The roles of microfilament-associated proteins, drebrins, in brain morphogenesis: a review

Tomoaki Shirao
Department of Neurobiology and Behavior, Gunma University School of Medicine,
Maebashi 371-8511, Japan.
Running title: The roles of drebrins in brain morphogenesis

Correspondence: Tomoaki Shirao,
Department of Neurobiology and Behavior, Gunma University Graduate School of Medicine,
3-39-22 Showamachi, Maebashi, Gunma 371-8511, Japan.
Tel : 027-220-8050
Fax: 027-220-8053
e-mail tshirao@med.gunma-u.ac.jp

Summary

The cytoskeleton has been suggested to be one of the important endogenous factors that control neuronal morphogenesis. Analysis of the developmental changes in the protein composition of the brain led to the discovery of novel developmentally regulated actin-binding proteins, drebrins. Drebrins exhibit a number of characteristics that one might expect for an intracellular regulator of neuronal morphogenesis. Drebrin has three isoforms and the mRNA of each isoform is transcribed from a single gene through alternative RNA splicing mechanisms. The expression pattern of each isoform is regulated spatially and temporally in the developing brain. Drebrin and tropomyosin competitively bind to actin filaments, and the exclusion of tropomyosin from actin filaments by overexpression of drebrin in fibroblasts results in the appearance of thick, curving bundles of actin filaments and the formation of cell processes. Taken together, these data indicate that drebrin is one of the intracellular regulators of the neuronal morphogenesis.

Key words: drebrin, actin-binding protein, central nervous system, neurite outgrowth, development

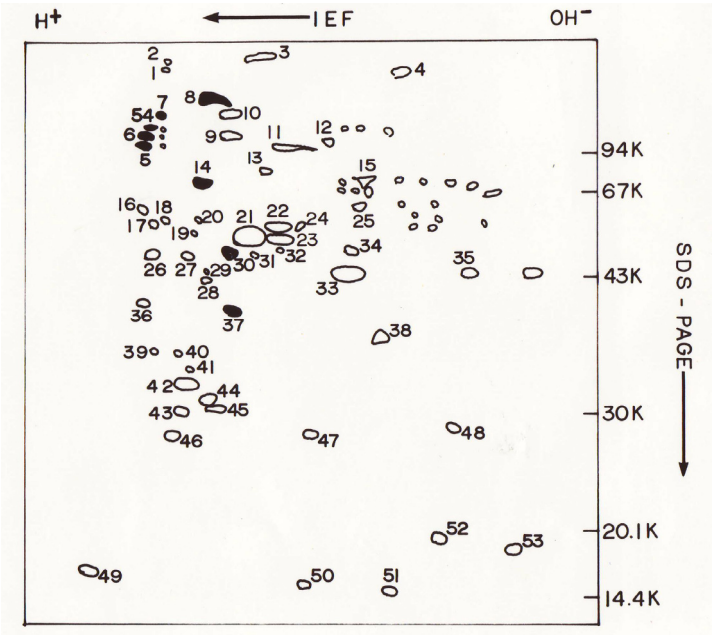
The outgrowth of neuronal cell processes, axons and dendrites, is the first step in the formation of a neuronal network in the brain. The cytoskeleton has been suggested to be one of the important endogenous factors that control the elaborate morphology of neuronal processes (1-6). The expression of intracellular regulators of this neuronal morphogenesis is most likely arranged spatially and temporally during development of the brain. A variety of data implicate the microtubule-associated proteins in neuronal morphogenesis (7-12). On the other hand, the regulatory mechanism for the microfilament system in neuronal morphogenesis has not been clarified, although a number of actin-binding proteins were identified in the brain with properties similar to those of other nonmuscle tissues (13-15).

This paper first describes the developmental changes in the protein composition of the brain and how this type of analysis led to the discovery of the novel developmentally regulated actin-binding proteins, drebrins. Drebrins exhibit a number of characteristics that one might expect for an intracellular regulator of neuronal morphogenesis. The main part of this paper is dedicated to reviewing recent studies on drebrins and what the findings suggest is drebrin's role in neuronal morphogenesis.

Temporally arranged protein expression in the developing brain

Two-dimensional gel electrophoresis is a good means of analyzing proteins from tissue, and for determining those proteins that are expressed in a

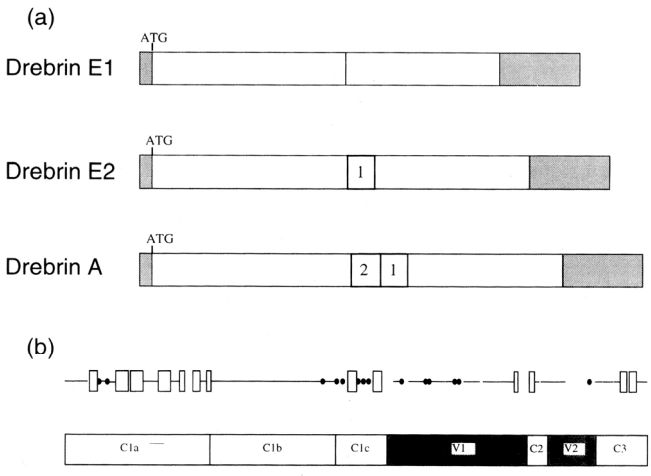
temporally and spatially arranged manner. Changes in the protein composition of the optic tectum of chicken brain during development are summarized in Fig. 1. Although many protein spots were detected in the electrophoretogram, fifty-four principal spots were numbered (as illustrated in Fig. 1) and analyzed for changes in their staining intensities during development. Spot 21 is b-tubulin, spots 22 and 23 are a-tubulin, and spot 33 is actin. Most of the proteins were found early in development (4-day chick embryo) and their expression remained unchanged until adulthood. There were eight proteins (spots 5, 6, 7, 8, 14, 30, 37 and 54) that showed remarkable changes in their staining intensities during embryonic development[16]. They are shown as black spots in Fig. 1. These eight proteins were divided into three groups. The proteins in Group I (spots 8, 14, 30 and 54) appeared in the course of development, then increased and were present in the adult brain. Proteins 8 and 14 are low and middle molecular weight neurofilament proteins. The proteins in this group are nervous system-specific proteins. The proteins in Group II (spots 7 and 37) were found not only in the brain but also in the liver of embryos, and they decreased during development. We suggest that these proteins are involved in some developmental function, but are not specific for the nervous system.



Group III contains proteins 5 and 6, named drebrins E1 and E2, respectively. Drebrin E1 is abundant at the developmental stage when neurons are migrating, and drebrin E2 is abundant at the stage when neurons are extending their cell processes. The developmental changes in the expression levels of drebrins occurred earlier in the rostral region of the optic tectum than in the more caudal region, which is consistent with the rostrocaudal gradient of development in the optic tectum (17). Furthermore, the developmental changes in drebrin expression are delayed in the cerebellum, in accordance with the later development of the cerebellum, as compared to the optic tectum (18). This stage specific expression is one of the characteristics that one might expect for a regulator of neuronal morphogenesis.

Developmental regulation in the expression of drebrin

Cloning of drebrin cDNAs has shown that the amino acid sequences of drebrins E1 and E2 are identical except for a 43 amino-acid sequence (Ins 1) inserted into the middle portion of the drebrin E1 sequence (Fig. 2a) (19). Moreover, it was determined that protein 54 is another protein isoform of drebrins. This protein has another 46 amino-acid sequence (Ins2) upstream of the Ins 1 insertion (20). Protein 54 was named drebrin A due to the later finding that this isoform is predominantly expressed in adulthood. A single gene pattern on genomic Southern blotting, and genomic and cDNA sequence analysis, indicating that Ins 1 and Ins 2 are separate independent exons of the drebrin gene, revealed that the three drebrin mRNAs are transcribed from a single gene through alternative RNA splicing mechanisms (20). Immunohistochemistry and in situ hybridization have demonstrated that drebrin E1 is accumulated first in migrating neurons. Shortly after neurons cease migration, they stop generating drebrin E1 mRNA. In parallel, drebrin E2 is accumulated in the growing cell processes of neurons. There is a temporal correlation between expression of drebrin E1 and cell migration, and between expression of drebrin E2 and growth of neuronal cell processes. The increase in drebrin A expression begins with the start of maturation of the neuronal network, which marks the terminal differentiation of synaptic connections between axons and dendrites (21).



Phylogenetic conservation of drebrins

Drebrins have been detected in the brains of various species by means of immunohistochemistry and immunoblotting techniques. Although the molecular weights of drebrins differ between isoforms and between species, their isoelectric points are similar (approximately 4.5). The cDNAs for drebrin have been cloned in chicken, rat and man (19,20,22-24). 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 any other known protein sequences. Drebrins in all species are classified into an embryonic form that lacks the Ins 2 sequence, and into an adult form that contains the Ins 2 sequence. Chicken has two embryonic isoforms, drebrins E1 and E2, and one adult isoform, drebrin A. In mammals, however, only one

embryonic isoform, drebrin E, and one adult isoform, drebrin A, have been reported. The apparent molecular weights of chicken drebrins E1, E2 and A are 95, 100, and 110 kDa, as judged on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), respectively, although the molecular cloning of chicken drebrins disclosed that their molecular weights, according to their deduced amino acid sequences, are 62,165, 66,553 and 71,532, respectively. This discrepancy between the apparent molecular weight on SDS-PAGE and the molecular weight of deduced amino acids is conserved between species, and is likely due to the acidic nature of drebrins. For example, the deduced molecular weight of drebrin A in rat is 77,471, which is smaller than its apparent molecular weight determined on SDS-PAGE (140 kd). The overall amino acid identity of the optimal aligned sequences of drebrin A between chicken and rat 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 % (Fig. 2b). These regions are also conserved in man, there being very close homology to rat drebrins.

Actin-binding activity of drebrin and interaction with other actin-binding proteins

Ishikawa et al. (25) recently reported that rat drebrin E bound to actin filaments at a stoichiometry of 1:5, with a dissociation constant (Kd) of 1.2 x 10^{-7} M. Rat drebrin E does not exhibit actin-nucleating, actin-severing or actin-capping activity, nor does it crosslink actin filaments. Rat drebrin E did not affect the activity of gelsolin, filamin or caldesmon, but it inhibited the actin-binding activity of tropomyosin and a-actinin (25). The dissociation constant of brain tropomyosin is 5 x 10^{-6} M, which is smaller than that of drebrin E. Tropomyosin bound to actin filaments at a molar ratio of 0.11 and 0.007 in the absence and presence of drebrin E, respectively. On the other hand, drebrin E bound to actin filaments at a molar ratio of 0.097 and 0.031 in the absence and presence of tropomyosin, respectively. Therefore, although there was almost complete inhibition of tropomyosin's actin-binding activity by drebrin, the reduction of the binding of drebrin by tropomyosin was only 70%. An interesting possibility is that drebrin has two actin-binding sites, one of which is tropomyosin-sensitive and the other of which is a tropomyosin-insensitive actin-binding site.

Electron microscopy revealed that actin filaments that bound drebrin (referred to as DB-actin filaments) were straight, while control actin filaments were kinky and folded in the absence of ATP in the solution in which they were suspended. When side-binding proteins such as tropomyosin or caldesmon bound to actin filaments, they became straight (26,27). It is probable therefore that drebrin may bind to the side of actin filaments. These results suggest that drebrin and tropomyosin might competitively bind to actin filaments via similar actin-binding sites.

Drebrin E inhibited not only the actin-binding activity of a-actinin but also its actin crosslinking activity. α-Actinin bound to actin filaments at a molar ratio of 0.038 in the absence of drebrin. This value gradually decreased to 0.014 when the concentration of drebrin was increased to 2.6 μM. Surprisingly, a low-speed-centrifugation assay demonstrated that drebrin completely inhibited the actin-bundling activity of a-actinin at the above concentration (2.6 μM), but at the same concentration reduced the actin-binding of a-actinin by only 40 %. One possible explanation for this is that inhibition of actin binding at one of the two actin-binding sites allows drebrin to inhibit actin-crosslinking activity. Drebrin must act at two actin-binding sites to inhibit actin-binding activity. Another possible explanation is that drebrin may directly bind to a-actinin causing conformational changes of or breaking the dimer-form of a-actinin, resulting in the loss of actin-bundling activity of a-actinin.

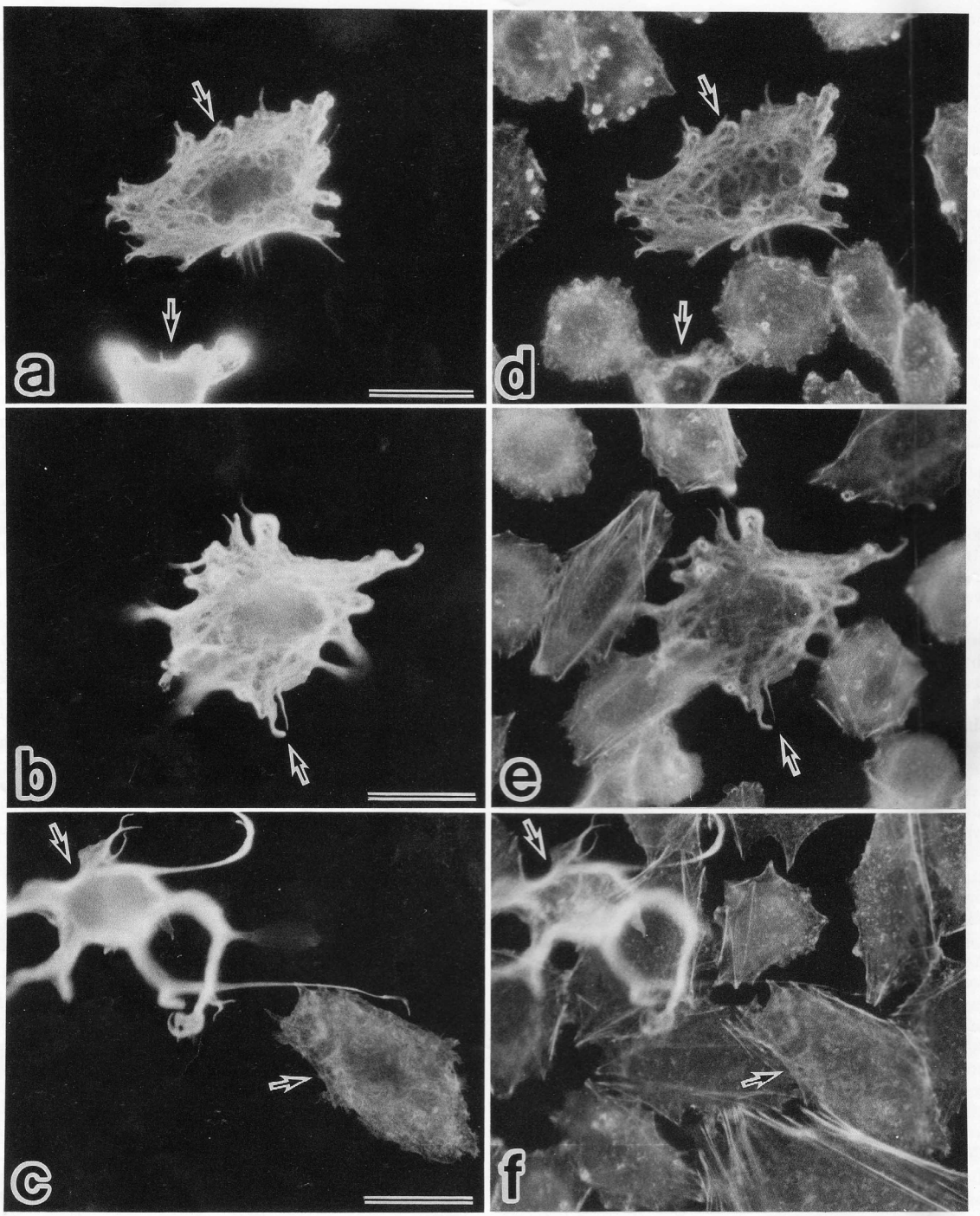
Compartmentalization of drebrins within cells

In the optic tectum and cerebellum of adult chicken brain, drebrin-like immunoreactivity, detected using a monoclonal antibody which does not distinguish between drebrin isoforms, is highly concentrated at synapses (28,29). Moreover, immunoelectron microscopy demonstrated that drebrin-like immunoreactivity is localized at the postsynaptic sites of dendrites (29). As Western blot analysis of tissue from the above regions of the brain has indicated predominant expression of drebrin A but not other isoforms of drebrins, it follows that drebrin A is characteristically localized at the postsynaptic sites of dendrites. A small population of neuronal cell bodies in rat brain is also immunostained with the drebrin-specific antibodies. Since Western blot analysis has indicated that the rat brain contains a trace amount of drebrin E in addition to drebrin A in the adult, the drebrin-like immunoreactivity within cell bodies may reflect the presence of drebrin E. Drebrin E is a major population of the drebrin isoforms during development. In the developing brain, drebrin-like immunoreactivity is observed in the cell bodies, growing axons and dendrites (28). This temporally regulated compartmentalization is a conserved feature of drebrins in other species.

Cultured human neuroblastoma cells express drebrin E. When they are cultured in a control medium, the cell morphology of neuroblastoma cells is similar to that of fibroblasts. In these cells, drebrin-like immunoreactive small dots are scattered sparsely in the cytoplasm and more abundantly around the nucleus. In contrast, neuroblastoma cells, caused to differentiate by the addition of retinoic acid, extend cell processes with a few branches. Immunocytochemical analysis of these differentiated neuroblastoma cells demonstrated that drebrin E together with actin filaments accumulated mostly in the submembranous cortical cytoplasm of the cell bodies and cell processes ┌30┌. This distribution of drebrins and actin filaments was very similar to that observed in primary cultured neurons.

Process formation activity of drebrin

This group has reported that the expression of drebrins in transfected fibroblasts resulted in the formation of cell processes (31,32). Immunocytochemistry demonstrated that drebrin A together with actin filaments was concentrated in the cell processes. In the cell bodies of transfectants that expressed drebrin A at high levels, thick, curving bundles of DB-actin filaments were observed, but straight actin filaments (stress fibers) had disappeared (31) (Fig. 3). In contrast, normal cultured fibroblasts



contained stress fibers, but not such thick, curving bundles of actin. In normal fibroblasts, the level of drebrin expression was low, and tropomyosin was localized in actin stress fibers. In drebrin-transfected cells, immunostaining of tropomyosin became diffuse and faint, and was not co-localized with actin filaments. Since tropomyosin is co-localized with actin stress fibers but not with actin filaments in ruffling membranes or microspikes (33,34), tropomyosin may be involved in the stability of actin filaments in vivo. Therefore it is suggested that, in transfected cells, expressed drebrin causes tropomyosin to dissociate from actin filaments by competing for its binding sites, and thereby may reduce the stability of actin filaments. Consequently this might provide a more plastic environment, allowing the formation of the cell processes on the transfected cells.

Correlation of drebrin expression with synaptic plasticity

During the sensitive period in early postnatal development, the functional architecture of the cat visual cortex is highly susceptible to alterations of the visual environment (35). Imamura et al. (36) reported that the expression patterns of drebrins are correlated in time with the changes in the level of physiologically-defined cortical plasticity. At 1 - 3 weeks, the beginning of the sensitive period for ocular dominance columns, drebrin was concentrated in layer IV of the cat visual cortex. Drebrin-like immunoreactivity dramatically decreased around the end of the sensitive period (about 3 months of age). In the adult visual cortex, drebrin-like immunoreactivity was very low. In contrast, in the brain of dark-reared cats, in which the onset of the sensitive period is delayed, drebrin expression persists in the mature visual cortex at a level similar to that in the developing brain. Furthermore, in the adult rat olfactory bulb, which maintains high plastic ability of synapses throughout life, there is expression of high levels of drebrins (unpublished data). The temporal and spatial correlation of drebrin expression with synaptic plasticity, together with the findings on the process formation activity, suggests that drebrins most likely play a role in synaptic plasticity.

What is the nature of drebrin-bound (DB) actin filaments?

Some cultured fibroblast cell lines express a trace amount of drebrin E[31]. Stress fibers, which bind tropomyosin, within these cells are not immunostained with a drebrin-specific antibody, but adhesion plaques and dot-like aggregates of actin filaments scattered in the cytoplasm are immunostained with the antibody. This indicates that there are at least two types of actin filaments in living cells, actin filaments that bind tropomyosin (referred to as TB-actin filaments) and DB-actin filaments. It has been reported that TB-actin filaments are resistant to actin-destabilizing proteins, such as gelsolin[27] and actin depolymerizing factor[37], which are found in brain tissue. In contrast, DB-actin filaments are depolymerized by gelsolin[25]. This suggests that DB-actin filaments might be more unstable than TB-actin filaments. However, when cultured cells were exposed to a

different reagent that depolymerizes actin filaments (Cytochalasin D), stress fibers (bundles of TB-actin filaments) disappeared but DB-actin filaments remained stable. Although it is generally agreed that Cytochalasin D slows the rate of filament polymerization by inhibiting the rate of elongation, consequently inhibiting the formation of stress fibers, the precise molecular mechanism has not been clarified (38,39). Therefore it is not known how drebrin protects actin filaments from the effect of Cytochalasin D, but it is clear that DB-actin filaments have a unique nature different from that of TB-actin filaments.

How does drebrin regulate neuronal morphogenesis?

It is tempting to speculate that neurons may consist of rigid and plastic domains of cell compartments, and that neuronal morphogenesis is regulated by the balance between rigidity and plasticity at specific domains of cell compartments. For example, outgrowth and branching of neurites may occur at the plastic domains of cell bodies and neurites, respectively. The rigidity and plasticity at these domains might depend on the nature of actin filaments. As judged on staining with Coomassie blue, the level of drebrin in the fraction of embryonic brain extract that co-precipitated with actin filaments is higher than that of tropomyosin. Thus, a major fraction of actin filaments in growing neurons, which are plastic in morphogenesis, seems to be associated with drebrins. It has been reported that morphological changes in dendritic spines are involved in synaptic plasticity (13). Dendritic spines contain high levels of drebrin and actin filaments (40). These data suggest that DB-actin filaments might be related to the plasticity of cell compartments and thereby regulate neuronal morphogenesis. Probably the greatest need for future research on drebrins is to explore their function in living animals. Gene targeting experiments on drebrin are currently in progress, and may give us a clearer idea as to the physiological functions of drebrins and DB-actin filaments.

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Legends

Fig. 1.

Composite diagram of major proteins of the developing brain. The proteins were separated by two-dimensional gel electrophoresis using the procedure of O'Farrell [41]. Fifty-four major spots were numbered. The filled spots are those spots that showed remarkable changes in their staining intensities (expression levels) during development. Adapted from ref. 16.

Fig. 2.

(a) Schematic representation of mRNAs for chicken drebrins E1, E2, and A. Each mRNA structure was predicted from a drebrin cDNA clone. Open and shaded rectangles represent coding and non-coding regions, respectively. 1: ins1 sequence. 2: ins2 sequence. (b) The secondary structure was predicted by the method of Chou and Fasman42. The predicted γ-structure is indicated by rectangles, the a-helical structure by lines, and the γ-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 included. (b) Adapted from Ref. 20.

Fig. 3.

Thick, curving bundles of actin in transfected CHO cells. CHO cells were transfected with a drebrin A expression plasmid. After fixation, the cells were incubated with a 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 localization of actin filaments. Visualization was performed with filters specific for FITC (a, b, c) and rhodamine (d, e, f). Arrows indicate transfected cells that expressed drebrin A. Bars, 20μm.