzed by Western blot to determine whether the cells express ETS-1, PEA3, and ERM proteins and to estimate their relative amounts in the E10 and MFLM-4 cells. For ETS-1, an immunoreactive doublet at 62 kDa is identified in both cell lines (Fig. 6*a*). For PEA3, a doublet at 72 kDa is observed in the E10 cell line, whereas only a single 72-kDa immunoreactive band is observed in the MFLM-4 cell line (Fig. 6*b*). The higher molecular weight bands seen in ETS-1 and PEA3 likely represent phosphorylated forms of these transcription factors; similar doublets for ETS-1 associated with ETS-1 phosphorylation have been described previously (32, 33).

Because ERM protein could not be detected by Western blot in these samples using available antibodies, we used immunoprecipitation followed by Western blot analysis. A single immunoreactive band at 82 kDa is identified in both cell lines (Fig. 6*c*).

ETS Transcription Family Proteins Bind to the Caveolin-1 Promoter in E10 Cells but Not MFLM-4 Cells—Supershift experiments were performed to determine the identity of the nuclear protein complex. As shown in Fig. 8*a*, nuclear protein complex from the E10 cells is supershifted by anti-ETS-1, -PEA3, and -ERM. Both anti-ETS-1 and anti-PEA3 used together and the combination of all three antibodies together supershift the complex to a greater degree than any single antibody. Control IgG does not supershift the complex. In contrast, the nuclear protein complex from the MFLM-4 cell line is not supershifted by anti-ETS-1, -PEA3, -ERM, IgG, or the combination of the three antibodies (Fig. 8*b*).

Immunodepletion and EMSA Analyses Confirm That ETS-1, PEA3, and ERM Contribute to the Formation of the DNA-Protein Complex—We next examined the effect of immunodepletion of E10 nuclear extracts with anti-ETS-1, anti-PEA3, or ERM antibody on binding to the ETS consensus site. Nuclear extract recovered from the second cycle of depletion was first analyzed by immunoblot assay indicating that at least 90% of ETS-1 protein, 80% of PEA3, and 70% of ERM are removed from the E10 extract (Fig. 7, *a–c*). In the E10 nuclear extract, a single immunoreactive band is seen for ETS-1 and PEA3 in contrast to the doublet seen in our previous E10 nuclear extract analyzed by immunoblot assay (shown in Fig. 6). A possible explanation for these differences is that there is variability in the growth state of the cells isolated for nuclear extract, which influences the level of basal phosphorylation as reported previously. In astrocytes at confluence, for example, ETS-1 is largely unphosphorylated, whereas in exponentially growing cells there is a substantial increase in the phosphorylated form (32).

The results of the EMSA performed with the immunodepleted extracts are shown in Fig. 8*c*. Compared with control IgG-treated extracts (Fig. 8*c*, *1st lane*), nuclear protein extracts depleted of ETS-1, PEA3, or ERM have decreased DNA binding. Densitometry of these data normalized to the IgG control value shows that ETS-1 binding is decreased about 15%, whereas extracts immunodepleted of PEA3 and ERM are each decreased about 25%. Given that the immunodepletions are incomplete, these may actually account for all of the binding. The competition, supershift, and immunodepletion experiments suggest therefore that ETS-1, PEA3, and ERM can form the DNA-protein complex in the E10 cell line.

DISCUSSION

In this study we show that an Ets *cis*-element strongly enhances expression of the caveolin-1 gene in a lung epithelial cell line, but has only minimal effects on caveolin-1 expression in a lung endothelial cell line, and that three members of the ETS family of winged helix-loop-helix transcription factors recognize and bind to the Ets *cis*-element in the epithelial cell line but not the endothelial cell line. The comparison of these two cell lines was undertaken because of our previous observations on the expression patterns of caveolin-1α in fetal lung showing that endothelial cell expression commences very early in lung development, whereas epithelial expression commences sometime after birth (4), suggesting that the regulatory mechanisms of caveolin-1 gene transcription are markedly different in these cell types.

The lung is unusual in that two cell populations with exceedingly large surface areas, alveolar epithelial type I cells and alveolar capillary endothelial cells, express very high concentrations of caveolin-1 mRNA and protein. These epithelial and endothelial cells reside adjacent to each other to form the alveolar septae, often separated only by their single or sometimes fused basement membranes. Thus the cells would be expected to encounter many similar stimuli that affect gene expression, including that of caveolin-1. We hypothesized that the selective expression of intracellular molecules such as transcription factors or their cofactors was likely to account for differences in caveolin-1 in these two cell populations. We therefore anticipated finding a key transcription factor, such as an epithelial specific forkhead protein, that would be expressed in epithelial but not endothelial cells.

We provide evidence, however, that epithelial expression is regulated by ETS family members and that forkhead proteins are not likely to be involved. Based on analysis of promoter deletion fragments and mutational analyses, we first identified a *cis*-element containing the core Ets family binding site se-

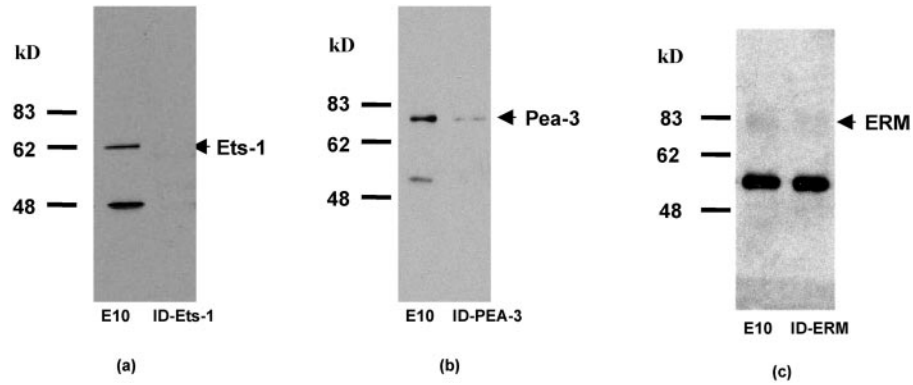


FIG. 7. **Western blot analyses of E10 nuclear extracts showing immunodepletion efficiency of ETS-1, PEA3, and ERM.** *a*, analysis of 10 μg of nuclear protein/lane of E10 and E10 nuclear extract immunodepleted of ETS-1. E10 nuclear extract recovered from the second cycle of depletion was 90% depleted of ETS-1 protein (*b*). Analysis of 30 μg of nuclear protein/lane of E10 and E10 nuclear extract immunodepleted of PEA3. E10 nuclear extract recovered from the second cycle of depletion was 80% depleted of PEA3 protein. *c*, analysis of 10 μg of nuclear protein/lane of E10 nuclear extract immunoprecipitated for ERM and then immunodepleted of ERM. E10 nuclear extract recovered from the second cycle of depletion was 70% depleted of ERM protein.

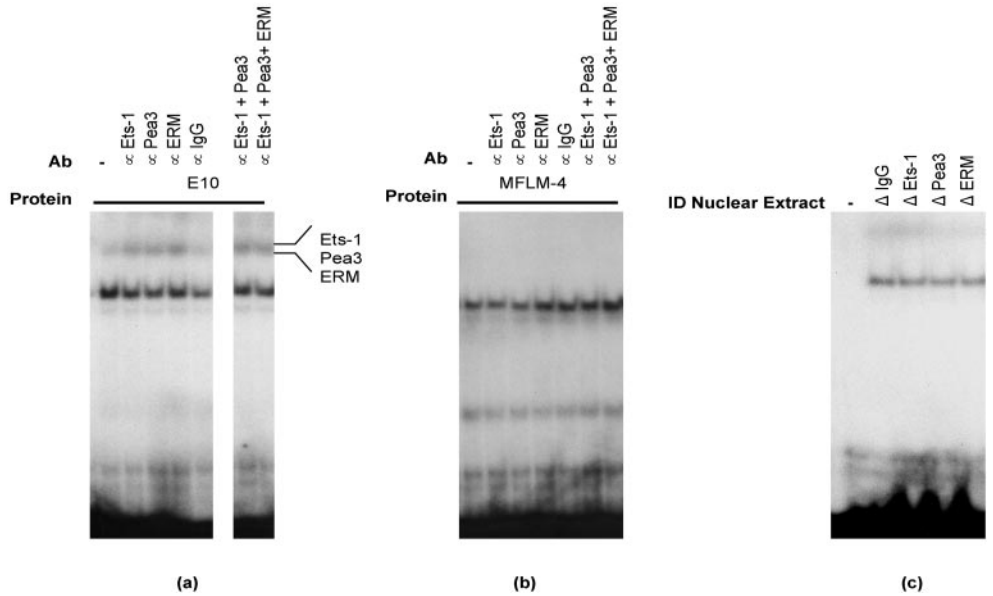


FIG. 8. **EMSA supershifts show that ETS-1, PEA3, and ERM from E10, but not MFLM-4, cells bind to the Ets oligonucleotide.** *a*, supershift analyses of the complexes formed by E10 nuclear extract and labeled Ets probe. Antibodies (denoted as α) for ETS-1, PEA3, and ERM or control IgG (4 μl) were incubated at RT for 20 min with the nuclear extracts before adding labeled probe. Both nuclear extract alone (no antibody) and control IgG have equivalent bands in the supershifted region as assessed by densitometry. In contrast, complexes are markedly supershifted by anti-ETS-1, PEA3, and ERM as compared with controls. The antibodies used in combination supershift the complex to a greater degree than any antibody alone. *b*, supershift analyses of the complexes formed by MFLM-4 nuclear extract and labeled Ets probe. Nuclear protein complex was not supershifted in the presence of anti-ETS-1, PEA3, ERM, control IgG, or the antibodies used in combination. *c*, immunodepletion (depleted noted as Δ) of ETS-1, PEA3, and ERM (10 μg) decreases binding of E10 nuclear proteins to the Ets oligonucleotide compared with control IgG.

quence, 5′-GGA(A/T)-3′, that preferentially activates the caveolin-1 promoter in the epithelial cell line. In contrast, *cis*-elements containing the forkhead family binding site sequences did not activate caveolin-1 transcription in either cell line.

The identification of the ETS family member(s) responsible for transcriptional activation in the epithelial cell line is not straightforward, given that the ETS family consists of more than 30 proteins. These proteins can function either to activate or repress transcription depending on promoter context, cell type, or stage of cellular differentiation and growth cycle, and different proteins can activate the same gene promoter. In A549 human lung carcinoma cells, for example, ETS-2 activates the GM-CSF promoter, whereas in Jurkat T cells ETS-1 activates this promoter (34), clearly demonstrating cell type-specific regulation.

In addition, the binding characteristics of ETS proteins are complex. ETS proteins can function as both transcriptional activators and repressors (35), and it is not uncommon for many ETS family members to bind to the same 11-bp extended consensus DNA binding sequence. Nitric-oxide synthase 2 that catalyzes nitric oxide production, for instance, can be regulated by several ETS factors. ESE-1 and ETS-2 increase nitric-oxide synthase 2 promoter activity, whereas ELK-3 represses nitric-oxide synthase 2 via an inhibitory domain (36). Other ETS domain proteins including ETS-1, ETS-2, PEA3, and ERM are known to have inhibitory regions flanking the Ets domain that affects their transcriptional and DNA binding activities (27, 28, 31).

ETS proteins can also interact with coregulatory proteins either through protein-protein or protein-DNA interactions and/or undergo post-translational modifications such as phosphorylation, dephosphorylation, or acetylation that modulate their ability to activate transcription and promoter recognition specificity (27, 28, 31). Many ETS proteins, for instance, require cooperation with other transcription factors, such as NF-EM5, Sp1, Ap1, or SRF, to be active (37). Some ETS proteins

including ETS1, ETS2, PEA3, and ERM require activation by either a Ras-dependent or -independent phosphorylation pathway (27, 38), whereas ELF-1 activation requires phosphorylation and glycosylation (39). Whereas all ETS proteins bind a central GGA motif, individual ETS proteins select specific nucleotides over an 11-base sequence. Specificity is thought to be maintained, at least partly, through these coregulatory proteins and post-translational modifications of both ETS proteins and their partners (27).

Given the complexity of the binding and transactivating properties of ETS proteins, we were not surprised to find that gel shift analysis shows that nuclear extracts from both cell lines bind equally to the Ets-specific oligonucleotide even though the Ets *cis*-element preferentially increases expression in the epithelial cell line. To further identify the ETS transcription factors responsible for transactivating the caveolin-1 promoter in the lung epithelial cell line, we selected for study those proteins that are known to be expressed in epithelial cells in the lung or to be involved in lung tumorigenesis, which included ETS-1, PEA3, ERM, PDEF, ELF-3, and ESE-3.

It is notable that *Ets-1, Pea3*, and *Erm* have been shown by *in situ* hybridization analyses and other methods to be expressed simultaneously in the same population of lung epithelial cells. *ETS-1* is known to be expressed in developing and adult lung, lymphoid organs, brain, and vascular endothelial cells and is thought to play a key role in vascular development and angiogenesis (40, 41). The molecular characteristics of ETS-1, the founding member of the family, include the conserved DNA binding domain and a second conserved domain called the pointed domain that functions in homodimerization, heterodimerization, and transcriptional repression (27) and thus can activate or repress gene promoters. An ETS-1 target important in lung development is *Sprouty2* (*Spry2*), a gene localized predominantly at the distal epithelium during development in the same cell population expressing *Pea3* and *Erm* (42–44). *Spry2* is transcriptionally regulated by a *cis*-element that binds ETS-1, thus providing indirect evidence that ETS-1 is expressed in distal fetal epithelium. In mice deficient in Ets-1, there is an increase in T cell apoptosis and defects in B cell function, but there is no defect in the lung or vascular development (27, 28, 40).

PEA3 (E1AF/ETV4) is the first member of a subfamily of ETS proteins known as the PEA3 group, which also includes ERM and ER81. PEA3 group members have nearly identical Ets domains and additional homologous sequences. Although PEA3 and ERM bind with similar affinities to some target genes, they are not functionally equivalent. Animals with a null mutation in the ERM gene die in early embryogenesis, whereas those lacking PEA3 live a normal life span (44). PEA3 is mainly expressed in epithelial cells and is preferentially expressed at sites of epithelium-mesenchymal interactions (27). During lung organogenesis, PEA3 is expressed in both the epithelium and mesenchyme of the distal buds in the developing bronchial tree. Like PEA3, ERM is restricted to the distal bronchial tree during development but is expressed exclusively in the epithelium (44).

Although PDEF has not been reported previously to be expressed in the lung, it is expressed in other epithelia including trachea, breast, and prostate and, like caveolin-1, is overexpressed in human breast tumors (45). ESE-1/ELF-3/ESX/jen/ERT is also expressed in the lung and in cell lines of epithelial origin but is not expressed in hematopoietic cells (46). The human *ESE-1*/*ELF-3* gene is localized to 1q32.2, a region that is amplified in epithelial tumors of the lung, breast, and prostate (47). Most interesting, ESE-1/ELF-3 is overexpressed in lung adenocarcinomas but not in small cell or squamous cell cancers of the lung. ESE-3 is expressed in the lung and is thought to be involved in the branching morphogenesis of the lung, in the oncogenesis of epithelial derived tumors such as bronchogenic tumors, and in the pathogenesis of asthma (48). Because the lung expresses many ETS family members as noted above, understanding their roles in lung biology and in lung tumors is a challenging undertaking, not the least of which is to determine which proteins are expressed by each of the more than 40 cell types that make up the normal lung.

Three ETS family members, *Ets-1, Pea3,* and *Erm*, were found to be expressed in both the E10 and MFLM-4 cell lines, yet they bind specifically to the ETS site in the epithelial but not the endothelial cell line. There were no differences between the cell lines in *Ets-1, Pea3,* and Erm mRNA expression levels and protein abundance. However, for PEA3 we detected two immunoreactive bands in the E10 cells and only one in the MFLM-4 cells. When E10 nuclear extracts were immunodepleted of ETS-1, PEA3, or ERM, each had decreased binding to the Ets *cis*-element in EMSA studies, suggesting that all three ETS proteins contribute to caveolin-1 regulation in epithelial cells. These data do not rule out the possibility that other ETS proteins may bind and transactivate or inhibit the caveolin-1 gene either through direct protein-protein interaction or by competing for binding to the Ets site in these cell lines. This possibility may be reflected by the residual binding we observed using immunodepleted nuclear protein extracts.

We are left with the question of what directs epithelial cell-specific caveolin expression because all three proteins are found in both cell lines but bind and activate the caveolin-1 promoter only in the epithelial cell line. There are several possible explanations. A coactivator protein may be present in the epithelial cell line that increases the affinity of these ETS proteins to their binding site; alternatively, the state of phosphorylation of the ETS proteins may be different in the epithelial cell line, and/or inhibitory proteins may be present in the endothelial cell line. These hypotheses require critical testing in our model.

There is an extensive literature that links expression of both ETS proteins and caveolin-1 to cancer. Expression profiles comparing mouse lung cancers with those of human lung adenocarcinomas show that caveolin-1 is one of the few genes whose expression is uniformly decreased in both murine and human lung cancers (49). Furthermore, in chemically induced murine lung adenocarcinomas, *Ets-2* expression is markedly decreased as shown by QRT-PCR (49). Overexpression of the *ETS-1* gene has been associated with metastases of pulmonary adenocarcinomas and many other human tumors, most of which are epithelial in origin (27, 41). PEA3 overexpression has been reported in various cancer cells, including non-small cell lung cancers, breast, colorectal, oral squamous cell, and ovarian, and current evidence suggests that it contributes to cancer cell invasiveness (50, 51).

At the molecular level, ETS proteins have been shown to regulate genes implicated in cell invasiveness and tumorigenesis such as MMP-1 and matrilysin (50). Furthermore, Ras-responsive ETS elements are thought to be activated in tumors and promote oncogenesis. Pea3 transactivates the Muc4/SMC promoter by a Ras and MEKK1 kinase pathway, which may contribute to mammary tumor metastases (51).

Likewise, there is clear evidence that expression of caveolin-1 is altered in cancers; in fact, this has led to the proposal that caveolin-1 has the characteristics of a tumor suppressor gene, although this is debated because caveolin−/− animals do not have increased spontaneous tumors. In lung adenocarcinoma gene microarray studies, caveolin-1 is markedly down-regulated (49, 52), and caveolin-1 expression is down-regulated in cells that

have been transformed by oncogenes (53). Furthermore, re-expression of caveolin-1 in caveolin−/− cells partially reverses the transformed hyperproliferative phenotype (5). The fact that mice carrying null mutations in the caveolin-1 gene do not acquire more spontaneous tumors than controls or acquire tumors at a younger age, however, argues strongly that caveolin-1 is not a direct tumor suppressor gene. If caveolin-1 acts as a tumor suppressor gene in mice, it does so with low suppressor activity.

In contrast, some studies of caveolin-1 in tumor tissues indicate a possible tumor-promoting effect of caveolin-1 (54). Several reports indicate that caveolin-1 expression is up-regulated in tumors that become or have already metastasized, thus suggesting a role for the protein in the multiple steps that allow detachment, invasion, motility, anoikis, and seeding into a new tissue domain. In lung carcinoma cell lines (CL cells), up-regulation of caveolin-1 mRNA and protein correlates with increased metastatic capability; expression of caveolin-1 in the less invasive CL cells that were caveolin-1-negative increases their ability to metastasize (55). In human prostate, pancreatic ductal tumors, and lung adenocarcinomas, sustained expression of caveolin-1 has been reported to be a negative prognosticator of clinical outcome (54–56).

These observations lead to an obvious interest in the molecular transcriptional regulation of caveolin-1, which in some way appears to influence lung tumor progression. We now show that three ETS proteins bind to an Ets *cis*-element that enhances transactivation of the caveolin-1 promoter in a lung epithelial cell line. We therefore hypothesize that the ETS protein driving expression of caveolin-1 in lung epithelial cells may itself be up-regulated as lung carcinomas progress toward metastases. Additional studies will focus on the role of ETS-1, PEA3, and ERM in caveolin-1 regulation by using expression vectors, cancer-derived cell lines, and human tumors. We believe this is the first report on the transcriptional regulation of caveolin-1 in lung cell lines representative of alveolar type I and lung endothelial cells. Because of its putative role in lung and other epithelial derived carcinomas, exploring the role of ETS proteins as regulators of caveolin in human lung tumors will be important and perhaps allow the identification of therapeutic targets not currently known.

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