Cbln3, a Novel Member of the Precerebellin Family that Binds Specifically to Cbln1

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Precerebellin (Cbln1) is the precursor of the brain-specific hexade-capeptide cerebellin. Although cerebellin has properties of a conventional neuropeptide, its function is controversial because Cbln1 has structural features characteristic of circulating atypical collagens. Cbln1 is related to the three subunits of the complement C1q complex. Therefore, we hypothesized that Cbln1 participated in analogous heteromeric complexes with precerebellin-related proteins. Using LexA-Cbln1 as bait in a yeast two-hybrid screen, we isolated a cDNA encoding a novel Cbln1-related protein, designated Cbln3. The gene encoding *cbln*3 had the same intron–exon structure as *cbln*1 but mapped to a different mouse chromosome (14). The deduced amino acid sequence of Cbln3 was 55% identical to Cbln1 and also contained a C1q signature domain and signal sequence for secretion. In addition to binding avidly to Cbln3, Cbln1 also formed homomeric complexes. In contrast, Cbln3 homomeric association was weak. These interactions exhibited specificity because C1qB bound to neither Cbln1 nor Cbln3. Like *cbln*1, *cbln*3 was expressed in the cerebellum and dorsal cochlear nucleus in which it was detected in granule neurons. Because Cbln1 and Cbln3 are coexpressed in the brain and interact avidly, they may function as a secreted heteromeric complex *in vivo*.

Key words: cerebellum; dorsal cochlear nucleus; granule cells; yeast two-hybrid; C1q signature domain; gene mapping

Cerebellin is a brain-specific hexadecapeptide (Slemmon et al., 1984) that is structurally conserved from chicken to man (Slemmon et al., 1984; Morgan et al., 1988; Yiangou et al., 1989). This neuropeptide is most abundant in the cerebellum and dorsal cochlear nucleus (DCoN), although it can be detected in other brain regions at relatively low levels (Mugnaini and Morgan, 1987; Burnet et al., 1988; Morgan et al., 1988). Antibodies against cerebellin specifically stain Purkinje cells in the cerebellum and cartwheel neurons in the DCoN (Slemmon et al., 1985; Mugnaini and Morgan, 1987). Because neurons within the DCoN and cerebellum have similar lineages and the two brain regions have analogous organization, we suggested that cerebellin functioned as a conventional neuropeptide in these structures (Mugnaini and Morgan, 1987). Indeed, cerebellin is enriched in the synaptosomal fraction (Slemmon et al., 1984) and is released in a calcium-dependent manner after depolarization (Burnet et al., 1988). Furthermore, cerebellin can elicit norepinephrine secretion from the adrenal gland (Mazzocchi et al., 1999; Albertin et al., 2000). However, the precise function of cerebellin is controversial.

Like many neuropeptides, cerebellin is derived from a precursor, named precerebellin or Cbln1 (Urade et al., 1991). Although precerebellin has no collagen motif, the C-terminal two-thirds of the protein shows significant structural similarity to the globular (noncollagen) domains of several atypical collagens, including the complement C1q subunits (Urade et al., 1991). Such proteins are not typically thought to undergo selective processing to yield neuroactive peptides. Rather, the globular domains of the atypical collagens are important for the appropriate alignment of three polypeptide subunits that permits the subsequent formation of the collagen triple helix (Brass et al., 1991). Because most of the C1q signature domain proteins exist as homomeric or heteromeric complexes, we hypothesized that Cbln1 may be part of a protein complex that consisted of related polypeptides. Indeed, a protein (Cbln2) similar to Cbln1 has been identified (Wada and Ohtani, 1991; Kavety et al., 1994), indicating the existence of a precerebellin gene family. However, the expression pattern of *cbln*2 is different from that of *cbln*1, implying that they could not participate in heteromeric complexes. For example, whereas *cbln*1 is expressed at high levels in the adult cerebellum, *cbln*2 is nearly undetectable (Wada and Ohtani, 1991). In contrast, *cbln*2 is expressed at relatively high levels in extracerebellar brain areas and in the fetal nervous system, whereas *cbln*1 is either absent or only expressed at very low levels (Urade et al., 1991; Wada and Ohtani, 1991). Therefore, we hypothesized that additional Cbln1-related proteins existed that bound to Cbln1.

Here we report the cloning of Cbln3, a third member of the precerebellin family. This protein was isolated through its binding to LexA-Cbln1 in a yeast two-hybrid screen. Not only did Cbln3 bind to Cbln1 but the two were also coexpressed at high levels in the cerebellum and DCoN. This casts doubt on the role of cerebellin as a classical neuropeptide modulator. Rather, these findings suggest that the precerebellins are secreted proteins that function as heteromeric complexes.

MATERIALS AND METHODS

Yeast two-hybrid cDNA library screening and analysis of protein–protein interactions. A mouse cerebellar cDNA library (Kurschner and Morgan, 1995) was constructed in the yeast *Escherichia* coli shuttle vector pSD10a that carries the yeast URA3 gene as selectable marker (Dalton and Treisman, 1992). This permits the production of cDNA-encoded proteins as fusions to the transactivation domain of the VP16 protein of the *Herpes simplex* virus. A LexA-Cbln1 fusion protein was used as bait to screen the cerebellar VP16 fusion library in yeast. Because the signal peptide is likely processed from Cbln1 to yield the mature protein, the first 10 amino acids of precerebellin were deleted. This truncated cDNA was cloned into the Y.LexA vector that carries the yeast TRP1 gene as a selectable marker (a gift from Dr. Steven Dalton, University of Adelaide, Adelaide, Australia). The expression of LexA-Cbln1 and the VP16 fusions was under the control of a GAL10-CYC1 hybrid promoter, which rendered expression galactose-inducible and glucose-repressible.

The budding yeast *Saccharomyces cerevisiae* strain S260 (URA3⁻ TRP1⁻), which contains a genomic *lex*A-operator-*lac*Z reporter gene integrated into the URA3 locus (Kurschner and Morgan, 1996), was first transformed with the *lex*A-*cbln*1 construct. Transformants that survived on tryptophan-deficient medium were then transformed with the plasmid library DNA, plated onto Hybond-N filters (Amersham Pharmacia Biotech, Arlington Heights, IL), and selected on Trp⁻ Ura⁻ plates. Filters

with cotransformed colonies were transferred to galactose medium to induce the expression of the fusion proteins. LacZ-positive colonies were identified in a β-galactosidase assay in which 5-bromo-4-chloro-3-indolyl-β-D-galactopyranosidase was used as substrate (Breeden and Nasmyth, 1985; Dalton and Treisman, 1992). Positive colonies were streaked out, and the activation of the reporter gene was confirmed. Plasmids were rescued by transforming ElectroMax *E. coli* competent cells (Life Technologies, Gaithersburg, MD) with total yeast DNA extracts. Protein interaction was further confirmed by cotransformation of the yeast with purified plasmid DNA. The cDNA inserts were sequenced using automated DNA sequencing.

DNA sequencing. Sequencing reactions were performed by the Center for Biotechnology at St. Jude Children's Research Hospital on template DNA using dye-terminator cycle sequencing-ready reaction kits with Ampli Taq DNA polymerase FS (Perkin-Elmer/Applied Biosystems, Inc., Foster City, CA) and synthetic oligonucleotides. Samples were electrophoresed, detected, and analyzed on a Perkin-Elmer/Applied Biosystems, Inc. model 373 DNA sequencer.

5′ Rapid amplification of cDNA ends and reverse transcription-PCR. To obtain additional sequence from the 5′ end of *cbln*3 mRNA, 5′ rapid amplification of cDNA ends (RACE) (Frohman et al., 1988) was performed on adult mouse cerebellum total RNA using the Gibco 5′ RACE System, version 2.0 (Life Technologies). The first-strand cDNA was synthesized using a gene-specific antisense oligonucleotide (GSP1a, GGA-AGCAGCACAGAGCTTG). Two other gene-specific primers (GSP1b, ACCACGTGGAATCGGAAGCTG and GSP2, ACGAAGCAGCCCG-AGGTCCGATC) in combination with the 5′ abridged universal amplification primer were used for two consecutive rounds of nested PCR. The PCR products were separated in agarose gels, extracted using the GIA-quick Gel Extraction Kit (Qiagen, Hilden, Germany), cloned into pBluescript KS(−) (Stratagene, La Jolla, CA), and sequenced. The longest cDNA sequence assembled from the 5′ RACE product and cDNA isolated from the library was deposited in GenBank (accession number AF218379). Based on the new sequence information, cDNAs corresponding to the full-length protein and the predicted mature protein were amplified and cloned into the pSD10a and Y.Lex vectors for interaction analysis.

Northern blot analysis. Total RNA from various mouse tissues at different developmental stages was extracted using RNAzol B (TEL-TEST, Friendswood, TX). RNA (10 μg/sample) was denatured and separated in 1% agarose gels containing 0.41 M formaldehyde and 1× 4-morpholinepropanesulfonic acid (MOPS) running buffer (MOPS 0.02 M, sodium acetate 8 mM, and EDTA 1 mM; pH 7.0). RNAs were transferred onto Hybond-N membranes (Amersham Pharmacia Biotech) using a downward alkaline blotting system (Chomczynski, 1992) and fixed to the membrane by UV-cross-linking. ^{32}P-labeled probes were synthesized using the Mega-prime DNA labeling system (Amersham Pharmacia Biotech). The 5′ RACE product (371 bp) of *cbln*3 and a 324 bp cDNA fragment corresponding to nucleotides 147–471 of *cbln*1 (GenBank accession number 164680) (Kavety and Morgan, 1998) were used as templates. Hybridization was performed using the QuikHyb hybridization solution (Stratagene) according to the instructions of the manufacturer. After hybridization and washing, blots were exposed to Kodak X-OMAT AR film (Eastman Kodak, Rochester, NY) for autoradiography. To control for sample loading, blots were stripped and rehybridized with probes specific for the mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (GenBank accession number W34118) and the mouse β-actin (GenBank accession number AA138737).

In situ hybridization. Postnatal, adult, and time-pregnant (for embryonic tissue) mice were perfused transcardinally with 4% paraformaldehyde under pentobarbital anesthesia. Brains were dissected, post-fixed at 4°C for 4 hr, and cryoprotected in 25% sucrose in 0.1 M phosphate buffer at 4°C until used. For sectioning, brains were mounted in tissue freezing medium (Triangle Biomedical Sciences) at −54°C and warmed to −23°C. Frozen sections were cut at 12.5 μm on a cryostat and thaw mounted onto Fisherbrand Superfrost Plus microscope slides and kept at −20°C until used. *In situ* hybridization was performed following the protocol described by Simmons et al. (1989) with slight modifications (Soares et al., 1998). Slides were hybridized at 55°C for 12–16 hr with ^{33}P-labeled sense or antisense riboprobes (10^6 cpm/slide), which were synthesized in the presence of ^{33}P-UTP via *in vitro* transcription using T3 or T7 RNA polymerases (Roche, Indianapolis, IN). The templates were linearized with pBluescript KS containing a 166 bp cDNA fragment corresponding to codons 30–84 of *cbln*3 in two different orientations. Sense probes served as specificity controls. After hybridization, ribonuclease A treatment and high-stringency washes, slides were exposed to x-ray film and then to autoradiography emulsion (NTB2; Eastman Kodak) for 3–5 d. After development of the emulsion, sections were counterstained with cresyl violet, dehydrated, and cover-slipped for microscopic observation.

Genomic DNA cloning and sequencing. A bacteriophage P1-based mouse genomic DNA library was subjected to PCR screening (Genome Systems, St. Louis, MO). Primers used were GSP2 as described and GSP3 (GG-GAGTGCCTGGTGGTCTGTGAG) corresponding to codons 34–42 of *cbln*3. DNA from two positive clones was analyzed by Southern hybridization after digestion with several restriction endonucleases. The downward transfer to Hybond-N membrane was performed according to Chomczynski (1992). Two PCR-amplified genomic fragments were used as probes. The 5′ probe, amplified using GSP2 and GSP3 as primers, spans

intron 1 of *cbln*3. The 3′ probe, amplified with GSP4 (CTTCCCACTCT-GAGGACCCAAG) and GSP5 (CCAAATAGCGCTGAGCAGGAAG), is in the 3′ untranslated region of *cbln*3. Hybridization was performed in QuikHyb (Stratagene). After a high-stringency wash at 60°C in a solution containing 0.2× SSC and 0.1% SDS, the membrane was exposed to Kodak X-OMAT AR film. A 5.5 kb *Eco*RI fragment encompassing the *cbln*3 coding sequence was identified on the Southern blots and cloned into pBluescript KS. Sequence analysis was conducted after subcloning and, sometimes, using gene-specific oligonucleotide as primers. Sequence data was deposited in GenBank (accession number AF218380).

Gene mapping. The Jackson Laboratory (Bar Harbor, ME) interspecific backcross panel (C57BL/6JEi × SPRET/Ei)F1 × SPRET/Ei called Jackson BSS (Rowe et al., 1994) was used to determine the chromosomal localization of *cbln*3 in the mouse. Two PCR primers were chosen to amplify a 200 bp genomic DNA that showed polymorphism between C57BL/6J and *Mus* spretus. The 5′ primer, GSP4, spans the stop codon (TGA) of *cbln*3, and the 3′ primer GSP6 (GTCTAGAGGTTCCG-TAGCTCTG) is located 200 bp downstream and corresponds to a sequence in the 3′ untranslated and alternatively spliced region. Genomic DNA (10 ng/reaction) from different animals was amplified under the following conditions: 94°C for 5 min; 35 cycles of 94°C for 30 sec, 56°C for 30 sec, and 72°C for 30 sec; and 72°C for 10 min. The identity of the PCR products was confirmed by DNA sequencing. To detect single-strand conformation polymorphism, PCR products were labeled with [$α$-^{32}P]dATP and 3 μl of PCR products were denatured in 9 μl of 95% formamide, 10 mM NaOH, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF first at 95°C for 5 min and then at 4°C for 5 min, before loading on a 0.5× mutation detection electrophoresis gel (FMC BioProducts, Rockland, ME) and running at 5 W for 16 hr at 4°C in 0.6× Tris borate-EDTA.

RESULTS

Cloning of *cbln*3

A yeast two-hybrid screen was used to isolate candidate proteins that physically interacted with Cbln1. A mouse cerebellar cDNA library that produces VP16-fusion proteins was screened using LexA-Cbln1 as bait. Of 11 positive clones that were isolated and sequenced, five encoded Cbln1 itself. This indicated that Cbln1 was capable of homomeric interaction. Two other overlapping clones contained an open-reading frame corresponding to a novel polypeptide that was similar to both Cbln1 and Cbln2 (Fig. 1). The rest of the coding sequence along with 45 nucleotides of the 5′ untranslated sequence was obtained by 5′ RACE. This protein, designated Cbln3, is predicted to consist of 197 amino acids, with a calculated molecular weight of 21.1 kDa and an isoelectric point of 6.35. The first 21 residues resemble a typical signal peptide. Overall, Cbln3 is 55.1% identical and 67.6% similar to mouse Cbln1 and 49.1% identical and 61.7% similar to rat Cbln2. In the cerebellin motif, Cbln3 is quite different from Cbln1 and Cbln2, the latter two being identical except for one amino acid. Therefore, Cbln3 is not the precursor for cerebellin. Notably, many of the aromatic amino acid residues that are highly conserved among C1q signature domain-containing proteins are also conserved in Cbln3.

Protein–protein interactions measured using a yeast two-hybrid assay

Results from the cDNA library screening suggested that Cbln1 bound to itself and to truncated Cbln3. To determine whether full-length Cbln3 exhibited homomeric or heteromeric binding, protein–protein interactions were studied in the yeast two-hybrid system. cDNAs corresponding to the full-length and the predicted mature Cbln3 polypeptides were cloned into both Y.LexA and pSD10a vectors. As shown in Table 1, both full-length and mature Cbln3 interacted with Cbln1. However, unlike Cbln1, Cbln3 only displayed marginal homomeric binding. To establish the specificity of these precerebellin interactions, the globular domain of the distantly related C1q B-chain was cloned and tested for its ability to undergo homomerization and to bind to Cbln3. Although C1qB did self-associate, as predicted, it did not bind to Cbln3. Together, these data suggest that Cbln3 preferentially forms heteromers with Cbln1.

Expression profiles of *cbln*1 and *cbln*3

Previous studies have shown that *cbln*1 and *cbln*2 exhibit different expression profiles in the rat (Urade et al., 1991; Wada and Ohtani, 1991), suggesting that they cannot interact *in vivo*. To qualify Cbln3
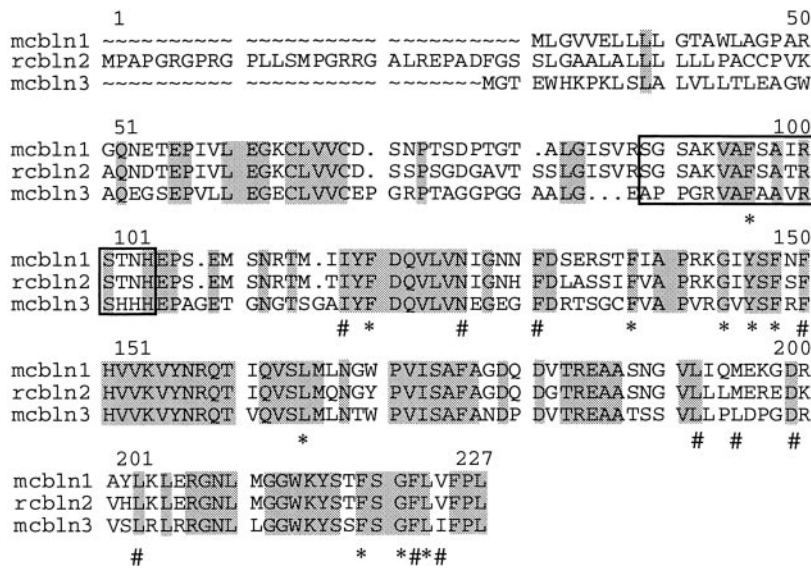
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            1                                                       50
mcbln1  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ MLGVVELLLL GTAWLAGPAR
rcbln2  MPAPGRGPRG PLLSMPGRRG ALREPADFGS SLGAALALLL LLLPACCPVK
mcbln3  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~MGT EWHKPKLSLA LVLLTLEAGW

            51                                                      100
mcbln1  GQNETEPIVL EGKCLVVCD. SNPTSDPTGT .ALGISVRSG SAKVAFSAIR
rcbln2  AQNDTEPIVL EGKCLVVCD. SSPSGDGAVT SSLGISVRSG SAKVAFSATR
mcbln3  AQEGSEPVLL EGECLVVCEP GRPTAGGPGG AALG...BAP PGRVAFAAVR
                                                          *

            101                                                     150
mcbln1  STNHEPS.EM SNRTM.IIYF DQVLVNIGNN FDSERSTFIA PRKGIYSFNF
rcbln2  STNHEPS.EM SNRTM.TIYF DQVLVNIGNH FDLASSIFVA PRKGIYSFSF
mcbln3  SHHHEPAGET GNGTSGAIYF DQVLVNEGEG FDRTSGCFVA PVRGVYSFRF
               # *         #          #         *      * * * #

            151                                                     200
mcbln1  HVVKVYNRQT IQVSLMLNGW PVISAFAGDQ DVTREAASNG VLIQMEKGDK
rcbln2  HVVKVYNRQT IQVSLMQNGY PVISAFAGDQ DGTREAASNG VLLLMEREDK
mcbln3  HVVKVYNRQT VQVSLMLNTW PVISAFANDP DVTREAATSS VLLPLDPGDR
                         *                            # # #

            201                       227
mcbln1  AYLKLERGNL MGGWKYSTFS GFLVFPL
rcbln2  VHLKLERGNL MGGWKYSTFS GFLVFPL
mcbln3  VSLRLRRGNL LGGWKYSSFS GFLIFPL
          #                *  *#*#
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Figure 1. Amino acid sequence alignment of precerebellin-related proteins. The sequence alignment for mouse Cbln1 (*mcbln1*), rat Cbln2 (*rcbln2*), and mouse Cbln3 (*mcbln3*) was generated using PILEUP (GCG Wisconsin Package). Because only an incomplete mouse *cbln*2 clone has been isolated (Kavety et al., 1994), the rat Cbln2 sequence was used to provide a complete comparison of structure. Residues that are identical in all three family members are *shaded*. *Asterisks* and *pound signs* indicate identical and highly conserved residues, respectively, found in the C1q signature domain. The cerebellin peptide motif is contained within the *boxed area*.

Table 1. Homomeric and heteromeric interactions of Cbln1 and Cbln3

LexA fusion	VP16 fusion	β-Galactosidase activity
Cbln1	Cbln1	++
Cbln1	Cbln3	+
Cbln3	Cbln1	++
Cbln1	Cbln3(Δ21N)	++
Cbln3(Δ21N)	Cbln1	++
Cbln3	Cbln3	+/−
Cbln3(Δ21N)	Cbln3(Δ21N)	+/−
C1qB	Cbln3	−
C1qB	C1qB	++

In each two-hybrid assay, S260 was cotransformed with two constructs, one coding for a LexA fusion protein and the other a VP16 fusion protein. For Cbln1, the predicted mature protein (Cbln1, codons 11–193) was used. For Cbln3, either the full-length polypeptide (Cbln3) or one without the presumed signal peptide [Cbln3(Δ21N)] was included in the constructs. The globular domain of C1q B-chain (C1qB) served as specificity control. β-Galactosidase activity, an index for the avidity of interaction between the two hybrids, was assessed. ++, Highest activity, yeast colonies turned dark blue within 1 hr; +, moderate activity, yeast colonies turned blue between 1 and 2 hr; +/−, trace activity, yeast colonies just above background at 2 hr; −, no activity, yeast colonies white at 2 hr. None of these constructs when expressed alone produced blue colonies (data not included).

as a natural partner for Cbln1, it is necessary to demonstrate that they are coexpressed. Therefore, the expression of *cbln*1 and *cbln*3 was determined in the mouse by Northern blot analysis.

As shown in Figure 2, in adult mice *cbln*3 was highly expressed in cerebellum but was undetectable in forebrain, spinal cord, and a range of non-neural organs. The same result was obtained for *cbln*1, except that low levels of *cbln*1 transcripts were also detected in forebrain and spinal cord (Fig. 2*A*). During development, the expression of *cbln*3 generally paralleled that of *cbln*1 in the cerebellum. However, *cbln*3 was not expressed until postnatal day 7 (P7), whereas *cbln*1 was already present at low to moderate levels before P7 in cerebellum as well as in the forebrain (Fig. 2*B*). These data indicated that *cbln*1 and *cbln*3 were coexpressed in the adult cerebellum, although *cbln*3 expression was more restricted.

In situ hybridization

The precise location of *cbln*3 expression in the brain was determined by *in situ* hybridization. Whereas the *cbln*3 sense probe generated no signal above background (Fig. 3*B*), the antisense probe gave robust hybridization in the cerebellum (Fig. 3*D,F*). In the adult, the internal granule cell layer (IGL) was the predominant site of *cbln*3 expression (Fig. 3*F*). Grain density over Purkinje

neurons was indistinguishable from background, suggesting that they did not express *cbln*3. In sections from a P8 mouse, no expression of *cbln*3 was observed in the external granule layer (EGL) (Fig. 3*D*). This region contains proliferating granule cell progenitors and postmitotic, premigratory granule cells. In contrast, granule cells that had ceased division and migrated into the IGL did express *cbln*3 (Fig. 3*D*). Consistent with Northern blot analyses, there was no specific hybridization signal for *cbln*3 in embryonic day 12.5/E7 (E12.5), E15.5, or P1 mice (data not shown). However, *in situ* hybridization revealed that *cbln*3 was expressed in the adult DCoN (Fig. 3*H*). Thus, the two brain regions that have the highest levels of *cbln*1 mRNA, and cerebellin peptide also expressed the highest levels of *cbln*3 mRNA.

Genomic structure and chromosomal location of *cbln*3

To study the genomic structure of *cbln*3, a PAC clone was isolated, from which a 5.5 kb *Eco*RI fragment that hybridized with *cbln*3 probes was subcloned. Sequence analysis revealed a gene with three coding exons separated by two small introns of 325 and 289 bp, respectively (Fig. 4). The relative positions of these introns, between codons CAG (Gln) and GTA/G (Val) in *cbln*3 are the same as in *cbln*1 (Kavety et al., 1994). There was no alternative ATG between the predicted start codon and the nearest in-frame stop codon.

One approach to elucidate the function of *cbln*3 is to identify pathological phenotypes in mice that carry naturally occurring mutations in this gene. Therefore, we first determined the chromosomal localization of *cbln*3 in the mouse genome. We took advantage of an established gene mapping panel, the Jackson BSS backcross (Rowe et al., 1994), that consists of 94 offspring of a backcross between two distantly related mouse strains, Mus musculus (C57BL/6) and M. spretus. First, a DNA polymorphism was identified in the 3′ UTR of the *cbln*3 gene using single-strand conformation polymorphism between the two strains (Treadaway and Zuo, 1998). Subsequently, we determined the pattern of segregation of the two strains at the *cbln*3 locus in the Jackson BSS panel. An identical pattern of segregation is expected for genes that are located in a closely linked genomic segment, whereas a nonconcordant (or unrelated) pattern of segregation is expected for genes that are located on different chromosomes or far apart on the same chromosome. By comparing the pattern obtained from the *cbln*3 gene with other known markers in the genome, we were able to place *cbln*3 in the vicinity of six other markers on chromosome 14 (Fig. 5). The DNA marker *D14Mit5* displays an identical segregation pattern to *cbln*3, suggesting that *D14Mit5* and *cbln*3 are extremely close. All but 3 of 94 backcrosses showed a linkage between *otx*2 and *cbln*3, indicating that these genes are separated by ~3 centimorgan (cM). Two independent primer pairs (*cbln*3-1/2 and
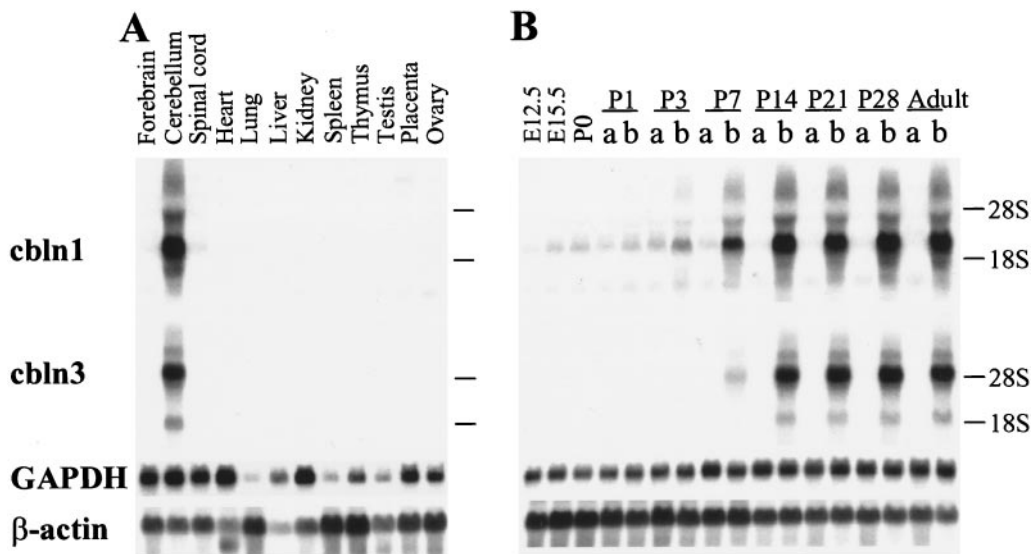
Figure 2. Northern blot analysis of *cbln*1 and *cbln*3 expression. *A*, Distribution of *cbln*1 and *cbln*3 transcripts in different adult tissues and organs. *B*, Expression of *cbln*1 and *cbln*3 in the mouse brain during development. For E12.5, E15.5, and P0 (the day of birth), whole brains were used. For postnatal (*P*) time points, cerebellum (*b*) and the rest of the brain (*a*) were analyzed separately. Adult mice were 2 months old. GAPDH and β-actin probes were used as controls for RNA loading and transfer after stripping of the blots.

*cbln*3-3/4) derived from *cbln*3 were used to screen the Jackson BSS backcross. The mapping results were identical for both sets of primers (data not shown). Using a similar strategy, we also confirmed the position of *cbln*1 on mouse chromosome 8 (data not shown).

DISCUSSION

Here we describe the cloning and characterization of *cbln*3, a new member of the precerebellin family. The structural similarities between Cbln1 and Cbln3 and their coexpression in the cerebellum and DCoN suggest that they serve related or identical functions. Furthermore, the demonstration that Cbln3 interacts selectively with Cbln1 indicates that, like some members of the atypical collagen superfamily, these proteins form a heteromeric complex. Because both Cbln1 and Cbln3 possess signal sequences for secretion, it is further hypothesized that the precerebellin-containing complexes are either released into the extracellular milieu or undergo intercellular trafficking.

Based on protein sequence comparisons, *cbln*1 is more closely related to *cbln*2 than to *cbln*3. Within the region of the proteins that contain the cerebellin peptide motif, there is only a single amino acid difference between Cbln1 and Cbln2. In contrast, in Cbln3 only 7 of 16 amino acids are identical to cerebellin. Nevertheless, the three molecules constitute a small gene family that is part of a larger superfamily that contains collagens and collagen-like proteins. This is exemplified by the presence of conserved aromatic amino acids within all three precerebellins that conform to a consensus motif sometimes referred to as the C1q signature domain or aromatic zipper (Smith et al., 1994). This region typically spans about 130 amino acids and is located at the C terminus of the protein. In the case of the precerebellins, this domain begins within the cerebellin motif and extends to the C terminus. Noticeably, although multiple clones encoding Cbln1 and Cbln3 were obtained in the two-hybrid screen, in all cases the C1q signature domain was intact. For example, of the five *cbln*1 clones isolated, four encoded full-length protein and one lacked 54 amino acids at the N terminus. Similarly, the two *cbln*3 clones lacked 29 and 35 amino acids from the N terminus, respectively. This suggested that integrity of the C1q signature domains in Cbln1 and Cbln3 is required for homomeric and heteromeric binding.

The precerebellins are related to a superfamily of atypical collagens that include collagens type VIII and X (Muragaki et al., 1992; Elima et al., 1993), the A, B, and C subunits of the comple-

ment C1q complex (Petry et al., 1989, 1992), hibernation-associated serum proteins (Takamatsu et al., 1993), and a saccule-specific collagen (Davis et al., 1995). Within the atypical collagens, the globular (C1q signature) domain is responsible for the initial assembly of trimeric complexes. This initial interaction brings the subunits into correct alignment, thereby permitting the single collagen domain in each subunit to associate in a triple helix (Brass et al., 1991). In some cases, such as collagen X, the trimer consists of three identical chains. However, in other instances, such as C1q, the complex is composed of three distinct subunits. Therefore, individual globular head groups not only align protomers but also discriminate different molecular entities to ensure the correct subunit stoichiometry in the complex. The precerebellins are capable of forming homomeric and heteromeric complexes. Thus, Cbln1 can oligomerize and bind to Cbln3. In contrast, although Cbln3 binds avidly to Cbln1, its homomeric interaction is weak. Therefore, we hypothesize that the function of Cbln3 is dependent on its incorporation into a complex with Cbln1. Furthermore, because this complex is most likely a trimer, there may be yet another member of the precerebellin family to be identified.

Some of the atypical collagens are components of the extracellular matrix. For example, collagen type X assembles into a lattice structure in which the globular head groups associate with each other to form the nodules while the collagen helices form the interstices (Kwan et al., 1991). However, other superfamily members, such as the hibernation proteins and the complement C1q complex, circulate in the blood and may bind to membrane receptors. Because none of the precerebellins contain a collagen motif, it seems unlikely that they are conventional components of the extracellular matrix. Recently, the crystal structure of the C1q signature domain of another superfamily member, ACRP30/adipoQ, was solved (Shapiro and Scherer, 1998). This protein is synthesized by adipose tissue but is released into the plasma. Remarkably, ACRP30 was found to have a similar three-dimensional structure to tumor necrosis factor-α (TNFα). This is despite the fact that TNFα is not an atypical collagen, and only a few of the amino acids in the C1q signature domain are conserved in TNFα. However, these and additional amino acids are conserved between TNFα and the precerebellins. Therefore, it is conceivable that the precerebellin complexes interact with a membrane receptor and activate an intracellular signal transduction cascade in a manner analogous to TNFα.
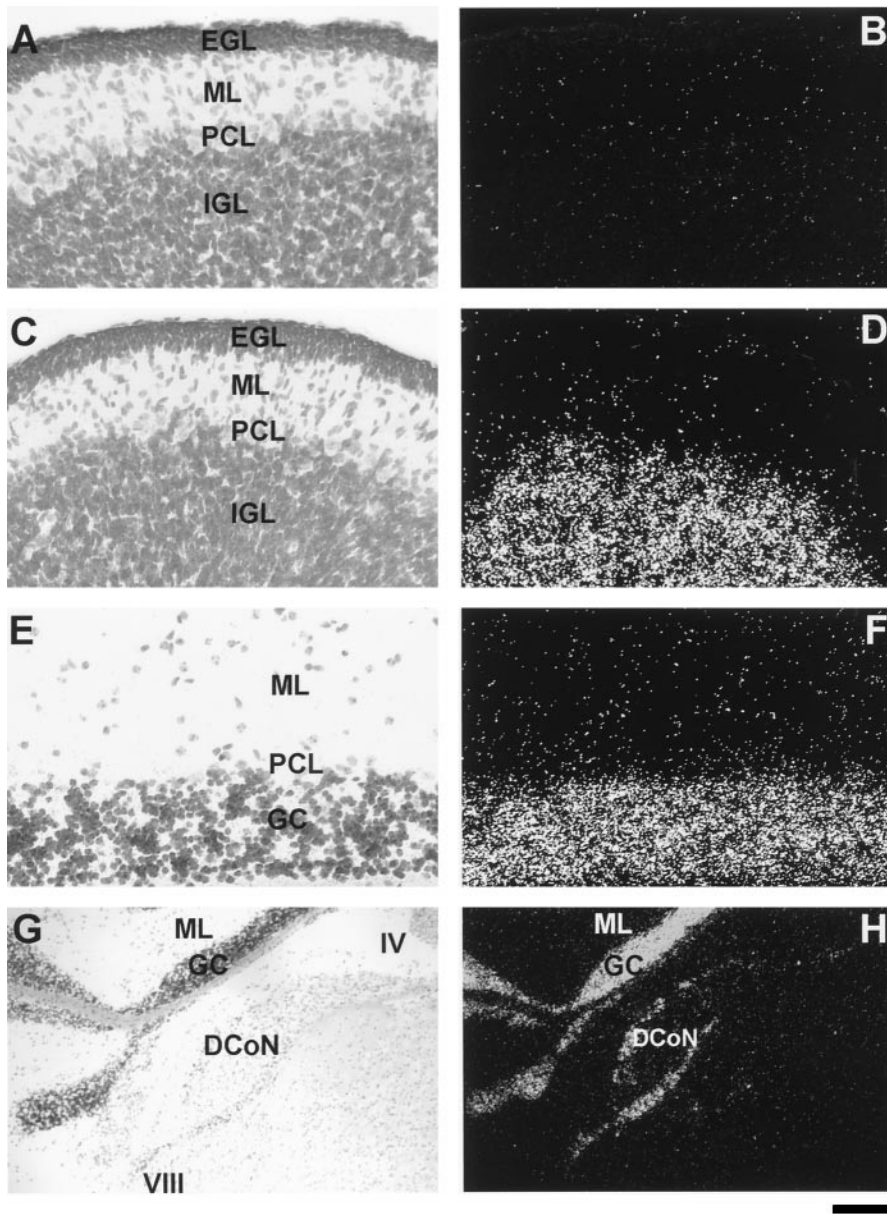
Figure 3. *In situ* hybridization of *cbln*3 in mouse cerebellum and dorsal cochlear nucleus. Saggital sections of a P8 mouse brain (*A–D*) and coronal sections of an adult mouse cerebellum (*E, F*) and dorsal cochlear nucleus (*G, H*) were labeled with a sense (*A, B*) or antisense (*C–H*) riboprobe specific for *cbln*3. Both bright-field (*A, C, E, G*) and dark-field (*B, D, F, H*) views were acquired using a digital camera. *ML*, Molecular layer; *PCL*, Purkinje cell layer; *IV*, fourth ventricle; *VIII*; nucleus of the VIII cranial nerve; *EGL*, external granule layer; *IGL*, internal granule layer; *DCoN*, dorsal cochlear nucleus. Scale bar: *A–F*, 50 μm; *G, H*, 250 μm.

The levels of cerebellin are much reduced in strains of mutant mice that have developmental anomalies that involve a loss of cerebellar granule neurons (Morgan et al., 1988; Slemmon et al., 1988). In addition, cerebellin is depleted in cerebella from patients that died of olivopontocerebellar atrophy and Shy-Drager syndrome (Mizuno et al., 1995). However, to date cerebellin has not been causally linked to any neurological disorder. Furthermore, the
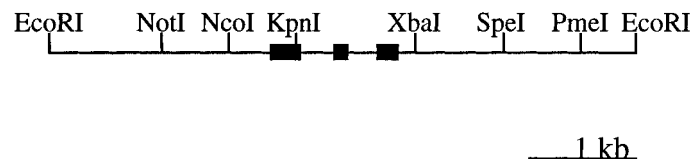

Figure 4. Structure and physical map of the *cbln*3 gene. A 5.54 kb *Eco*RI fragment from a mouse genomic PAC clone was identified by Southern hybridization as described in Materials and Methods. This fragment was then subcloned and sequenced (GenBank accession number AF218380). The coding sequences are represented by the *black boxes*. The first intron is located between codons 92 and 93, and the second between codons 132 and 133. The cleavage sites for restriction endonucleases *Eco*RI, *Kpn*I, *Nco*I, *Not*I, *Pme*I, *Spe*I, and *Xba*I were identified using the MAP program in the GCG Wisconsin Package.

position of *cbln*1 on mouse chromosome 8 (syntenic with human 19p13.2) has not been linked to known disorders in mice or man (Kavety et al., 1994). The observation that cerebellin levels are reduced in several multiple systems atrophy disorders that impact the cerebellum may simply be a reflection of loss of the neurons that generate the peptide. The complex hereditary patterns of these diseases and their variable phenotypes have made it difficult to identify the gene mutations involved. However, one of the precerebellin genes could potentially contribute to the pathobiology of such disorders.

*cbln*1, *cbln*2, and *cbln*3 are independent genes that map to the mouse chromosomes 8, 18, and 14, respectively. Given their levels of homology, these genes have probably arisen by gene duplication, as also suggested by their identical intron–exon organization. Two potentially relevant mouse mutations, *agitans* (*ag*) and *wabbler-lethal* (*wl*), map to chromosome 14, near the *hairless* locus (*hr*). Both mutations exhibit degeneration in many parts of the nervous system, including the cerebellum (Hoecker et al., 1954; Luse et al., 1967; Carroll et al., 1992). The *agitans* mutant mouse is probably extinct, but *ag* homozygotes were reported to exhibit retarded growth, generalized tremor, and ataxia (Hoecker et al., 1954). Furthermore, Purkinje cell atrophy was noted in some regions of
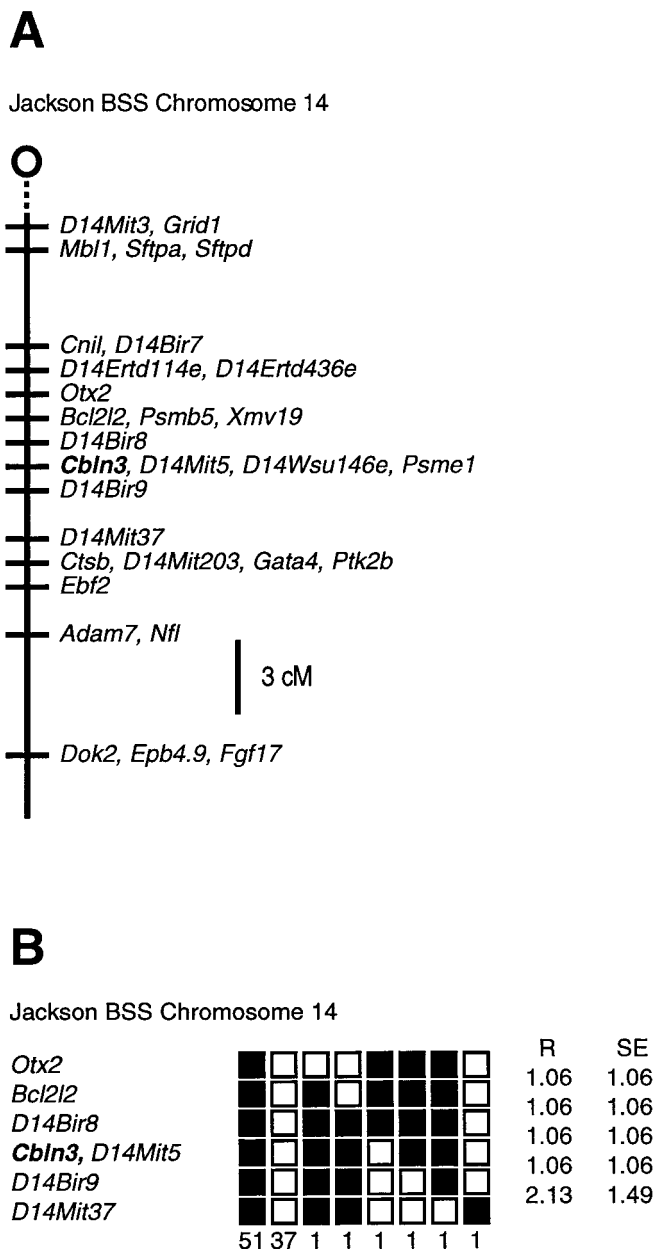
A

B

Figure 5. Chromosomal location of cbln3. Top, Map figure from the Jackson BSS backcross showing part of chromosome 14. The map is depicted with the centromere toward the top. A 3 cM scale bar is shown to the right. Loci mapping to the same position are listed in alphabetical order. Raw data from The Jackson Laboratory were obtained from http://www.jax.org/resources/documents/cmdata. Bottom, Haplotype figure from the Jackson BSS backcross showing part of chromosome 14 with loci linked to cbln3. Loci are listed in order with the most proximal at the top. The black boxes represent the C57BL6/JEi allele, and the white boxes represent the SPRET/Ei allele. The number of animals with each haplotype is given at the bottom of each column of boxes. The percent recombination between adjacent loci is given to the right, with the SE for each recombination (R).

the cerebellum of ag homozygotes. Therefore, cbln3 may be a candidate for ag.

Although they are coexpressed in the cerebellum and DCoN in adult mice, cbln1 but not cbln3 is also expressed in the forebrain and in the developing nervous system. Thus, if the precerebellins function as heteromers, this would imply that Cbln1 must be partnered with another family member in some regions of the fetal and adult brain. Cbln2 is expressed in the cerebellum during early development and is present outside the cerebellum in the adult (Wada and Ohtani, 1991). Thus, the pattern of cbln2 expression is complementary to that of cbln3 but overlaps that of cbln1. There-

fore, it is possible that Cbln1 may partner with Cbln2 in early development and in areas of the adult brain in which Cbln3 is not expressed. In the case of the cerebellum, there may also be a switch from Cbln1–Cbln2 complexes in fetal life to Cbln1–Cbln3 complexes in the neonate and adult. Moreover, because Cbln3 is generated by granule neurons of the IGL, this switch must occur at the time that the cells move from the premigratory zone of the EGL to their final position.

The biological function of cerebellin in the CNS is still unknown. The calcium-dependent release of the peptide from cerebellar synaptosomes and its effect on secretion from the adrenal gland suggests a function similar to conventional neuropeptide modulators. However, the present data suggest that the function of Cbln1 is exerted through a complex composed of several precerebellins. Nevertheless, the precise cleavage of Cbln1 to yield the cerebellin peptide in many diverse species suggests that this processing may be of functional relevance and not just a fortuitous event. For example, TNFα, which shares structural similarities with C1q signature domain proteins, is released from the cell membrane after specific cleavage by a disintegrin metalloproteinase (Black et al., 1997). Thus, proteolytic processing of precerebellins might contribute to their release. Alternatively, processing may occur in the target cell, in which case the peptide may fulfill a role in transcellular signaling. Currently gene-targeting experiments are being conducted to investigate these possibilities.

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