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Advances in Brief

A Novel Member of the NF2/ERM/4.1 Superfamily with Growth Suppressing Properties in Lung Cancer[1]

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Abstract

A novel putative tumor suppressor gene and member of the NF2/ERM/4.1 superfamily was isolated using Differential Display PCR (DDPCR) on primary lung tumors. When reintroduced into nonexpressing non-small cell lung carcinoma cell lines, this gene, named *DAL-1* (for *D*ifferentially expressed in *A*denocarcinoma of the *L*ung), was shown to suppress growth. In addition, significantly reduced expression (>50%) of *DAL-1* was measured in 39 primary non-small cell lung carcinoma tumors as compared with patient-matched normal lung tissue. Immunocytochemical staining with a polyclonal anti-*DAL-1* antibody localized the protein to the plasma membrane, particularly at cell-cell contact points, a pattern reminiscent of other members of the protein 4.1 superfamily including ezrin and NF2. The data suggest *DAL-1* is a novel membrane-associated protein with potential to play an important role in the origin and progression of lung cancer.

Introduction

Lung cancer is at present the number one cause of cancer death for both men and women. The 88% mortality rate for NSCLC[3] has remained remarkably unchanged since 1985 despite advances in cytotoxic drug development, radiotherapy and patient management. An important step toward deciphering key intervention points for this disease is a clear understanding of its genetic pathobiology. Little information exists about the sequence of genetic events leading to lung cancer, especially for tumors such as adenocarcinomas that occur in the peripheral airways of the lung. Present theories suggest that as many as 10–20 events including alterations of oncogenes and tumor suppressor genes must have occurred by the time lung cancer becomes clinically evident (1). Although putative tumor suppressor genes involved in NSCLC have been localized by LOH analyses to chromosome bands 3p, 5q, 9p, 11p, 11q, and 17p (1, 2), many of the corresponding genes have yet to be characterized. In this study, an effort was undertaken to identify novel tumor suppressor genes whose alterations are important in the pathogenesis of NSCLC by studying expression. Using DDPCR technologies, a novel gene whose expression is diminished in both primary NSCLCs and NSCLC cell lines was identified. This gene, named *DAL-1* (for *D*ifferentially expressed in *A*denocarcinoma of the *L*ung) was determined to be a novel member of the NF2/ERM/4.1 superfamily.

The NF2/ERM/4.1 superfamily consists of a growing number of proteins which share a homologous M_r 30,000 NH_2-terminal membrane-attachment domain (recently renamed the FERM domain; Ref. 3), which suggests involvement in membrane-cytoskeletal interactions. One family member, NF2 or merlin (for moesin-ezrin-radixin-like protein; also called schwannomin) has been implicated in neurofibromatosis 2, a disease characterized by schwannomas and other central nervous system tumors (4). Expression of merlin in NIH3T3 cells was found to decrease their growth rate by 3-fold (5), which indicates that the NF2 protein possesses tumor suppressing properties. Because all of the superfamily proteins are in some fashion involved in maintaining the focal contact points between the plasma membrane and cytoskeleton, it seems reasonable that some members could be directly involved in the regulation of cell growth and/or tumor suppression by affecting cytoskeletal/membrane interactions. The *NF2* gene supports this theory and further suggests that cytoskeletal reorganization may represent a primary event in, rather than be a consequence of, tumorigenesis.

Protein 4.1, the founder protein for this superfamily was originally identified as a component of erythrocyte membranes. It has been shown to cross-link spectrin/actin dimers of the cytoskeleton with glycophorin molecules in the lipid bilayer (6). The major form for erythrocyte 4.1 is an M_r 80,000 doublet, although mammalian nonerythrocyte tissues show a diverse collection of 4.1 polypeptides ranging in size from M_r 30,000 to 210,000 (7).

An additional group of 4.1-related proteins are members of the ERM family which display 74% identity in their amino acid sequences. Ezrin (cytovillin/p81), a component of the microvillus cytoskeleton, has recently been shown to anchor cyclic AMP-dependent protein kinase (8). Radixin is an actin-modulating protein isolated from cell-to-cell adherens junctions (9), and moesin is a membrane-organizing protein localized near the plasma membrane (10). Other functionally diverse members of this superfamily include several protein tyrosine phosphatases (11) that attach to the plasma membrane by binding integral membrane proteins and to cytoskeletal elements by binding to other proteins. These protein tyrosine phosphatases share the homologous 4.1 FERM domain but contain a phosphatase domain in their COOH-terminal end.

This report describes the preliminary characterization of DAL-1, a novel member of this superfamily of proteins. DAL-1 shares the FERM domain common to other members but possesses a unique COOH-terminal sequence of as yet undetermined function. Its expression is frequently greatly reduced in primary NSCLC tumors and reintroduction into nonexpressing lung cancer cell lines results in significant reduction of cell growth. Anti-*DAL-1* antibody staining established the presence of *DAL-1* at the plasma membrane especially at regions of cell-cell contact. These findings suggest that *DAL-1* may be a new and important tumor suppressor gene in lung cancer, whose

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[3] The abbreviations used are: NSCLC, non-small cell lung carcinoma; SCLC, small cell lung carcinoma; LOH, loss of heterozygosity; DDPCR, Differential Display PCR; DAL, differentially expressed in adenocarcinoma of the lung; merlin, moesin-ezrin-radixin-like protein; NF2, neurofibromatosis 2; ERM, ezrin-radixin-moesin; TE, 10 mM Tris (pH 8)-1 mM EDTA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TBS, 20 mM Tris/0.5 M NaCl (pH 7.5); MAGUK, *m*embrane-*a*ssociated *gu*anylate *k*inase.

potential for clinical relevance to the diagnosis and prognosis of lung cancer should be studied further.

Materials and Methods

Tissues and Cell Lines. Primary NSCLC tumors and patient-matched normal lung tissues were obtained either from patients undergoing surgical resection at the University of California–San Diego Medical Center or from the Cooperative Human Tissue Network Western Division. After surgical removal, all of the samples were immediately snap-frozen in liquid nitrogen and stored at −80°C until further use. Each fresh lung tumor and accompanying normal tissue was histopathologically characterized at the time of surgery with informed consent, and the corresponding pathology reports have been obtained. Lung tumor cell lines A549, SK-LU-1, SK-MES-1, NCI-460, and SW900 and breast carcinoma cell line MCF-7 were obtained from American Type Culture Collection (Manassas, VA). LX-1 was obtained from the Deutsche Krebsforschungszentrum (Essen, Germany). All of the other cell lines were obtained as described in Wieland *et al.,* (12).

DDPCR. For DDPCR, normal and matching tumor tissues were ground on dry ice in a mortar, and RNA was isolated using TriZol reagent (Life Technologies, Inc.). Subsequently 25–50 μg of RNA was treated with DNase I (Boehringer Mannheim) in the presence of Rnase Inhibitor (Promega). Samples are precipitated and resuspended in TE. Total RNA (200 ng) was combined with poly-dT primer dT-VG (10 pmol/ml) and a cocktail containing dNTPs, 5× buffer, and Rnasin. Reverse transcriptase M-MLV (Life Technologies, Inc.) was added and the reaction incubated at 37°C for 1 h. PCR amplification of the generated cDNA fragments was performed with the addition of dNTPs, 10× PCR buffer, random 10mer oligonucleotides, additional poly(A)$^+$ primer ^{35}S-labeled ATP (Amersham), and AmpliTaq (Perkin-Elmer). Amplification was performed at 94°C for 30 s, 40°C for 2 min, and 72°C for 30 s for a total of 40 cycles. Products were separated on a 7% polyacrylamide sequencing gel and dried onto Whatman paper where the radioactively-amplified fragments were visualized by autoradiography. Fragments determined to be differentially expressed were eluted but cutting out the piece of gel/paper containing the band and placing it in an Eppendorf tube containing 300μl of double-distilled water. After precipitation, the eluted fragment was reamplified, blunt ligated into Bluescript SK (Stratagene), and transformed into XL1-Blue competent cells (Stratagene). Individual colonies containing the cDNA fragment of interest were prepared for use as hybridization probes or sequenced for comparison with known expressed sequences.

Screening a Lung cDNA Library. A human lung 5′ STRETCH cDNA library with an average insert size of 1.5 kb (Clontech, Inc) was screened with the 750-bp DDPCR cDNA fragment. A total of 150,000 plaques were screened representing single coverage of the cDNA library. After individual plaque purification and confirmation, a total of seven plaques, designated 1C, 3A, 4A, 5A, 6C, 8B, and 10A, were isolated representing *DAL-1* cDNAs. DNA inserts ranging in size from 1.3–2.8-kb were isolated by *Eco*RI digestion from all of the plaques except 3A and cloned into the *Eco*RI site of Bluescript SK$^-$ (Stratagene).

Subsequently, a 920-bp *Eco*RI/*Pst*I fragment from the 5′ region of clone 4A was used to rescreen the original cDNA library. Three additional plaques with inserts ranging from 1.0–1.9-kb were isolated, representing 5′ cDNA sequences of *DAL-1*. These inserts were also cloned into the *Eco*RI site of Bluescript SK$^-$ and designated 1–1B3.1, 1–2A3, and 5–1B1. All of the clones were restriction map-aligned using *Hin*dIII, *Bgl*II and *Pst*I. The restriction map was used to subclone individual cDNA subfragments into Bluescript for sequencing with the Sequenase v2.0 kit (Amersham Pharmacia) using either the T3 or T7 sequencing primers. ^{35}S sequencing reactions were electrophoresed on 6% polyacrylamide gels (National Diagnositcs) at a constant 80 W. Gels were dried and exposed to Kodak BioMax MR film. This sequencing revealed the presence of an open reading frame with appropriate start and stop codons fully contained within the 1.85-kb insert of clone 2A3.

Isolation of *DAL-1* P1 Clones and Chromosomal Localization of *DAL-1*. The 750-bp DDPCR cDNA fragment of *DAL-1* was screened against a human P1s library by Genome Systems, Inc (St. Louis). Two P1 clones were identified. Filter addresses for these P1 were P1–141-M20 and P1-M210-H5. The 96 well addresses are DMPC-HFF#1–560-G10 and DMPC-HFF#1–210-H5. Cursory restriction enzyme mapping with *Pst*I and *Bam*HI, followed by hybridization with the 750-bp cDNA fragment confirmed these P1 as related to *DAL-1*. These experiments also indicated that P1–210-H5 contained an approximate 100-kb insert representing the complete genomic *DAL-1* locus while P1–560-G10 contained a 68-kb insert lacking a portion of the 5′ region of *DAL-1*.

Purified DNA from P1 clone DMPC-HFF#1–210-H5 was labeled with digoxigenin dUTP by nick translation. Labeled probe was combined with sheared human DNA and hybridized to normal metaphase chromosomes derived from phytohemaglutin A stimulated peripheral blood lymphocytes in a solution containing 50% formamide, 10% dextran sulfate, and 2× SSC. Specific hybridization signals were detected by incubating the hybridized slides in fluoresceinated antidigoxigenin atibodies and Texas red avidin followed by counterstaining with 4′,6-diamidino-2-phenylindole. The initial experiment resulted in specific labeling of the terminus of the short arm of a group E chromosome that was believed to be chromosome 18 on the basis of 4′,6-diamidino-2-phenylindole staining. A second experiment was conducted in which a biotin-labeled probe that is specific for the centromere of chromosome 18 (D18Z1) was cohybridized with clone 8298. This experiment resulted in the specific labeling of the centromere in red and the short arm in green of chromosome 18. A total of 80 metaphase cells were analyzed, and 74 exhibited specific labeling.

Northern Analysis of *DAL-1* RNA. RNA was extracted from NSCLC cell lines and primary NSCLC tumors with matching normal tissue using TriZol reagent (Life Technologies, Inc.). Cells grown in culture to 70–80% confluency were lysed directly on the plates by the addition of the TriZol reagent. For tissue samples, a frozen piece of tissue was powderized using a mortar and pestle on dry ice and added directly to TriZol. Total RNA was extracted following manufacturer's protocols. In the case of primary tissue, an additional spin was added after the addition of TriZol to pellet insoluble material that might trap genomic DNA. The isolated total RNA was redissolved in diethylpyrocarbonate-treated (Sigma) water and quantitated by spectrophotometry.

Approximately 10 μg of RNA was loaded onto 1.0% agarose-formaldehyde gels and electrophoresed at 80 V for 3–4 h in 1× 4-morpholinepropanesulfonic acid (0.4 M 4-morpholinepropanesulfonic acid/100 mM NaOAc/10 mM EDTA). Separated lanes were transferred to Hybond-N+ (Amersham Pharmacia) in 20× SSC (1× SSC = 150 mM NaCl/15 mM sodium citrate). Filters were prehybridized and hybridized at 65°C in $NaPO_4$ buffer [0.5 M $NaPO_4$ (pH 7.2)/7% SDS/10 mM EDTA]. The 750-bp DDPCR fragment was random prime-labeled with [^{32}P]CTP (3000 Ci/mmol; Amersham), and hybridized filters were washed as described previously (13). Bands were visualized by autoradiography on Kodak Biomax MR film. Hybridization of the same filters with a probe for GAPDH was used to control for RNA loading. Loss of expression for *DAL-1* was calculated by densitometric comparison of the band intensities for *DAL-1* in normal lung tissue and matched tumor tissue after normalization against the signal for GAPDH. Normal/tumor pairs in which the tumor *DAL-1* message was decreased by more than a factor of 5 as compared with the normal sample were considered to be lacking or decreased in mRNA expression for this novel gene.

Production of a *DAL-1*-specific Antibody. A *Pst*I fragment from clone 2A3,, representing the 3′ most 600-bp of this cDNA clone was isolated and recloned into Bluescript SK$^-$ (Stratagene). This fragment was chosen because it is unique to *DAL-1*. Subsequently, this fragment inserted in frame into a *Sma*I/*Eco*RI linearized pGEX-KG expression vector. pGEX-KG is a derivative of the GST-fusion vector pGEX-2T (Pharmacia) in which the polylinker region has been expanded to include additional cloning sites (14). Chemically competent XL2 Blue cells (Stratagene) were transformed with the ligated products and grown on Luria Broth/Amp plates. Recombinant vector products were isolated and the orientation and conservation of the translational frame for the Glutathione-*S*-Transferase (GST)-*DAL-1* fused fragments confirmed by sequencing (Sequenase 2.0 kit; US Biochemical/Amersham) with the 5′pGEX sequencing primer (5′-GGGCTGGCAAGCCACGTTTGGTG-3′; Pharmacia) located in the 3′ region of the GST protein sequence.

The resulting expression vector, pGEX-600, was transformed into BL21 cells (Pharmacia) and the expressed GST-*DAL-1* fusion protein purified from a 1-liter culture using Pharmacia's Bulk GST Purification kit following supplied protocols. Expression of the fusion protein was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside (100 mM) for 3–5 h, followed by sonication and centrifugation of insoluble material. Supernatant was added to 50% glutathione Sepharose 4B slurry (Pharmacia) and incubated overnight, allowing for GST-DAL-1 protein binding to the matrix. Bound fusion protein

was eluted from the matrix, and its purity was assessed by Comassie staining of the product on a 15% polyacrylamide gel. The pGEX-600 fusion protein was estimated to be M_r 72,000.

The pGEX-600 fusion protein was used as antigen by HTI BioProducts, Inc. (Ramona, CA) for the production of a rabbit polyclonal *DAL-1* antibody. A standard 70-day protocol including preimmunization bleed, four immunizations per rabbit, and three production bleeds was performed on two rabbits. After the initial confirmation of antibody production, an additional antigen boost and subsequent serum collection was performed. The polyclonal *DAL-1* antibody produced was immunoaffinity-purified from serum by column chromatography using CNBr-activated Sepharose 4B (Pharmacia) coupled to the antigenic pGEX-600 fusion protein. Bound antibody was eluted with glyine buffer [0.5 M glycine and 0.15 M NaCl (pH 3.0)] into Eppendorf tubes containing 0.1 volume of 1 M Tris (pH 9.0). Titration of the *DAL-1* antibody against the *DAL-1*-expressing NSCLC cell line NCI-460 determined a 1:2000 dilution should be used for Western analyses.

Western Analysis of DAL-1 Protein. Cell lines from which protein was extracted were initially rinsed with PBS, then lysed in 1 ml of prechilled radioimmunoprecipitation assay buffer [150 mM NaCl, 1.0% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris (pH 8.0)] containing protease inhibitors (phenylmethylsulfonyl fluoride, Leupeptin, Aprotinin, and Pepstatin), allowed to incubate on ice for 10 min, and scraped into an Eppendorf tube. After centrifugation for 10 min at 10,000 rpm at 4°C, the lysate supernatant was assayed for protein concentration using Bio-Rad Protein Assay reagent, aliquoted, and stored at −80°C.

For Western analysis, 10–40 μg of protein was boiled for 5 min in loading buffer containing 10% SDS and 6 M DTT for denaturation. Samples were electrophoresed on an 8–10% SDS-polyacrylamide gel using the Mini-PROTEAN II electrophoresis system (Bio-Rad) and subsequently transferred to Immobilon polyvinylidene difluoride membrane (Millipore) using the Mini Trans-Blot Cell apparatus (Bio-Rad). Western analysis was performed with the polyclonal *DAL-1* antibody at a dilution of 1:2000 using the Immun-Star Chemiluminescent Protein Detection System (Bio-Rad) following the manufacturer's suggested protocol except that filters were blocked in 5% nonfat dry milk in TBS. In addition, primary and secondary antibodies were diluted in 1% dry milk in TBS. Bands were detected by exposure of Kodak BioMax ML film for 1–7 min at room temperature.

Transfection of *DAL-1*. Cell lines NCI-460, SK-LU-1, SK-MES-1, A549, and MCF-7 were grown to 50–70% confluency. Using the pcDNA-2A3 construct, each cell line was transfected using Calcium-Phosphate complex formation in several independent experiments as were the Bluescript plasmid (Stratagene) and the pcDNA3 plasmid as separate control transfections. After 4 h of incubation, cells were grown for 48 h in unselected medium, after which G418 (Geneticin; Life Technologies, Inc.) selective agent was added to a concentration of 400 υg/ml. After 10–14 days of selection, plates were trypsinized, and G418-resistant cells were counted. The effect of the re-expression of *DAL-1* was measured by comparing the number of surviving cells for the *DAL-1* containing pcDNA-2A3 plasmid transfection *versus* the number of cells surviving after transfection with the control pcDNA3 plasmid.

Immunocytochemistry. The cellular location of DAL-1 protein was examined in NCI-460 using the rabbit polyclonal *DAL-1*-specific antibody (1:500 dilution) and immunocytochemical detection methods. Analysis of β-catenin was performed with an mouse monoclonal β-catenin antibody (Transduction Laboratories) used at a 1:500 dilution. NCI-460 cells were grown on coverslips to 50% confluency, fixed in methanol for 10 min at −20°C, and washed in HBSS supplemented with 5% donor calf serum and 10% sodium azide. Primary antibody was added to the fixed cells and incubated for 30–40 min at room temperature. The appropriate species-specific secondary antibody, labeled with either fluorescein (for *DAL-1*, antirabbit IgG; Southern Biotechnologies) or rhodamine (for β-catenin, antimouse IgG; Southern Biotechnologies) was added at a dilution of 1:100 in HBSS with 5% goat serum and allowed to complex at room temperature for 1 h. During the final 10 min of the secondary incubation, Hoechst solution (at 1:5000) was added to stain the nuclei. Coverslips were rinsed in HBSS, placed cell-side down on a microscope slide in Di-amino-bicyclo-octane (2.5% in glycerol; Sigma), and secured. Stained cells were visualized following exposure to the appropriate wavelength on an Olympus fluorescent microscope and photographed using Kodak Elite II, ASA 1600 film.

Accession Number. The GenBank accession number for *DAL-1* is AF069072.

Results

Identification and cDNA Sequence Analysis of *DAL-1*. DDPCR technology was used to identify genes whose expression is altered in NSCLC. Using a combination of a poly-dT primer whose 3′ sequence is TVG, where V = G, C, or A, and 10 random 10mer oligonucleotide primers, DDPCR fingerprinting was performed on two independent adenocarcinomas (Adeno 1204 and Adeno 1373) and their matched normal tissues (data not shown). All of the PCR amplifications were performed in duplicate and only those changes that were consistent in both normal lanes or both tumor lanes were isolated as potential differentially expressed sequence tags. These experiments originally identified 10 partial cDNA fragments whose expression profiles were altered presumably as a result of the tumorigenic process. Northern analysis confirmed the altered expression of four. One of these four, a 750-bp fragment named *DAL-1*, was identified as missing in the Adeno1373 tumor sample. Loss of expression of the corresponding 4.4-kb human mRNA species of *DAL-1* in the Adeno tumor 1373 and other primary tumors was confirmed by Northern analysis using the 750-bp DDPCR fragment as a probe (data not shown). Confirmation of the loss of expression suggests that this novel gene may be involved in the pathogenesis of lung tumors.

An oligo dT-primed adult human lung cDNA library (Clontech, Inc) was screened with the 750-bp DDPCR fragment to isolate a full length cDNA clone for *DAL-1*. Tertiary screens produced four clones—10A, 6C, 5A, and 4A—ranging in size from 1.3 to 2.6 kb (Fig. 1). Preliminary restriction enzyme mapping and sequencing indicated homology for all of the clones 3′ of the *Pst*I site. Rescreening of the cDNA library using a 5′ clone 4A fragment identified three additional clones—1B3, 1B1, and 2A3—ranging in size from 1050 to 1850 bp. The structure of clone 2A3 suggests that clone 4A may be a splice variant of *DAL-1*. Sequencing of the 3′-most extending clone 5A, which contains only untranslated sequence, did not reveal a poly-adenylation signal.

Sequence analysis of clone 2A3 revealed an open reading frame from nucleotide 73 to 1903, with the first ATG at base 394 and a stop codon at base 1903. Nucleotides representative of a Kozak consensus sequence are found flanking the start codon (5′-AGCATGC-3′). Approximately 320 bp of sequence 5′ of the start exhibits a CpG content of 62% (244 C+G:393 bp) with 34 CpG and 47 GpC doublets. In addition, sites for characteristic methylation sensitive (*Hha*I and *Hpa*II) and GpC-rich recognition site restriction enzymes (*Sac*II, BssHII, *Sma*I) have been identified in this region. The presence of such sequences may indicate that *DAL-1* expression is subject to regulation by methylation in its promoter region and allows for the possibility that aberrant methylation could be involved in the loss of *DAL-1* expression in NSCLC. Such 5′ methylation has been shown to inactivate transcription of p16 in 20% of the primary NSCLCs analyzed (15). The 5′ region of *DAL-1* is currently being scrutinized for its methylation status in both normal lung and tumor tissue.

Chromosomal Localization of *DAL-1*. Southern hybridization of the 750-bp DDPCR-generated probe against a panel of somatic cell hybrids containing single human chromosomes in either a hamster or a mouse background located the *DAL-1* locus to human chromosome 18 (data not shown). To more precisely map the position of this gene, purified *DAL-1* P1 DNA (P1–210-H5) was labeled with digoxigenin dUTP by nick translation, and a probe specific for the centromere of chromosome 18 (D18Z1) was labeled with biotin. Cohybridization of these probes resulted in specific labeling of the centromere in red and the short arm of chromosome 18 in green (data not shown). Observations of specifically hybridized chromosomes 18 demonstrated that

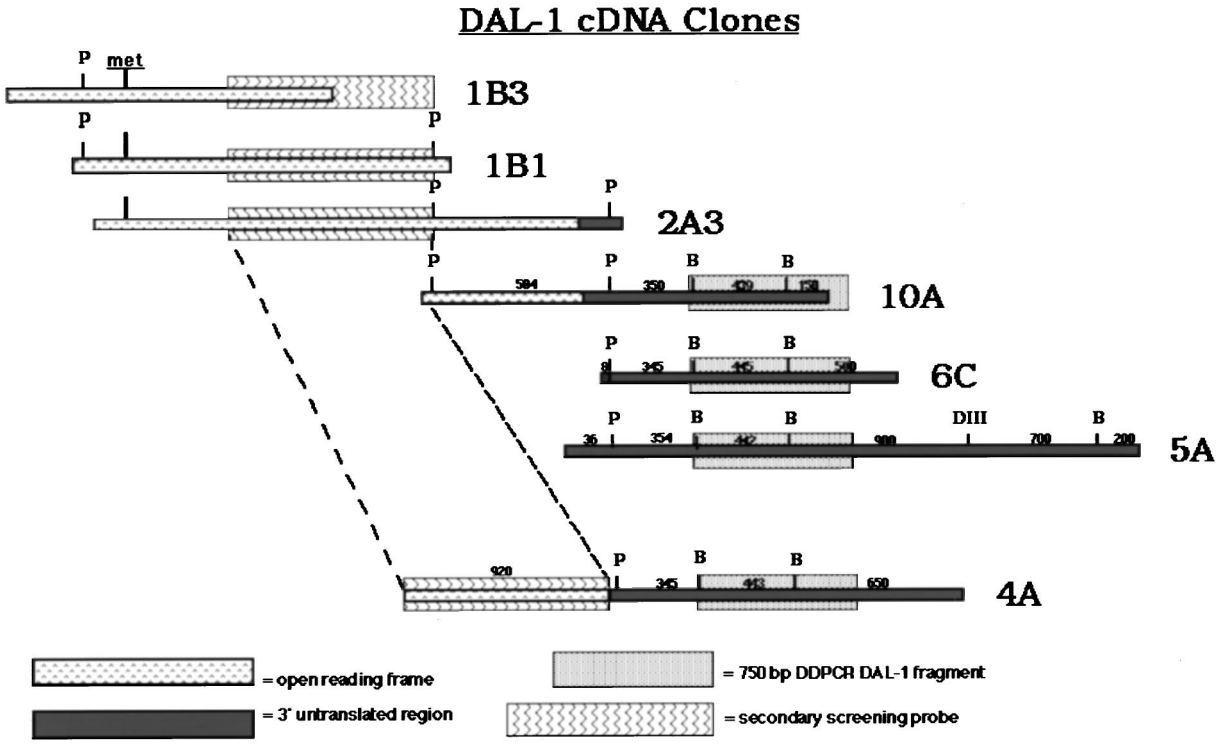

Fig. 1. Characterization of cDNA clones isolated from a human lung cDNA library after two hybridization screens with fragments of the *DAL-1* gene. Clones *1B3 through 5A* represent the full-length *DAL-1* cDNA. Clone *2A3* contains a full-length open reading frame and was used to make expression constructs. Clone *4A* depicts a potential *DAL-1* splice variant isolated using the 750-bp DDPCR fragment.

P1–210-H5 and, therefore, *DAL-1* is located at the p terminus of this chromosome in an area corresponding to band 18p11.3 (Genome Systems, Inc).

Tissue Expression Pattern of *DAL-1*. Evolutionary conservation and tissue expression profiles are often excellent indicators of the relative importance of genes to the normal functioning of cells. Northern analysis of a panel of adult mouse tissue revealed that the 4.4-kb message of DAL-1 was actively expressed in the brain, testis, small intestine, and kidney (Fig. 2). Lower levels of this mRNA were also discovered in the lung and pancreas, although no signal was detected in the liver or skeletal muscle (data not shown). The murine *DAL-1* mRNA species appears similar in size to that identified in human tissue. Interestingly, testis expressed a second smaller mRNA, and brain tissue seemed to contain several alternate species, which suggests that differential splicing may occur for *DAL-1* expression in several tissues.

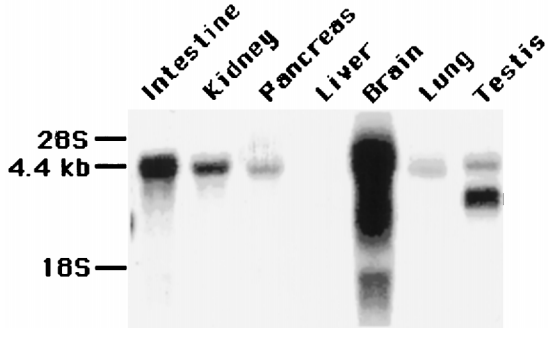

Fig. 2. Tissue expression pattern for *DAL-1* mRNA mouse. The 4.4-kb *DAL-1* mRNA is visualized by hybridization with the 750-bp DDPCR fragment for *DAL-1*. No expression was detected in skeletal muscle (not shown) or liver. Single mRNA species were found in kidney, pancreas, intestine, and lung. Multiples transcripts for this gene were identified in the testis and the brain.

***DAL-1* Homology to the NF2/ERM/4.1 Superfamily.** The 1509-bp open reading frame in *DAL-1* cDNA clone 2A3 predicts a 503 amino acid protein with a hydrophobic NH_2-terminal domain and a hydrophilic COOH terminus. Comparison of this protein with sequence databases revealed 73% identity of amino acids 132–468 to the NH_2-terminal region of the human protein, protein 4.1 (Fig. 3*A*) including the position of the start codon. Membrane protein 4.1 is known to interact with actin and spectrin by cross-linking spectrin dimers in the plane of the membrane and by helping to anchor the cytoskeleton to the overlying lipid bilayer (6). The NH_2-terminal region of identity between protein 4.1 and DAL-1 corresponds to the FERM domain. The predicted size of the *DAL-1* domain is similar to the M_r 30,000 domain found in protein 4.1, ezrin/radixin/moesin proteins (ERM), merlin, and talin. The remaining COOH-terminal amino acids for *DAL-1* share no significant homology to other known 4.1 family members or sequences deposited in GenBank or SwissProt. *DAL-1's* lack of a spectrin/actin binding site makes its structure similar to the singular *Drosophila* 4.1 homologue, *coracle* (16). Curiously, a short region of homology exists between amino acids in the COOH-terminal region of protein 4.1 and approximately 100 codons in *DAL-1*'s predicted 3′ untranslated region (Fig. 3*B*). This homology exists in an alternate reading frame, which suggests that evolutionary divergence was responsible for creating a premature stop codon or that this sequence is translated in an as yet undiscovered alternate splice variant. Most interesting to the potential function of the DAL-1 protein is the discovery of a potential ATP/GTP binding motif A (P loop) at amino acids 405–412 (AIQHEGKT; Ref. 17). The [AG]-x (4)-G-K-[ST] motif is purported to form a flexible loop that interacts with one of the phosphate groups of ATP or GTP (18). This motif potentially represents a unique feature for the COOH-terminal region of this novel protein.

Plotting the evolutionary relationship of DAL-1, protein 4.1, and other family member proteins reveals DAL-1 to be most closely

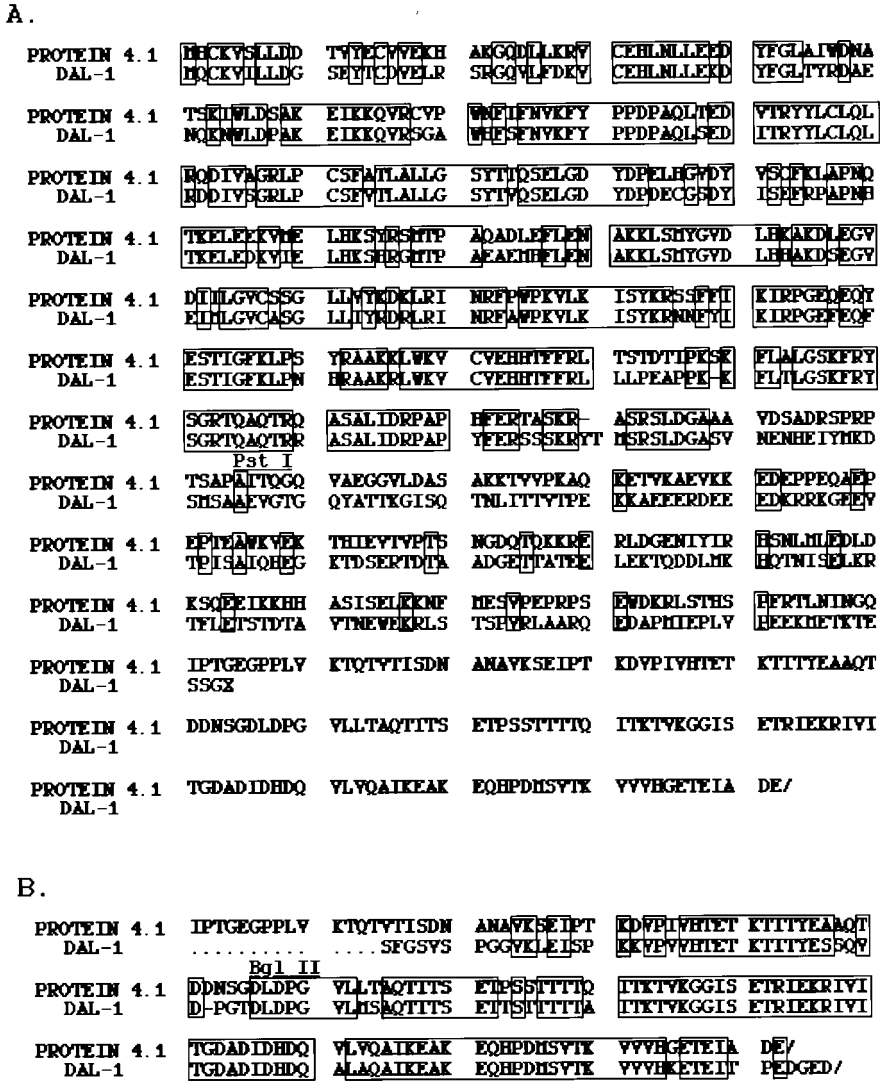

Fig. 3. Homology of cytoskeletal membrane protein 4.1 with the predicted amino acid sequence for *DAL-1*. *A*, the 1503-bp open reading frame in *DAL-1* cDNA clone 2A3 predicts a 503 amino acid protein. Comparison with available databases revealed 73% identity of amino acids 132–468 to the NH_2-terminal region of the human membrane protein 4.1 corresponding to the hydrophobic glycophorin binding domain. The remaining 3′ coding amino acids for *DAL-1* share no significant homology to other 4.1 family members or sequences deposited in GenBank or SwissProt. A potential ATP/GTP binding motif A (*P loop*) occurs at amino acids 405–412 (AIQHEGKT). *B*, An alternate translational frame for sequences in the 3′ untranslated region of *DAL-1* shares amino acid homology to the COOH-terminal region of protein 4.1.

related to protein 4.1 followed by the ERM family and NF2. Of particular relevance to *DAL-1*'s potential as a tumor suppressor gene in lung cancer is its similarity to merlin, the product of the *NF2* tumor suppressor gene. Merlin functions in the GTPase/Ras pathway at the surface of the cell membrane (4) allowing for the possibility that *DAL-1* may serve a similar function and is a related but novel membrane-associated tumor suppressor gene.

Northern Analysis in Primary NSCLC Tumors and Cell Lines. Expression of *DAL-1* was analyzed by Northern hybridization of total RNA extracted from a series of 39 primary non-small cell lung tumors and matching normal lung tissue. Filters were hybridized with either the original DDPCR 750-bp cDNA fragment known to be positioned in the 3′ untranslated region or the 1.85-kb insert from clone 2A3 containing the coding region for *DAL-1*. Alterations in *DAL-1* expression were assessed after densitometric normalization of band intensities using GAPDH expression to control for RNA loading. A numerically determined 5-fold or greater reduction in tumor signal when compared with matching normal tissue was used to indicate significant reduction of *DAL-1* message. In our series of tumors, 54% (21 of 39) showed greatly reduced levels of *DAL-1* message (Fig. 4*A*). No subtype specificity was apparent as adenocarcinomas (40%; 6 of 15), squamous (69%; 11 of 16), large cell (100%;1 of 1) and nonspecified NSCLCs (43%; 3 of 7) were all affected by between a 5- and 1000-fold reduction in signal. Because no enrichment for tumor cells was performed, these findings may represent an underestimate of the frequency of *DAL-1* reduction, especially in NSCLC tumors where the quantity of infiltrating leukocytes or stromal cells is sufficient enough to obscure the tumor-related level of gene expression.

To verify that the reduction of *DAL-1* expression in lung tumors was specifically associated with tumorigenesis and not with a varying presence of intermixed normal stroma in the tumor, a series of NSCLC and SCLC cell lines were analyzed. Of 10 NSCLC cell lines surveyed—represented by adenocarcinoma subtypes Calu3, A427, A549, LX-1, LXF289, SK-LU-1, and SK-LC-12, squamous subtypes SK-MES-1 and SW900, and undifferentiated subtype NCI-460—only NCI-460 and SW900 retained measurable levels of *DAL-1* mRNA (Fig. 4*B*). Such detectable levels of mRNA indicate that *DAL-1* expression is not confined to normal stromal components of the lung. Of interest was the discovery that *DAL-1* expression was observed in all of the six SCLC cell lines analyzed, albeit at greatly varying levels (data not shown). It would be interesting if altered *DAL-1* expression was a NSCLC *versus* SCLC tumor-specific marker and/or if the lack of expression in NSCLC is directly related to the cell of origin for this lung tumor subtype. In addition, several other tumor cell lines including those from breast (MCF-7), colon (RKO), pancreas (Hs766T), head and neck (UMSCC5 and UMSCC10b), and neuroblastoma (MCIX) tumors also lacked endogenous *DAL-1* ex-

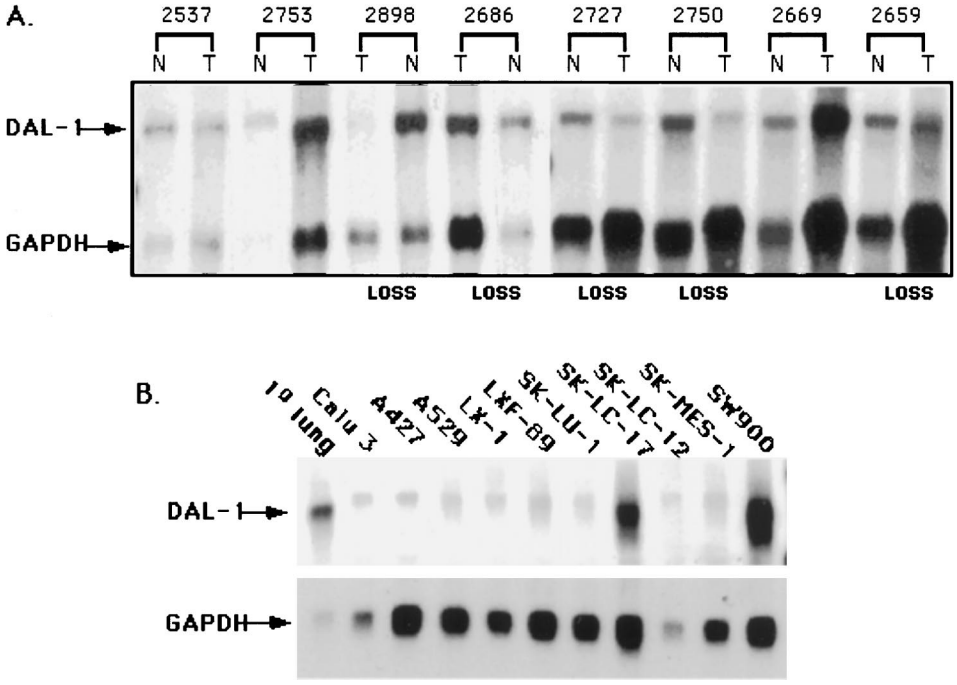

Fig. 4. Analysis of *DAL-1* expression in primary NSCLCs and cell lines. *A*, total RNA was extracted from a series of 39 primary NSCLC tumors and patient-matched normal lung tissue. Expression of *DAL-1* was analyzed by Northern hybridization with either the original DDPCR 750-bp cDNA fragment positioned in the 3′ untranslated region or the 1.85-kb insert from library clone 2A3 containing the open reading frame for *DAL-1*. Loss of *DAL-1* expression was assessed after the normalization of band intensities using GAPDH expression to control for RNA lane loading. *B*, poly(A)+ RNA Northern analysis of NSCLC cell lines and a normal human lung lane as a positive control. Hybridization with a *DAL-1*-specific probe revealed expression was missing in all of the cell lines analyzed except small cell carcinoma cell line SK-LC-17 and squamous cell carcinoma SW900.

pression, which indicates that its loss may be involved in the pathogenesis of tumors a variety of tissues (data not shown).

Growth Suppression Associated with *DAL-1* Expression. Identification of *DAL-1* as a gene whose expression is lost in lung tumorigenesis is a strong indicator that this gene could function normally as a growth inhibitor in lung tissue. Further evidence in support of a tumor suppressing capability for *DAL-1* comes from the discovery of LOH at a frequency of 38% for NSCLCs in the 18p11.3 region containing the *DAL-1* locus (19). Direct evidence that *DAL-1* can function to suppress growth of tumorigenic cell lines null for, or expressing low levels of, the endogenous *DAL-1* gene product was obtained. Reintroduction of *DAL-1* expression was analyzed using *DAL-1*-null NSCLC cell lines SK-LU-1 and SK-MES-1, low expressor A549, *DAL-1*-null breast carcinoma cell line MCF-7, and *DAL-1*-positive NSCLC cell line NCI-460. Using a pCDNA3 construct that contained the coding region represented by cDNA clone 2A3 (pCDNA3–2A3), each cell line was transfected in several independent experiments along with separate control transfections with the pCDNA3 vector alone. After a 10–14-day selection in Geneticin (G418), G418-resistant surviving cells were counted. As shown in Fig. 5*A*, the introduction of *DAL-1* into SK-LU-1, SK-MES-1, and A549 resulted in significantly reduced cell growth (26 ± 3%, 35.7 ± 5.5%, and 40 ± 8.2%, respectively) as compared with the pCDNA3 control (100%). For the endogenously expressing cell line NCI-460, no significant growth reduction was measured (88.4 ± 6.1%). In MCF-7, a less dramatic but significant reduced cell growth of 53.9 ± 4.5% was observed.

Protein extracts isolated from G418-resistant cells transfected with pCDNA3 or pCDNA3–2A3, were analyzed by Western analysis with a polyclonal antibody raised against the COOH terminus of *DAL-1*. As shown in Fig. 5*B*, the endogenous protein product for *DAL-1* is detected as an approximate M_r 110,000 protein in the NCI-460 cell line as well as in the glioblastoma cell line D247MG. As predicted from previous Northern analyses, no endogenous protein is detected in pCDNA3-only transfected SK-LU-1, SK-MES-1, and MCF-7 cells. However, a M_r 65,000 protein species is detected in protein extracts from corresponding cells after transfection with the pCDNA3–2A3 construct and G418 selection. The expected pCDNA-2A3-associated M_r 65,000 protein of *DAL-1* was not detected in transfected NCI-460 cells despite confirmation of stable transfection via Southern analysis (data not shown). It is not clear from these experiments whether transfected *DAL-1* expression of the transgene is somehow regulated by the high amount of endogenous DAL-1 protein in NCI-460 or whether plasmid integration may have inhibited expression.

Size differentials detected between the endogenous protein and transfected DAL-1 protein could result from either extensive post-translational modification of endogenous DAL-1 protein or from homo- or heterodimerization of the protein such as has been shown for ezrin (20). Reverse transcription-PCR analyses of overlapping *DAL-1* cDNA fragments from normal lung and NCI-460 RNA as well as 5′- and 3′- rapid amplification of cDNA ends has failed to detect fragments larger than that expected for the 2A3 cDNA-supporting clone 2A3 as a representative full-length *DAL-1* cDNA.

Localization of the DAL-1 Protein to Cell-Cell Junctions. To begin to understand how DAL-1 exercises its growth-suppressing properties, the cellular location of the protein was examined using the endogenously expressing NSCLC cell line NCI-460. NCI-460 cells, grown on coverslips, were fixed and incubated with the polyclonal *DAL-1* antibody. Resulting antibody-protein complexes were detected with a fluorescein-labeled antirabbit IgG secondary antibody. As seen in Fig. 6*A*, DAL-1 protein was clearly visible at the interface of NCI-460 cells in direct contact with one another. This “honeycomb” pattern of staining indicates DAL-1 protein is integral to the process of cell-cell contact and/or adhesion. Minor punctate staining was also seen around the edges of cell patches further suggesting a possible role in cell attachment.

The DAL-1 staining pattern is similar to that shown previously for other members of the protein 4.1 superfamily including ezrin, radixin, moesin, and merlin (21). In addition, several proteins known to be located at cell-cell contact points including β-catenin and E-cadherin share this staining pattern (22). β-catenin binds directly to the E-cadherin transmembrane adhesion molecules forming adherens junctions. The extent to which DAL-1 shares protein location with β-catenin was examined by coincubating NCI-460 cells with the DAL-1

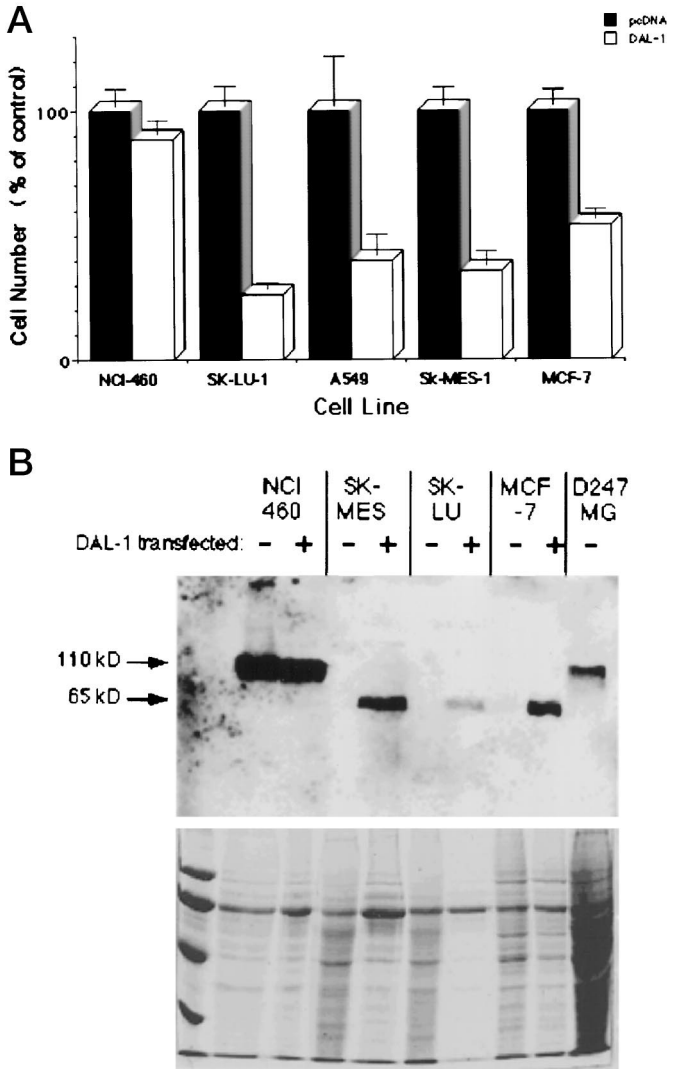

Fig. 5. Transfection of *DAL-1* cDNA into negative NSCLC cell lines and breast carcinoma cell line MCF-7. *A*, cell lines NCI-460, SK-LU-1, SK-MES-1, A549, and MCF-7 were grown to 50–70% confluency and transfected with either the control vector pCDNA3 or the *DAL-1* containing pCDNA-2A3. After 10–14 days of selection in G418, the effect of the re-expression of *DAL-1* was measured by comparing the number of surviving cells containing the pCDNA-2A3 plasmid *versus* the number of cells surviving after transfection with the control pCDNA3 plasmid. Re-expression of *DAL-1* into SK-LU-1, SK-MES-1, and A549 resulted in statistically significant reduced cell growth (26 ± 3%, 35.7 ± 5.5%, and 40 ± 8.2%, respectively) as compared with the pCDNA3 control. For the endogenously expressing cell line NCI-460, no significant growth reduction was discovered (88.4 ± 6.1%). In MCF-7, a less dramatic but significant reduction of cell growth (53.9 ± 4.5%) was measured. *B*, protein extracts isolated from G418-resistant cells transfected with either the control vector pcDNA3 or pcDNA3–2A3 were isolated and analyzed by Western analysis with a polyclonal antibody raised against the COOH-terminus of *DAL-1*. Endogenous DAL-1 protein is detected as an approximate M_r 110,000 protein (*110kD*) in the NCI-460 cell line as well as in the glioblastoma cell line D247MG. A M_r 65,000 protein (*65kD*) species, corresponding to the transfected pcDNA3–2A3 DAL-1 construct, is detected only in protein extracts isolated after transfection and G418 selection (+ *Lane*). As predicted from previous Northern analysis, no endogenous protein is produced in NSCLC cell lines SK-LU-1 and SK-MES-1 and the breast carcinoma cell line MCF-7. pCDNA3–2A3 DAL-1 protein was not detected in the transfected NCI-460 cells despite confirmation of transfection via Southern analysis (data not shown).

antibody and a mouse monoclonal antibody for β-catenin. Each antibody-protein complex was detected with the appropriate species-specific fluorescent-labeled secondary antibody. β-catenin staining is detected wherever cell-cell contact occurs, consistent with its known function (Fig. 6*B*). Colocalization of DAL-1 and β-catenin proteins at cell-cell contact points is shown in Fig. 6*C*, where double exposure produces a yellow-orange signal. Fig. 6*C* also illustrates higher and more diffuse levels of cytoplasmic *DAL-1* and β-catenin staining in cells that are dividing. Interfaces between dividing and nondividing cells retain localized immunoreactivity. This complex staining pattern may indicate that *DAL-1* exerts its growth-suppressing properties through regulation of attachment of cells either to one another or to another surface.

Discussion

Efforts to shape the future in prevention, diagnosis, and treatment of lung cancer will, in part, depend on the generation of specific genetic markers capable of distinguishing and characterizing individual lung tumor subtypes. Such genes or markers might then aid in disease management by assessing survival or predicting response to therapeutic approaches. We report the isolation of a novel member of the protein 4.1 superfamily whose expression is absent in a large proportion of primary NSCLC tumors. This gene, named *DAL-1*, shares significant identity in it's NH_2-terminal 300 amino acids to the FERM domain identified in all currently known 4.1 superfamily members. The COOH-terminal region of *DAL-1*, represented by a 600bp *Pst*I fragment in cDNA clone 2A3, shares no significant homology to any other family member. Analysis of the predicted *DAL-1* protein sequence using software available through the ExPasy web site[4] revealed no known functional or specific binding domains in the COOH-terminal region except an 8 amino acid sequence (AIQHEGKT) representing a potential ATP/GTP binding domain. In addition, potential O-glycosylation sites were found at amino acids Asn 540 and Asn 350.

Sequences 3′ of the predicted *DAL-1* stop codon when translated in an alternative reading frame, share protein sequence identity with the COOH terminus of protein 4.1 (see Fig. 2*B*). The maintenance of this sequence may simply indicate that *DAL-1* is recently diverged from protein 4.1 and, therefore, more evolutionarily related to it than the other 4.1 superfamily proteins. A subfamily of *DAL-1/4.1* genes (*4.1G*, *ELP41L1*, *NLB*, and so forth) sharing close sequence identity have recently been identified (23, 24). Alternatively, the isolated *DAL-1* cDNA may represent a splice variant that results in a variant stop codon. This might explain the differences in apparent protein molecular weight that were detected between the endogenous DAL-1 protein and the protein resulting from transfection of clone 2A3 (Fig. 5). Many important structural proteins are expressed not as single polypeptides but rather as families of closely related structural isoforms perhaps as an adaptation to accommodate varying form and function in specialized cell and tissue types. Key intracellular proteins (*e.g.*, actin and tubulin), extracellular matrix proteins (*e.g.*, fibronectin and collagen) and membrane skeletal proteins (*e.g.*, α-spectrin) all exist as families of closely related proteins. Furthermore, recent investigations on the membrane-associated tumor suppressor gene *NF2*, discovered various alternatively spliced transcripts for this gene in breast and colon tissues (25). Given the relationship of *DAL-1* to protein 4.1 and that a major mechanism of modification for protein 4.1 is alternative RNA splicing affecting both the 5′ and 3′ coding regions of the gene (7), one can envision such splicing variations for *DAL-1*. At least one potential splice variant for *DAL-1* was identified from the cDNA library screen (clone 4A, Fig. 1) in which the 600-bp COOH-terminal sequence was spliced out. It will be important to determine the extent of differential splicing and the level of expression of these variants in all of the cell types in which *DAL-1* is lost as a function of tumorigenesis.

Mapping of *DAL-1* to chromosome band 18p11.3 is interesting because at present no strong molecular genetic evidence exists to

[4] http//:expasy.hcuge.ch/

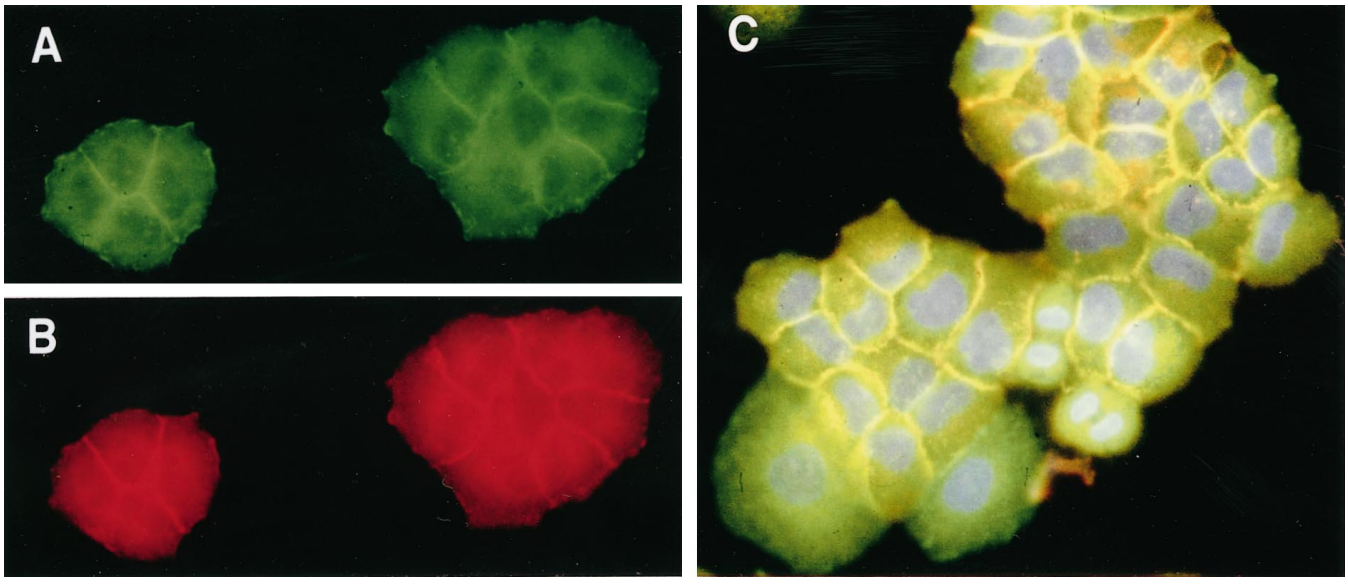

Fig. 6. Immunocytochemical colocalization of DAL-1 and β-catenin at the plasma membrane. Methanol-fixed NCI-460 cells were incubated with the polyclonal anti-DAL-1 antibody. Resulting antibody-protein complexes were detected with a fluorescein-labeled antirabbit IgG secondary antibody. In *A*, DAL-1 protein was clearly visible at the interface of NCI-460 cells in direct contact with one another. This "honeycomb" pattern of staining suggests that DAL-1 protein is integral to the process of cell-cell contact and/or adhesion. Minor punctate staining was also seen around the edges of the cell. In *B*, staining of NCI-460 cells with a mouse anti-β-catenin antibody localizes this protein wherever cell-cell contact occurs, consistent with its known function. *C* shows the extent of overlap for DAL-1 and β-catenin where simultaneous fluorescence produces a yellow-orange signal wherever proteins are detected. Nuclei are shown stained by Hoechst solution. This pattern of staining indicates that DAL-1 and β-catenin are both expressed at the contact points between cells. Note two dividing cells in the *lower right quadrant* of *C*.

support a role for genes on chromosome band 18p in NSCLC. However, several cytogenetic analyses on primary NSCLC tumor samples have revealed that chromosome 18 is one of several chromosomes preferentially lost as a result of tumorigenesis. In these NSCLC tumors, chromosome 18 was lost in approximately 35% of the 63 samples analyzed. Other significant losses were measured for chromosomes 9, 13, 21, and 22 (26). Another study of pleural effusions containing metastatic or invasive NSCLC tumor cells detected chromosome 18 loss in 45% of samples studied (27). These losses were not restricted to any particular histological subtype of lung cancer. In addition, chromosome 18 structural rearrangements have been found to be rare events, suggesting that loss of a gene(s) on this chromosome is the important event in the tumorigenic process. LOH studies on chromosome 18p have provided the initial significant molecular evidence for the location of a gene(s) on chromosome 18p involved in NSCLC tumorigenesis with significant LOH frequency of 38% being measured for primary NSCLC tumors in band 18p11.3 (19).

Transfection and subsequent re-expression of the DAL-1 protein in null NSCLC cell lines is the first functional evidence that this novel member of the Protein 4.1 superfamily can act as a suppressor of tumorigenic growth in NSCLC cell lines and the breast carcinoma cell line MCF-7. What needs to be determined is the mechanism by which it exerts this tumor suppressing function. Immunocytochemical staining with a polyclonal *DAL-1* antibody localized the protein to the junction between cells as well as to specific focal regions of attachment to the plate surface. On the basis of this staining pattern and its identity with that of a family of membrane-associated protein species, it is reasonable to hypothesize that *DAL-1* may function to organize the membrane and its associated cytoplasmic components, facilitating their interaction in a variety of pathways. The loss of *DAL-1* may disrupt a signal transduction pathway, alter anchorage dependence, or upset cell cycle regulation by disrupting links between the membrane and the cytoskeleton. Considering their secondary structure homologies and their cellular localization, it has been suggested that the protein 4.1-related proteins ezrin, radixin, and moesin may also be involved in dynamic changes in cell shape.

Recently a family of MAGUK proteins have been identified that may provide an important link between the inter- and intracellular signaling events controlling growth and the cytoskeletal framework needed to stage these events. One such human MAGUK, hDlg, has been cloned and found to possess a cytoplasmic FERM-like binding domain in addition to three PDZ domains, an SH3 domain, and the guanylate kinase-homologous region as part of its peptide core (28). That these intermediary proteins can be directly involved in suppression of growth and tumorigenesis is illustrated by the interaction of the hDlg-PDZ2 with the adenomatous polyposis coli (APC) tumor suppressor gene (29). In *Drosophila*, dlg is localized to septate junctions, precisely where the 4.1-homologue coracle is located. As dlg functions as a tumor suppressor, it is intriguing to consider that their interaction is required for suppression. Consequently, a mutation in one of these two proteins would lead to loss of interaction and thus loss of tumor suppression. If DAL-1 was found associated with a MAGUK or other PDZ domain-containing protein, it could be envisioned that loss of its expression in tumors might disrupt the normal scaffolding that provides the stability and framework for several signaling pathways.

The immunocytochemical staining described for *DAL-1* is also reminiscent of that detected for β-catenin. Curiously, β-catenin has also been shown to bind *APC* although to a different region of APC than the hDlg binding motif (29). Although there is at present no binding domain that might potentially place DAL-1 at these adherens junctions, it is interesting to postulate that the loss of *DAL-1* may indirectly affect the formation of appropriate cell contact, thereby creating a more fluid, less controllable environment in which the cell might experience a loss of proper growth control. This is in fact what was seen for antisense oligonucleotides to ezrin/radixin/moesin proteins that suppressed expression and induced the destruction of both cell-cell and cell-substrate adhesion (21).

Regardless of its exact mechanism of action, it is clear from the data presented here that DAL-1 is a novel membrane-associated protein that has the potential to play an important role in maintaining the normal growth of lung cells and potentially breast cells as well. The

search for the mechanism by which DAL-1 exerts its growth suppressing properties, a more detailed molecular investigation of its potential splice variants and tumor-related mutations and its related clinical usefulness as a marker for NSCLC prognosis is presently underway.

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A Novel Member of the NF2/ERM/4.1 Superfamily with Growth Suppressing Properties in Lung Cancer

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