Lung-endothelial cell adhesion molecule-1 (Lu-ECAM-1) is an endothelial cell surface molecule that mediates adhesion of metastatic melanoma cells to lung endothelium. Here we analyze the organization of the Lu-ECAM-1 protein complex, report the sequence of Lu-ECAM-1 cDNAs, and reveal a novel function of the protein. Lu-ECAM-1 immunopurified from bovine aortic endothelial cells (BAEC) consists of tightly associated glycoproteins of 90, 38, and 32 kDa, with minor components of 130 and 120 kDa. We present evidence that all of these protein species are encoded by a single open reading frame whose initial translation product is proteolytically processed to yield the other products. Correct processing in vitro was demonstrated by transfection of the longest cDNA into human embryonic kidney 293 cells; immunoblot analysis showed that the 120-kDa precursor gave rise to 90- and 38-kDa products. RNA blots of BAEC mRNA detected messages in agreement with the sizes of the cDNA clones in addition to several of high molecular weight. DNA blot analysis showed that Lu-ECAM-1 is conserved throughout its length in all mammals tested, usually as a single or low copy gene. In the bovine, Lu-ECAM-1 protein is 88% identical to a calcium-dependent chloride channel described recently in tracheal epithelium, Ca-CC. Probes for Lu-ECAM-1 mRNA and protein confirmed the presence of a homolog in this tissue. We show that messages for both proteins are present in lung while only Ca-CC is present in trachea and only Lu-ECAM-1 is present in BAEC. These results suggest that endothelial cells express a chloride channel that is related to, but distinct from, that expressed in tracheal epithelium. They further suggest that an adhesion molecule can also be a chloride channel.

The preference of metastasizing tumor cells for certain organs may be explained if such cells fortuitously recognize and adhere to organ-specific, endothelial cell-surface molecules. In studying this hypothesis, much emphasis has been placed on the role of members of the classic families of cell-cell adhesion molecules including selectins, the immunoglobulin superfamily, and integrins (1–3). The contribution of these adhesion molecules to organ preference of metastasis was suggested by a number of reports describing the presence of such molecules on endothelia of various tissues and vessel calibers and denoting corresponding ligands on malignant cells of tumors of various tissue origins (1–7). In our laboratory, a different approach was chosen to testing the contribution of specific endothelial cell adhesion molecules to organ preference of metastasis. It relied on an endothelial cell culture system that could be modulated by growing unspecific, large vessel-derived endothelial cells (e.g. bovine aortic endothelial cells (BAEC)) on organ-specific matrix to express phenotypic traits of the microvasculature of that organ (8, 9). Tumor cells with distinct metastatic dissemination patterns were then evaluated for adhesion to these endothelial cells, observing that tumor cells only bound in large numbers to those endothelial cells that were modulated with matrix from the preferred organ site of metastasis (9). To identify the adhesion-receptor/ligand pair that mediated binding of lung-metastatic melanoma cells to lung matrix-modulated BAEC, monoclonal antibodies directed against endothelial luminal membrane vesicles were produced that inhibited the specific adhesion of melanoma cells to endothelium (10, 11). Such a mAb was 6D3. The antibody selectively recognized endothelia of pleural and subpleural venules and, to a lesser extent, endothelia of peribronchiolar and parenchymal venules of mouse lung (11, 12). This vascular distribution was highly correlated with the position of emerging tumor colonies three weeks after intravenous inoculation of B16-F10 melanoma cells; i.e. most metastases were observed in pleural and subpleural tissues (12). The purified antibody effectively increased the clearance of B16-F10 cells from murine lungs, exerting its most dramatic effect during the first 30 min after intravenous inoculation of tumor cells (13). Accordingly this antibody was efficient in preventing metastatic colonization of the lungs by B16-F10 melanoma cells (11, 13).

Purification of the endothelial cell adhesion molecule was performed by immunoprecipitation from extracts of lung matrix-modulated BAEC using mAb 6D3 (11). A major component at 90 kDa was identified and termed lung-endothelial cell adhesion molecule-1 (Lu-ECAM-1; Ref. 11). The purified molecule promotes strong adhesion of B16-F10 cells under static and hydrodynamic conditions (14) and, in soluble form, competi-

1 The abbreviations used are: BAEC, bovine aortic endothelial cells; KLH, keyhole limpet hemocyanin; DST, disuccinimidyl tartarate; RT-PCR, reverse transcription-polymerase chain reaction; RACE, rapid amplification of cDNA ends; HER293, human embryonic kidney 293; CMV, cytomegalovirus; ORF, open reading frame; mAb, monoclonal antibody; bp, base pair(s); nt, nucleotide(s); kb, kilobase(s); wtLu-ECAM-1, wild-type Lu-ECAM-1; rLu-ECAM-1, recombinant Lu-ECAM-1; PBS, phosphate-buffered saline.
tively inhibits adhesion of these tumor cells to immobilized Lu-ECAM-1 (11, 13). Here, we identify other components of the Lu-ECAM-1 complex and define their interrelationships. The sequences of cDNAs that encode these proteins are reported, and it is shown that the Lu-ECAM-1 gene is conserved in humans as well as other mammals. Lu-ECAM-1 is not a member of any of the classic families of cell-cell adhesion molecules introduced above but is a homolog of the recently described calcium-activated chloride channel, Ca-CC (15). In light of this homology, our previous characterization of sites of expression of the Lu-ECAM-1 complex is extended and compared with the localization of Ca-CC. The dual adhesion/chloride channel function of Lu-ECAM-1 is novel and intriguing and is discussed here in the context of channel regulation and importance to metastasis.

EXPERIMENTAL PROCEDURES

Immunopurification and Protein Analysis—Lu-ECAM-1 was immuno-purified from BAEC and lung with mAb 6D3 as described by Zhu et al. (11). Proteins were fractionated by SDS-PAGE (10%) and stained with silver (see Fig. 1) or transferred to polyvinylidene difluoride membrane (Bio-Rad) and sequenced at the Northwestern or Harvard University protein sequencing facilities. Molecular weights were determined relative to unsized markers (Life Technologies, Inc. and Molecular Probes). For deglycosylation, immunopurified proteins were treated with recombinant N-glycosidase F according to the manufacturer (N-Glycanase; Genzyme, Cambridge, MA).

Isolation of Lu-ECAM-1 cDNAs—The general strategy for assembly of Lu-ECAM-1 cDNA Clone 1 from fragments obtained by PCR is schematized in Fig. 2. First, the amino-terminal and internal protein sequences of the 38-kDa component (see Table I) were used to design degenerate primers for primary and nested PCR using reverse transcribed BaEC total RNA as template. Upstream primers corresponded to amino acids 685–693 and 698–705. Downstream antisense primers corresponded to amino acids 829–832 and 852–846. A product overlapping a 520-bp was obtained and sequenced (see Fig. 2, P1). From this sequence, nondegenerate primers for 3′-RACE were designed (nt 2562–2588 and 2590–2611 of Clone 1, GenBankTM Data Bank accession number AF001261; 3′ Amplifier RACE kit, CLONTECH). An ~750-bp product was obtained and sequenced (see Fig. 2, P2). Nondegenerate primers were designed for the first round of 5′-RACE (nt 2643–2619 and 2520–2496) using the Amersham Pharmacia Biotech (Munich, Germany) ML100 gel and DNA ligase. A product of ~900 bp was obtained and sequenced (Fig. 2, P3). Its 5′ end (nt 1572) lacked a Kozak-context ATG (17) and signal sequence and so appeared to be an abortive reverse transcription product. A further 5′-RACE reaction using an internal primer (nt 1599–1572) was performed to obtain the entire 5′ end (see Fig. 2, P4), containing a Kozak-context ATG, a signal sequence, and the amino-terminal sequence of the 90-kDa protein (see Fig. 3). To reconstitute the cDNA, these overlapping PCR products were assembled into one ORF by an overlap-extension strategy using a high fidelity Taq/Phu polymerase combination (Stratagene) (18). The order of assembly was ([P1 + P2] + P3) + P4. Products were cloned into a T-vector (Invitrogen) and sequenced. To obtain other clones, total RNA was isolated from BAEC by the guanidinium chloride procedure (19) and used to prepare a cDNA library in Lambda ZAP II (Stratagene). Internal peptide sequences of the 90-kDa component were used to design degenerate primers for RT-PCR with BaEC RNA as template. Primers corresponding to amino acids 143–148 and 310–305 (Fig. 3) yielded an ~520-bp product. This product was used to screen the library by filter hybridization. Oligonucleotide primers were prepared by Life Technologies, Inc., and DNA sequencing was by the Sanger method. Sequences were analyzed with GCC software (University of Wisconsin).

Immunological Methods—Polyclonal antibodies were generated against peptides based on the cDNA sequences or against purified Lu-ECAM-1 components (Table II). Antibodies CU11 and CU8 were generated against synthetic peptides. Peptides were synthesized at the Dept. of Genetic Engineering (University of Illinois, Urbana), partially purified by HPLC, and conjugated to KLH using the Activated Immuno-nogen Conjugation kit (Pierce). Antibody CU19 was prepared after cloning the BovBi-Sty1 fragment containing the 3′ end of Clone 3 (amino acids 624–799) into the PfuUl site of pcRSET A (Invitrogen) to produce a fusion protein bearing a six-histidine metal affinity tag. The protein was purified over nickel-resin (Invitrogen) and conjugated to KLH. Rabbit polyclonal antisera were prepared by Cocalico Biologica (Reamstown, PA). Polyclonal antibodies R4 and R41 were prepared by immunizing rats with the 90- and 38-kDa bands, respectively, excised from polyacrylamide gels, pulverized, and mixed with complete Freund’s adjuvant. Immunoblots were performed according to standard methods (20) using horseradish peroxidase-conjugated secondary antibodies (Life Technologies, Inc.) and chemiluminescent reagents (ECL, Amersham Corp., or Super Signal, Pierce). For biotinylation, BAEC were treated with NHS (long arm)-biotin (Vector Laboratories, Inc., Burlingame, CA) as recommended by the manufacturer. Disuccinimidyl tartarate (DST, Pierce) cross-linking was as described (21). Proteins were immunoprecipitated from lysates, analyzed by SDS-PAGE, transferred to nitrocellulose, and detected using avidin-horseradish peroxidase and chemiluminescent reagents.

Expression of Lu-ECAM-1 cDNA in HEK293 Cells and in vitro—For expression in HEK293 cells, Lu-ECAM-1 cDNA was placed under control of a tetracycline-regulated promoter in pTet-Splice (Life Technologies, Inc.). The construction was accomplished in two steps. A PCR product was first generated that corresponded to the 3′ end of Clone 1 cDNA (nt 2391 to 2780). The 5′ primer was 5′-ACTGAATTCTCAAGACAGTCACCTGAGGAACTC-3′ and contained an EcoRI restriction site. The 3′ primer was 5′-TCTACTAGTAGCTTATGACTGAACAGAG-3′ and contained a SpeI site. The product was cleaved with SpeI and EcoRI and then cloned into corresponding sites in pTet-Splce. A plasmid clone was selected and sequenced to confirm the absence of mutations. The construct was co-transfected with pGEM-3Zf into the tumor cell line HeLa to substitute the Lu-ECAM-1 ORF, the 2.3-kb EcoRI-BglII fragment was excised from Clone 3 and inserted into the plasmid. The resulting plasmid, pTet-Splce-Lu-ECAM-1, was then transfected into HEK293 cells along with pTet-tTAK, which encodes a transcriptional activator specific for the pTet-Splce-vector. Transfection was by the LipofectAMINE (Life Technologies, Inc.) method according to the manufacturer’s instructions. Cells were harvested 24 h after the start of transfection. Control cells were transfected in parallel with a plasmid expressing rat dipeptidyl peptidase IV under control of the CMV promoter (ReCMV, Invitrogen) (22). For in vitro expression, Lu-ECAM-1 cDNA Clone 1 was placed under control of the phage T7 promoter in pGEM3. The EcoRI-SpeI fragment containing Lu-ECAM-1 was excised from pTet-Splce and inserted into the EcoRI and XbaI sites of pGEM3. Plasmid DNA was transfected using the TNT-coupled transcription-translation system (Promega) and [35S]methionine (Amersham Life Science, Inc.). Glycosylation was accomplished with canine pancreatic microsomes (Amersham Life Science, Inc.).

DNA and RNA Blots—The genomic DNA blot was hybridized for 14 h at 37 °C in 5 × SSC, 50% formamide, 4 × Denhardt’s solution, 10% polyethylene glycol (MW 1000), and 0.1% sodium dodecyl sulfate (SDS) DNA. The blot was stripped after exposure by boiling in 0.1 × SSC. For RNA blots, lung and spleen mRNA preparations were purchased from CLONTECH. Alternatively, mRNA was prepared from confluent BAEC and freshly scraped epithelium from bovine trachea using the Fast Track system (Invitrogen, San Diego, CA). mRNA was electrophoresed on a 1.2% agarose-formaldehyde gel, blotted onto Nitran (Schleicher & Schuell) membrane, and hybridi- zed to 65 °C with specific cDNA probe. (Stratagene). 32P-labeled probes were prepared by random priming (Promega kit, Life Technologies, Inc.). Radioisotopes were purchased from Amersham Life Science, Inc.

Immunohistochemistry—Formalin-fixed sections of bovine trachea were first boiled for ten min in 4 × urea in a microwave oven and then probed with polyclonal antibody R4. The sections were incubated with biotinylated donkey anti-rat IgG and Neutravidin-peroxidase conjugate (Molecular Probes). The peroxidase conjugate was detected using diaminobenzidine as substrate and the slides were counterstained with hematoxylin. Lung sections were prepared and probed with mAb 6D3 according to Zhu et al. (12) except that a biotinylated secondary antibody was used followed by Neutravidin-peroxidase conjugate.

RT-PCR Analysis—For RT-PCR analysis of bovine lung, spleen, tracheal epithelium, and cultured BAEC, 500 ng of each mRNA was reverse transcribed with random oligonucleotide primers and Super- script II reverse transcriptase (Life Technologies, Inc.) in a 20-μl reaction volume. Primers specific for Lu-ECAM-1 cDNA (LU primers) or Ca-CC cDNA (TC primers) were selected such that the last one, two, or three nucleotides were complementary only to the Lu-ECAM-1 or the Ca-CC cDNA sequence, respectively. The selectivity of the primers was confirmed in control experiments with a Lu-ECAM-1 cDNA clone. The primer sequences and their locations in the respective cDNA sequence are given in Table III. The cycling protocol was as follows: 94 °C, 20 s; 55 °C, 10 s; and 72 °C, 10 s, for 35 cycles with a time increment of 2 s/cycle for annealing and extension times and a final extension step at 72 °C for 10 min.
Clones—Lu-ECAM-1 cDNAs were obtained using degenerate 252–2438 but diverged at the 3′ component. Clone 2 was identical to Clone 1 from nucleotide 55/32-kDa components. Its length was 3.3 kb, and it encodes entirely by PCR using primers based on sequences of the 38-kDa polypeptides (see “Experimental Procedures”). PCR primers based on peptide sequences from the 90-, 38-, and 32-kDa proteins were obtained (Figs. 2 and 3). The first, Clone 1, was generated directly (Fig. 3). Clone 4 encodes a truncated, 321-amino acid version of Lu-ECAM-1 that may be secreted. Its derived amino acid sequence is identical to that of the other ORFs up to amino acid 303 (Fig. 3). The ORFs of all four cDNA clones contain the amino-terminal sequence of the 90-kDa component of Lu-ECAM-1 following a presumptive signal sequence of 21 amino acids. The amino-terminal sequence of the 38- and 32-kDa components lies near the carboxyl termini of the ORFs of Clones 1, 2, and 3 (Table I and Fig. 3, arrowhead). However, only the ORF of Clone 1 contains the sequence of the internal peptide (Table I and Fig. 3, residues 828–851) derived from the 38-kDa component.

Hybridization of a genomic DNA blot with a Lu-ECAM-1 probe (EcoRI-BglII fragment) detected signals in all mammals tested (Fig. 4). However, searches of genetic data banks for similarity to Lu-ECAM-1 revealed only one extensive match, the chloride channel Ca-CC (GenBankTM/EBI Data Bank accession number U36455). Within their ORFs, the Lu-ECAM-1 and Ca-CC represent products of different genes. Only one other significant match was detected in the data banks, a swine intestine partial cDNA (55% identity over 58 amino acid residues; GenBankTM/EBI Data Bank accession number F15082).

Peptide Derivation

Table I

| Peptide sequences derived from immunopurified proteins |
|---------------------------------------------------------|
| Total Protein | Derivation (kDa protein) | Location in Clone 1 ORF |
|---------------------------------------------------------|
| SMNLININGDYGIVIAIN | 90 | 1–18 |
| QSFDQAVSVVANFVY | 90 | 74–89 |
| RHAELGGFIDNEYV | 90 | 134–149 |
| DQFYFISR | 90 | 150–157 |
| SVSVDIMSSDFQNTSPMTMNM | 90 | 251–274 |
| LFWMQQAELLYIQVIKEG | 90 | 306–324 |
| VLVPGYYVNGKIIKNPPPEPVDLDKAK | 32 and 38 | 683–710 |
| KEDYIQLSWATPGNV | 38 | 752–766 |
| FYISVQAINELISEVSHVQA | 38 | 828–851 |

RESULTS

Immunopurification of the Lu-ECAM-1 Complex—The Lu-ECAM-1 complex was immunopurified from BAEC and analyzed by SDS-PAGE and silver-staining (Fig. 1). Major bands corresponded to sizes of 90, 55, 38, and 32 kDa. The amino-terminal sequence of each of the polypeptides was determined after resolution on SDS-PAGE and transfer to Immobilon. The terminal sequence of each of the polypeptides was determined by Edman degradation after transfer to polyvinylidene difluoride membrane. The 55-kDa band was determined to be an IgG contaminant.

Character of the Predicted Protein—All of the ORFs predict hydrophobic amino termini (residues 21 to –1) with the features of a cleavable signal sequence (24) preceding the mature
amino terminus determined by protein sequencing (Fig. 3, double underline). Four other generally hydrophobic regions (Fig. 3, underline) were proposed by Cunningham et al. (15) to comprise transmembrane segments in the cognate Ca-CC molecule. In particular, the hydrophobic segment starting at residue 595 conforms well to the established criteria for a helical transmembrane segments, containing 23 amino acid residues with a total transfer free energy of 36.3 kcal/mol (25). Nine potential sites exist for asparagine-linked glycosylation (Fig. 3, asterisks).

**Organization of Lu-ECAM-1 Components**—The relationship between the high and low molecular weight Lu-ECAM-1 components was explored using antibodies that recognize distinct epitopes. To determine which components share the epitope for the mAb used for immunoprecipitation, affinity purified Lu-ECAM-1 was probed on an immunoblot with mAb 6D3. This antibody detected the 90-kDa component but not the 38- or 32-kDa components, indicating that the smaller components lack the epitope and are instead tightly complexed with the larger components (Fig. 5A). In addition, 6D3 detected two larger bands migrating at approximately 120- and 130-kDa, respectively, that were not clearly visible by silver staining.

To determine which parts of the cDNAs correspond to which proteins, polyclonal antibodies were generated against synthetic peptides based on the cDNA sequences or against chimeric proteins obtained by expression of cDNAs in bacteria (Table II). CU11 (against amino acid residues 22–38) recognized the larger components but not the 38- and 32-kDa components (Fig. 5A). CU19 was raised against a region of the predicted protein containing the amino terminus of the 38- and 32-kDa components and a small part of the putative carboxyl terminus of the 90-kDa component, amino acids 618–767. It strongly detected the 38- and 32-kDa, as well as the 130- and 120-kDa, components but only weakly detected the 90-kDa component (Fig. 5A). These results suggest a model in which the initial translation product of Clone 1, manifested by the 120- and 130-kDa components, is processed to yield the 90-, 38-, and 32-kDa components, as depicted in Fig. 2.

The relationship between the larger and smaller Lu-ECAM-1 components was further explored by generating antibodies...
TABLE II

| Antibody | Antigen | Proteins detected |
|----------|---------|-------------------|
| 6D3      | Lu-ECAM-1<sup>a</sup> | 90, 120, 130 |
| CU11     | Amino acids 23–40<sup>b</sup> | 90, 120, 130 |
| CU8      | Amino acids 514–530<sup>b</sup> | 90, 120, 130 |
| CU19     | Amino acids 618–767<sup>c</sup> | 32, 38, 90<sup>d</sup>, 120, 130 |
| R4       | 90-kDa protein | 90, 120, 130 |
| R41      | 38-kDa protein | 32, 38, 120, 130 |

<sup>a</sup> Whole protein.

<sup>b</sup> Synthetic peptide: amino acid positions in Clone 1 ORF.

<sup>c</sup> Bacterially expressed fusion protein.

<sup>d</sup> Weak.

against gel-purified 90- and 38-kDa protein bands. By the proposed model, an antibody against one of these bands should not recognize the other band while either antibody should recognize the 120- and 130-kDa bands. R4, a polyclonal anti-90-kDa protein antibody, detected the 90-kDa band as well as the 130- and 120-kDa bands but not the 38- or 32-kDa bands (Fig. 5A). Similarly, R41, an anti-38-kDa protein antibody, recognized the 38-, 32-, 120-, and 130-kDa proteins but did not detect the 90-kDa band. These results again indicate that the 90-kDa protein has no epitope in common with the 38- or 32-kDa proteins. On the other hand, the 120- and 130-kDa proteins have sequence in common with both the 90-kDa protein and the 38- and 32-kDa proteins. These results also show that the 38- and 32-kDa proteins are antigenically related, as are the 120- and 130-kDa proteins. These proteins may thus represent pairs of alternate glycoforms. Indeed, treatment of the Lu-ECAM-1 protein complex with N-glycosidase F reduced the 38- and 32-kDa bands to a common band of 22 kDa while the 90-kDa band was reduced to 77 kDa (Fig. 5B). Products of exactly these sizes are expected following processing of the initial translation product of Clone 1. Unexpectedly, the 77-kDa band is a doublet, suggesting that deglycosylation may have been incomplete. The 120- and 130-kDa bands were not detected in this experiment.

The 38- and 32-kDa components of Lu-ECAM-1 are not recognized by 6D3 and so are likely noncovalently complexed with the other components (Fig. 5A). The complex is resistant to dissociation by high salt, detergent, and EDTA, all routinely employed in the washing of immunobeads, but dissociates readily when boiled in SDS in the presence or absence of reducing agents (Fig. 6A). To visualize this complex and to determine whether the association is intra- or extracellular, we treated BAEC with DST, a hydrophilic reagent that restricts chemical cross-linking to the extracellular moieties of proteins that are in close contact (21). Lu-ECAM-1 was then immunoprecipitated from cell lysates with mAb 6D3 and immunoblotted using R4 and R41. Both antibodies detected a novel band migrating at ~140-kDa in the cross-linked sample (Fig.
6B). This observation, together with the N-glycosylation data (Fig. 5B) and the fact that all Lu-ECAM-1 components were detected by BAEC surface biotinylation (Fig. 6C), is consistent with an extracellular association.

The presence of multiple Lu-ECAM-1-related mRNAs in BAEC greatly complicates the task of matching a particular mRNA with its protein product. To study the genesis of one product in isolation, we expressed Clone 1 by *in vitro* translation and later by transfection into HEK293 cells. *In vitro* translation produced the expected primary translation product of 101 kDa; this product shifted to about 120 kDa in the presence of pancreatic microsomes, which allow rudimentary glycosylation (Fig. 7A). Placed under control of a tetracycline-regulated promoter and transfected into HEK293 cells, Clone 1 produced similar results to BAEC (Fig. 7B). Antipeptide antibody CU8 (Table II), which reacts with the 90-, 120-, and 130-kDa proteins in BAEC, and R41 both detected a 120-kDa band, the presumptive precursor. In addition, CU8 detected a 90-kDa band, and R41 detected a 38-kDa band. Thus, BAEC-like processing of Lu-ECAM-1 occurs in a heterologous cell system. The absence of 130- and 32-kDa bands in HEK293 cells may be due to a cell-type-specific difference in glycosylation.

**RNA Blot Analysis of Lu-ECAM-1 Expression**—To confirm that the isolated cDNA clones indeed represent BAEC mRNAs of the expected lengths, we extracted RNA from BAEC and hybridized it with a probe comprising most of the Lu-ECAM-1 ORF, the EcoRI-BglII fragment (Fig. 8A, lane 1, and Fig. 2). Bands of the expected sizes were observed in addition to several others. The 3.3-kb band corresponds in size to Clones 1 and 2, and the 2.8-kb band corresponds in size to Clone 3 (Fig. 8A, lane 1). The band at 1.3 kb is consistent with the 1.3-kb Clone 4. Since Clone 4 is identical to Clone 1 only up to nucleotide 1155, the 1.3-kb band should not be, and was not, detected by probes from the latter half of Clone 1, including the XhoI-BglII fragment and a PCR product consisting of nt 2565–3323 (data not shown). Several prominent higher molecular weight bands can also be seen in lane 1, at 6, 8.5, and 10 kb. These RNAs appear to contain all of the sequence information of the cloned cDNAs since any probe derived from the cDNAs detects them as well. Determination of the structures of these RNAs awaits further cloning and sequence analysis.

**Tissue Distribution of Lu-ECAM-1 Expression**—Earlier work from our laboratory showed that, in addition to BAEC, Lu-ECAM-1 is expressed in venular endothelia of the lungs and, to a lesser extent, in spleen (11). Here, we extend those results by further probing the respiratory tree with anti-Lu-ECAM-1 antibodies. Strong signals were detected in endothelia of small to medium size venules and in the respiratory epithelia of bronchi and trachea (Fig. 9, A, B, and C, respectively). The staining pattern in bronchial epithelium reveals a predominantly vesicular expression (Fig. 9B), while that in tracheal epithelium seems restricted to the apical plasma membrane (Fig. 9C). This staining pattern was subsequently compared with mRNA expression in these same tissues. Northern hybridization of trachea, the site of Ca-CC expression, revealed a 3-kb band, as reported (Fig. 8A, lane 3; Ref. 15). Lung contained messages of 10, 8.5, 6, 3, and 1.3 kb (Fig. 8A, lane 2) and spleen, messages of 10, 8.5, and 6 kb (Fig. 8A, lane 4). These results suggest that both Lu-ECAM-1 and Ca-CC are expressed in lung, although the overwhelming signal from the 3-kb message made it impossible to ascertain from longer exposures whether the 3.3- and 2.8-kb bands detected in BAEC were also present. Moreover, because of the high degree of sequence identity between these homologs, our probe could not discriminate Lu-ECAM-1 from Ca-CC expression. Therefore, we designed PCR primers that could distinguish between Lu-ECAM-1 and Ca-CC and performed RT-PCR on the RNA samples analyzed above (Table III). The data show that BAEC and spleen express only Lu-ECAM-1, trachea expresses only Ca-CC, and lung expresses both of these messages (Fig. 8B). It was not possible to ascertain from these data whether staining of bronchus reflects expression of Lu-ECAM-1 or Ca-CC. However, it is more likely that Ca-CC is expressed in bronchus given the continuity of tracheal with bronchial epithelium.

Finally, to investigate whether Lu-ECAM-1 is processed in lung as in BAEC, we immunoprecipitated lysates of BAEC and lung with mAb 6D3 and performed an immunoblot analysis. The expected 90-, 38-, and 32-kDa components co-precipitated from lung as from BAEC (Fig. 8C). The ~90-kDa protein from lung, however, migrated somewhat more rapidly than that from BAEC and, on a higher resolution gel, proved to be a doublet. These mobility differences were eliminated by deglycosylation (data not shown).

**Adhesion of B16-F10 Melanoma Cells to rLu-ECAM-1**—Adhesion of lung-metastatic B16-F10 melanoma cells to rLu-ECAM-1 was compared with that of wtLu-ECAM-1 in a stand-
Evidence that we have indeed cloned Lu-ECAM-1 is based on direct protein sequencing and on immunological data. The longest cDNA contains within its principal ORF the sequences of nine different peptides derived from purified Lu-ECAM-1. Furthermore, antibodies raised against synthetic peptides predicted by the ORF recognize the same bands as antibodies raised against the isolated protein components. The proposed linear arrangement of the 90-kDa and 38/32-kDa components in Clone 1 cDNA is supported by the following observations. (a) The 90- and 38/32-kDa Lu-ECAM-1 components are structurally and antigenically distinct, but both are related to the 120- and 130-kDa forms. (b) The predicted protein sequence is continuous, and all peptide sequences from the 90-kDa component are in a different part of the ORF than those from the 38/32-kDa components. (c) Deglycosylation of the proteins purified from BAEC reduces their apparent sizes to those predicted by the ORF. (d) 5'-RACE using the 38/32-kDa sequences detects only a cDNA that also contains coding sequences of the 90-kDa protein. (e) RNA blot analysis using the 38/32-kDa sequences as a probe detects only messages that are also detected by 90-kDa-specific probes. (f) Transfection of Clone 1 into HEK293 cells produces similar cleavage products to those seen in BAEC. The subcellular location at which the cleavage occurs and the responsible enzyme are unknown. The cleavage site, just before residue 683, is preceded by a lysine, and the region has a high surface probability, suggesting that it may be exposed to cleavage by trypsin-type proteases. The distance, if any, between the carboxyl terminus of the 90-kDa component and the amino terminus of the 38- and 32-kDa components is unknown; the carboxyl terminus of the 90-kDa component has proven refractory to amino acid analysis. The 38- and 32-kDa proteins are closely related and appear to be alternate glycoforms since deglycosylation reduces both to a single band of 22 kDa. The same may be true of the 130- and 120-kDa proteins.

What is the nature of the association between the proteins? The complex dissociates when heated in SDS in the absence of reducing agents, and the 38/32-kDa moiety lacks cysteines, implying that noncovalent bonds bind the complex together. The carboxyl-terminal region of Clone 1 represented by p38/p32 also lacks an obvious transmembrane segment and so likely resides on the plasma membrane only by virtue of its association with the 90-kDa protein. This extracellular association is supported by the cross-linking and biotinylation experiments. The surface-biotinylation of the 120-kDa precursor indicates that processing is not required for transport to the cell surface.

The transmembrane topology of Lu-ECAM-1 cannot be precisely predicted. Although only one hydrophobic region strictly conforms to the criteria established for alpha-helical transmembrane segments (25), there is evidence that more than one such region exists. The deglycosylation data indicate that the sequence following this transmembrane segment is strongly asparagine-glycosylated and therefore extracellular. On the other hand, the cleavage of the amino-terminal signal sequence indicates that the amino terminus should also be extracellular (26). In addition, the epitope recognized by mAb 6D3, which was raised against an extracellular region of Lu-ECAM-1, maps to the sequence prior to the canonical transmembrane segment. Thus, the protein should traverse the membrane at least twice. Indeed, Cunningham et al. have proposed as many as four transmembrane segments for Ca-CC (15). In any case, Lu-ECAM-1 would still have a long amino-terminal extracellular region, over 300 amino acid residues. This characteristic, as well as the density, spacing, and conservation of the cys-

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Fig. 9. Immunohistochemical analysis of lung and trachea. A and B, formalin-fixed tissue sections of lung were incubated with mAb 6D3 and stained. A, arrowheads, venular endothelium. B, major bronchus; arrow, intracellular vesicles; arrowhead, apical surface. C, tracheal sections were incubated with R4 polyclonal antibody. Arrow, apical surface of superficial epithelial cells, bars: 100 μm (A) and 50 μm (B and C).

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2 H. Lin and B. U. Pauli, unpublished observation.
cells was recorded as described (13). Mean ± S.D. are from three experiments. *p < 0.01.

The proteins between positions 166 and 206, is most typical of ligand-gated channels (Fig. 3) (27). Further biochemical investigation will be required to conclusively determine the transmembrane distribution of Lu-ECAM-1.

Although these data account well for the genesis and maturation of the protein encoded by the longest Lu-ECAM-1 ORF, less is known of the fate of the smaller products encoded by the other cDNAs. These cDNAs are likely alternative splice products from a single gene: the DNA sequences of Clones 2 and 3 are identical to that of Clone 1 for most of their lengths and then diverge at the same point. An intron mapped to this location in the gene may be responsible for this divergence.3 The proteins encoded by Clones 2 and 3 contain the same processing site as the product of Clone 1; presumably they are processed similarly. However the carboxyl-terminal products would be very small, 10- and 13-kDa, respectively, and immuno-bLOTS from 15% gels failed to detect products of the expected sizes. It may be that these products are either unstable or have no affinity for the 90-kDa component and hence do not co-purify. Clone 4 is predicted to encode a secreted protein of about 35 kDa. This protein would not have been detected in the present work; it is predicted to lack the epitope recognized by mAb 6D3 (100 μg/ml). Nonimmune mouse IgG (100 μg/ml) had no effect on binding. Unbound cells were removed by 3 cycles of flicking and washing in PBS. The percent bound tumor cells was recorded as described (13). The mean ± S.D. are from three experiments. *p < 0.01.

The very high sequence identity between the chloride channel Ca-CC and the adhesion molecule Lu-ECAM-1 argues for a conservation of function. The combination of channel and adhesion functions in one molecule has been reported previously. For example, the unc-105 gene product of Caenorhabditis elegans is an epithelial sodium channel of the degenerin family that interacts with type IV collagen (31). This feature is thought to bestow mechanosensitivity to the channel, opening it in response to stretching. Similarly, in mammalian brain, the β-2 subunit of a voltage-gated sodium channel contains an Ig-like cell-adhesion motif that may interact with extracellular matrix (32). For Lu-ECAM-1, the functional association between adhesion and channel properties is unknown. For instance, does the channel respond to ligand interaction? Isolation and identification of the melanoma cell ligand and characterization of its binding domain on Lu-ECAM-1 may provide insight into this question.

### Table III

| Primer name | Polarity | Specificity | Nucleotide sequence | Location |
|-------------|----------|-------------|---------------------|----------|
| LU-1        | Upstream | Lu-ECAM-1   | ATGTCAACCTAATCTCTGTAT | 741–764  |
| TC-1        | Upstream | Ca-CC       | ATGTCAACCTAATCTCTGTAC | 576–599  |
| LU-2        | Upstream | Lu-ECAM-1   | CACAGCAGGCGTGTAGAA | 827–846  |
| TC-2        | Upstream | Ca-CC       | CACAGCAGGCGTGTAGAG | 659–678  |
| LU-3        | Downstream | Lu-ECAM-1 | TGTAGTTGAGACTCCAC | 974–953  |
| TC-3        | Downstream | Ca-CC | TGTAGTTGAGACTCCAC | 806–877  |
| LU-4        | Downstream | Lu-ECAM-1 | GGAGGTATTTTGGAAAGTCA | 1044–1054 |
| TC-4        | Downstream | Ca-CC | GGAGGTATTTTGGAAAGTCA | 876–856  |

* Nucleotide sequence is given in 5'→3' orientation.

b Location is given as nucleotide number of Lu-ECAM-1 cDNA sequence or Ca-CC cDNA sequence (15).

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3 R. C. Elble, unpublished data.

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![Figure 10](image_url)

**FIG. 10. Adhesion of B16-F10 melanoma cells to rLu-ECAM-1.** Wells of 96-well plates were coated with 100 μg/ml either wLu-ECAM-1 or rLu-ECAM-1 (overnight, 4 °C). Wells were washed with PBS (3 times) and blocked with 1% bovine serum albumin in PBS (2 h, 37 °C). Wells were then seeded with B16-F10 melanoma cells (1 × 10^5 in 100 μl of PBS/well) and incubated for 20 min at 37 °C in the absence or presence of anti-Lu-ECAM-1 mAb 6D3 (100 μg/ml). Nonimmune mouse IgG (100 μg/ml) had no effect on binding. Unbound cells were removed by 3 cycles of flicking and washing in PBS. The percent bound tumor cells was recorded as described (13). The mean ± S.D. are from three experiments. *p < 0.01.
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