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Formation of Thick, Curving Bundles of Actin by Drebrin A Expressed in Fibroblasts

TOMOAKI SHIRAO,*,†,[1] KENSUKE HAYASHI,* RYOKI ISHIKAWA,‡ KAORU ISA,* HIDEO ASADA,† KEIROU IKEDA,† AND KEIICHI UYEMURA†

*Department of Neurobiology and Behavior and ‡Department of Pharmacology, Gunma University School of Medicine, Maebashi 371; and †Department of Physiology, Keio University School of Medicine, Tokyo 160, Japan

Drebrin A is a neuron-specific protein, the expression of which is regulated during development. Upon transfection of fibroblasts with drebrin A cDNA, the protein is expressed at high levels in fibroblasts and the outgrowth of highly branched, neurite-like cell processes is induced. In this report, we describe a biochemical examination of the binding of drebrin A to actin filaments. We also demonstrate by an immunocytochemical method that, when drebrin A is expressed in transfected cells, it binds to actin filaments and is concentrated in cell processes. Furthermore, we provide evidence that thick, curving bundles of actin together with drebrin are formed in some of the transfected cells. Our results suggest that the actin filaments that bind drebrin might be a novel class of actin filaments and might play a role in neuronal morphogenesis. © 1994 Academic Press, Inc.

INTRODUCTION

The outgrowth of neurites, axons, and dendrites is the first step in formation of a neuronal network in the central nervous system. Many proteins are specifically expressed in association with the outgrowth of neurites [1–7]. The cytoskeleton has been suggested to be one of the important endogenous factors that control the elaborate morphology of neuronal processes [8–13]. It has been reported that expression of tau protein in fibroblasts results in formation of thick bundles of microtubules that are arranged parallel to each other and extend into cell processes [14]. Furthermore, overexpression of tau protein in insect ovarian Sf9 cells induces the formation of long, straight cell processes that resemble neuronal axons [15, 16]. These studies indicate that a microtubule-associated protein, tau, may play a role in axonal elongation.

Drebrins were first identified in extracts of embryonic chicken brain, after two-dimensional gel electrophoresis, as developmentally regulated acidic proteins, and their expression was shown to be closely related to neuronal development [17, 18]. Three isoforms, namely, two embryonic types (E1 and E2) and an adult type (A), are generated by alternative RNA splicing from a single gene for drebrin in the chicken brain [19, 20]. In the rat and human, only one type of embryonic drebrin (drebrin E) has been identified to date. Drebrin A is a neuron-specific protein and is localized in dendrites [21], but embryonic types of drebrin are expressed in non-neuronal tissues as well as in the nervous system [18]. A comparison of deduced amino acid sequences of drebrins from the chicken, rat, and human demonstrated that drebrins are a novel family of proteins, common to both birds and mammals [19, 20, 22–24]. The molecular weight predicted from the cDNA for rat drebrin A is 77,471, but the apparent molecular mass of drebrin A indicated by its mobility during sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) is about 140 kDa. A similar discrepancy is observed in the case of the proteins from the chicken and human. The significance of drebrins in cell morphogenesis was first demonstrated by transfection of fibroblasts with cDNA for drebrin A. Transient expression of neuron-specific drebrin A in the fibroblasts induced the formation of highly branched, neurite-like cell processes [22]. Since immunocytochemical staining indicated that the level of expression of actin in the process-bearing transfectants increased slightly, we postulated that drebrin A might affect the formation of cell processes by regulating the formation of actin filaments. However, because of the limited ability of the avidin–biotin complex method to reveal the immunoreactivity of drebrin, the association of drebrin with actin filaments could not be determined. In the present study, using immunofluores-

[1] To whom correspondence and reprint requests should be addressed at Department of Neurobiology and Behavior, Gunma University School of Medicine, 3-39-22 Showamachi, Maebashi, Gunma 371, Japan. Fax: 81-272-35-8669. e-mail: tshirao@sb.gunma-u.ac.jp.

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cence staining, we examined the binding of drebrin A to actin filaments *in vitro* and the effect of drebrin A, expressed in transfected cells, on actin filaments.

MATERIALS AND METHODS

Chemicals and antibodies. Fluorescein isothiocyanate (FITC)- and peroxidase-conjugated, affinity-purified antibodies raised in goat against mouse IgG and peroxidase-conjugated, affinity-purified antibodies raised in goat against rabbit IgG were purchased from Cappel (Durham, NC). Rhodamine-conjugated phalloidin was purchased from Molecular Probes, Inc. (Eugene, OR). Vectastain ABC kit for the avidin–biotin–peroxidase complex method was purchased from Vector Laboratories, Inc. (Burlingame, CA). F-12 nutrient mixture was purchased from Gibco (Grand Island, NY). Other chemicals were purchased from Wako (Osaka, Japan). Monoclonal antibody (MAb) M2F6 was prepared previously as a drebrin-specific antibody [18]. Antiserum DAS1 was raised in a rabbit by immunization with synthetic peptides. The peptides synthesized were peptide 1 (residues 319–335: Gly–Arg–Pro–Tyr–Cys–Pro–Phe–Ile–Lys–Ala–Ser–Asp–Ser–Gly–Pro–Ser–Ser), peptide 2 (residues 342–353: Ser–Pro–Pro–Arg–Thr–Pro–Phe–Pro–Tyr–Ile–Thr–Cys), and peptide 3 (residues 354–363: His–Arg–Thr–Pro–Asn–Leu–Ser–Ser–Ser–Leu), which are unique to drebrin A [22].

Assay for actin-binding activity. Drebrin A was partially purified from adult rat brain as follows. Three-month-old adult Wistar rats were anesthetized with ether. The animals were sacrificed by decapitation and the isolated brain tissues were homogenized in 5 vol of buffer A that contained 20 m*M* Tris–HCl (pH 7.6), 100 m*M* KCl, 1 m*M* $MgCl_2$, 5 m*M* EGTA, 10 m*M* 2-mercaptoethanol, 0.5 m*M* phenylmethylsufonyl fluoride, and 0.5 μg/ml leupeptin. The homogenate was centrifuged at 100,000*g* for 1 h. Since drebrins are heat-stable, the crude supernatant was then heated in a boiling-water bath for 3 min, cooled on ice for 20 min, and centrifuged at 100,000*g* for 1 h. The supernatant fraction was used as partially purified drebrin A. Actin was purified from chicken skeletal muscle as described by Matsumura and Lin [25].

The partially purified preparation of drebrin A was mixed with actin filaments (final concentration, 0.5 mg/ml) in 100 m*M* NaCl and 20 m*M* Tris–HCl (pH 7.6). After a 30-min incubation at room temperature, the mixture was centrifuged in an Airfuge (Beckman, Palo Alto, CA) at 140,000*g* for 25 min. The pellet was resuspended in the original volume, and equal volumes of the supernatant and the solution of the resuspended pellet were analyzed by SDS–PAGE on a 10% gel with subsequent immunoblotting.

Immunoblotting. Proteins on a gel were transferred to an Immobilon-P membrane (Millipore, Bedford, MA) as described previously [18]. For detection of specific proteins, the membrane was probed with MAb M2F6 (diluted 1:1) or with antiserum DAS1 (diluted 1:500). Immunoblots were then developed with peroxidase-conjugated antibodies against mouse or rabbit IgG and 3,3′-diaminobenzidine.

Culture and transfection of cells. Mouse L cells and Chinese hamster ovary cells (CHO-K1) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and in F-12 nutrient mixture supplemented with 10% FBS, respectively, in an atmosphere of 5% CO_2 in air at 37°C. For immunocytochemistry, cells were plated on 60-mm tissue culture plates that contained glass coverslips which had been coated with poly-L-lysine (0.1 mg/ml). The cells were transfected with plasmid MIW-DA that contained a full-length cDNA for rat drebrin A, oriented in the sense direction, and a β-actin promoter [22] by the standard calcium phosphate precipitation method [26]. As controls, L cells were transfected with plasmid MIW-C that contained a drebrin cDNA oriented in the antisense direction relative to the promoter. After 18 h, the cells were rinsed with DMEM plus 10% FBS and they were maintained in the same medium for 1 further day. Transfected cells were then fixed in 4% paraformaldehyde in 0.1 *M* sodium phosphate buffer (pH 7.4) for 30 min at room temperature.

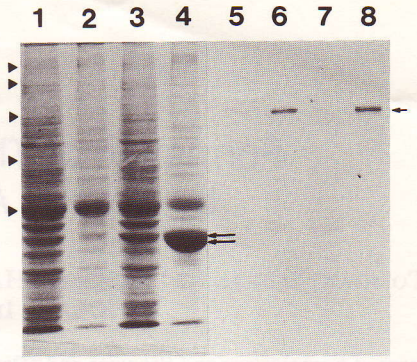

FIG. 1. Cosedimentation of drebrin A with actin filaments. A crude supernatant from adult rat brain was incubated without (lanes 1, 2, 5, and 6) and with (lanes 3, 4, 7, and 8) F-actin at room temperature for 60 min and then the mixture was centrifuged at 140,000*g* for 25 min. The supernatant (lanes 1, 3, 5, and 7) and the pellet (lanes 2, 4, 6, and 8) were processed for electrophoresis and electroblotted onto an Immobilon-P membrane. Proteins in lanes 1–4 were stained with Coomassie brilliant blue G 250 and proteins in lanes 5–8 were immunostained with MAb M2F6. The single arrow and double arrows indicate drebrin A and actin, respectively. Triangles show molecular weights of standards (212, 170, 116, 76, and 53 kDa, from top to bottom). Note that the band of drebrin A in lane 6 was more strongly immunostained than that in lane 5.

Immunocytochemistry. To label drebrin, fixed cells on coverslips were permeabilized with 0.1% Triton X-100 in 0.02 *M* sodium phosphate-buffered saline (PBS), pH 7.4, for 15 min, blocked by PBS with 3% BSA for 60 min, and reacted with MAb M2F6 for 60 min. The immunoreactivity was visualized by the avidin–biotin–peroxidase complex method as described previously [18]. After photographs of the cells had been taken, the percentage of immunostained cells was determined. For double-labeling of drebrin and actin filaments, cells were incubated with MAb M2F6 and then with a mixture of FITC-conjugated, affinity-purified goat antibodies against mouse IgG (diluted 1:100) and rhodamine-conjugated phalloidin (diluted 1:2000). To control for fluorescent "cross-bleed," parallel coverslips, after incubation with primary antibody, were stained with the second antibody alone or with rhodamine-conjugated phalloidin alone. To control for second-antibody specificity, parallel coverslips were stained with each second antibody alone. All procedures were performed at room temperature.

RESULTS

Actin-Binding Activity of Drebrin A

Drebrin A from the adult rat brain was recovered in the crude supernatant after centrifugation of the extract at 100,000*g* and almost all the drebrin A in this crude supernatant was pelleted by centrifugation of 140,000*g* (lanes 5 and 6 in Fig. 1). The crude supernatant that contained drebrin A was boiled and recentrifuged at 100,000*g*. Drebrin A in the resultant supernatant did

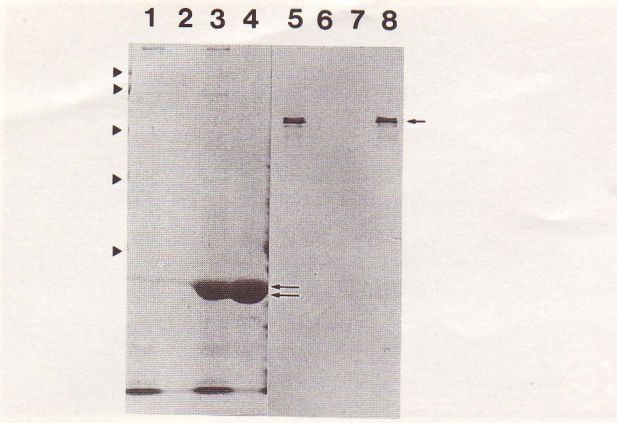

FIG. 2. Cosedimentation with actin filaments of drebrin A after heat treatment. Partially purified drebrin A from adult rat brain was incubated without (lanes 1, 2, 5, and 6) and with (lanes 3, 4, 7, and 8) F-actin at room temperature for 60 min and then the mixture was centrifuged at 140,000*g* for 25 min. The supernatant (lanes 1, 3, 5, and 7) and the pellet (lanes 2, 4, 6, and 8) were processed for electrophoresis and electroblotted onto an Immobilon-P membrane. Proteins in lanes 1–4 were stained with Coomassie brilliant blue G 250 and proteins in lanes 5–8 were immunostained with MAb M2F6. The single arrow and double arrows indicate drebrin A and actin, respectively. Triangles indicate marker proteins (see legend to Fig. 1). Note that the band of drebrin A was strongly immunostained in lanes 5 and 8.

not sediment upon centrifugation at 140,000*g* (lanes 5 and 6 in Fig. 2), and it was used as partially purified drebrin A for the analysis of actin-binding activity. As shown in lane 8 of Fig. 2, after incubation of partially purified drebrin A with actin filaments, drebrin A cosedimented with actin filaments upon centrifugation at 140,000*g*.

Expression of Drebrin A in L Cells

When L cells were transfected with plasmid MIW-DA, immunoreactivity of drebrin was detected in 5 to 10% of the cells, as previously reported [22]. Drebrin-specific immunoreactivity was detected neither in nontransfected L cells nor in control cells that had been transfected with plasmid MIW-C (Fig. 3d). The shapes of most immunopositive cells (type I transfectants, indicated by single arrows in Fig. 3) were similar to those of control cells, but about 5% of immunopositive cells (type II transfectants, indicated by double arrows in Fig. 3) formed highly branched cell processes, as described previously [22]. All of the type II transfectants were strongly immunostained for drebrin. They were often distributed as single cells (Figs. 3a and 3c) while type I transfectants were usually seen in groups. The majority of the cell processes of type II transfectants were not very long (usually they were not longer than two cell diameters). However, some cells had long cell processes (Fig. 3c). As shown in Fig. 3, almost all the type I transfectants were less strongly immunostained than type II transfectants, but a very small fraction of the type I transfectants (indicated by a large arrow in Fig. 3b) were as strongly immunostained as type II transfectants.

Drebrin and Actin Filaments in Transfected L Cells

Analysis by fluorescence microscopy of type II transfectants that had been double-stained with the drebrin-specific antibody MAb M2F6 and phalloidin revealed that the patterns of staining of drebrin A and actin filaments were very similar and that both were concentrated in the cell processes (Figs. 4a and 4d). We observed that thick bundles of actin filaments, which were strongly immunostained with the drebrin-specific antibody, were present in the proximal thick portions of the cell processes. Such thick bundles of actin were most prominent in the type I transfectants that expressed drebrin A at high levels. However, the relative number of these cells was very low. The thick bundles of actin were usually curved and bifurcated and they sometimes even formed a circle. Their thickness changed from place to place (Figs. 4b and 4e). Nontransfected fibroblasts contained straight bundles of actin, known as stress fibers (arrowheads in Fig. 4f), but they did not contain any curving or bifurcated bundles of actin. In the type I transfectants that expressed drebrin A at low levels, there were no thick, curving bundles of actin but patchy dot-like aggregates of actin filaments were distributed diffusely in the cytoplasm. These aggregates were immunostained with MAb M2F6 (arrows in Figs. 4c and 4f). Sometimes actin filaments at the periphery of the type I transfectants were not immunostained with MAb M2F6 (double arrows in Fig. 4).

Drebrin and Actin Filaments in Transfected CHO Cells

Immunoblotting analysis revealed that one protein band in an extract of CHO cells could be immunostained with MAb M2F6 (lane 4 in Fig. 5) although this protein was not immunostained with antiserum DAS1, which was specific to drebrin A (lane 7 in Fig. 5). The pattern of migration of this protein was similar to that of rat drebrin E. When CHO cells were transfected with plasmid MIW-DA and drebrin A was expressed at high levels, the cells formed cell processes similar to those of the type II transfectants of L cells, although the processes were not as highly branched as those of the transfected L cells. Figure 6 shows a range of phenotypes of transfected CHO cells that expressed drebrins at high levels. We frequently observed thick, curving bundles of actin, even in the cell bodies of the type II transfectants of CHO cells, in contrast to those of L cells. Figures 7b and 7e show high-magnification fluorescence micrographs of transfected CHO cells that had been double-

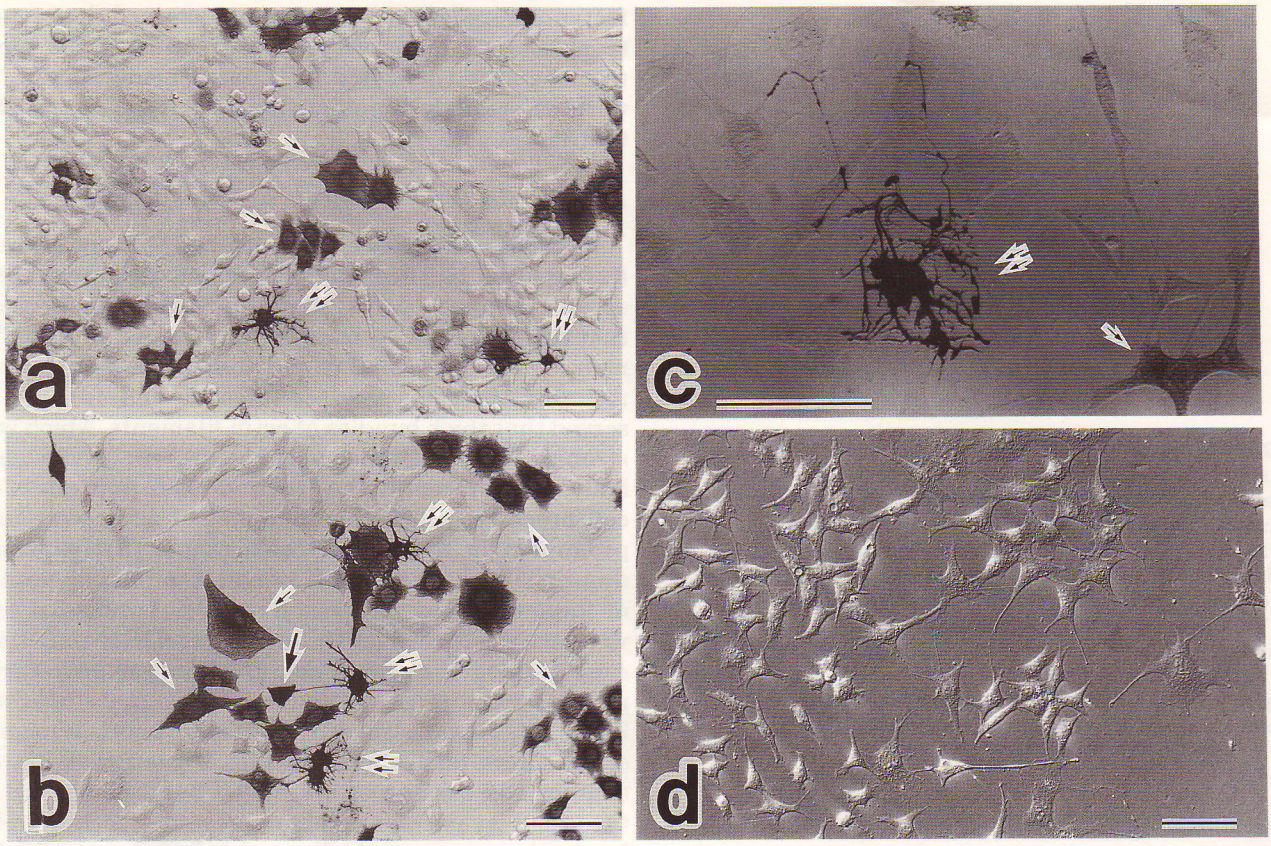

FIG. 3. Light micrographs of transfected L cells. L cells were transfected with plasmid MIW-DA. After fixation, cells were incubated with drebrin-specific monoclonal antibody M2F6. The immunoreactivity was visualized by the avidin–biotin–peroxidase complex method and the cells were photographed under an inverted microscope equipped with Hoffman Module optics. Note that type II transfectants (indicated by double arrows) were more intensely immunostained than most of the type I transfectants (indicated by single arrows). Some of the cell processes were very long (c). Strong immunoreactivity for drebrin was also present in a few of the type I transfectants [indicated by a large arrow in (b)]. Bars, 50 μm.

stained with MAb M2F6 and phalloidin. Thick, curving bundles of actin together with drebrin are evident within the cells. Since control CHO cells were also faintly immunostained with MAb M2F6, we could not discriminate type I transfectants that expressed drebrin A at low levels from nontransfected cells. Many prominent stress fibers were frequently observed after staining with phalloidin of cells that expressed drebrin at control levels. These stress fibers in CHO cells were not immunostained with MAb M2F6 but some of the patchy dot-like aggregates of actin filaments were immunostained with this monoclonal antibody (Figs. 7c and 7f).

DISCUSSION

The results presented here demonstrate that drebrin A bound to actin filaments and that the actin filaments that bound drebrin A (referred to as DB-actin filaments) were concentrated in the highly branched cell processes of drebrin-expressing transfectants. Furthermore, we also observed that DB-actin filaments formed thick, curving bundles in the cell bodies and in the proximal thick portions of cell processes of the transfectants that expressed drebrin A at high levels. The limitations of the immunofluorescence method prevented us from determining whether DB-actin filaments also formed thick, curving bundles in the highly branched thin cell processes of transfectants.

In a previous report we suggested that drebrin A had actin-binding activity since actin could be copurified with drebrins by immunoaffinity chromatography on a column with bound drebrin-specific antibodies. In the present study, the actin-binding capacity of drebrin A was demonstrated by cosedimentation of partially purified drebrin A with actin filaments. Moreover, most of the drebrin A in a crude supernatant from adult rat brain was pelleted by centrifugation of 140,000*g* without the addition of actin filaments, while partially purified

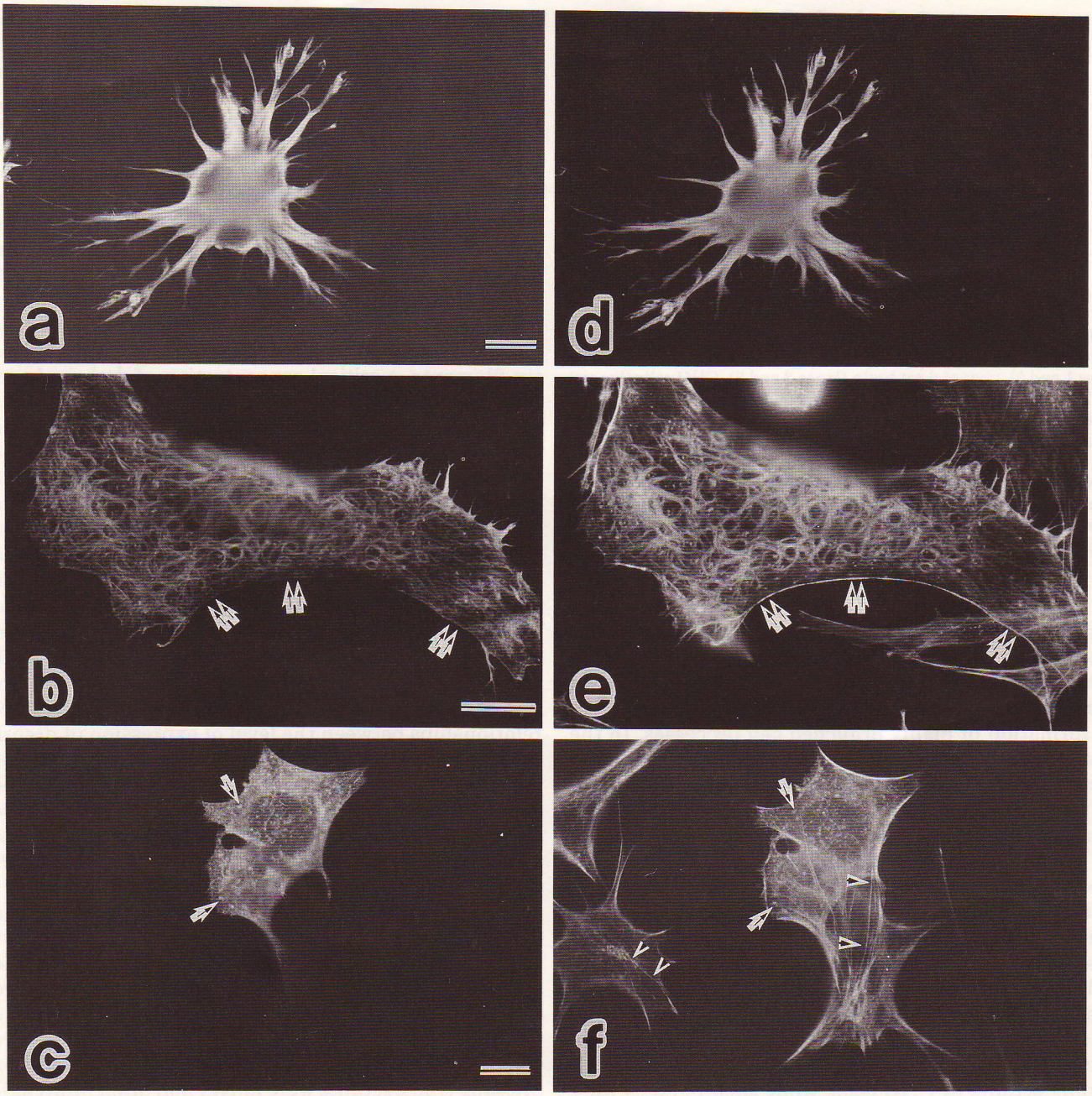

FIG. 4. Double-staining analysis of drebrin and actin in transfected L cells. L cells were transfected with plasmid MIW-DA. After fixation, cells were incubated with drebrin-specific monoclonal antibody. The cells then were incubated with FITC-conjugated goat antibodies against mouse IgG to demonstrate the localization of drebrin and with rhodamine-conjugated phalloidin to demonstrate the actin filaments. Staining was visualized with filters specific for FITC (a, b, c) and rhodamine (d, e, f). Note that drebrin-like immunoreactivity was colocalized with actin filaments, except in the case of actin filaments at the periphery of a cell [double arrows in (b) and (e)]. The single arrows in (c) and (f) indicate examples of patchy dot-like aggregates of actin filaments that were colocalized with drebrin-like immunoreactivity. The arrowheads in (f) indicate examples of stress fibers in normal cells. Bars, 20 μm.

drebrin A was not. This result suggests that most of the drebrin A may originally have been bound to actin filaments *in vivo*. We have recently succeeded in purifying drebrin E from embryonic rat brain and demonstrated that drebrin E can bind directly to actin filaments (submitted for publication). However, drebrin A in its native form has not yet been purified to homogeneity. Therefore, at present, it is not yet clear whether drebrin A

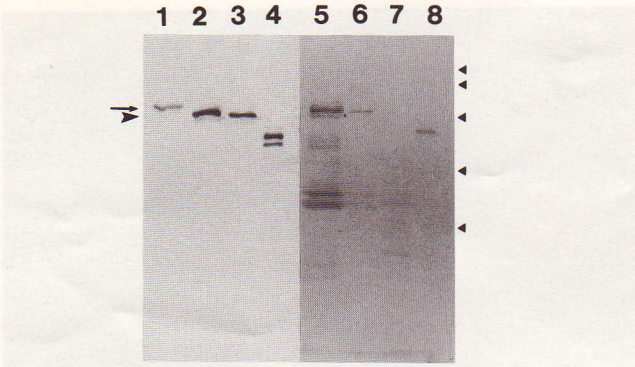

FIG. 5. The presence of drebrin E in control CHO cells. Immunoblotting analysis was carried out using extracts of adult rat brain (lanes 1 and 5), neonatal rat brain (lanes 2 and 6), CHO cells (lanes 3 and 7), and the brain of an 11-day chick embryo (lanes 4 and 8). Each extract (25 μg of protein in each case) was subjected to electrophoresis and processed for immunoblotting with MAb M2F6 (lanes 1–4) and with antiserum DAS1 (lanes 5–8). A large arrowhead and an arrow indicate rat drebrin E and drebrin A, respectively. Note that the mobility of a immunostained band in the case of the CHO-cell extract (lane 3) was similar to that of rat drebrin E (lane 2). Triangles indicate marker proteins (see legend to Fig. 1).

binds to actin molecules directly or via some other component associated with actin filaments.

In a previous study we demonstrated that high-level expression of drebrin A in transfected L cells resulted in the formation of highly branched cell processes. In the present study we used CHO cells in addition to L cells since CHO cells have a more homogeneous cell morphology than L cells and they contain well-developed stress fibers. The changes in morphology of transfectants of the two cell lines were similar, although the cell processes from the type II transfectants of CHO cells were not as highly branched as those from transfected L cells. In a previous study [22] we showed that drebrin A, expressed in type II transfectants of L cells, accumulated in the submembranous region of the cell bodies. In the present study, we demonstrated that thick, curving bundles, visualized by immunostaining with a drebrin-specific antibody, were formed in the cell bodies of type II transfectants of CHO cells, while such bundles were not formed in the cell bodies of type II transfectants of L cells. Since immunocytochemical and immunoblotting analyses revealed that CHO cells express drebrin E, in contrast to L cells that do not express any detectable level of any drebrin [22], the minor differences in terms of the morphology of cell processes might be explained by the expression of endogenous drebrin E in CHO cells, in addition to the expression of exogenous drebrin A.

Double staining of drebrin and actin filaments in transfected L cells and CHO cells demonstrated that drebrin A was colocalized with actin filaments. The actin-binding activity of drebrin A *in vitro* and the colocalization of drebrin A with actin filaments in the transfectants indicates strongly that drebrin A actually bound to actin filaments and formed DB-actin filaments *in situ*. In the transfectants that expressed drebrin A at high levels, thick, curving bundles of actin filaments were formed, while the straight bundles of actin filaments (stress fibers) had disappeared. By contrast, normal cultured fibroblasts contain stress fibers, but they never contain thick, curving bundles of actin under our culture conditions. These data suggest that the binding of drebrin A to actin filaments might be directly related to the formation of thick, curving bundles of actin.

Since the low-level expression of drebrin A did not induce the formation of thick, curved bundles of DB-actin filaments, a critical concentration of drebrin A may be necessary for the formation of these bundles. In a previous report we suggested that a critical concentration of drebrin A seemed necessary for the formation of highly branched cell processes [22]. Since actin filaments that did not bind drebrin sometimes remained at the periphery of type I transfectants, it appeared that cells did not form any highly branched cell processes until all of the actin filaments in the cell had been converted to DB-actin filaments. Although, in a previous study, we established several stable lines of transformed cells, all expressed drebrin A only at low levels. High-level expression of drebrin A may block the proliferation of cells. Therefore, at present, possible quantitative relationships between the level of expression of drebrin and the formation of thick bundles of actin and between the level of expression of drebrin and the formation of cell processes remain to be clarified. However, neither thick bundles of actin nor highly branched cell processes were ever observed in control fibroblasts, which did not express drebrin A.

When cultured cells were exposed to a reagent that depolymerizes actin filaments, such as cytochalasin D [27], each cell had a few short retraction processes around the collapsed cell body. These retraction processes usually did not extend beyond the original edge of the cell and seldom bifurcated. In contrast to these retraction processes, the cell processes from drebrin-expressing transfectants were longer and often bifurcated. Moreover, the distribution of actin filaments in process-bearing transfectants was completely different from that in cells that had been treated with cytochalasin D. The highly branched cell processes of transfectants were entirely filled with DB-actin filaments while the retraction processes contained only patchy dot-like aggregates of short actin filaments, which were scattered in the cytoplasm [28]. These data indicate that the cell processes of transfectants were different from retraction processes that are caused by the destruction of actin filaments. Using time-lapse video recording, we will determine in future experiments whether drebrin-

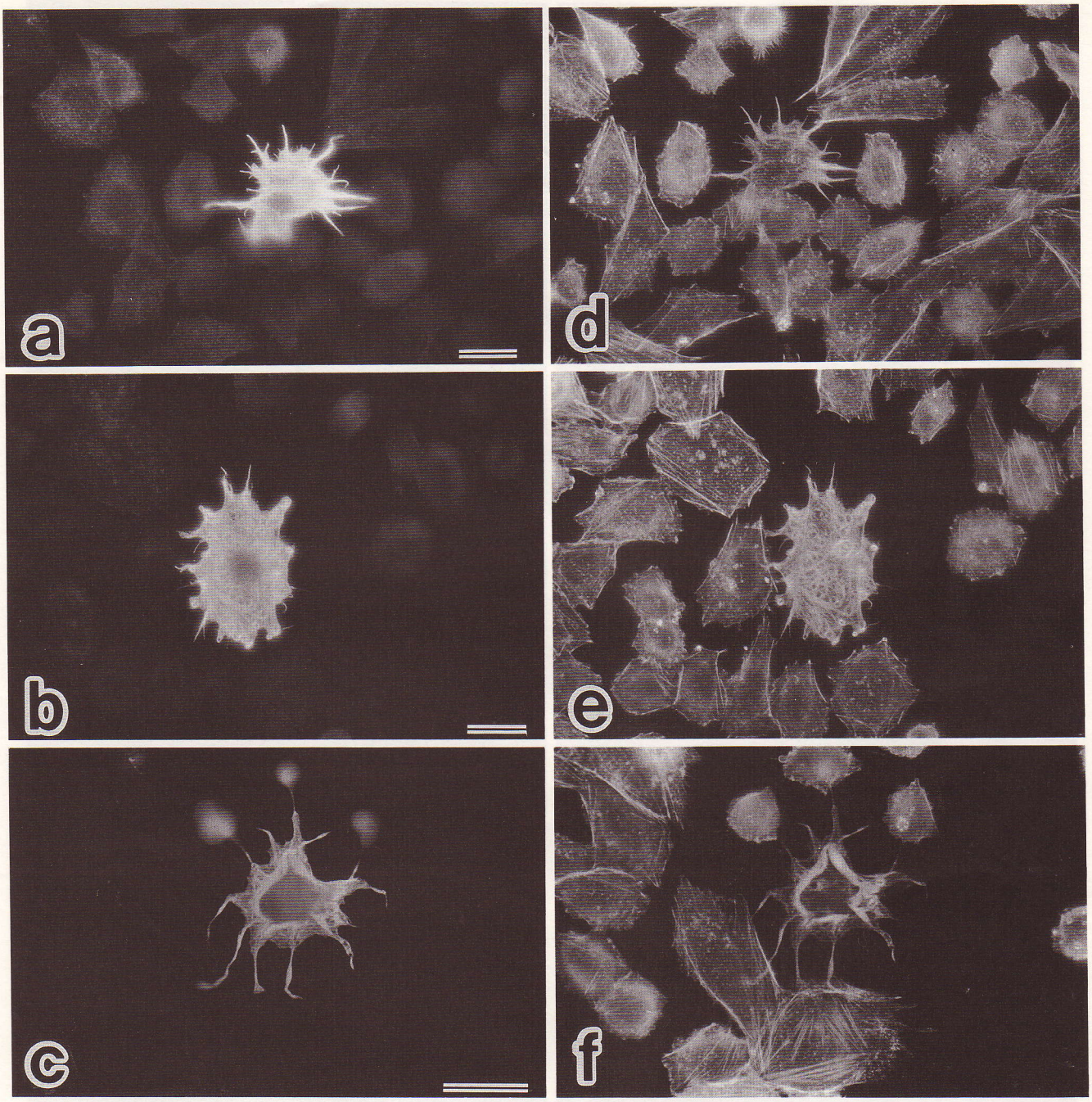

FIG. 6. Double-staining analysis of drebrin and actin in transfected CHO cells. CHO cells were transfected and stained as described in the legend to Fig. 4. Staining was visualized with filters specific for FITC (a, b, c) and rhodamine (d, e, f). Note that drebrin-like immunoreactivity on thick, curved bundles was colocalized with actin filaments. Bars, 20 μm.

expressing transfectants actively extend cell processes to achieve their novel morphology.

We have already demonstrated the high-level expression of drebrin in developing neurons and its accumulation in growing neurites by two-dimensional gel electrophoresis and immunohistochemistry [17, 18]. Fisher *et al.* recently reported detection of messenger RNA for drebrin E in osteoblasts [24]. Osteocytes in bone exhibit highly branched, dendrite-like cell processes that reside in channels called canaliculi. Fisher *et al.* demonstrated, using a cell culture system, that the level of the messenger RNA was elevated when osteoblasts were producing

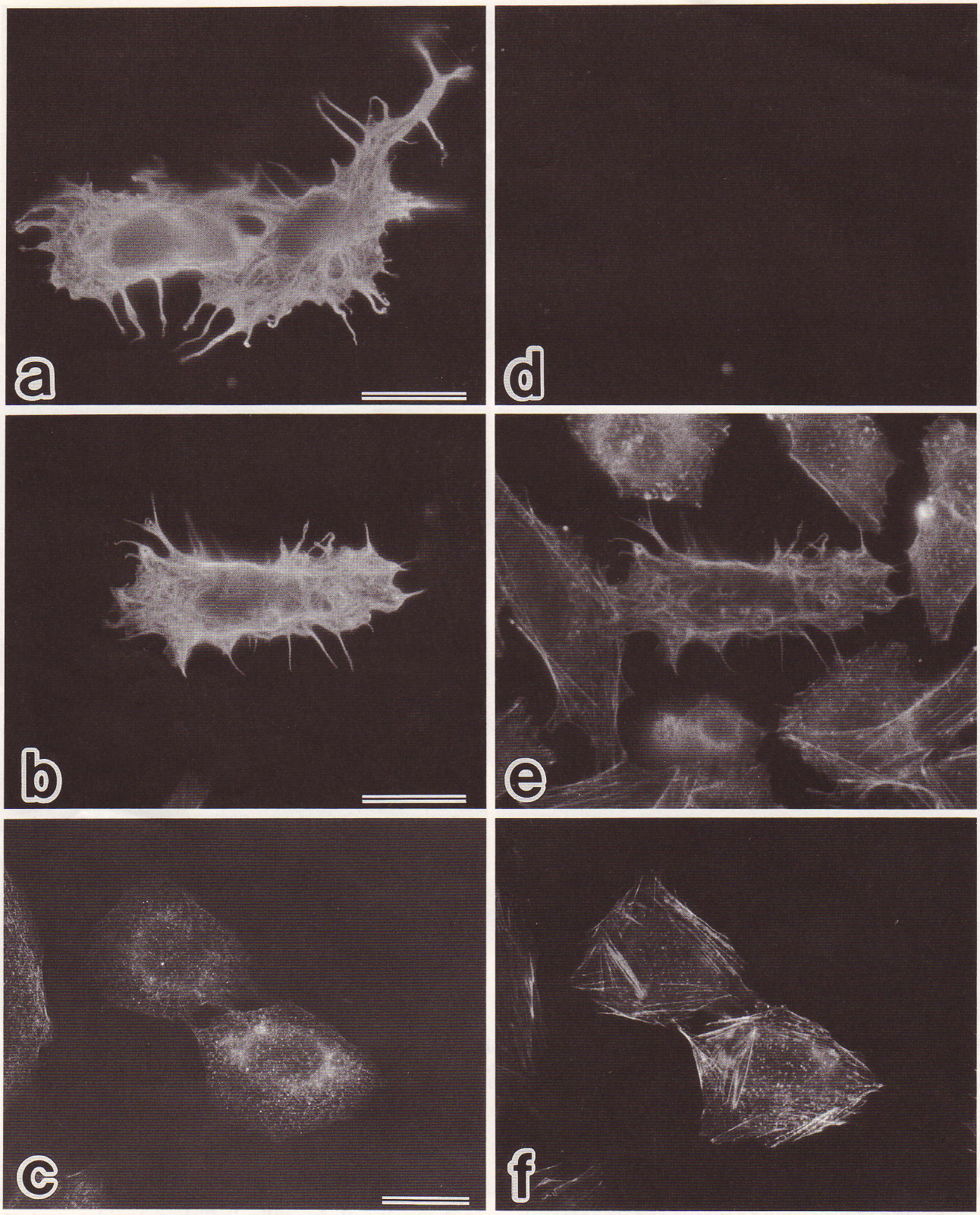

FIG. 7. High-magnification fluorescence micrographs of transfected and control CHO cells. CHO cells transfected with cDNA for drebrin A (a, b, d, e) and control CHO cells (c, f) were fixed and incubated with drebrin-specific monoclonal antibody. The cells were then incubated with FITC-conjugated goat antibodies against mouse IgG and with rhodamine-conjugated phalloidin, but the cells for control analysis of fluorescence "cross-bleed" (a, d) were incubated with FITC-conjugated goat antibodies against mouse IgG alone. Staining was visualized with filters specific for FITC (a, b, c) and rhodamine (d, e, f). The photograph in (c) was exposed longer than those in (a) and (b) to record the weak immunofluorescence of drebrin in control cells. Note that drebrin-like immunoreactivity on thick, curved bundles was colocalized with actin filaments (b, e). Bars, 20 μm.

cell processes and decreased when osteoblasts reduced the rate of formation of new cell processes in culture. These data also indicate that cells may use DB-actin filaments in the formation of cell processes. At present, the nature of DB-actin filaments is unknown. However, our results suggest that DB-actin filaments might be considered a new class of actin filaments and that this novel class of actin filaments might be directly related to the formation of highly branched cell processes from drebrin A-expressing cells. It will be of interest to analyze DB-actin filaments biochemically and to elucidate their role in the molecular mechanism of the outgrowth of neurites from developing neurons.

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