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Molecular cloning of a developmentally regulated brain protein, chicken drebrin A and its expression by alternative splicing of the drebrin gene

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Drebrins are developmentally regulated proteins found in the chicken brain and are classified into three forms, E1, E2 and A. Previously we isolated two cDNAs corresponding to the embryonic drebrin mRNAs from a chick embryo cDNA library. They differed in that an internal 129-nucleotide sequence, designated ins1, was inserted in the cDNA encoding drebrin E2 and was deleted in the other cDNA encoding drebrin E1. To search for the cDNA clone encoding drebrin A, a cDNA library of 1-day-old chick brains was screened using embryonic drebrin cDNA fragments as probes. Consequently, a novel cDNA was isolated, the sequence of which was entirely identical with that of drebrin E2 except for the insertion of a 138-nucleotide sequence, designated ins2, in the 5' direction immediately upstream from ins1. Since the translation product of the entire coding region was similar to that of drebrin A, this cDNA should correspond to the mRNA for drebrin A. Sequencing analysis of three drebrin cDNAs clearly indicated that the heterogeneity of chicken drebrins was caused by the insertion or deletion of the two sequences, ins1 and ins2. The amino-terminal half region including ins2 and two short sequences in the carboxyl-terminal region of the predicted drebrin A were highly evolutionarily conserved. Cloning and sequencing of the drebrin gene revealed that ins1 and ins2 were independently encoded by separate exons and three drebrin isoforms were thought to arise by alternative splicing from a single drebrin gene. The difference in the time course of expression and tissue distribution of each drebrin suggests that the machinery of alternative splicing site selection of the drebrin gene is regulated in a developmental stage-dependent and tissue-specific manner.

INTRODUCTION

The organization of the nervous system is accomplished by the events which are precisely programmed during development. These events, including neurogenesis, cell migration, outgrowth of axons and dendrites and synapse formation, are likely to depend upon developmental stage-dependent and cell type-specific expression of a variety of molecules. Such molecules include homoeodomain proteins[6,38] and intermediate filament proteins[19], as well as cell adhesion and axonal surface glycoproteins[10,15,40]. We previously demonstrated that drebrin is one of these molecules and may contribute in part to the molecular mechanisms of neural development[16,31,35].

Drebrins are acidic, cytosolic proteins first detected in the developing chick brain, then classified into two embryonic forms (E1 and E2) and one adult form (A). Expression of each is independently regulated during development, although it has been shown that by analysis of peptide mapping[31] and cross-reactivity of monoclonal antibodies for drebrins[32] their primary structures are closely related. The expression of drebrins in the chick optic tectum has been surveyed in detail by two-dimensional gel electrophoresis[31]. Drebrin E1 and E2 appear transiently at the developmental stages corresponding to the beginning of the neuronal differentiation and outgrowth of the processes, respectively. They are widespread throughout the brain at certain embryonic stages, but are absent in the adult brain. On the other hand, drebrin A appears in parallel with further maturation of the neurons and remains in the adult brain[32]. All drebrins are distributed in the cytoplasm of the neurons, drebrin A being particularly localized in the dendrites[33].

Two forms of immunoreactive molecules are also present in the brains of mammals, including rat[34], cat[13] and human (Toda et al., in preparation). Although they

are slightly larger than chicken drebrins, conversion from one form (drebrin E) to the other (drebrin A) occurs during development[34] in the same way as it does in chicken drebrins. Their wide interspecific distribution suggests that drebrins play a general role in neural organization. Imamura et al.[13] have reported that drebrins in the cat visual cortex are dramatically decreased around the end of the sensitive period for ocular dominance plasticity. These observations suggest that drebrins are also related to neural plasticity.

The significance of drebrins in the cell morphogenesis was first demonstrated by transfection of fibroblasts with the rat drebrin A expression plasmid[36]. Transient expression of drebrin A induces the formation of highly branched neurite-like cell processes in these non-neuronal cells. Drebrin A is concentrated on the inner face of the cell membrane. Another aspect of drebrins is that they can interact with actin filaments[36]. An antiserum raised against the actin-binding protein purified from the rat brain[20] specifically reacts with drebrins, and vice versa (Maekawa et al., unpublished observations). In fact, it has been recently revealed that drebrins are colocalized with actin filaments in the neurons and neuroblastoma cells (Asada et al., submitted). These results suggest that drebrin A modifies the interaction between the cytoskeletal networks and the cell membrane in the neurons.

Although the biological significance of heterogeneity of drebrins remains to be established, their independent changes in expression during development suggest that each play a unique role at a particular developmental stage. We proposed that drebrins E1, E2 and A play roles in cell migration, outgrowth of neuronal processes and plasticity of dendrites, respectively. These functions should be caused by the different molecular structures of the three isoforms. However, the structural differences between the embryonic and adult forms, as well as their genomic organization, have not yet been determined. It is necessary to elucidate their structural relationships, in order to investigate the biological functions of each drebrin.

We previously isolated cDNAs corresponding to the two embryonic drebrins E1 and E2 from a 10-day chick embryo cDNA library[16]. Both sequences are entirely identical except for an internal 129-bp sequence, designated ins1, that is inserted into drebrin E2 cDNA, and deleted in drebrin E1 cDNA. An antiserum against a synthetic peptide of the portion of ins1 specifically reacts with drebrin E2 and A, but not drebrin E1. Therefore, the sequence of ins1 is also included in drebrin A mRNA, on the assumptions that drebrin A is not derived from a posttranslational modification of the embryonic drebrin, rather that a unique mRNA

encoding drebrin A exists. In this study, we isolated cDNA clone for drebrin A by screening a cDNA library constructed from the newly hatched chick brain, in which drebrin A was the most abundant form. Furthermore, we estimated the genomic organization of three drebrin isoforms by cloning and sequencing the drebrin gene. Using the cDNA fragment as a probe, we detected drebrin transcripts in various tissues from several developmental stages of the chick.

MATERIALS AND METHODS

RNA isolation

The tissues of chick embryos (5 and 11 days of incubation) and postnatal 1-day-old chicks (White Leghorn) were rinsed with ice-cold phosphate-buffered saline, pH 7.4, then quickly frozen in liquid nitrogen. Total RNA was extracted by the single-step method with acid guanidinium thiocyanate-phenol-chloroform[3]. Poly(A)$^+$ RNA was purified by two elutions through an oligo(dT) cellulose (Collaborative Research) column[28].

cDNA library construction and screening

The cDNA was synthesized from poly(A)$^+$ RNA of postnatal 1-day chick brains by conventional oligo (dT)-primed reverse transcription using a cDNA synthesis kit (Pharmacia LKB Biotechnology). The double-stranded cDNAs ligated with an EcoRI adaptor were fractionated by size, and fragments longer than 0.5 kb were ligated to EcoRI digested λgt10 arms (Bethesda Research Labs) then packaged into phage particles using a commercial in vitro packaging system (Gigapack II gold, Stratagene Cloning Systems).

The unamplified cDNA library was screened by the standard method[12,28] using drebrin E2 cDNA, Dcw17 and an 80-base-pair (bp) HinfI fragment within the ins1 sequence of Dcw17 (Fig. 2) as probes. To isolate genomic clones for the drebrin gene, a λEMBL3 chicken genomic library (Clonetech Labs) was screened using Dcw17 as a probe. The probe was labeled with [α-^{32}P]dCTP using the multiprime labeling kit (Amersham) and hybridized to the recombinant phages transferred to nylon membrane (Hybond-N, Amersham). Hybridization was performed in 5 × Denhardt's solution[28] containing 6× SSC, 1% sodium dodecyl sulfate (SDS) and 1×10^5 cpm/ml of drebrin cDNA probe (spec. act. 5×10^8 cpm/μg cDNA) at 68°C for 16–24 h. Membranes were washed twice in 2 × SSC containing 1% SDS at 42°C for 30 min. The signals were detected by autoradiography using an intensifying screen at −70°C.

DNA sequencing

DNA fragments were subcloned into M13mp18 and sequentially deleted subclones were prepared by the method of Yanisch-Perron et al.[41]. DNA sequencing was performed in both directions by the dideoxy chain termination method of Sanger et al.[29]. The universal sequencing primers conjugated with four fluorescent dyes (−21m13, Applied Biosystems Industries) was anealed with the single-stranded M13 DNA then extended using a modified T7 DNA polymerase (Sequenase Ver.2.0, United States Biochemical Co.) or Taq DNA polymerase (AmpliTaq, Perkin-Elmer Cetus Instruments). The sequencing reaction products were run on a DNA sequencer (373A, Applied Biosystems Industries). Sequence data were assembled and analyzed using the GENETYX software package (Software Development Co.).

RNA polymerase chain reaction

Poly(A)+ RNA prepared from postnatal 1-day chick brains was converted into cDNA using oligo(dT) primer and Moloney murine leukemia virus reverse transcriptase (Bethesda Research Labs). The cDNA fragment was amplified using a GeneAmp DNA amplification kit (Perkin-Elmer Cetus Instruments). The oligonucleotide primers used were as follows: the upstream primer (UP1), 5′-GCAACATCCCGACCCCGC-GATGGCTGGCGT-3′; downstream primer (DP5), 5′-TTTGGGGTGCGGTGGCAGGTGATATAGGG G-3′. The polymerase chain reaction (PCR) was performed in a programmable heat block (PC-700, Astec), set to heat the sample to 95°C for 1 min, cool it to 55°C over 1 min, and heat it to 72°C for 2 min. After 40 amplification cycles, the PCR product was separated on a 1% agarose gel and detected by ethidium bromide staining.

In vitro transcription and translation

The cDNA fragments containing the full-length drebrin open reading frame sequences were subcloned into pGEM11Z (Promega Co.) for preparation of the sense-stranded cRNA of each drebrin. The template DNA linearized at the XbaI site was transcribed by T7 RNA polymerase (Takara Shuzo Co.). The transcripts were then translated in a rabbit reticulocyte lysate pretreated with micrococcal nuclease (Promega Co.) in the presence of a complete amino acid mixture at 37°C for 60 min. The translation products were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) according to the method of Leammli[18] and analyzed by
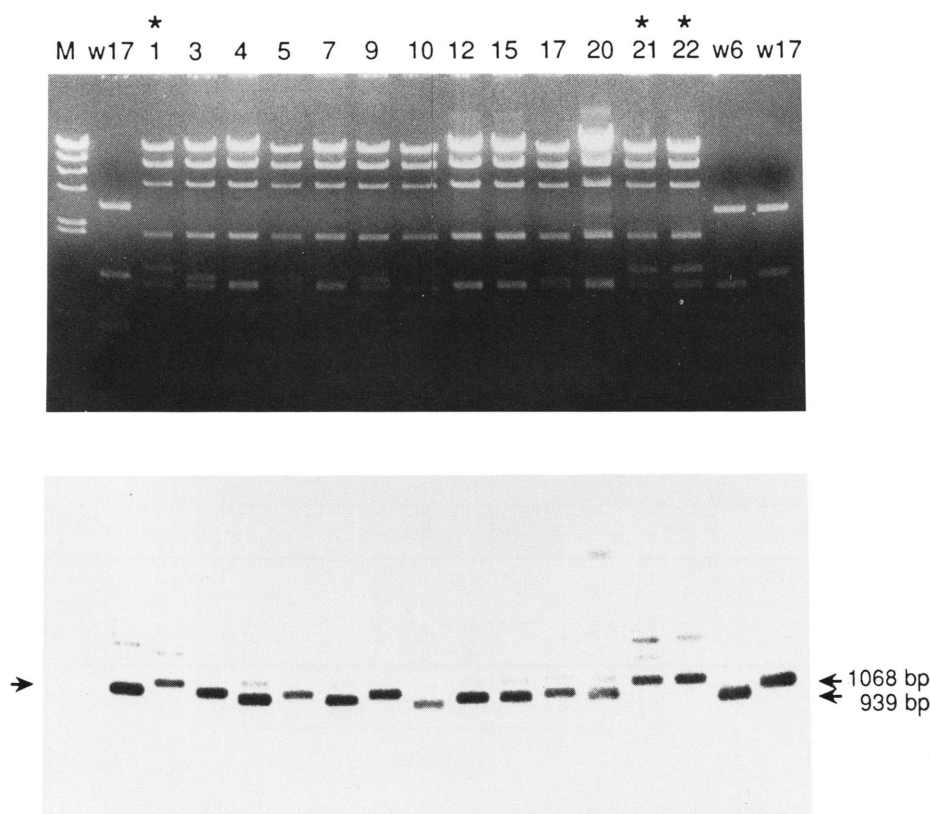
Fig. 1. Restriction analysis of drebrin cDNAs. The fragments of drebrin cDNA clones digested by EcoRI and SmaI were electrophoresed on a 1% agarose gel (upper panel), then transferred to a nylon membrane and probed with a radiolabeled 3′ PstI–EcoRI fragment of Dcw17 (lower panel). The two embryonic drebrin cDNAs (w6 and w17) digested by EcoRI and SmaI were used as guides. (The lengths of the hybridized bands were 939 and 1,068 bp, respectively.) The λDNA fragments digested by HindIII (M) were used as molecular weight markers.

immunoblotting as described previously[8,32] with a slight modification. The ECL western blotting detection system (Amersham) was used to detect horseradish peroxidase-labeled products.

Ribonuclease protection assay

A 680-bp *Ava* I fragment of the novel drebrin cDNA, Dcb21, containing the ins1 and ins2 sequences (Fig. 2) was subcloned into plasmid pGEM4Z (Promega Co.). From the template DNA linearized at the *Eco*RI site, the antisense cRNA probe was synthesized using T7 RNA polymerase in the presence of $[\alpha^{-32}P]UTP$ (spec. act. $0.5 \times 10^8 cpm/\mu g$ cRNA) and purified through acrylamide gel electrophoresis. The ribonuclease protection assay was performed using a commercially available system (RPAII kit, Ambion). Five μg of total RNA was hybridized with 0.5 ng of cRNA probe at 45°C for 16–24 h, then digested with RNase A and T1. The ratio of sample RNA to the probe was determined prior to the experiments. The protected bands were separated on a 3.5% denaturing acrylamide gel and detected by autoradiography. The intensity of the pro-

tected bands was quantified by an image analyzer (BAS2000, Fujix).

RESULTS

Isolation of a novel drebrin cDNA

Independent recombinant phages, 1×10^6, containing the 1-day-old chick brain cDNA library were screened with drebrin E2 cDNA, Dcw17, and an 80-bp *Hin*fI fragment of ins1 as probes. Twenty-two positive clones that hybridized with both probes were isolated. To eliminate cDNA clones that differ from the embryonic drebrin cDNAs, restriction analysis was performed using Dcw17 as a guide. The restriction enzyme *Sma*I chosen as it provided several digestion profiles. The digested fragments were electrophoresed on an agarose gel, transferred to a nylon membrane and probed with the radiolabeled 3′ *Pst*I–*Eco*RI fragment of Dcw17 (Fig. 2). The digestion profiles from three cDNA clones differed from that of Dcw17. Dcb1, 21 and 22 contained a significantly longer internal *Sma*I fragment compared with that of Dcw17 (Fig. 1). To
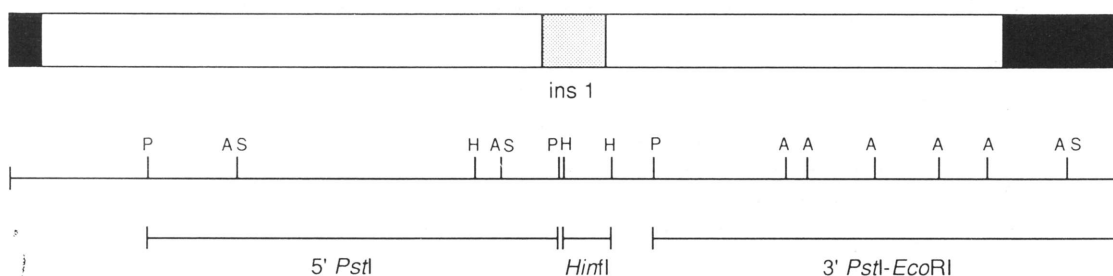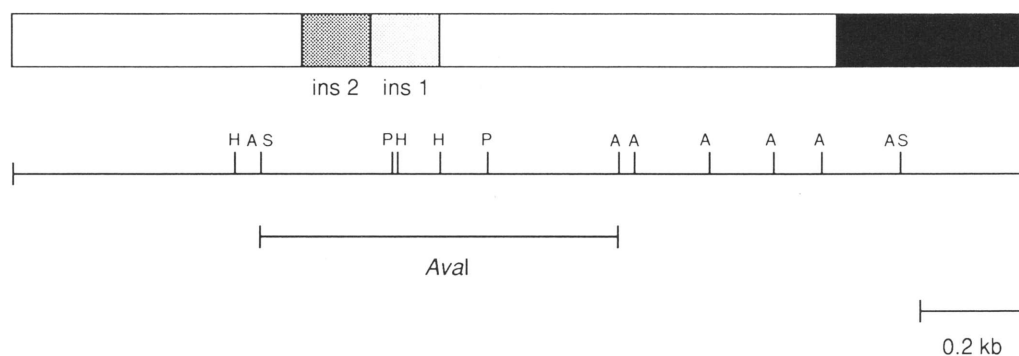
Fig. 2. Structures of drebrin cDNAs. The black rectangles indicate 5′- and 3′-non-coding regions. The shaded rectangles indicate the insertion sequences, ins1 and 2. Restriction sites in Dcw17 previously isolated and Dcb21 are shown for *Ava* I (A), *Hin*fI (H), *Pst* I (P), and *Sma* I (S). The fragments used as probes were indicated below the restriction maps.

further characterize these clones, the longest clone, Dcb21, was chosen for determining the nucleotide sequence and for comparison with those of embryonic drebrin cDNAs.

Nucleotide and deduced amino acid sequences of a novel drebrin cDNA

The nucleotide and deduced amino acid sequences of Dcb21 (1,919 bp) are shown in Fig. 3. Dcb21 started at position 457 in Dcw17. The 3'-non-coding region of Dcb21 was 137-bp longer than that of Dcw17. Although Dcb21 ended with eight repeats of adenine, poly(A) addition signal sequence[27] was not present. Alignment of these two cDNAs revealed that they were identical except that Dcb21 included an insertion of a 138-bp sequence, designated ins2, in the 5' direction immediately upstream from ins1 (at position 550–687). The precise insertion point was determined through comparison with the genomic clone (see below). The sequence of ins2 was deduced to 46 amino acid residues. As the insertion of ins2 did not occur with an in-frame, the first amino acid residue of ins1 (residue 230) was substituted from glycine to cysteine.

Dcb21 did not cover the entire sequence of drebrin cDNA. The 5'-non-coding region and the sequence encoding the amino-terminus were missing. Several further attempts to isolate cDNA clones that extended more toward the 5' direction than Dcb21 were unsuccessful. To confirm whether the putative initiation site of mRNA corresponding to Dcb21 is the same as that of Dcw17, we constructed a plasmid DNA, Dcb21f, containing the full-length open reading frame of Dcb21, by inserting the ins2 sequence into Dcw17. The cDNA fragment was amplified from poly(A)+ RNA of the 1-day-old chick brain by PCR using the sequence surrounding the initiation codon of Dcw17, UP1 (at position 41–70 in Dcw17), as an upstream primer and part of ins2 sequence, DP5 (at position 638–667 in Dcb21), as a downstream primer. The PCR product was electrophoresed on a 1% agarose gel along with the DNA fragment amplified from Dcb21f by another PCR reaction using the same primers. As shown in Fig. 4, the length of the amplified DNA fragment from poly(A)+ RNA was the same as that of cDNA fragment amplified from Dcb21f (1,083-bp in length). We determined by DNA sequencing, that this amplified cDNA frag-

Fig. 3. The nucleotide and deduced amino acid sequences of a novel drebrin cDNA. (a) Partial nucleotide sequence (from 1 to 456) and the deduced amino acid sequence of Dcw17. (b) The nucleotide sequence and deduced amino acid sequence of Dcb21. The insertion sequences, ins1 and ins2 are indicated by light and dark shading, respectively. The oligonucleotide primers used in the PCR assay are underlined.

Fig. 4. PCR assay for drebrin cDNA. The PCR products were electrophoresed on a 1% agarose gel and stained with ethidium bromide. Lanes: (1) the DNA fragment amplified from postnatal 1-day chick brain cDNA using the oligonucleotide primers, UP1 and DP5 (see Materials and Methods); (2) the DNA fragment amplified from Dcb21f with the same primers. The λDNA fragments digested by HindIII (M) were used as molecular weight markers.

ment was identical in sequence to Dcw17 up to the point of insertion. Therefore, the novel cDNA for drebrin should contain the same putative initiation codon and the following coding region as those for Dcw17.

In vitro transcription and translation of the entire coding regions of drebrin cDNAs

We were unable to obtain the amino-terminal sequences of drebrins by directly protein sequencing electro-eluted drebrins, suggesting that the amino-termini are blocked. To confirm that the predicted initiation ATG codon is correct, we subcloned each type of drebrin cDNA containing a 5′-non-coding region and a full-length open reading frame into the pGEM11Z vector. The sense-stranded transcript of each drebrin was synthesized, then translated in the rabbit reticulocyte lysate system. Immunoblot analysis using the anti-drebrin monoclonal antibody, M2F6[32], showed that the translation products and chick drebrins were indistinguishable on SDS-PAGE (Fig. 5), although the additional product that was larger than d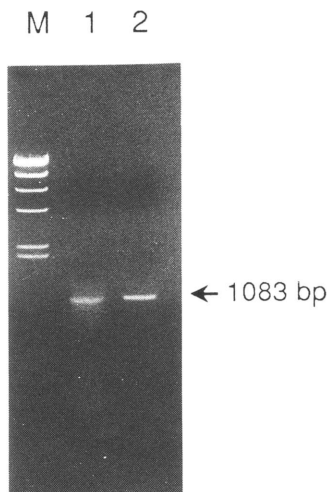rebrin A was also translated from Dcb21 for some unknown reason. Therefore, the ATG codon at position 60–62 of Dcw17 should be the real translation initiation site for each drebrin mRNA and Dcb21 seems to correspond to drebrin A mRNA.

An open reading frame for drebrin A was composed of 652 amino acid residues with a predicted molecular weight of 71,532 Da, which was smaller than its assessed molecular weight on SDS-PAGE (110 kDa).

This discrepancy seems to be due to a similar cause to that in embryonic drebrins[16].

Homology between chicken and rat drebrin A and secondary structure prediction

Computer-aided homology searches of the two available protein sequence databases (NBRF-PDB and SWISS-PROT) revealed that the deduced amino acid sequences of drebrins showed no significant homology with known protein sequences. Although they partially resembled several glutamate-rich proteins, such as caldesmon and the middle subunit of neurofilament protein, these homologies appear to be a consequence of the glutamate-rich composition of the respective proteins. Interspecies comparison of the deduced amino acid sequences of drebrin A showed a high degree of similarity. The overall amino acid identity of the optimal aligned sequences between chicken and rat[36] is 68%. In particular, the homology of the amino-terminal half (domain C1, residues 1–361) and two short regions in the carboxyl-terminal region (domain C2, residues 521–539 and domain C3, residues 596–653) is greater than 80% (Figs. 6 and 7). These regions are also conserved in human (Toda et al., in preparation).

As shown in Fig. 7, the hydropathy plot and the secondary structure prediction suggest that the large homologous region, domain C1 can be subdivided into three. The amino-terminal domain of drebrin A (domain C1a, residues 1–164) is relatively hydrophobic and has the potential to form repeating stretches of β-structure that are separated by short stretches of α-helix and β-turns. By analogy with other proteins

Fig. 5. In vitro transcription and translation of three drebrin cDNAs. The sense-stranded transcript of each drebrin cDNA containing a full-length open reading frame was translated in the rabbit reticulocyte lysate system. The translation product was then electrophoresed on a 7.5% SDS–polyacrylamide gel, blotted and stained with the anti-drebrin monoclonal antibody, M2F6. Lanes: (1 and 5) embryonic day 11 chick brain homogenate; (2) Dcw6; (3) Dcw17; (4) Dcb21.

that exhibit regions of β-structure, such as myelin basic protein[21] and synapsin I[22], domain C1a may organize into a relatively stable β-sheet and form a globular core. This region is followed by a hydrophilic, elongated α-helical region (domain C1b, residues 164–303). The sequence of ins2 is included in domain C1c (residues 316–361), in which β-turns are concentrated. An unusual aspect of the amino acid sequence is that a stretch of 8 serine residues between the series of two proline residues within the middle portion of ins2, is conserved in the rat, with a substitution of proline at residue 331 to serine. We propose that this highly conserved domain, domain C1 contributes to expression and regulation of biological function of drebrins.

We have previously observed that three drebrins are phosphorylated with [^{32}P]orthophosphate in the cultured embryonic optic tectum (Shirao, unpublished observations) and have speculated that phosphorylation of drebrins is an essential step for regulation of their functions. Several consensus sequences for phosphorylation sites[25,39] conserved between two species are found in these regions. One potential recognition site for cAMP-dependent protein kinase lies within the ins2 insertion sequence (at position 351–353). Two sites for Ca^{2+}, calmodulin-dependent protein kinase II are at positions 139–142 and 299–302. One site for protein kinase C is at position 69–71. Two possible glycine residues for *N*-myristoylation (at positions 3

```
  1'  MAGVGFAAHRLELLASYQDVIGEDSPTDWALYTYEDGSDDLKLAASGGGGLLELSGHFEI
      ****.*..*******.*..** *.*..*******************.*** *******
  1"  MAGVSFSGHRLELLAAYEEVIREESAADWALYTYEDGSDDLKLAASGEGGLQELSGHFEN

 61'  QKVMYGFCSVKEPQAVLPKYVLVNWVGEDVPDARKCACASHVAKIAEFFQGVDVIVNASS
      **********..**.****** .****************.***************
 61"  QKVMYGFCSVKDSQAALPKYVLINWVGEDVPDARKCACASHVAKVAEFFQGVDVIVNASS

121'  VEDIDPGAIGQRLSNGLARVSSPVLHRLRLREDENAEPVGTTYQKTDATVEMKRLNREQF
      *****.*************.*********************** .*****.*****
121"  VEDIDAGAIGQRLSNGLARLSSPVLHRLRLREDENAEPVGTTYQKTDAAVEMKRINREQF

181'  WEQAKKEEELRKEEERKKALDARLRFEQERMEQERLEQEERERRYREREEQIEEHRRKQQ
      ******************************** *************.**********
181"  WEQAKKEEELRKEEERKKALDARLRFEQERMEQERQEQEERERRYREREQQIEEHRRKQQ

241'  SMEAEEARQRLKEQSIFGEQQEEDDRQQLRKSESEVEEAAAIIAQRPDNPREFFKQQERV
      *.*****.***.*****.*.*.. *.************************.*****
241"  SLEAEEAKRRLKDQSIFGDQRDEEEESQMKKSESEVEEAAAIIAQRPDNPREFFRQQERV

301'  ASGSGD---AISPGSHRTGRLHCPFIKTADSGPRSSSSSSSSSPPRTPFPYITCHRTPNLS
      **.**.    * **.**.** ****** .****.*********************
301"  ASASGGSCDAPSPFNHRPGRPYCPFIKASDSGPSSSSSSSSSSPPRTPFPYITCHRTPNLS

358'  SFFFCSQSD-YRKVSAAG---CSPCESSPASTPLGEQRTRAPAEETPATPKDSPSPSTQV
      * .***. * .*..... **..**.****..** .** .* *.. * .*.*....
361"  SSLPCSHLDSHRRMAPTPIPTRSPSDSSTASTPITEQIERALDEVTSSQPPPPPPPPPPA

414'  AEPAATEQHWPFPG--PEDKAAEPPGDEPDPDPRPAWTAGADVLGDLVTLEPSEPSPAPA
      .*.......   . *.*.*..* ...  *......*.  *  .. .  .
421"  QEAQESAPRLDGEEVCKEAKVAAAPQVWAGCAEEPPRAQEPPLLQSSPT---EDLMCTES

472'  ASEPQPVETPGVAEPLIELWQSDGAAPAATSTWPLPDTPAGPPVPPEEG----------
      .... ...*. .........  ....*.. .*.* . ...*.. *.*...
478"  PEQAVLAASPEPDASVTSVADAHAADTIETTT-ATTATTIADNVTPAAASLIDLWPGNGE

521'  -----------------------------------------TLLGLDELPEPPATFC
                                               .**..**********
537"  EASTPQAEPRVPTPPSGAEASLAEVPLLNEAAQEPLPPVGEGCANLLNFDELPEPPATFC

537'  DAEQHEEVEEEEEEEEEATAGEPHPTGLGYQEGYQEGPEVPPITNGEMGPKDGTAGRGEQ
      *.*...* *  ....  ....  .....*.. .****  ..*.** .*
597"  DPEEEAEGEPLAASQVLTMPSALEEVDQVLEQELEPEPHL--LTNGETTQKEGT----QQ

597'  ASEGYFSQSQEEEAPPPEEPSAKAPQPVFYNKPPEIDITCWDTDPLPEEEESFGGGL
      ************  ...***.****.****************.** .*****.*.**
651"  ASEGYFSQSQEEEFAQSEEPCAKAPPPVFYNKPPEIDITCWDADPVPEEEEGFEGGD
```

Fig. 6. Comparison of the deduced amino acid sequences of chicken (top line) and rat drebrin A (bottom line). The identical amino acids and substitutable amino acids are indicated by asterisks and dots, respectively. Underlining indicates the conserved potential sites of phosphorylation. The conserved possible glycine residues for *N*-myristoylation are indicated by outlined characters.
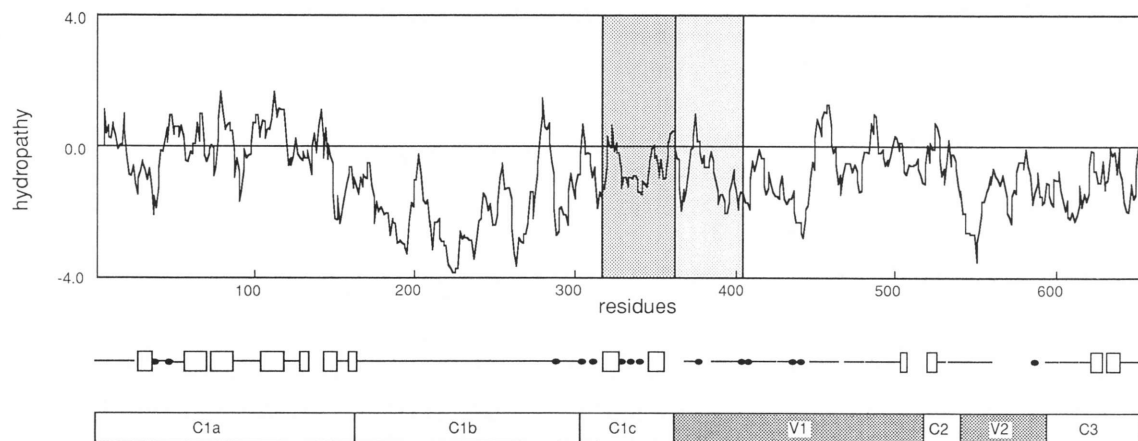
Fig. 7. Hydropathy profile and secondary structure prediction for drebrin A. The hydrophobicity plot of the deduced amino acid sequence of drebrin A was generated by the method of Kyte and Doolittle[17] with a span setting of 9 residues. Shading indicates the insertion sequences, ins1 and 2. The secondary structure, shown below the hydropathy plot, was predicted by the method of Chou and Fasman[4]. The predicted β-structure is indicated by rectangles, the α-helical structure by lines, the β-turn by dots. A diagram of the conserved (C1,2 and 3) and non-conserved (V1 and 2) domains in the predicted drebrin A molecule is also indicated.

and 130)[9,37] are also conserved between species, suggesting the possibility that drebrins may be acylated after exposure of the glycine residue by endoproteolytic proccessing, as is found in the picornavirus capsid protein VP4[5].

The central region of drebrin A including ins1 (domain V1, residues 362–519) has the potential to form repeating stretches of α-helix. The sequence of domain V1 is diverged between chicken and rat, although the ins1 (residues 362–404) sequence shows a weaker resemblance. The proline-rich feature of domain V1 (21%) is retained in the two species.

The carboxyl-terminal region (residues 521–653) is hydrophilic for most of its length and contains two highly conserved domains, C2 and C3, separated by a non-homologous sequence (domain V2, residues 540–595). A prominent sequence consisting of a stretch of 9

glutamate residues in domain V2 of chicken drebrin A is not conserved in the rat. Since protein motifs in domains C2 and 3, identified by the search with the PROSITE protein database, are not conserved among the species, at present we do not have significant evidence supporting the importance of these conserved domains.

Genomic organization of three drebrins

According to the profiles of genomic Southern blots[16], we proposed that each drebrin mRNA is transcribed from a single gene that should include both the ins1 and ins2 sequences. To isolate a drebrin genomic clone, the chicken genomic library (1.7×10^6 plaques) was screened with drebrin E2 cDNA, Dcw17 as a probe. As previously reported[35], one positive clone, designated eDcg5, containing 15.4k-bp insert was iso-
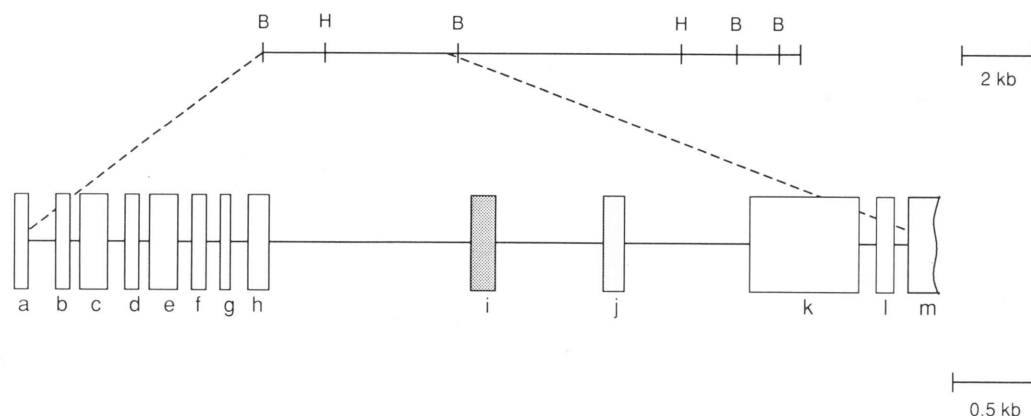

Fig. 8. Restriction map and structural organization of the drebrin genomic clone, eDcg5. The upper line indicates the relative sites for BamHI (B) and HindIII (H). The bottom line shows the region of which the sequence is partially determined. Exons are indicated by rectangles and are labeled a to m. Shaded rectangles, i and j, indicate the exons for ins2 and ins1, respectively.

a

GATCCAGAAGGTGATGTACGGCTTCTGCAGCGTCAAGGAGCCCCAGGCCGTGCTCCCCAAATATGTCCTTGTCAATTGGgtgagtggggcaacgtggggacacctcctgctgagcccgga
  I Q K V M Y G F C S V K E P Q A V L P K Y V L V N W
ctcctgt

b - e

gctcagcacacccctggctgatgtagGTGGGTGAGGATGTGCCTGACGCCCGCAAgTGTGCCTGTGCCAGCCACGTGGCCAAGATCGCAGAGTTCTTCCAGgtgagtgatgccgggggatg
                    V G E D V P D A R K C A C A S H V A K I A E F F Q
gggactgggcctgccggagggggggtctctgatgggtttttgcccccaccgcccccagGGTGTGGATGTTATCGTCAATGCCAGCAGCGTGGAGGACATTGACCCGGGGGCCATCGGGCAGC
                      G V D V I V N A S S V E D I D P G A I G Q
GGCTCTCCAAtGGGCTGGCGCGCGTCTCCAGCCCCGTGCTGCACCGCCTGCGGCTGCGcGAGGACGAGAATGCCGAGCCCGTGgtaacgtccccgtggccacgtccctatggccacggtg
R L S N G L A R V S S P V L H R L R L R E D E N A E P V
tcccccgtgccgatcccagccctacccctgccaccctctgactgtgctccatcccagCACGACCTACCAGAAAACCGACGCCACCGTGGAGATGAAGCGGCTCAACCGGGAGCAGTTC
                               G T T Y Q K T D A T V E M K R L N R E Q F
TGGGAACAAGCCAAGgtgggcagcgagggatggagaggggacgggtgccccggcgtgggctgatggccccatgtcccgtccctgcagAAAGAGGAGGAGTTGCGCAAGGAGGAGGAGAGGA
W E Q A K   K E E E L R K E E E R
AAAAGGCGTTGGATGCGCGGCTGCGGTTCGAGCAGGACGCGCATGGAGCAGGAGCGGCTGGAGCAGGAGGAGCGCGAGAGGCGCTACCGGGAGCGCGAGGAGCAGATCGAGGAGCACAGgc
K K A L D A R L R F E Q E R M E Q E R L E Q E E R E R R Y R E R E E Q I E E H R
atggggcgagccg

f

gggtgtggggtgtcaggggtgctgggggccgggggtagccacgctcagctctgctcctggccccattgcagGAGGAAGCAGCAGAGCATGGAGGCGGAGGAGGCCCGGCAGCGCCTGAAG
                                            R K Q Q S M E A E E A R Q R L K
GAGCAGTCCATCTTTgtaagtgccatttggagctgggaac
E Q S I F

g

gtggtgctgtcggggggtgatggtgtcaggagtgatggtgccgggggatgatggtgccgtatctttgcagGGGGAGCAGCAAGAGGAGGACGACAGGCAGCAGCTCCGGAAATCAGAGTCAG
                                         G E Q Q E E D D R Q Q L R K S E S
AGGTGGAGgtgagctcagcacccagagctgatcggggcctggtgtggggcagccccctac
E V E

h

tgcccgagctgggggttccagccttgcttctccctgcagGAGGCCGCTGCCATCATTGCCCAGCGACCTGACAACCCCCGGGAGTTCTTCAAGCAGCAGGAGCGGGTGGCATCGGGCAGC
                              E A A A I I A Q R P D N P R E F F K Q Q E R V A S G S
GGCGACGCCATCTCGCCGGGCAGCCATAGGACAGgtgaggggctgagggcacaggggaggtgcagtacgtgcgagttcg
G D A I S P G S H R T

i

tgtgccttggggcagcactgtgtgttttgctgtgggctgtgctgtgtggttcctgctgtagggatgcgctgtgcctgagcagaaagatggcccccgaggctgtgtcatggcccc`ttgccc
acccggccttggtgctgcctcaccgtcctgccgtccttctgtcctgccgtcctgccgtcctgctgtcagtgctgtcagtgctgcctccctgccgtcctcccatcccggtgcctctgtgt
cccggccccatccatggccactctgagcagggactgacccatctctttgctctctctctctcctccgtccgtctgtctgtctttctctcggcagGTCGTCTGCACTGTCCTTTCATAAAGACA
                                                      G R L H C P F I K T
GCTGACAGTGGGCCGCCTTCTTCTTCTTCCTCCTCCTCCTCCCCCCCGCGGACCCCTTTCCCCTATATCACCTGCCACCGCACCCCAAATCTCTCCTCTTTCTTCCCATgtaggtagctg
 A D S G P P S S S S S S S P P R T P F P Y I T C H R T P N L S S F F P
ggagccccctgggcacgcagccccttggccctgcagccctccccagccctgctgcttgtggggactggggtgcaaggacagagctgtgggatcagcagccaaccacagggggccaaggggca
cctcctgtgctctgtggtgcatcagaaagtcaaatgggacagggcagagccgtgcccagggacgtgatgctgtgcccgtggggcagcgagggatggacgaggctgtgtccaggaccacgt
ggagctgtgcctatggggcagaaggggcacacggtgctgtgcccctccagccagataaagtgggcaccgtgtggtgctgcagagagcggaggctctgccagggctcggtggggcagccgt
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ggtatgtggcaatgtgcgtctcacagctttcggccagactg

j

cgccggggcgctgggggcctgctccctccccgttcaggtgttcacccccccagtgcaggcggttcccccccaagccatgtctccgttgtctgcagGCAGCCAGTCCGACTACCGAAAGGTTTCG
                                                         C S Q S D Y R K V S
GCAGCGGGCTGCAGCCCCTGCGAGTCCAGCCCGGCCTCCACGCCGCTGGGCGAGCAGCGCACCCGCGCCCCGGCtGAAGAGACGCCGGCAACGCCCAAAGgtgggcgagggggctggcgct
A A G C S P C E S S P A S T P L G E Q R T R A P A E E T P A T P K
gcctgctgca

k

gtcctactgcatctccagagaacaccatcgctgcccacccatcccacccacccttcaccattccccagcccaaagctctctccatctgcccccctgtgctcagccatcctcagggtgggc
tctgaccgctgttccccccctcccaccttgcagACTCCCCCAGCCCCAGCACCCAGGTGGCAGAGCCGGCAGCGACTGAGCAGCACTGGCCCTTCCCTGGGCCTGAAGACAAAGCTGCA
                                        D S P S P S T Q V A E P A A T E Q H W P F P G P E D K A A
GAGCCGCCGGGGGACGAGCCCGACCCCGACCCCAGGCCGGCGTGGACAGCGGGGGCTGACGTGCTGGGGGACCTGGTGACCCTGGAGCCCTCCGAGCCATCCCCAGCGCCCGCTGCGTCC
E P P G D E P D P D P R P A W T A G A D V L G D L V T L E P S E P S A P A A S
GAACCCCAGCCCGTGGAGACACCGGTGTGGCCGAGCCCCTCATCGAGCTGTGGCAGAGTGATGGCGGCCCCCGCTGCCACCAGCACCTGGCCCCTGCCCGACACCCCCGCGGGACCA
E P Q P V E T P G V A E P L I E L W Q S D G A A P A A T S T W P L P D T P A G P
CCGGTCCCCCCCGAGGAGGGCACGCTGCTGGGCCTGGACGAGCTGCCCGAGCCCCCCGCCACgTTCTGTGACGCGGAGCAGCACGAGGAGGTGGAAGAAGAAGAGGAGGAGGAGGAGGCC
P V P P E E G T L L G L D E L P E P P A T F C D A E Q H E E V E E E E E A
ACgGCAggTGAGCCCCATCCCACAGGGCTGGGCTACCAGGAGGGCTACCAAGAAGGCCCCGAGGTGCCCCCCATCACCAATGGGGAGATGGGCCCCAAGGACGGCACGGCGGGCCGTGGC
T A G E P H P T G L G Y Q E G Y Q E G P E V P P I T N G E M G P K D G T A G R G
GAGCAGgtgagcaccgtgagg
E Q

l

gccgcaccccaaatcccgcagGCCAGCGAGGGCTACTTCAGCCAGTCCCAGGAGGAGGAGGCCCCGCCGCCCGAGGAGCCGTCGGCCAAAGCCCCGCAGCCCGTCTTCTACAACAAGCCG
                     A S E G Y F S Q S Q E E A P P P E E P S A K A P Q P V F Y N K P
CCAGgtgagtgcgcaggggcaggaatggtggggggggtggtcctccac
P

m

gggtgcccccctaaccccatccccacagAGATCGACATCACGTGCTGGGACACGGACCCGCTGCCCGAGGAGGAGGAGAGCTTCGGGGGCGGCCTGTAGGCCCAGCGCAGCCTGGAGGCCC
              E I D I T C W D T D P L P E E E E S F G G G L *
CGCCGGCGCGGGGGTGTGAGCGGCCGTGGCCGGGGGCCCCCTCCCCGCTGGGGCCCCGCAGGATGGGAGCCGGGGGGGGCCGCCCCGCAGCACCCC

Fig. 9. Nucleotide sequence of the drebrin genomic clone, eDcg5. The DNA sequences of exons a to m are shown, along with some of their flanking sequences. The deduced amino acid sequences show below the nucleotide sequences of the exons.

lated (Fig. 8). The relative cleavage sites for two restriction enzymes (*Hin*dIII and *Bam*HI) were consistent with the results of genomic Southern blotting. DNA fragments carrying exons were detected by hybridization with a 5' *Pst*I fragment or a 3' *Pst*I–*Eco*RI fragment of Dcw17 (Fig. 2). The *Hin*dIII fragment (11.2 kb) hybridized with the 3' *Pst*I–*Eco*RI fragment, but not the 5' *Pst*I fragment. The *Bam*HI fragment (5.6 kb) hybridized with both fragments, suggesting that it carries most of the exons for drebrins. Thus, the sequence of the 5' *Bam*HI fragment was partially determined and the exact location of the exons was confirmed through comparison with the cDNA sequences.

The eDcg5 insert started from the *Sau*3AI site in Dcw17 (at position 236) and carried the remaining exons. The determined nucleotide sequence of the exons and their flanking regions are shown in Fig. 9, which covered the sequence from position 236 in Dcw17 to 1,680 in Dcb21. Within this region, all the exons were flanked by appropriate splice sites[23,30] and the drebrin gene consisted of at least 13 exons, named a to m. Strikingly, the sequences of ins1 and 2 were independently encoded in the individual exons, i and j, respectively, separated from the neighboring exons by the insertion of relatively long introns. Since Southern blot data and the structure of the genomic clone reported here are consistent with there being a single drebrin gene in the chicken genome, the mechanism for generating three drebrin mRNAs from the single drebrin gene should be according to a common mechanism for an alternative splicing that is the inclusion or exclusion of individual exons[1]. The sequences 5' upstream and 3' downstream from the ins2 exon resemble consensus sites for intron acceptor and donor, respectively[23]. These splice junctions should be used for inclusion of ins2 only in the drebrin A mRNA. Similarly, alternative use of the splice acceptor and donor in the boundaries of the ins1 exon should result in inclusion and exclusion of ins1 in the drebrin E2 and A mRNAs and the drebrin E1 mRNA, respectively.

Temporal and spatial regulation of splicing site selection of the drebrin gene

Although drebrin mRNAs were detected by Northern blotting with either ins1 or ins2 as a probe (data not shown), the three drebrin mRNAs were indistinguishable due to the limitation of resolution. To estimate the expression of each drebrin mRNA in the various tissues during development, we detected each drebrin transcript by ribonuclease protection assay using the antisense-stranded cRNA probe of a 680-bp *Ava*I fragment of Dcb21 (Fig. 2). As shown in Fig. 10,

three protected bands corresponding to drebrin E1, E2 and A were detected (337, 466 and 680 bases in length, respectively). The protected bands corresponding to the drebrin E1 and E2 mRNAs were detected in the whole body of the 5-day chick embryo. The expression level of drebrin E1 was slightly higher than that of drebrin E2. The protected band of the drebrin A mRNA was not detected at this developmental stage. In the 11-day chick embryo, drebrin E2 mRNA was more abundant than the others and distributed in the variety of the tissues investigated, except for the liver. The expression level of drebrin E1 was relatively low at this stage and the tissue distribution of drebrin E1 mRNA was similar to that of drebrin E2 mRNA. Although the origins of the protected bands in these non-neural tissues were not identified, it has been shown that embryonic drebrins also localize within the intestinal longitudinal muscular layer and skeletal muscle in addition to the peripheral nerve cells[32], suggesting that embryonic drebrins are also expressed in the non-neuronal cells. In contrast to the widespread distribution of embryonic drebrin mRNAs, the protected band corresponding to drebrin A mRNA was restricted in the neural tissues. In the postnatal 1-day chick,
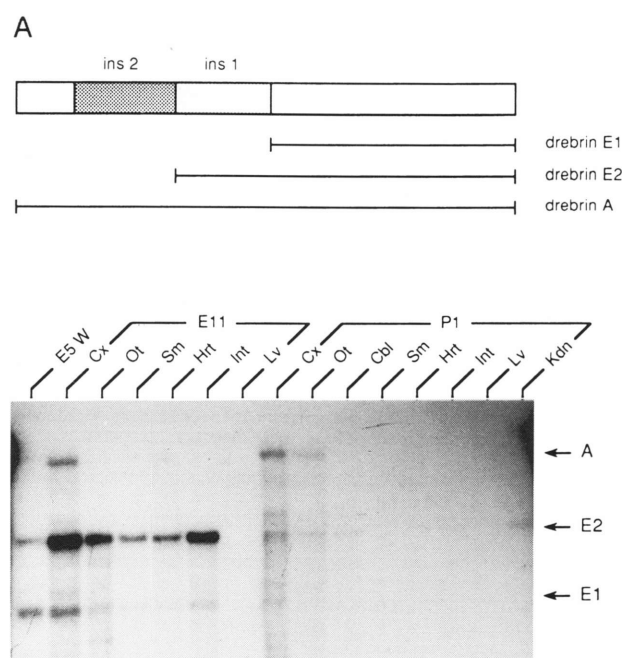
Fig. 10. Ribonuclease protection assay for each drebrin transcript. Total RNAs were isolated from the whole body of the 5-day chick embryo (E5 W) and various tissues of the 11-day of chick embryo (E11) and postnatal 1-day chick (P1). The protected fragments corresponding to drebrin E1, E2 and A mRNAs (337, 466 and 680 bases in length, respectively) were detected in 10 μg of total RNAs. The protected fragments were electrophoresed on a 3.5% denaturing acrylamide gel and detected by autoradiography (A). The intensity of the protected bands was quantified using a image analyzer (B). Cx, cerebral cortex; Ot, optic tectum; Cbl, cerebellum; Sm, skeletal muscle; Hrt, heart; Int, intestine; Lv, liver; Kdn, kidney.

B

drebrin E1

drebrin E2

drebrin A

Fig. 10 (*continued*).

pared with that in the cortex and optic tectum. The ratio of the intensity was 1.0 : 7.8 : 4.0 (cerebellum : cerebral cortex : optic tectum). On the other hand, considerable amounts of drebrin E1 and E2 mRNAs still remain in the cerebellum, at ratios of 1.0 : 3.5 : 1.5 and 1.0 : 2.7 : 1.5, respectively. These results coincide with the fact that the migration, the growth of the dendrites and the formation of synapses continue in the 1-day postnatal cerebellum[14].

DISCUSSION

We isolated part of a novel cDNA clone that was distinct from the two cDNAs for the embryonic drebrins previously reported. The nucleotide sequence of this cDNA was entirely identical to that of drebrin E2 except for the internal 138-bp insertion, designated ins2. The translation product of the transcript containing the full-length coding region showed the same molecular weight as chicken drebrin A, estimated by SDS–PAGE. Moreover, the developmental change in the amount of the mRNA containing both ins1 and 2 and its distribution analyzed by the ribonuclease protection assay is consistent with that of drebrin A detected by two-dimensional gel electrophoresis[31] and immunoblotting[32]. Therefore, this novel drebrin cDNA containing the two insertion sequences, ins1 and ins2, should correspond to the mRNA encoding the remainder of the isoforms, drebrin A.

Immunoblots have shown that drebrins are classified into three forms in the chicken[32]. Molecular cloning of three types of drebrin cDNAs indicated that the heterogeneity of chicken drebrins can be explained by insertion or deletion of the two sequences, ins1 and 2, that is, drebrin E1 mRNA excludes both ins1 and 2, drebrin E2 mRNA includes ins1, but not ins2, and drebrin A mRNA includes both these insertion sequences. In mammals, two isoforms (E and A) of drebrins have been detected using a monoclonal antibody to chicken drebrins. We recently isolated and characterized the adult form of drebrins from a rat hippocampal cDNA library[36]. Since the sequence of ins2 was well conserved between chicken and rat, it is proposed that this insertion also results in the heterogeneity in rat drebrins. We have no evidence supporting further insertional sequences in chicken drebrins. Only a single product was amplified by PCR from the total cDNAs of the chick brain between the oligonucleotide primers, UP1 and DP5, suggesting that no additional sequence is inserted or deleted at least in this region.

three drebrin mRNAs were detected almost solely in the neural tissues, although drebrin E2 mRNA is still detectable in the kidney. In the cortex, the expression levels of drebrin E2 and A were almost even and that of drebrin E1 was considerably low. The amounts of the protected bands correlated well with the amounts of proteins[31] and immunoreactivity[32], suggesting that changes in the amounts of drebrins are mainly regulated at the levels of their mRNAs. The level of drebrin A mRNA in the cerebellum was slightly low, as com-

Differences in expression and localization of three drebrin isoforms

One of characteristics of drebrins is that their expression is regulated developmentally. Each of three drebrin isoforms shows unique changes in expression during neuronal differentiation[32]. Drebrin E1 appears in postmitotic and premigratory cells. Drebrin E2 replaces drebrin E1 in migratory cells. Drebrin A accumulates in postmigratory, mature neurons. These observations give rise to the notion that the three drebrin isoforms have different roles. The cellular and subcellular distribution of the three isoforms also support this hypothesis.

Each drebrin isoform shows significant differences in its tissue distribution. Immunoblots[32] and the ribonuclease protection assay reported here revealed that embryonic drebrins were widely distributed in a variety of tissues except for the liver at certain developmental stages, whereas drebrin A was detected only in the neural tissues at the latter stage. These observations suggest that embryonic drebrins also have other roles in non-neuronal cells, whereas the function of drebrin A seems to be restricted to the neural tissues.

The subcellular localization also varies among drebrins. We previously reported changes in their localization in the cerebellar granule cells during differentiation[32,35]. First, drebrin E1 distributes uniformly within the cell somata of postmitotic, premigratory granule cells, then drebrin E2 accumulates in the growing dendrites of migratory and postmigratory cells and finally, drebrin A localizes in the postsynaptic sites of the granule cell dendrites. It has been shown that drebrin A is concentrated on the submembranous region in the neurons[33] and fibroblasts transfected with the drebrin A expression plasmid[36], despite being hydrophilic, showed typical characteristics of a cytosol protein. These observations suggest that drebrin A interacts with the plasma membrane directly, or indirectly through binding with a membrane or a submembraneous protein. Considering its effect on the transfected fibroblasts[36], it is reasonable to suppose that drebrin A is concentrated on the inner surface of the cell membrane where it is then involved in control of the cell shape. The interaction of submembraneous proteins and microfilaments is thought to be important for the control of cellular morphogenesis[2]. The morphological diversity of the neurons and the plastic changes in their processes should be regulated by complex, 3-dimensional networks of these proteins. Since drebrins are colocalized with actin filaments in the neurons and neuroblast cells (Asada et al., submitted), drebrin A might be one of key components of the submembraneous networks. Precise biochemical analysis of the interaction of drebrin A with the other submembraneous components will be needed to confirm this hypothesis. Moreover, to understand the molecular basis of differences in the subcellular localization among the three isoforms, it would be of interest in future investigations to use cultured cells transfected with the expression plasmid carrying the embryonic forms and the site-specific deletion mutants.

Temporal and spatial regulated alternative splice site selection of the drebrin gene

In this study, we demonstrated that three drebrin mRNAs were transcribed from a single drebrin gene by alternative splicing. The restriction profiles of the genomic clone, eDcg5, were consistent with the results of genomic Southern blots[16]. Therefore, the possibility of the existence of an additional drebrin gene closely related to this clone is considered unlikely.

A large number of genes that express several related, but structurally distinct mature mRNAs have been reported[1]. These alternative splice site selections are often subject to tissue-specific and/or developmental control. In some instances, functional differences among the protein isoforms generated by these mechanisms have been also discussed. For example, the diversity in the N-CAM polypeptide structure achieved by alternative splicing correlates with distinct stages of neuronal differentiation[26]. N-CAM-140 and -120 are expressed from the time of neural tube formation, whereas N-CAM-180 is the first to appear on postmigratory cells. These three N-CAM polypeptides have identical amino-terminal extracellular domains but truncated carboxyl-terminal domains. N-CAM-180 and -140 are integral membrane proteins, whereas N-CAM-120 is attached to the membrane via a GPI lipid anchor and can be released spontaneously from the membrane[11]. The diversity of MAP2 is also generated by alternative splicing in a developmental stage-specific manner[24]. The high-molecular weight form of MAP2 is expressed in the dendrites, where its mRNA is also located. In contrast, the low-molecular weight form, MAP2c, which is particularly abundant in the developing brain, lacks the cross-linking sidearm domain and dendritic targeting signal. Therefore, the high- and low-molecular weight forms of MAP2 might regulate the stability of microtubules in a different manner. Although the biological significance of the structures of the drebrin isoforms raised by alternative splicing is not yet understood, their expression and distribution are strictly different. Therefore, it is proposed that alternative splicing is important for drebrins to play appropriate roles suited to the various requirements of neural development.

Although the molecular mechanisms underlying the alternative splice site selection are for the most part unknown, the *cis*-active elements required to decide the splicing pattern seem to exist near the splice junction. Emeson et al.[7] have demonstrated that the tissue-specific alternative splice site selection of the calcitonin/calcitonin gene-related peptide (CGRP) primary transcript was primarily regulated by *cis*-active element(s) near the calcitonin-specific 3'-splice junction. They suggested that tissue-specific *trans*-acting factor(s) may bind to the element(s) and inhibit the calcitonin splice acceptor site in the CGRP-producing cells. Similar factor(s) expressed in a tissue-specific and a stage-dependent manner may regulate the alternative splice site selection of the drebrin gene. Because of its stringent spatial and temporal regulation, the drebrin gene should provide a useful model system for examining the molecular mechanisms of alternative splicing.

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