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CAST: a novel protein of the cytomatrix at the active zone of synapses that forms a ternary complex with RIM1 and Munc13-1

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The cytomatrix at the active zone (CAZ) has been implicated in defining the site of Ca^{2+}-dependent exocytosis of neurotransmitter. We have identified here a novel CAZ protein of ~120 kD from rat brain and named it CAST (CAZ-associated structural protein). CAST had no transmembrane segment, but had four coiled-coil domains and a putative COOH-terminal consensus motif for binding to PDZ domains. CAST was localized at the CAZ of conventional synapses of mouse brain. CAST bound directly RIM1 and indirectly Munc13-1, presumably through RIM1, forming a ternary complex. RIM1 and Munc13-1 are CAZ proteins implicated in Ca^{2+}-dependent exocytosis of neurotransmitters. Bassoon, another CAZ protein, was also associated with this ternary complex. These results suggest that a network of protein–protein interactions among the CAZ proteins exists at the CAZ. At the early stages of synapse formation, CAST was expressed and partly colocalized with bassoon in the axon shaft and the growth cone. The vesicles immunoisolated by antibassoon antibody–coupled beads contained not only bassoon but also CAST and RIM1. These results suggest that these CAZ proteins are at least partly transported on the same vesicles during synapse formation.

Introduction

The active zone beneath the presynaptic membrane is the principal site for Ca^{2+}-dependent exocytosis of neurotransmitter (Landis et al., 1988). Synaptic vesicles dock to the active zone and fuse with the plasma membrane, resulting in exocytosis of neurotransmitter (Burns and Augustine, 1995). Although various proteins involved in synaptic vesicle fusion have thus far been isolated and characterized (Südhof, 1995; De Camilli and Takei, 1996), the molecular mechanisms by which synaptic vesicles are properly localized at the active zone still remain unclear. It has been assumed that the cytomatrix at the active zone (CAZ)* is involved in determining the site of synaptic vesicle fusion (Landis et al., 1988; Hirokawa et al., 1989; Gotow et al., 1991; Dresbach et al., 2001). Thus, identifying and characterizing molecular components of the CAZ is a crucial step for understanding its organization and the targeting mechanism of synaptic vesicles to the active zone. However, only four CAZ proteins have thus far been characterized. (1) Bassoon is a 420-kD protein containing two NH_2-terminal zinc fingers, several coiled-coil domains, and a stretch of polyglutamines at its COOH terminus (tom Dieck et al., 1998). Bassoon is localized at the CAZ of excitatory and inhibitory synapses (tom Dieck et al., 1998; Richter et al., 1999) as well as at the base of retinal ribbon synapses (Brandstatter et al., 1999). (2) Piccolo is a 500-kD protein with zinc fingers structurally related to bassoon, but it also has PDZ and C2 domains (Cases-Langhoff et al., 1996; Fenster et al., 2000). The piccolo zinc fingers are shown to directly interact with PRA1, the dual prenylated Rab3A and VAMP2/synaptobrevin II receptor (Martincic et al., 1997; Bucci et al., 1999; Fenster et al., 2000). Rab3A is a small G protein and VAMP2/synaptobrevin II is a component of the SNARE

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*Abbreviations used in this paper: APMSF, α-amidinophenyl-methanesulfonyl fluoride hydrochloride; CAST, CAZ-associated structural protein; CAZ, cytomatrix at the active zone; P2, crude membrane; P100, pellet; pBS, pBluescript-SK; PSD, postsynaptic density; S100, supernatant; SAP, synapse-associated protein.

Key words: synapse; active zone; Munc13-1; bassoon; RIM

complex, both of which are implicated in Ca^{2+}-dependent exocytosis of neurotransmitter (Söllner et al., 1993; Geppert et al., 1997; Takai et al., 2001). Aczonin has been characterized in chick and mouse and is an ortholog of piccolo (Wang et al., 1999). (3) Munc13-1 is a mammalian ortholog of *Caenorhabditis elegans* UNC-13 (Maruyama and Brenner, 1991; Brose et al., 1995). Munc13-1 contains one C1 and two C2 domains, which mediate phorbol ester and diacylglycerol binding, and phospholipid-dependent Ca^{2+} binding, respectively. Munc13-1 is implicated in Ca^{2+}-dependent exocytosis of neurotransmitter through regulating vesicle priming (Betz et al., 1998; Augustin et al., 1999) and interacts with many other components of the synaptic exocytosis machinery, such as Doc2 (Orita et al., 1997; Duncan et al., 1999), Munc18 (Verhage et al., 1997), and syntaxin (Betz et al., 1997). Munc13-1 has two other isoforms, Munc13-2 and -3, but it is currently unclear whether the other members are localized at the CAZ. (4) RIM1 is a 180-kD protein containing two NH_2-terminal zinc fingers, one PDZ, and two C2 domains. RIM1 has originally been isolated as a putative downstream effector of Rab3A (Wang et al., 1997). RIM1 binds not only Rab3A but also cAMP-GEFII (Ozaki et al., 2000), RIM-BPs (Wang et al., 2000), Munc13-1 (Betz et al., 2001), synaptotagmin (Coppola et al., 2001), Ca^{2+} channel (Coppola et al., 2001), and α-liprin (Schoch et al., 2002). It has recently been shown that RIM1 regulates Ca^{2+}-dependent exocytosis of neurotransmitter through regulating vesicle priming (Castillo et al., 2002; Schoch et al., 2002). Another member of the RIM family, RIM2, has also been identified (Ozaki et al., 2000; Wang et al., 2000). RIM2 is highly homologous to RIM1 and expressed mainly in the brain. These four CAZ proteins with multiple domains seem to function as scaffolds at the CAZ, but the temporal and spatial regulation of these proteins in the formation and maintenance of the CAZ as well as molecular interactions among the CAZ proteins are largely unknown.

To further investigate the protein composition at the synaptic junction, we attempted to isolate novel synaptic proteins by a new method. In this method, we biochemically obtained the crude membrane (P2) and postsynaptic density (PSD) fractions from rat brain, extracted proteins from each fraction, and subjected them to a column chromatography, followed by SDS-PAGE and protein staining. We then searched for proteins more concentrated in the PSD fraction than in the P2 fraction by comparing each corresponding protein band. We identified 20 proteins, most of which were known to be synaptic proteins, but one of them was a novel protein of ~120 kD. The protein was localized at the CAZ, and we named it CAST (a novel CAZ-associated structural protein). We moreover found that CAST directly bound RIM1 and indirectly bound Munc13-1, forming a ternary complex. We characterize here this novel CAZ protein and discuss its possible function at the CAZ.

Results

Identification of CAST and molecular cloning of its cDNA

To isolate proteins concentrated in the synaptic junction, proteins were extracted from the P2 and PSD fractions of rat brain by a urea-based buffer, and each extract was then subjected to Mono Q column chromatography. Each fraction was subjected to SDS-PAGE, followed by protein staining with silver. When each protein band was compared between the P2 and PSD fractions, 20 protein bands were more concentrated in the PSD than in the P2 fraction (unpublished data). Some of these protein bands are shown in Fig. 1. The corresponding bands for the 20 proteins were cut out from the gels and analyzed by MALDI-TOF mass spectrometry. When a database was searched, most spectra had significant matches to known proteins, such as bassoon (tom Dieck et al., 1998), synamon/shank 1A (Naisbitt et al., 1999; Yao et al., 1999), and synapse-associated protein (SAP) 102 (Muller et al., 1996; unpublished data). These proteins are enriched in the PSD or presynaptic plasma membrane fraction, indicating that this approach is effective to identify new proteins enriched in the synaptic junction.

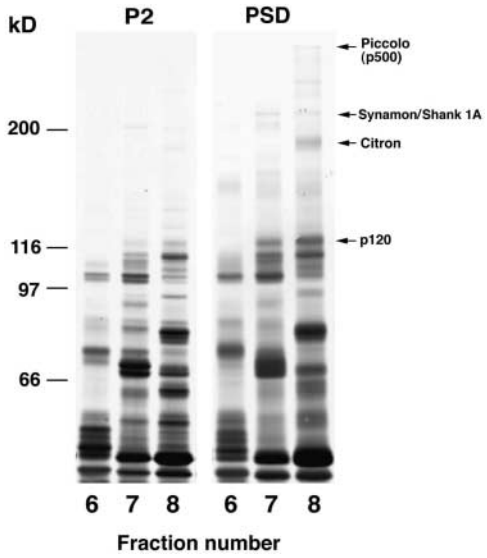

Figure 1. **Mono Q column chromatographies of the P2 and PSD fractions.** Proteins were extracted from the P2 and PSD fractions and each extract was subjected to Mono Q column chromatography. An aliquot (20 μl) of each eluted fraction was subjected to 7.5% SDS-PAGE, followed by protein staining with silver. Fractions 6, 7, and 8 of each chromatography are shown. These fractions contained piccolo, synamon/shank 1A, citron, and p120 (CAST). These results are representative of three independent experiments.

Two spectra for ~500- and ~120-kD bands (p500 and p120) had no significant matches in the database. Thus, we determined the partial aa sequences of p500 and p120. Computer homology search revealed that the two peptides derived from p500 were contained within the aa sequence of piccolo (Fenster et al., 2000). The four peptides derived from p120 were contained in the primary sequence deduced from a human cDNA (KIAA0378). However, this cDNA appeared to lack its NH_2-terminal portion and its function has been unknown. We obtained the full-length cDNA of p120, which encoded the protein consisting of 957 aa with a calculated molecular weight of 110,616 (Fig. 2 A). We named this protein CAST. CAST had no transmembrane segment but had four coiled-coil domains. The COOH-terminal three aa (IWA) was a putative consensus motif for binding to PDZ domains (Songyang et al., 1997). To con-

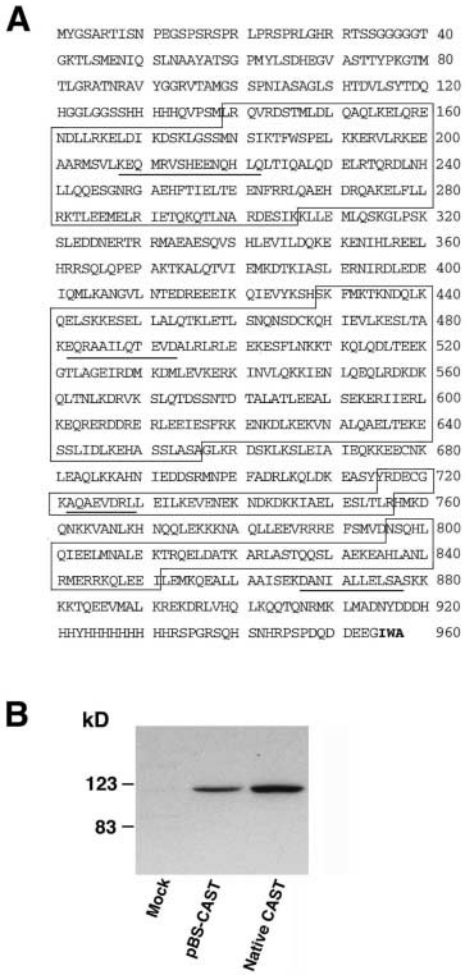

Figure 2. **Full-length sequence of CAST.** (A) Deduced aa sequence of CAST. Underlines indicate the aa sequences of the four peptide peaks. Boxes indicate coiled-coil domains. The putative consensus motif for binding to PDZ domains is shown in bold letters. These sequence data are available from GenBank/EMBL/DDBJ under accession no. AY049038. (B) Western blot analysis of recombinant CAST. The pBluescript-SK (pBS) vector containing CAST or pBS alone was in vitro translated in the rabbit reticulocyte lysate system. The lysates (5 μl each) and the homogenate of rat brain (10 μg of protein) were analyzed by Western blotting using the anti–CAST-1 Ab. This result is representative of three independent experiments.

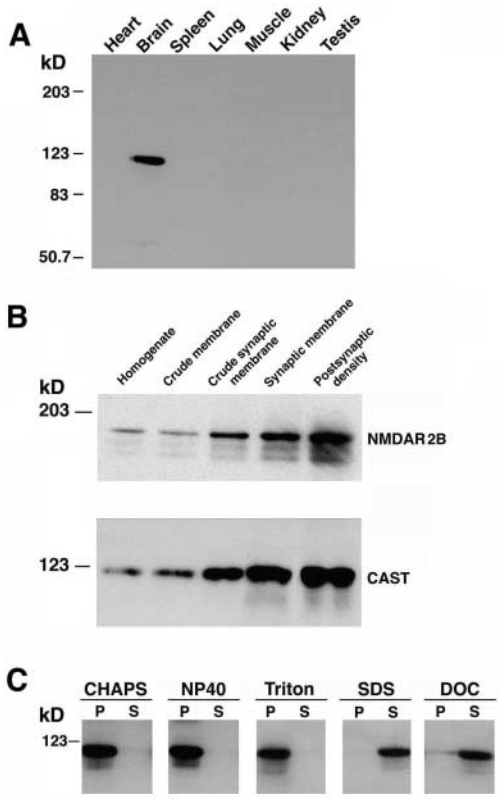

Figure 3. **Tissue and subcellular distribution of CAST and its solubilization property by various detergents.** (A) Tissue distribution. The homogenates of various rat tissues (20 μg of protein each) were analyzed by Western blotting using the anti–CAST-1 Ab. (B) Subcellular distribution. The homogenate of rat brain was subjected to subcellular fractionation. An aliquot of each fraction (5 μg of protein each) was analyzed by Western blotting using the anti-NMDA receptor 2B or anti–CAST-1 Ab. NMDAR2B, the NMDA receptor 2B. (C) Insolubility of CAST by nonionic and zwitter ionic detergents. Proteins were extracted from the P2 fraction with the indicated detergents and then separated into the soluble and pellet fractions. An aliquot (25 μl) of each fraction was analyzed by Western blotting using the anti–CAST-1 Ab. P, pellet fraction; S, soluble fraction. These results are representative of three independent experiments.

firm whether this clone encoded the full-length cDNA, we constructed an expression vector with the cDNA and expressed the protein by an in vitro translation system. Western blot analysis using a polyclonal antibody (Ab) (anti–CAST-1 Ab) indicated that the expressed protein showed mobility similar to that of native CAST on SDS-PAGE (Fig. 2 B). Thus, we concluded that this clone encoded the full-length cDNA of CAST.

Tissue and subcellular distribution of CAST

Western blot analysis showed that the anti–CAST-1 Ab recognized a protein band of ~120 kD in rat brain, but not in other rat tissues including heart, spleen, lung, muscle, kidney, and testis (Fig. 3 A). This result indicates that CAST is mainly expressed in the brain. Subcellular distribution analysis in rat brain showed that CAST was concentrated in the PSD fraction (Fig. 3 B). The subcellular distribution pattern of CAST was similar to that of the NMDA receptor. In addition, CAST was resistant to solubilization by CHAPS, a zwitter ionic detergent, and NP-40 and Triton X-100, nonionic detergents, although CAST was solubilized by SDS and deoxycholate (Fig. 3 C). These results indicate that CAST is a synaptic protein and tightly associated with the cytoskeletal structure.

Localization of CAST at the CAZ

We then examined the spatial distribution of CAST in a sagittal section of adult mouse brain using another anti-CAST polyclonal Ab (anti–CAST-2 Ab). A widespread immunoreactivity of CAST was detected throughout the central nervous system, including the hippocampus, the cortex, the cerebellum, and the olfactory bulb (Fig. 4 A). Consistently, Western blot analysis revealed that CAST was expressed in the various rat brain regions, such as the hippocampus, the cortex, the cerebellum, the amygdala, and the olfactory bulb (unpublished data). We next examined the precise localization of CAST in the hippocampus by immunohistochemistry. The

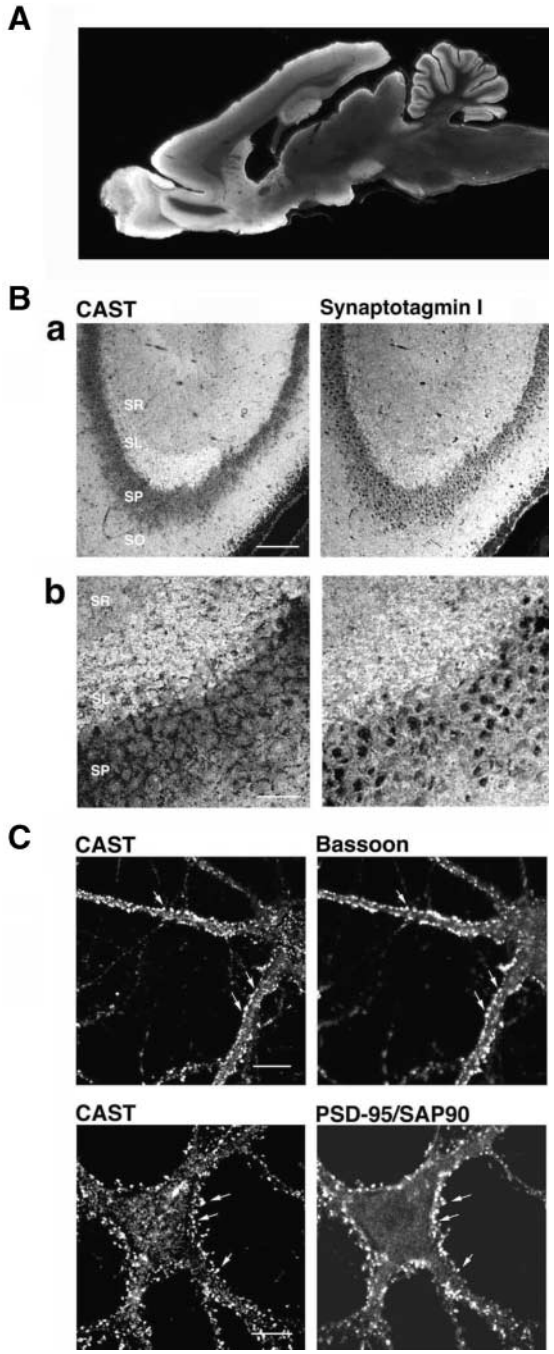

Figure 4. **Synaptic localization of CAST.** (A) Gross distribution of CAST in mouse brain. (B) CA3 region of mouse hippocampus. The sections were doubly stained using the anti–CAST-2 and anti–synaptotagmin I Abs. (a) Low magnification. (b) High magnification. SR, stratum radiatum; SL, stratum lucidum; SP, stratum pyramidale; SO, stratum oriens. Bars: (a) 100 μm; (b) 30 μm. (C) Primary cultured rat hippocampal neurons. Neurons at 21 d of culture were doubly stained using the anti–CAST-2 and antibassoon Abs or anti–PSD-95/SAP90 Ab. Arrows indicate the colocalized signals. Bars, 10 μm. These results are representative of three independent experiments.

staining pattern of CAST was compared with that of synaptotagmin I, a synaptic vesicle protein (Matthew et al., 1981). In the CA3 region of the hippocampus, CAST showed the most intense signal in the stratum lucidum where the synapses are formed between the mossy fiber terminals and the

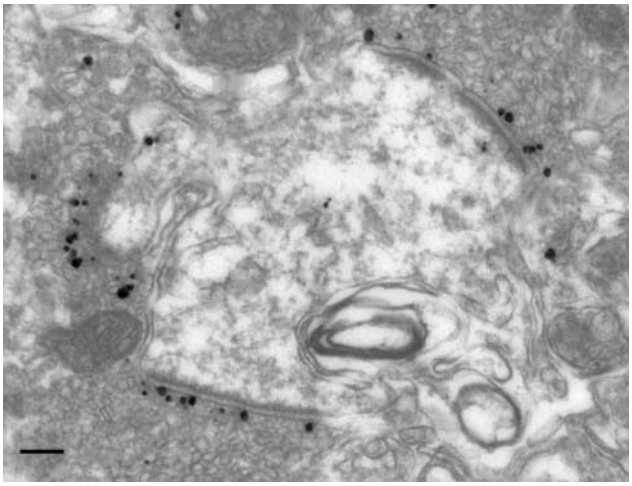

Figure 5. **Localization of CAST at the CAZ in mouse hippocampus.** The sections were reacted with the anti–CAST-2 Ab, incubated with immunogold particles (1.4 nm) conjugated with goat IgG against rabbit IgG, and silver enhanced, followed by electron microscopic analysis. Bar, 100 nm. The result is representative of three independent experiments.

dendrites of pyramidal cells (Fig. 4 B). The staining pattern of CAST was similar to that of synaptotagmin I. In primary cultured rat hippocampal neurons, CAST was also colocalized with bassoon and PSD-95/SAP90 (Fig. 4 C). Immunoelectron microscopic analysis of mouse brain showed that the immunoreactivity of CAST was detected in the presynaptic nerve terminals in the stratum lucidum of the CA3 region (Fig. 5). The CAST signal was concentrated at the CAZ. These results indicate that CAST is localized at the CAZ.

A ternary complex of CAST with RIM1 and Munc13-1

To explore a function of CAST at the CAZ, we examined the binding of CAST to other CAZ proteins. For this purpose, we first immunoprecipitated CAST by its Ab from the extract of the P2 fraction of rat brain. Among the proteins examined, RIM1 and bassoon were coimmunoprecipitated with CAST (Fig. 6 A, a). Neither Munc13-1 nor PSD-95/SAP90 was coimmunoprecipitated with CAST. Our anti-RIM1 Ab could not be used for immunoprecipitation, but our antibassoon Ab could. CAST and RIM1 were coimmunoprecipitated with bassoon by the antibassoon Ab (Fig. 6 A, b). Furthermore, Munc13-1 was coimmunoprecipitated with bassoon. Because it has been shown that Munc13-1 directly binds RIM1 (Betz et al., 2001), the RIM1–Munc13-1 complex might be coimmunoprecipitated with bassoon. These results suggest that CAST is associated with other CAZ proteins, including at least RIM1, bassoon, and Munc13-1, although it is unknown why Munc13-1 was not coimmunoprecipitated with CAST by its Ab.

We next confirmed the binding of CAST, RIM1, bassoon, and Munc13-1 by the cosedimentation assay. The extract of the P2 fraction was incubated with the Myc–CAST-coupled or Myc–RIM1-coupled affinity beads. Native RIM1 and bassoon bound to the Myc–CAST-coupled affinity beads and native CAST and bassoon bound to the Myc–RIM1-coupled affinity beads (Fig. 6 B). Moreover, native Munc13-1 bound not only to the Myc–

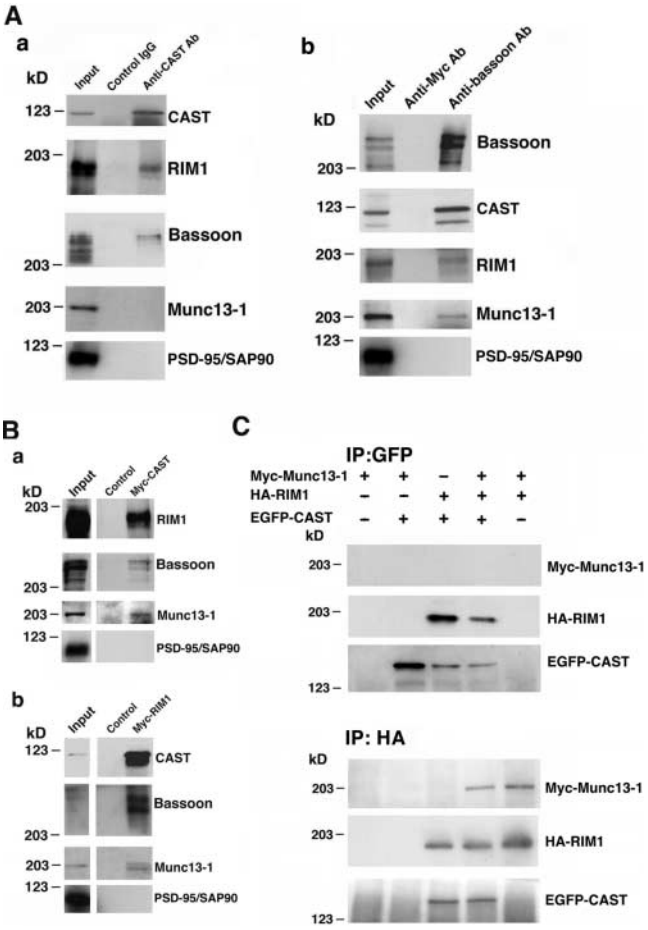

Figure 6. **Binding of CAST, RIM1, bassoon, and Munc13-1.** (A) Coimmunoprecipitation of other CAZ proteins with CAST. The extract of the P2 fraction was subjected to immunoprecipitation by the anti–CAST-1 or antibassoon Ab. The immunoprecipitate was analyzed by Western blotting using various Abs against the indicated proteins: (a) by the anti-CAST Ab and (b) by the antibassoon Ab. Input contains 5% of the extract used for the assay. (B) Cosedimentation of other CAZ proteins with CAST. The extract of the P2 fraction was incubated with the control or Myc–CAST- or Myc–RIM1-coupled affinity beads. Proteins that bound to the beads were analyzed by Western blotting using the anti-RIM1, anti-CAST, anti–Munc13-1, or antibassoon Ab. Bound Myc–CAST and Myc–RIM1 were detected by the anti-Myc Ab (unpublished data). (a) The Myc–CAST-coupled affinity beads. (b) The Myc–RIM1-coupled affinity beads. Input contains 5% of the extract used for the assay. (C) Ternary complex formation of CAST with RIM1 and Munc13-1. Each expression plasmid of EGFP–CAST-1, HA–RIM1, or Myc–Munc13-1 was transfected into HEK293 cells. Each protein was extracted and then mixed in the indicated combinations, followed by immunoprecipitation using the anti-GFP or anti-HA Ab. The immunoprecipitates were then analyzed by Western blotting using the indicated Abs. IP, immunoprecipitation. These results are representative of three independent experiments.

RIM1-coupled affinity beads but also to the CAST-coupled affinity beads (Fig. 6 B).

Because it was not clear from these results whether the binding of CAST to other CAZ proteins is direct or indirect, we examined whether CAST forms a complex directly with RIM1 and Munc13-1. We could not examine whether CAST forms a complex with bassoon, because bassoon is a very large protein and transfection of its cDNA has not been done (tom Dieck et al., 1998). We transfected each expression plasmid of CAST, RIM1, or Munc13-1 into HEK293 cells, extracted each protein, and mixed them in various combinations, followed by immunoprecipitation using the anti-GFP Ab for CAST or the anti-HA Ab for RIM1. RIM1 was coimmunoprecipitated with CAST by the anti-GFP Ab for CAST (Fig. 6 C). Munc13-1 was not, however, coimmunoprecipitated with CAST in the presence or absence of RIM1. Conversely, CAST and/or Munc13-1 were coimmunoprecipitated with RIM1 by the anti-HA Ab. These results, together with the earlier observation that Munc13-1 directly binds RIM1 (Betz et al., 2001), indicate that CAST forms a ternary complex with at least RIM1 and Munc13-1 by directly binding RIM1 and indirectly binding Munc13-1. It is currently unclear why Munc13-1 is not coimmunoprecipitated with CAST by its Ab, but the immunoprecipitation of CAST might affect the binding of RIM1 and Munc13-1, which is in part consistent with the result in Fig. 6 A, a. In addition, bassoon appears to be associated with the ternary complex of CAST, RIM1, and Munc13-1, but it remains to be clarified how bassoon interacts with this complex.

We finally confirmed the direct binding of CAST and RIM1 in a heterologous expression system. EGFP–CAST-1 (full length) or Myc–RIM1 (full length) was expressed in HEK293 cells. EGFP–CAST-1 formed large aggregates (Fig. 7 A, a) and was recovered in the Triton X-100–insoluble fraction (Fig. 7 B, a). In contrast, Myc–RIM1 was mainly distributed in the nucleus (Fig. 7 A, a) and recovered in the Triton X-100–soluble fraction (Fig. 7 B, b). EGFP was distributed throughout the cytoplasm (Fig. 7 A, a). When both EGFP–CAST-1 and Myc–RIM1 were expressed, CAST formed large aggregates again and RIM1 was colocalized with CAST at the aggregates (Fig. 7 A, b). Moreover, RIM1, as well as CAST, was recovered in the Triton X-100–insoluble fraction (Fig. 7 B, c). In contrast, PSD-95/SAP90, which contains three PDZ domains, was not colocalized with CAST (Fig. 7 A, c). These results indicate that CAST forms aggregates and recruits RIM1 to the Triton X-100–insoluble structure and provide another line of evidence for the direct binding of CAST and RIM1.

Because CAST has a putative COOH-terminal consensus motif for binding to PDZ domains (Fig. 2) (Songyang et al., 1997) and RIM1 has one PDZ domain (Wang et al., 1997), we examined the direct binding of CAST and RIM1 through the COOH-terminal consensus motif and the PDZ domain by the pull-down assay. The extract of HEK293 cells expressing Myc–RIM1 was incubated with glutathione-Sepharose beads containing various GST fusion proteins of CAST (Fig. 8 A, a). Myc–RIM1 stoichiometrically bound to GST–CAST-4 containing the COOH-terminal consensus motif, but not to other GST fusion proteins (Fig. 8 A, b and c), indicating that the COOH-terminal consensus motif of CAST was essential for its binding to RIM1. We then confirmed that CAST binds to the PDZ domain of RIM1. The extract of HEK293 cells expressing the Myc-tagged COOH terminus of CAST (Myc–CAST-4) was incubated with glutathione-Sepharose beads containing various GST fusion proteins of the PDZ domains of RIM1 and PSD-95/SAP90. Myc–CAST-4 bound to the GST fusion protein containing the PDZ domain of RIM1, but not to GST alone or the

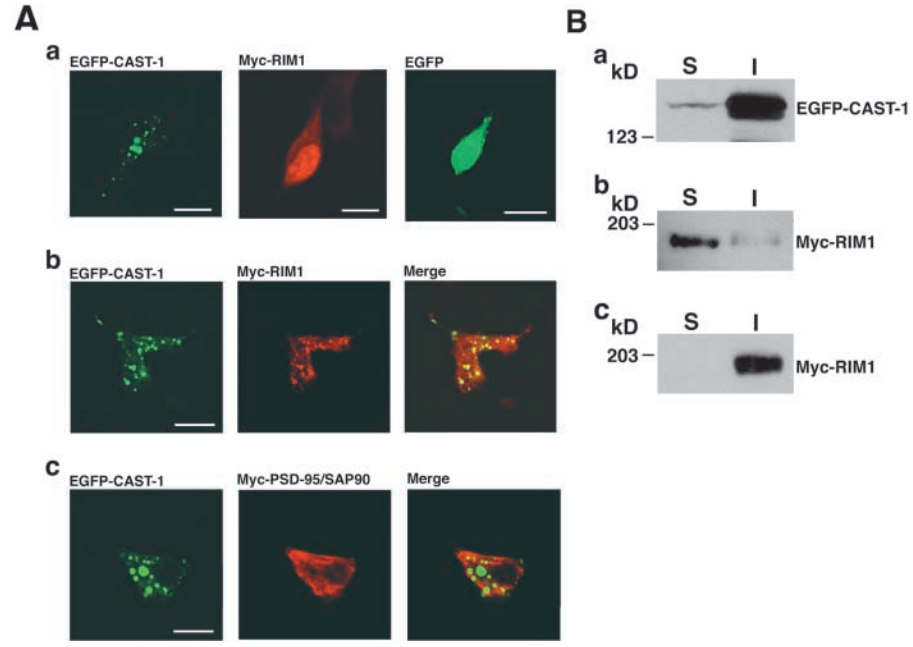

Figure 7. **Aggregation of CAST and recruitment of RIM1 to the aggregates.** (A) Colocalization of RIM1 with CAST at the aggregates of CAST. HEK293 cells were transfected with pEGFP–CAST-1 alone, pCMV-Tag3–RIM1 alone, or both. At 36–48 h after the transfection, the cells were fixed. CAST and RIM1 were visualized by direct fluorescence of EGFP and by staining using the anti-Myc Ab and the Cy3-conjugated secondary Ab, respectively. (a) Cells expressing EGFP–CAST-1 alone, Myc–RIM1 alone, or EGFP alone. (b) Cells expressing both EGFP–CAST-1 and Myc–RIM1. (c) Cells expressing both EGFP–CAST-1 and Myc–PSD-95/SAP90. Bars, 8 μm. (B) Recruitment of RIM1 to the Triton X-100–insoluble fraction. The cells expressing EGFP–CAST-1 alone, Myc–RIM1 alone, or both were lysed and centrifuged to collect the Triton X-100–soluble and –insoluble fractions. A comparable amount of each fraction was analyzed by Western blotting using the anti–CAST-1 and anti-Myc Abs. (a) EGFP–CAST-1 alone. (b) Myc–RIM1 alone. (c) Both EGFP–CAST-1 and Myc–RIM1. S, soluble fraction; I, insoluble fraction. These results are representative of three independent experiments.

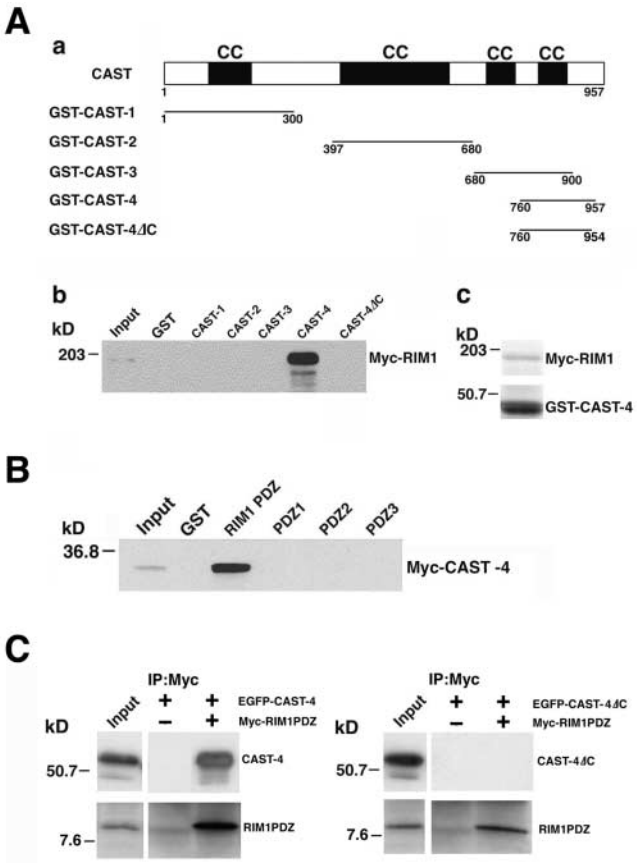

Figure 8. **Direct binding of CAST and RIM1.** (A) The RIM1-binding site of CAST. (a) GST constructs of CAST. CC, coiled-coil domain. (b) Direct binding of RIM1 to the COOH terminus of CAST. The GST fusion proteins containing various regions of CAST in panel a as well as GST alone were immobilized to glutathione-Sepharose beads. The extract of HEK293 cells expressing Myc–RIM1 was then incubated with the beads. Proteins that bound to the beads were analyzed by Western blotting using the anti-Myc Ab. The GST fusion proteins were loaded equally, which was assessed by protein staining with Coomassie brilliant blue. (c) Stoichiometric binding of RIM1 and CAST. The extract of HEK293 cells expressing Myc–RIM1 was incubated with glutathione-Sepharose beads containing GST–CAST-4. Proteins that bound to the beads were subjected to 10% SDS-PAGE, followed by protein staining with Coomassie brilliant blue. (B) The CAST-binding site of RIM1. The GST fusion proteins containing the PDZ domain of RIM1 and three PDZ domains (PDZ1, -2, and -3) of PSD-95/SAP90 as well as GST alone were immobilized to glutathione-Sepharose beads. The extract of HEK293 cells expressing Myc–CAST-4 was then incubated with the beads. Proteins that bound to the beads were analyzed by Western blotting using the anti-Myc Ab. The GST fusion proteins were loaded equally, which was assessed by protein staining with Coomassie brilliant blue. (C) Requirement of the last three aa (IWA) of CAST for its binding to the PDZ domain of RIM1. Each expression plasmid of EGFP–CAST-4, EGFP–CAST-4ΔC, or Myc–RIM1 PDZ was transfected into HEK293 cells. Each protein was extracted and then mixed in the indicated combinations, followed by immunoprecipitation using the anti-Myc Ab. The immunoprecipitates were then analyzed by Western blotting using the anti-GFP and anti-Myc Abs. These results are representative of three independent experiments.

GST fusion proteins containing each of the three PDZ domains of PSD-95/SAP90 (Fig. 8 B). Finally, we confirmed the specific binding of the COOH-terminal consensus motif of CAST and the PDZ domain of RIM1 by the immunoprecipitation assay (Fig. 8 C). These results indicate that the binding of CAST and RIM1 is mediated through the COOH-terminal consensus motif and the PDZ domain.

Mechanism of the localization of CAST and RIM1 in neurons

We then examined the mechanism of the localization of CAST and RIM1 in primary cultured rat hippocampal neurons by expressing their various mutants. The various CAST

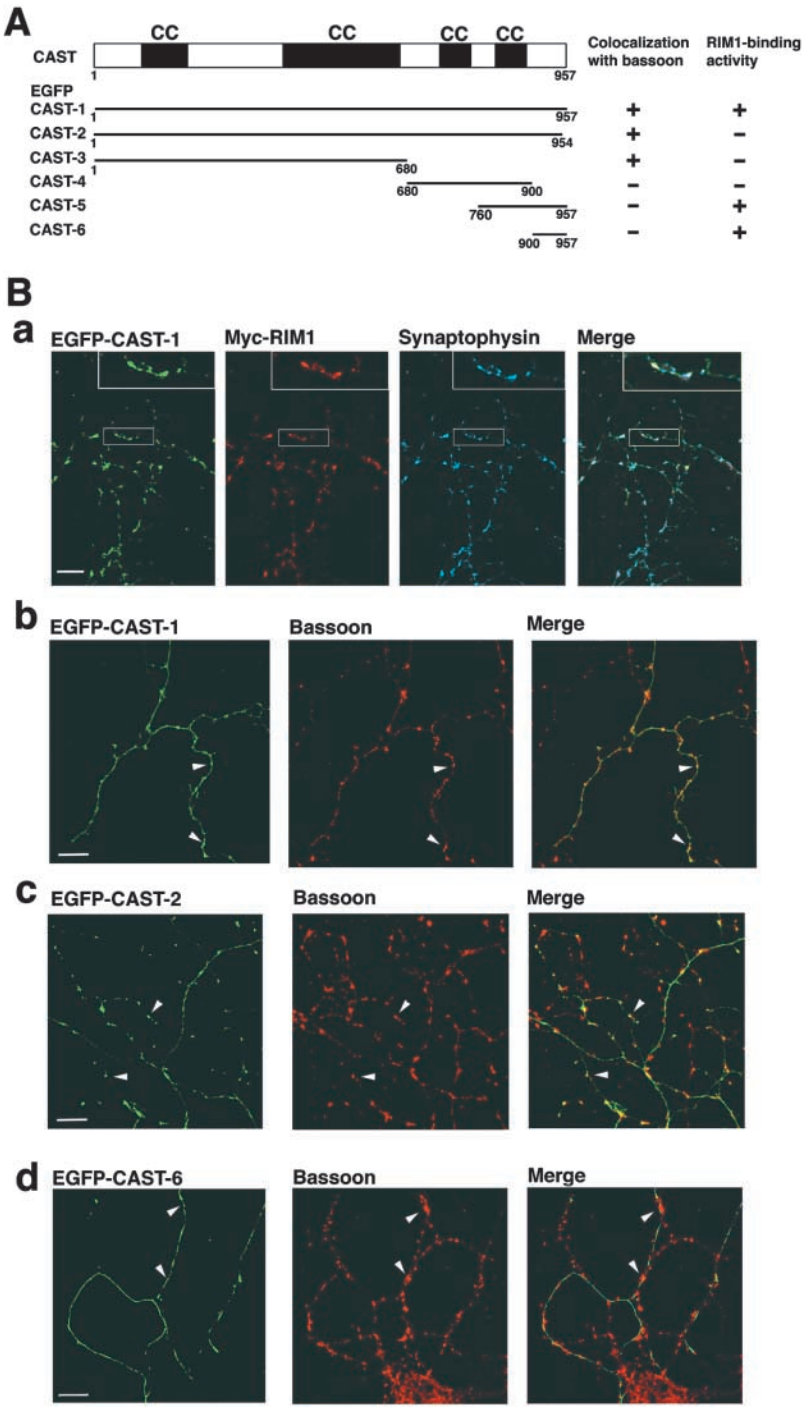

Figure 9. **RIM1-independent colocalization of CAST with bassoon in primary cultured rat hippocampal neurons.** (A) pEGFP constructs of CAST. The activities of CAST for colocalization with bassoon and for binding to RIM1 are summarized. The lysate of HEK293 cells expressing each CAST mutant was incubated with glutathione-Sepharose beads containing GST–RIM1 PDZ. After the beads were extensively washed, the bound proteins were analyzed by Western blotting. CC, coiled-coil domain. (B) Localization of the CAST mutants. Neurons at 7 d of culture were transfected with the indicated expression vectors, and stained with the anti-Myc, antibassoon, and anti-synaptophysin Abs at 9–11 d of culture. The EGFP signal was detected by FITC channel. Arrows indicate the sites where the signals of bassoon are clustered. Bars, 10 μm. These results are representative of three independent experiments.

mutants are schematically shown in Fig. 9 A, and their activities for their colocalization with bassoon (see below) and binding to RIM1 (unpublished data) are also summarized in the figure. The RIM1-binding activity of the CAST mutants was estimated by the pull-down assay (see Materials and methods). We first expressed both EGFP–CAST-1 and Myc–RIM1 in cultured neurons. Both proteins were colocalized at the synaptic boutons as estimated by the localization of synaptophysin, a well-known synaptic protein (Wiedenmann and Franke, 1985; Fig. 9 B, a). Bassoon was also colocalized there (unpublished data), consistent with the earlier observation that it is synaptically localized in cultured neurons (tom Dieck et al., 1998). When EGFP–CAST-1 alone was expressed, it colocalized with bassoon (Fig. 9 B, b). EGFP–CAST-2, which lacks only the COOH-terminal three aa (IWA) and does not bind RIM1, colocalized with bassoon (Fig. 9 B, c). In contrast, EGFP–CAST-6, which has the COOH-terminal three aa (IWA) and binds RIM1 but lacks the most NH_2-terminal region, was diffusely distributed and not clustered, as compared with bassoon (Fig. 9 B, d). EGFP–CAST-3, which lacks the third and fourth coiled-coil domains and the COOH-terminal three aa (IWA), colocalized with bassoon, whereas EGFP–CAST-4, which lacks the first and second coiled-coil domains and the COOH-terminal three aa (IWA) but contains the third and fourth coiled-coil domains, did not colocalize with bassoon

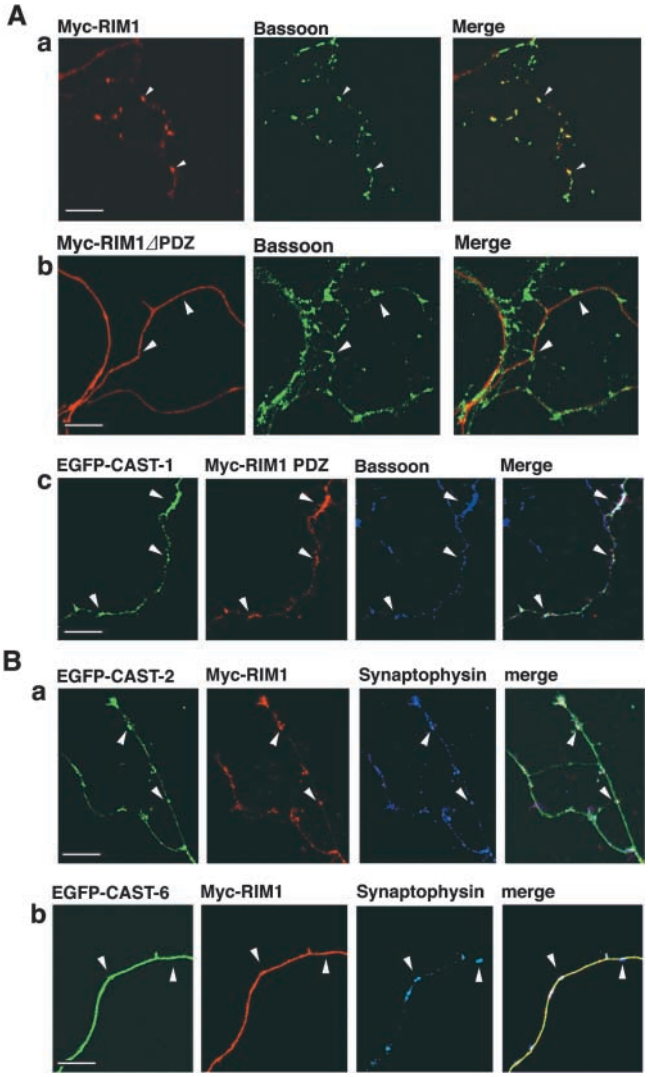

Figure 10. **Mechanism of localization of RIM1 in primary cultured rat hippocampal neurons.** (A) Colocalization of RIM1 with bassoon in cultured neurons. Neurons at 7 d of culture were transfected with the indicated expression vectors, and stained with the anti-Myc and antibassoon Abs at 9–11 d of culture. The EGFP signal was detected by FITC channel. Arrowheads indicate the sites where the signals of bassoon are clustered. Bars, 10 μm. (B) Effect of overexpression of CAST on the localization of RIM1 in cultured neurons. Neurons at 7 d of culture were cotransfected with the expression vectors of Myc–RIM1 and EGFP–CAST-2 or EGFP–CAST-6, and stained with the anti-Myc and anti-synaptophysin Abs at 9–11 d of culture. The EGFP signal was detected by FITC channel. Arrowheads indicate the sites where the signals of synaptophysin are clustered. Bars, 10 μm. These results are representative of three independent experiments.

(unpublished data). EGFP–CAST-5, which contains only the fourth coiled-coil domain and the COOH-terminal three aa (IWA), did not colocalize with bassoon either (unpublished data). These results indicate that CAST is synaptically localized in cultured neurons through its NH_2-terminal half containing at least the first and second coiled-coil domains in a manner independent of RIM1.

As for the mechanism of the localization of RIM1 in neurons, Myc–RIM1 was colocalized with bassoon (Fig. 10 A, a) and CAST (unpublished data), when it was expressed in cultured neurons. In contrast, a Myc-tagged deletion mutant (Myc–RIM1ΔPDZ), which lacks the PDZ domain and does not bind to CAST, was diffusely distributed and not clustered, as compared with bassoon (Fig. 10 A, b). When the Myc-tagged PDZ domains of RIM1 (Myc–RIM1 PDZ) and EGFP–CAST-1 were coexpressed, they were colocalized with bassoon (Fig. 10 A, c). An essentially similar result was obtained when Myc–RIM1 PDZ alone was expressed (unpublished data). Thus, the PDZ domain of RIM1 appears to play a role, at least partly, in the localization of RIM1 in cultured neurons.

We finally examined the role of CAST in the localization of RIM1. When EGFP–CAST-2 and Myc–RIM1 were coexpressed in cultured neurons, both the proteins were colocalized with synaptophysin (Fig. 10 B, a), consistent with the results in Fig. 9 B, suggesting that Myc–RIM1 binds endogenous CAST and/or other presynaptic proteins. In contrast, when EGFP–CAST-6 and Myc–RIM1 were coexpressed in cultured neurons, both the proteins were diffusely localized and not clustered, as compared with synaptophysin (Fig. 10 B, b). However, Myc–RIM1 was often colocalized with synaptophysin even when EGFP–CAST-6 was diffusely localized (unpublished data). Taken together, it is likely that CAST plays a role, at least partly, in the localization of RIM1 in cultured neurons, but that another presynaptic protein(s) is additionally involved in this localization of RIM1.

Temporal and spatial localization of CAST during synapse formation

In the last set of experiments, we examined the temporal and spatial localization of CAST during synapse formation, using young primary cultured rat hippocampal neurons as well as rat brain tissue. Western blot analysis using rat brain homogenates of various developmental stages showed that the expression of CAST, as well as of RIM1, bassoon, and Munc13-1, was detected from early stages, and the levels of expression of these CAZ proteins did not significantly change during the developmental stages tested, although those of synaptophysin and PSD-95/SAP90 were sharply increased (Fig. 11 A).

Immunofluorescence microscopic analysis of CAST in cultured neurons revealed that after 2 d of culture, when minor processes appeared, the immunoreactivity of CAST was detected as dotty signals in the cell body and fine processes, which was similar to that of bassoon (unpublished data). After 3–4 d of culture, when the axonal outgrowth was observed, the immunoreactivity of CAST was detected as dotty signals in the axon shaft and the growth cone (Fig. 11 B), which were partly colocalized with the signals of bassoon.

Because our anti-RIM1 Ab could not be used for immunofluorescence microscopic analysis of RIM1, we examined the localization of exogenously expressed RIM1 at the early stages of synapse formation. When EGFP–CAST-1 alone was first expressed, it was colocalized with bassoon (Fig. 11 C, a), consistent with the results in Fig. 11 B. Moreover, EGFP–CAST-2, which lacks only the COOH-terminal three aa (IWA) and does not bind RIM1, was also colocalized with bassoon (unpublished data). When Myc–RIM1 alone was expressed, it was indeed colocalized with bassoon and CAST (Fig. 11 C, b and c). In contrast, Myc–RIM1ΔPDZ was diffusely localized and not clustered, as

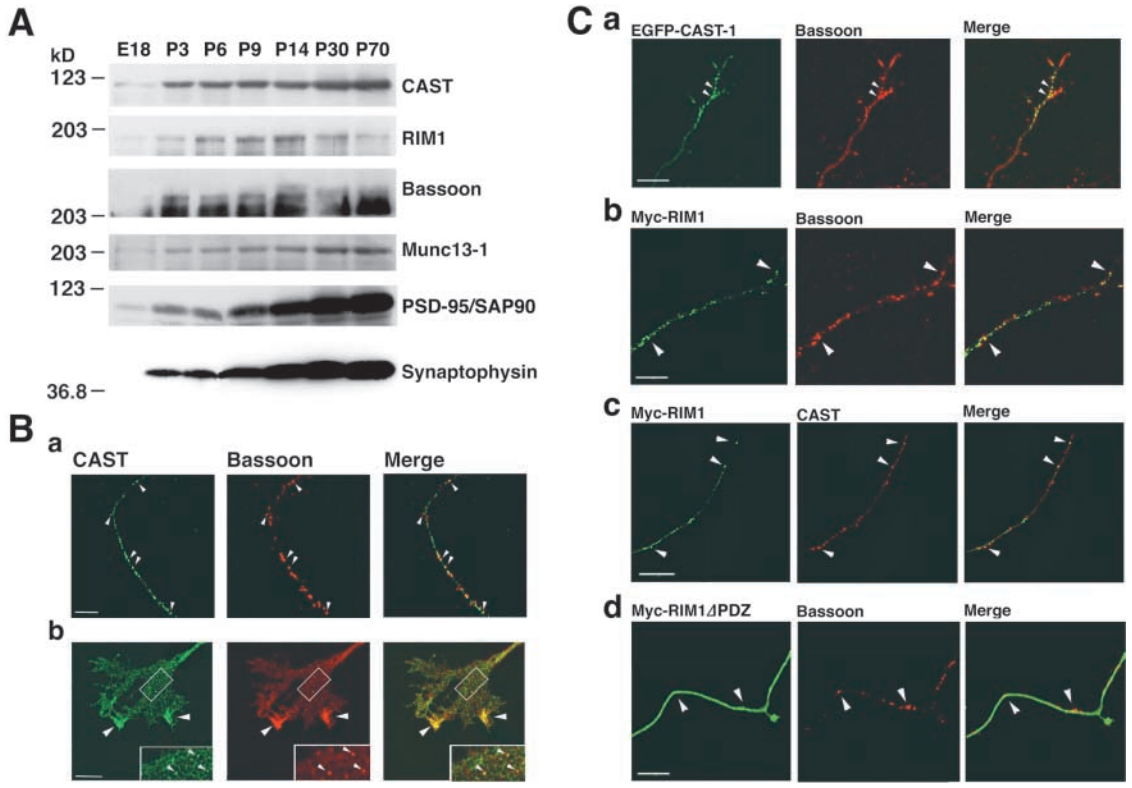

Figure 11. **Expression and localization of CAST at the early stages of synapse formation.** (A) Temporal expression of the CAZ proteins during synapse formation. The homogenates (20 μg of protein each) from rat brain tissues at embryonic day (E) 18, through postnatal day (P) 70 were analyzed by Western blotting using various Abs against the indicated proteins. (B) Localization of CAST at the early stage. Neurons at 3 d of culture were stained with the anti-CAST and antibassoon Abs. (a) The axon shaft. (b) The growth cone. Arrowheads indicate the colocalized signals. Bars, 10 μm. (C) Localization of exogenously expressed CAST and RIM1 at the early stage. Neurons at 1 d of culture were transfected with the indicated expression vectors and stained with the anti-Myc, antibassoon, and anti-CAST Abs at 3 d of culture. The EGFP signal was detected by FITC channel. Arrowheads indicate the sites where the signals of endogenous bassoon or CAST are clustered. Bars, 10 μm. These results are representative of three independent experiments.

compared with bassoon (Fig. 11 C, d), suggesting that the PDZ domain of RIM1 is at least partly required for its clustering with bassoon at the early stages.

The expression of CAST at the early stages of synapse formation and its overlapping dotty signals with those of bassoon allowed us to speculate that CAST could be associated with vesicular membranes. To clarify the nature of the CAST signals, we first performed a sucrose gradient centrifugation assay using E18 rat brain. E18 rat brain homogenate was hypotonically lysed and subjected to ultracentrifugation at 100,000 *g* to obtain the supernatant (S100) and pellet (P100) fractions. Like bassoon (Zhai et al., 2001), CAST, RIM1, and Munc13-1 were mainly detected in the P100 fraction (Fig. 12 A). The P100 fraction was then layered on a discontinuous sucrose gradient of 0.3, 0.8, and 1.2 M. After the centrifugation, fractions were collected and analyzed by Western blotting. CAST, bassoon, RIM1, and Munc13-1, as well as synaptophysin, were found in 0.3 and 0.8 M layers (Fig. 12 A), containing light membranes (Zhai et al., 2001). The essentially similar results were obtained by continuous sucrose gradient (0.3–1.2 M) ultracentrifugation (Fig. 12 B). CAST, bassoon, RIM1, and Munc13-1 were detected in the fractions similar to those of synaptophysin. Importantly, when the P100 fraction was treated with Triton X-100 before the centrifugation, CAST, as well as the other CAZ proteins, was recovered near the bottom fraction, whereas synaptophysin was recovered near the top fraction (Fig. 12 B). These results suggest that the similar behavior of CAST, RIM1, and Munc13-1 to that of bassoon and synaptophysin on sucrose gradient centrifugation is dependent on membrane integrity and that not only bassoon, but also CAST, RIM1, and Munc13-1, is associated with the light membranes.

It has recently been reported that bassoon and piccolo are associated with precursor vesicles for the active zone, which resemble classic dense core vesicles with a diameter of ~80 nm (Zhai et al., 2001). To clarify that CAST is also associated with the same vesicles, we immunoisolated the vesicles from the light membrane fraction by the antibassoon Ab (Zhai et al., 2001). Beads coated with irrelevant IgG, the antibassoon Ab, or the anti–CAST-2 Ab were incubated with the light membrane fraction and the bound proteins were analyzed by Western blotting using indicated Abs. CAST, but not synaptophysin, was coimmunoisolated with bassoon by the antibassoon Ab–coupled beads (Fig. 12 C). In addition, RIM1, but not Munc13-1, was coimmunoisolated. Consistently, bassoon and RIM1, but not Munc13-1, were coimmunoisolated with CAST by the anti–CAST-2 Ab–coupled beads. Together with the earlier observation that bassoon is a good marker for the precursor vesicles for the active zone (Zhai et al., 2001), our biochemical and cell biological results suggest that at least some portions of CAST and RIM1 might also be associated with the same vesicles as

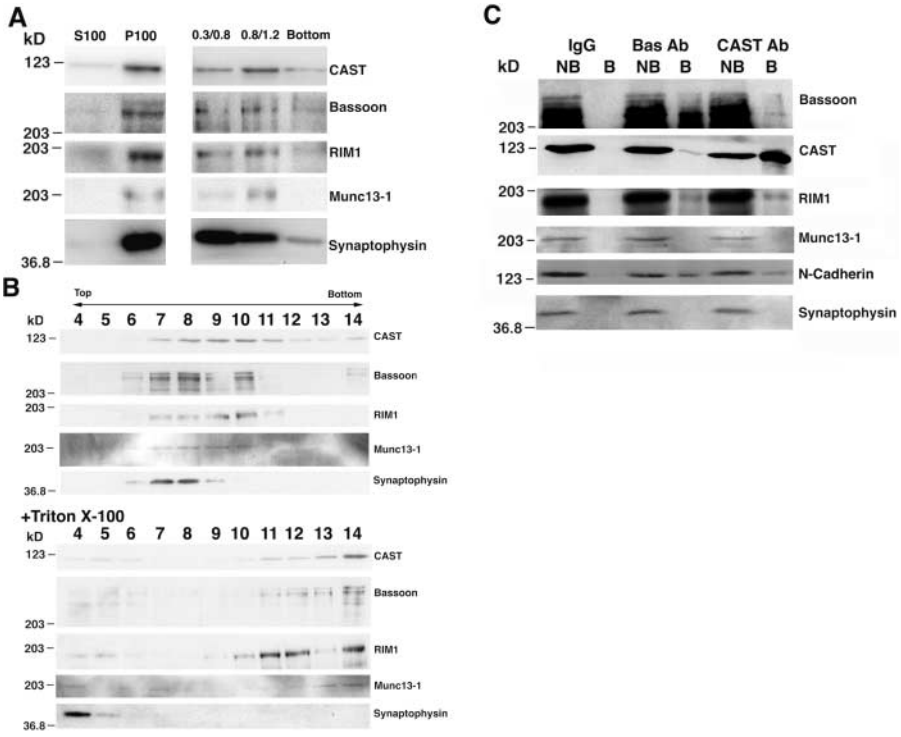

Figure 12. **Biochemical analysis of association of the CAZ proteins with the same vesicles.** (A) Presence of the CAZ proteins in the light membrane fraction. (Left) P100 and S100 fractions from hypotonically lysed E18 rat brain homogenates were analyzed by Western blotting using various Abs against the indicated proteins. (Right) The P100 fraction was layered on a discontinuous sucrose gradient of 0.3, 0.8, and 1.2 M. After the centrifugation, fractions between 0.3 and 0.8 M and between 0.8 and 1.2 M, and the bottom fraction were collected and analyzed by Western blotting using various Abs against the indicated proteins. (B) Association of the CAZ proteins with membrane structures. The P100 fraction was layered on a continuous sucrose gradient of 0.3–1.2 M. After the centrifugation, fractions (360 μl of each fraction) were taken from the top of the gradient to the bottom, and then analyzed by Western blotting using various Abs against the indicated proteins. Fraction 14 is the bottom fraction. Membrane disruption was performed using 2% (wt/vol) Triton X-100 before the centrifugation. (C) Presence of the CAZ proteins on the bassoon-associated vesicles. The light membrane fraction was subjected to immunoisolation by beads coated with control IgG, the antibassoon Ab, or the anti-CAST Ab. The bound proteins were analyzed by Western blotting using various Abs against the indicated proteins. IgG, control IgG; Bas Ab, antibassoon Ab; CAST Ab, anti-CAST Ab; NB, nonbound fraction; B, bound fraction. These results are representative of three independent experiments.

those transporting bassoon. It remains unknown whether Munc13-1 is associated with the same vesicles but dissociates from the vesicles during the immunoisolation procedure or whether it is associated with different vesicles.

Discussion

CAST as a CAZ protein

We propose here that CAST is a novel CAZ protein according to the following several lines of evidence. (1) CAST is concentrated in the PSD fraction. Originally, the PSD fraction has been thought to contain mainly postsynaptic proteins (Kennedy 1997). However, it has been shown that presynaptic proteins are also included in this fraction (Langnaese et al., 1996). Consistently, bassoon (tom Dieck et al., 1998), piccolo/aczonin (Cases-Langhoff et al., 1996; Wang et al., 1999), and RIM1 (Wang et al., 1997) are concentrated in the PSD fraction. (2) CAST is tightly associated with the detergent-insoluble structure. This property is similar to those of the other CAZ proteins. (3) CAST is electron microscopically localized at the CAZ of conventional synapses. (4) CAST forms a ternary complex with at least RIM1 and Munc13-1, and bassoon is associated with this complex. Taken together, these findings indicate that CAST is a new component of the CAZ.

A possible function of CAST

We postulate that CAST plays a role at least partly in the localization of RIM1 in neurons according to the following several lines of evidence. (1) In HEK293 cells, CAST forms aggregates and recruits RIM1 to the Triton X-100–insoluble structure. (2) In primary cultured rat hippocampal neurons, exogenously expressed CAST is synaptically colocalized with synaptophysin and bassoon in a manner independent of RIM1. (3) Overexpression of the RIM1-binding domain of CAST inhibits, not completely but partly, the localization of RIM1 in cultured neurons. It is currently unknown why the overexpression of the RIM1-binding domain of CAST does not completely inhibit the localization of RIM1 in cultured neurons, but many proteins, including Rab3A (Wang et al., 1997), cAMP-GEFII (Ozaki et al., 2000), RIM-BPs (Wang et al., 2000), Munc13-1 (Betz et al., 2001), synaptotagmin (Coppola et al., 2001), Ca^{2+} channel (Coppola et al., 2001), and α-liprin (Schoch et al., 2002), have been shown to bind the zinc fingers, C2 domains, and other regions of RIM1. Therefore, some of these CAZ or presynaptic proteins in addition to CAST are also involved in the localization of RIM1. Although it remains unknown which CAZ proteins other than CAST are involved in the localization of RIM1 at the CAZ, α-liprin (Serra-Pages et al., 1998), which binds the C2B domain of RIM1 in vitro (Schoch et al., 2002), may be of particular interest because its ortholog in *C. elegans*, SYD-2, has been shown to be essential for normal active zone function (Zhen and Jin, 1999). In SYD-2 mutant animals, the active zone is significantly lengthened. Currently, however, it is unclear whether α-liprin is a CAZ protein. If α-liprin might also be a component of the CAZ proteins involved in the localization of RIM1 through its binding to the C2B domain, Myc–RIM1ΔPDZ would be colocalized with bassoon. However, Myc–RIM1ΔPDZ is diffusely distributed in cultured neurons. This may just be due to the limited amount of endogenous α-liprin. All in all,

molecular determinants involved in scaffolding of the CAZ proteins at the CAZ may be more complex than envisaged.

Recently, an ortholog of RIM (UNC-10) in *C. elegans* has been identified and characterized (Koushika et al., 2001). RIM regulates vesicle priming, but the organization of the active zone is intact in RIM mutant animals. Consistently, the recent studies from RIM1 knockout mice demonstrate that RIM1 plays important roles in synaptic plasticity through regulating vesicle priming, but that the structural alteration of the active zone is not observed in RIM1 knockout mice (Castillo et al., 2002; Schoch et al., 2002). Thus, RIM1 appears not to be essential for the formation and/or maintenance of the CAZ structure. It may be noted that bassoon or piccolo/aczonin is not evolutionarily conserved in *C. elegans* (Garner et al., 2000), suggesting that these proteins are not essential for the formation and/or maintenance of the CAZ structure. In contrast, with a database search, we have identified a putative ortholog of CAST in *C. elegans* (F42A6.9; GenBank/EMBL/DDBJ accession no. AF038613). This hypothetical protein shows ~20% aa identity to CAST (unpublished data). Moreover, it is intriguing that the COOH-terminal three aa (IWA) of CAST are conserved in the hypothetical protein. CAST may be essential for the formation and/or maintenance of the CAZ structure.

We have demonstrated here that CAST forms a ternary complex with at least RIM1 and Munc13-1. We have moreover shown here that bassoon is associated with this complex. At present, we do not know whether bassoon directly binds CAST, RIM1, and/or Munc13-1. Because bassoon is a very large protein with several protein–protein interaction domains, it may have a potency to interact with many presynaptic proteins. The physiological significance of the ternary complex of CAST, RIM1, and Munc13-1 or the association of bassoon with the ternary complex is currently unclear, but, to our knowledge, we have provided here, for the first time, the evidence that a network of protein–protein interactions among the CAZ proteins exists in vivo. Genetic ablation of the CAST gene in mice as well as *C. elegans* might provide us with some clues for our understanding of the molecular mechanism underlying the assembly of the CAZ.

Implication of CAST in synapse formation

It has been suggested that the active zone might be formed by the incorporation of preassembled, macromolecular complexes into the presynaptic membrane (Vaughn, 1989; Roos and Kelly, 2000). Consistently, GFP-tagged VAMP2/synaptobrevin II has been shown to cluster at the newly forming active zone together with other presynaptic proteins, such as synapsin I, SV2, and Ca^{2+} channel (Ahmari et al., 2000). These clusters are thought to be cytoplasmic transport packets for presynaptic proteins. It is currently unknown whether the CAZ proteins are contained in the clusters. Recently, bassoon- and piccolo-associated dense core vesicles have been discovered and characterized (Zhai et al., 2001). Because bassoon and piccolo are almost always found at nascent synapses, the dense core vesicles are thought to be precursor vesicles for the active zone (Zhai et al., 2001), although the presence of other CAZ proteins on the vesicles has not been studied. We have shown here that CAST, as well as bassoon, is expressed at the early stages of synapse formation in primary cultured rat hippocampal neurons. Our present study further suggests that not only CAST, but also RIM1, is at least partly associated with the same vesicles as those transporting bassoon. Taken together with the earlier observation (Zhai et al., 2001), it is likely that the CAZ proteins are expressed and associated with the vesicles at the early stages of synapse formation and then correctly transported into newly forming synapses, followed by fusion with the plasma membrane, which might result in the formation of the new active zone.

Materials and methods

Purification of synaptic proteins

Subcellular fractionation of rat brain was performed as described by Cohen et al. (1977). Proteins were extracted from the P2 or PSD fraction (1.4 mg of protein each) with 900 μl of an extraction buffer (20 mM Tris-Cl, pH 8.0, 7 M urea, 2 M thiourea, 0.9% CHAPS, 1 mM DTT, 10 μM α-amidinophenyl-methanesulfonyl fluoride hydrochloride [APMSF], and 10 μg/ml leupeptin) at RT for 30 min. 450 μl of a dilution buffer (20 mM Tris-Cl, pH 8.0, and 1 mM DTT) was added to the extract. The filtrated extract (~1 ml) for the P2 or PSD fraction was then applied on a Mono Q column (1.6 mm × 50 mm; Amersham Biosciences) equilibrated with 2 ml of buffer A (20 mM Tris-Cl, pH 8.0, 0.6% CHAPS, 1 mM DTT, and 10 μM APMSF). After the column was washed with 2 ml of buffer A, elution was performed with a 2.4-ml linear gradient of NaCl (0–0.5 M) in buffer A, and fractions of 100 μl each were collected. All the fractions were subjected to SDS-PAGE, followed by protein staining with Coomassie brilliant blue. The bands more concentrated in the PSD fraction than those in the P2 fraction were cut out from the gels and stored at −20°C until use.

Determination of partial aa sequence of CAST and molecular cloning of its cDNA

The Mono Q fraction containing CAST (600 μl) was subjected to 7.5% SDS-PAGE. A protein band corresponding to CAST was cut out from the gel, digested with a lysyl endopeptidase, and analyzed as previously described (Imazumi et al., 1994). Aa sequences of the four peptides were determined. Computer homology search revealed that all four peptides were contained within the primary sequence deduced from a human cDNA fragment (KIAA0378). The cDNA fragment was obtained from Kazusa DNA Research Institute for a probe. To obtain full-length CAST, a rat hippocampus cDNA library in λZAPII (Stratagene) was screened with the probe. The full length in pBluescript-SK (pBS; Stratagene) was used as the template for PCR to prepare various constructs.

Abs

A rabbit antiserum against CAST was raised against GST–KIAA0378-1, aa 30–182 (anti–CAST-1), or GST–KIAA0378-2, aa 183–308 (anti–CAST-2). The antiserum was affinity purified as previously described (Takeuchi et al., 1997). The polyclonal anti–NMDA receptor 2B (Chemicon), monoclonal synaptophysin (Chemicon), monoclonal anti-Myc (9E10) (Roche), monoclonal anti–Munc13-1 (Synaptic Systems), monoclonal antibassoon (StressGen Biotechnologies), monoclonal anti–synaptotagmin I (Wako Pure Chemical Industries), monoclonal anti-RIM1 (Transduction Laboratories), and monoclonal anti–PSD-95/SAP90 (Transduction Laboratories) Abs were purchased from commercial sources.

Solubilization of CAST from the P2 fraction of rat brain

Proteins were extracted from the P2 fraction (500 μg of protein) of rat brain with 500 μl of an extraction buffer (20 mM Tris-Cl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10 μg/ml of leupeptin, and 10 μM APMSF), containing an indicated detergent at a concentration of 1% (wt/vol), at RT for 30 min. The extract was centrifuged at 100,000 *g* at RT for 30 min to obtain the soluble and pellet fractions.

Neuron culture and transfection

Primary cultured rat hippocampal neurons were prepared as previously described (Bito et al., 1996). Immunofluorescence microscopy was performed as previously described (Takeuchi et al., 1997). In the case of endogenous CAST, methanol was used for fixation. Neurons were transfected with the indicated expression vectors as previously described (Boudin et al., 2000).

Expression vectors

Expression vectors were constructed in pGEX (Amersham Biosciences), pCIneo-Myc (Hirao et al., 1998), pCMV-HA (Irie et al., 1997), or pEGFPC1 (CLONTECH Laboratories, Inc.) using standard molecular biological methods. The expression vector containing the human RIM1 cDNA (pCMV-Tag3-RIM1, full length, aa 1488) was kindly supplied from Dr. S. Seino (Chiba University, Chiba, Japan). pCIneo-Myc–RIM1 and –RIM1 PDZ (aa 568–695) and pCMV-HA–RIM1 were constructed from the RIM1 cDNA and used for transfection. pCIneo-Myc–RIM1ΔPDZ was constructed by connecting the NH_2-terminal (aa 1–576) and COOH-terminal (aa 696–1488) regions of RIM1 at an EcoRI site. The PDZ domain of RIM1 was also subcloned in pGEX and pCMV-Myc (Irie et al., 1997) for pull-down and immunoprecipitation assays, respectively. pGEX-PDZ1, -2, and -3 for PSD-95/SAP90 and pCMV-Myc–PSD-95/SAP90 (full length) were obtained as previously described (Irie et al., 1997). pEFBos-Myc–Munc13-1 was obtained as previously described (Orita et al., 1997). The GST fusion proteins were purified according to the manufacturer's protocol (Amersham Biosciences).

Immunoprecipitation

Proteins were extracted from the P2 fraction (4 mg of protein) with 1% deoxycholate, followed by dilution and dialysis with a Triton X-100–based buffer as previously described (Luo et al., 1997). The extract was then incubated with 2.5 μg of the control IgG, the anti–CAST-1, anti-Myc, or anti-bassoon Ab at 4°C for 2 h. After the beads were extensively washed with buffer B (20 mM Tris-Cl, pH 7.5, 100 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 1% [wt/vol] Triton X-100, 10 μg/ml leupeptin, and 10 μM APMSF), the bound proteins were eluted by boiling the beads in an SDS sample buffer (60 mM Tris-Cl, pH 6.7, 3% SDS, 2% [vol/vol] 2-mercaptoethanol, and 5% glycerol) for 5 min. The samples were then analyzed by Western blotting. For analysis of ternary complex formation of CAST, RIM1, and Munc13-1, immunoprecipitation was performed as follows. Each expression plasmid of EGFP–CAST-1, HA–RIM1, or Myc–Munc13-1 was transfected into HEK293 cells. Each protein was extracted with buffer B and then mixed in various combinations. After incubation at 4°C overnight, immunoprecipitation was performed as described using 2.0 μg of the anti-GFP or anti-HA Ab. The samples were then analyzed by Western blotting. For analysis of the interaction of the PDZ domain of RIM1 and the COOH terminus of CAST, immunoprecipitation was also performed as described above using the indicated plasmids.

Assay for cosedimentation of CAST and RIM1

HEK293 cells expressing Myc–CAST and Myc–RIM1 were lysed in 600 μl of buffer C (20 mM Tris-Cl, pH 7.5, 75 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 0.4% [wt/vol] Triton X-100, 0.1% SDS, 10 μg/ml leupeptin, 10 μM APMSF) and buffer B at RT for 30 min, respectively. The sample was centrifuged at 100,000 *g* at 4°C for 30 min to collect the supernatant. The supernatant was incubated with 8 μg of the anti-Myc Ab coupled with protein A–Sepharose beads to prepare the Myc–CAST- or Myc–RIM1-coupled affinity beads. As for the control beads, native HEK293 cells were used under the same conditions. Proteins were extracted from the P2 fraction (5 mg of protein) with buffer C. The extract (~2.8 ml) was incubated with the control, Myc–CAST-coupled affinity beads, or Myc–RIM1-coupled affinity beads at 4°C overnight. After the beads were extensively washed with buffer C, the bound proteins were eluted by boiling the beads in the SDS sample buffer for 5 min. The samples were then analyzed by Western blotting.

Expression of CAST and RIM1 in HEK293 cells

HEK293 cells in 6-cm dishes were transfected with the indicated expression vectors by lipofectAMINE 2000 (Invitrogen). At 24 h after the transfection, one fifth of the cells were replated on cover glasses, followed by immunofluorescence microscopic analysis (Takeuchi et al., 1997). The remainder of cells was replated on a 10-cm dish and further incubated for 48 h. The cells were collected and proteins were extracted from the cells with 500 μl of buffer B. The sample was centrifuged at 10,000 *g* at 4°C for 30 min to collect the supernatant and pellet fractions, which were kept as the Triton X-100–soluble and –insoluble fractions, respectively.

Assay for pull-down of CAST and RIM1

HEK293 cells expressing Myc–RIM1 or Myc–CAST-4 in 10-cm dishes were lysed in 2.0 ml of buffer B at 4°C for 30 min. The sample was centrifuged at 100,000 *g* at 4°C for 30 min to collect the supernatant. The supernatant was divided into five fractions. Each extract was then incubated with 50 μl of glutathione-Sepharose beads containing the indicated GST fusion proteins (~1 μg of protein each) at 4°C overnight. After the beads were extensively washed with a wash buffer (20 mM Tris-Cl, pH 7.5, 100 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 0.02% SDS, and 0.4% [wt/vol] Triton X-100), the bound proteins were eluted by boiling the beads in the SDS sample buffer for 5 min. The samples were then analyzed by Western blotting. Pull-down assay was performed as described above to estimate the RIM1-binding activity of CAST mutants. In brief, the lysate of HEK293 cells expressing each CAST mutant was incubated with 20 μl of glutathione-Sepharose beads containing GST–RIM1 PDZ at 4°C overnight. After the beads were extensively washed with buffer B, the bound proteins were eluted by boiling the beads in the SDS sample buffer for 5 min. The samples were then analyzed by Western blotting.

Sucrose gradient ultracentrifugation and immunoisolation

Discontinuous sucrose gradient ultracentrifugation was performed as previously described with slight modifications (Zhai et al., 2001). In brief, E18 rat brain was homogenized in a homogenization buffer (5 mM Hepes, pH 7.4, 0.5 mM EDTA, 0.3 M sucrose, and a protease inhibitor cocktail [Roche]). The homogenate was centrifuged at 800 *g* for 20 min, and the crude membrane in the supernatant was hypotonically lysed by adding nine volumes of H_2O. The crude membrane was then centrifuged at 100,000 *g* for 1 h. The pellet is referred to as P100 and the supernatant is referred to as S100. The P100 fraction was then layered on a discontinuous sucrose gradient of 0.3, 0.8, and 1.2 M. After the centrifugation at 350,000 *g* for 3 h, fractions between 0.3 and 0.8 M and between 0.8 and 1.2 M and the bottom fraction were collected and analyzed by Western blotting. Continuous sucrose gradient ultracentrifugation (0.3–1.2 M sucrose) was performed as previously described (Fujita et al., 1998). Fractions (360 μl of each fraction) were taken from the top of the gradient to the bottom. Fraction 14 is the bottom fraction.

Immunoisolation of vesicles was performed as previously described (Zhai et al., 2001). In brief, tosylated superparamagnetic beads (Dynabeads M-500 Subcellular; Dynal Inc.) were incubated with a goat anti–rabbit or anti–mouse linker IgG (Jackson ImmunoResearch Laboratories) at 10 μg/mg beads in 0.2 M phosphate buffer at pH 7.4 overnight. For all subsequent steps, beads were collected with a magnetic device (MPC; Dynal Inc.). Beads were washed with PBS containing 0.1% BSA and blocked with a Tris blocking buffer (0.2 M Tris-Cl, pH 8.8, and 0.1% BSA) at 37°C for 4 h. The linker IgG-coupled beads were then incubated at 4°C overnight with control IgG, the anti–CAST-2 Ab, or the antibassoon Ab at a concentration of 10 μg/mg beads in an incubation buffer (PBS, 2 mM EDTA, and 5% FBS). The Ab-coupled beads were incubated with the light membrane fraction at 4°C overnight. The beads were then collected and washed five times with the incubation buffer and three times with PBS at 5 min each and kept as a bound fraction. Supernatants were kept as a nonbound fraction. The bound and nonbound fractions were analyzed by Western blotting.

Other procedures

Protein concentrations were determined as previously described (Takeuchi et al., 1997). MALDI-TOF mass spectrometry was performed as previously described (Jensen et al., 1996). In vitro translation was performed with the reticulocyte lysate system (Promega). The prestained markers used in Western blotting and Coomassie brilliant blue staining were myosin (203 kD), β-galactosidase (123 kD), BSA (83 kD), ovalbumin (50.7 kD), and carbonic anhydrase (36.8 kD). The standard markers used in silver staining were myosin (200 kD), β-galactosidase (116 kD), phosphorylase b (97 kD), and BSA (66 kD). Preparation and staining of a sagittal section of adult mouse brain was performed as previously described (Shigemoto et al., 1993). Immunohistochemical analysis of mouse hippocampus was performed as previously described (Kawabe et al., 1999). Electron microscopic analysis of mouse hippocampus was performed as previously described (Kinoshita et al., 1998).

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