Loss of the Putative Tumor Suppressor Band 4.1B/Dal1 Gene Is Dispensable for Normal Development and Does Not Predispose to Cancer

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Received 17 June 2005/Returned for modification 11 July 2005/Accepted 23 August 2005

The band 4.1 proteins are cytoskeletal proteins, harboring a conserved FERM domain highly homologous to the N-terminal FERM domain of ezrin, radixin, moesin, and merlin. Recently, a truncated form of the 4.1B protein, termed Dal-1, was identified in a screen as down regulated in adenocarcinoma of the lung and was mapped to chromosome 18p11.3, which is lost in 38% of primary non-small cell lung carcinoma tumors. Analysis of several meningiomas has shown that Dal-1 expression was lost in 76% of the tumors. To further elucidate the function of the 4.1B/Dal-1 gene in development and tumorigenesis we generated mice deficient for this allele. The 4.1B/Dal-1 null mice develop normally and are fertile. Rates of cellular proliferation and apoptosis in brain, mammary, and lung tissues from the 4.1B/Dal-1 null mice were indistinguishable from those seen with wild-type mice. Aging studies indicate that these mice do not have a propensity to develop tumors. Analysis of fibroblasts from these mice demonstrated that the growth characteristics and kinetics of these cells were not different from those of cells from the wild-type mice. These findings indicate that the 4.1B gene is not required for normal development and that 4.1B/Dal-1 does not function as a tumor suppressor gene.

The 4.1 protein family consists of the 4.1R, 4.1G, 4.1N, and 4.1B proteins. These proteins are encoded by individual genes, have similar structural features, and display distinct tissue expression patterns (16). The 4.1R protein is expressed mainly in erythrocytes, 4.1G shows a broad general expression pattern, 4.1N displays a neuronal pattern, and 4.1B is found to be most highly expressed in the brain (14). All the 4.1 proteins have a conserved structure with an N-terminal FERM domain, a spectrin-actin binding domain, and a C-terminal domain (reviewed in reference 21). The FERM domain is found in several proteins, including ezrin, radixin, moesin, and the product of the *NF2* tumor suppressor gene—merlin (1). While the function of the 4.1 proteins is not well understood, various data suggest that they play multiple roles in the structural integrity of cell shape, nuclear architecture, and protein localization at the cell membrane, among other functions (21). The 4.1 proteins are alternatively spliced, and the different isoforms are expressed in a tissue-specific fashion, which further confounds the elucidation of their function. A mutation in 4.1R, found in hereditary elliptocytosis, results in decreased levels of expression and abnormally shaped red blood cells (22). This has also been confirmed in mice deficient for band 4.1R protein (20). The band 4.1N protein has been recently shown to bind to PIKE, an

activator of nuclear PI3-kinase (27, 28). To elucidate the function of the band 4.1B protein, efforts have been made to identify interacting proteins. These efforts have led to the identification of CD44, β II-spectrin, 14-3-3 family members, and caspr-paranodin and caspr2 as binding partners of 4.1B (2, 3, 5, 17, 29).

Recently, a truncated form of the 4.1B protein was found in a screen to identify gene products down regulated in adenocarcinoma of the lung (24). This isoform, termed Dal-1, differs from protein 4.1B, as it lacks the unique N-terminal domain, parts of the spectrin-actin binding domain and unique internal domain, and the C-terminal domain. Reintroduction of Dal-1 into non-small cell lung carcinoma, breast carcinoma, and meningioma cell lines resulted in growth suppression (5, 24). Subsequent work has found loss at the chromosome 18p11.3 region, to which Dal-1 is mapped, in 38% of primary non-small cell lung carcinoma tumors (24). In addition, analysis of several meningiomas has shown that Dal-1 expression was lost in 76% of the tumors, at the protein level (6). This may indicate that loss of Dal-1 is an early step in meningioma tumorigenesis (4, 15).

To further elucidate the function of the 4.1B/Dal-1 and determine whether it may play a role in development and tumorigenesis we have generated a mouse deficient for the 4.1B gene. Due to extensive alternative splicing we targeted the second coding exon of the 4.1B locus, which contains sequences coding for the initiating codon of Dal1 (22). We show here that the targeting strategy eliminated the expression of all splice forms of protein 4.1B. Mice that are homozygotes for the targeted allele were viable and born at the expected Mendelian ratio. The mice develop normally, and their survival rate is similar to that of wild-type littermates. Furthermore, these

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FIG. 1. Generation and characterization of 4.1B-deficient mice. (A) Schematic depiction of the wild-type 4.1B allele, targeting vector and targeted 4.1B loci. K = KpnI, St = StuI, SI = SalI, P1 and P2 = probes 1 and 2, and G2, G4, and P1225 = genotyping primers. (B) Representative Southern blot analysis for the presence of a properly targeted 4.1B allele in ES cells. Clone 22 carries the modified allele. (C) Genotyping of 4.1B^{+/+} (WT), 4.1B^{+/-}, and 4.1B^{-/-} mice by PCR analysis. 310 = wild-type band, 610 = mutant band. (D) Western blot analysis of 4.1B in protein extracts prepared from brains and lungs of wild-type, 4.1B^{+/-}, and 4.1B^{-/-} mice. An actin antibody was employed to control for equivalent protein loading on the gel.

mice did not show an increased disposition towards the development of tumors. Examination of mammary epithelium, brain, and lung tissue from the 4.1B/Dal-1 null mice indicates that the ratios of proliferating and apoptotic cells are similar to those of wild-type littermates. Mouse embryo fibroblasts (MEFs) derived from the mice did not display any growth advantage in comparison to MEFs from wild-type littermates. Collectively, these data show that the protein 4.1B/Dal-1 is dispensable for normal development and that it is not directly involved in the onset of tumorigenesis.

MATERIALS AND METHODS

Generation and characterization of 4.1B/Dal-1 knockout mice. All animal experiments complied with all relevant federal guidelines and were approved by the institutional animal care and use committee. The animals were housed in a facility accredited by the American Association for Laboratory Animal Care.

A clone harboring 4.1B exon 3 was isolated from a 129Sv/J genomic library, using exon 3 as a probe. The clone was cut with StuI, and a 5.5-kb 5' fragment and a 3.8-kb 3' fragment were subcloned into pBlueScript SK. PCR primers with NheI and SalI sites were used to amplify a 3.7-kb 5' arm, and primers with NotI

and SalI sites were used to amplify a 3.5-kb 3' arm. Both arms were inserted into pBlueScript. A neomycin selection cassette was inserted in to the SalI site, replacing the sequence between +15 and +145 of exon 3. The entire insert was then subcloned into pPNT to insert a thymidine kinase-negative selection cassette at the 5' end. A 40- μ g volume of linearized vector was electroporated into J1 embryonic stem (ES) cells, and clones were selected in 400 μ g/ml of G418 (Gibco). Clones were picked, expanded, and examined by Southern blot analysis of genomic DNA cut with KpnI by use of probes P1 and P2 (see Fig. 1A). Two positive clones were injected into blastocysts to generate chimeric mice.

PCR genotyping of 4.1B-deficient mice. After initial verification of genotype by Southern blotting, the mice were genotyped by PCR. The following primer sequences were employed: G2 (5'-CGC CAC CGT CTG AGC AGC-3'), G4 (5'-GCA CGT TTG GTA GCA GTT CCC-3'), and Puro1255 (5'-GCA CGA CCC CAT GCA TCG-3'). Reaction conditions were 94°C for 1 min, 94°C for 1 min, 61°C for 1 min, and 72°C for 45 s. The wild-type band was 310 bp, and the mutant band was 670 bp.

Western blot analysis. For Western blotting, tissues were homogenized in extraction buffer (10 mM Tris-HCl [pH 8], 150 mM NaCl, 1% sodium dodecyl sulfate, 0.5% NP-40, 0.1% sodium deoxycholate, 1 mM NaVO₄, and protease inhibitors) using a Dounce homogenizer. Protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to Immobilon filters (Millipore), blocked in 5% milk in Tris-buffered saline with 0.2% Tween 20, hybridized with primary and secondary antibodies, developed with ECL (Phar-

macia), and exposed to film. The primary antibodies used to detect 4.1B were a rabbit polyclonal generated against a glutathione *S*-transferase fusion to residues P612-L804 of rat KIAA0987 (26) and a goat polyclonal raised against a peptide derived from the unique U2 region of 4.1B (14).

Immunohistochemistry. Sections from brain, lung, and mammary tissue were harvested and sectioned as previously described (9, 13). The sections were stained with an antibody specific to the Ki-67 antigen (NCL-L-Ki67-MM1; Novocastra), employing a M.O.M. blocking kit (PK-2200; Vector Labs). To assess cell death, the sections were stained with a rabbit monoclonal antibody specific to cleaved caspase 3 (clone 5A1; Cell Signaling Technology). Biotinylated secondary antibodies were used, and the sections were then incubated with Vectastain ABC reagent (PK-6100; Vector Laboratories) and developed with NovaRed substrate (SK-4800; Vector Laboratories). The sections were counterstained with hematoxylin (Sigma). Images were obtained with a Zeiss Axiovert microscope using a 63 $\times$ oil-immersion objective. For quantification, Ki-67 or cleaved caspase 3-positive cells were scored and compared to the total number of nuclei in the frame. In each group at least three animals were scored, and five separate frames were counted from each slide and averaged.

Histology. Tissues were collected and fixed in 10% neutral buffered formalin. The various organs were separated and embedded in paraffin, cut in 4- μ m sections, and stained with hematoxylin and eosin. Blood samples were collected from capillaries, and hematocrit levels were determined by centrifugation. Blood smears were stained with Wright-Giemsa stain and examined microscopically.

Cell culture and proliferation assays. MEFs were prepared from embryonic day 13.5 embryos, as previously described (18). For all assays passage 3 cells were employed. For cell proliferation assays, 3×10^4 cells/well were plated in 12-well dishes and fed with Dulbecco's modified Eagle's medium—10% IFS or Dulbecco's modified Eagle's medium—1% IFS every other day. The numbers of cells were counted daily. The 3T3 cell assays and assays of growth at low and high density were done as previously described (18).

RESULTS

Generation and characterization of 4.1B/Dal-1 knockout mice. To assess the role of 4.1B/Dal-1 in development and cancer we generated mice deficient for 4.1B/Dal-1. We targeted exon 3, which at the onset of these studies was reported to be the first coding exon for Dal-1 (22). Thus, the neomycin resistance cassette was targeted to exon 3 (the second coding exon of the 4.1B locus), replacing the 3' half of the exon (Fig. 1A). The rationale behind this strategy was that exon 3 could not splice into exon 4, resulting in abortive splicing and, if translation did initiate, in early truncation of translated product. The targeting vector was introduced into ES cells, and several neomycin-resistant colonies were isolated. Correctly targeted ES cell clones were identified by Southern blot analysis (Fig. 1B). Two independent clones were injected into blastocysts, and several chimeric mice were generated that transmitted the targeted allele through the germ line. Mice heterozygous for the targeted 4.1B/Dal-1 allele were then mated, and mice carrying both targeted alleles of 4.1B/Dal-1 were born at the expected Mendelian ratio. To examine the expression of 4.1B/Dal-1 in these animals we performed Western blot analysis on protein extracts prepared from brain and lung, in which 4.1B is highly expressed, of the 4.1B^{-/-} mice. As can be seen in Fig. 1C, the expression of 4.1B is completely lost in the 4.1B^{-/-} animals. None of the 4.1B isoforms could be detected, including the Dal-1 isoform. This was verified by the use of two independent antibodies, raised against the U2 and U3 domains

of 4.1B (14, 26). These domains are reported to be present in all splice forms of 4.1B, including Dal-1. Thus, the 4.1B^{-/-} mice do not express Dal-1 or any other isoform of the 4.1B protein.

Animals deficient for 4.1B/Dal-1 develop normally and do not show a predisposition towards cancer. The 4.1B^{-/-} mice were born at the expected Mendelian ratio. The 4.1B^{-/-} mice were fertile and produced normal-sized litters of 4.1B-deficient mice. We carefully examined the gross anatomy and histology of the mice and found no differences from the wild type. Close histological preparations from tissue expressing high levels of 4.1B, including lung, brain, and kidney tissue, are shown in Fig. 2. In all organs and tissue examined no obvious pathological abnormalities were detected, and the phenotypes of the 4.1B^{-/-} mice were indistinguishable from those of their wild-type littermates. The kidneys displayed a normal structure, as did the lungs, which displayed normal alveolar and bronchiolar architecture. Careful examination of the brain found no indication of structural abnormalities, size differences, layering defects, or cellular defects.

The 4.1B^{-/-} mice developed normally, and comparison of aging groups of these mice to the wild-type littermates also showed no difference in the longevity of these two groups (Fig. 2B). Furthermore, the animals did not show a predisposition towards the development of tumors, above the background levels observed in the littermates. These findings indicate that 4.1B/Dal-1 does not function as a tumor suppressor gene in these mice.

4.1B/Dal-1^{-/-} mice do not display altered cell proliferation or cell death rates in brain, lung, or mammary tissue. Dal-1 was originally isolated as down regulated in adenocarcinoma of the lung (24). It is also highly expressed in the brain and was shown to be up regulated in the mammary epithelium specifically during pregnancy (13). We therefore assessed whether the loss of 4.1B resulted in alterations in cell proliferation or cell death in these tissues *in vivo*. We examined sections of brain, lung, and mammary epithelium from the 4.1B/Dal-1^{-/-} mice, by immunohistochemistry, and compared them to sections from wild-type littermates. Staining for the Ki-67 antigen was employed as a marker for cellular proliferation (19), and staining for cleaved caspase 3 served as a marker for apoptotic cells. When examining the numbers of Ki-67-positive cells in mammary epithelium from mature virgin 4.1B/Dal-1^{-/-} mice and their wild-type littermates, we found that the proliferative indices for the mammary epithelium were 0.08% and 0.1%, respectively. Similarly, comparing lung tissue from these groups we found that the numbers of proliferating cells in the lung epithelium were 0.08% in the wild-type and 0.07% in the 4.1B/Dal-1^{-/-} mice. Close examination of the various regions of the brain failed to detect any significant differences between the wild-type and 4.1B/Dal-1^{-/-} mice. As an example, the proliferative indices in inner layer of the cerebellum were 0.09% and 0.07%, respectively. Overall, none of the differences

FIG. 2. Normal histology and survival of 4.1B^{-/-} mice. Histological appearance of tissue sections from adult 4.1B^{-/-} (A, C, E, and G) and wild-type (B, D, F, and H) mice. Representative $\times 10$ -magnification sections stained with hematoxylin-eosin are shown. (A to D) Coronal sections through hippocampus (A to B) and cerebellum (C to D) are shown. (E to H) Lung (E to F) and kidney (G to H) sections are shown. (I) The survival rate of 4.1B^{-/-} mice ($n = 16$) versus wild-type (W.T.) litter mates ($n = 16$) over time is represented by a Kaplan-Meier plot.

FIG. 3. Cell proliferation and cell death in the brain, lung, and mammary tissue of 4.1B^{-/-} mice. (A) Immunohistochemical analysis of Ki-67-positive cells in cerebellum, lung, and mammary tissue of 4.1B^{-/-} versus wild-type mice. (B) Cell proliferation rates in the brain, lung, and mammary epithelium of 4.1B^{-/-} versus wild-type littermates. (C) Cell death rates in the brain, lung, and mammary epithelium of 4.1B^{-/-} versus wild-type littermates. The sections are representative of three different animals from each group. The differences are not statistically significant.

FIG. 4. Growth characteristics of 4.1B^{-/-} MEFs. (A) Cell proliferation as measured by increases in cell numbers over time at both high (10%) and low (1%) serum levels. (B) Cell proliferation on a 3T3 cell assay. Cumulative proliferation ratios were measured for passages 3 through 8. (C) Focus formation assay of cell cultures grown for 21 days. Cells were stained with crystal violet, and the presence of foci was scored visually. All data are representative of three independent experiments done with at least two independent MEF clones from each genotype. The differences are not statistically significant.

were statistically significant (Fig. 3A and 3B). This indicates that the loss of 4.1B/Dal-1 does not result in increased cellular proliferation in the brain, lung, or mammary epithelium *in vivo*. Likewise, when comparing numbers of apoptotic cells in the lung and mammary epithelium, we did not observe significant differences in the apoptotic rates between the two groups of mice. For the lung tissue the apoptotic index was 0.06% in wild-type versus 0.4% in 4.1B/Dal-1^{-/-} mice. For the mammary tissue the apoptotic index was around 0.01% in both groups of mice. For the brain tissue the apoptotic index was 0.02% in wild-type versus 0.015% in 4.1B/Dal-1^{-/-} mice (Fig. 3C). Again, these differences are not significant. This indicates that the loss of 4.1B/Dal-1 does not affect the rate of apoptosis in the lung or mammary epithelium *in vivo*.

Characterization of 4.1B/Dal-1^{-/-} MEFs. As previous work has suggested that 4.1B/Dal-1 can function as a growth suppressor, we assessed the effect of 4.1B deficiency on growth parameters of MEFs derived from the 4.1B^{-/-} mice. Comparing the growth kinetics of the 4.1B-deficient MEFs to the wild-type MEFs by counting cell numbers daily, we found no significant differences in cell numbers between the two groups (Fig. 4A). This indicates that there is no difference in the proliferation rates of the two MEF groups. In addition, we analyzed the ability of the cells to proliferate at low (1%) levels of serum in the growth media, an ability usually found in transformed cells. In both groups of MEFs the cells arrested and did not proliferate under these conditions (Fig. 4A). We also examined the ability of MEFs to proliferate for an extended number of population doublings and escape senescence, a property attributed to immortalized cells. Comparing the MEFs prepared from the different genetic backgrounds in a 3T3 cell assay indicated that the 4.1B-deficient MEFs behaved similarly to the wild-type MEFs, as both cell populations proliferated in culture for similar numbers of population doublings (Fig. 4B).

Finally, we examined the ability of 4.1B null MEFs to form foci when grown to high cell densities. As can be seen in Fig. 3C, these MEFs did not form colonies and persisted as a monolayer culture over 21 days of culture at high density, in similarity to wild-type MEFs. These findings indicate that the 4.1B MEFs are not transformed, as they do not display any characteristic of transformed cells *in vitro*.

DISCUSSION

As recent evidence had suggested that 4.1B/Dal-1 is a putative tumor suppressor gene, involved in the pathogenesis of meningioma and adenocarcinoma of the lung, we set out to verify the consequences of 4.1B deficiency in an animal model. Mice that are deficient for 4.1B develop and age normally. They do not demonstrate an increased rate of any type of cancer, and life span is similar to that of their wild-type littermates. Furthermore, these mice do not display any significant differences in cell proliferation or cell death in various cell types in the brain, including neurons, or in lung or mammary epithelium.

Dal-1 was originally identified by differential-display PCR as down regulated in adenocarcinoma of the lung (24). It is a truncated form of protein 4.1B, and its expression was also found to be down regulated in meningiomas (5). However, this

reduction in Dal-1 expression could be coincidental or even secondary to a primary lesion leading to tumorigenesis. Events of chromosomal loss at chromosome 18p have been found to be a frequent occurrence in lung, brain, and breast tumors and in meningiomas (4, 5, 15, 23, 24). Alas, studies examining the remaining allele of 4.1B have failed to detect any mutations (4, 7, 12). It is possible that other mechanisms may account for the lack of mutations, such as epigenetic modifications of the 4.1B allele. Further studies will be required to determine whether indeed this is the case. Another possibility is that the losses observed for 18p11.3 represent an event of loss of heterozygosity for a different gene, at this locus, which functions as a tumor suppressor gene. Possible candidates include the protein tyrosine phosphatase PTPN2 and the thymidylate synthase gene (8, 10, 11, 25).

To assess the potential growth-suppressive ability of 4.1B we examined brain, lung, and mammary tissue sections of 4.1B/Dal-1^{-/-} mice for changes in cell proliferation or death rates. In addition, we also employed MEFs from the 4.1B-deficient animals. Our findings indicate that loss of 4.1B does not result in deregulation of cell growth, as the growth characteristics of various cell types in the 4.1B^{-/-} brain, lung, or mammary tissues and MEFs were indistinguishable from wild-type cells. This indicates that 4.1B does not function as a growth suppressor in the cells and tissues examined. We have also recently found that while 4.1B/Dal-1 is not expressed in virgin murine mammary gland epithelium, it is dramatically up regulated during pregnancy. Close examination of the 4.1B^{-/-} mice indicates that 4.1B loss does not affect cell proliferation in virgin, lactating, or involuting mammary glands but results in a significant increase in mammary epithelial cell proliferation specifically only during pregnancy (13). These findings indicate that 4.1B might play a role in regulating mammary epithelial cell proliferation during pregnancy. However, we have not detected an increased rate of mammary tumors in multiparous 4.1B/Dal-1^{-/-} females (J. L. Kissil, unpublished results).

As the loss of 4.1B alone in the mouse did not lead to increased rates of cancer it cannot be classified as a tumor suppressor gene, at least not in our model system. It is possible that loss of 4.1B may alter the spectrum of tumors in the background of additional cancer-promoting mutations. This is currently under investigation.

ACKNOWLEDGMENTS

We thank the Albert R. Taxin Center for support. J.L.K. was supported in part by Commonwealth Universal Research Enhancement Program grant ME03-190. J.H.M. was supported by PO1HL 66105.

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