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Role of actin cytoskeleton in dendritic spine morphogenesis

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Running title (38 letters): Actin in dendritic spine morphogenesis

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Abstract (237 words)

Dendritic spines are the postsynaptic receptive regions of most excitatory synapses, and their morphological plasticity play a pivotal role in higher brain functions, such as learning and memory. The dynamics of spine morphology is due to the actin cytoskeleton concentrated highly in spines. Filopodia, which are thin and headless protrusions, are thought to be precursors of dendritic spines. Drebrin, a spine-resident side-binding protein of filamentous actin (F-actin), is responsible for recruiting F-actin and PSD-95 into filopodia, and is suggested to govern spine morphogenesis. Interestingly, some recent studies on neurological disorders accompanied by cognitive deficits suggested that the loss of drebrin from dendritic spines is a common pathognomonic feature of synaptic dysfunction. In this review, to understand the importance of actin-binding proteins in spine morphogenesis, we first outline the well-established knowledge pertaining to the actin cytoskeleton in non-neuronal cells, such as the mechanism of regulation by small GTPases, the equilibrium between globular actin (G-actin) and F-actin, and the distinct roles of various actin-binding proteins. Then, we review the dynamic changes in the localization of drebrin during synaptogenesis and in response to glutamate receptor activation. Because side-binding proteins are located upstream of the regulatory pathway for actin organization via other actin-binding proteins, we discuss the significance of drebrin in the regulatory mechanism of spine morphology through the reorganization of the actin cytoskeleton. In addition, we discuss the possible involvement of an actin-myosin interaction in the morphological plasticity of spines.

Key words: spine formation, spine morphology, actin, actin-binding protein, drebrin, synaptic activity, actin-myosin interaction

CONTENTS

1 Introduction

Neurons show characteristic morphological changes during development. They extend axons and terminate on small protrusions of various shapes on a dendrite, which are called dendritic spines. Dendritic spines are the postsynaptic receptive regions of most excitatory synapses (Harris and Kater, 1994). Because morphological studies of spines by the autopsy of dementia patients demonstrate the correlations between brain dysfunction and abnormal spine morphology (Purpura et al., 1982; Wisniewski et al., 1991; Irwin et al., 2000), it has been believed for a long time that spine morphology is crucial for understanding higher brain functions, such as learning and memory. Although synaptic function cannot be elucidated directly from spine shapes, the regulatory mechanisms of spine morphogenesis and the dynamics of spine morphology will provide essential information on the developmental and regulatory mechanisms of higher brain functions.

Spines have not yet been observed to emerge on dendrites of immature neurons. Instead, immature neurons have many thin headless protrusions, called dendritic filopodia, on their dendrites (Fig. 1A). Newly born filopodia lack the postsynaptic machinery necessary for matured synaptic function. When the brain receives much information on circumstances, the number of filopodia rapidly decreases and the number of bulbous spines simultaneously increases (Fig. 1B). Dendritic spines are fully equipped with postsynaptic machineries, such as neurotransmitter receptors, scaffold proteins anchoring the receptors, intracellular signaling molecules, and actin-binding proteins endowing the actin cytoskeleton with spine-specific characteristics (Fig. 2). Hence, dendritic spines can respond to extracellular signals and show morphological plasticity.

Because filopodia and spines are similar in terms of the presence of small protrusions (0.5 μm ~ 8 μm) on dendritic shafts and of the lack of microtubules and intermediate filaments (Kaech et al., 1997; Kaech et al., 2001), there are occasionally some confusions in terminology which lead to

the difference between dendritic filopodia and dendritic spines. In this review, the term "filopodia" will apply to all thin headless protrusions on dendritic shafts, and the term "spine" will apply to all other protrusions on dendritic shafts. In addition, filopodia at the tips of axonal and dendritic growth cones are excluded from "filopodia" in this review, because they differ from dendritic filopodia (Fiala et al., 1998; Portera-Cailliau et al., 2003) in terms of their mobility and fine structures.

Dendritic spines observed in fixed brain tissue shows various shapes, and are generally classified into three types: the thin type having a slender neck and a small head, the mushroom type having a short neck and a relatively large head, and the stubby type having no neck (Fig. 1C). In living neurons, spine shapes easily interchange between the above three types. In other words, spine morphologies are snapshots of dynamic morphological changes. Therefore, not only the spine morphology but also its dynamic change should be elucidated to understand synaptic functions.

What machineries are involved in the motility and dynamics of dendritic spines? Luo et al. (1996) were the first to suggest the significance of actin cytoskeleton in spine formation. The overexpression of a constitutively active Rac1, a regulatory signal of the actin cytoskeleton, facilitates spine formation. Three years later, we showed the enlargement of the spine by the overexpression of a neuron-specific actin-binding protein, drebrin A, in cultured neurons (Hayashi and Shirao, 1999). This is the first observation demonstrating that the manipulation of a single actin-binding protein in neuron alters spine morphology. Numerous findings related to actin organization in dendritic spines have rapidly emerged after these initial studies, which clearly demonstrated that the actin cytoskeleton plays a pivotal role in spine morphology (for review, see Shirao and Sekino, 2001; Ethell and Pasquale, 2005).

The purpose of this review is to provide comprehensive knowledge of actin elucidated in non-neuronal cells and to propose the importance of the

actin-binding protein in the regulation of spine morphology.

2. Structural elements of dendritic spines: Actin cytoskeleton and PSD

To understand the molecular mechanisms of dynamic changes in spine morphology, we first focus on the two major structural elements of dendritic spines: actin cytoskeleton and postsynaptic density (PSD) (Fig. 2).

Actin is one of most abundant proteins in neurons as well as in muscle cells. Filamentous actin (F-actin) forms bundles and networks that are components of the actin cytoskeleton. The characteristics of the actin cytoskeleton have been well elucidated in non-neuronal cells. Actin plays pivotal roles in regulating cell structures by modulating their network and bundle structures, and in producing the motile force of cells. For example, cellular processes, such as filopodia and microvilli, contain F-actin bundles, and the cortical submembranous region of cells contains F-actin networks. In lamellipodia, both bundles and network of F-actin are observed. Bundle structures are thought to be associated with cell movement. The motile force is regulated by the activity of myosin ATPase or the modulation of its own polymerization and depolymerization states of F-actin. In neuron, F-actin is particularly enriched in dendritic spines (Matus et al., 1982; Cohen et al., 1985) and forms a complex network to support structures of dendritic spines. Immunoelectron microscopic studies show that bundles of F-actins are observed in the necks of dendritic spines, and that lattices (network) of F-actins are observed in the spine head (Landis and Reese, 1983). The dendritic spine continuously changes its morphology by modulating actin dynamics, and the activity of glutamate receptors is involved in this morphological change (Matus, 1999).

PSD, the other structural element of dendritic spines, is an electron-dense detergent-resistant structure found just beneath the postsynaptic membrane of dendritic spines (for review, see Kennedy, 1997). PSD consists of various scaffold proteins including several membrane-associated guanylate kinases

(MAGUKs) and many PDZ proteins (Kim et al., 1995). PSD-95, the prototypical PDZ protein, is an abundant MAGUK in PSD (Cho et al., 1992; Kistner et al., 1993). Since the local assembly of PSD-95 spatially and temporally correlates with spine morphogenesis, it was thought that PSD-95 governs spine morphogenesis. However, the initiation of spine morphogenesis precedes the synaptic assembly of PSD-95 (Okabe et al., 2001). Furthermore, mutant mice lacking PSD-95 expression exhibit normal spine morphology (Migaud et al., 1998). These suggest that PSD-95 does not govern spine morphogenesis. On the other hand, the disruption of actin cytoskeleton during spine morphogenesis results in the global disassembly of synaptic structural elements (Allison et al., 2000; Zhang and Benson, 2001), suggesting that the actin cytoskeleton tethers PSD scaffold proteins to postsynaptic sites and govern spine morphogenesis. Thus, it is apparent that the actin cytoskeleton is more responsible than PSD for spine morphogenesis.

3. Overview of actin cytoskeleton

To provide better understanding of the mechanisms that regulate "dynamic changes in dendritic spine morphology", we outline the molecular aspects of actin dynamics.

3.1 Presence of globular and filamentous actins in living cells

Actin is present in monomeric (G-actin; globular actin) and filamentous forms in living cells. *In vitro* experiments showed that pure actin molecules mostly polymerize into double helical filaments under physiological salt conditions. G-actin is observed predominantly under low salt conditions *in vitro*, instead of under physiological salt conditions. How can large amount of G-actin be present *in vivo*? G-actin-binding proteins, such as thymosin β4

(Safer et al., 1990; Safer and Nachmias, 1994), sequester G-actin and suppress the assembly of G-actin into F-actin. Moreover, G-actin/F-actin equilibrium in dendritic spines is regulated in an activity-dependent manner (Okamoto et al., 2004). The mechanism of this regulation may depend on the presence of a sequestered G-actin pool, which enables site-directed F-actin polymerization in response to appropriate cell stimulation, such as synaptic activity.

3.2 F-actin "treadmilling"

F-actin has a structural polarity, and the two ends of F-actin lengthen and shorten at different rates (Woodrum et al. 1975). The difference in the critical monomer concentrations of both ends leads to F-actin treadmilling, a remarkable feature of F-actin, resulting in continuous exchanges of actin molecules in F-actin. Under physiological conditions, the net assembly and disassembly of actin molecules are observed at the barbed and pointed ends, respectively, when the concentration of G-actin is between the critical concentrations of both ends (Wegner, 1982). When the rate of association at the barbed end is equal to the rate of dissociation at the pointed end, the length of F-actin remains constant during treadmilling (steady-state treadmilling). The steady-state treadmilling of pure actin polymers is much slower *in vitro* than that of F-actin *in vivo* (Pollard, 1986; Zigmond, 1993). The presence of ADF/cofilin (Nishida et al., 1984; Yonezawa et al., 1985) accelerates the treadmilling *in vivo*, which is sufficiently rapid enough to be comparable to that observed in motile lamellipodia (Theriot and Mitchison, 1991; Small et al., 1995). This is because ADF/cofilin increases the rate of actin dissociation from the pointed ends, which is the rate-limiting step of steady-state F-actin treadmilling (Edelstein-Keshet and Ermentrout, 2000). This accelerated treadmilling can be responsible for the actin-based motility of lamellipodia (Carlier et al., 1997; Carlier and Pantaloni, 1997).

Actin-related motility has been observed in dendritic spines of neurons (Fischer et al., 1998). Fluorescence recovery after photobleaching (FRAP) analysis has demonstrated the rapid turnover of F-actin in dendritic spines (Star et al., 2002), suggesting that most F-actin in dendritic spines undergo treadmilling.

The pharmacological manipulation of actin turnover results in morphological changes of dendritic spines (Allison et al., 1998; Fischer et al., 2000; Zhang and Benson, 2001). This suggests that F-actin treadmilling plays an important role in regulating the morphology and motility of dendritic spines as well as a physiological role in regulating those of non-neuronal cells (Edelstein-Keshet and Ermentrout, 2000). On the other hand, actin dynamics in dendritic spines involves the formation of new actin filaments, which is proposed in dendritic nucleation model (Machesky and Way, 1998; Mullins et al., 1998), and the trafficking of fractionated short F-actin (Sekino et al., 2006) in addition to treadmilling. Thus, the dynamics of actin in dendrites is regulated by various molecular mechanisms downstream of cell surface signals.

3.3 Role of small GTPases

Most external signals, affecting the organization of F-actins in non-neuronal cells, converge inside the cells on Rho GTPases, such as RhoA, Rac1, and cdc42 (Hall, 1998; Settleman, 1999). These GTPases act as molecular switches existing in the active guanosine triphosphate (GTP)-bound and inactive guanosine diphosphate (GDP)-bound states (Van Aelst and D'Souza-Schorey, 1997; Hall, 1998), and modify actin-binding proteins. The modified actin-binding proteins finally change the actin organization.

In neurons, rapid morphological changes in the peripheral region of dendritic spines resemble the actin-based ruffling motion of lamellipodia, a

typical actin-based motility in many cell types. The major regulator of lamellipodial activity in non-neuronal cells is a small GTPase, Rac (Small et al., 2002; Katoh et al., 2006). Also a glutamate-receptor-mediated RhoA-GTPase-dependent signaling pathway directly controls F-actin reorganization and spine morphology (Schubert et al., 2006). These observations suggest that small-GTPases induce F-actin reorganization by modifying actin-binding proteins, resulting in the morphological changes of dendritic spines. However, it is not yet clarified which actin-binding proteins are involved in this pathway, and what types of organizational changes are predominantly involved in spine morphogenesis.

4 Actin-binding proteins in dendritic spine

4.1 Various types of actin-binding proteins

As mentioned previously, F-actins *in vivo* are composed of actin polymers and their associated proteins. A set of actin-binding proteins determine the characteristic organizations of F-actins, such as bundles or networks. Therefore, each F-actin behaves differently and forms a unique structure according to its binding proteins. In the skeletal muscle, F-actin is composed of actin polymers completely covered with tropomyosin along its entire length. The pointed ends of the filaments are completely capped with tropomodulin (Weber et al., 1999) and their barbed ends are inserted into the complex formed with α-actinin at the Z-band (Blanchard et al., 1989). The set of actin-binding proteins make F-actin in muscle so stable that no F-actin treadmilling is observed.

In contrast, F-actin in neurons organizes various structures, such as networks, and straight or tangled bundles in a subcellular location-dependent manner. For example, F-actin networks are found in spine heads and straight bundles of F-actin are found in spine necks (Landis

and Reese, 1983). Each F-actin organization is accompanied by a certain set of actin-binding proteins, suggesting that the composition of actin-binding proteins determines the region-specific organization of F-actin. Interestingly, the subcellular localization of actin-binding proteins can be changed by extracellular signals, such as synaptic activity (Sekino et al., 2006). This suggests that the local organization of F-actin within a neuron is interchangeable from one to another by changing the composition of actin-binding proteins in an activity-dependent manner.

Many actin-binding proteins have been identified in dendritic spines, such as Arp2/3, cortactin, ADF/cofilin, profilin, gelsolin, drebrin and neurabin (for review, see Ethell and Pasquale, 2005). Thus, we will provide an overview of several types of actin-binding protein that have essential roles in the regulation of F-actin organization.

4.1.1 Proteins regulating F-actin length

Some actin-binding proteins control F-actin length by severing F-actins, and some by regulating the dynamics of treadmilling.

Gelsolin decreases F-actin length in a Ca^{2+}-dependent manner. Gelsolin has a strong actin-severing activity, and can cap the barbed end of F-actin as well as nucleate filament formation (Janmey et al., 1985). F-actin severing is the initial and direct step for F-actin shortening, and the resultant increase in the number of pointed end, in combination with barbed end capping, further promotes F-actin shortening. The gelsolin activity is up-regulated by Ca^{2+} binding (Janmey et al., 1985), and inhibited by polyphosphoinositide binding (Janmey and Stossel, 1987). Thus, gelsolin mediates F-actin reorganization induced by NMDA receptor activation and Ca^{2+} influx (Star et al., 2002).

ADF/cofilin decreases F-actin length by depolymerizing F-actin in a Ca^{2+}-independent and pH-dependent manner (Bamburg et al., 1980; Nishida et al., 1984; Yonezawa et al., 1985). ADF/cofilin binds to both F-actin and

G-actin. It severs F-actin and sequesters G-actin (Maciver et al., 1991). However, the depolymerization activity of ADF/cofilin is mainly derived from their ability to increase the rate of dissociation from the pointed end of F-actin (Carlier et al., 1997). This leads to the acceleration of F-actin treadmilling *in vivo*, as described above.

Adducin is a membrane-skeletal protein localized at spectrin-actin junctions. Adducin caps the barbed end of F-actin and stabilizes them. In addition, it promotes the association of spectrin with F-actin (Gardner and Bennett, 1987; Mische et al., 1987; Kuhlman et al., 1996). It has been reported that the knockout of β-adducin leads to synaptic dysfunction, although the spine morphology is normal (Rabenstein et al., 2005). The destabilization of submembranous F-actin might cause synaptic dysfunction.

Profilin is a multifunctional G-actin binding protein. Profilin increases the exchange of ADP for ATP bound to actin, resulting in promoting F-actin polymerization (Goldschmidt-Clermont et al. 1992; Kovar et al. 2006). On the other hand, the sequestering of G-actin by profilin inhibits F-actin polymerization. In addition, profilin acts as an adaptor protein linking transmembrane signaling to the actin cytoskeleton (Carlsson et al., 1977). Matus's group has shown, using cultured hippocampal neurons expressing GFP-tagged profilin subtypes, that profilin II but not profilin I is targeted to spine heads in an activity-dependent manner and stabilizes spine morphology (Ackermann and Matus, 2003). Furthermore, it has recently been shown that profilin II forms a complex with RhoA-specific kinase (ROCK) and regulates F-actin organization in a RhoA activity-dependent manner (Schubert et al., 2006).

4.1.2 Cross-linking proteins of F-actin

Cross-linking proteins organize F-actin into bundles or networks. α-Actinin is observed in dendritic spines (Wyszynski et al., 1998). α-Actinin was originally discovered as a component of the Z-disc in skeletal muscle

(Masaki et al., 1967). At low α-actinin concentrations, isotropic networks of F-actins are formed, whereas at higher α-actinin concentrations, bundles of F-actin are formed (Wachsstock et al., 1993). α-Actinin in dendritic spines is thought to modulate NMDA receptor function by cross-linking them with F-actin (Krupp et al., 1999).

Fascin is highly concentrated in growth cone filopodia (Edwards and Bryan, 1995; Sasaki et al., 1996), but not in dendritic filopodia. Fascin was originally purified as an actin bundling protein from sea urchin eggs (Bryan and Kane, 1978) and Hela cells (Yamashiro-Matsumura and Matsumura, 1985, 1986). It has been reported that the suppression of fascin expression causes the loss of filopodia and abnormal morphology of the remaining filopodia of mouse melanoma cells (Vignjevic et al., 2006).

The Arp2/3 complex enhances actin nucleation and causes the branching and cross-linking of F-actin (Mullins et al., 1998; Welch et al., 1998). Thus, nascent F-actins, whose pointed ends are capped by the Arp2/3 complex, are integrated into the actin network at the front of lamellipodia (Svitkina and Borisy, 1999). Since the regulatory proteins of the Arp2/3 complex, such as WAVE1 and cortactin, are involved in spine formation (Hering and Sheng, 2003; Kim et al., 2006), Arp2/3-dependent actin network formation may play a role in spine formation (Rao and Craig, 2000).

Neurabin-I and neurabin-II/ spinophilin bundle F-actin and modulate the organization of the actin cytoskeleton in dendritic spines. They bind to protein phosphatase 1 (PP1) localized highly in dendritic spines (Ouimet et al., 1995) and may serve as the substrates for PP1 (Allen et al., 1997; Satoh et al., 1998). Neurabin I was originally identified as a neuronal F-actin-binding protein, and has been shown to be concentrated in the developing spines and the lamellipodia of the growth cone of immature neurons (Nakanishi et al., 1997). On the other hand, neurabin-II/spinophilin is a ubiquitous isoform of neurabin-I (Satoh et al., 1998; Feng et al., 2000).

4.1.3 Side-binding proteins of F-actin

The roles of side-binding proteins in F-actin organization tend to be undervalued compared with those of other actin-binding proteins, because they do not directly alter actin dynamics. Instead, they often alter the stability and mechanical properties of F-actin by binding along the sides of F-actin, and give a unique character to F-actin. Side-binding proteins, such as drebrin and tropomyosin, alter F-actin configuration from kinky to straight at the single-filament level (Ishikawa et al., 1989a; Ishikawa et al., 1994). They sometimes prevent filaments from interacting with other actin-binding proteins. For example, gelsolin cannot sever the tropomyosin-binding F-actin (Ishikawa et al., 1989a, b), since tropomyosin blocks gelsolin from binding to F-actin. In contrast, drebrin-binding F-actin (DBF-actin) can be severed by gelsolin (Ishikawa et al., 1994), because drebrin does not block gelsolin binding on F-actin, but rather inhibits the binding of tropomyosin on F-actin. Interestingly, the binding of drebrin and tropomyosin on F-actin are mutually excusive (Ishikawa et al., 1994).

Thus, side-binding proteins strongly modify the structural property of F-actin, and regulate interactions of many actin-binding proteins with F-actin. In this regard, regulation by side binding proteins might be upstream of the regulation by other actin-binding proteins. In this review, we will use the term "DBF-actin" to present a unique F-actin that is characterized by drebrin binding.

4.1.4 Myosin II as actin-based molecular motor

Motor proteins are included in the actin-binding proteins. They move organelles along F-actin or move F-actins themselves. Myosin is an ATP-driven, actin-based molecular motor (Sellers, 2000). Immunoelectron microscopic studies showed that myosin is observed in dendritic spines (Morales and Fifkova, 1989). There are various types of myosin in neurons (Bridgman, 2004). Among them, myosins I, V, and VI have been observed in

the lamellipodia and filopodia of non-neuronal cells, and implicated in the transport processes of proteins. Therefore, their roles in dendritic spines have been extensively studied, and it have been shown that myosin VI is involved in dendritic spine formation (Osterweil et al., 2005) and that myosin V functions in the trafficking of spine-resident proteins (Yoshimura et al., 2006).

On the other hand, the role of myosin II in dendritic spines has been undervalued because myosin II is thought to be involved in events faster than the transport of small organelles and the slow morphological change of spines. However, we have shown that nonmuscle myosin IIB exists in dendritic spines (Cheng et al., 2000). In addition, the inhibition of myosin II ATPase activity results in the elongation of spines (Ryu et al., 2006). Furthermore, in growth cone filopodia, there is a continuous flow of F-actin from the tip to the base in a myosin II-dependent manner (Ishikawa et al., 2003; Medeiros et al., 2006). Therefore, the role of myosin II in dendritic spines will be extensively analyzed in near future.

Myosin II activity is regulated in two ways. One regulatory mechanism is phosphorylation. Actin-activated myosin II ATPase activity is inhibited by the myosin light chain (MLC). The phosphorylation of serine-19 of MLC relieves this inhibition, and thus activates ATPase (Adelstein et al., 1973; Kamm and Stull, 1985; Moussavi et al., 1993). This regulatory mechanism is called myosin-linked regulation, and is only observed in smooth muscle and nonmuscle cells. The other regulatory mechanism is in F-actin. In skeletal muscles, myosin II (thick filaments) cannot bind to F-actin (thin filaments) at low Ca^{2+} concentrations, since tropomyosin blocks the myosin binding sites of F-actin. When Ca^{2+} concentration increases, the troponin complex dislocates tropomyosin on F-actin, and the myosin binding sites of F-actin are exposed. Then myosin II can bind to F-actin, and produces the motive force by ATP hydrolysis. Similar regulations are observed in cardiac and smooth muscles, and in nonmuscle cells, such as the platelet contractile

system (Cohen et al., 1973). This regulatory system is called actin-linked regulation (Ebashi and Endo, 1968; Ishikawa et al., 1995).

Myosin IIB (Kiehart, 1990; Kawamoto and Adelstein, 1991) is present in large amounts in PSD (Jordan et al., 2004; Peng et al., 2004) and DBF-actin preparations (Hayashi et al. 1996; Cheng et al. 2000). Drebrin can be considered a neuronal tropomyosin, since drebrin is a side-binding protein, modulating the interaction between myosin II and F-actin (Hayashi et al., 1996), similarly to tropomyosin. Therefore, we propose that drebrin functions in spine morphogenesis via actin-linked regulation. However, a Ca^{2+} sensor protein in dendritic spines, corresponding to troponin in skeletal muscles, has not yet been clarified. Calponin is a Ca^{2+}-binding protein (Wills et al. 1994), and is involved in the actin-linked regulation of smooth muscles (Takahashi et al., 1986; Abe et al., 1990). Since acidic calponin is present in dendritic spines (Agassandian et al., 2000; Ferhat et al., 2003; Rami et al., 2006), it will be interesting to examine whether acidic calponin functions as a Ca^{2+} sensor in the actin-linked regulation of spine morphogenesis.

On the other hand, small GTPase is thought to function in spine morphogenesis via myosin-linked regulation. Because MLC phosphorylation is an indirect target of activated small GTPase, small GTPase enhances dendritic spine formation by activating MLC (Zhang et al., 2005).

4.2 Drebrin A as neuron-specific side-binding protein

To understand the role of side-binding proteins in spine morphogenesis and spine morphology, we will focus on drebrin A, a typical side-binding protein found in dendritic spines. As a neuron-specific actin-binding protein, drebrin is the first to have been extensively studied for its role in spine morphogenesis and spine morphology (for review, see Shirao, 1995; Shirao and Sekino, 2001). Drebrin was originally identified as a developmentally regulated brain protein using two-dimensional gel electrophoresis (Shirao and Obata, 1985, 1986; Shirao et al. 1988; Kojima et al. 1988). There are two

major drebrin isoforms, drebrins E and A (Shirao et al., 1989, 1992), which are generated by the alternative splicing of the drebrin gene (*dbn*1) (Kojima et al., 1993; Jin et al., 2002). Drebrin E is a ubiquitous isoform, and predominates in the developing brain (Shirao and Obata, 1986; Kojima et al., 1993). In chicken, drebrin E is further subdivided into drebrins E1 and E2 (Shirao and Obata, 1985, 1986; Shirao et al., 1990; Kojima et al., 1988). On the other hand, drebrin A is a neuron-specific isoform, and predominates in the adult brain (Shirao and Obata, 1986; Kojima et al., 1993; Aoki et al., 2005). In addition to these major isoforms, a minor isoform of truncated drebrin A, s-drebrin A, has also been identified at the mRNA level (Jin et al., 2002).

Up to now, no clear differences in biochemical nature between the drebrin isoforms have been reported. Drebrin has an ADF/cofilin homology (ADF-H) domain in the N-terminal region (residues 8-134 of rat drebrin E) (Lappalainen et al., 1998), but this domain is not necessary for the F-actin-binding activity of drebrin. The 85-amino-acid sequence in the N-terminal region (residues 233-317 of rat drebrin E), different from ADF-H domain, is sufficient for the strong binding of drebrin to F-actin (Hayashi et al. 1999). It is likely that drebrin does not bind to G-actin despite of the presence of ADF-H domain, because the binding of drebrin is saturated at the molar ratio of 5 actin molecules to 1 drebrin molecule (Ishikawa et al., 1994).

Transfection experiments using non-neuronal cells showed that DBF-actin forms thick, winding actin bundles, which are clearly different from straight stress fibers, whose dominant actin-binding proteins are tropomyosin and α-actinin (Shirao et al. 1992; Shirao et al., 1994). DBF-actin is not similar to the F-actin of either non-neuronal cells (Lazarides, 1976; Yamashiro-Matsumura and Matsumura, 1986) or growth cone filopodia, in which the dominant actin-binding protein is fascin. Typical bundles of DBF-actin are occasionally observed in the cell bodies and base of apical

dendrites of intact neurons as well as in the transfected cells (unpublished observation).

The difference of drebrin A from drebrin E is the insertion of 46 amino acid residues (named ins2) in the middle portion of drebrin E (between residue 318 and 319 of drebrin E). Ins2 residues are highly conserved between species (100% among mammals, and 84% between chicken and mammals) (Kojima et al., 1993), but their physiological role is not yet elucidated. In developing neurons where drebrin E predominates, drebrin is distributed throughout the entire cell (Tanaka et al., 2000; Shirao and Sekino, 2001). In contrast, in mature neurons where drebrin A predominates, drebrin mainly localizes at dendritic spines and occasionally at cell bodies and dendritic shafts, but never at presynaptic terminals (Shirao et al., 1987; Hayashi et al., 1996; Aoki et al., 2005). Transfection experiments have demonstrated that drebrin A expressed in mature cultured neurons accumulates spontaneously in dendritic spines (Hayashi and Shirao, 1999). Thus, the actin cytoskeleton of dendritic spines is distinguished from that of dendritic shafts by its association with drebrin A.

Immunoelectron microscopic studies have shown that about 70% of spines contain detectable drebrin level under normal conditions *in vivo* (Aoki et al., 2005). However, the acute inhibition of NMDA receptors significantly increases the number of such drebrin-positive spines (Fujisawa et al., 2006). This suggests that the targeting of drebrin A to each dendritic spine is regulated in an activity-dependent manner. Furthermore, drebrin A level is markedly reduced in the brains of Alzheimer's disease (Harigaya et al., 1996; Hatanpaa et al., 1999; Counts et al., 2006) and Down syndrome (Shim and Lubec, 2002) (for review, see Kojima and Shirao, 2007). Studies using Alzheimer's disease animal model also indicated that drebrin is involved in the pathogenesis of Alzheimer's disease (Calon et al., 2004; Mahadomrongkul et al., 2005; Zhao et al., 2006; Lacor et al., 2007). In addition, the drebrin gene (*dbn*1) has a cross talking points of basic

helix-loop-helix–PAS transcriptional factors related to Down syndrome pathology, such as NXF (activation) and Sim2 (repression), in its promoter regions (Ooe et al., 2004). Together, it is suggested that drebrin localization at dendritic spines is closely related to synaptic function.

5 Reorganization of actin cytoskeleton in spine formation

5.1 Filopodium as spine precursor

It has been proposed that dendritic filopodia serve as precursors of dendritic spines during neuronal development (Dailey and Smith, 1996; Ziv and Smith, 1996), although it is possible that dendritic filopodia may facilitate the establishment of shaft synapses (Fiala et al., 1998). The development of presynaptic terminals has been fully described from the initial moment upon contact between an axon and a dendrite (Friedman et al., 2000). On the other hand, the initial step of postsynaptic development has not yet been clarified. The presence of a transitional stage in spine formation involving the conversion of a dynamic filopodium to a stable spine has been suggested (Dailey and Smith, 1996; Dunaevsky et al., 1999). We have shown that filopodia in immature neurons are classified into two distinct types depending on the cluster formation of DBF-actin: diffuse type and cluster type (Takahashi et al., 2003) (Fig. 3).

Diffuse-type filopodia are dominant in early postnatal developmental stages. In this period, drebrin E expression predominates and drebrin A expression is hardly detected. Biochemical data have shown that part of drebrin does not bind to actin filaments in early postnatal developmental stages (Aoki et al., 2005). This is consistent with the diffuse distribution of drebrin in diffuse-type filopodia. In parallel with neuronal maturation, cluster-type filopodia become dominant over diffuse-type filopodia. A cluster-type filopodium, similar to a mature spine, has a single cluster of

DBF-actin. Although few diffuse-type filopodia have presynaptic contacts, most cluster-type filopodia have presynaptic contacts (Takahashi et al., 2003). This suggests that cluster-type filopodia are functionally more mature than diffuse-type filopodia. On the other hand, although most mature spines have PSD-95 clusters, a significant number of cluster-type filopodia have no PSD-95 clusters. This clearly shows the difference between cluster-type filopodia and mature spines. Together, we conclude that cluster-type filopodia represent the transitional stage from filopodia to spines (Fig. 3).

5.2 Roles of drebrin A in spine morphogenesis

5.2.1 Appearance of drebrin A at nascent axo-dendritic contact sites

We have recently shown the drebrin-A accumulation in the submembranous region of nascent axo-dendritic contact sites in dendrites *in vivo* by immunoelectron microscopy (Aoki et al., 2005). This finding strongly supports our idea that drebrin A expression is involved in the initial step of synaptogenesis. The submembranous localization of drebrin A at nascent synapses may correspond to the initial step of DBF-actin formation in cluster-type filopodia.

However, it has recently been reported that the 54-amino-acid sequence (residues 173-227 of rat drebrin E), which is different from the actin-binding domain (residues 233-317) (Hayashi et al. 1999), is sufficient for the submembranous localization of drebrin (Xu and Stamnes, 2006). Furthermore, clusters are observed in the core region of spine heads in mature neurons, whereas they are observed in the submembranous region of nascent axo-dendritic contact sites. Therefore, the submembranous localization of drebrin A in early postnatal stages might play a different role from the cluster formation of DBF-actin during spine maturation.

There are several studies that suggested roles of drebrin as an adhesion molecule. Drebrin E is enriched specifically in adherens junctions, and

forms a distinct F-actin-anchoring system from a vinculin-based one in non-neuronal cells (Peitsch et al,. 1999). Furthermore, transfection experiments showed that cell-substratum adhesion is stabilized when drebrin A is overexpressed (Ikeda et al., 1995, 1996). The most interesting finding related to the functional roles of drebrin in the submembranous region is that drebrin can be recovered as a binding partner of connexin-43 (Cx43) from mouse brain homogenate and that drebrin E is required for maintaining Cx43-containing gap junctions in their functional state (Butkevich et al., 2004). Because gap junctions are observed in contact sites between developing neurites of cultured neurons (Koss et al. 2003), drebrin A may be detected at these gap junctions.

5.2.2 Recruitment of other spine-resident proteins

Although the importance of F-actin in the development and maintenance of young synapses has already been shown (Zhang and Benson, 2001), the regulatory mechanisms for F-actin assembly in postsynaptic sites remain to be elucidated. We have demonstrated that the knockdown of drebrin-A expression in postnatal developing neurons suppresses the clustering of F-actin and PSD-95, and decreases the density of cluster-type filopodia, resulting in the inhibition of spine formation (Takahashi et al., 2003, 2006). This indicates that the developmentally regulated expression of drebrin-A governs spine formation via the accumulation of DBF-actin at postsynaptic sites.

How does drebrin A contribute to spine formation? A knockdown experiment on drebrin A suggested that drebrin-A expression facilitates DBF-actin formation, resulting in the accumulation of spine-resident proteins. However, a transfection experiment of drebrin-A cDNA in immature neurons showed that the premature expression of drebrin A induces the accumulation of DBF-actin and PSD-95 within filopodia, but their accumulation is not restricted at postsynaptic sites (Mizui et al., 2005).

Consequently, large filopodia called as megapodia are formed instead of cluster-type filopodia and spines (Fig. 4). These findings clearly show that the developmentally regulated upregulation of drebrin A plays a pivotal role in the formation of DBF-actin and in its accumulation with PSD-95 in immature filopodia; however, such upregulation is not sufficient for inducing the morphological changes of diffuse-type filopodia into cluster-type filopodia or spines (Mizui et al., 2005). Therefore, spine formation requires some other factors, such as synaptic activity, in addition to drebrin A expression.

5.3 Activity-dependent translocation of drebrin A in dendritic spines

The involvement of F-actin in structural plasticity in mature spines has already been highlighted. Synaptic activity induces the actin reorganization of discrete spatial and temporal patterns, each of which is driven by a distinct Ca^{2+} entry source (Furuyashiki et al., 2002). Prolonged NMDA receptor activity induces the local F-actin disappearance at synapses (Halpain et al., 1998). However, it has not been fully elucidated how F-actin changes its organization in an activity-dependent manner. The activity-dependent change of actin-binding proteins is a highly possible mechanism of F-actin reorganization. Some actin-binding proteins, such as profilin (Ackermann and Matus, 2003) and cofilin (Fukazawa et al., 2003), accumulate in dendritic spines, and others, such as cortactin (Hering and Sheng, 2003) and SPAR (Pak and Sheng, 2003), disappear from dendritic spines in an activity-dependent manner. Because these actin-binding proteins regulate the polymerization or nucleation of F-actin, the modulation of F-actin length by the polymerization or nucleation is probably involved in the activity-dependent reorganization of F-actin.

On the other hand, we have proposed that the translocation of fractionated short DBF-actin is also involved in the activity-dependent reorganization of F-actin in dendritic spines. A prolonged activation of the NMDA receptor induces the translocation of drebrin clusters from dendritic

spines to their parent dendrites. Intriguingly, glutamate treatment neither depolymerizes F-actin, nor dissociates drebrin from F-actin (Sekino et al., 2006). How can F-actin in dendritic spines be specifically fractionated? As mentioned in section 4.1.3, Ca^{2+}-activated gelsolin preferentially severs DBF-actin. Therefore, even if the increase of intracellular Ca^{2+} concentration by a prolonged activation of the NMDA receptor activates gelsolin within entire cells, F-actin only within dendritic spines seems to be severed by gelsolin. Thus, the fractionated DBF-actin translocates from dendritic spines to parent dendrites by an unidentified mechanism. As a result, non-DBF actin predominates in dendritic spines, and composes the actin cytoskeleton similarly to that in developing dendritic filopodia, which change their shapes more dynamically than mature spines. Taken together, we propose that NMDA receptor activity promotes the morphological change of spines by changing a set of side-binding proteins of F-actin in dendritic spines. An actin-myosin interaction is possibly involved in the translocation of DBF-actin, because drebrin regulates myosin ATPase activity (See section 4.1.4).

6 Conclusions

Because of the correlation between brain dysfunction and spine abnormality, it has been thought that spine morphology is crucial for understanding higher brain function. Nowadays, it is common knowledge that various spine shapes are snapshots of dynamic morphological changes of spines. The dynamics of spine morphology is due to the actin cytoskeleton being highly concentrated in spines. Many findings on the actin cytoskeleton of dendritic spines have emerged in the last decade. The regulatory mechanisms of actin dynamics in spines have been highlighted because they are closely related to synaptic and brain functions. It has also been shown that a spine-resident actin-binding protein, drebrin A, is responsible for recruiting F-actin and PSD-95 in filopodia, resulting in spine formation. Drebrin is classified as a

side-binding protein among actin-binding proteins. Because side-binding proteins control the association of various actin-binding proteins with F-actin, they provide a unique feature of F-actin in a subcellular-location-specific manner. The facts that drebrin A is highly localized in dendritic spines and is dislodged by NMDA activity indicate the importance of the side-binding-protein-dependent reorganization of F-actin in the morphological plasticity of spines. Thus, we propose the importance of the actin-binding protein in the regulation of spine morphology, and the possibility that an actin-myosin interaction is involved in synaptic plasticity. More detailed study of actin-binding proteins in the spine is surely the most promising road that will lead to a breakthrough in our understanding of how the regulatory mechanism of dynamic F-actin organization is associated with higher brain function.

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Figure legends

Figure 1. Morphology of dendritic protrusions, filopodia and spines. Dendrites of GFP-transfected hippocampal neurons cultured for 7 (A) and 21 (B) days. At the immature stage (7days), dendritic protrusions are very thin and long; these protrusions are called dendritic filopodia. In contrast, at the mature stage (21 days), dendrites are covered by dendritic spines, which commonly have an expanded head and a narrow neck. (C) Schematic representation of morphologies of filopodium and three types of dendritic spine: thin type, stubby type and mushroom type. Black disks represent the PSD structure and chains of red circles represent F-actin.

Figure 2. Ultrastructure of asymmetric synapse. The left panel shows a representative electron microscopic image of an asymmetric synapse immunostained with the anti-drebrin antibody. The right panel shows a schematic of an asymmetric synapse. The presynaptic terminal is filled with a number of synaptic vesicles and occasionally with mitochondria. The dendritic spine is composed of an actin cytoskeleton with actin-binding proteins and a PSD structure containing transmitter receptors.

Figure 3. Schematic of spine formation. During synaptogenesis, dendrites are bristled with many diffuse-type filopodia in which drebrin is diffusely distributed. After an axon terminal establishes contact with filopodia, drebrin clusters with F-actin at a postsynaptic site and forms cluster-type filopodia. PSD-95 cluster formation follows drebrin-actin cluster formation. The drebrin-actin complex tethers the postsynaptic machinery and is crucial for the maturation of dendritic spines.

Figure 4. Hippocampal neuron overexpressing GFP-drebrin A (DA). GFP-DA cDNA vectors were microinjected into the nuclei of neurons at 7

days *in vitro* (DIV), and fixed at 9 DIV for immunocytochemical staining. GFP-DA (green) accumulated in dendritic protrusions and formed large abnormal structures. PSD-95 (red) also accumulated in abnormal dendritic protrusions. Blue indicates synapsin I, a pre-synaptic terminal marker. Scale bar, 20 μm.

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