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

Yen K. Tran, Oliver Bögler, Karen M. Gorse, Ilse Wieland, Mark R. Green, and Irene F. Newsham

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 Differentially expressed in Adenocarcinoma of the Lung), 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 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 Differentially expressed in Adenocarcinoma of the Lung) 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 M9 30,000 NH2-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 glycoporphin molecules in the lipid bilayer (6). The major form for erythrocyte 4.1 is an M9 80,000 doublet, although mammalian nonerythrocyte tissues show a diverse collection of 4.1 polypeptides ranging in size from M9 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...
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 Deutshe 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 32P-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 sequence of the 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, blunted ligated into Bluescript SK (Strategene), and transformed into XL1-Blue competent cells (Strategene). 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 with a library with an average insert size of 1.5 kb (Clontech, Inc) was screened with ddNTPs sequencing probes or sequenced for comparison with known expressed sequences.

Subsequently, a 920-bp EcoRIPsed 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 EcoRI site of Bluescript SK− and designated 1–1B3, 1–2A3, and 5–1B1. All of the clones were restriction map-aligned using HindIII, BglII and PstI. 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. 32P sequencing reactions were electrophoresed on 6% polyacrylamide gels (National Diagnostics) 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 P1 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 array DMPC-HFF#1–560-G10 and DMPC–HFF#1–210-H5. Curative reason enzyme extraction with PstI and BamHI, 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 antibodies 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 17. Hybridization was repeated with the basis of 4′,6-diamidino-2-phenylindole staining. A second experiment was conducted in which a cDNA 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% agarose formaldehyde gels and electrophoresed at 80 V for 3–4 h in 1× 4-morpholinopropansulfonic acid (0.4 M 4-morpholinopropansulfonic 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 NaPO4 buffer [0.5 mM NaPO4 (pH 7.2)/7% SDS/10 mM EDTA]. The 750-bp DDPCR fragment was random prime-labeled with [32P]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 bands 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 fragment from clone 2A3, representing the 3′ most 600-bp of this cDNA clone was isolated and recloned into Bluescript SK− (Strategene). This fragment was chosen because it is unique to DAL-1. Subsequently, this fragment was inserted into the SmallEcoRIPsar 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 (Strategene) 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′-GGGCGTGCAACGCATCGTGTCG-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-β-D-thiogalactoside (100 μM) 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 \( M_c \), 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 glycine 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% NP-40, 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 0.1 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 Western Chemiluminescent Protein Detection System (Bio-Rad) following the manufacturer’s suggested protocol except that filters were blocked in 5% nonfat 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 (Stratagene) and the pcDNA3 plasmid as separate control transfections. After centrifugation for 10 min at 10,000 rpm at 4°C, the lysate supernatant was visualized following exposure to the appropriate wavelength on an Olympus fluorescent microscope and photographed using Kodak Elite II, ASA 1600 film.

**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 (0.5% 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.

**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-primer 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 Psrl 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. Nucleotide 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 doubles. In addition, sites for characteristic methylation sensitive (Hhal and HpaII) and GpC-rich recognition site restriction enzymes (SacII, BssHII, Smal) 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 digoxigienin 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...
P1–210-H5 and, therefore, \( \text{DAL-1} \) is located at the \( p \) terminus of this chromosome in an area corresponding to band 18p11.3 (Genome Systems, Inc).

### Tissue Expression Pattern of \( \text{DAL-1} \)

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 \( \text{DAL-1} \) 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 \( \text{DAL-1} \) 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 \( \text{DAL-1} \) expression in several tissues.

### DAL-1 Homology to the NF2/ERM/4.1 Superfamily

The 1509-bp open reading frame in \( \text{DAL-1} \) cDNA clone 2A3 predicts a 503 amino acid protein with a hydrophobic \( \text{NH}_2 \)-terminal domain and a hydrophilic COOH terminus. Comparison of this protein with sequence databases revealed 73% identity of amino acids 132–468 to the \( \text{NH}_2 \)-terminal region of the human protein, protein 4.1 (Fig. 3A) 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 \( \text{NH}_2 \)-terminal region of identity between protein 4.1 and \( \text{DAL-1} \) corresponds to the FERM domain. The predicted size of the \( \text{DAL-1} \) 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 \( \text{DAL-1} \) share no significant homology to other known 4.1 family members or sequences deposited in GenBank or SwissProt. \( \text{DAL-1} \)’s lack of a spectrin/actin binding site makes its structure similar to the singular \( \text{Drosophila} \) 4.1 homologue, \( \text{coracle} \) (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 \( \text{DAL-1} \)’s predicted 3’ untranslated region (Fig. 3B). 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 \( \text{DAL-1} \) protein is the discovery of a potential ATP/GTP binding motif A (P loop) at amino acids 405–412 (\( \text{AIQHEGKT}; \) 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 \( \text{DAL-1} \), protein 4.1, and other family member proteins reveals \( \text{DAL-1} \) to be most closely...
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. 4A). 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. 4B). 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 (HaS766T), head and neck (UMSCC5 and UMSCC10b), and neuroblastoma (MCIX) tumors also lacked endogenous DAL-1 ex-
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. 5A, 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 blotting with a polyclonal antibody raised against the COOH terminus of DAL-1. As shown in Fig. 5B, the endogenous protein product for DAL-1 is detected as an approximate Mr 65,000 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 Mr 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 Mr 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. 6A, 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 pcDNA-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 Mr 110,000 protein (110kD) in the NCI-460 cell line as well as in the glioblastoma cell line D247MG. A Mr 65,000 protein (65kD) species, corresponding to the transfected pCDNA-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. pCDNA-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. 6B). Co-localization of DAL-1 and β-catenin proteins at cell-cell contact points is shown in Fig. 6C, where double exposure produces a yellow-orange signal. Fig. 6C 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 NH2-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 PsI 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 site4 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. 2B). 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/
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.

Acknowledgments

We thank Dr. Gerrit Los (University of California–San Diego, La Jolla, CA) for making RNA from various cell lines available for Northern analysis. We acknowledge Douglas Daub for technical assistance and Dr. Ray Colello for assistance with the fluorescent microscopy.

References


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

Yen K. Tran, Oliver Bögler, Karen M. Gorse, et al.