Isolation and Characterization of cDNAs Encoding Human Brain Ankyrins
Reveal a Family of Alternatively Spliced Genes

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Abstract. Ankyrins are a family of membrane-associated proteins that can be divided into two immunologically distinct groups: (a) erythrocyte-related isoforms (ankyrinR) that have polarized distributions in particular cell types; and (b) brain-related isoforms (ankyrinB) that display a broader distribution. In this paper, we report the isolation and sequences of cDNAs related to two ankyrinB isoforms, human brain ankyrin 1 and 2, and show that these isoforms are produced from alternatively spliced mRNAs of a single gene. Human brain ankyrin 1 and 2 share a common NH2-terminus that is similar to human erythrocyte ankyrins, with the most striking conservation occurring between areas composed of a repeated 33-amino acid motif and between areas corresponding to the central portion of the spectrin-binding domain. In contrast, COOH-terminal sequences of brain ankyrin 1 and 2 are distinct from one another and from human erythrocyte ankyrins, and thus are candidates to mediate protein interactions that distinguish these isoforms. The brain ankyrin 2 cDNA sequence includes a stop codon and encodes a polypeptide with a predicted molecular mass of 202 kD, which is similar to the $M_r$ of the major form of ankyrin in adult bovine brain membranes. Moreover, an antibody raised against the conserved NH2-terminal domain of brain ankyrin cross-reacts with a single $M_r = 220$ kD polypeptide in adult human brain. These results strongly suggest that the amino acid sequence of brain ankyrin 2 determined in this report represents the complete coding sequence of the major form of ankyrin in adult human brain. In contrast, the brain ankyrin 1 cDNAs encode only part of a larger isoform. An immunoreactive polypeptide of $M_r = 440$ kD, which is evident in brain tissue of young rats, is a candidate to be encoded by brain ankyrin 1 mRNA. The COOH-terminal portion of brain ankyrin 1 includes 15 contiguous copies of a novel 12-amino acid repeat. Analysis of DNA from a panel of human/rodent cell hybrids linked this human brain ankyrin gene to chromosome 4. This result, coupled with previous reports assigning the human erythrocyte ankyrin gene to chromosome 8, demonstrates that human brain and erythrocyte ankyrins are encoded by distinct members of a multigene family.
ankyrinB forms are expressed primarily in neurons, and have been localized at specialized cell domains such as the node of Ranvier (24). Several ion channels colocalize with ankyrinB isoforms in specialized membrane domains and interact with erythrocyte ankyrin in vitro. These include the voltage-dependent sodium channel of brain (35), and the anion exchanger (14) and Na⁺/K⁺ ATPase of kidney (23, 29, 30). In contrast, isoforms of the other group, ankyrinA, react better with antibodies against bovine brain ankyrin. In brain, ankyrinA is present in both glial and neuron cells, and is not concentrated at the node of Ranvier (24). The membrane attachment sites for ankyrinA forms are not yet established. Current candidates, however, include ABGP-205 (36) and a broadly distributed membrane glycoprotein termed Pgp-1, gp-85, or CD44 antigen (21).

cDNA sequences encoding human erythrocyte ankyrins (ankyrinB isoforms) have recently been reported (25, 26), and the two well-characterized isoforms of erythrocyte ankyrin, protein 2.2 and 2.1, were shown to result from alternative mRNA splicing (26). As a first step towards identifying the structural bases underlying functional differences between ankyrinB and ankyrinA forms, we have isolated cDNAs encoding human ankyrinB isoforms. In this paper, we report the sequences of these cDNAs and show that they represent portions of two alternatively-spliced mRNAs encoding isoforms with distinct COOH-terminal sequences. These brain isoforms, which we designate brain ankyrin 1 and 2, are encoded by a gene linked to chromosome 4, an assignment distinct from the human erythrocyte ankyrin gene, which has previously been linked to chromosome 8 (25, 27). Thus, we conclude that in humans, there is a family of at least two ankyrin genes, each encoding multiple isoforms by alternative mRNA splicing.

**Materials and Methods**

cDNA Isolation

Unless specified, molecular cloning methods used were essentially as described by Sambrook et al. (32). cDNA clones were isolated from a human brain stem expression library (lambda gt11/oligo-dT primed) graciously provided by Dr. C. Lutz-Freyermuth and Dr. J. Keene (Department of Microbiology, Duke University). Screens were performed using antibodies against human erythrocyte ankyrin 2.1 (5), antibodies against bovine brain ankyrin (24), or various 32P-labeled cDNA fragments (1-9 x 10⁶ cpm/µg) prepared by randomly primed DNA synthesis (Multiprime System; Amerham Corp., Arlington Heights, IL). All hybridizations were performed at 65°C using 10-20 ng/ml probe DNA, 0.1 x SSC, 0.5% SDS. Filters were washed once with 2 x SSC, 0.5% SDS for 15 min at 25°C, once in 1 x SSC, 1.0% SDS for 15 min at 65°C, and then twice in 0.1 x SSC, 1.0% SDS for 15 min at 65°C.

DNA Sequence Analysis

cDNA fragments were subcloned into plasmids pGEM-4Z (Promega Biotech, Madison, WI) or pBluescript SK(+) (Stratagene Cloning Systems, La Jolla, CA). Nucleotide sequences from these templates or from sets of unidirectional Exo III deletions (Exo III/MungBean System; Stratagene Cloning Systems) were determined using the dideoxy chain termination method (33) and T7 polymerase (Sequenase; United States Biochemical Corp., Cleveland, OH). Sequence spanning the EcoRI site was confirmed directly from linear template fragments amplified from lambda 1 by the polymerase chain reaction (Perkin Elmer Cetus, Norwalk, CT).

Southern and Northern Blot Analyses

Isolation of genomic DNA from cultured cells and Southern blot analysis were performed as described previously (31). Blots were hybridized to 32P-cDNA probes encoding brain (bp 583-2,192) or erythrocyte ankyrin repeats (bp 997-2,379) (26). Prepared blots of DNA from human/hamster cell hybrids were obtained from BIOS Corp. (New Haven, CT) and were hybridized to the brain ankyrin probe (bp 583-2,192). These blots were washed twice in 2 x SSC, 0.5% SDS for 10 min at 25°C, once in 1 x SSC, 1.0% SDS for 15 min at 65°C, and then twice in 0.1 x SSC, 1.0% SDS for 15 min at 65°C.

Total RNA was isolated using RNAzol (Cinna/Biotex, Houston, TX). Northern blots were performed as described (18). Probes used in Fig. 9 were derived from bp 583-2,192 (brain ankyrin) and bp 997-2,379 (erythrocyte ankyrin; 26).

Immunoblot Analyses

Proteins associated with crude membrane fractions (9) and erythrocyte ghost proteins (2) were prepared as described. The amount of membrane proteins loaded in each lane was normalized relative to the weight of the tissue homogenized. SDS-PAGE was performed using 0.2% SDS with the buffers of Fairbanks et al. (15) and 3.5-17% exponential gradient gels. Immunoblot analyses using 125I-labeled protein A to detect antibodies were performed as described (9). All blots were incubated with antibodies at 1 µg/ml. Protein A was labeled with Na125I using chloramine T as an oxidant (20).

Bacterial Expression of Brain Ankyrin

A bacterial strain expressing a portion of human brain ankyrin was created by transforming E. coli JM109(DE3) with pBrank, a T7 polymerase-based expression vector constructed by ligating the upstream EcoRI fragment of lambda 1 into GEMEX-1 (Promega Biotech). To purify brain ankyrin fusion protein, 2.5 liters of exponentially growing cells were induced with 0.5 mM isopropyl-B-D-thiogalactoside for 1 h and collected by centrifugation (2,000 g, 10 min). Cells were washed in 100 mM NaCl, 10 mM sodium phosphate, pH 7.4, repelleted, and then resuspended in 30 ml containing 4 mg/ml lysozyme (Sigma Chemical Co., St. Louis, MO), 50 mM sodium phosphate, pH 8.4, 1 mM NaEDTA, 25% (wt/vol) sucrose, and protease inhibitors (5 µg/ml leupeptin, 5 µg/ml pepstatin, 0.05% (vol/vol) diisopropylfluorophosphate, 10 mM benzamidine). After 10 min on ice, DNase (United States Biochemical Corp.) and MgCl2 were added to final concentrations of 25 µg/ml and 10 mM respectively. After an additional 10 min on ice, cells were lysed by the addition of 30 ml lysis buffer: 10% (wt/vol) deoxycholate, 1.0% (vol/vol) Triton X-100, 200 mM NaCl, 20 mM sodium phosphate, pH 7.4, 2 mM NaEDTA, 1 mM DTT. This lysate was then passed three times through a 20 gauge needle, and inclusion bodies containing brain ankyrin collected by centrifugation (3,500 g, 10 min). Inclusion bodies were washed once with lysis buffer, and a second time using 0.4% (vol/vol) Triton X-100, 10 mM sodium phosphate, pH 7.4, 1 mM NaEDTA, and protease inhibitors. The final pellet was dissolved in 8 M urea, 10 mM sodium phosphate, pH 7.4, 1 mM NaEDTA, 1 mM MgCl2, and then diluted to a 6 M urea before loading on to a Mono Q sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) column. Proteins were eluted using a linear gradient of 10-500 mM NaBr dissolved in 6 M urea, 10 mM sodium phosphate, pH 7.4, 1 mM NaEDTA, 10 mM glycine, 1 mM sodium azide, 1 mM DTT, pH 7.4. Fractions were analyzed by SDS-PAGE and peak fractions pooled to yield ~4 mg purified protein.

Antibodies

Procedures used for the affinity purification of antibodies were described by Davis and Bennett (12). Antiserum against human brain ankyrin was collected from rabbits immunized with recombinant fusion protein expressed in bacteria (see above). To remove antibodies cross-reacting with erythrocyte ankyrin or with the viral (gene 10) portion of the injected antigen, this antiserum was passed over columns containing sepharose-linked human erythrocyte ankyrin 2.1 and gene 10 protein. Antibodies specific for brain ankyrin were then collected from the eluate on an antigen affinity column and eluted with 4 M MgCl2. Antibodies were dialyzed against 20% (wt/vol) sucrose, 150 mM NaCl, 10 mM sodium phosphate, pH 7.4, 1 mM NaEDTA, 1 mM sodium azide, and stored frozen at -70°C. Antibodies against human brain ankyrin 2.1 (5), against the portion of erythrocyte ankyrin 2.1 absent from spliced variant 2.2 (26), and against bovine brain ankyrin (24) have been described.

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Results

Isolation of Human Brain Ankyrin cDNAs Related to Two Alternatively Spliced mRNAs

To obtain cDNA clones encoding human brain ankyrin, a human brain stem expression library was screened using two antibodies as probes: (a) an antibody raised against human erythrocyte ankyrin isoform 2.1, that had previously been shown to recognize ankyrin in bovine and rat brains (9, 24); and (b) an antibody raised against purified bovine brain ankyrin (24). From these screens, one clone reacting with the erythrocyte antibody, lambda 1, and one clone reacting with the brain antibody, lambda 2, were recovered (Fig. 1B). Additional screens of the same library using DNA probes derived from lambda 1, lambda 2, or clones recovered in subsequent rounds produced the series of overlapping clones shown in Fig. 1B.

The 5'-most 24 bp of lambda 2 were identical to a segment within lambda 1, suggesting that lambda 1 and lambda 2 represented portions of alternatively spliced mRNAs with common 5' but unique 3' sequences. The presence of alternatively spliced transcripts was confirmed by the nucleotide sequences of independent clones lambda 110 and lambda 5. The overlapping portion of lambda 110 (1,630 bp) was an exact match with lambda 1 and included 1,211 bp 5' and 419 bp 3' to the apparent splice site. Lambda 5 contained a 423-bp match with lambda 2, and extended the 24 bp of identity with lambda 1 in the 5' direction by over 2,500 bp. It is of interest that the point of divergence among these sequences occurs at exactly the same point (nucleotide 4,344), and that the alternative sequences give rise in both cases to an open reading frame (see below). Given the extent of identity shared by lambda 1 and lambda 110 (1,630 bp) and shared by lambda 5, lambda 1, and lambda 2 (3,086 bp), it is unlikely that either of these two linear sequences reflect a chimera created by the artificial juxtaposition of sequences from different mRNAs. A restriction map summarizing the organizations of these cDNAs is shown in Fig. 1A. Based on their relationship to lambda 1 and lambda 2, these cDNAs and the proteins they encode (see below) have been designated brain ankyrin 1 and brain ankyrin 2.

Consistent with alternative splicing, a cDNA probe derived from sequences shared by both brain ankyrin 1 and 2 (bp 2,841-4,763; Fig. 1A) hybridized to multiple brain mRNAs isolated from 10-d-old rats: three major mRNAs of 7, 9, and ~13 kb, and a minor form of 4 kb (Fig. 2; lane a). Hybridization of another common probe (bp 583-2,192), however, revealed only the two largest mRNAs (Fig. 2, lane d), indicating that the smaller transcripts lack these upstream sequences and thus are either additional alternatively spliced mRNAs whose organizations are not represented by the cDNAs in this report or closely related species transcribed from a distinct gene. A probe representing the unique 3' portion of lambda 1 (bp 4,344-5,028) recognized

Figure 1. Structure of human brain ankyrin cDNAs. (A) Composite restriction maps of alternatively spliced brain ankyrin cDNAs, created from the overlapping clones shown in B. Sequences present only in brain ankyrin 2 are shown by an open box; those unique to brain ankyrin 1 are cross-hatched. The regions encoding the 33-amino acid repeats present in both isoforms (R1-R22) and the 12-amino acid repeats unique to brain ankyrin 1 (r1-r15) are indicated. The position of the stop codon (UAA) marking the end of translation of brain ankyrin 2 is noted. All restriction sites are in agreement with those predicted by the nucleotide sequence (Fig. 3). Restriction sites: B, BamHI; E, EcoRI; H, HindIII; K, KpnI; P, PstI; S, SalI; V, PvuII. (B) Brain ankyrin cDNA clones. Arrows indicate the direction (strand) and extent of nucleotide sequence obtained during individual determinations.
only the largest mRNA (apparent size of 13 kb estimated by extrapolation of migration of smaller standards) (Fig. 2, lane b), indicating that brain ankyrin 1 is encoded by a transcript of this size. Surprisingly, a probe expected to be specific for brain ankyrin 2 (bp 4,351-4,930) revealed mRNAs (Fig. 2, lane c) with sizes of 7, 9, and ~13 kb that are similar to those revealed by the common probe in lane a. Thus, from these results, it is not clear whether brain ankyrin 2 is encoded by a 9-kb or ~13-kb transcript. The fact that probes derived from the distinctive 3' regions of brain ankyrin 1 and 2 both hybridize with a message of ~13 kb, suggests the possibility that this mRNA encodes a protein containing sequences related to COOH-terminal portions of both brain ankyrin 1 and 2.

**Human Brain Ankyrin Sequences**

The nucleotide sequences of brain ankyrin cDNAs were determined on both strands and are shown in Fig. 3. Brain ankyrin 2 was composed of 6,179 bp and contained a large open reading frame encoding a 1,839 amino acid protein. Computer-assisted comparison of brain ankyrin 2 and human erythrocyte ankyrin 2.1 (Fig. 4, bottom) revealed a strong central diagonal indicative of similar linear organizations, with the most significant conservation (>70% identity) occurring between regions corresponding to the central portions of the membrane and spectrin-binding domains of erythrocyte ankyrin. In contrast to these highly conserved regions, several segments of brain ankyrin 2 were less similar to erythrocyte ankyrin and thus represent candidates to mediate interactions that distinguish brain and erythrocyte isofoms: (a) the NH2-terminal region (amino acids 1-34), which includes a short stretch of basic residues (amino acids 22-28) similar to nuclear localization sequences (arg-arg-lys-arg-pro-lys-lys); (b) the segment linking the conserved portions of the membrane and spectrin-binding domains (amino acids 817-950) which includes a stretch of threonines (amino acids 817-828; thr-glu-glu-val-(thr)5-ile-thr); and (c) the COOH-terminal region (amino acids 1,515-1,839), whose position corresponds to the protease-sensitive domain of erythrocyte ankyrin that has previously been shown to be involved in regulation of binding (see Discussion).

A striking feature shared by both brain ankyrin 1 and 2 is the presence of a repeated 33-amino acid motif that has also been found in human erythrocyte ankyrins and in a number of proteins involved in cell differentiation, cell cycle control, and transcription (Fig. 5, and 26). Human brain ankyrins, like erythrocyte isoforms, contain 22 contiguous copies of this motif. In brain ankyrins, 16 of the 33 residues in each repeat are highly conserved (present in at least two-third of the brain repeats), with the remainder being more variable (Fig. 5 A; consensus). Computer-assisted comparison of individual brain and erythrocyte repeats revealed that 21 of the 22 brain repeats are most similar to the corresponding erythrocyte repeat (Fig. 5 A). Brain repeats nearer the COOH-terminal end of membrane-binding domain (R12-R22) were, in general, less similar to their erythrocyte counterparts than brain repeats nearer the NH2-terminus (Fig. 5 C). The 33 amino acid periodicity of the repeats is rigorously conserved in both brain and erythrocyte ankyrins, with the only deviations being the fourth repeat, which has only 29 residues in both, and an additional 8 amino acids after the fifth repeat in the brain sequence.

The alternative nucleotide sequence unique to brain ankyrin 1 was determined on both strands and is shown in Fig. 3. This sequence, beginning at bp 4,345 (Fig. 3, arrow), contained an open reading frame that was an extension of the frame used for brain ankyrin 2. The amino acid sequence deduced from this reading frame was unlike the corresponding regions of erythrocyte ankyrin 2.1 (Fig. 4, top) and brain ankyrin 2 (comparison not shown). The open reading frame encoding brain ankyrin 1 extended to the 3' end of the cDNAs isolated, and thus the brain ankyrin 1 sequence shown in Fig. 3 represents only a portion of a larger sequence. Based on the sequence presented here, it is clear that brain ankyrin 1 must have a theoretical molecular mass of >227 kD.

An interesting feature of the brain ankyrin 1 alternative sequence is the presence of 15 tandem copies of a 12-amino acid motif (Fig. 6). The sequence of this motif is very highly conserved, with 11 of the 12 residues being found in greater than two-third of the repeats. A search of GenBank and NBRF libraries for sequences related to this motif revealed no significant matches. Thus, potential role(s) of these repeats are currently unclear and remain the focus of future study.
Despite the high level of overall protein similarity shared by the membrane and spectrin-binding portions of human brain and erythrocyte ankyrins, the complete lack of any significant blocks of nucleotide identity strongly suggested that these isoforms are the products of different genes. In support of this view, probes derived from corresponding 33-amino acid repeats of brain and erythrocyte ankyrin cDNAs hybridized to distinct genomic DNA fragments (Fig. 7), the number of which indicated that both brain and erythrocyte repeats are encoded by multiple exons. To absolutely rule out the possibility that brain and erythrocyte ankyrins are produced by alternative splicing of mutually exclusive exons within a large single gene, we determined the chromosomal linkage of the human brain ankyrin gene by analysis of a panel of human/hamster cell hybrids with known karyotype. Hybridization of a brain ankyrin cDNA probe to genomic DNA isolated from normal human and hamster cells and digested with EcoRI produced patterns that allowed the human gene to be distinguished from its hamster counterpart. Comparison of these patterns with those obtained using DNA from 25 human/hamster cell hybrids showed that brain ankyrin sequences were linked to human chromosome 4; hybridization patterns and karyotypes of only a representative sample of lines screened are shown in Fig. 8. Previous reports have linked the erythrocyte ankyrin gene to human chromosome 8 (25, 27). Thus, taken together, these results confirm that human brain and erythrocyte ankyrins are encoded by different genes. This conclusion is also supported by linkage studies in mouse assigning erythrocyte and brain ankyrin sequences to different chromosomes (L. Peters, C. Birkenmeier, R. Bronson, R. White, S. Lux, E. Otto, V. Bennett, A. Higgins, and J. Barker, manuscript in preparation).

**Members of Human Ankyrin Gene Family Show Distinct Patterns of Expression**

Under stringent conditions, probes prepared from the 33-amino acid repeat regions of human brain and erythrocyte ankyrin cDNAs hybridized to distinct transcripts on northern blots of RNA isolated from various tissues of adult rats (Fig. 9). The brain probe revealed a major mRNA of 9 kb and a minor one of ~13 kb in rat brain (Fig. 9 A, lane a1; also see Fig. 2, lane d). The relative amount of the 13-kb message is reduced compared to the blot of Fig. 2. The difference between experiments of Fig. 2 and Fig. 9 is that 10-d-old rats were used in Fig. 2, while adult rats were used for Fig. 9. The reduced amounts of the ~13 kb message relative to the 9-kb message in adult animals was a consistent result in at least three independent experiments and reflects differences in developmental expression of the 9- and 13-kb messages (Kunimoto, M., E. Otto, and V. Bennett, manuscript in preparation). The erythrocyte probe detected transcripts in both spleen (7 and 8.5 kb) and brain (9 kb). The sizes of the erythrocyte-related mRNAs are similar to those reported for human tissues (25). The erythrocyte-related transcript in brain was also detected using an erythrocyte ankyrin probe derived from unique COOH-terminal sequences (data not shown); thus, this 9-kb transcript can not be the same as the one detected using the brain probe and most likely represents expression of erythrocyte ankyrin mRNA in brain tissue. Consistent with this conclusion, recent findings have shown that mutant mice deficient in erythrocyte ankyrin have normal amounts of the brain-related transcript, but are missing the erythrocyte-related 9-kb transcript in brain (L. Peters, C. Birkenmeier, R. Bronson, R. White, S. Lux, E. Otto, V. Bennett, A. Higgins, and J. Barker, manuscript in preparation).

Tenfold longer exposures of these northern blots revealed several additional mRNAs whose sizes were distinct from any of the major transcripts described above. The brain ankyrin probe revealed a transcript in kidney that was slightly larger than the 9-kb transcript detected in brain, while the erythrocyte ankyrin probe revealed a small transcript in liver (2 kb) and a kidney mRNA whose size was intermediate to the 7- and 8.5-kb transcripts detected in spleen. It is possible that these transcripts represent alternatively spliced mRNAs that are transcribed from the brain and erythrocyte ankyrin genes, but accumulate at much lower levels than those detected by short exposure. Given the abundance of immunoreactive forms of ankyrin in kidney (11), however, a likely alternative is that these mRNAs are produced by distinct gene(s) having some sequence similarity to brain and erythrocyte ankyrin genes. Thus, it is likely that transcription of brain ankyrin is brain-specific, although very low levels of expression in other tissues cannot be ruled out.

To further characterize human brain ankyrins, amino acids 191-942 (shared by both brain ankyrin 1 and 2) were expressed in bacteria as the NH2-terminal portion of a larger fusion protein that also contained sequences of a bacteriophage T7 gene 10 protein. Antibodies were prepared that reacted specifically with brain ankyrin but not erythrocyte ankyrin of T7 gene 10 protein by passage over affinity columns containing purified human erythrocyte ankyrin 2.1 and nonrecombinant gene 10 protein before isolation using the brain ankyrin fusion protein as an immunoadsorbant. Immunoblot analyses using these antibodies revealed brain polypeptides of two sizes in 16-d-old rats, $M_r = 220$ kD and $M_r = \sim 440$ kD. Consistent with Northern blots, these proteins were associated with brain membranes, but were not detected with membranes from spleen, kidney, or liver (Fig. 10 A, panel b). A single polypeptide of 220 kD was detected with membranes from temporal cortex of adult human brain (Fig. 10 B). The absence of the 440-kD ankyrin isoform in this sample from adult brain reflects the fact that this form of ankyrin is greatly reduced in adults and most likely is not a species difference between rats and humans (Kunimoto, M., E. Otto, and V. Bennett, manuscript in preparation). In contrast to the antibody against recombinant brain ankyrin, an antibody against a portion of the unique COOH-terminus of erythrocyte ankyrin detected proteins of 215 kD in spleen (human erythrocyte ankyrin 2.1) and brain, and a 150-kD protein in liver. The liver protein reacted poorly with an antibody against entire erythrocyte ankyrin 2.1, suggesting that it is only weakly related (data not shown). Kidney samples contained small amounts of a polypeptide comigrating with erythrocyte ankyrin that cross-reacted with the erythrocyte ankyrin antibodies. The kidney polypeptide is most likely the result of contamination with circulating erythrocytes, since kidneys perfused to remove erythrocytes do not contain an immunoreactive form of ankyrin of this size (11, 13). Kidneys perfused to remove erythrocytes and with protease
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uniquetobrainankyrin1beginatthearrowandareshown onlines3 and4. Amino acids identical to those of human erythrocyte ankyrin are shown at the bottom of Figure 3. Nucleotide and deduced amino acid sequences of human brain ankyrin DNAs. Nucleotide sequence of the composite cDNA (26) in an aligned comparison using the University of Wisconsin Genetics Computer Program BESTFIT are shown as capital letters.

Figure 3. Nucleotide and deduced amino acid sequences of human brain ankyrin cDNAs. Nucleotide sequence of the composite cDNA (26) in an aligned comparison using the University of Wisconsin Genetics Computer Program BESTFIT are shown as capital letters.
The beginning of each 33-amino acid repeat (RL-R22) is indicated above line 1; the beginning of each 12-amino acid repeat unique to brain ankryin 1 (rl-r15) is shown below line 4. The eight amino acids interrupting the periodicity of the 33-amino acid repeats are indicated on line 5. The beginning of each 33-amino acid repeat (R1-R22) is indicated above line 1; the beginning of each 12-amino acid repeat unique to brain ankryin 1 (rl-r15) is shown below line 4. The eight amino acids interrupting the periodicity of the 33-amino acid repeats are indicated on line 5.
Figure 4. Graphic comparisons of human brain and erythrocyte ankyrins. Dot matrix plots comparing the deduced amino acid sequences of brain ankyrin 1 (top vertical axis) and brain ankyrin 2 (bottom vertical axis) to erythrocyte ankyrin 2.1 (horizontal axis) (26). Comparison was made using MacVector (International Biotechnologies, Inc., New Haven, CT); each dot represents a match (70% minimum identity) between windows of eight amino acids. Arrows mark the position at which brain ankyrin 1 and 2 alternative sequences begin (see Fig. 3). The boundaries and functions of the protease-resistant domains of erythrocyte ankyrin are indicated.

Erythrocyte Ankyrin

In this paper, we report that sequences of cDNAs encoding two human brain ankyrins, one of which is closely related to bovine brain ankyrins that are members of a class of ankyrins referred to as ankyrinB. First, an antibody raised against purified bovine brain ankyrin reacted strongly with the recombinant protein expressed by lambda 2 (see above); while conversely, purified bovine brain ankyrins could be completely immunoprecipitated by saturating concentrations of the antibody raised against recombinant human brain ankyrin (data not shown). Because the portion of brain ankyrin 2 encoded within lambda 2 and the portion of brain ankyrin 2 used to raise antibody are nonoverlapping, these results indicate that bovine ankyrinB forms and brain ankyrin 2 share multiple epitopes. Second, human and rat brain proteins reacting with the antibody against recombinant human brain ankyrin have an $M_r$ of 220 kD that is similar to that reported for bovine brain ankyrins, which include two major polypeptides of $M_r = 220$ and 210 kD that are nearly identical by peptide map (10). Thus, based on its overall similarity to these bovine brain ankyrin isoforms, human brain ankyrin 2 is classified as an ankyrinB isoform (see Discussion for nomenclature considerations).

Discussion

In this paper, we report that sequences of cDNAs encoding two human brain ankyrins, one of which is closely related to the ankyrinB isoforms previously isolated and characterized from adult bovine brain (9, 10, 24). These two human isoforms, designated brain ankyrin 1 and 2, are translated from alternatively spliced mRNAs and share common membrane and spectrin-binding domains joined to unique COOH-terminal sequences. Antibodies raised against a recombinant protein segment common to brain ankyrin 1 and 2 revealed two rat proteins that were associated with membranes from rat brain but not from other tissues. One of these, a polypeptide of $M_r = 220$ kD, was similar in mass to that predicted for human brain ankyrin 2 and is likely to represent the major form of ankyrin in adult brain. The other, a polypeptide with an $M_r$ of $\approx 440$ kD, is a candidate to be encoded by the brain ankyrin 1 alternative mRNA, and is present in low sequence of brain ankyrin 2 (202 kD). Optimal alignment of brain ankyrin 2 and erythrocyte ankyrin predicted a membrane-binding domain for brain ankyrin 2 whose theoretical molecular mass (93–95 kD) was very similar to the $M_r = 93–95$ kD of the membrane-binding domain of bovine brain ankyrin described previously (10). Thus, the first AUG codon used in Fig. 3 is, or is very close to, the start site of translation. Good correlation between the electrophoretic mobility ($M_r = 72$ kD) and theoretical molecular mass (89 kD) for the highly homologous membrane-binding domain of erythrocyte ankyrin supports the validity of this conclusion. Thus, differences between the apparent size based on SDS-electrophoresis and actual molecular weight of brain ankyrin are likely to be due to features of the remainder of the molecule. Consistent with this hypothesis, the spectrin-binding domain of erythrocyte ankyrin has an $M_r = 72$ kD based on electrophoretic mobility that is 16% larger than the 62 kD predicted from the cDNA sequence (25, 26). The discrepancy in apparent size of the spectrin-binding domain is most likely not due to posttranslational modifications since a recombinant polypeptide expressed in bacteria also has an $M_r = 72$ kD (our unpublished data).

Inhibitors to reduce proteolysis do contain polypeptides of $M_r = 190$ kD crossreacting with erythrocyte ankyrin and of $M_r = 220$ kD crossreacting with brain ankyrin (11), which may have been degraded in these preparations. Although both brain and erythrocyte ankyrins are expressed in brain, erythrocyte-related ankyrin was especially abundant in cerebellum (Fig. 10, compare lanes 1 and 2 in b and c).

The $M_r$ of the 220-kD human brain ankyrin (deduced from electrophoretic migration; Fig. 10 B) was 10% larger than the molecular mass predicted from the amino acid sequence of brain ankyrin 2 (202 kD). Optimal alignment of brain ankyrin 2 and erythrocyte ankyrin predicted a membrane-binding domain for brain ankyrin 2 whose theoretical molecular mass (93–95 kD) was very similar to the $M_r = 93–95$ kD of the membrane-binding domain of bovine brain ankyrin described previously (10). Thus, the first AUG codon used in Fig. 3 is, or is very close to, the start site of translation. Good correlation between the electrophoretic mobility ($M_r = 72$ kD) and theoretical molecular mass (89 kD) for the highly homologous membrane-binding domain of erythrocyte ankyrin supports the validity of this conclusion. Thus, differences between the apparent size based on SDS-electrophoresis and actual molecular weight of brain ankyrin are likely to be due to features of the remainder of the molecule. Consistent with this hypothesis, the spectrin-binding domain of erythrocyte ankyrin has an $M_r = 72$ kD based on electrophoretic mobility that is 16% larger than the 62 kD predicted from the cDNA sequence (25, 26). The discrepancy in apparent size of the spectrin-binding domain is most likely not due to posttranslational modifications since a recombinant polypeptide expressed in bacteria also has an $M_r = 72$ kD (our unpublished data).

Two results indicate that human brain ankyrin 2 is closely related to bovine brain ankyrins that are members of a class of ankyrins referred to as ankyrinB. First, an antibody raised against purified bovine brain ankyrin reacted strongly with the recombinant protein expressed by lambda 2 (see above); while conversely, purified bovine brain ankyrins could be completely immunoprecipitated by saturating concentrations of the antibody raised against recombinant human brain ankyrin (data not shown). Because the portion of brain ankyrin 2 encoded within lambda 2 and the portion of brain ankyrin 2 used to raise antibody are nonoverlapping, these results indicate that bovine ankyrinB forms and brain ankyrin 2 share multiple epitopes. Second, human and rat brain proteins reacting with the antibody against recombinant human brain ankyrin have an $M_r$ of 220 kD that is similar to that reported for bovine brain ankyrins, which include two major polypeptides of $M_r = 220$ and 210 kD that are nearly identical by peptide map (10). Thus, based on its overall similarity to these bovine brain ankyrin isoforms, human brain ankyrin 2 is classified as an ankyrinB isoform (see Discussion for nomenclature considerations).
amounts in adult brain tissue. The 3’ portion of brain ankyrin 1 has been partially cloned, with current available sequence sufficient for only 227 kD, suggesting that ~5.8 kb of cDNA remain to be characterized.

Analysis of DNA from a panel of human/hamster cell hybrids revealed that brain ankyrins 1 and 2 and erythrocyte ankyrin isoforms are encoded by different genes: the brain ankyrin gene is linked to human chromosome 4 (Fig. 8), and the erythrocyte ankyrin gene is linked to human chromosome 8 (25, 27). Recent reports by Lux et al. (26) have shown that erythrocyte ankyrin isoforms 2.1 and 2.2 are produced by alternative splicing of the erythrocyte ankyrin gene. Thus, the existence of multiple genes, each capable of encoding alternatively spliced mRNAs, represents a potential source for considerable ankyrin isoform diversity. Currently, the magnitude of this diversity includes at least five isoforms: two from the brain ankyrin gene (1 and 2) and three from the erythrocyte ankyrin gene (2.1 and 2.2 and a highly basic alternative COOH-terminal sequence noted by Lambert et al. [25]). However, detection of additional mRNAs in brain and other tissues, using various brain and erythrocyte ankyrin probes (Figs. 2 and 9), indicates the existence of additional isoforms related to additional genes and/or alternatively spliced mRNAs.

A rational nomenclature has not yet evolved to deal with the existence of ankyrins encoded by distinct genes each with...
a variety of spliced variants and expressed in multiple tissues. A useful simplification is that ankyrins would be described by the gene that encodes them rather than the tissue where they are expressed. One possibility is that the genes would be assigned numbers according to the order in which they were cloned. According to this system, erythrocyte ankyrin would be ankyrin 1, and brain ankyrin would be ankyrin 2. Alternatively, the genes could be designated by mnemonic letters based on the tissue or cell where the ankyrin is highly expressed, or by some other distinguishing feature. Erythrocyte ankyrin could be referred to as ankyrinR (R for red cell and restricted), since it exhibits a restricted distribution in brain where it is most highly expressed in cerebellar neurons, and brain ankyrin as ankyrinB (B for brain and broadly distributed), since it is broadly distributed in brain tissue. The various spliced products of these genes could be referred to by their SDS gels: brain ankyrin 2 in this report would be 220 kD ankyrinB or 220 kD ankyrin 2 depending on the preference for letters or numbers.

A striking feature of the membrane-binding domains of ankyrinR and ankyrinB is the presence of a repeated 33-amino acid motif that is also present in a number of diverse proteins of broad phylogenetic distribution. Originally detected within the products of cell cycle control proteins cdc 10 of Schizosaccharomyces pombe and SW16 of Saccharomyces cerevisiae (6), this motif has also been noted in several proteins involved in cell differentiation. Four of these proteins, Drosophila Notch (37), Xenopus xotch (8), and Caenorhabditis elegans lin-12 (39) and glp-1 (38) encode proteins with similar organization: each is a transmembrane protein with an extracellular domain containing multiple copies of an EGF-related motif and an intracellular domain containing six copies of the 33 amino acid motif. This motif has also been found within fem-1, a protein involved in sex determination of both germine and somatic tissues in C. elegans (34). This protein also contains six copies of this repeat; however, it lacks the EGF-like repeats and is predicted to be a soluble intracellular protein. Most recently, this motif has been found in the precursors of the transcription factor NF-kappaB (17, 22). The functional significance of this motif is not yet clear. The processes in which these proteins are involved, however, suggest a possible role in protein–protein interactions. Consistent with this idea, it has recently been shown that ankyrinR repeats bind to the anion exchanger with high affinity (12).

Both ankyrins contain 22 copies of the 33 amino acid motif, with 21 of the ankyrinR repeats being most similar to the corresponding ankyrinB repeat. Taken together, these results strongly suggest that the genes encoding these ankyrin isoforms arose by duplication of an ancestral gene that also contained 22 copies of this motif. Such strict conservation is consistent with a relatively recent duplication event. However, given that these genes also contain several areas of almost complete sequence divergence, have distinct patterns of expression, and are linked to different chromosomes, it is more likely that this duplication occurred long ago and that the areas of strict conservation reflect functional requirements. It is of interest in this regard that the minimum amount of sequence for stable folding encompasses at least nine repeats for erythrocyte ankyrin based on studies with recombinant proteins (Davis, L., E. Otto, and V. Bennett, unpublished data). The 22 repeats thus are not independently folded, and are likely to be assembled as an integrated unit.

The membrane-binding domain of erythrocyte ankyrin is approximately spherical in shape based on physical properties (12). Such a conformation requires that the repeats be packed into a sphere or compact helix, but not as an extended rod as is the case with many proteins containing multiple amino acid repeats. Conservation of 22 repeats may reflect the maximum number of repeats that can be packed into a sphere.
Several regions of human ankyrins show almost complete sequence divergence, and are thus likely candidates to explain the functional differences between these isoforms: (a) the NH2-terminal region; (b) the region linking the membrane- and spectrin-binding domains; and (c) the COOH-terminal region. In support of this view, previous studies have demonstrated the functional significance of two of these regions in ankyrinR. The COOH-terminal region of ankyrinR contains several domains involved in regulation of binding. Deletion of one of these domains by alternative splicing produces a smaller form of ankyrin with increased affinities for spectrin and increased association with the anion exchanger.

Figure 8. Linkage of human brain ankyrin to chromosome 4 using human/hamster cell hybrids. (A) Southern blot of genomic DNAs isolated from a panel of human/rodent cell hybrids (BIOS, Corp.) and hybridized to 32P-labeled brain ankyrin cDNA. Each lane contained 8 μg DNA from human cells, hamster cells, hybrid line 803, hybrid line 967, hybrid line 968, or hybrid line 683. Hybrid lines shown represent only a portion (4 out of 25) of those screened; data from lines not shown, however, were consistent with linkage of brain ankyrin to chromosome 4. (B) Human chromosomes present in cell lines shown in A. Karyotypes were performed by BIOS, Corp.

Figure 9. Accumulation of brain and erythrocyte ankyrin mRNAs in rat tissues. Duplicate Northern blots of adult rat RNA were hybridized to 32P-cDNA probes encoding 33-amino acid repeats of brain (A) and erythrocyte (B) ankyrins. Each lane contained 7.5 g of poly A+ RNA isolated from adult brain (lane 1), spleen (lane 2), kidney (lane 3), or liver (lane 4). For each probe, short exposures (a) and tenfold longer exposures of selected lanes (b, primed numbers) are shown. Spleens were isolated from phenylhydrazine-treated rats; all other tissues were isolated from untreated rats. Relative to weight loaded, the amount of ~13 kb transcript in adult brain is lower than in 10-d-old rats (compare with Fig. 2, and see text). Nucleotide boundaries of the DNA fragments used as probes are described in Materials and Methods. As a control for RNA integrity, hybridization of blots of these RNA samples to a human alpha adducin cDNA probe produced intense discrete signals for all tissues (data not shown).
in erythrocyte membranes (19,26). This smaller ankyrin, known as protein 2.2, also contains a binding site for a major class of unidentified proteins in kidney microsomes that are not recognized by the larger form of ankyrin (11). Thus, the COOH-terminal region of ankyrinB not only modulates binding affinities but defines specificity in binding to membrane sites as well. The lack of sequence similarity between the two forms of ankyrinB in this region suggests that these alternatively spliced variants may also differ in the membrane sites that they recognize. Sequences at the NH2-terminus of the spectrin-binding domain of ankyrinB have been shown to be required for high affinity binding, also suggesting the unique functional potential of the corresponding region of ankyrinB (12).

The proteins that are associated with the various forms of ankyrinB are not yet established, although it is likely that they will differ from proteins coupled to ankyrinR. We have expressed a portion of the membrane-binding domain of human brain ankyrin in bacteria and have used the recombinant protein to identify at least 10 new ankyrin-binding proteins from brain membranes (Davis, J., E. Otto, and V. Bennett, unpublished results). Thus, human brain ankyrin cDNA and expressed proteins should provide useful tools for isolating and characterizing ankyrin-binding proteins.

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