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Molecular cloning of a cDNA for the developmentally regulated brain protein, drebrin

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A λgt11 cDNA library from 10-day-old chicken embryo was screened immunologically using an antiserum against drebrins E1, E2 and A, proteins previously designated S5, S6 and S54, respectively. A cDNA clone for a common domain of drebrin was isolated. Northern blot analysis of chicken brain indicated that drebrin mRNAs are about 2.7 kilobases in molecular size and that expression of these proteins is developmentally regulated.

Drebrins E1, E2 and A are developmentally regulated proteins, which were previously designated as proteins S5, S6 and S54, respectively[10,11]. Drebrins E1, E2 and A migrate with apparent molecular weights of 95, 100 and 110 kDa on sodium dodecyl sulfate (SDS)–polyacrylamide gels, and their isoelectric points are about 4.5[10]. All of them are localized in the cytosol of neurons[11], but the time course of appearance for each protein differs completely from one another[10]. Specifically, drebrin E1 is first expressed immediately after neurons have finished their final mitosis[11]. While these neurons are extending their axons and dendrites, drebrin E1 is displaced by drebrin E2[10]. The amount of drebrin E2 declines as drebrin A appears in parallel with further maturation of the nervous system. Finally, in mature neurons, drebrin A is localized in certain types of dendrites[11,12]. It is proposed that drebrin A is involved in modification of the structure of dendrites[12]. Drebrins E1, E2 and A have a high degree of structural homology as determined by peptide mapping of purified drebrins[10]. It therefore would be interesting to determine whether each of these drebrin types is coded by a unique mRNA or whether the 3 forms arise individually as a result of posttranslational modifications of a single protein precursor. In this study, we have isolated a cDNA for a common polypeptide domain shared by drebrins E1, E2 and A and investigated their respective mRNAs using Northern blot analysis. We report here molecular sizes of drebrin mRNAs and developmental change of the amount of these mRNAs.

A cDNA library constructed in the λgt11 vector using 10-day-old whole-chicken embryo tissue (Clontec Labs, CA) was screened using a rabbit antiserum to drebrin, designated RS6 which recognizes the common polypeptide domain shared by drebrins E1, E2 and A. Preparation and characterization of this antiserum has been previously described[11]. The cDNA library was infected, plated on *E. coli* Y1090 at a density of 50,000 phage per 15-cm plate, and incubated at 42 °C for 3 h. The plates were overlaid with a dry nitrocellulose filter, previously saturated with 10 mM isopropyl β-D-thiogalactoside, and then incubated at 37 °C for 12 h. The standard protocol of Young and Davis[14] was followed for incubation of the

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Fig. 1. Restriction map of the cDNA insert from the clone gDcw1. The cDNA was mapped by single digestion with endonucleases. Digested DNA was electrophoresed on 1% agarose gels or 5% acrylamide gels. The vertical lines indicate the restriction endonucleases site. E, *Eco*RI; Sm, *Sma*I; P, *Pst*I; A, *Alu*I; S, *Sac*II; M, *Mbo*I. The following restriction endonucleases do not cut within the sequence covered by the cDNA: *Hin*dIII, *Bam*HI, *Sph*I, *Kpn*I, *Sac*I and *Xho*I.

filters in the anti-drebrin antiserum RS6 (1:1000 dilution in Tris-buffered saline (50 mM Tris, pH 7.4, 150 mM NaCl) with 5% bovine serum albumin). This antiserum had been preabsorbed by incubation with *E. coli* Y1089 lysate[13]. Filters were washed twice in Tris-buffered saline, and incubated in Tris-buffered saline with ^{125}I-labeled *Staphylococcus aureus* protein A. After washing, the filters were dried and exposed to Kodak X-Omat AR film with an intensifying screen at −70 °C.

One putative drebrin clone (gDcw1) was identified among 2×10^5 clones that were screened. After plaque purification, the cDNA insert was excised by digestion with restriction endonuclease *Eco*RI and its size was estimated to be 1.4 kb pairs. Restriction endonuclease analysis of the cDNA insert is shown in Fig. 1. Using the restriction endonucleases *Kpn*I and *Sst*I, which have restriction sites only within λgt11 vector, as well as *Pst*I, orientation of the cDNA was determined by mapping the entire gDcw1 DNA.

This clone was further characterized as drebrin cDNA using 4 monoclonal antibodies (MAbs) M2A6, M2E7, M2F6 and M2H1, each of which recognizes a different epitope in the common domain of drebrins E1, E2 and A, to identify the drebrin fusion protein in λ-lysogens. The production and character-

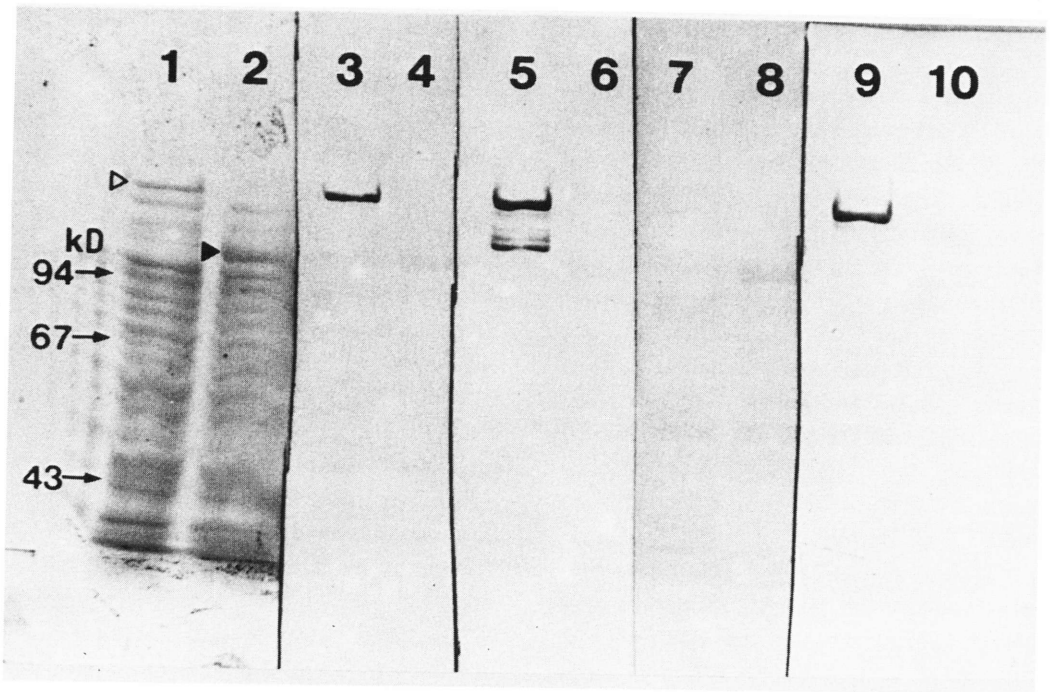

Fig. 2. Western blot analysis of the fusion protein encoded in clone gDcw1. Extract of induced lysogen (0.5 mg of protein in each lane) were electrophoresed on an SDS–polyacrylamide gel (8%). Lanes 1, 3, 5, 7 and 9 are proteins from the gDcw1 lysogen; and lanes 2, 4, 6, 8 and 10 are from the intact λgt11 lysogen. After the electrophoresis the proteins were transferred to a nitrocellulose sheet by electroblotting for 12 h at 200 mA[9]. The sheet was cut into 5 strips and all strips (except that for lanes 1 and 2) were incubated in Tris-buffered saline (pH 7.4) containing 5% bovine serum albumin for 60 min. Each strip was then further incubated with a monoclonal antibody (see below) in the same buffer for 90 min. After washing, the sheets were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG in the above buffer for 60 min. The blots were developed using 0.05% 4-chloro-1-naphthol and 0.01% hydrogen peroxide[6]. Proteins in lane 1 and 2 were stained with Amido black. Lanes 3 and 4, MAb M2A6; lanes 5 and 6, MAb M2E7; lanes 7 and 8, MAb M2H1; lanes 9 and 10, MAb M2F6. The open triangle indicates the fusion protein and the closed triangle indicates β-galactosidase protein.

ization of these monoclonal antibodies have been previously described[11].

gDcw1 and intact λgt11 lysogens were established in *E. coli* Y1089. Each of these lysogens was grown at 32 °C to $O.D._{600}$ = 0.5 and incubated at 42 °C for 20 min. Isopropyl β-D-thiogalactoside was then added to 1 mM and the culture was incubated for 2 h at 37 °C. The induced bacteria were recovered by centrifugation, resuspended in phosphate-buffered saline (pH 7.4), and sonicated. Then the lysate was electrophoresed on an SDS–polyacrylamide gel (8%) according to the procedure of Laemmli[7] and immunoblotted with each of the 4 monoclonal antibodies as described previously[11]. As shown in Fig. 2, MAbs M2A6, M2E7 and M2F6 specifically bound to a fusion protein of about 180 kDa (lanes 3, 5 and 9), which is not present in the λgt11 lysogen (lanes 4, 6 and 10). MAb M2H1 did not recognize any protein either in the gDcw1 or λgt11 lysogen (lanes 7 and 8). MAb M2E7 stained additional bands in the gDcw1 lysogen (lane 5). Since MAb M2E7 could recognize proteolytic fragments of purified drebrins generated with *Staphylococcus aureus* V8 protease (data not shown), these additional bands were thought to be degradation products of the fusion protein. These results indicate that this cDNA encodes at least a part of the common domain of drebrins E1, E2 and A.

In previous reports[10,11] the developmental changes of amounts of drebrin E1, E2 and A proteins have been investigated using two-dimensional gel electrophoresis and immunoblot analysis. In the chick embryonic optic tectum, drebrin E1 is known to increase from embryonic day 4 to day 7, after which it decreases. Drebrin E2 increases from day 7 to day 9, and decreases after day 18. Drebrin A appears by day 11, and then increases. In order to determine whether the appearance of these mRNAs corresponds to the developmental regulation of the drebrin proteins, Northern blot analyses of poly(A)$^+$ RNAs from the optic tecta of day 7, day 11 and day 17 chicken embryos were carried out. Total RNAs were extracted from the brain tissue by the guanidine hydrochloride procedure[2] and poly(A)$^+$ RNAs were isolated by chromatography on oligo-dT cellulose columns[1]. RNAs were fractionated on a denaturing agarose gel[5,8], blotted to a nylon membrane filter and hybridized with [^{32}P]cDNA insert from gDcw1 labeled by random priming with DNA polymerase I[3].

As shown in Fig. 3, the probe hybridized to a band of 2.7 kb in molecular size. Although abundance of the hybridized mRNA species was different at each developmental stage, the molecular size of the hybridized mRNA species was similar in day 7, day 11 and day 17 embryos. Possibly each type of drebrin is encoded by a unique mRNA that shares homology to the other drebrin mRNAs, and its developmental expression is regulated independently. However, it is

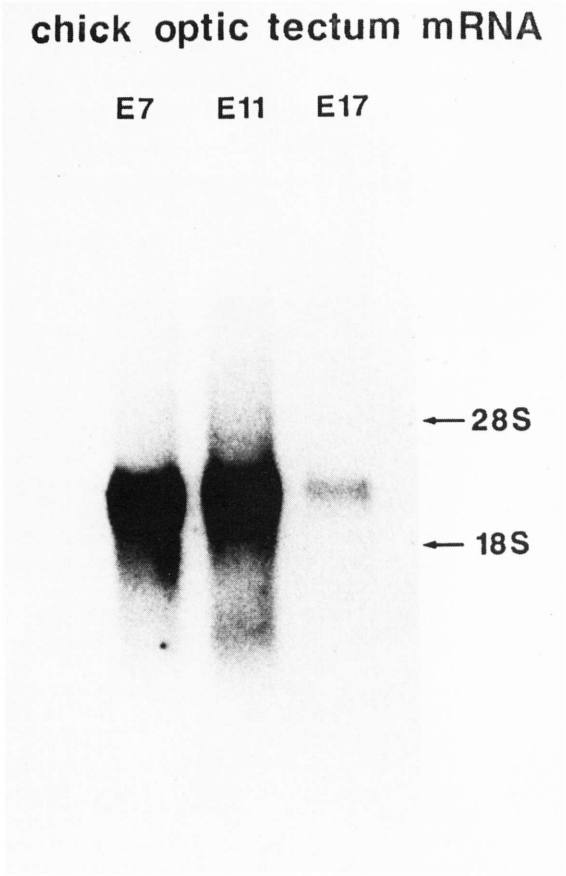

Fig. 3. Northern blot analysis of poly(A)$^+$ RNA from chicken optic tectum. Five μg of poly(A)$^+$ RNA from optic tecta of 7-day chicken embryo (lane E7), 11-day chicken embryo (lane E11), and 17-day chicken embryo (lane E17) were denatured with 6% formaldehyde, electrophoresed on a 1% agarose gel under conditions of denaturation, and transferred to a nylon membrane filter. Approximately 5 × 10^5 cpm of ^{32}P-labeled cDNA (1 × 10^8 cpm/μg) were added to hybridization buffer containing 6 × SSC, 10 × Denhardt's solution and 1% SDS. Following hybridization for 12 h at 68 °C, the filter was washed twice in 0.1 × SSC and 1% SDS for 1 h at 42 °C. The filter was exposed to X-ray film at −70 °C in the presence of an intensifying screen for 1 week. rRNAs served as molecular size standards.

not possible to distinguish size difference of 120–240 b which would be expected for the mRNAs of drebrin species with 5000–10,000 Da differences in size. In Fig. 3 the intensity of the band in each lane reflects the total amount of all 3 drebrin mRNAs; consequently the smaller amount of drebrin mRNAs in day 17 embryo compared with those in day 7 and day 11 embryos is consistent with the smaller amount of drebrin proteins as demonstrated previously in two-dimensional gel electrophoresis of the chick optic tectum[10]. Since we have recently used gDcw1 as a probe to isolate from the same cDNA library another cDNA clone, which does not possess the *Pst*I site in the 5′ terminal region in Fig. 1 (Kojima et al. in preparation), the possibility that each type of drebrin is encoded by a unique mRNA is high. In order to confirm this explanation, however, we have to await S1 nuclease mapping experiments for the occurrence of 3 drebrin mRNAs.

The isolation of a cDNA encoding the common domain of drebrin will now allow direct determination of drebrin mRNAs in different tissues and at different stages of development. And this cDNA will also make it possible to isolate the drebrin gene, and open up approaches for the study of the molecular bases of the developmental regulation and the cell-specificity of its expression.

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