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The Brain Link Protein-1 (*BRAL1*): cDNA Cloning, Genomic Structure, and Characterization as a Novel Link Protein Expressed in Adult Brain

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We report here molecular cloning and expression analysis of the gene for a novel human brain link protein-1 (*BRAL1*) which is predominantly expressed in brain. The predicted open reading frame of human brain link protein-1 encoded a polypeptide of 340 amino acids containing three protein modules, the immunoglobulin-like fold and proteoglycan tandem repeat 1 and 2 domains, with an estimated mass of 38 kDa. The brain link protein-1 mRNA was exclusively present in brain. When analyzed during mouse development, it was detected solely in the adult brain. Concomitant expression pattern of mRNAs for brain link protein-1 and various lectican proteoglycans in brain suggests a possibility that brain link protein-1 functions to stabilize the binding between hyaluronan and brevican. The human *BRAL1* gene contained 7 exons and spanned ~6 kb. The entire immunoglobulin-like fold was encoded by a single exon and the proteoglycan tandem repeat 1 and 2 domains were encoded by a single and two exons, respectively. The deduced amino acid sequence of human brain link protein-1 exhibited 45% identity with human cartilage link protein-1 (*CRTL1*), previously reported as link protein to stabilize aggregates of aggrecan and hyaluronan in cartilage. These results suggest that brain link protein-1 may have distinct function from cartilage link protein-1 and play specific roles, especially in the adult brain. © 2000 Academic Press

Key Words: **link protein; lectican proteoglycan; hyaluronan; brain.**

The DNA sequences of human, mouse, and rat cDNAs of BRAL1 and that of human genomic DNA of BRAL1 gene were submitted to DDBJ/EMBL/GenBank/DNA database through DDBJ under Accession Nos. AB049054 to AB049063.

Abbreviations used: EST, expressed sequence tag; RACE, rapid amplification of cDNA ends; PTR, proteoglycan tandem repeat; PCR, polymerase chain reaction; Ig, immunoglobulin like.

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An increasing number of hyaluronan (HA)-binding extracellular matrix and membrane molecules have been identified and are now referred to as the link module superfamily (1–4). The link module superfamily possesses a common structural domain of about 100 amino acids, from which the family name was derived. The chondroitin sulfate proteoglycans (CSPGs), e.g., aggrecan, versican/PG-M, neurocan, and brevican, display a high degree of homology in their N- and C-terminal globular domains. Their N-terminal homologous domain contains an immunoglobulin-like fold and two link modules, mediating their binding to hyaluronan. At the C-terminus, there is an epidermal growth factor (EGF)-like repeat, a C-type lectin-like domain, and a complement regulatory protein-like domain. These globular domains are connected by the central region, which contains attachment sites for chondroitin sulfate chains and differs in size. These proteoglycans were recently termed either lecticans (5) or hyalectans (6). Aggrecan and versican are ubiquitously expressed in extracellular matrix, whereas neurocan and brevican are present specifically in the central nervous system (1, 2). Four members of the lecticans showed differential expression patterns in developing rat brain from embryonic day 14 to eight month postnatal. (7). CD44 is a major and ubiquitous receptor for HA that anchors the extracellular matrix to a variety of cells (8). LYVE-1 is a newly recognized homologue of CD44, which is a lymphocyte-specific receptor for HA (9). TSG-6 is a 35-kDa protein that has been found to be secreted by a number of cell/tissue

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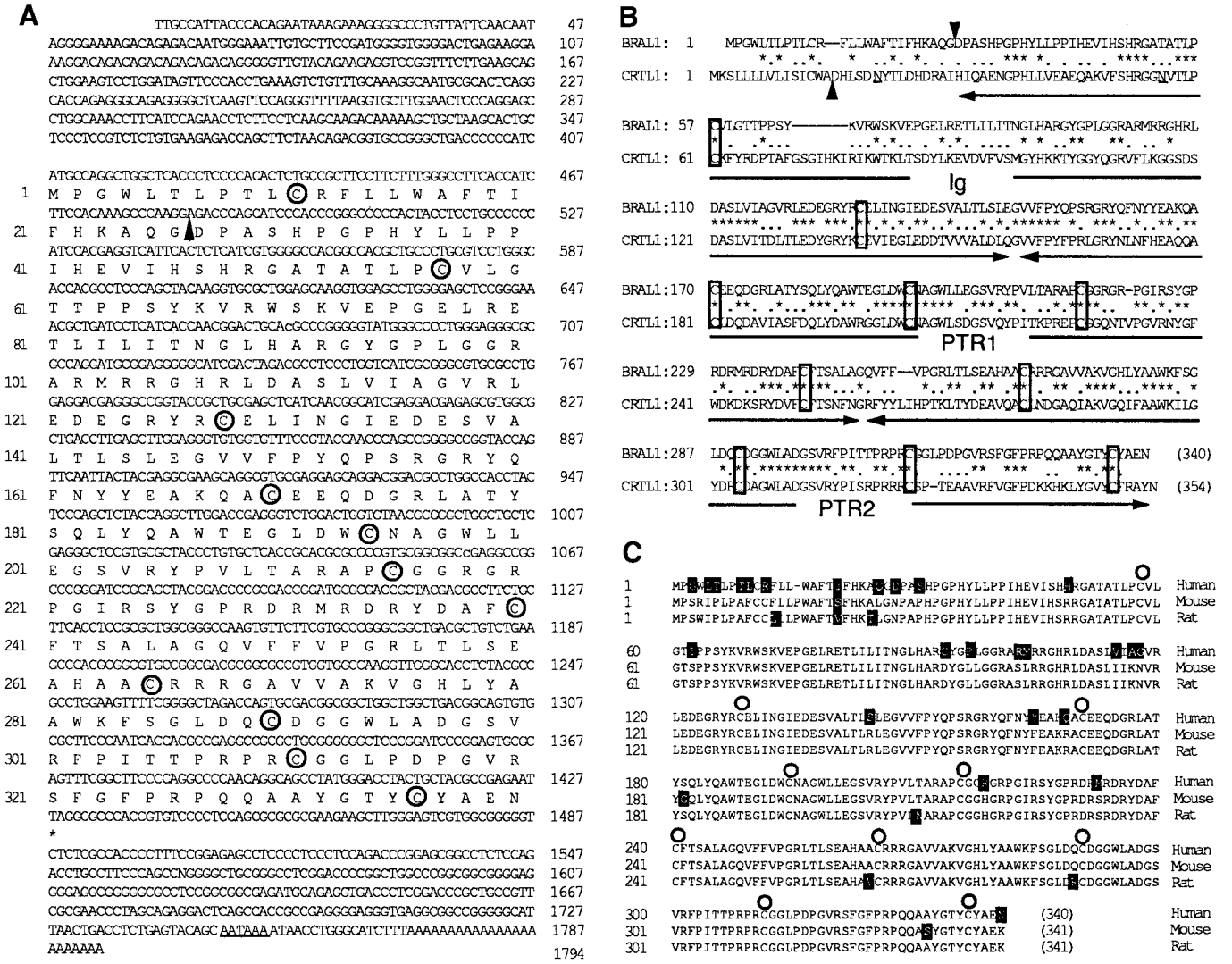

FIG. 1. (A) Nucleotide and deduced amino acid sequences of human *BRAL1* cDNA. The stop codon, TAG, is indicated by an asterisk. Conserved cysteinyl residues are circled. Putative cleavage site for signal peptidase is indicated by an arrowhead. (B) Comparison of the deduced amino acid sequences of human *CRTL1* and *BRAL1*. Identical amino acid residues are marked by asterisks. Horizontal arrows indicate the extension of the three modules. Putative signal peptidase cleavage site is indicated by the arrowhead. Sites of the potential N-linked glycosylation are underlined. Note that the ten cysteinyl residues (blocked) are all conserved in the two sequences. (C) Comparison of deduced amino acid sequences of the human, mouse, and rat brain link protein-1. Shaded amino acid residues highlight non-identical amino acid residues among three species.

types in response to inflammatory mediators and growth factors (10).

The cartilage link protein-1 is a major structural component of cartilage and is present in various connective tissues (4). It stabilizes the aggrecan/hyaluronan aggregate by binding to both components. Analysis of knockout mice of the cartilage link protein-1 gene demonstrated that the cartilage link protein-1 is important for the formation of proteoglycan aggregates and normal organization of hypertrophic chondrocytes (11). Contrary to the fact that the lecticans contain at least four members having distinct functions, the link protein has been thought to be encoded by a single gene to date. In the present study, we describe the cDNA cloning, genomic structure, and characterization of another novel link protein, *BRAL1*.

MATERIALS AND METHODS

Isolation of cDNA and genomic clones. A human (GenBank Accession No. AI370911), a mouse (GenBank Accession No. AA967720), and a rat EST cDNA clone (GenBank Accession No. AA145729) were supplied by Research Genetics, Inc. (Huntsville, AL). A 914 bp BanHI/NotI fragment from the 5′ end of the rat EST clone was randomly labeled with [α-^{32}P]-dCTP (Amersham Pharmacia, Uppsala, Sweden) and used to screen an oligo dT-primed mouse adult

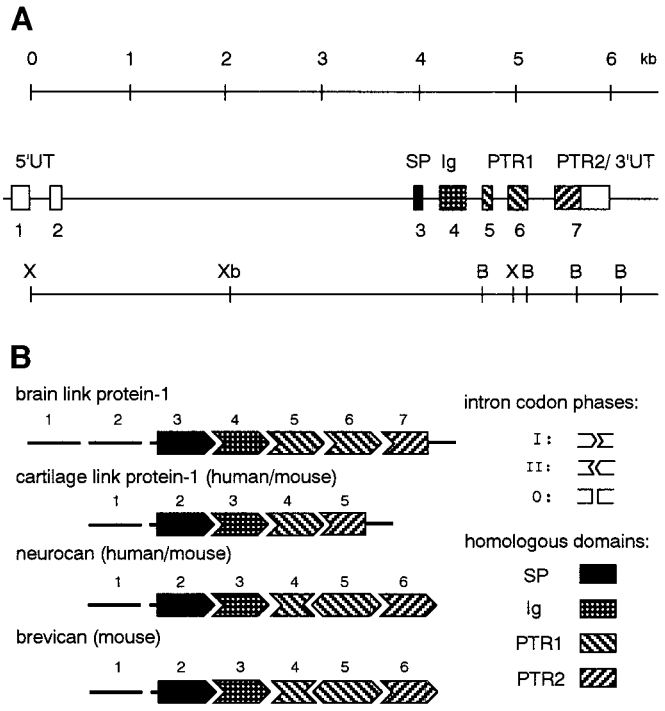

FIG. 2. (A) Exon organization of the brain link protein-1 gene. The *BRAL1* gene contained 7 exons. UT, untranslated region; SP, signal peptide; IG, immunoglobulin-like; PTR, proteoglycan tandem repeat. X, Xho; Xb, Xba; B, Bam HI (B) Alignment of the genomic exon organization of the human *BRAL1* with the genes of the human *CRTL1*, human and mouse neurocan, and mouse brevican. The codon phases of the intron boundaries are indicated: 0 indicates boundaries of introns inserted between codons; and I and II indicate introns inserted after the first or second nucleotide of a codon, respectively. Abbreviations are as above.

brain cDNA library (ML1042a; Clontech, CA) and rat adult brain cDNA library (RL5003t; Clontech, CA) as described (12). A human *BRAL1* EST clone (GenBank Accession No. AI370911) was used to probe a human genomic library constructed in the EMBL3 SP6/T7 vector (HL1067j: Clontech, CA). A 20-kb genomic clone (MH322) was isolated and further characterized by restriction mapping analysis and by Southern blot hybridization.

5′ Cap site. 5′ RACE was performed by using a Cap Site cDNA kit for human brain (Nippon gene, Toyama, Japan) according to the manufacturer's protocol. Primers for the first PCR were 5′-CAAGGTACGCCACAGCGTATG-3′ (1RC primer) and 5′-GAGAGTGAATGACCTCGTGGATGG-3′ (HLP2 RC1 primer). The nested PCR primers were 5′-GTACGCCACAGCGTATGATGC-3′ (2RC primer) and 5′-GCTTTGTGGAAGATGGTGAAGGC-3′ (HLP2 RC2 primer). One micro L of the original concentration of cDNA was used for the first PCR. PCRs were performed with rTaq DNA polymerase (Takara, Kyoto, Japan) in a reaction volume of 20 μl. Amplifications were carried out for both the first and nested PCRs by using an initial denaturation at 94°C (2 min) followed by 35 cycles of 94°C (30 s), 58°C (30 s), and 72°C (1 min 30 s), and final extension at 72°C (2 min). Southern hybridization using the ^{32}P-labeled 5′UTR fragment of the human *BRAL1* EST clone was performed for each PCR product to confirm the specific amplification.

Sequence analysis. Clones were inserted into pBluescript SK vector (Stratagene, CA) or into pCRII vector (Invitrogen, CA), and both DNA strands were sequenced by using an ABI BigDye terminator cycle sequencing kit (Perkin Elmer, CA). The sequences were analyzed with an ABI 310 capillary automatic sequencer (Perkin Elmer, CA). Comparison of deduced amino acid sequences were performed with CLUSTAL W program (13).

RNA preparation and Northern blot analysis. Total RNA was isolated from rat costal cartilage and brain using 1 ml of TRIZOL reagent (Gibco BRL, MD) according to the manufacturer's instruction. For Northern analyses RNA was electrophoretically separated, blotted onto Hybond-N membrane (Amersham Pharmacia, Uppsala, Sweden), UV cross-linked, and probed in Church buffer (14) at 65°C. Filters were washed in 2 × SSC/1% SDS and 0.4 × SSC/1% SDS at 65°C and exposed to an X-ray film for 1 day at −80°C. For quantification, the filter was exposed to an imaging plate. The plate was analyzed with a Bioimage Analyzer (BAS 2000; Fuji Film, Tokyo, Japan).

RESULTS AND DISCUSSION

Isolation of Brain Link Protein-1 cDNA

The screening of EST cDNA library was performed by BLAST search with an amino acid sequence of mouse brevican (156–355; GenBank Accession No. X87096) protein tandem repeat as a query (15). A rat EST cDNA clone (GenBank Accession No. AI145729) was cloned with an e-value of 3e-31. Using the rat EST cDNA sequence, a human (GenBank Accession No. AI370911) and a mouse (GenBank Accession No. AA967720) EST cDNA were also cloned. The human EST cDNA clone contained a full-length coding se-

TABLE 1

Exon–Intron Boundaries of the Human Brain Link Protein-1 Gene

Exon No.	Domain	Exon size (bp/aa)	Splice donor	Intron size (bp)	Splice acceptor	Codon phase	Amino acid
1	5′ UT	242	CAGAG gtgagt	482	gaacag GGGCT		
2	5′ UT	140	AACAG gtaggc	~4000	ctgcag ACGGT		
3	SP	109/29	C CCA G gtaaga	231	gcccag CA TCC	I	Ala 29
4	Ig	354/118	G GAG G gtgagg	188	ccctag GT GTG	I	Gly 147
5	PTR1-1	117/39	C CAG G gtgagc	133	cgccag CT TGG	I	Ala 186
6	PTR1-2	183/61	G GCG G gtgagg	317	ccccag GC CAA	I	Gly 247
7	PTR2/3′ UT	>284 + 324/93					

Note. Exon sequences are in capital letters and divided into codon triplets; intron sequences are in lowercase letters. Codon phases and abbreviations are the same as in Fig. 2.

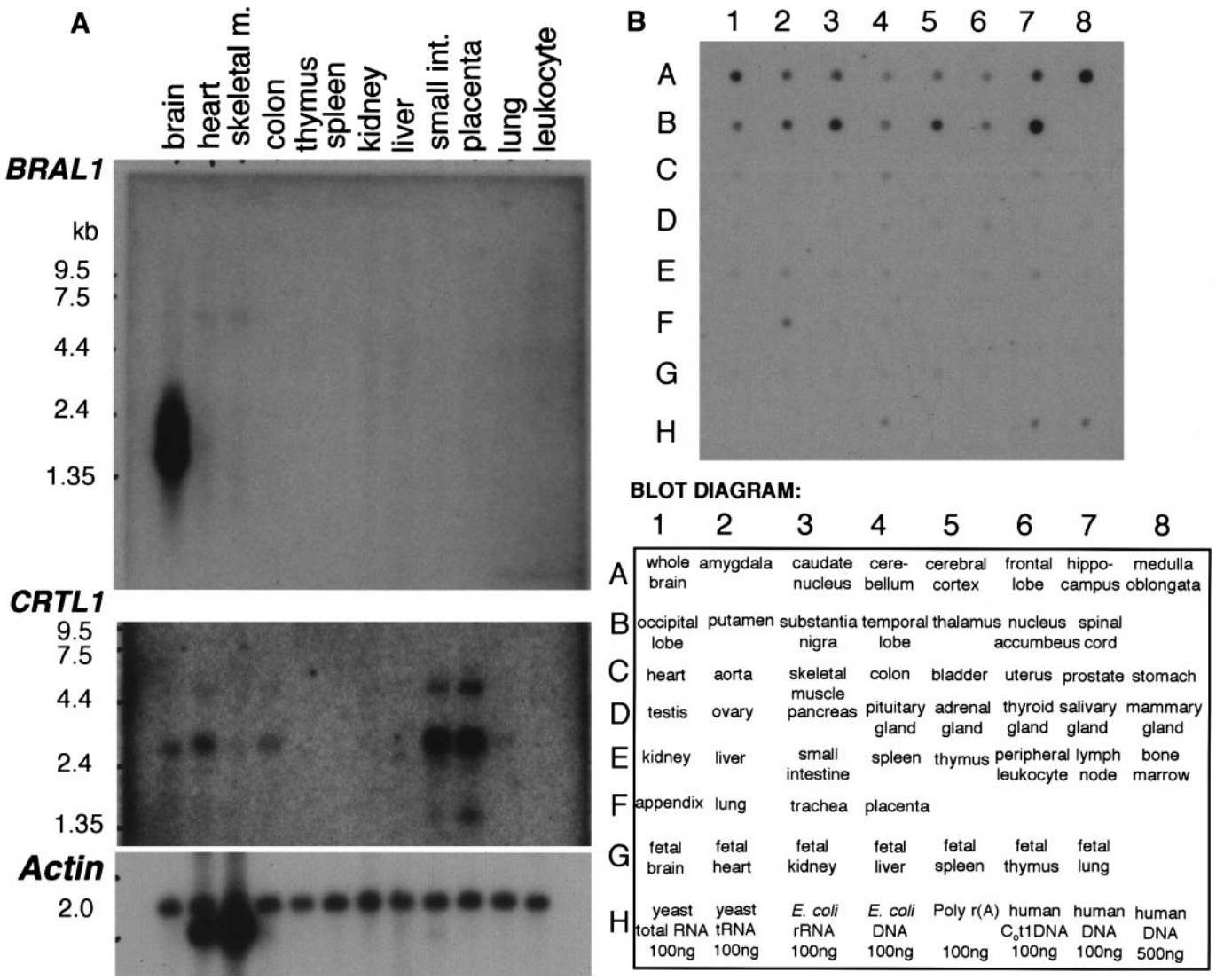

FIG. 3. Northern blot analysis of *BRAL1* and *CRTL1*. (A) Expression of mRNA for *BRAL1* and *CRTL1* in adult human tissues. Multiple Tissue Northern filter (Clontech) containing 1 μg of poly(A)+ RNA isolated from the indicated tissue sources was hybridized consecutively with the cDNAs of *BRAL1* and *CRTL1* coding sequences and GAPDH. (B) Expression of mRNA for *BRAL1* in human adult tissues. Human RNA Master Blot (Clontech) containing a normalized load of 100–500 ng of each poly(A)+ RNA isolated from various tissues was hybridized with cDNA of *BRAL1*.

quence and terminated with a poly (A) tail. To determine the 5′ end of the human mRNA, we conducted 5′ RACE using the human brain "Cap Site cDNA" (Nippon gene, Toyama, JAPAN). The PCR products that hybridized as a single band with the EST cDNA probe were cloned and the inserts were sequenced (data not shown). The 5′ sequences of the four clones obtained started from exactly the same nucleotide and were also confirmed in the genomic DNA that is described below. The screening of mouse adult brain cDNA library resulted in the isolation of three overlapping clones (SL1–SL3), extending to 5′ direction. To obtain rat cDNA clones extending in the 3′ direction, we screened a brain cDNA library from adult rat. Two more clones, RB3 and RB4, were isolated. The complete sequence analysis of the human cDNA clone demonstrated an open reading frame of 1020 bp. The deduced amino acid sequence contained 340 residues with a calculated molecular mass of 38 kDa for the mature human protein. The deduced amino acid sequence of the human cDNA showed a high degree of homology to N-terminal part of lectican proteoglycans (40–45%, refs. 16–20). However, they were clearly different from these molecules. The polypeptide started with a typical hydrophobic signal peptide sequence, and the PSORT II program (http://psort.nibb.ac.jp:8800/) predicted its cleavage site at position 26/27 (21 Fig. 1A). A consensus polyadenylation signal, AATAAA, was located 23 bp upstream of the poly (A)+ tail. The deduced amino acid sequence of the human cDNA exhibited 45% identity

with the human cartilage link protein-1 (22). Furthermore, the relative locations of the cysteinyl residues were exactly the same as those of the cartilage link protein-1 (Fig. 1B). From the structure of the predicted protein and its gene expression as described later, we designated the novel gene brain link protein-1 (*BRAL1*). The amino acid sequence deduced from the human *BRAL1* cDNAs showed 91% identity to that deduced from the both mouse and rat *bral1* cDNAs, indicating a strong conservation across different species (Fig. 1C).

Structure of Human Brain Link Protein-1

The link protein contains three protein modules, the Ig fold, PTR1 domain, and PTR2 domain. Alignment of the cartilage link protein-1 and predicted brain link protein-1 polypeptides showed that they were quite similar, especially in PTR1 (57%) and in PTR2 (54%). The amino acid sequences of the Ig domains were less homologous (38%) than those of the PTR domains (Fig. 1B). Ten cysteinyl residues, which define five disulfide bonds and are thought to be very important to maintain the domain structure, were well conserved. X-ray and neutron solution scattering studies showed that a ternary complex with the aggrecan G1 domain, cartilage link protein-1, and hyaluronan is formed by side-by-side contact between G1 and cartilage link protein-1 (23). The alignment of 59 PTR sequences of the link module super family and predictions of secondary structure implicated several basic residues important for hyaluronan binding. The arginine residue at position 169 in cartilage link protein-1 is conserved in all the PTRs (24). The arginine residue was shown by site-directed mutagenesis to be important for hyaluronan binding (25). The arginine residue was also conserved at the relative position 158 in brain link protein-1. The human cartilage link protein-1 has two putative N-linked glycosylation sites (Asn at position 21 and 56) in the Ig fold, and these sites are conserved among several species. However, the all human, mouse, and rat brain link protein-1 lacked the recognition sequence at the two corresponding positions.

Intron/Exon Structure of the Human BRAL1 Gene

Restriction mapping analysis, Southern blot hybridization and partial sequence analysis of the MH322 clone revealed that the mRNA coding for *BRAL1* was organized in 7 exons, which spanned ~6 kb of genomic DNA (Fig. 2A). The transcriptional start site was confirmed by 5′RACE as described above. The first and second exons contained only the untranslated sequence. The last 24 nt of the 5′ untranslated sequence and the coding sequence of the signal peptide were contained in exon 3. The *BRAL1*, which is comprised of one immunoglobulin (Ig) fold and two proteoglycan tandem repeat (PTR) domains, was encoded by four exons. A single exon encoded the entire Ig fold; two exons, the first PTR; and one exon, the second PTR. The following 3′ untranslated region was present in the last exon analyzed. All exon–intron junctions followed the GT/AG rule (Table 1). All codon phases of those introns that separated modular domains showed phase I interruption, as was the case for *CRTL1* (26). The *BRAL1* gene structure reflects the modular protein structure. Each of these modules was encoded by a single or two exons. The structure of *BRAL1* conforms closely to the structure of the previously known link module superfamily genes in the number of protein-coding exons and the location of splice sites (Fig. 2B, refs. 27–30). Our data demonstrate the closely conserved gene structure of all members of the link module superfamily, which is in keeping with the apparent functional similarity between the products of these genes.

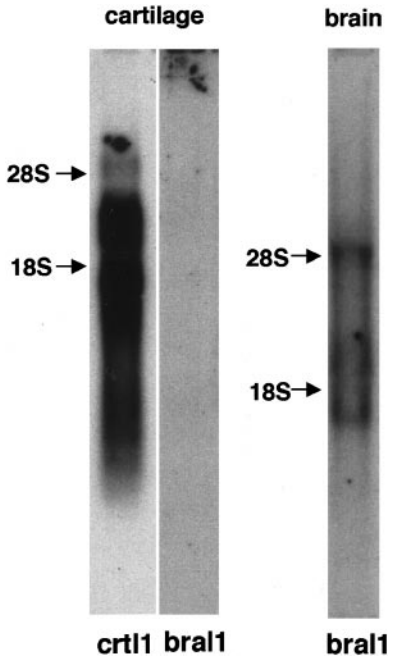

FIG. 4. Northern blot analysis of *bral1* and *crtl1* in cartilage. Fifteen micrograms of total RNA was isolated from 6-week-old rat costal cartilage and probed with *bral1* and *crtl1* cDNAs. Brain blot is shown as a probe control for *bral1*.

Tissue Distribution of Brain Link Protein-1 mRNA

Since the EST cDNA clone was initially isolated from a rat brain cDNA library, we further examined the expression of human *BRAL1* mRNA in various tissues by using commercially available Northern and Master Blot (Clontech, CA). As shown in Fig. 3A, *BRAL1* mRNA 1.8 and 2.2 kb in size were detected solely in the adult brain. By contrast, *CRTL1* mRNA was broadly expressed in many tissues and weakly expressed in the brain. Thus, the expression pattern of mRNA for

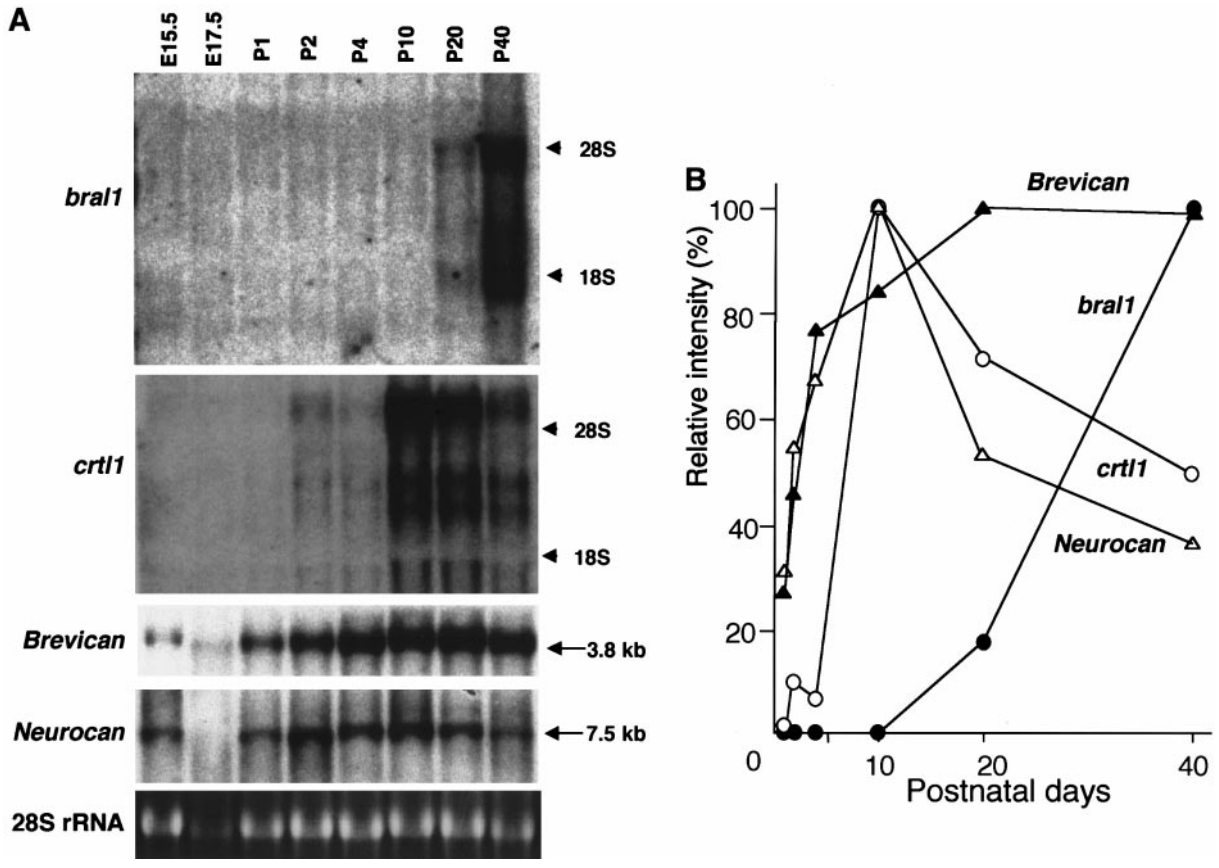

FIG. 5. Northern blot analysis of *bral1, crtl1,* neurocan and brevican in developing brain. (A) Fifteen micrograms of total RNA isolated from different days of mouse brain were loaded on the agarose gel and transferred to the membrane, which was hybridized consecutively with cDNAs for *bral1* and *crtl1*, neurocan and brevican. To show the quality and integrity of RNA, 28S rRNA was visualized by ethidium bromide staining. (B) Relative levels of expression of link proteins and lecticans mRNA were plotted after normalization to 28S rRNA.

BRAL1 in the adult human was quite different from that of the mRNA for *CRTL1*. The human RNA Master Blot, which contained normalized loading of 100–500 ng of each poly (A)+ RNA per dot showed that the *BRAL1* had very little expression in the fetal brain (Fig. 3B). *crtl1* is known to be abundantly expressed in cartilage and to play a critical role in chondrocyte differentiation and maturation (4, 11). Total RNA was prepared from rat costal cartilage, and hybridized with the *crtl1* and *bral1* cDNA probes. The *crtl1* showed a prominent expression in cartilage (Fig. 4). On the other hand, *bral1* mRNA was not detected in cartilage when it was detected in the control brain. We suggest, therefore, that the two link proteins play distinct roles in adult tissues.

Brain Link Protein-1 mRNA Expression in Developing Brain

Since the *BRAL1* seems to be expressed specifically in brain, we studied the temporal expression pattern of mouse *bral1* in developing brain up to 40 days of age by Northern blot and compared results with those of *crtl1*, brevican and neurocan. Milev *et al.* have examined the expression of hyaluronan-binding chondroitin sulfate proteoglycans in developing rat brain quantitatively using specific antibodies recognizing the respective non homologous domain (7). They showed the individual proteoglycans and their isoforms are differentially regulated both in the embryonic and postnatal period. Their expression patterns were subdivided into two groups. One of the brain specific lectican proteoglycan, neurocan, reached a peak in the early postnatal period and declined thereafter. The other brain specific lectican proteoglycan, brevican, increased steadily after birth to reach an adult level. The *bral1* mRNA expression starts at day 20, when neuronal pathways are already well established, and increase notably thereafter (Fig. 5). Temporal expression pattern of *bral1* mRNA in brain coincides rather with that of brevican than that of neurocan. These results indicate that brain link protein-1 is most likely functioning at least to stabilize the binding between hyaluronan and brain-specific lecticans such as brevican. However, there is a subtle discrepancy of expression between brevican and

brain link protein-1 in the early postnatal days. Further investigation is necessary to elucidate the functional relationship of brain link protein-1 and lecticans. Taken together, the results suggest the importance of the brain link protein-1 for maintenance and function of adult brain.

The New Gene of Link Protein

Molecular cloning has enabled the identification of a family of lecticans/hyalectans that share structural and functional similarities at both the genomic and protein levels. This family currently contains four distinct genes as described above. The structural features of lecticans/hyalectans are made with the tridomain of the core protein and the glycosaminoglycan side chains attached to the central domain. The major functional roles of lecticans/hyalectans would be to bind hyaluronan at their N-termini. Different splice variants and various degrees of glycation and glycosylation of the central domain also provide various functions. Differential expression of lecticans/hyalectans in the developing brain implies different functions for them in central nervous system development.

In contrast, the link protein gene has known as a single gene and supposed to stabilize the aggregates of lecticans/hyalectans and hyaluronan. No other link protein gene had been reported until now. Here, we have reported for the first time a new link protein gene that is expressed solely in the central nervous system. We have designated the new link protein gene as *BRAL1*. The finding of *BRAL1* will provide an idea that the link proteins have individual diverse functions as do the lecticans/hyalectans.

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