Identification of a Third Protein 4.1 Tumor Suppressor, Protein 4.1R, in Meningioma Pathogenesis

Victoria A. Robb[1], Wen Li[1], Philippe Gascard[2], Arie Perry[3], Narla Mohandas[4], and David H. Gutmann[1]

Departments of [1] Neurology and [3]Neuropathology, Washington University School of Medicine, St. Louis, MO 63110; [2] Department of Subcellular Structure, Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, California 94720; [4]New York Blood Center, New York, New York 10021

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Address correspondence to: David H. Gutmann, MD-PhD

Department of Neurology

Box 8111; 660 S. Euclid Avenue

St. Louis, MO 63110

Phone: 314-362-7379

FAX: 314-362-2388

E-mail: gutmannd@neuro.wustl.edu

Abstract

Meningiomas are common tumors of the central nervous system, however, the mechanisms underlying their pathogenesis are largely undefined. Two members of the Protein 4.1 superfamily, the neurofibromatosis 2 (NF2) gene product (merlin/schwannomin) and Protein 4.1B have been implicated as meningioma tumor suppressors. In this report, we demonstrate that another Protein 4.1 family member, Protein 4.1R, also functions as a meningioma tumor suppressor. Based on the assignment of the Protein 4.1R gene to chromosome 1p32-36, a common region of deletion observed in meningiomas, we analyzed Protein 4.1R expression in meningioma cell lines and surgical tumor specimens. We observed loss of Protein 4.1R ~~protein~~ expression in two meningioma cell lines (IOMM-Lee, CH157-MN) by Western blotting as well as in 6 of 15 sporadic meningiomas by immunohistochemistry (IHC). Analysis of a subset of these sporadic meningiomas by fluorescent *in situ* hybridization (FISH) with a Protein 4.1R-specific probe demonstrated 100% concordance with the IHC results. In support of a meningioma tumor suppressor function, overexpression of Protein 4.1R resulted in suppression of IOMM-Lee and CH157MN cell proliferation. Similar to the Protein 4.1B and merlin meningioma tumor suppressors, Protein 4.1R localization in the membrane fraction increased significantly under conditions of growth arrest *in vitro*. Lastly, Protein 4.1R interacted with some known merlin/Protein 4.1B interactors such as CD44 and βII-spectrin, but did not associate with the Protein 4.1B interactors 14-3-3 and PRMT3 or the merlin binding proteins SCHIP-1 and HRS. Collectively, these results suggest that Protein 4.1R functions as an important tumor suppressor ~~important~~ in the molecular pathogenesis of meningioma.

Introduction

The two most common central nervous system tumors affecting adults are the astrocytoma (glioma) and the meningioma. Compared to the glioma, relatively little is known about the molecular genetic events important in the molecular pathogenesis and malignant progression of sporadic meningiomas. One of the most common events associated with meningioma tumorigenesis is chromosome 22q deletion and inactivation of the neurofibromatosis 2 (*NF2*) gene (Ruttledge et al., 1994; Gutmann et al., 1997; Huynh et al., 1997; Leone et al., 1999; Ueki et al., 1999). Additional events observed in meningiomas include chromosome 1p, 3p, 6q, 10q and 14q deletions (Leone et al., 1999; Weber et al., 1997; Menon et al., 1997; Simon et al., 1995; Perry et al, 1996). These regions are thought to contain tumor suppressor genes involved in malignant progression, since these deletions are frequently seen in higher-grade meningiomas (Schneider et al., 1995; Simon et al., 1995; Weber et al., 1997; Lamszus et al., 1999; Leone et al., 1999; Cai et al., 2001a). In addition, p16 (CDKN2A) inactivation and PS6K (17q23) amplification likely represent late alterations associated with anaplasia (Tse et al., 1998; Bostrom et al., 2001; Cai et al., 2001b). (I am not sure that this sentence really fits in the overall theme of this paragraph but it's a very personal point of view). Chromosomal gains involving 12q, 15q, 17q, and 20q have also been reported (Simon et al., 1995).

Since 50% of individuals affected with ~~NF2~~ *NF2* (be consistent with the font throughout the text, italic or not) develop meningiomas, *NF2* inactivation has been hypothesized to represent a critical initiating event in ~~NF2~~ *NF2*-associated meningioma formation. Moreover, loss of merlin appears to be an early event in sporadic meningioma pathogenesis (Stemmer-Rachamimov et al., 1998; Perry et al., 2001) and conditional inactivation of ~~*Nf2*~~ *NF2* in

leptomeningeal cells in mice leads to meningeal cell hyperplasia and subsequent meningioma formation (Kalamarides et al., 2002). The *NF2* gene encodes a 595 amino acid cytoplasmic protein (merlin or schwannomin) structurally related to the Protein 4.1 family of molecules. As a negative growth regulator, merlin suppresses cell proliferation when overexpressed in meningioma cell lines (Gutmann et al, 2001a) or *NF2*-deficient meningioma primary cells (Ikeda et al., 1999). Collectively, these data suggest that merlin is a critical growth regulator for leptomeningeal cells and *NF2* inactivation occurs early in the development of meningiomas.

Recent work from our laboratory has implicated another Protein 4.1 tumor suppressor, Protein 4.1B, in the molecular pathogenesis of these tumors (Gutmann et al., 2000; Perry et al., 2000; Perry et al., 2001). Protein 4.1B loss of heterozygosity (LOH) is a common genetic alteration in meningiomas, regardless of histological grade, suggesting that Protein 4.1B inactivation, like *NF2* loss may also be an early event in meningioma tumorigenesis (Perry et al., 2000, Gutmann et al., 2000). Similar to merlin, ~~re-expression~~ rescue of Protein 4.1B expression in meningioma cells resulted in reduced growth and alterations in actin cytoskeleton organization and function (Gutmann et al., 2001a). The region required for meningioma cell growth suppression was mapped to a 503 amino acid fragment termed DAL-1 (Differentially expressed in Adenocarcinoma of the Lung). Both full-length Protein 4.1B and DAL-1 bound differentially to merlin interacting proteins. Whereas Protein 4.1B and DAL-1 associated with βII-spectrin (fodrin) as well as ezrin, radixin, and moesin, they did not bind to merlin-specific interactors, such as schwannomin-interacting protein-1 (SCHIP-1) and hepatocyte growth factor tyrosine kinase regulated substrate (HRS; Gutmann et al., 2001, Robb VA & Gutmann DH, unpublished observations, 2002). In contrast, Protein 4.1B and DAL-1, but not merlin, interacted with 14-3-3 proteins (Yu and Robb et al., 2002) and PRMT3 (Singh et al., in press). These results suggest

that Protein 4.1B is a distinct meningioma tumor suppressor with a unique set of interacting proteins.

The fact that at least two members of ~~from~~ the Protein 4.1 superfamily are tumor suppressors relevant to meningioma pathogenesis raises the intriguing possibility that other members of the Protein 4.1 superfamily might also participate in meningioma growth regulation. Recent observations have indicated that Protein 4.1R may play a role in tumor pathogenesis. Similar to the *NF2* and Protein 4.1B/DAL-1 inactivation in a wide variety of cancers, the Protein 4.1R gene was mutated, silenced, or had its intronic sequence changed in 14 of 72 neuroblastomas studied (Huang et al., 2001). The Protein 4.1R gene maps to chromosome 1p, a region whose loss is the second most common genetic aberration observed in meningiomas (Simon et al., 1995; Bostrom et al., 1997; Sulman et al, 1998). Protein 4.1R, like other 4.1 molecules, contains six structurally distinct regions, including an amino terminal FERM (Four.1, Ezrin, Radixin, Moesin) domain, a spectrin-actin binding (SAB) region, and a C-terminal domain (CTD) separated by three unique regions (U1, U2, and U3). ~~In the brain, Protein 4.1R has been localized to specific neuronal populations including granule cells of the cerebellum and dentate gyrus (Walensky et al., 1998). Nervous system~~ Expression of ~~the~~ two classes of ~~predominant 4.1R~~ protein 4.1R isoforms, Protein 4.1R80 and 4.1R135, differing by the presence or absence of the unique U1 region, results from the use of two distinct initiation codons present in ~~and~~ alternative exons ~~splicing~~ (Tang et al., 1990 Huang et al., 1993; review Conboy, 2000). In the brain, Protein 4.1R has been localized to specific neuronal populations including granule cells of the cerebellum and dentate gyrus (Walensky et al., 1998).

Protein 4.1R was originally identified as an abundant protein in human erythrocytes that localizes to the membrane cytoskeleton and stabilizes red blood cell shape (Takakuwa et al.,

2001). Consistent with this role, decreased Protein 4.1R expression, resulting from a chromosomal mutation, ~~results in~~ leads to hereditary elliptocytosis, a disorder characterized by pronounced hemolysis, splenomegaly, and abnormally shaped red blood cells (Tchernia et al., 1981). This abnormal erythrocyte phenotype has also been documented in Protein 4.1R null mice (Shi et al., 1999). Consistent with a role in membrane stabilization, Protein 4.1R has been shown to mediate membrane-cytoskeleton interactions through interactions with integral membrane proteins. Protein 4.1R interacts with Band 3 protein (Pasternack et al., 1985), glycophorin C and glycophorin D (Hemming et al., 1994, Marfatia et al., 1995), p55 (Marfatia et al., 1995, Pasternack et al., 1985), CD44 (Nunomura et al., 1997) and calmodulin (Tanaka et al., 1991) though N-terminal FERM domain sequences. Protein 4.1R also binds spectrin, actin and tubulin through its SABD (Ohanian et al., 1984; Correas et al., 1988) and with FKBP13 (13 kDa FK506-binding protein; Walensky et al., 1998), tight junction protein ZO-2 (Mattagasingh et al.,), and protein NuMA (Mattagasingh et al., other paper) through the CTD. As a result of these interactions, Protein 4.1R plays an important structural and regulatory role in the stabilization and assembly of the cell membrane. Unlike merlin and Protein 4.1B, Protein 4.1R is also expressed in the nucleus (De Carcer et al., 1995, Krauss et al., 1997) and the centrosome (Krauss, 1997).

In an effort to gain insight into the function of Protein 4.1R as a meningioma tumor suppressor, we examined the ability of Protein 4.1R to mediate interactions and functions previously demonstrated for Protein 4.1B and merlin. In this study, we demonstrated loss of Protein 4.1R expression in 40% of sporadic meningiomas analyzed by immunohistochemistry (IHC) and fluorescent *in situ* hybridization (FISH). Consistent with a meningioma tumor suppressor function, overexpression of Protein 4.1R resulted in suppression of meningioma cell

proliferation. Similar to Protein 4.1B and merlin, Protein 4.1R expression was increased in membrane fractions during growth arrest. Lastly, Protein 4.1R only interacts with a subset of known merlin/Protein 4.1B interactors, including CD44 and βII-spectrin. Collectively, these results suggest that Protein 4.1R functions as a unique tumor suppressor important in the molecular pathogenesis of meningioma.

Methodology

Colony suppression assay. The colony suppression assay was performed by transfecting IOMM-Lee or CH157-MN cells (at approximately 60% confluency) with equimolar amounts of pSV2 (vector), pcDNA3.myc.DAL-1, pSV2.myc.Protein 4.1R80, or pSV2.myc.Protein 4.1R135. Quadruplicate 60mm plates for each transfection were grown for two weeks in the presence geneticin. The number of colonies was counted after Cresyl violet staining with the mean and standard deviation determined for each condition. This experiment was repeated at least 4 times with similar results.

Immunohistochemistry. Paraffin sections were deparaffinized and endogenous peroxidase activity was quenched with 3% peroxide. Antigen retrieval was accomplished by boiling slides for 10 min in 10nM sodium citrate. Sections were then blocked with 1% BSA, and incubated overnight with the primary antibody (rabbit polyclonal anti-Protein 4.1R ~~x~~ diluted 1:6000) at 4C. The Protein 4.1R antibody (generated by Drs. Loren Walensky and Solomon H. Snyder, The Johns Hopkins University School of Medicine, Baltimore, MD) was raised in rabbit against a histidine (His)-tagged recombinant protein corresponding to ~~a~~ mouse 4.1R exon 13-encoded peptide. Secondary anti-rabbit biotinylated antibodies were used at 1:200 and slides were developed using DAB as the chromagen. Slides were photographed at 100 or 400 x magnification using a X microscope.

Fluorescent in situ hybridization (FISH). Dual-color FISH experiments were performed as previously described (Singh *et al.*, 2002). Samples were deparaffinized, boiled in citrate buffer

for 20 min, and digested with pepsin (4 mg/mL) at 37C for 30 min. Slides were then washed in 2 x standard saline citrate (SSC) and dried. A fluorescein-labeled probe targeting the Protein 4.1R gene (PAC212P9, GenBank Accession #AL009181) and a rhodamine-labeled 1q42 probe were paired for each hybridization and applied to the slide at a concentration of X, followed by simultaneous denaturation of the probe and target at 90C for 30 min. Overnight hybridization at 37C took place in a humidified chamber. Slides were washed in 50% formamide / 1 x SSC for 5 min, 2 x SCC for 5 minutes, and air-dried. DAPI (0.5 µL/mL, Insitus Laboratories) was used as a nuclear counterstain, and the sections were viewed under an X fluorescent microscope. Only sections showing sufficient hybridization efficiency (> 90% nuclei with signals) were evaluated.

Western blotting. Frozen tumor specimens were homogenized in NP40 lysis buffer containing protease inhibitors and the protein concentration was determined by the Biorad method (Biorad Laboratories, California). To determine protein expression within various cell lines, C6, IOMM-Lee, and CH157-MN cells were grown to confluency, lysed in NP40, and protein concentration was determined. Electrophoresis was performed on 100µg of each sample loaded on 8% SDS-PAGE gels. Proteins were transferred onto Immobilon membranes (Millipore, Massachusetts) for Western blotting with DAL-1 (3A-1, 1:2000), merlin (C-18, 1:1000) and Protein 4.1R (1:6000). Western blots were developed using horseradish peroxidase-conjugated secondary antibodies (1:20,000) and ECL chemiluminescence reagent (Amersham, New Jersey).

Because CH157 and IOMM-Lee cells have low transfection efficiencies, RT4 cells were transfected with 2µg of pSV2 (vector), pcDNA3.myc.DAL-1, pSV2.myc.Protein 4.1R80, and pSV2.HA.Protein 4.1R135 to determine protein expression. Cells were lysed and protein concentration was determined as above. Electrophoresis was performed on 50µg of lysate

loaded on 8% SDS-PAGE gels. Proteins were transferred onto Immobilon membranes for Western blotting analysis using anti-c ~~with~~ myc (1:2000) ~~and~~ or anti HA (1:1000) antibodies. Western blots were developed using horseradish peroxidase-conjugated mouse secondary antibodies (1:20,000) and ECL chemiluminescence reagent.

Immunofluorescence. RT4 cells were transfected with 2µg of pSV2 (vector), pcDNA3.myc.DAL-1, pSV2.myc.Protein 4.1R80, and pSV2.HA.Protein 4.1R135 (sometimes you talk about a myc tagged 135 construct, sometimes about a HA tagged 135 construct did you actually use two different R135 constructs depending on the experiments, I am confused since you talk only about a c-myc secondary antibody latter in this paragraph ?). After 48 hours, cells were fixed with 4% paraformaldehyde, permeabilized with 0.25% Triton, washed, blocked with PBS containing 10% goat serum and 1% BSA, and incubated overnight with an anti-myc antibody (1:200). Immunofluorescence was achieved using a CY3-conjugated mouse secondary antibody (1:20,000) and counterstained with Bodipy-Phalloidin.

Isolation of nuclear and membrane fractions. C6 cells were plated in 6-well dishes at approximately 10^5 cells / well. After 24-48 hours, the cells were trypsinized, washed with PBS, and resuspended in hypotonic lysis buffer (10mM KCl, 10mM Tris, pH 7.4, 1.5mM $MgCl_2$, 1mM PMSF) plus protease inhibitors, incubated on ice for 15 min, and homogenized. Lysates were centrifuged at 4000 rpm for 5 min and the pellet containing the nuclear fraction was saved. The supernatant was ultracentrifuged at 100,000 x g for 40 min at 4C and the pellet containing the membrane fraction was saved. The pellet was lysed in NP40 lysis buffer and protein concentration was determined using the Biorad BCA kit (Pierce). Electrophoresis was

performed on equal amounts of each sample loaded on 8% SDS-PAGE gels. Samples were transferred onto Immobilon membranes and subjected to immunohistochemical analysis as described above.

GST-fusion protein affinity chromatography. GST-14-3-3, GST-PRMT3, GST-βII-spectrin, and GST-HRS were generated as described previously (Yu and Robb et al., 2002; Singh et al., in press, Gutmann et al., 2001a; Gutmann et al., 2001b). Briefly, constructs were cloned into the pGEX.3X vector (Pharmacia), verified by sequencing, and transformed into DE3 (BL21) competent cells for GST fusion protein production. Bacterial cultures were induced overnight with 0.5mM IPTG at room temperature, lysed, and the GST fusion proteins isolated on glutathione-agarose beads (Sigma) for the interaction experiments. Each fusion protein was newly isolated for each experiment and verified by SDS-PAGE with Coomassie blue visualization. Protein production of DAL-1 and Protein 4.1R was accomplished by the TnT method (Promega) according to the protocol supplied by the manufacturer. *In vitro* ~~transcribed and~~ translated proteins were synthesized in the presence of ^{35}S-methionine and confirmed by SDS-PAGE electrophoresis and autoradiography. Radiolabeled proteins were incubated with equimolar amounts of GST-fusion proteins immobilized on glutathione beads for 4h at 4C in NET buffer (25mM Tris pH7.5, 100mM NaCl, 3mM EDTA) containing 1% BSA. An aliquot of the unbound fraction was saved and the beads were washed twice with NET buffer plus 0.1% Triton X-100 and twice with NET buffer plus 0.05% Triton X-100. The unbound and bound fractions were eluted in Laemmli buffer, boiled for 5 minutes, separated by SDS-PAGE electrophoresis, and analyzed by autoradiography. In all experiments, no significant binding to

immobilized GST alone was ~~not~~ observed. Each interaction experiment has been repeated at least three times with identical results.

Immunoprecipitation. RT4 cells were transfected with 2µg pSV2 (empty vector), pcDNA3.myc.DAL-1, pSV2.myc.Protein 4.1R80, or pSV2.HA.Protein 4.1R135 and 1µg of pcDNA3.hisA.CD44 or pcDNA3.hisA.SCHIP. After 48 hours, cells were lysed in NP40 lysis buffer plus protease inhibitors. Ni-NTA beads (Qiagen) were added and samples were rotated at 4C for 2 hours, washed, eluted in 2x Laemmli sample buffer, loaded on 8% SDS-PAGE gels, and separated by electrophoresis. Bound proteins were then identified by Western blot analysis using either anti-HA (diluted 1:500) or anti-myc (diluted 1:100) antibodies as described above.

Results

Protein 4.1R Loss is Associated with Sporadic Meningiomas

The Protein 4.1R gene has been mapped to chromosome 1p32-36 and has structural similarities to Protein 4.1B based on previously mapped domains with high sequence similarity: an N-terminal FERM domain, a spectrin-actin-binding (SAB) domain, a C-terminal domain (CTD) and three unique regions (U1, U2, and U3; Fig. 1a). The alignment of Protein 4.1R80 and Protein 4.1R135 demonstrates that Protein 4.1R135 contains unique sequences not present in Protein 4.1R80 as a result of splicing events consisting of inclusion or exclusion of alternative start sites (Fig. 1a, this figure shows quite some mistakes, please look at the alignment of the four mouse 4.1 proteins for sequences and boundaries of the 4.1 protein domains in the Parra et al., JBC 2000 paper, among the mistakes there is an incorrect assignment of a part of the CTD to the U3 region and incorrect splicing events in the U2 and U3 regions between 4.1R80 and 4.1R135, see below and my comments highlighted in red in figure 1). Protein 4.1R135 contains an N-terminal U1 domain, ~~as well as additional sequences within the U2 and U3 domains~~ (this is incorrect, the only difference between 4.1R80 and 4.1R135 is the presence or absence of the U1 region. You may find a very short exon (exon 15, 36bp long in 4.1B and 4.1N, I actually don't remember its size in 4.1R) at the 3' end of the U2 region specifically expressed in some brain 4.1R isoforms regardless of the presence or absence of the U1 region. The U3 region is absent in both isofoms; the exons encoding the U3 region, exons 17a and 17b, being found only in muscle and/or epithelial tissues, thus in 4.1R80 and 4.1R135 the CTD immediately follows the SAB domain: see my comments highlighted in red in figure 1). A Protein 4.1R specific polyclonal antibody directed against part of the unique U2 sequence detected both 80 kDa and 135 kDa

4.1R proteins (Protein 4.1R80 and Protein 4.1R135) in whole mouse brain as well as C6 glioma cell lysates (Fig. 1b) (see my comments in Figure legend). Protein 4.1R80 and Protein 4.1R135 expression was also detected in the three additional glioma cell lines (mouse B8 as well as human U87 and U373 cells; data not shown). However, Protein 4.1R ~~protein~~ was not expressed in two meningioma cell lines, CH157-MN and IOMM-Lee, by Western blotting.

Previous studies have demonstrated that both Protein 4.1B and merlin are expressed in normal human leptomeningeal cells and that merlin and Protein 4.1B loss is a common and early event in sporadic meningiomas (Perry et al., 2000). To determine whether Protein 4.1R expression was lost in primary meningioma tumor specimens, we analyzed normal human leptomeninges and 15 sporadic meningiomas classified according to World Health Organization grading criteria (Kleihues et al., 2002) using a Protein 4.1R-specific antibody. The abundant expression of Protein 4.1R in both blood vessels and erythrocytes precluded the examination of Protein 4.1R protein expression in fresh surgical specimens by Western immunoblotting. As a result, analysis of paraffin-imbedded human meningioma samples for Protein 4.1R ~~protein~~ expression was accomplished by IHC. Protein 4.1R was detected in normal human leptomeningeal tissues and within the lining of the blood vessel wall (Fig. 2a). However, immunohistochemical analysis of the meningioma tumors demonstrated that approximately 40% of the tumors lacked Protein 4.1R expression. The tumor shown in Figure 2b is a representative 4.1R-immunopositive meningioma, whereas Figure 2c illustrates a 4.1R-immunonegative meningioma. The frequency of Protein 4.1R loss stratified by tumor grade is summarized in Fig. 2e. Protein 4.1R loss was a common and consistent event (40%) in all tumor grades examined. A subset of these meningiomas was analyzed by fluorescent in situ hybridization (FISH) with a Protein 4.1R-specific probe. FISH analysis resulted in 100% concordance with the IHC data

(data not shown). A representative FISH hybridization on the same tumor specimen as shown in Figure 2c (Fig. 2d) demonstrates Protein 4.1R deletion. Collectively, this data suggests that loss of Protein 4.1R may be an early and common event in meningioma pathogenesis.

Protein 4.1R Suppresses Meningioma Cell Proliferation

Since Protein 4.1R loss was a common event in meningioma pathogenesis, we next wished to determine whether Protein 4.1R overexpression could suppress meningioma cell growth using a clonogenic assay. In these experiments, equimolar amounts of pSV2 (vector), pcDNA3.myc.DAL-1, pSV2.myc.Protein 4.1R80 or pSV2.myc.Protein 4.1R135 were transfected into the Protein 4.1R-negative meningioma cell lines IOMM-Lee or CH157-MN and selected in geneticin. Due to the low transfection efficiency of the meningioma cell lines, schwannoma RT4 cells were transfected with each construct to demonstrate protein production *in vivo*. Immunoblotting of transfected RT4 cell lysates demonstrates the protein expression of Protein 4.1R80, Protein 4.1R135, and DAL-1 *in vivo* (Fig. 3a). Similar to DAL-1, overexpression of either Protein 4.1R80 or Protein 4.1R135 resulted in a 40% reduction in colony number in both IOMM-Lee and CH157-MN cell lines (Figs. 3b and 3c), suggesting that 4.1R can suppress meningioma cell proliferation and that the domains required for this growth suppression are contained within Protein 4.1R80 sequences. In Fig 3a, the level of expression of DAL-1 is much higher than that of the two 4.1R isoforms but their antiproliferative effects shown in 3b and 3c seem very similar. Does it mean that 4.1R is a more potent tumor suppressor than 4.1B or that a low level of overexpression of 4.1 proteins is sufficient to allow maximum antiproliferative properties ? I think that this should be addressed somehow here or even better in the discussion.

Protein 4.1R is Recruited to the Membrane Under Conditions of Growth Arrest In Vitro.

Based on our observation that Protein 4.1R expression is lost in 40% of human meningiomas examined and functions as a tumor suppressor in Protein 4.1R-deficient meningioma cell lines, we sought to determine whether the subcellular localization of Protein 4.1R was similar to that of merlin / Protein 4.1B. Previously, it was shown that both endogenous and exogenously overexpressed Protein 4.1B or merlin localized to the cell membrane (Tran et al., 1999; Gutmann et al., 2001a; Gonzalez-Agosti et al., 1996; Scherer et al., 1996; Parra et al., 2000). Protein 4.1R has been shown to be an abundant protein in the membrane cytoskeleton as well as the nucleus (Takakuwa et al., 2001; De Carcer et al., 1995; den Bakker et al., 1995; Krauss et al., 1997; Gascard et al., 1998; Gascard et al., 1999). To determine the subcellular distribution of endogenous Protein 4.1R, C6 glioma cell fractions, representing plasma membrane or nuclear components, were isolated and analyzed by Western immunoblotting with specific antibodies (Fig 4a). Protein 4.1B expression was abundant in the membrane fraction and at insignificant levels in the nuclear fraction. However, Protein 4.1R was expressed in both the nuclear and membrane fractions. To confirm this observation, RT4 cells were transfected with equimolar amounts of pSV2 (vector), pcDNA3.myc.DAL-1, or pSV2.myc.Protein 4.1R80 and immunostained with a myc-specific antibody (Fig. 4b). Overexpression of DAL-1 resulted in a predominately cytosolic localization, whereas Protein 4.1R was detected prominently at the nucleus.

As Protein 4.1R is localized to the membrane and nucleus, we explored the possibility that Protein 4.1R localization may be regulated by cellular growth arrest cues. Growth inhibition *in vitro* is observed in response to serum starvation and contact inhibition. Under conditions of increased confluency or serum starvation, merlin expression is increased in the membrane of

NIH3T3 cells (Shaw et al, 1998). To determine whether merlin, DAL-1 and Protein 4.1R were regulated in a similar manner, total C6 cell lysates were isolated from subconfluent and serum starved cultures and the plasma membrane fractions were analyzed by Western immunoblotting with specific antibodies (Fig. 4c). Similar to merlin, under serum free conditions, both Protein 4.1R and Protein 4.1B expression was increased in cell membrane fractions, suggesting that under growth arrest conditions these tumor suppressors are redistributed to the plasma membrane ~~whether~~ ???? where they may interact with critical effector proteins.

Protein 4.1R Interacts with a Subset of Merlin/Protein 4.1B Interactors

Because Protein 4.1R shares both functional and structural similarities with Protein 4.1B and merlin, we examined the ability of Protein 4.1R to bind known Protein 4.1B / merlin interactors that may be implicated in growth regulation. Previous studies have shown that merlin interacts with the hyaluronadate receptor CD44 (Morrison et al., 2001), βII spectrin (fodrin, Scoles et al., 1998), schwannomin interacting protein-1 (SCHIP-1, Goutebroze et al, 2000) and hepatocyte growth factor-regulated tyrosine kinase substrate (HRS, Scoles et al., 2000; Gutmann et al, 2001b). The DAL-1 fragment of Protein 4.1B also interacts with CD44 (Robb VA & Gutmann DH; unpublished observations), and βII-spectrin (Gutmann et al, 2001a), but uniquely interacts with 14-3-3 proteins (Yu and Robb et al, 2002), and PRMT3 (Singh et al, manuscript in preparation). To determine whether Protein 4.1R interacts with CD44 and SCHIP-1 *in vivo*, RT4 cells were co-transfected and interacting proteins were immunoprecipitated. Similar to DAL-1 and merlin, both Protein 4.1R80 and Protein 4.1R135 interact with CD44 (Fig. 5a) Such an interaction has been previously reported by Nunomura et al., JBC 1997. SCHIP???? To determine the ability of Protein 4.1R80 to bind to other Protein 4.1B and merlin interactors *in*

vitro, we utilized GST affinity chromatography (Fig. 5b). Similar to Protein 4.1B/DAL-1 and merlin, Protein 4.1R80 exhibits significant binding to βII-spectrin. However, Protein 4.1R80 does not interact with 14-3-3 proteins or PRMT3. HRS??? These results collectively suggest that Protein 4.1R shares only some of the binding partners implicated in merlin or Protein 4.1B tumor suppressor function.

Discussion

Traditionally, 4.1 proteins are thought to be important in the maintenance of cell shape and regulation of actin cytoskeleton-mediated processes, such as cell adhesion and motility (Bretscher et al., 2002). Prototypic Protein 4.1 molecules bind cell surface glycoproteins as well actin and actin-binding proteins (Ohanian et al., 1984; Correas et al., 1988). Protein 4.1R alterations in erythrocytes result in dramatic cell shape abnormalities and the human disorder elliptocytosis (Tchernia et al., 1981). Similarly, Protein 4.1 molecules also link cell surface glycoproteins, like CD44, to the actin cytoskeleton (Nunomura et al., 1997; Morrison et al., 2001). Recently, the function of Protein 4.1 molecules has been expanded to include growth regulation with the identification of two Protein 4.1 tumor suppressors.

Work from a number of independent laboratories has demonstrated that the Protein 4.1 molecules, merlin and Protein 4.1B, function as negative growth regulators in the pathogenesis of a diverse number of human cancers (Tran et al., 1999; Yana et al., 1995; Bryan et al, 1996; Allione et al, 1998). Given the high levels of expression of both merlin and Protein 4.1B in the central nervous system coupled with the observation that individuals with *NF2* loss develop meningiomas, our laboratory and others have previously shown that *NF2* and Protein 4.1B inactivation is a common early event in meningioma formation. Both merlin and Protein 4.1B are expressed in normal leptomeninges and loss of expression is observed in approximately 60% of sporadic meningiomas (Gutmann et al, 1997; Gutmann et al., 2000; Perry et al., 2000). In addition, rescue ~~replacement~~ of merlin or Protein 4.1B expression in deficient human meningioma cells results in growth suppression (Gutmann et al, 2001a; Ikeda et al., 1999) ~~and~~ while conditional inactivation of ~~*Nf2*~~ *NF2* in leptomeningeal cells in mice results in meningioma

formation (Kalamarides et al., 2002). Collectively, these data suggest that merlin and Protein 4.1B are critical growth regulators for leptomeningeal cells.

The finding that two members of the Protein 4.1 superfamily have negative growth regulatory properties and function as tumor suppressors raised the possibility that other structurally related proteins may also function as growth regulators in meningeal cells. Based on the chromosomal localization of Protein 4.1R to chromosome 1p, a location where genetic aberration is associated with meningioma pathogenesis, we explored the possibility that Protein 4.1R may also represent a candidate tumor suppressor gene. Similar to merlin and Protein 4.1B, Protein 4.1R is also expressed in normal human leptomeninges and is inactivated at ~~on~~ both ~~the~~ protein and DNA levels as analyzed by IHC and FISH, respectively. In the meningioma tumors examined, loss of Protein 4.1R expression was observed in 6 of 15 tumors and was a common event (40%) in all tumor grades. The frequency of Protein 4.1R loss is significantly ~~less~~ lower than ~~we~~ that previously observed by us for either merlin or Protein 4.1B, suggesting that this genetic alteration may be a less frequent event in meningioma pathogenesis. This result is consistent with the finding that chromosome 1p loss is the second most common genetic aberration observed in meningiomas (Simon et al., 1995; Bostrom et al., 1997; Sulman et al, 1998). Similar to Protein 4.1B and merlin, Protein 4.1R loss was irrespective of WHO tumor classification, indicating that loss of Protein 4.1R expression is an early event in meningioma pathogenesis. Given the small number of meningiomas examined in this study, it is premature to confidently determine the true frequency of Protein 4.1R loss compared with that of Protein 4.1B or *NF2*. Studies are in progress to determine the relationship between Protein 4.1R, Protein 4.1B, and *NF2* loss in sporadic meningiomas stratified by WHO grade. Previous studies of Protein 4.1 loss have shown that epigenetic events, such as DNA methylation, are common ???

be more specific in your statement so that the rationale behind the experiment described in the following sentence becomes more clear (Garinis et al., 2002). To explore this possibility in the two human meningioma cell lines lacking Protein 4.1R expression, we treated cells with increasing doses of the DNA methylation inhibitor (5-Aza-2'-Deoxycytidine), but did not observe Protein 4.1R re-expression (Li W and Gutmann DH, unpublished results 2002). Further work will be required to determine the mechanism underlying Protein 4.1R loss in meningiomas.

Since Protein 4.1R loss was a common event in human meningioma tumors, we wished to determine whether Protein 4.1R re-expression in deficient meningioma cells could suppress cell growth. Previous work has demonstrated that overexpression of either merlin or Protein 4.1B in meningioma cell lines resulted in suppression of cell proliferation (Gutmann et al., 2001a). In a similar fashion, overexpression of either Protein 4.1R80 or Protein 4.1R135 resulted in a suppression of meningioma cell proliferation, suggesting that the residues required for growth suppression are contained within Protein 4.1R80. Protein 4.1R80 contains the N-terminal FERM domain, the spectrin-actin-binding (SAB) domain, and the C-terminal domain (CTD), but is missing the U1 domain ~~and sequences within the U2 and U3 domains~~ present in Protein 4.1R135. This result is very similar to that observed with Protein 4.1B, in which the DAL-1 fragment can suppress cell growth. Studies are underway to determine the minimal domain of Protein 4.1R required for growth suppression in an effort to determine its mechanism of action.

Significant insights into the mechanism of merlin growth suppression have derived from an examination of its binding partners. Because Protein 4.1R shares both functional and structural similarities with Protein 4.1B and merlin, we examined the ability of Protein 4.1R to bind known Protein 4.1B/DAL-1 interactors. Both merlin and Protein 4.1B have been shown to

interact with CD44 (Morrison et al., 2001; Robb VA & Gutmann DH, unpublished observations 2002). CD44 is a widely expressed cell surface hyaluronadate receptor, which plays a key role in mediating cell migration and adhesion. Moreover, recent work has demonstrated that the transduction of growth inhibition signals from the extracellular matrix is dependent on merlin's interaction with CD44 (Morrison et al., 2001). Under growth permissive conditions, merlin is phosphorylated, resulting in an "open" and "inactive" merlin molecule incapable of negatively regulating cell growth. In contrast, when cells are stimulated to undergo growth arrest by cell contact or specific extracellular matrix cues (e.g., high molecular weight hyaluronic acid), merlin exists in a hypophosphorylated state, resulting in molecules in the "closed" conformation (Morrison et al., 2001). This would favor binding of merlin to the cytoplasmic tail of CD44 to promote cell growth suppression (Sherman & Gutmann, 2001). Thus, the phosphorylation state of merlin and merlin's ability to interact with CD44 is modulated by growth arrest signals such as confluency and serum deprivation. As demonstrated here and by others (Nunomura et al., 1997), Protein 4.1R also interacts with CD44. We also demonstrate that Protein 4.1R localizes to the cell membrane and that, similar to Protein 4.1B and merlin (Scherer et al., 1996; Gutmann et al., 2001a), Protein 4.1R is recruited to the cell membrane under conditions of growth arrest. It is tempting to postulate that Protein 4.1R redistributes to the cell membrane to interact with molecules like CD44 to negatively regulate cell growth, as it has been described for merlin (Morrison et al., 2001). Further work will be required to determine whether the regulated binding of Protein 4.1R to CD44 is important for Protein 4.1R growth suppression.

Similar to our previous analysis of Protein 4.1B, Protein 4.1R interacts with only a subset of molecules that bind merlin or Protein 4.1B. Protein 4.1R, like merlin and Protein 4.1B, also interacts with βII spectrin (Scoles et al., 1998; Gutmann et al, 2001). However, Protein 4.1R

does not associate with the Protein 4.1B-specific interactors 14-3-3 (Yu and Robb et al., 2002) or PRMT3 (Singh et al., in press), or merlin-specific interactors SCHIP-1 and HRS (Still in Progress). These results suggest that Protein 4.1R associates with unique effector proteins that may be specific to its function as a negative growth regulator. Protein 4.1R is highly expressed in the nucleus and binds the nuclear mitotic apparatus protein NuMA (Mattagajasingh et al., 1999), a non-histone protein that exits the nucleus during mitosis to become associated with mitotic spindle structures. This unique property of Protein 4.1R may be important for the transduction of signals from the cell membrane to the nucleus. Future studies on Protein 4.1R interactors will be necessary to determine which of its binding partners are important for transducing its growth signal.

Our report demonstrating that a third Protein 4.1 family member, Protein 4.1R, functions as a tumor suppressor in the molecular pathogenesis of meningiomas expands the number of Protein 4.1 molecules implicated in growth regulation. The observation that all three Protein 4.1 tumor suppressors (4.1R, 4.1B, and merlin) associate with CD44 and concentrate at the cell membrane during cell growth arrest *in vitro* suggest that these molecules initiate their signaling at the plasma membrane (Bretscher et al., 2002; Sherman & Gutmann 2001). Their individual mechanisms of action might reflect the diversity of their binding partners (Morrison et al., 2001; Scoles et al., 1998; Goutebroze et al, 2000; Scoles et al., 2000; Gutmann et al, 2001a; Gutmann et al, 2001b; Yu and Robb et al, 2002; Singh et al, manuscript in preparation) and the regulation of their association with cell membrane proteins, including lipid modifications (Sechi et al., 2000; Hamada et al., 2000; An et al., 2001) and phosphorylation (Matsui et al., 1998; Hirao et al., 1996; Takakuwa 2001). Future experiments aimed at determining the minimal region required for Protein 4.1R growth suppression and identifying specific binding proteins that

transduce the growth signal should provide insights into the mechanism of Protein 4.1R growth suppression.

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Figure Legends

Figure 1. Comparison of Protein 4.1R80 and Protein 4.1R135. (a) The alignment of Protein 4.1R135 (top) and 4.1R80 (bottom) demonstrates that Protein R135 contains a unique sequence not present in Protein 4.1R80 as the result of the use of an alternative start site ~~and splicing events.~~ Functional domains, based on previously mapped areas of homology, in common with both isoforms of Protein 4.1R are: an N-terminal FERM domain, a spectrin-actin-binding domain (SAB), a C-terminal domain (CTD) and three unique regions (U1, U2, and U3; Figure 1a). Protein 4.1R135 also contains a N-terminal U1 domain~~, as well as additional sequences within the U2 and U3 domains~~ (please see my comments about this in the manuscript). (b) A Protein 4.1R specific polyclonal antibody against mouse exon 13-encoded peptide detects various isoforms of ~~proteins of 80 and 135 kDa, corresponding to~~ Protein 4.1R80 and Protein 4.1R135, in whole brain lysates as well as C6 glioma cell lysates (I modified a bit the sentence since you detect several bands not only two bands). Protein 4.1R expression is lost in two meningioma cell lines ~~examined by Western blotting,~~ CH157MN and IOMM-Lee.

Figure 2. Representative examples of Protein 4.1R expression in meningiomas. Immunohistochemistry was performed on paraffin sections using a Protein 4.1R specific antibody (1:6000 dilution) with DAB as the chromagen. Protein 4.1R is expressed in normal meninges (a, 100 x magnification) and tumor 2-831-332 (b, 400 x magnification), whereas loss of Protein 4.1R expression is observed in tumor 4-318-421 (c). Representative fluorescence in situ hybridization (FISH) analysis confirming chromosome 1p loss in the same meningioma pictured in c (d, x magnification). A dual hybridization was performed using a Protein 4.1R specific probe (green) and a 1q42-specific probe (red). Of five meningiomas analyzed by IHC,

there was 100% concordance by FISH analysis. (e) Table summarizing results from immunohistochemical analysis of 15 meningiomas stratified by WHO tumor grade. I think it is a good idea to add the fractions in Fig 2e (see modified figure).

Figure 3. Growth suppression in meningioma cell lines CH157MN and IOMM-Lee. (a) Immunoblotting of RT4 cell lysates transfected with equimolar amounts of various protein 4.1 constructs to demonstrate *in vivo* protein expression. You should explain what the minus lanes correspond to, non transfected cell lines, transfected with empty vector, other ? Also the expression of DAL-1 is much higher than that of 4.1R80 and 4.1R135 but their antiproliferative properties seem very similar. (b) Protein 4.1R80, Protein 4.1R135, or DAL-1 overexpression in CH157-MN cells resulted in growth suppression. CH157-MN cells were transfected with equimolar amounts of pSV2 (vector), pcDNA3.myc.DAL-1, pSV2.myc.Protein 4.1R80 or pSV2.HA.Protein 4.1R135. After 2 weeks in culture under geneticin selection, individual colonies were counted on quadruplicate plates per transfection. The mean and standard deviation for each transfection is shown. Asterisk denotes $P<0.01$ using the Student's t-test. (c) Similar results were obtained in another meningioma cell line, IOMM-Lee.

Figure 4. Subcellular localization of Protein 4.1R and DAL-1. (a) Total cell lysates isolated from C6 glioma fractions representing plasma membrane or nucleus components were analyzed by Western immunoblotting with specific antibodies. (b) RT4 cells were transfected with equimolar amounts of constructs and analyzed by immunofluorescence. Photographs were taken at 400x magnification. Upper panels depict protein localization; lower panels show specific staining merged with the phalloidin counterstain.

Figure 5. Protein 4.1R binds to some known interactors of DAL-1 and merlin. (a) DAL-1, Protein 4.1R80, and Protein 4.1R135 co-immunoprecipitates with CD44. RT4 cells were transfected with 2µg pSV2 (vector), pcDNA3.myc.DAL-1, pSV2.myc.Protein 4.1R80, or pSV2.HA.Protein 4.1R135 and 0.5µg of pcDNA3.hisA.CD44. Cells were lysed and CD44 complexes were isolated by Ni-NTA beads. For each sample, the supernatant (S), bound fraction (B), and total cell lysate (C) are shown. Bound proteins were identified by Western blot analysis using either anti-HA (1:500) or anti-myc (1:100) antibodies. DAL-1, Protein 4.1R80, and Protein 4.1R135 exhibited significant binding to CD44. SCHIP-1 ? (b) DAL-1 and Protein 4.1R80 binding to 14-3-3, PRMT3, and fodrin were investigated by GST affinity interactions as described in the Materials and Methods section. The bound and supernatant fractions are shown for each representative interaction. DAL-1 exhibited significant binding to 14-3-3, PRMT3, and fodrin. In contrast, Protein 4.1R80 bound only to fodrin. HRS. No significant binding to GST alone was ~~not~~ observed.

```
MTTEKSLVTEAENSQHQQKEEGEEAINSGQQEPQQEESCQTAAEGDNWCEHKL
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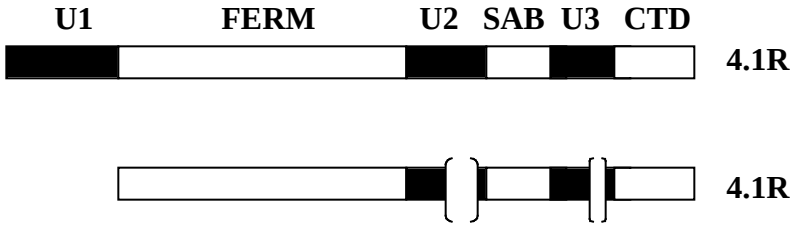


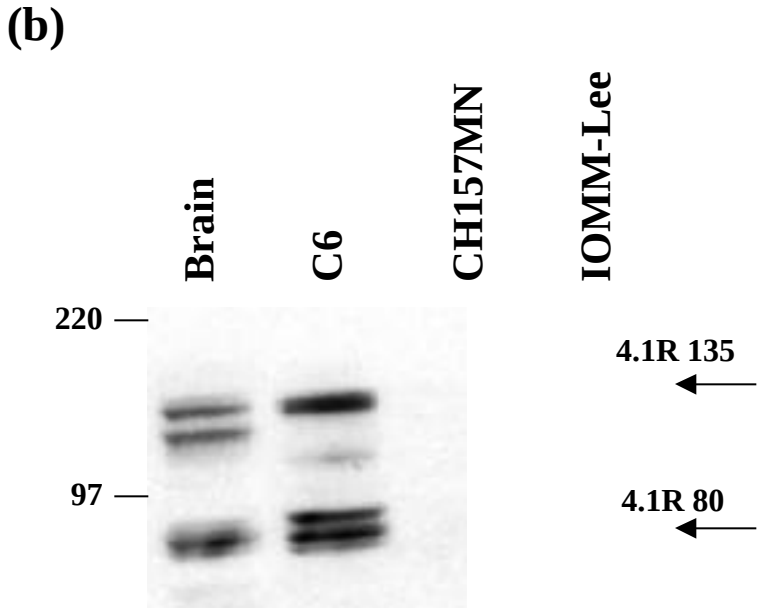
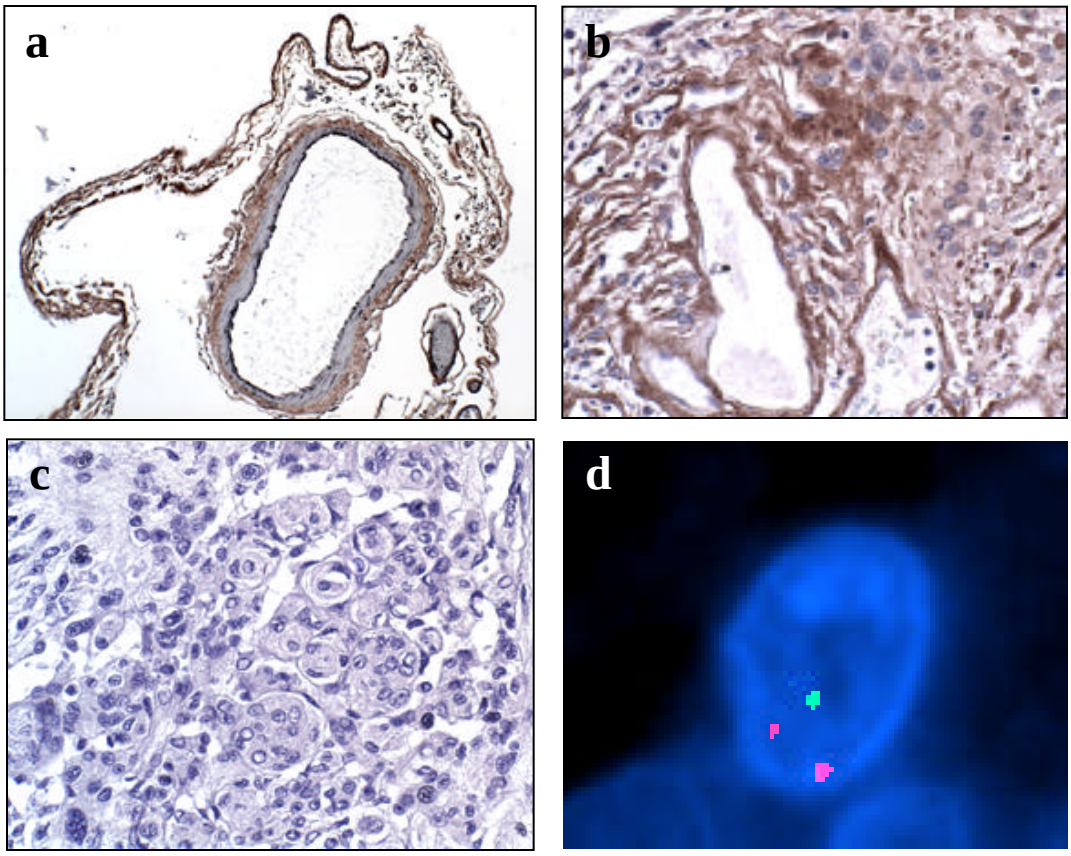


Figure 1



(e)

Tumor Grade	Number of Negative Tumors (%)
WHO grade I	2/5 (40%)
WHO grade II	2/5 (40%)
WHO grade III	2/5 (40%)

Figure 2

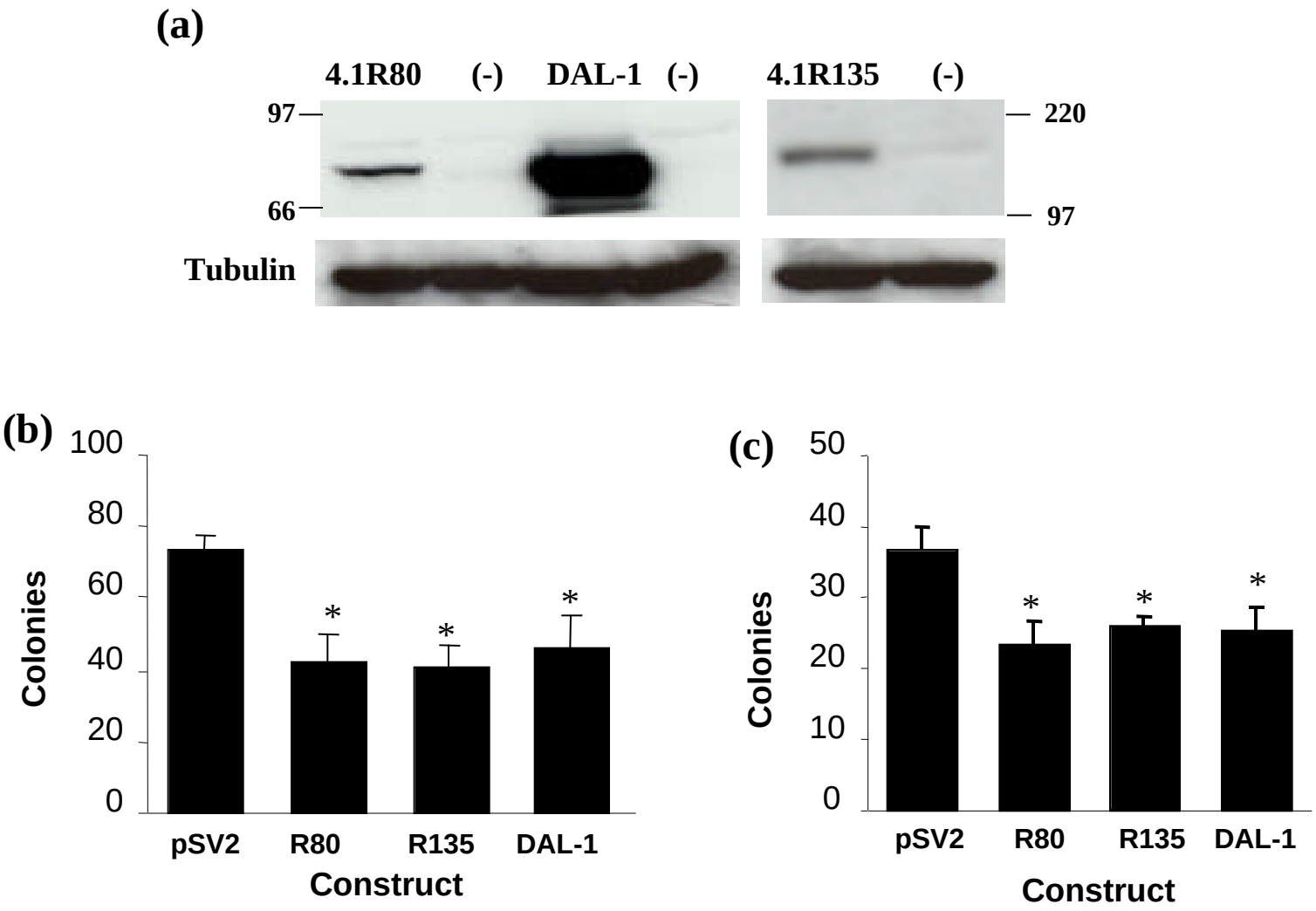
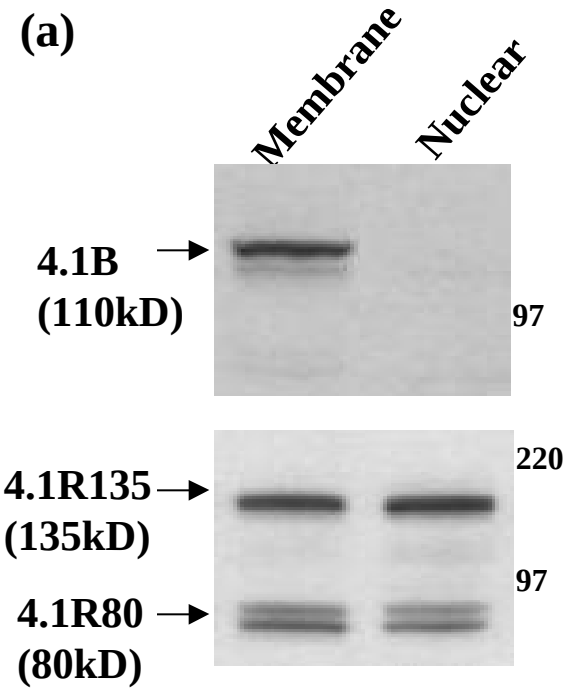
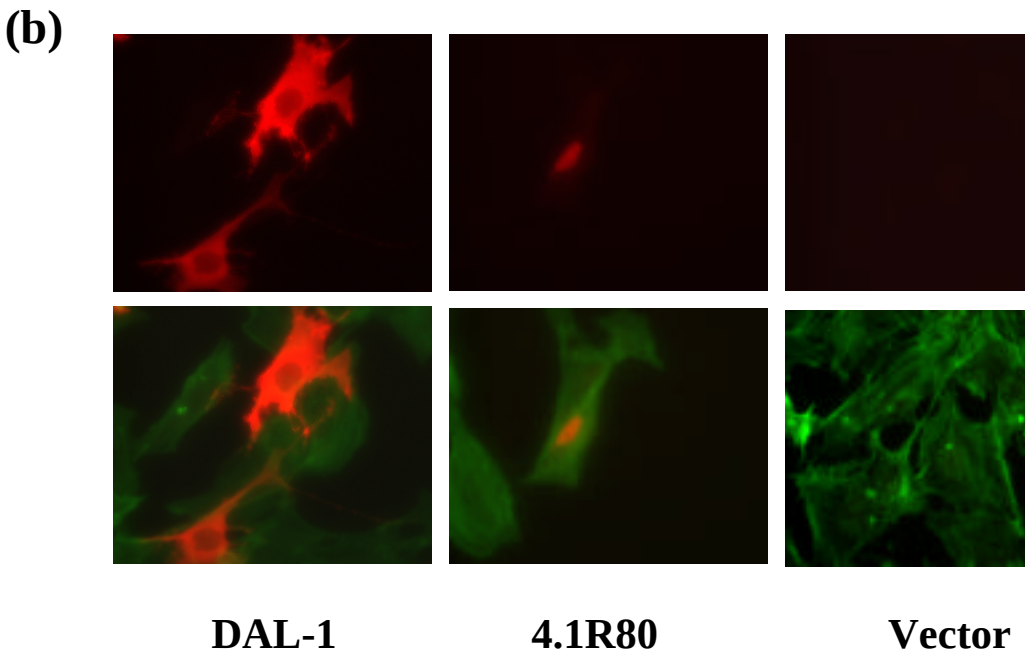

(a)
4.1R80
(-)
DAL-1
(-)
4.1R135
(-)
97
66
220
97
Tubulin
(b)
Colonies
100
80
60
40
20
0
*
*
*
pSV2
R80
R135
DAL-1
Construct
(c)
Colonies
50
40
30
20
10
0
*
*
*
pSV2
R80
R135
DAL-1
Construct

Figure 3


(a)
Membrane
Nuclear
4.1B
(110kD)
97
4.1R135
(135kD)
220
97
4.1R80
(80kD)


(b)
DAL-1
4.1R80
Vector

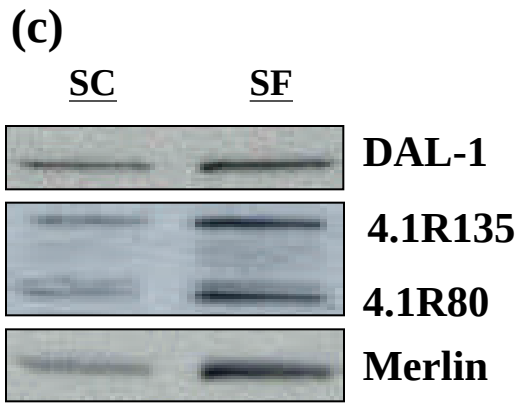

(c)
SC
SF
DAL-1
4.1R135
4.1R80
Merlin

Figure 4

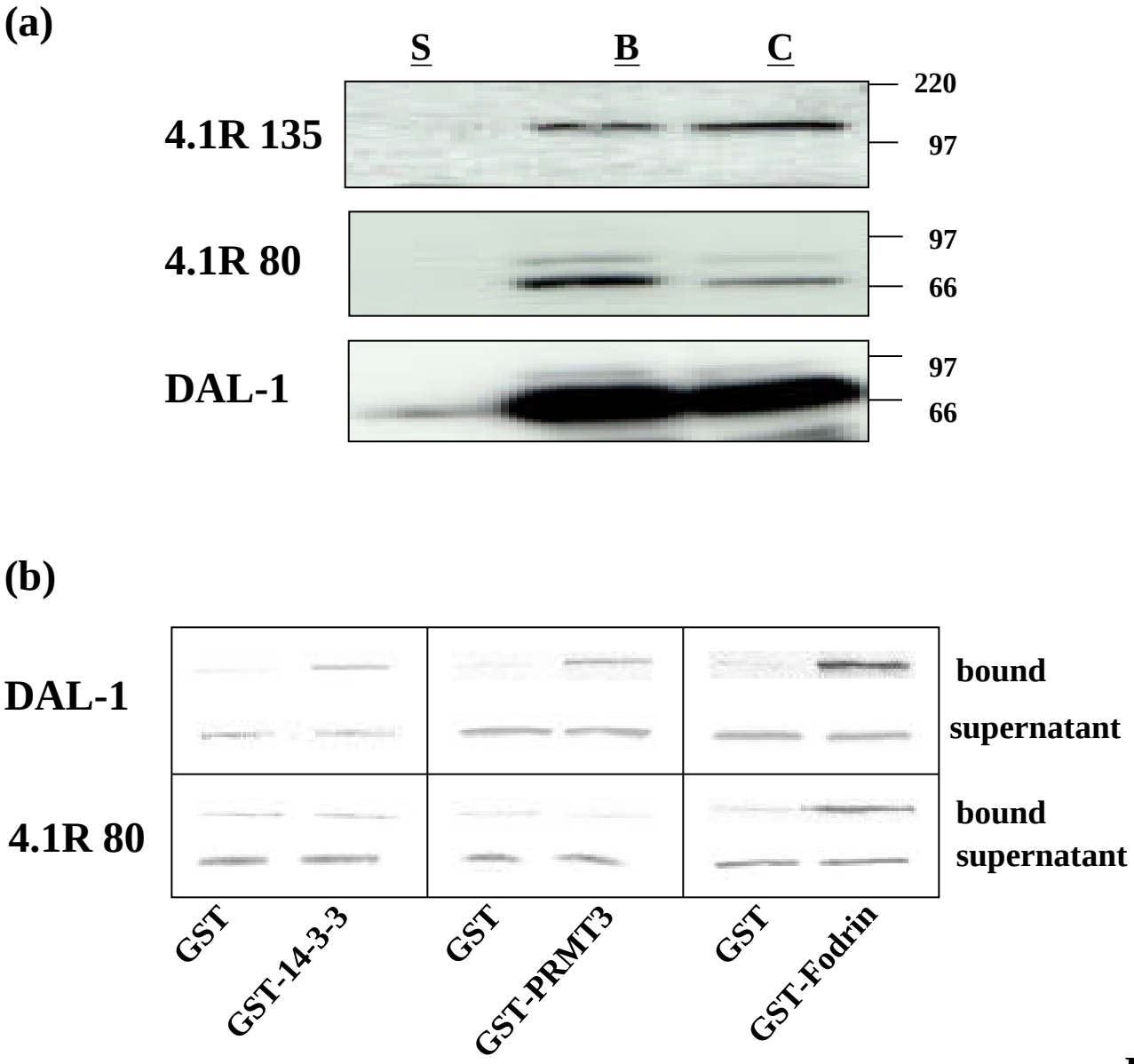

(a)
S
B
C
4.1R 135
220
97
4.1R 80
97
66
DAL-1
97
66
(b)
DAL-1
bound
supernatant
4.1R 80
bound
supernatant
GST
GST-14-3-3
GST
GST-PRMT3
GST
GST-Fodrin

Figure 5