**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 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 Major = 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 Major = 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.
ankyrinβ 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 ankyrinβ 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, ankyrinα, react better with antibodies against bovine brain ankyrin. In brain, ankyrinα is present in both glial and neuron cells, and is not concentrated at the node of Ranvier (24). The membrane attachment sites for ankyrinα 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 (ankyrinα 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 ankyrinα and ankyrinβ forms, we have isolated cDNAs encoding human ankyrinα 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 gtl1/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 10^6 cpm/μg) prepared by randomly primed DNA synthesis (Multiprime System; Amersham Corp., Arlington Heights, IL). All hybridizations were performed at 65°C using 5x SSC, 0.3% SDS. Filters were washed twice with 5x SSC, 0.3% SDS for 10 min at 25°C, once in 2x SSC, 0.3% SDS for 15 min at 54°C; and then twice in 0.1x SSC, 0.3% SDS for 15 min at 65°C. Total RNA was isolated using RNAzol (Cinna/Biotech, 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 eliminating 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 β-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, re pelleted, 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) diisopropyl fluorophosphate, 10 mM benzamidine). After 10 min on ice, DNAase (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: 1.0% (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 derived from by 583-2,192 (brain ankyrin) and by 997-2,379 (erythrocyte ankyrin).

Antibodies

Procedures used for the affinity purification of antibodies were described by Davis and Bennett (12). Antisera 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 T7 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 erythrocyte ankyrin 2.1, against the portion of erythrocyte ankyrin 2.1 absent from spliced variant 2.2 (26), and against bovine brain ankyrin (24) have been described.

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-labeled 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 2x SSC, 0.5% SDS for 10 min at 25°C, once in 1x SSC, 1.0% SDS for 15 min at 65°C; and then twice in 0.1x SSC, 1.0% SDS for 15 min at 65°C. The Journal of Cell Biology, Volume 114, 1991
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, Bam-HI; 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.
Figure 2. Alternatively spliced mRNAs related to brain ankyrins 1 and 2. Northern blot of oligo-dT selected RNA isolated from whole brains of 10-d-old rats. Each lane contained 10 μg of RNA and was hybridized to 32P-labeled probes derived from bp 2,841–4,763 (lane a), bp 4,344–5,028 of brain ankyrin 1 (lane b), bp 4,351–4,930 of brain ankyrin 2 (lane c), or bp 583–2,192 (lane d).

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 sequenc- e 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 organiza-

tions, 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 isoforms: (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)6 -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-thirds of the repeat sequences), 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 repeat sequences 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 28 residues in both, and an additional 6 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-thirds 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.
Distinct Patterns of Expression

Members of Human Ankyrin Gene Family Show Distinct Patterns of Expression

Brain and Erythrocyte Ankyrins Are Encoded by Different Genes

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).

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, = 220 kD and M, = ~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. 10A, panel b). A single polypeptide of 220 kD was detected with membranes from temporal cortex of adult human brain (Fig. 10B). 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
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 Group program BESTFIT are shown as capital letters.

**Nucleotide Sequence:**

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TCACAACTGTCCAAAATGTGAGAGACGAGATGCTGACAAAAGGAGCACGAGAGACTCTAGGCATCTGAGAGGGAGGTGAGAGCTGAGAGGAGACGAGAGATGCTGACAAAGGAAGGAGGAGACGAGAGATGCTGACAAAGG
LCRAARaAGHLDVeeyyLkg1GCJH1CTNHG1MNLA1HLRAaRREG
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**Amino Acid Sequence:**

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FTKRiFvRkK1195
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Further details and alignment are provided in the image. The figure illustrates the nucleotide and amino acid sequences with specific annotations and alignments.
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 ankyrin 1 (rl-r15) is shown below line 4. The eight amino acids interrupting the periodicity of the 33-amino-acid repeats are indicated.
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.

Discussion

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 ankyrin B. 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 ankyrin B 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 $M_r = 220$ 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 ankyrin B 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 chromo-
some 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 al-
ternatively spliced mRNAs, represents a potential source for
considerable ankyrin isoform diversity. Currently, the mag-
nitude of this diversity includes at least five isoforms: two
from the brain ankyrin gene (1 and 2) and three from the
erthrocyte ankyrin gene (2.1 and 2.2 and a highly basic al-
ternative 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

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**Figure 5.** 33-amino acid repeats present in ankyrins and diverse proteins involved in cell differentiation, cell cycle control, and transcrip-
tion. (A) Optimal alignment of human brain ankyrin repeats
(R1-R22). Amino acids conserved in at least two-thirds of the
repeats are stippled and summarized in a consensus. The erythro-
cyte ankyrin repeat that is most similar to each brain ankyrin repeat
is indicated within parentheses and was determined using the
University of Wisconsin Genetics Computer Group program
BESTFIT. (B) Similarly derived consensus sequences of repeats in
human erythrocyte ankyrin (26); Drosophila Notch (37); Xenopus
xotch (8); C. elegans lin-12 (39), glp-1 (38), and fem-1 (34); human
NF-kB precursor (22); and yeast SWI6 (6) and cdc10 (1). (C) Com-
parison of corresponding brain and erythrocyte ankyrin repeats.
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 ankyrin\textsubscript{R} (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 ankyrin\textsubscript{B} (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 \( M \) on SDS gels: brain ankyrin 2 in this report would be 220 kD ankyrin\textsubscript{B} or 220 kD ankyrin 2 depending on the preference for letters or numbers.

A striking feature of the membrane-binding domains of ankyrin\textsubscript{R} and ankyrin\textsubscript{B} 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 \textit{Schizosaccharomyces pombe} and SW16 of \textit{Saccharomyces cerevisiae} (6), this motif has also been noted in several proteins involved in cell differentiation. Four of these proteins, \textit{Drosophila} Notch (37), \textit{Xenopus} notch (8), and \textit{Caenorhabditis elegans} \textit{lin-12} (39) and \textit{glp-1} (38) encode proteins with similar organization: each is a transmembrane protein with an extracellular domain containing multiple copies of a EGF-related motif and an intracellular domain containing six copies of the 33 amino acid motif. This motif has also been found within \textit{fred-1}, a protein involved in sex determination of both germline and somatic tissues in \textit{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 ankyrin\textsubscript{R} 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 ankyrin\textsubscript{R} repeats being most similar to the corresponding ankyrin\textsubscript{B} 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.

**Figure 6.** 12-amino acid repeats of brain ankyrin 1. Optimal alignment of brain ankyrin 1 repeats (r1–r15). Amino acids conserved in at least two-thirds of the repeats are stippled and summarized in a consensus.

**Figure 7.** Genomic organization of human brain and erythrocyte ankyrins. Duplicate Southern blots of HeLa DNA were hybridized to \textsuperscript{32}P-cDNA probes encoding 33-amino acid repeats of brain (a) and erythrocyte (b) ankyrins. Each lane contained 10 \( \mu \)g of DNA that was digested with EcoRl (lane 1), PvuII (lane 2), or BamHl (lane 3). Nucleotide boundaries of the DNA fragments used as probes are described in Materials and Methods.
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 (26) (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 ~7 kb transcript in adult brain is lower than in 10-d-old rats (compare with Fig. 2, and see text).]
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 ankyrinR (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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