Molecular and Functional Characterization of Protein 4.1B, a Novel Member of the Protein 4.1 Family with High Level, Focal Expression in Brain*

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Brain-enriched isoforms of skeletal proteins in the spectrin and ankyrin gene families have been described. Here we characterize protein 4.1B, a novel homolog of erythrocyte protein 4.1R that is encoded by a distinct gene. In situ hybridization revealed high level, focal expression of 4.1B mRNA in select neuronal populations within the mouse brain, including Purkinje cells of the cerebellum, pyramidal cells in hippocampal regions CA1-3, thalamic nuclei, and olfactory bulb. Expression was also detected in adrenal gland, kidney, testis, and heart. 4.1B protein exhibits high homology to the membrane binding, spectrin-actin binding, and C-terminal domains of 4.1R, including motifs for interaction with NuMA and FKBP13. cDNA characterization and Western blot analysis revealed multiple spliceoforms of protein 4.1B, with functionally relevant heterogeneity in the spectrin-actin and NuMA binding domains. Regulated alternative splicing events led to expression of unique 4.1B isoforms in brain and muscle; only the latter possessed a functional spectrin-actin binding domain. By immunofluorescence, 4.1B was localized specifically at the plasma membrane in regions of cell-cell contact. Together these results indicate that 4.1B transcription is selectively regulated among neuronal populations and that alternative splicing regulates expression of 4.1B isoforms possessing critical functional domains typical of other protein 4.1 family members.

The protein 4.1 family comprises a group of skeletal proteins structurally related to the erythrocyte membrane skeletal protein, 4.1R, that plays a critical role in determining the morphology and mechanical stability of the red cell plasma membrane. These proteins are characterized by the presence of three main conserved structural/functional domains. A 20-kDa N-terminal membrane binding domain (MBD); also called the FERM domain (1), possesses binding sites for the cytoplasmic tails of integral membrane proteins such as band 3 (2, 3), glycophorin C (4, 5), CD44 (6), and Drosophila neurexin (7). This domain also binds to p55 (5) and calmodulin (8), the latter interaction being important for regulating the affinity of 4.1R-band 3 and 4.1R-CD44 interactions (6, 9). An internal 8–10-kDa domain contains the critical spectrin-actin binding activity required for membrane stability (10–13), and the C-terminal 22–24-kDa domain has recently been reported to bind the immunophilin FKBP13 (14) and NuMA (15). The prototypical protein 4.1R has been characterized most extensively in the erythrocyte, where it plays a critical role in maintaining the erythrocyte's morphology and mechanical integrity. Consequently, deficiency of protein 4.1R yields an elliptocytic morphology and decreased membrane strength, leading to cellular fragmentation and hemolytic anemia (reviewed in Ref. 16).

The complement of 4.1 proteins expressed in brain and other nonerythroid cells is structurally more complex and functionally less well understood than in erythroid cells. Western blot analysis suggests that multiple protein 4.1 isoforms are expressed in most nucleated cells (17, 18). This molecular heterogeneity is probably due not only to post-translational modifications but also to complex alternative pre-mRNA splicing pathways that insert or delete discrete peptides within the polypeptide backbone (19, 20). Several of the alternative splicing events are mediated in tissue-specific fashion, resulting in expression of unique protein 4.1R isoforms in muscle (21, 22), brain (20), and epithelial cells (22). Additionally, programmed changes in alternative splicing alter the repertoire of 4.1R isoforms synthesized during erythroid differentiation (23–25). Moreover, transcriptional regulation of three recently discovered 4.1 genes contributes further heterogeneity to the complement of 4.1 isoforms expressed in a given tissue. Among these are protein 4.1G, a widely expressed homolog (14, 26); protein 4.1N, a neuronal homolog (27); and protein 4.1B, the novel brain-enriched homolog reported here.

The brain is a particularly rich source of protein 4.1 isoforms. By in situ hybridization analysis, 4.1R, 4.1G, 4.1N, and 4.1B mRNAs are all expressed in distinct patterns within the brain. Several studies indicate that 4.1 protein(s) are likely to play important functional roles in the brain. For example, 4.1R has been localized to specific neuronal populations including granulocytes, spectrin-actin binding; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; PBS, phosphate-buffered saline; BSA, bovine serum albumin; kb, kilobase(s); Epb4.1l3, erythrocyte protein band 4.1-like gene 3 (designated as protein 4.1B in this paper).
uclei of the cerebellum and dentate gyrus (28). Consistent with this localization, behavioral studies of protein 4.1R-deficient knock-out mice have demonstrated neurological defects in fine motor coordination and spatial learning (28). A second important 4.1 protein in brain is 4.1N, which is found in most neurons of the brain, with expression detected at the earliest stage of postmitotic differentiation (27). Immunofluorescence experiments show that 4.1N protein is enriched at regions of synaptic contact between neurons, where it could potentially play an important role in synaptic architecture and function (27). In addition to these recent reports, earlier biochemical studies indicated that at least one brain isoform of 4.1 possesses spectrin-actin binding activity, strongly suggesting that one function of brain 4.1 is analogous to its role in the red cell (29). However, the relationship of that biochemical activity to the newly described 4.1 isoforms is unknown.

Functional studies of 4.1 proteins in other nonerythroid, nonneuronal cells indicate that selected 4.1 isoforms are imported into the nucleus, where they may play a general structural role in nuclear architecture (30–33) and/or may interact with splicing factors (34). 4.1 proteins have also been reported to interact with the immunophilin FKBP13 (14), with CD44 (6), with the Drosophila protein neurexin (7), and with pCln, a protein potentially involved in volume regulation through association with chloride channel protein(s) (35). This diversity of interactions suggests that 4.1 proteins play multiple functional roles in addition to their well known function in stabilizing the red cell membrane.

Several classes of neurons in the brain, including Purkinje cells in the cerebellum and the majority of thalamic nuclei, appear to exhibit little or no expression of the previously described 4.1 protein family members (27, 28). In this paper, we present a detailed characterization of a fourth member of the protein 4.1 family, 4.1B. This protein is expressed in Purkinje cells and thalamic nuclei as part of a region-specific expression profile that is distinct from that of the other 4.1 genes in the brain. Conserved structural features of 4.1B are presented as well as evidence that alternative splicing events govern expression of tissue-specific 4.1B isoforms with different functional properties. These studies suggest that 4.1B protein(s) play a critical structural role in a subset of neurons in the brain.

MATERIALS AND METHODS

Isolation of 4.1B cDNA—The dbEST data base (National Center for Biotechnology Information, or NCBI) was screened for novel clones with homology to 4.1R, 4.1G, and 4.1N, resulting in identification of clone L26705. This cDNA spanned the C-terminal coding sequences plus the 3'-untranslated region of a novel mouse cDNA designated as protein 4.1B. Primers designed from this sequence were used to amplify mouse 4.1B sequences (ultimately corresponding to nucleotides 2973–4019 of mouse 4.1B cDNA, accession number AF152247) for use as a hybridization probe. Two long cDNAs of 3.9 and 3.5 kb, isolated from a mouse brain cDNA library (Stratagene, La Jolla, CA), allowed us to deduce the full-length mouse brain 4.1B primary structure. The translation initiation site was assigned based on the following criteria: it is the first AUG in the cDNA, it is situated in a favorable Kozak consensus sequence, and it encodes an N-terminal peptide (MTTE) that is identical to the N-terminal sequence of 4.1G, 4.1N, and high molecular weight isoforms of 4.1R. The mouse 4.1B gene designation is Efpb,113 (36).

RNA Blot Analysis—4.1B mRNA size was determined by hybridization to Multiple Tissue Northern blots (CLONTECH, Palo Alto, CA). The probe used for Northern blot analysis of 4.1B mRNA was derived from the 3'-untranslated region of 4.1B, not overlapping the coding region. This probe was gene-specific for 4.1B, i.e. it did not cross-hybridize with other 4.1 mRNA. Controls were performed using the ubiquitin probe supplied by the manufacturer.

In Situ Hybridization—Experiments were carried out using digoxigenin-labeled probes corresponding to the 3'-untranslated region of mouse 4.1B cDNA (accession number AF152247; nucleotides 2973–4019). Fresh frozen 20 μm cryostat sections of whole mount embryos were laid onto Superfrost Plus slides (Fisher) and processed as described (14). Control sections were hybridized with identical quantities of sense cRNA, and no signal was observed. The in situ hybridization protocol has been used to discriminate the localizations of transcripts sharing as much as 85–90% nucleic acid identity (37).

RT-PCR Experiments—Total RNA from mouse tissues was prepared and transcribed into cDNA using specific antisense primers as described (19). Two μl of cDNA was amplified in a 25-μl PCR containing Taq polymerase buffer, 50 pmol each of sense and antisense primers, 0.2 μM dNTPs, and 0.625 units of Taq polymerase. Thirty-five cycles of amplification were performed using an automated Perkin-Elmer Cetus 9700 thermal cycler under the following conditions: denaturation for 30 s at 94 °C, annealing for 30 s at 60 °C, and extension for 1 min at 72 °C. DNA fragments were analyzed by 5% polyacrylamide gel electrophoresis. The identity of PCR products was confirmed by DNA sequence analysis. For PCR of 4.1B SAB domain sequences, the following primers were used to amplify mouse 4.1B sequences: sense strand, 5′-GAAGAGAAGGCAAGAAAGAAAGGCT-3′; antisense strand, 5′-CCACCGTGTATCGACACCT-3′; antisense strand, 5′-CAGGAGCAGATAAGGTCTAGGGT-3′. For PCR of mouse 4.1B C-terminal domain sequences, the following primers were used: sense strand 5′-GCCCGACAGATACAGTCTGAAACCACT-3′; antisense strand, 5′-CAGGAGCAGATAGAAGTGACTTTG-3′.

Antibodies—The synthetic peptide N-TRKGISQTLNLLTTVPEKK-C was used as an immunogen to raise goat anti-4.1B antibodies. This peptide represents a portion of the unique U2 domain that is conserved between human and mouse 4.1B but not mouse 4.1R homology with 4.1R, 4.1G, or 4.1N. Before use on Western blots, the antibody was affinity-purified using the immunizing peptide coupled to a Sulfolink column (Pierce). Preimmune IgG affinity purified from the same goat was used as control blots. Brain extracts were also probed with rabbit polyclonal antibodies to the unique U1 domains of 4.1L (14) and 4.1N (27). In some experiments, antibodies were preabsorbed by incubation with 2 μg of the appropriate 4.1 recombinant proteins expressed in the pET22b vector (Novagen, Madison, WI) before blotting.

Mouse Tissue Preparation—A mouse was perfused free of blood via left ventricular/ascending aortic perfusion with PBS containing 0.5 mM di-isopropl fluorophosphate. The brain was immediately harvested and homogenized in ice-cold lysis buffer (100 mM NaCl, 50 mM Trisma, pH 7.4, 2% SDS, 1% Triton X-100, 1 mM EDTA, 2 mM Pefabloc, 5 mM benzamidine, 2 μg/ml pepstatin A, 5 μg/ml leupeptin, 5 μg/ml aprotinin). Tissue was left on ice for 30 min with intermittent vortexing. The tissue lysate was denatured by boiling with an equal volume of 2X SDS denaturing buffer containing 100 mM dithiothreitol. An aliquot of denatured lysate was assayed for protein concentration using DC reagent (Bio-Rad).

Western Blot Analysis—Detection of endogenous mouse brain 4.1B proteins was performed using fresh brain extracts isolated as described above. 50 μg of protein was loaded on one in a 7.5% polyacrylamide gel. For control experiments to detect the epitope-tagged 4.1B isoform expressed in transfected COS-7 cells, 1.5 × 105 cells were grown in two 150-mm culture dishes for 24 h and transfected for 8 h using 30 μl of Liposome-Plus (Life Technologies, Gaithersburg, MD). Approximately 5 μg of DNA/dish. 48 h after transfection, cells were washed in PBS, scraped off the plates in 750 μl of ice cold radioimmunoprecipitation buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% IGEFA CA-630 (Sigma), 0.1% SDS, 2 mM Pefabloc (Roche Molecular Biochemicals), 5 mM benzamidine, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 2 μg/ml pepstatin A), and lysed for 30 min on ice with intermittent vortexing. The supernatant was cleared of particulate materials by centrifugation. 100 μl of protein A-agarose beads, preincubated overnight with 10 μg of anti-hemagglutinin epitope tag antibody, was added and allowed to bind for 6 h at 4°C. Beads were spun down and washed in radioimmunoprecipitation buffer, and associated proteins were denatured by boiling in SDS-PAGE sample buffer and resolved by polyacrylamide gel electrophoresis.

Proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA) using a semidyde electroblotter (Integrated Separation Systems Inc., Natick, MA). Nonspecific binding sites on the membranes were blocked by incubation for 1 h at room temperature in TBS (10 mM Tris-HCl, pH 7.4, 150 mM NaCl) containing 0.5% Tween 20, 4% nonfat dry milk, 1% BSA, and 0.02% sodium azide. The blocking buffer also included either 2% donkey serum (4.1G, 4.1N) or 2% rabbit serum (4.1B) to minimize non-specific binding of secondary antibodies. Blots were then probed overnight at 4°C with either anti-4.1B antibody at 0.3 μg/ml, anti-4.1G antibody at 0.025 μg/ml, or anti-4.1N antibody at 0.025 μg/ml (all dilutions in TBS buffer). Immunoreactive bands were visualized after incubation with secondary antibodies coupled to horseradish peroxidase, using either a 1:3000 dilution of donkey anti-rabbit IgG (Amersham Pharmacia Biotech) for 4.1N

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and 4.1G, or a 1:80,000 dilution of rabbit anti-goat IgG (Sigma) for 4.1B, together with the Renaissance chemiluminescence detection kit (NEN Life Science Products).

Immunofluorescence of Endogenous 4.1B in Cultured PC12 Cells—Rat PC12 cells were grown for 6 days on two-well Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL) in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated horse serum, 10% heat-inactivated fetal calf serum, and 1% penicillin/streptomycin (Life Technologies). Cells were washed twice in PBS, fixed for 30 min at room temperature in PBS plus 4% paraformaldehyde, and permeabilized for 10 min at room temperature in PBS plus 0.5% Triton X-100. After an extensive wash in PBS, samples were blocked for 1 h at room temperature in PBS plus 10 mg/ml BSA (PBS/BSA) plus 10% goat serum. Prior to incubation with primary antibodies, samples processed for 4.1B staining were incubated at room temperature with an avidin-biotin blocking kit according to the manufacturer (Vector Laboratories, Burlingame, CA). After blocking, cells were incubated for 1 h in PBS/BSA containing 10 μg/ml of either affinity-purified goat anti-human 4.1B antibody or preimmune serum from the same goat. After incubation with primary antibodies, cells were washed in PBS plus 0.1 mg/ml BSA and then incubated for 1 h at room temperature with biotin-SP-conjugated donkey anti-goat IgGs (Jackson Immunoresearch Laboratories, West Grove, PA) diluted 1:2000, followed by fluorescein (5-(4,6-dichlorotiazin-2-
yclamin) fluorescein-conjugated streptavidin (Jackson Immunoresearch Laboratories, West Grove, PA) diluted 1:15,000. After extensive washing in PBS plus 0.1 mg/ml BSA, samples were mounted in Vectashield containing DAPI (Vector Laboratories, Burlingame, CA) and analyzed with a Zeiss Axiosvert 135 microscope.

Measurement of Spectrin-Actin Binding Activity by Falling Ball Viscometry—For these assays, peptides from the human 4.1 proteins were utilized to allow comparison with previous functional studies. Recombinant human protein 4.1R SAB bearing a C-terminal hexahistidine tag had the sequence MEPTEAWKRRLDGENYIRHSNLMLEDLD-KSGERIKHKHASIELEKKNPMESEVPEPSEWDKLSTFIQFPRTLN-GREGGTLHRIHHHHHHH, corresponding approximately to the coding domains of exons 16 (single underline) and 17 (double underline) as used in previous binding assays (10) but now including a hexahistidine tag. The sequence of the homologous SAB domain in human protein 4.1B was as follows: MGNSLIKRIKGENVYVKHSNLMLEEKTQDLMKQTNISELKRTFLETSTDITAVTNWEDKRLSTSPYGG-RHIIIIIIHIIIIII, including the C-terminal hexahistidine tag. The single underline indicates sequences homologous to those encoded by 4.1R exon 16, while the double underline represents the first 45 residues encoded by the paralog of 4.1R exon 17. This construct includes all residues with homology to the 4.1R SAB domain defined previously. The N-terminal methionine was added to facilitate translation initiation, and the “G” and “GGR” sequences at each end derive from restriction sites used in construction of the clone.

Recombinant proteins were purified by Cobalt column chromatography (CLONTECH, Palo Alto, CA) using 200 mM imidazole as the elution buffer. Spectrin was purified from human red cells (38, 39) and non-muscle actin derived from human platelets was obtained from Cytoskeleton (Denver, CO). Monomeric actin was polymerized at high concentration (5 mg/ml) to kinetically favor formation of many short filaments, and the “G” and “GGR” sequences at each end derive from restriction sites used in construction of the clone.

RESULTS

Cloning of 4.1B cDNA from Mouse Brain—A novel mouse brain cDNA, representing a new member of the protein 4.1 family of skeletal proteins, was identified and isolated by a combination of computer analysis of genetic data bases and traditional cDNA library screening (see “Materials and Methods”). This new 4.1 family member was designated as 4.1B, reflecting its high level expression in brain as well as its broad expression in a number of other tissues. The 4.1B gene was mapped to mouse chromosome 17 and assigned the gene designation Epp4.113 (36).

The predicted protein product of this 4.1B cDNA is 103 kDa in size. In overall domain structure, it closely resembles 4.1G, 4.1N, and 4.1R, including three regions of high homology to known functional domains of protein 4.1R (Fig. 1). The most extensive conserved sequence spans residues 118–454 of 4.1B and exhibits 74% identity to the MBD of 4.1R (also called the FERM domain due to the homology of this region in 4.1 with ezrin, radixin, and moesin). As defined by this phylogenetic approach, the MBD extends approximately 38 amino acids C-terminal to the domain boundary originally defined by chymotryptic digests (42). The MBD of 4.1R interacts with integral proteins such as band 3, glycophorin C, and CD44. While the precise binding motifs for some of these ligands has not been defined, the overall high sequence homology of 4.1B suggests a similar function involving interaction with one or more integral proteins in the brain. However, a key peptide required for 4.1R binding to band 3, the LEEDY sequence at residues 247–251 (Fig. 1B; Ref. 43), is altered to LEKDY in 4.1B. It therefore seems likely that the integral protein targets for 4.1B binding may differ somewhat from those of 4.1R. It should be noted that the membrane binding domain of these 4.1 proteins is less homologous to ERM proteins ezrin, radixin, and moesin (~35% amino acid identity).

Another conserved domain in 4.1B corresponds to the SAB domain, whose structure and function has been studied predominantly in mature red blood cells. In the prototypical 4.1R,
alternative splicing controls expression of two functionally different SAB domain structures: a high affinity isoform that binds strongly to spectrin and actin and is abundant in red cells and a low affinity isoform that lacks a key 21-amino acid peptide and is expressed in nonerythroid cells and early erythroid progenitors. Analysis of 4.1B expression has revealed a similar phenomenon in which alternative splicing can generate structurally different SAB domains. Specifically, brain 4.1B exhibits ~50% identity with the C-terminal 43 amino acids of the minimal SAB domain of 4.1R but lacks the critical N-terminal 21-amino acid peptide. In contrast, an isoform possessing a complete functional SAB domain is expressed in skeletal and heart muscle (see also Figs. 4 and 7).

The C-terminal region of 4.1B constitutes a third highly conserved domain that is found not only in the other mammalian 4.1 proteins depicted in Fig. 1 but also in the related Drosophila 4.1 protein (44). This domain of 4.1B is highly homologous to the NuMA binding domain of 4.1R (15) and the FKBP13 binding domain of 4.1G (14). More specifically, 4.1B and 4.1R are approximately 93% identical over the C-terminal 59 residues that constitute the NuMA binding domain (4.1R residue 800 to the C terminus in Fig. 1B; Ref. 15). Similarly, 4.1B and 4.1G exhibit 80% identity in the overlapping C-terminal 92 residues that correspond to the FKBP13 binding domain (4.1G residue 897 to the C terminus (14)).

In addition to these conserved domains, protein 4.1B also possesses several unique domains whose primary sequence is not shared with other 4.1 proteins. These unique regions are designated as U1, U2, and U3 (Fig. 1A). U1 represents the N-terminal “headpiece” located upstream of the membrane binding domain in not only 4.1B but also in the other three members of the family. 4.1B has little or no homology to the other 4.1 proteins in this region, aside from the initial MTTE peptide. Unique domain U2 corresponds to the “spacer” region between the membrane binding and SAB domains. Again, this domain is present in all four 4.1 proteins, but the primary sequence is poorly conserved, and the function is not known.

Finally, the U3 represents another region whose position is conserved among the 4.1 proteins but whose primary sequence is not. The amino acid content of this region in 4.1B exhibits some similarities to the large insert in the 440-kDa isoform of human ankyrin B (45); both are highly charged with a particular abundance of glutamate and aspartate residues and an acidic pI, and both are highly enriched in serine and threonine residues. The functional significance of this similarity remains to be explored.

An apparent human ortholog of the mouse 4.1B, designated as DAL1, was recently reported (46). The respective 4.1B and DAL1 genes map to regions of conserved synteny in human chromosome 17 and mouse chromosome 18 (36, 46). Moreover, DAL1 protein is nearly identical in a 600-amino acid region of overlap with mouse 4.1B, extending from the methionine at 4.1B position 118 to a point C-terminal to the spectrin-actin binding domain (46). However, the predicted DAL1 protein lacks the N- and C-terminal domains of mouse 4.1B, apparently due to frameshifts in the DAL1 cDNA sequence (46).

Tissue-specific Expression of 4.1B mRNA—Northern blot analysis of various tissues was performed with a 4.1B cDNA probe. As shown in Fig. 2A, the brain exhibited high level expression of a 4.4-kb 4.1B mRNA and somewhat lower levels of a 2.8-kb transcript. Both of these transcripts were also observed in placenta, while kidney, heart, and lung expressed predominantly the larger transcript. Low expression was observed in pancreas and skeletal muscle, with no detectable 4.1B mRNA in liver. While the 4.4-kb mRNA is consistent with the length of our mouse brain 4.1B cDNA sequence (GenBank accession no. AF152247), the structure of the 2.8-kb mRNA is less well understood. Northern blot experiments with region-specific probes indicated that the smaller transcript contains sequences homologous to the coding domain for SAB and the C-terminal domain, as well as the 3′-untranslated region; however, no hybridization was observed to a MBD probe (data not shown).

A more extensive survey of 4.1B mRNA expression was per-
formed by two additional approaches. First, a 4.1B probe was hybridized to a dot blot containing RNA from many human tissues (Fig. 2B). High expression was observed in many regions of the brain (rows A and B), and very high expression was observed also in adrenal gland (coordinate D5). Little or no expression was detected in erythropoietic tissues (bone marrow, E8; fetal liver, G4), where 4.1R expression is highest (26).

To obtain a higher resolution of 4.1B expression patterns, we performed in situ hybridization. Consistent with the results of the Northern blots, Fig. 3A demonstrates that 4.1B transcripts are most abundant in brain. Lower expression was also detected in testis, adrenal gland, and kidney. Examination of higher resolution hybridization signals in brain for 4.1B, as well as for 4.1R and 4.1N, revealed focal expression patterns in various neuronal populations (Fig. 3B). In the cerebellum, 4.1B was expressed specifically in Purkinje cells. In contrast, 4.1R and 4.1N are expressed in the granule cell layer, with no detectable expression in the Purkinje cells. Distinct expression patterns were also observed in the hippocampus. Whereas 4.1B expression was restricted to pyramidal cells of the CA1–3 region, 4.1R was localized exclusively to granule cells of the dentate gyrus. A third pattern was observed with 4.1N, which was expressed both in the dentate gyrus and CA1–3 regions. 4.1B also exhibited strong expression in the thalamic nuclei, where there is no detectable 4.1R, 4.1G, or 4.1N expression (data not shown).

Lower levels of 4.1B transcript were also detected in select peripheral tissues, including the testis, adrenal gland, kidney, and gastrointestinal tract (Fig. 3A). Examination of these peripheral tissues at higher power revealed 4.1B expression in discrete cell populations (data not shown): In the testis, highest expression occurs in sexually mature mice, with 4.1B transcripts predominantly localized to spermatocytes (undergoing meiosis) in the seminiferous tubules. In the adrenal gland, transcripts localize to the medullary chromaffin cells; the cellular components of the adrenal cortex are negative. The kidney demonstrates striking regional expression, with 4.1B transcripts restricted to the convoluted tubule epithelia of the cortex; the epithelial cells of the straight tubules, loops of Henle, and collecting tubules in the kidney medulla are negative. In addition to detection of high level 4.1B expression in select neurons of the central nervous system, the enteric neurons of the gastrointestinal tract are also positive for 4.1B transcript.

Alternative Splicing in 4.1B Pre-mRNA—The prototypical protein 4.1R gene utilizes alternative pre-mRNA splicing to mediate tissue-restricted expression of several distinct 4.1R protein isoforms. Of particular importance, the complete SAB domain is encoded by alternative exon 16 plus constitutive exon 17, and its structure and function is regulated by alternative splicing of exon 16 to produce isoforms that bind with high affinity (e.g. in mature red blood cells) or low affinity (e.g. in T lymphocytes) to spectrin and actin. To explore whether variations in SAB structure exist among protein 4.1B isoforms, we employed RT-PCR techniques to characterize this region of 4.1B mRNA isolated from various tissues. As shown in Fig. 4A,
this experiment yielded three distinct DNA bands in tissue-specific patterns, indicative of regulated alternative splicing of discrete exons in the 4.1B gene. Tissues such as kidney (lane 3) yielded only a single PCR product corresponding to a “default” splicing pattern in 4.1B that encodes a truncated SAB domain. The shortened SAB domain lacks a critical N-terminal peptide and would not be expected to exhibit high affinity interactions with spectrin. In contrast, amplification of RNA from heart (lane 2) and skeletal muscle (results not shown) generated two products. Sequence analysis of the larger product revealed that it encodes a complete SAB domain, including a 66-nucleotide paralog of 4.1R exon 16. The deduced primary amino acid sequences of the truncated and intact SAB domains of 4.1B are shown below the gel in Fig. 4A. Finally, PCR analysis of brain 4.1B mRNA revealed expression of a unique isoform that includes a novel 36-nucleotide exon, provisionally assigned as exon 15 due to its relative position in the gene. The 12-amino acid peptide encoded by this exon (Fig. 4A) is not homologous to any sequence of the 4.1R. Together these results strongly suggest that alternative splicing mediates muscle-specific expression of 4.1B isoforms that can interact with spectrin and actin, whereas 4.1B isoforms expressed in brain and other tissues are likely to exhibit distinct function(s).

Alternative splicing also generates structural heterogeneity in the C-terminal region of 4.1B mRNAs predicted to encode binding sites for NuMA (15) and FKBP13 (14). Two classes of 4.1B-related ESTs were found in data base searches. One class encoded the full C-terminal domain and contains the putative binding sites for NuMA and FKBP13. Representative clones of this class were isolated from mouse testis (L26705) and from a human mixed fetal lung/testis/B-cell library (AI377940 and AA928508). A second class of clones exhibited a deletion of 117 nucleotides that encompasses the normal C terminus and normal stop codon. Representative ESTs of this class were reported from mouse embryo (AI466582) and bowel (AI50619) and from human fetal kidney (AA340302) and the mixed fetal lung/testis/B-cell library (AA905681). Clones of this type will lack 36 C-terminal amino acids and will terminate translation at a new site in the 3’-untranslated region after the addition of a single glutamate residue (Fig. 4B). Such isoform(s) should be incapable of binding NuMA or FKBP13. At the genomic level, the corresponding region of 4.1R is encoded by exon 21. Analysis of 4.1B genomic DNA sequences has revealed that this portion of 4.1B gene structure is highly conserved, i.e. the 117-nucleotide sequence represents the paralogous exon in the 3’ region of the 4.1B gene (results not shown). However, while 4.1R exon 21 appears to be constitutively spliced, the data above indicate that its counterpart in the 4.1B gene is alternatively spliced.

To explore the tissue specificity of this alternative splicing event, RNA from several mouse tissues was amplified with 4.1B-specific oligonucleotide primers flanking the C-terminal region. As shown in Fig. 4B, there is considerable tissue variation in the relative exon 21 inclusion/exclusion ratio. Brain and heart RNA exhibited predominantly inclusion of this alternative exon, while kidney mostly skipped this exon. This result implies that alternative pre-mRNA splicing may regulate tissue-specific functional differences in NuMA and/or FKBP binding ability among the 4.1B isoforms expressed in individual cell types.

**Detection of 4.1B Protein Isoforms** — The existence of alternative splicing events in the protein 4.1B transcript predicts that multiple protein isoforms should be expressed from the 4.1B gene. To directly investigate 4.1 protein expression, we performed immunoblot analysis of mouse tissues using an affinity-purified anti-peptide antibody raised against a portion of the unique U2 region of the protein. Fig. 5 shows that prominent immunoreactive protein doublets of 148/144 and 128/124 kDa were expressed in mouse brain (lane 1). These 4.1B immunoreactive proteins were not observed in control experiments using preimmune IgG (lane 3), or antibody to protein 4.1G (lane 6), supporting their identity as 4.1B specific polypeptides.

The apparent size of the endogenous 4.1B bands in brain was larger than predicted from the cDNA sequence. A similar observation has been made previously for other members of the 4.1 family; 4.1R (19, 20), 4.1G (14, 26), and 4.1N (27) all exhibit apparent sizes significantly larger than the size calculated from the known sequence. In order to ascertain directly the apparent size of a known 4.1B protein, we epitope-tagged the brain 4.1B isoform illustrated in Fig. 1, transfected it into cultured cells, and detected the product via immunoblot analysis using a tag-specific antibody. The transfected 4.1B (Fig. 5B, lane 2) approximately co-migrated with the prominent 148-kDa endogenous band detected among endogenous brain proteins (lane 1). As a negative control, lane 3 shows that no protein was detected with an anti-tag antibody in cells transfected with empty vector (lane 3).

**Intracellular Localization of Protein 4.1B** — Protein 4.1B isoforms have been detected in various intracellular locations including the plasma membrane, the cytoplasm, and the nucleus, as well as centrosomes and spindle poles of mitotic cells (15, 30, 31, 47, 48). To investigate the intracellular localization of protein 4.1B, we performed immunofluorescence microscopy on cultured PC12 cells using affinity-purified anti-4.1B antibody. 4.1B was prominently localized to the plasma membrane at regions of cell-cell contact (Fig. 6A). Isolated cells, in con-
trast, exhibited little distinct 4.1B staining (Fig. 6A, inset). Regions of the cells corresponding to the nucleus (Fig. 6B) did not contain any detectable 4.1B. As a control, preimmune serum showed no plasma membrane (Fig. 6C).

**Spectrin-actin Binding Function of Muscle-specific 4.1B Isoform(s)**—The studies above demonstrated that brain 4.1B possesses a truncated SAB domain, whereas muscle isoform(s) of 4.1B contain an intact SAB domain. To test whether the latter 4.1B protein domain retains the functional ability to interact with spectrin and actin in ternary complexes, a falling ball viscometry assay was performed. This assay is based on the observation that spectrin binds to actin with low affinity in the absence of protein 4.1R and that the addition of intact 4.1R or its SAB domain significantly enhances spectrin-actin binding (in the context of a ternary complex that includes 4.1R), leading to increased apparent viscosity of the solution. Recombinant human 4.1R or 4.1B SAB domains were mixed in vitro with spectrin and actin and then incubated to allow formation of ternary complexes. Fig. 7 shows that the prototypical SAB domain of 4.1R promotes a concentration dependent increase in viscosity of a solution containing micromolar concentrations of spectrin and actin. 4.1B also clearly demonstrated the ability to promote gelation of a spectrin-actin mixture in a concentration-dependent manner, although with reduced efficiency compared with protein 4.1R.

**DISCUSSION**

Protein 4.1B represents a novel brain-enriched member of the 4.1 family, encoded by a unique gene on mouse chromosome 17 and human chromosome 18 (36). 4.1B represents the fourth member of a gene family that shares homology with the prototypical 4.1R protein in red blood cells but exhibits distinct tissue-specific expression patterns. High level, focal expression of 4.1B was observed in brain, particularly in Purkinje cells of the cerebellum, pyramidal cells of regions CA1–3 of the hippocampus, and neurons of the thalamic nuclei and olfactory bulb. Region-specific regulation of 4.1B transcription among different classes of neurons is thus a hallmark of 4.1B gene expression. Nonneuronal sites of expression, including testis, adrenal, kidney, and heart, suggest that additional transcription controls must exist in these cell types. Regulation of 4.1B gene expression is mediated also at the level of alternative pre-mRNA splicing. Tissue-specific splicing events appear to control synthesis of unique 4.1B isoforms in brain and heart/skeletal muscle; the latter isoform(s) contain a functionally competent spectrin-actin-binding domain encoded in part by a paralog of alternative exon 16 of the 4.1R gene. 4.1B protein isoforms exhibit many of the well characterized features of the prototypical protein 4.1R characterized earlier. The presence of conserved functional domains suggests that 4.1B proteins function in the skeletal architecture of specific neurons in brain in a manner analogous to the role of 4.1R in red blood cells. In particular, the membrane binding domain of 4.1 proteins has been shown to interact with integral proteins such as band 3 (2, 3), glycophorin C (4, 5), CD44 (6), and Drosophila neurexin (7). The high conservation of 4.1B sequences in this region (74% identity to 4.1R over 330 amino acids), indicates that 4.1B probably interacts with the cytoplasmic tails of one or more integral membrane proteins in specific neurons. Likewise, the C-terminal domain is also highly conserved, indicating functional similarities among 4.1 proteins that may include interactions with previously described C-terminal targets including immunophilin FKBP13 (14) and NuMA (15). As mentioned above, a classical erythroid-type spectrin-actin binding domain is expressed only in muscle due to the exclusion of exon 16 in other cell types. However, a novel 12-amino acid peptide motif is substituted into this region uniquely in brain, creating a modified domain that may bind alternative neuronal targets, perhaps brain-specific isoform(s) of a nonerythroid spectrin. Indeed, the existence of 4.1 homologs in brain that can bind to spectrin has already been demonstrated (49), although the precise relationship between those proteins and the recently cloned 4.1 homologs needs to be defined. Finally, each of the protein 4.1 genes also encodes unique domains that are not shared with other family members; such differences may encode novel functions not yet appreciated.

Region-specific expression of 4.1 family genes in selected neuronal classes indicates the need for exquisite transcriptional controls probably involving multiple promoter elements. This hypothesis is based on in situ hybridization experiments...
that reveal unique expression patterns for each of the 4.1 genes in the brain (Refs. 14 and 27 and Fig. 3). For example, the distribution of 4.1 mRNAs in hippocampus exhibits at least three distinct expression patterns; while 4.1R is restricted to the dentate gyrus and 4.1B to the CA1–3 region of the hippocampus, 4.1N is found in both dentate gyrus and CA1–3. In other regions, 4.1B and 4.1N exhibited a complementary but not mutually exclusive distribution; 4.1N is present in virtually all neurons of the brain with the prominent exception of Purkinje cells in the cerebellum and the majority of thalamic nuclei (27), while 4.1B is expressed strongly in these neurons. Together these observations suggest that transcriptional regulation of 4.1 family members in the brain is a complex process involving multiple region-specific promoters. A similar regulatory mechanism probably controls regional expression among ankyrin family genes expressed in the brain (50–53). For example, ankyrin G knockout mice, generated via a specific 5’ sequence deletion, selectively lose expression of ankyrin G in some brain regions but not others (54). This observation indicates that multiple promoter elements must control ankyrin expression in distinct neuronal populations.

Regulated alternative splicing is also a fundamental feature of gene expression shared by both the 4.1 and ankyrin gene families. Protein 4.1R (20), 4.1B (this paper), and 4.1N 2 all exhibit brain-specific splicing patterns indicative of unique brain isoforms encoded by each of these genes. Similarly, neural specific isoforms of ankyrin G and ankyrin B have been reported (52). These results support earlier observations that the brain is a rich source of tissue-specific alternative pre-mRNA splicing (e.g. Refs. 55 and 56) and suggest that the unique 4.1 isoforms generated by such splicing events must play roles in the brain that are distinct from their functions in nonneuronal cells.

Together, these carefully regulated transcription and alternative splicing processes cooperate to facilitate tissue- and region-specific expression of a diverse repertoire of structural proteins in the 4.1 and ankyrin families. A subset of these proteins are likely to exhibit neuron- and intracellular compartment-specific functions. Elucidating the specific roles for the diverse 4.1 proteins in the brain, including their distinct binding partners and intracellular distributions, should provide important insights into neuronal cytoskeletal architecture and the relevance of such structure to cell function.

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