The Prototypical 4.1R-10-kDa Domain and the 4.1G-10-kDa Paralog Mediate Fodrin-Actin Complex Formation*

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Aikaterini Kontrogianni-Konstantopoulos‡§, Carole S. Frye¶, Edward J. Benz, Jr.‡‡,**, and Shu-Ching Huang‡‡‡§§

From the ‡Department of Medicine, The Johns Hopkins University School of Medicine and the §Department of Molecular Biology and Genetics, The Johns Hopkins University, Baltimore, Maryland 21205, the ¶Dana-Farber Cancer Institute, the **Department of Medicine, Brigham and Women’s Hospital, the Department of Pediatrics, Children’s Hospital of Boston, and Harvard Medical School, Boston, Massachusetts 02115, and the ‡‡Department of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

A complex family of 4.1R isoforms has been identified in non-erythroid tissues. In this study we characterized the exonic composition of brain 4.1R-10-kDa or spectrin/actin binding (SAB) domain and identified the minimal sequences required to stimulate fodrin/F-actin association. Adult rat brain expresses predominantly 4.1R mRNAs that carry an extended SAB, consisting of the alternative exons 14/15/16 and part of the constitutive exon 17. Exon 16 along with sequences carried by exon 17 is necessary and sufficient to induce formation of fodrin-actin-4.1R ternary complexes. The ability of the respective SAB domains of 4.1R homologs to sediment fodrin/actin was also investigated. 4.1G-SAB stimulates association of fodrin/actin, although with an ∼2-fold reduced efficiency compared with 4.1R-10-kDa, whereas 4.1N and 4.1B do not. Sequencing of the corresponding domains revealed that 4.1G-SAB carries a cassette that shares significant homology with 4.1R exon 16, whereas the respective sequence is divergent in 4.1N and absent from brain 4.1B. An ∼150-kDa 4.1R and an ∼160-kDa 4.1G isoforms are present in PC12 lysates that occur in vivo in a supramolecular complex with fodrin and F-actin. Moreover, proteins 4.1R and 4.1G are distributed underneath the plasma membrane in PC12 cells. Collectively, these observations suggest that brain 4.1R and 4.1G may modulate the membrane mechanical properties of neuronal cells by promoting fodrin/actin association.

Erythroid protein 4.1R is an 80-kDa phosphoprotein that plays a pivotal role in the structural organization and maintenance of the red blood cell cytoskeleton (1). Specifically, it stabilizes the spectrin-actin complexes (2–5) and mediates the attachment of the underlying cytoskeleton to the overlying lipid bilayer through interactions with integral membrane proteins, such as band 3 (6) and glycophorin C (7, 8). Abnormalities in 4.1R are associated with congenital red cell defects leading to severe membrane fragmentation and hereditary elliptocytosis (9).

The major functions of erythroid 4.1R have been well described. In brief, the 30-kDa NH2-terminal domain interacts with transmembrane proteins including glycophorin C, calmodulin, p55, and band 3 (6–8), whereas the 10-kDa or SAB1 domain is required for the formation and stabilization of spectrin-actin complexes (10, 11). In the erythrocyte system, the SAB domain is encoded by the alternatively spliced exon 16 (63 nts/21 amino acids) and the majority of the constitutive exon 17 (135 nts/45 amino acids out of 177 nts) (12, 13). The spectrin/actin/4.1R interactions have been extensively studied in red blood cells, indicating that inclusion of exon 16 along with amino acid residues carried by exon 17 are critical for spectrin/actin/4.1R binary association, actin/4.1R direct interaction, and spectrin-actin-4.1R ternary complex formation (11).

During the past 10 years, it has been shown that the prototypical erythroid 4.1R is only one of the multiple isoforms that arise from the single 4.1R gene through extensive alternative pre-mRNA splicing (14–17). Consequently, discrete internal sequence motifs are preferentially included or omitted in different non-erythroid tissues and organs, giving rise to a complex family of 4.1R modular isoforms. Moreover, three novel 4.1-like genes were recently added to the already complex NF2/ERM/4.1 gene family, namely 4.1G, 4.1N, and 4.1B (18).

4.1G is widely expressed among different tissues and organs (19), whereas 4.1N and 4.1B appear to be confined in the peripheral and central neurons and the brain, respectively (19–24). A significant degree of homology within the 30-, 10-, and 22/24-kDa domains is shared by 4.1R and the three 4.1-like genes, whereas the NH2-terminus and intervening sequences appear diverse and highly specific. Specifically, a 73% identity is shared between 4.1R- and 4.1G-SAB domains (19), whereas a 36% similarity exists between the respective 4.1R and 4.1N domains (22). Furthermore, brain 4.1B exhibits an ∼50% homology within the COOH-terminal 45 residues of 4.1R-10-kDa carried by exon 17 (23, 24).

Spectrin is a major component of the erythrocyte cytoskeleton, where it plays an essential role in the maintenance of the mechanical stability and shape of the cell membrane (25). Erythroid spectrin consists of two subunits, encoded by α1 and

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§ Supported by a National Institutes of Health post-doctoral training grant. Current address: Dept. of Physiology, University of Maryland, Baltimore, MD 20541.

¶ Ts whom correspondence should be addressed: Dept. of Adult Oncology, Dana-Farber Cancer Inst., D1420B, 44 Binney St., Boston, MA 02115. Tel.: 617-632-6965; Fax: 617-632-2662; E-mail: Shu-Ching_Huang@dfci.harvard.edu.

1 The abbreviations used are: SAB, spectrin/actin binding; nts, nucleotides; GST, glutathione S-transferase; PCR, polymerase chain reaction; RT, reverse transcription; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; PBS, phosphate-buffered saline; Ab, antibodies; NGF, nerve growth factor.
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\(\beta\) genes, that are aligned side to side to form heterodimers, which in turn assemble by head to head association to form tetramers (\(\alpha(\beta)\)). In 1981, however, Goodman et al. (26) demonstrated the presence of spectrin-like proteins in several non-erythroid cells and tissues. Up until this day, five distinct genes encoding non-erythroid spectrin-like molecules have been described in mammals, including \(\alpha I\), \(\beta II\), \(\beta III\), \(\beta IV\), and \(\beta V\) (27–30). \(\alpha I\) and \(\beta II\) spectrins have an apparent molecular mass of \(~240\) and \(~235\) kDa, respectively, and are particularly abundant in brain giving rise to \(\alpha II(\beta)\) brain spectrin or fodrin tetramers (31–34).

Initial observations by Burns et al. (35) indicated that fodrin, F-actin, and erythroid-4.1R form a ternary complex, linking the fodrin tetramers into a hexagonal lattice. Furthermore, Tyler et al. (36) and Liu et al. (37) demonstrated that \(\beta\)-fodrin carries binding sites for protein 4.1R and actin at its NH\(2\) terminus (reviewed in Ref. 38).

In the present study we endeavored to decipher the minimal sequences within brain 4.1R-SAB domain required for fodrin-actin-4.1R ternary complex formation. Moreover, we assessed the ability of the respective domains of the 4.1-homologs, 4.1G, 4.1N, and 4.1B, to induce fodrin/actin association in vitro. Finally, we investigated the potential \(in vivo\) association of 4.1 proteins with fodrin tetramers and actin in the neuronal PC12 cell line. Our results suggest that brain 4.1R and 4.1G may be essential for the maintenance of the shape and membrane structural integrity of neural cells, through their involvement in the formation and/or stabilization of the fodrin-actin complexes. To the contrary, 4.1N and 4.1B appear to have distinct functional activities in neuronal tissues from stabilizing the cytoskeletal fodrin/actin network.

EXPERIMENTAL PROCEDURES

RNA Isolation and RT-PCR Analysis of Non-erythroid 4.1R-SAB Domain—Mouse and rat tissue samples were dissected after perfusion of the animals via the left ventricular ascending aorta with \(1\times\) PBS, immediately frozen in liquid \(N\)\(_2\), and ground to fine powder. Total RNA from several tissues and two cell lines (PC12 and MOLT4) was prepared using Trizol Reagent according to the manufacturer’s instructions (Life Technologies, Inc.). Subsequently, \(5\mu\)g of total RNA were subjected to reverse transcription-polymerase chain reaction (RT-PCR) following standard experimental protocols. The primers used in the PCR amplification reaction were chosen to flank the 4.1R-SAB domain and were as follows: primer a (within exon 13), 5’-1815-1847, 5’-AAGGATACCA- TCATGTCGTCATCGCCGAGCAGGATCGTGGGCCC-3’ (sense) and primer b (located in exon 17), 5’-AAGGATACCATCGATCTCTCCTGGGATGATCGCTCTCAGC-3’ (antisense); and primer c, 5’-ACCTGCTGACCACTGCCCACC-3’ (sense) and primer d, 5’-ACGTCGACTGAGGGCAGCC-3’ (antisense); and for generation of 4.1B-10-kDa, 5’-ACGTCGACTGAGGGCAGCC-3’ (sense) and 5’-ACCTGCTGACCACTGCCCACC-3’ (antisense).

To obtain the complete sequence of brain 4.1G-, 4.1N-, and 4.1B-10-kDa domains (Fig. 4A) the following primer sets that were flanking the respective 4.1B-SAB domains were used: 4.1G-sense 5’-GCTGAGTGGAGAGACCACGGGAGATGTAGAGAGCCT-3’ (antisense); for production of 4.1R-10-kDa, 5’-ACGTTCAGAGGAGGAGACTGTAACGCTTGCATGATGGTTGTCGAGTGA-3’ (antisense); and for generation of 4.1B-10-kDa, 5’-ACGTTCAGAGGAGGAGACTGTAACGCTTGCATGATGGTTGTCGAGTGA-3’ (antisense) and 5’-ACGTTCAGAGGAGGAGACTGTAACGCTTGCATGATGGTTGTCGAGTGA-3’ (antisense). Subsequently, the amplified fragments were subcloned into the bacterial expression vector pGEX-2T (Amersham Pharmacia Biotech) and verified by sequencing (T7 Sequenase version 2.0 kit, U. S. Biochemical Corp.). The recombinant polypeptides were overexpressed by isopropyl-1-thio-\(\beta\)-galactopyranoside induction and purified from bacterial lysate supernatant by affinity chromatographic use of protease inhibitors (39) was included in all peptide preparations. The GST fusion polypeptides were subjected to \(12\%\) SDS-PAGE and either visualized with Coomassie Blue staining or electrotransferred to polyvinylidene difluoride membrane for immunoblotting.

Purity of the protein was assayed by 8% SDS-PAGE. In all purification steps \(1\%\) KI were added in situ, were added for binding buffer to remove any unreacted label with the use of Dispo-quantitation kit (Amersham Pharmacia Biotech) for 2 min on ice. To stop the reaction, 40 \(\mu\)l of \(s\) pipette tip were added and added sequentially. Iodinated fodrin was extensively dialyzed against TKE/KCl buffer. The pooled fractions from the sucrose gradient were dialyzed either against storage buffer (1 mM Na\(_2\)HPO\(_4\), 3 mM NaN\(_3\), 0.1 mM DTT, pH 8.0) or binding buffer (130 mM Na\(_2\)SO\(_4\), 0.1 mM CaCl\(_2\), 0.1 mM DTT, pH 8.2), and sedimentation through a linear sucrose gradient (5–20%) in TKE/KCl buffer. The purity of the protein was assayed by \(8\%\) SDS-PAGE. In all purification steps 1 \(\mu\)M phenylmethylsulfonyl fluoride protease inhibitor was included.

Iodination of brain fodrin was essentially carried out as described previously (41). In brief, rat brains (~10 g) were dissolved in homogenization buffer (10 mM imidazole HCl, 5 mM EDTA, 5 mM Na\(_2\)HPO\(_4\), 1 mM EGTA, pH 7.3) after removal of the meninges and major blood vessels. Fodrin was extracted in a high salt buffer (10 mM Tris-HCl, 50 mM KCl, 3 mM Na\(_2\)HPO\(_4\), 0.1 mM MgCl\(_2\), 0.1 mM DTT, pH 8.2), (NH\(_4\))\(_2\)SO\(_4\)-precipitated, and gel-filtered through Sepharose 4B-CI, previously equilibrated with TKE/KCl (10 mM Tris-HCl, 700 mM KCl, 0.5 mM EDTA, 0.1 mM DTT, pH 8.2). Subsequently, the iodin-rich fractions, as assessed by \(8\%\) SDS-PAGE, were combined and followed by a second (NH\(_4\))\(_2\)SO\(_4\) precipitation, dialysis against a low salt buffer (10 mM Tris-HCl, 3 mM Na\(_2\)HPO\(_4\), 0.1 mM CaCl\(_2\), 0.1 mM DTT, pH 8.2), and sedimentation through a linear sucrose gradient (5–20%) in TKE/KCl buffer. The purity of the protein was assayed by \(8\%\) SDS-PAGE. In all purification steps 1 mM phenylmethylsulfonyl fluoride protease inhibitor was included.

4.1-mediated Fodrin/Actin Association

Commercially obtained muscle G-actin was polymerized at a concentration of 1 mg/ml, according to the manufacturer’s instructions (Cytoskeleton, Denver, CO). Purified brain fodrin, recombinant GST fusion polypeptides, and F-actin were subsequently dialyzed against several changes of binding buffer at 4 °C. Co-sedimentation assays were performed in 50 \(\mu\)l of binding buffer in the presence of 0.2 \(\mu\)M fodrin, 5.7 \(\mu\)M F-actin, and 1.4

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μM either of GST-4.1R, GST-4.1G, GST-4.1N, GST-4.1B, or GST-alone polypeptides. The reaction mixtures were incubated at room temperature for 45 min and then centrifuged at 4 °C for 1 h at 100,000 × g in a Beckman 42.2Ti rotor. Equivalent portions of supernatants and pellets were subsequently fractionated by 10% SDS-PAGE.

For quantitative binding studies 0.2 μM radioiodinated fodrin was allowed to interact with 5.7 μM F-actin in the presence of increasing amounts of either recombinant GST-4.1R-10-kDa or GST-4.1G-10-kDa polypeptides (0.14–1.4 μM). The pelleted protein complexes were subsequently counted in a counter, whereas correction for fodrin/F-actin sedimentation was made by subtracting the counts pelleted in the absence of GST-4.1R or GST-4.1G peptides. These experiments were conducted in duplicate and repeated twice, producing values with a range difference of ≤5%.

Cell Culture and Confocal Microscopy—Rat Pheochromocytoma cells (PC12) (21) were grown on poly-L-lysine-covered coverslips in RPMI supplemented with 5% fetal bovine serum, 10% horse serum, and 100 units of penicillin/streptomycin, at 37 °C with 5% CO2 atmosphere in a humidified incubator. Adhered cells were washed 3× with PBS and fixed in freshly prepared 4% paraformaldehyde in PBS for 20 min at room temperature. Subsequently, cells were rinsed twice with PBS and permeabilized with 0.5% Triton X-100 in PBS for 15 min at room temperature. Unreactive groups were blocked by incubation with 10% normal goat serum in PBS for 1 h at room temperature. In single labeling experiments the following primary antibodies were utilized: anti-GST-4.1R (1:100), anti-GST-4.1G (1:100), and anti-fodrin (1:600) plus 3 μg/ml bovine serum albumin incubated at 37 °C for 2 h. Following extensive washes in PBS, cells were counterstained either with goat anti-rabbit fluorescein isothiocyanate or goat anti-rabbit Texas Red IgGs (1:50, Pierce) at 37 °C for 1 h and extensively washed with PBS. In double labeling experiments, after the first set of staining, PC12 cells were blocked for 20 min at 4 °C with 10 μg/ml unjugated goat anti-rabbit Fab fragment (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in PBS and then processed for the second set of immunolabeling. Subsequently, coverslips were mounted with Vectashield (Vector Laboratories, Burlingame, CA) and analyzed either under a Nikon microphot FXA microscope (Garden City, NY) or a laser scanning confocal system (Noran Instruments, Middleton, WI) combines a Zeiss Axioskop microscope (Thornwood, NY) through a 100× oil immersion objective.

Immunoprecipitation and Immunoblotting—Immunoprecipitation assays were performed using PC12 cell extracts as described previously (44) with some modifications. About 4 × 106 cells were collected by scraping with a rubber policeman, washed 3× in ice-cold PBS, resuspended in lysis buffer (50 mM Tris-HCl, 125 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 0.2 mM EDTA, 5 mM iodoacetamide, pH 7.5) plus protease inhibitors (39), and given 20 strokes in a scraping with a rubber policeman, washed 3× with 0.1% SDS, 0.5% sodium deoxycholate, 0.2 mM EDTA, 5 mM iodoacetamide. The lysates were centrifuged at 14,000 × g for 20 min at 4 °C, and the supernatant was collected. Protein content was determined using a standard Bradford assay (Pierce), and 750 μg of total lysate were precleared with 150 μl of a 50% suspension of protein A-Sepharose-6MB beads (Amersham Pharmacia Biotech) on a 4 °C rotor for 2 h. Pre-immune serum, anti-Exon1R, and anti-fodrin antibodies, containing 5 μg of IgGs, were allowed to interact with 150 μg of protein A-Sepharose-6MB in the presence of 1× PBS at 4 °C with gentle rocking for 4 h. Subsequently, the antibodies bound to the beads were incubated with the precleared PC12 extracts in a rocking platform at 4 °C for 6–8 h. Following the end of the incubation period, the samples were centrifuged for 15–20 s at 14,000 × g for 4 °C. The supernatants were collected and stored at −20 °C, and the beads were extensively washed with several changes of lysis buffer by rocking at 4 °C. At the end of the washings the proteins were solubilized in 100 μl of 4× Laemmli sample buffer and boiled for 5 min. The immunoprecipitates were analyzed by 8% SDS-PAGE and processed for immunoblotting with the indicated antibodies (see Results) using an ECL detection kit (Amersham Pharmacia Biotech).

RESULTS

Alternative Splicing Events within the 4.1R-SAB Domain of Non-erythroid Tissues—To characterize the splicing patterns within the non-erythroid 4.1R-SAB domain, total RNA from several tissues of rat or mouse origin was subjected to RT-PCR analysis followed by sequencing. Three alternatively spliced cassettes encoded by exons 14 (57 nts), 15 (42 nts), and 16 (63 nts) along with the majority of the constitutive exon 17 (135 out of 177 nts) compose the non-erythroid 4.1R-10-kDa. These exhibited complex splicing patterns resulting in the generation of multiple combinatorial products that were expressed in variable amounts among different non-erythroid tissues and cell lines (Fig. 1A). Interestingly, adult rat brain and undifferentiated PC12 cells contained a substantial amount of a bigger size PCR product that included all three alternatively spliced exons 14–16, although additional PCR bands were also detected that corresponded to different exonic combinations. Sequence comparison of exons 14–16 from several species, including human, mouse, rat, and bovine, revealed extensive evolutionary conservation of these motifs with only few nucleotide changes (Fig. 1B).

Sequences within Exons 16 and 17 Are Necessary and Sufficient to Promote Fodrin-Actin-4.1R Ternary Complex Formation—To identify the exons within the 4.1R-SAB required for stimulation of fodrin-actin-4.1R ternary complex formation, we generated various GST-4.1R-10-kDa recombinant peptides that carried distinct combinations of the alternative spliced cassettes 14–16 (Fig. 1A). The authenticity of the resultant fusion polypeptides was verified by immunoblot assays with the use of appropriate anti-4.1R antibodies (Fig. 2B).

Subsequently, the ability of recombinant GST-4.1R-10-kDa variants to promote fodrin/actin association was tested in a series of co-sedimentation assays (Fig. 2C). In all cases in which 4.1R-10-kDa peptides carried the alternatively spliced exon 16 along with the constitutive 45 residues carried by exon 17, a significant, almost complete, shift of fodrin from the supernatant to the pellet fraction was observed either in the presence or absence of exons 14 and 15. On the contrary, when the 63-nt cassette encoded by exon 16 was omitted, almost equivalent amounts of fodrin were detected in both supernatant and pellet fractions. These results were similar to control experiments in which the 4.1R-10-kDa was omitted. Thus, the 4.1R-SAB domain stimulates co-sedimentation of fodrin and F-actin in an exon 16-dependent manner, regardless of the presence of the nucleotide motifs encoded by the alternatively spliced cassettes 14 and 15. It is of interest to note that the combination of exons 14 and 15 exhibits stronger activity in stimulating the sedimentation of fodrin and actin even though the individual exon appears to suppress the binding when present alone. A graphic presentation of the percentage of pelleted fodrin following stimulation with the various 4.1R-10-kDa peptides is shown in Fig. 2D after quantitation of the respective bands using NIH Image Software.

To determine the minimal sequences within exon 17 necessary to promote fodrin-actin-4.1R ternary complex formation, we generated GST fusion peptides containing all three alternative exons 14/15/16 and varying lengths of the constitutive 45 residues that belong to exon 17. Specifically, three constructs were produced that either included the first 36, 26, or 10 residues that belong to exon 17. i.e. 4.1R-10kd-36 peptide) to induce fodrin/actin association (Fig. 1B). The authenticity of the resultant fusion polypeptides was verified by immunoblot assays with the use of appropriate anti-4.1R antibodies (Fig. 2B).

Subsequent co-sedimentation assays demonstrated that recombinant polypeptides carrying any COOH-terminal deletions either completely failed (i.e. 10kd-26 and 10kd-16 constructs) or showed very limited activity (i.e. 10kd-36 peptide) to induce fodrin/actin association (Fig. 3B). These observations indicated that the last COOH-terminal residues of 4.1R-SAB, within exon 17, are also critical in the formation of fodrin-actin-4.1R ternary complexes. Moreover, similar results were obtained when 4.1R GST fusion peptides carrying exon 16, but skipping exons 14 and 15, along with various COOH-terminal deletions of exon 17 (e.g. 10kd/Ex16, 10kd/Ex16-36 and 10kd/Ex16-26) were examined in a co-sedimentation assay (Fig. 3C).

Furthermore, a case of heterozygous hereditary elliptocytosis associated with partial deficiency of 4.1R has been reported in nine related French families (45). Molecular genetic analysis
revealed a single codon deletion in the 4.1R locus resulting in elimination of a lysine residue located either at position 447 or 448 within the 4.1R-10-kDa domain. The abnormal 4.1R protein that is produced fails to interact with spectrin-actin complexes within the erythrocyte. Consequently, we generated by a two-step PCR approach GST-4.1R-SAB polypeptides that were missing either of these lysine residues (Fig. 3A, 10kd-Lys), and we investigated their ability to induce fodrin/actin co-sedimentation. As shown in Fig. 3, B and C, the sole deletion of lysine 447 (10kd-Lys and 10kd/Ex16-Lys) abolishes ternary complex formation, indicating that amino acids proven to be important in stimulating formation of erythroid-4.1R-spectrin-actin complexes are also critical in promoting brain-4.1R/fodrin/actin association as well. Identical results were obtained when lysine 448 was omitted (data not shown).

Taken altogether, the results obtained in Figs. 2 and 3 indicate that sequences carried by both exons 16 and 17 are necessary and sufficient to stimulate fodrin/actin co-sedimentation.

Brain 4.1G- but Neither 4.1N- Nor 4.1B-SAB Domains May Induce Fodrin-Actin Complex Formation—To investigate the functional ability of the 4.1-homologs to polymerize fodrin and actin into pelletable polymers, GST fusion polypeptides of brain 4.1G-, 4.1N-, and 4.1B-SAB domains were generated (Fig. 4A) and tested in a co-sedimentation assay (Fig. 4B). Notably, the 4.1G-SAB paralog was able to stimulate sedimentation of fodrin-actin complexes, whereas 4.1N and 4.1B did not. Sequence comparison between 4.1R- and 4.1G-, 4.1N-, and 4.1B minimal SAB domains demonstrated that 4.1G-10-kDa carries a nucleotide cassette that shares significant homology with 4.1R-exon 16 (76%), whereas the respective sequence is highly divergent in 4.1N-10-kDa and absent from brain 4.1B-10-kDa (Fig. 5A). Furthermore, the lysine residue located at position 447, shown to be essential in the ability of erythroid and brain 4.1R-10-kDa to induce fodrin/actin association (Fig. 4B), is not conserved in the 4.1 homologs but is replaced either by leucine in 4.1G and 4.1N or methionine in 4.1B (Fig. 5A, arrow).

To compare the efficiency of recombinant 4.1R- and 4.1G-10-kDa domains to promote fodrin/actin association in vitro, we performed a series of quantitative co-sedimentation assays using increasing concentrations of either 4.1R- or 4.1G-SAB polypeptides (0.14–1.4 μM) and constant amounts of radioiodinated fodrin (1.4 μM) and filamentous actin (5.7 μM). As shown in Fig. 5B both 4.1R- and 4.1G-10-kDa polypeptides induced fodrin/actin sedimentation in a concentration-dependent manner. However, the prototypical 4.1R-SAB domain exhibited a saturating concentration of 0.28 μM, whereas 4.1G-10-kDa showed a respective value of 0.56 μM. This finding indicates that 4.1R may stimulate fodrin/actin association with a 2-fold higher efficiency compared with 4.1G at least in vitro.

Proteins 4.1R and 4.1G Are Present at Points of Cell-Cell Contact and Occur in Vivo in a Supramolecular Complex with Fodrin Tetramers in PC12 Cells—All the aforementioned studies examined the ability of 4.1R- and 4.1G-SAB domains to promote fodrin/actin complex formation in vitro. We also attempted a series of in vivo experiments in order to evaluate the significance of the results we obtained within the context of the
cell. Consequently, we analyzed the subcellular distribution of 4.1R and 4.1G by confocal microscopy (Figs. 6 and 7, respectively) as well as their potential in vivo association with fodrin polymers by co-immunoprecipitation assays (Fig. 8) in the PC12 neuronal cell line.

As shown in Fig. 6, two different anti-4.1R antibodies raised against either the alternatively spliced exon 16 (Fig. 6, A and E) or the 4.1R-16-kDa domain (Fig. 6B) revealed the presence of 4.1R at the periphery of undifferentiated PC12 cells and especially concentrated at sites of cell-cell contact. When any of...
the primary antibodies was omitted, no specific signal was detected (Fig. 6C). When an anti-fodrin antibody was utilized, which can identify both α and β isoforms (40), a rather uniform staining at the periphery of the cells was detected (Fig. 6, D and F). Superimposed images of 4.1R (Fig. 6E) and fodrin (Fig. 6F) revealed complete overlapping at areas of cell-cell contact (Fig. 6G, areas of co-staining appear yellow). This finding demonstrated that 4.1R and αβ-fodrin tetramers co-distribute underneath the plasma membrane in PC12 cells. Moreover, when PC12 cells were stained for the presence of 4.1G protein, 4.1G appeared to be highly concentrated underneath the plasma membrane and specifically at cell boundaries, as protein 4.1R and fodrin are (Fig. 7A, arrows). However, significant accumulations of 4.1G were also observed within the cytoplasm of PC12 cells, assuming a rather punctate distribution pattern (Fig. 7A, arrowheads), whereas any specific staining was eliminated when the primary antibody was omitted (Fig. 7B).

In order to discern whether native 4.1R can associate in vivo with fodrin and actin, we did a series of co-immunoprecipitation assays, utilizing PC12 cell extracts and anti-4.1R-Ex16, anti-fodrin, or control pre-immune IgGs (Fig. 8). Three identical blots were examined for the presence of protein 4.1R, αβ-fodrin, and actin. An ~150-kDa 4.1R isoform was readily detected in both anti-Exon16 and anti-fodrin immunoprecipitates (Fig. 8A). When the same fractions were analyzed for the presence of fodrin, two closely migrated polypeptides of ~240 and ~235 kDa, which correspond to α- and β-fodrin isoforms, respectively, were detected (Fig. 8B). Moreover, when a replica blot was examined for the presence of β-actin, an ~43-kDa immunoreactive polypeptide was identified (Fig. 8C). In control experiments where pre-immune serum was used in the place of primary antibodies, no immunoreactive band was precipitated (Fig. 8, A–C). Collectively, all these findings suggest that protein 4.1R occurs in vivo in a supramolecular complex with fodrin tetramers and actin filaments.

The ability of the 4.1G-SAB domain to induce fodrin-actin complex formation in vitro (Fig. 4B), even with a 2-fold reduced efficiency compared with 4.1R-10-kDa (Fig. 5B), prompted us to...
investigate the presence of 4.1G in the anti-fodrin immunoprecipitates (Fig. 8D). Indeed an ~160-kDa immunoreactive band was detected that corresponds to 4.1G, indicating that 4.1G may also interact in vivo with fodrin tetramers. However, when the same immunoprecipitates were tested for the presence of 4.1N and 4.1B, the respective proteins were absent (Fig. 8, E and F, respectively). The presence of 4.1G and the absence of 4.1N and 4.1B proteins from the fodrin immunoprecipitates are consistent with the results obtained from our in vitro co-sedimentation studies (Fig. 4B).

**DISCUSSION**

In the current study we identified the structural requirements of brain 4.1R-SAB domain for ternary complex formation with αβ-fodrin (brain spectrin) and F-actin in a series of in vitro binding assays. We also explored the functional ability of the 4.1 homologs G-, N-, and B-SAB domains to promote fodrin/actin co-sedimentation in vitro. Finally, we examined the intracellular distribution of 4.1R, 4.1G, and fodrin in the neuronal PC12 cell line, and we analyzed the potential in vivo association of 4.1R and 4.1G proteins with fodrin by a series of co-immunoprecipitation assays.

Characterization of the 4.1R-SAB domain among various non-erythroid tissues demonstrated the presence of multiple combinatorial products. In the case of adult rat brain and undifferentiated PC12 cells, a major mRNA isoform that included the alternatively spliced cassettes 14–16 was identified. Elucidation of the binding behavior of a nested series of truncated 4.1R-10-kDa peptides revealed that the alternatively spliced exon 16 along with sequences carried by the constitutive exon 17 (i.e. the last 10 COOH-terminal amino acids and lysine 447/448) are necessary and sufficient to promote fodrin/actin complex formation. These observations suggest that the minimal sequences of brain 4.1R-SAB required for fodrin/actin association are similar to those of erythroid 4.1R-10-kDa (10, 11) and argue for functional conservation of the respective domain in diverse tissues. However, when a potential in vivo association of 4.1R with skeletal muscle spectrin was exam-
ined, no such interaction was detected, even though the predominant 4.1R-SAB isotype in striated muscle consists of exons 16 and 17 (44). To the contrary, the skeletal muscle 4.1R-SAB domain directly interacts *in vitro* with the major sarcomeric proteins as follows: myosin heavy chain, α-actin, and the actin-associated protein tropomyosin (44). Thus, it is conceivable that among different tissues 4.1R-10-kDa may have evolved diverse functional activities, either behaving as a general linkage molecule that bridges the underlying cytoskeleton to the cell membrane (e.g. erythroid cells and brain) or assuming new roles through interactions with major tissue-specific proteins (e.g. skeletal muscle). A slightly enhanced fodrin/actin binding activity was observed when both exons 14 and 15 were present even though the individual exon appeared to suppress the binding. The biological significance of exons 14 and 15 in fodrin/actin interaction remains to be determined.

Prior studies (46, 47) as well as our findings indicated that fodrin tetramers are distributed mainly underneath the plasma membrane of undifferentiated PC12 cells. However, during NGF-induced differentiation fodrin is recruited to perinuclear, spot-like aggregates associated with intermediate filament proteins, including peripherin and neurofilament (46). On the other hand, during PC12 neuronal differentiation, a dramatic down-regulation of 4.1R-SAB mRNA species that include the alternatively spliced exons 14–16 was detected followed by a concomitant increase of a cassette that contains only the first 45 amino acid residues of the constitutive exon 17 (Fig. 1A, lane PC12+NGF). A possible scenario to explain this differential exonic composition of 4.1R-10-kDa between undifferentiated and differentiated PC12 cells is that in the former exon 16 in conjunction with sequences carried by exon 17 may serve to facilitate and strengthen the formation of fodrin-actin complexes and thereby rigidify the underlying cortical cytoskeleton. Down-regulation of the expression levels of the minimal fodrin/actin 4.1R-binding domain may permit the redistribution of fodrin and the dynamic reorganization of the cytoskeletal proteins involved in the morphological differentiation of neurons. This hypothesis is in agreement with unpublished observations from our laboratory illustrating that upon administration of NGF to PC12 cells, protein 4.1R departs from the cell membrane and massively translocates to the nucleus of the differentiated cells, whereas perinuclear accumulations are also observed that occasionally coincide with perinuclear fodrin aggregates.

Protein 4.1R shows a very diverse distribution pattern among different cell types and different phase of the cell cycle. We have demonstrated that 4.1R is present in the nucleus of interphase and the spindle and spindle poles of mitotic HeLa cells (39). Moreover, 4.1R is present both in the nuclear and cytoplasmic compartments of non-confluent Madin-Darby canine kidney cells, and it is concentrated in the tight junctions of confluent Madin-Darby canine kidney cells (48). Finally, 4.1R is present both in the cell periphery and in the cytoplasm exhibiting a rather diffuse staining in PC12 cultures of low density. However, as cells divide and contact each other, 4.1R accumulates underneath the plasma membrane and colocalizes with fodrin, at the cell boundaries. Thus, it becomes obvious that protein 4.1R shuttles extensively between the nucleus, the cytoplasm, and the cell membrane depending on the cell type and stage of cell cycle. The trigger or the mechanisms that allow protein 4.1R to shuttle between the different cellular compartments have not been identified yet and are now under investigation in our laboratory. It is reasonable to presume that the diverse distribution patterns that 4.1R assumes reflect a great diversity in the functional activities of this protein depending on the developmental stage and the tissue and/or organs that are expressed.

The recent identification of three novel members of the 4.1 gene superfamily (18) prompted us to explore the functional ability of their respective SAB domain paralogs to promote fodrin/actin association. Our findings indicated that only 4.1G could polymerize fodrin/actin into pelletable polymers, whereas neither 4.1N nor 4.1B were able to do so. These results are corroborated by the fact that 4.1G-SAB domain carries a nucleotide cassette that shares a significant degree of homology (76%) with 4.1R-exon 16 (19), whereas the respective sequence has been highly diversified in 4.1N-10-kDa (22) and omitted from 4.1B-10-kDa (23, 24). It is noteworthy to mention that structural characterization of the 4.1B-SAB domain in various adult mouse tissues has revealed the presence of a cassette that shares 50% homology with 4.1R-exon 16 in skeletal and cardiac muscle (23), which is largely skipped in the brain ortholog (Ref. 23 and this work). Furthermore, the lysine residue, located at position 447 within exon 17, shown to play an essential role in the ability of 4.1R-SAB to stimulate fodrin/actin co-sedimentation, is replaced either by a leucine in the cases of 4.1G and 4.1N or a methionine in the case of 4.1B. Interestingly, the lysine residue located at position 448 is retained in all 4.1 homologs, implying that the positively charged interface generated by the presence of both lysine residues is critical for fodrin-actin-4.1R ternary complex formation.

When the ability of 4.1R- and 4.1G-SAB domains to promote fodrin-actin complex formation was evaluated, the prototypical 4.1R-10-kDa demonstrated a 2-fold higher efficiency to stimulate fodrin/actin association, at least *in vitro*. However, both native 4.1R and 4.1G proteins appear to occur *in vivo* in a supramolecular complex with α-fodrin tetramers in a series of co-immunoprecipitation experiments. Whether 4.1R or 4.1G promotes formation of fodrin-actin complexes more efficiently in the physiological environment of PC12 cells is still a matter of speculation. Nevertheless, the relative amounts of the respective proteins available within the cell may dramatically affect the favorable recruitment of 4.1R or 4.1G in the formation of ternary complexes. The fact that 4.1N and 4.1B do not stimulate fodrin/actin association, even though they are present at the boundaries of undifferentiated PC12 cells (21, 23), is indicative of non-redundancy among the multiple members of the 4.1 gene superfamily. Conceivably, these proteins may be involved in the segregation and modulation of other protein complexes associated with the plasma membrane, in the structural organization of cell-cell contact regions to specialized domains, or in major signal transduction pathways that control cell growth and differentiation (49).

The presence and subcellular distribution of the diverse 4.1 members have been extensively studied in brain by several laboratories. 4.1N is essentially expressed in almost all central and peripheral neurons with the notable exception of the Purkinje cells of the cerebellum and the thalamic neurons (21, 22, 50). On the other hand, 4.1B is mostly enriched in Purkinje cells and thalamic neurons (24). Protein 4.1R also occurs in the brain where it is distributed in specific neuronal populations, including the granule cells of the cerebellum and the dentate gyrus (51). Finally, 4.1G is mainly localized to glia (52). These observations strongly suggest complementary intracellular localization patterns among the 4.1-protein members, although coincident distributions are not precluded. Therefore, distinct 4.1-proteins may possess different binding partners in specific types of neurons, including components of the neuronal membrane cytoskeleton, integral membrane channels, and trans-

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2 A. Kontogianni-Kostantopoulou, E. J. Benz, Jr., and S.-C. Huang, unpublished observations.
membrane receptors. In the case of 4.1R and 4.1G, an in vivo association with αβ-fodrin, a major component of the neuronal cytoskeleton, was established. Consequently, the challenge of unraveling the association with confocal microscopy (The Johns Hopkins University School of Medicine).