NeuroReport 3, 109–112 (1992)

Cloning of drebrin A and induction of neurite-like processes in drebrin-transfected cells

Tomoaki Shirao,*[,CA] Nobuhiko Kojima and Kunihiko Obata

Laboratory of Neurochemistry, National Institute for Physiological Sciences, Okazaki, Japan
*Present address: Dept of Physiology, School of Medicine, Keio University, Shinanomachi, Shinjuku-ku Tokyo 160, Japan

[CA] Corresponding Author

THE developmentally-regulated neuron-specific protein, drebrin A, is expressed first at the time of outgrowth and maturation of dendrites, and is localized within dendrites of the adult brain. A cDNA clone of adult rat drebrin A was isolated and sequenced. There is no overall homology with other reported protein sequences except chicken drebrins. We constructed the expression vector MIW-DA containing the drebrin A cDNA. Transfection of non-neuronal cells with MIW-DA induced the formation of highly branched neurite-like cell processes. In these process-bearing transfectants, expressed debrin A is concentrated in submembraneous regions of the cell. Furthermore, actin concentration is higher in these cells than other fibroblasts. These results suggest a possible role of drebrin A in neurite outgrowth.

Key words: Molecular cloning; Debrin; Nerve tissue proteins; Neurite-outgrowth; Dendrites; Developmental biology; Transfection; Cultured cells; Microfilaments

Introduction

Outgrowth of axons and dendrites is the first step in neuronal network formation in the central nervous system. Since neuron-specific proteins may play a role in outgrowth of axons and dendrites, transfection studies of non-neuronal cells with neuron-specific proteins, whose expression is associated with outgrowth of axons or dendrites, such as GAP-43,[1] MAP2,[2] and tau proteins,[3] have been done. However, the intracellular mechanism of neurite outgrowth is not yet clarified.

The developmentally-regulated neuron-specific protein, debrin A, is expressed first at the time of outgrowth and maturation of dendrites,[4–6] and is localized within dendrites of the adult brain.[7] In cat visual cortex, expression of drebrin A is most extensive during the sensitive period[8] for ocular dominance plasticity.[9] In this study we first cloned a complementary DNA encoding rat drebrin A and sought to clarify by transfection whether drebrin A plays a role in neurite-outgrowth.

Materials and Methods

The initial cDNA clone (gDrh24) was isolated from oligo(dT)-primed lambda-gt10 cDNA library prepared from poly(A)$^+$RNA isolated from the hippocampus of adult rat brain, using chicken drebrin E2 cDNA[10,11] as a probe. Further clones were then isolated from the same library using a 5′-end 100-bp fragment (bases 221 to 320) of gDrh24 as a probe. Selected clones were subcloned into M13mp18 and partially deleted by successive ExoIII exonuclease digestion. The nucleotide sequences were determined using the dideoxy chain-termination method with modified T7 DNA polymerase. All experiments were performed using standard procedures described elsewhere[11] or according to the manufacturer's instructions. Sequence data were assembled and analyzed using the programs of genetyx (Software Development Co., Tokyo).

The drebrin A expression plasmid MIW-DA and the control plasmid MIW-C were constructed by inserting the fragment (bases −53 to 2312) of rat drebrin A cDNA (Drh102) into plasmid MIW-HEP which has a β-actin promoter.[12] The plasmids MIW-DA and MIW-C had a drebrin cDNA oriented in the sense and anti-sense direction, respectively. Clonal cell line LD2B4, constitutively expressing drebrin A, was established after co-transfection of MIW-DA and pSTB-neo (a neomycin resistance expression plasmid)[13] into L cells by the calcium phosphate coprecipitation method,[14] using active Geneticin (400 μg ml^{-1})[15] (Sigma) for selection.

Cells or tissues were weighed, and directly homogenized with a micro tissue grinder in 10 volumes of sodium dodecyl sulfate (SDS)-sample buffer composed of 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 1 mM EDTA, 40 mM Tris, and 240 mM glycine at pH 8.5. These homogenates were electrophoresed on SDS-polyacrylamide gel (6%) according to the method of Laemmli.[16] Then the gel was electroblotted onto a nitrocellulose sheet, and immunostained with anti-drebrin monoclonal antibody M2F6 as described previously.[5] In some cases the gel was stained with coomassie blue.

L cells were transfected by the standard calcium phosphate coprecipitation method[14] with plasmid MIW-DA or MIW-C. Transfected cells were cultured for 48 h and then fixed in 3.7% formaldehyde for 30 min at 37°C. Fixed cells were permeabilized with 0.1% Triton X-100 in phosphate buffered saline and

```
-53                                                                                                  GTCGGTCCGCCCCGTGCGTGCGTCTGTCCGTTCGGCCTCCGTCCGGCCCCGCAGC
  1 ATGGCCGGCGTCAGCTTCAGCGGCCACCGCCTGGAGCTGCTGGCGGCGTACGAGGAGGTGATCCGGGAGGAGAGTGCAGCCGACTGGGCTCTGTACACTTACGAAGATGGCTCAGATGAC
  1 M  A  G  V  S  F  S  G  H  R  L  E  L  L  A  A  Y  E  E  V  I  R  E  E  S  A  A  D  W  A  L  Y  T  Y  E  D  G  S  D  D
121 CTCAAGCTTGCAGCATCAGGAGAAGGAGGCTTGCAGGAGCTTTCAGGCCACTTCGAGAACCAGAAAGTGATGTACGGTTTCTGCAGCGTCAAGGACTCCCAGGCTGCCTTGCCAAAATAT
 41 L  K  L  A  A  S  G  E  G  G  L  Q  E  L  S  G  H  F  E  N  Q  K  V  M  Y  G  F  C  S  V  K  D  S  Q  A  A  L  P  K  Y
241 GTGCTCATCAACTGGGTTGGTGAGGATGTGCCTGATGCCCGAAAATGTGCTTGTGCCAGCCATGTGGCCAAGGTGGCTGAATTCTTCCAGGGCGTTGACGTAATTGTGAATGCCAGCAGT
 81 V  L  I  N  W  V  G  E  D  V  P  D  A  R  K  C  A  C  A  S  H  V  A  K  V  A  E  F  F  Q  G  V  D  V  I  V  N  A  S  S
361 GTGGAGGATATCGATGCTGGCGCCATTGGGCAGCGGCTCTCCAATGGACTGGCTCGGCTCTCTAGCCCAGTGCTGCACCGCCTGCGCCTTCGGGAGGATGAGAATGCTGAGCCGGTGGGC
121 V  E  D  I  D  A  G  A  I  G  Q  R  L  S  N  G  L  A  R  L  S  S  P  V  L  H  R  L  R  L  R  E  D  E  N  A  E  P  V  G
481 ACCACCTACCAGAAGACGGATGCGGCCGTGGAGATGAAGCGGATTAACCGTGAGCAGTTTTGGGAGCAGGCCAAGAAGGAGGAGGAGCTGCGGAAGGAGGAGGAGCGGAAGAAGGCTTTG
161 T  T  Y  Q  K  T  D  A  A  V  E  M  K  R  I  N  R  E  Q  F  W  E  Q  A  K  K  E  E  E  L  R  K  E  E  E  R  K  K  A  L
601 GATGCCAGGCTCAGGTTTGAGCAGGAGCGGATGGAGCAGGAGCGGCAGGAGCAGGAGGAACGGGAACGGCGCTACCGAGAGCGGGAGCAGCAGATCGAGGAGCACAGGAGGAAACAGCAG
201 D  A  R  L  R  F  E  Q  E  R  M  E  Q  E  R  Q  E  Q  E  E  R  E  R  R  Y  R  E  R  E  Q  Q  I  E  E  H  R  R  K  Q  Q
721 AGTCTGGAAGCTGAAGAGGCCAAGAGGAGGTTAAAGGATCAGTCTATCTTTGGTGATCAGCGAGATGAAGAGGAAGAGTCCCAGATGAAGAAGTCGGAATCTGAGGTGGAGGAGGCAGCT
241 S  L  E  A  E  E  A  K  R  R  L  K  D  Q  S  I  F  G  D  Q  R  D  E  E  E  E  S  Q  M  K  K  S  E  S  E  V  E  E  A  A
841 GCCATCATTGCCCAGCGGCCTGATAACCCACGGGAGTTCTTCAGACAGCAGGAACGAGTGGCTTCAGCCTCTGGTGGCAGCTGTGACGCACCCTCGCCCTTCAACCACCGACCAGGTCGT
281 A  I  I  A  Q  R  P  D  N  P  R  E  F  F  R  Q  Q  E  R  V  A  S  A  S  G  G  S  C  D  A  P  S  P  F  N  H  R  P  G  R
961 CCGTACTGCCCTTTCATAAAGGCATCGGACAGTGGGCCTTCCTCCTCCTCTTCCTCCTCTTCCCCTCCACGGACTCCCTTTCCCTATATCACCTGCCACCGCACCCCAAACCTCTCT
321 P  Y  C  P  F  I  K  A  S  D  S  G  P  S  S  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
1081 TCCTCCCTCCCATGCAGTCACCTGGACAGCCACCGGAGGATGGCGCCCACTCCCATTCCCACCCGGAGCCCATCTGATTCCAGCACAGCCTCCACCCCCATCACGGAGCAGATCGAGAGG
361 S  S  L  P  C  S  H  L  D  S  H  R  R  M  A  P  T  P  I  P  T  R  S  P  S  D  S  S  T  A  S  T  P  I  T  E  Q  I  E  R
1201 GCCCTGGATGAGGTCACATCCTCGCAGCCTCCACCCCCACCTCCACCACCCCCACCAGCTCAAGAGGCCCAGGAGTCTGCTCCCAGACTGGACGGTGAAGAGGTCTGCAAGGAGGCCAAA
401 A  L  D  E  V  T  S  S  Q  P  P  P  P  P  P  P  P  P  P  P  A  Q  E  A  Q  E  S  A  P  R  L  D  G  E  E  V  C  K  E  A  K
1321 GTAGCAGCAGCTCCTCAGGTCTGGGCTGGCTGTGCAGAGGAGCCTCCTCGGGCACAGGAACCTCCCCTGTTGCAAAGCAGCCCCACGGAGGACTTGATGTGCACAGAATCTCCTGAGCAG
441 V  A  A  A  P  Q  V  W  A  G  C  A  E  E  P  P  R  A  Q  E  P  P  L  L  Q  S  S  P  T  E  D  L  M  C  T  E  S  P  E  Q
1441 GCTGTCCTGGCGGCCTCTCCAGAGCCTGATGCCTCTGTCACCTCAGTAGCTGATGCTCATGCAGCTGACACCATCGAGACCACCACTGCCACTACTGCCACCACTATTGCCGACAACGTC
481 A  V  L  A  A  S  P  E  P  D  A  S  V  T  S  V  A  D  A  H  A  A  D  T  I  E  T  T  T  A  T  T  A  T  T  I  A  D  N  V
1561 ACCCCTGCCGCCGCCAGCCTCATTGATCTGTGGCCTGGTAACGGGGAAGAGGCCTCAACGCCTCAGGCTGAACCCAGGGTGCCCACCCCACCCTCAGGTGCTGAGGCCTCCCTGGCAGAG
521 T  P  A  A  A  S  L  I  D  L  W  P  G  N  G  E  E  A  S  T  P  Q  A  E  P  R  V  P  T  P  P  S  G  A  E  A  S  L  A  E
1681 GTGCCTCTGTTGAATGAGGCAGCTCAGGAGCCGCTGCCACCAGTGGGCGAAGGCTGTGCCAACCTTCTTAATTTCGATGAGCTGCCAGAACCTCCAGCCACCTTCTGTGACCCAGAGGAG
561 V  P  L  L  N  E  A  A  Q  E  P  L  P  P  V  G  E  G  C  A  N  L  L  N  F  D  E  L  P  E  P  P  A  T  F  C  D  P  E  E
1801 GAAGCCGAAGGAGAGCCGCTGGCTGCCTCCCAGGTCCTAACTATGCCCTCAGCCCTAGAGGAGGTAGATCAGGTGTTGGAGCAGGAGTTGGAGCCAGAACCTCATCTGCTGACCAATGGA
601 E  A  E  G  E  P  L  A  A  S  Q  V  L  T  M  P  S  A  L  E  E  V  D  Q  V  L  E  Q  E  L  E  P  E  P  H  L  L  T  N  G
1921 GAGACCACTCAGAAGGAGGGGACCCAGCAGGCCAGCGAAGGATACTTCAGTCAGTCACAGGAGGAAGAATTCGCCCAATCAGAAGAGCCATGTGCAAAGGCTCCACCTCCTGTATTCTAC
641 E  T  T  Q  K  E  G  T  Q  Q  A  S  E  G  Y  F  S  Q  S  Q  E  E  E  F  A  Q  S  E  E  P  C  A  K  A  P  P  P  V  F  Y
2041 AACAAGCCTCCAGAAATCGACATCACCTGCTGGGATGCAGACCCAGTTCCTGAAGAGGAAGAGGGCTTCGAGGGTGGTGATTAGTAGTGGCGACTGCCCCCTGGCTGCCCTCGCCAAGGC
681 N  K  P  P  E  I  D  I  T  C  W  D  A  D  P  V  P  E  E  E  E  G  F  E  G  G  D  *
2161 TGCCCACCTGCAGTGCCTCTGGCCAGCCGGCTTGCAGCGCCAGCATTAGCAGCAGCCCCGCCTGGCTCCCACTCTGGATTCCGGCACTGGCCGGGGACCTGTCTGCTTCTCTACCCACAG
2281 GGCCTGACTTTTACAGCTTTTCTCTTTTTTTTAAAAGTTGATAGACTTGTACAATTGACTGGTTTTCCTCCCGTTGGTAGTTGAAACAATGTTGCAGATTCCACCCCCTTCCTGCCCAGA
2401 CCAGATTGTAGCTTAGTCCTTCCTGCTCAGCTGACCAGGGTGGAGGCCTCACCCTGCTTGGGACCTGGTGGGGGTCGGGGGTTCTGGTGGGAAAATGTTCCCCCCCATCACTTTTCCTAG
2521 TTTTATGTTTCTTGGAAAAAAAATATCACTTTGTATTCTCTATCCAGGGCTTCAGATATTTTGCACGAAATTTAAAACATGGCAATAAATGGCTCGTGGGCTCTG(A)n
```

FIG. 1. **The cDNA sequence of rat drebrin A and the deduced amino-acid sequence (three-letter code).** The complete nucleotide sequence determined is 2,665 bases long without the poly(A) tail, corresponding well with the size of the~2.7 kb mRNA, detected on northern blots. The AATAAA poly(A)-addition signal, which precedes a poly(A) tail by 16 nucleotides, and the highly homologous amino acid sequences with chicken drebrins are underlined. The termination codon is indicated by asterisk. The nucleotide and protein sequences are numbered on the left.

then incubated with anti-drebrin antibody M2F6 or anti-actin antibody (Amersham) for 60 min. In some cases, living transfected cells were first incubated with anti-drebrin antibody without fixation to examine whether or not drebrin immunoreactivity can be detected on the cell surface. After washing, the cells were fixed in the same manner. Immunoreactivity was visualized by the avidin-biotin-peroxidase complex method (Vectastain ABC kit, Vector Labs.) as described previously.[5] After reaction with diaminobenzidine, cells were photographed with an inverted microscope equipped with Hoffman Module optics.

For optical sectioning of transfectant cells, cells were first treated as described above. After incubation with anti-drebrin antibody M2F6, they were incubated with rhodamine-conjugated anti-mouse immunoglobulins (G + L) (1:100) (TAGO). Then they were imaged using confocal inverted microscopy.

Results and Discussion

Drebrins consist of three developmentally-regulated protein isoforms (so called E1, E2 and A forms) which are expressed from a single gene by alternative mRNA splicing mechanism in the chicken.[6] Adult rat brain expresses only drebrin A while drebrin E1 or E2 is observed in immature animals. Anti-chicken drebrin antiserum cross-reacts with rat drebrin A,[17] indicating a high homology between drebrin molecules. In order to obtain rat drebrin A cDNA, a lambda-gt 10 cDNA library of adult rat brain was screened using chicken drebrin E2 cDNA[10,11] as a probe. The longest drebrin cDNA (Drh 102) isolated in this study was 2.7 kb and bound to a broad band of 2.7 kb mRNA in northern blot analysis (data not shown), suggesting that Drh 102 covers the full length of the drebrin mRNA. The nucleotide sequence determined by the dideoxy-chain termination method defined an open reading frame encoding 707 amino acids (Fig. 1). The protein expressed in the transformant (see below) had the same migration pattern as rat drebrin A on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as assessed by western blotting (Fig. 2). The

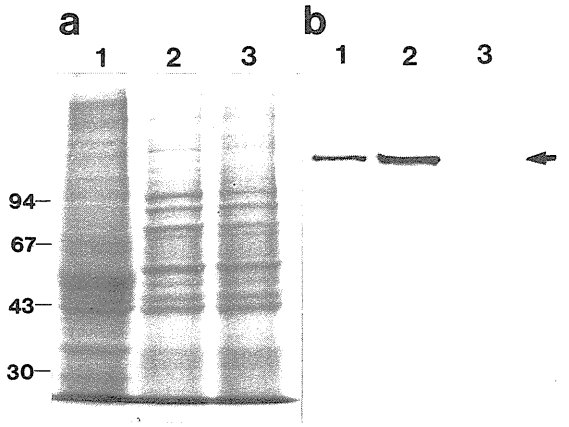

FIG. 2. **Identification of the protein of drebrin cDNA which was expressed in transformed L cells.** Extracts of tissue and cell homogenates were electrophoresed on sodium dodecyl sulfate-polyacrylamide gel. (**a**) is coomassie blue staining, and (**b**) is immunostaining with anti-drebrin antibody. The immunopositive protein was detected in the extract of transformed cell line LD2B4 (lane 2) at the same apparent molecular weight (arrow) as rat drebrin A in the adult hippocampus (lane 1). Native L cells (lane 3) did not express any immunopositive protein. Molecular weight markers are in kilodaltons and the position of drebrin A is indicated by an arrow.

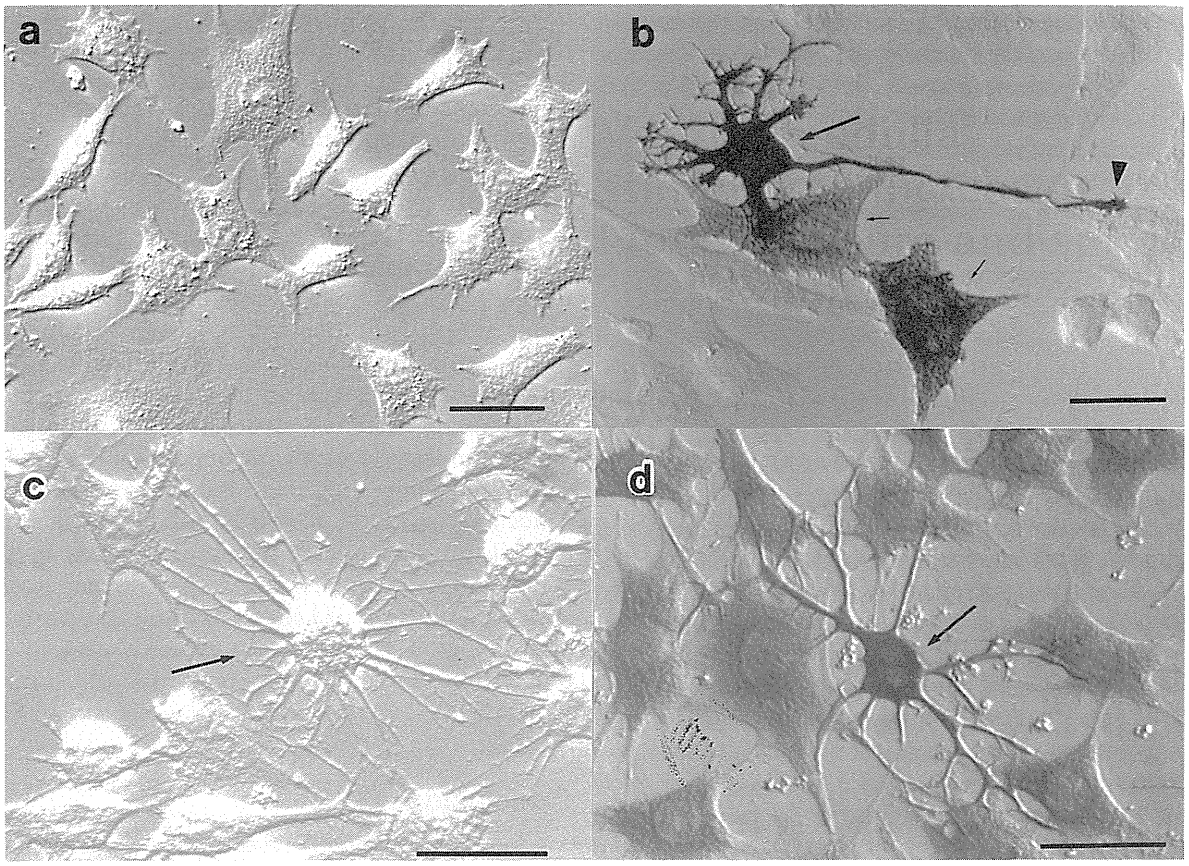

FIG. 3. **Expression of drebrin A causes highly branched neurite-like cell process outgrowth from L cells.** L cells were transfected by the standard calcium phosphate coprecipitation method[14] with the drebrin A expression plasmid MIW-DA (**b–d**) or plasmid MIW-C, which had a drebrin A cDNA oriented in the opposite direction, as a control (**a**). Cells in (**a**), (**b**) and (**c**) were subjected to immunostaining with the anti-drebrin antibody and cells in (**d**) were subjected to immunostaining with the anti-actin antibody. Highly branched process extension was seen only in cells transfected with MIW-DA (**b**) (thick arrow) but not in control cells transfected with MIW-C (**a**). Some long processes had growth cone-line swelling at their tips (arrowhead in **b**). Note that the process-bearing transfectants (thick arrow in **b**) are very intensely immunostained with anti-drebrin antibody. Anti-drebrin immunoreactivity was also present in some fibroblast-like cells, but staining was weaker (small arrows in **b**). The presence of these mildly drebrin-positive fibroblast-like transfectants suggest that drebrin concentration is important for displaying a neuron-like phenotype. The process-bearing transfectants were also immunostained with anti-actin antibody more intensely than other cells (thick arrow in **d**). Note that due to limitation of the avidin-biotin-peroxidase complex method subcellular localization of drebrin and actin was not demonstrated. In (**c**), a living cell with neurite-like cell-processes (thick arrow) was not immunostained with anti-drebrin antibody. Bars are 40 μm.

predicted molecular weight is 77,471, which is smaller than its apparent molecular weight on SDS-PAGE (140 kd). This discrepancy is similar to that in chicken drebrins.[11] Computer-aided sequence analysis revealed no overall homology with other protein sequences in NBRF Protein Identification Resource Files. A hydrophobicity analysis showed no membrane spanning domains. Homology of deduced amino acid sequences is 68% between rat and chicken drebrin A (data not shown), in particular, the homology of 354 amino acids in the *N*-terminal region and that of 58 amino acids in the C-terminal region was more than 80%.

Drebrin A was then expressed in non-neuronal cells, which do not have any endogenous drebrins or other neuron-specific molecules, by transfection technique. Expression vectors containing rat drebrin A cDNA with sense orientation (MIW-DA), or with anti-sense orientation (MIW-C) as a control, were used to transfect L cells. These transfected cells were analyzed by immunocytochemistry with a drebrin-specific monoclonal antibody M2F6.[5] Immunoreactivity was detected in 5 to 10% of the cells. Drebrin immunoreactivity was not detected in either non-transfected control L cells or in cells transfected with MIW-C. These immunonegative cells were essentially fibroblast-like in cell shape, with thin and flat cell bodies, with either a few or no processes extending from their perimeter (Fig. 3a). In comparison, immunopositive cells were either fibroblast-like or neuron-like in cell shape. Most of them were of the former type; however, 4 to 5% were neuron-like, with round, thick cell bodies and highly branched processes extending from the cell perimeter (Fig. 3b). Control L cells never had such highly branched processes. The majority of the processes were relatively short (usually not longer than two cell diameters); however some cells had one or two long cell processes with growth cone-like swellings at their tips (arrowhead in Fig. 3b). Immunocytochemistry indicated that all these neuron-like cells expressed drebrin A in larger amount than in fibroblast-like transfectants. This possibly suggests that a

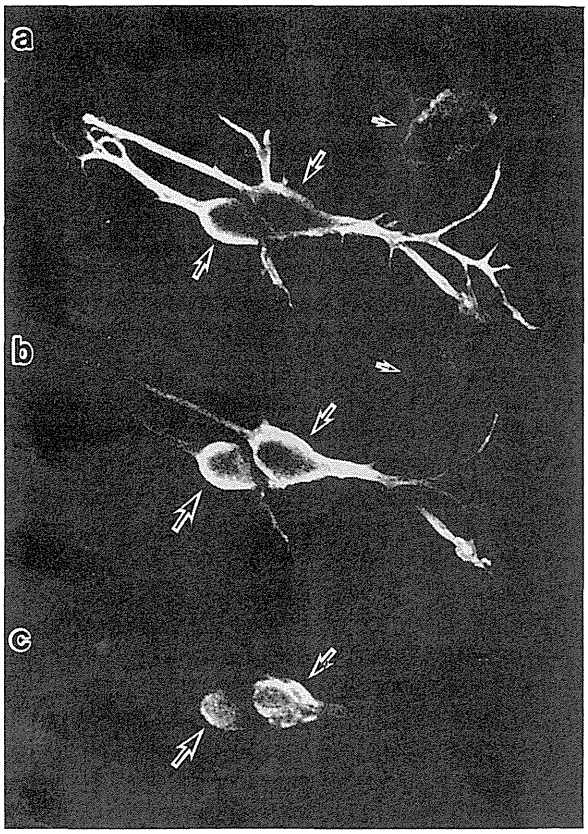

FIG. 4. **Optical sectioning of transfected L-cells expressing drebrin A.** The first image was taken at a focal plane close to a level near the dish surface on which the cells were grown (**a**). The other two images were taken at two additional focal planes at 5 μm intervals moving towards the top of the cells (**b, c**). The last image (**c**) was taken at a focal plane close to a level near the roof of cells. It is clear that intense fluorescence was associated with the cell membrane of the neuron-like cells (big arrows) but not of the fibroblast-like cells (small arrows).

critical concentration of drebrin is necessary for the expression of the neuron-like phenotype. A similar association of the process outgrowth with drebrin A expression was found when Swiss 3T3 cells were transfected with drebrin A expression vector MIW-DA (data not shown). Also of interest is that immunostaining with anti-actin antibody in the process-bearing cells was stronger than in the fibroblast-like transfectants and control fibroblast cells (Fig. 3d). It has recently been reported that the tau and tubulin system is involved in the formation of axon-like cell processes.[18] Our present results suggest that drebrin plays a role with actin in the formation of highly-branched neurite-like cell processes.

Confocal microscopy demonstrated that in neuron-like cells (big arrows in Fig. 4), strong immunoreactivity of drebrin A was associated with the cell membrane and weak immunoreactivity was in the cytosol. However in the fibroblast-like cells (small arrows in Fig. 4) the immunoreactivity was not associated with the cell membrane. Since no immunoreactivity was observed when living transfected cells were stained with anti-drebrin antibody (Fig. 3c), it appears that drebrin A is present in the cytoplasmic side of the membrane, but not exposed on the cell surface. Submembraneous proteins are thought to be important in the control of cell shape. A spectrin and actin meshwork undercoats the erythrocyte membrane, and abnormality of spectrin molecule results in erythrocytes with abnormal cell shape.[19] The neuron-specific, intracellular growth-associated protein called GAP-43 is concentrated near the growth cone plasma membrane.[20,21] Transfection of non-neuronal cells with GAP-43 enhanced the extension of filopodial processes from these cells, without outgrowth of neurites.[1] Since drebrin A is concentrated in the submembraneous region of the neuron and is able to bind to actin (data not shown), it may regulate the interaction between cell membrane and actin filaments, and induce outgrowth and arborization of dendrites and/or axons. It will be of interest in future investigations to dissect functional regions of drebrin A and to determine how the protein may interact with the cellular cytoskeleton to cause this particular morphological change. The sequence data in this publication have been submitted to the EMBL/GenBank/DDBJ Libraries under the accession number X59267.

Conclusions

Cloning of Drebrin A revealed that this protein is a novel neuron-specific protein that shares no homology with other reported proteins. Transient expression experiments using drebrin A cDNA demonstrated that expression of this protein induced the formation of highly-branched neurite-like cell processes in non-neuronal cells. Immunocytochemistry of these process-bearing cells suggested that drebrin A regulates the interaction between the cell membrane and actin filaments, and induces these highly-branched neurite-like cell processes.

References

1. Zuber MX, Goodman DW, Karns LR *et al. Science* **244**, 1193–1195 (1989).
2. Lewis SA, Ivanov IE, Lee G.-W *et al. Nature* **342**, 498–505 (1989).
3. Kanai Y, Takemura R, Oshima T *et al. J Cell Biol* **109**, 1173–1184 (1989).
4. Shirao T, Obata K. *J Neurochem* **44**, 1210–1216 (1985).
5. Shirao T, Obata K. *Dev Brain Res* **29**, 223–244 (1986).
6. Shirao T, Kojima N, Terada S *et al. Neurosci Res* **13**, S106–S111 (1990).
7. Shirao T, Inoue HK, Kano Y *et al. Brain Res* **413**, 374–378 (1987).
8. Hubel DH, Wiesel TN. *J Physiol* **206**, 419–436 (1970).
9. Imamura K, Shirao T, Mori K *et al. Neurosci Res* in press (1991).
10. Shirao T, Kojima N, Kato Y *et al. Mol Brain Res* **4**, 71–74 (1988).
11. Kojima N, Kato Y, Shirao T *et al. Mol Brain Res* **4**, 207–215 (1988).
12. Katoh KY, Takahashi Y, Hayashi S *et al. Cell Struct Funct* **12**, 575–580 (1987).
13. Suemori H, Kadokawa Y, Goto K *et al. Cell Dif Devl* **29**, 181–186 (1990).
14. Xie H. *Cell Struct Funct* **8**, 315–325 (1984).
15. Nagafuchi A. Shirayoshi Y, Okazaki K *et al. Nature* **329**, 341–343 (1987).
16. Laemmli UK. *Nature* **227**, 680–685 (1970).
17. Shirao T, Kojima N, Nabeta Y *et al. Proc Japan Acad* **65**, 169–172 (1989).
18. Knops J, Kosik K S, Lee G *et al. J Cell Biol* **114**, 725–733 (1991).
19. Goodman SR, Shiffer K. *Am J Physiol* **244**, C121–C141 (1983).
20. McGuire CB, Snipes GJ Norden, JJ. *Dev Brain Res* **41**, 277–291 (1988).
21. Goslin K, Schreyer DJ, Skene JHP *et al. Nature* **336**, 672–674 (1988).

ACKNOWLEDGEMENTS: Plasmids MIW-HEP and pSTB-neo were gifts from Dr. Agata and Dr. Nagafuchi, respectively. We thank Drs. H. Omori and S. Furuya for helpful advice. This work was supported by the Ministry of Education, Science and Culture of Japan and the Naito Foundation.

Received 11 October 1991; accepted 23 October 1991