

Physical and functional interaction of the active zone proteins, CAST, RIM1, and Bassoon, in neurotransmitter release

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We have recently isolated a novel cytomatrix at the active zone (CAZ)-associated protein, CAST, and found it directly binds another CAZ protein RIM1 and indirectly binds Munc13-1 through RIM1; RIM1 and Munc13-1 directly bind to each other and are implicated in priming of synaptic vesicles. Here, we show that all the CAZ proteins thus far known form a large molecular complex in the brain, including CAST, RIM1, Munc13-1, Bassoon, and Piccolo. RIM1 and Bassoon directly bind to the COOH terminus and central region of CAST, respectively,

forming a ternary complex. Piccolo, which is structurally related to Bassoon, also binds to the Bassoon-binding region of CAST. Moreover, the microinjected RIM1- or Bassoon-binding region of CAST impairs synaptic transmission in cultured superior cervical ganglion neurons. Furthermore, the CAST-binding domain of RIM1 or Bassoon also impairs synaptic transmission in the cultured neurons. These results indicate that CAST serves as a key component of the CAZ structure and is involved in neurotransmitter release by binding these CAZ proteins.

Introduction

Neurotransmitter release is precisely regulated at a specific site in nerve terminals, the so-called active zone (Landis et al., 1988). At an ultrastructural level, the active zone is characterized as a slightly electron-dense region beneath the presynaptic plasma membrane, and is composed of a meshwork of cytoskeleton (Landis et al., 1988; Hirokawa et al., 1989; Gotow et al., 1991; Dresbach et al., 2001). The meshwork or cytomatrix at the active zone (CAZ) extends from the presynaptic plasma membrane into the synaptic bouton where it is associated with synaptic vesicles. Thus, the CAZ appears to be involved in docking and priming of synaptic vesicles at the active zone, which are followed by fusion of the vesicles, eventually resulting in neurotransmitter release (Garner et al., 2000). The fusion of the vesicles is mediated by the formation of the SNARE complex of the synaptic vesicle protein VAMP2 and presynaptic plasma membrane protein syntaxin and SNAP-25 (Söllner et al., 1993; Jahn and Südhof, 1999).

Presently, the full molecular composition of the CAZ is unclear, but several CAZ-specific proteins including RIM1, Munc13-1, Bassoon, Piccolo/Aczonin, and CAST are thought to organize the molecular structure of the CAZ. RIM1 was originally identified as a target protein of the Rab3A small G protein (Wang et al., 1997). Rab3A is implicated in the docking of synaptic vesicles (Takai et al., 1996; Geppert et al., 1997). Munc13-1 binds both RIM1 and syntaxin and is implicated in priming of synaptic vesicles (Brose et al., 2000; Betz et al., 2001). Bassoon and Piccolo/Aczonin are very large (>400 kD) and structurally related CAZ proteins (tom Dieck et al., 1998; Wang et al., 1999; Fenster et al., 2000). Bassoon and Piccolo are almost always found at nascent synapses in cultured neurons, suggesting that they play important roles in the assembly of the active zone (Zhai et al., 2001; Shapira et al., 2003). Recent analysis of Bassoon knockout mice has revealed that Bassoon is

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Abbreviations used in this paper: BsnBD, Bassoon-binding domain; CasBD, CAST-binding domain; CAZ, cytomatrix at the active zone; EPSP, excitatory post-synaptic potential; His, hexahistidine; P2, crude membrane; PSD, post-synaptic density; RID, RIM-interacting domain; SCG, superior cervical ganglion; SM3, synaptic membrane 3.

essential not only for neurotransmitter release but also for formation of the retinal synaptic ribbon that corresponds to the CAZ of conventional synapses (Altrock et al., 2003; Dick et al., 2003).

CAST is a novel CAZ protein that forms a ternary complex with RIM1 and Munc13-1 through directly binding RIM1 (Ohtsuka et al., 2002). Moreover, RIM1 and Bassoon are both coimmunoprecipitated with CAST from the crude membrane (P2) fraction of rat brain (Ohtsuka et al., 2002). However, it is unknown how Bassoon is associated with this CAST-dependent molecular complex. The physiological significance of the complex formation of CAST with these CAZ proteins is also unknown. We demonstrate here that CAST directly binds not only RIM1 but also Bassoon and Piccolo, and that CAST is involved in neurotransmitter release by directly binding these CAZ proteins.

Results

CAST-dependent molecular complex at the presynaptic active zone

In our previous paper, we have demonstrated that the active zone proteins RIM1 and Bassoon are coimmunoprecipitated with CAST from the extract of the P2 fraction of rat brain (Ohtsuka et al., 2002). To obtain more insights into the molecular complex formed by CAZ proteins at the active zone, here we examined the association of other synaptic proteins with this CAST-dependent molecular complex *in vivo*. For this purpose, we used the synaptic membrane 3 (SM3) fraction as the starting material. This fraction, which contains relatively pure pre- and post-synaptic membranes and docked vesicles (Cohen et al., 1977), could be obtained by sucrose gradient ultracentrifugation of the synaptosomal membrane fraction. Then, we immunoprecipitated CAST by its Ab from the extract of the SM3 fraction of rat brain. Among the proteins examined, Bassoon, RIM1, and Munc13-1 were coimmunoprecipitated with CAST (Fig. 1 A, a). But, neither syntaxin, SNAP-25, VAMP2, Rab3, synaptophysin, CASK, N-cadherin, nor β -catenin was coimmunoprecipitated. Our previous report has shown that Munc13-1 does not coimmunoprecipitate with CAST when it is immunoprecipitated from the P2 fraction (Ohtsuka et al., 2002). Although the reason for this discrepancy is unknown, it is possible that because the epitope recognized by the present Ab is different from that of the previous Ab against CAST (Ohtsuka et al., 2002), the immunoprecipitation assay by the previous Ab might have affected the association of Munc13-1 with the CAST-dependent complex. Next, we immunoprecipitated Bassoon by its Ab. Then, CAST, RIM1, and Munc13-1 were coimmunoprecipitated with Bassoon (Fig. 1 A, b). But, again, none of the above synaptic proteins were coimmunoprecipitated. It should be noted that the bands seen in the panels of SNAP-25 and Rab3 may be light chains of the Abs for Myc and Bassoon used for the assay. These results suggest that synaptic proteins, at least those examined here, were not associated with the biochemically isolated CAZ protein complex. However, we cannot completely rule out the possibility that these results are due to limitations of the sensitivity of each Ab against these undetected proteins.

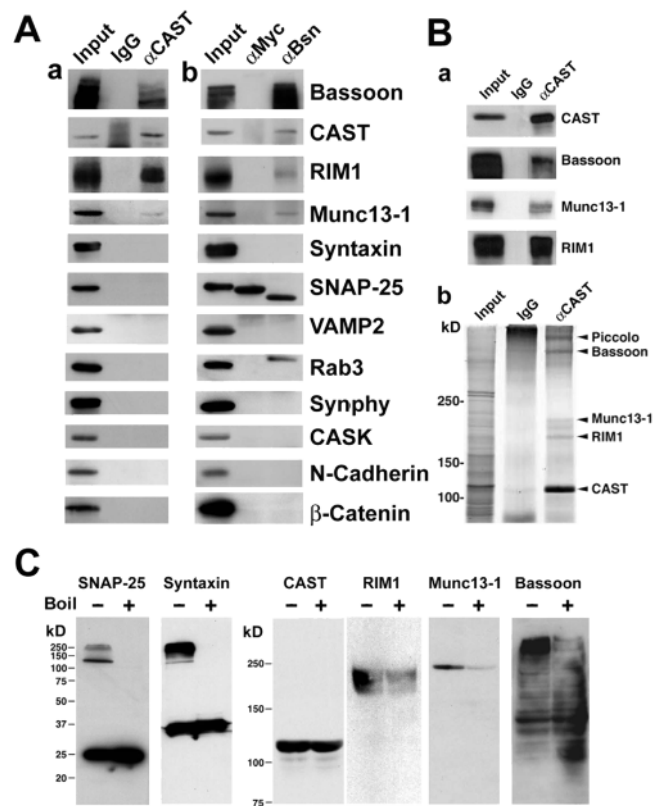


Figure 1. CAST-dependent molecular complex. (A) Coimmunoprecipitation of other synaptic proteins with the CAZ protein complex. The extract of the SM3 fraction was subjected to immunoprecipitation by the anti-CAST or anti-Bassoon Ab. Immunoprecipitates were analyzed by Western blotting using various Abs against the indicated proteins. (a) By the anti-CAST Ab. IgG, nonimmune rabbit IgG. (b) By the anti-Bassoon Ab. It should be noted that the bands seen in the panels of SNAP-25 and Rab3 may be light chains of the Abs for Myc and Bassoon used for the assay. Input contains 5% of the extract used for the assay. (B) Complex formation of the CAZ proteins. The extract of the PSD fraction was subjected to immunoprecipitation by the anti-CAST Ab, followed by Western blotting and protein staining with silver. (a) Western blotting. (b) Protein staining with silver. (C) SDS-resistant complex of the CAZ proteins. The SM3 fraction was directly solubilized in the SDS-sample buffer (see Materials and methods), boiled (+) at 100°C or not (-) at 37°C for 5 min, and immediately subjected to SDS-PAGE, followed by Western blotting using the indicated Abs. The results are representative of three independent experiments.

Of the known CAZ proteins, Piccolo/Aczonin is the largest with ~ 530 kD (Wang et al., 1999; Fenster et al., 2000). Because Piccolo and Bassoon are structurally related to each other (Wang et al., 1999; Fenster et al., 2000), we attempted to detect the presence of Piccolo in the immunoprecipitate using the anti-CAST Ab. To reduce the background, we used the post-synaptic density (PSD) fraction as the starting material, extracted it by a 1% SDS-containing buffer, and diluted the extract with a Triton X-100-based buffer, followed by immunoprecipitation. Consistent with both our present and earlier observations (Ohtsuka et al., 2002), Bassoon, RIM1, and Munc13-1 were coimmunoprecipitated with CAST from the extract of the PSD fraction (Fig. 1 B, a). As the Ab against Piccolo was not available to us, we then examined the immunoprecipitates by silver staining. Several bands

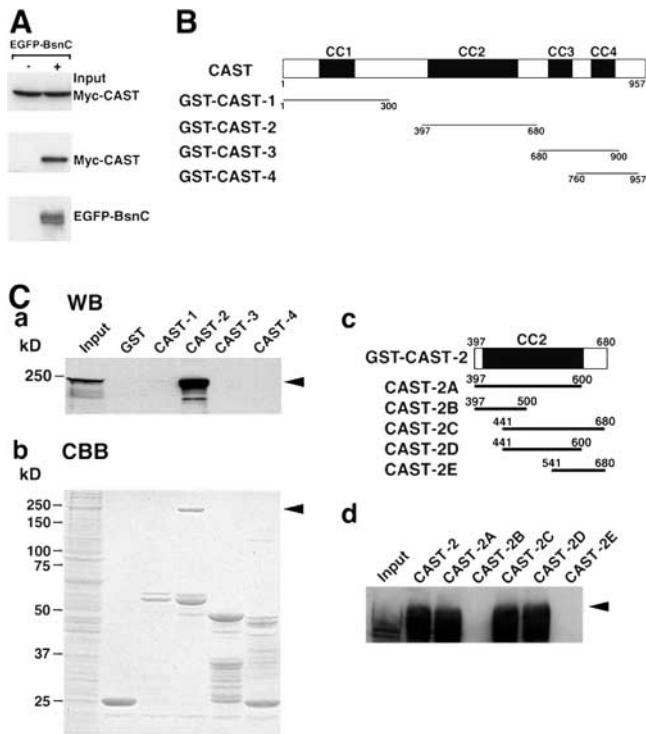


Figure 2. Bassoon-binding domain of CAST. (A) Coimmunoprecipitation of CAST with the COOH-terminal region of Bassoon. Expression plasmid of Myc-CAST or EGFP-BsnC was transfected into HEK293 cells, followed by immunoprecipitation using the anti-GFP Ab. (B) GST constructs of CAST. CC, coiled-coil domain. (C) The BsnBD of CAST. The extract of HEK293 cells expressing EGFP-BsnC was incubated with the beads containing the various GST-CAST constructs. Proteins that bound to the beads were analyzed by Western blotting using the anti-GFP Ab or by protein staining with Coomassie brilliant blue. Arrowheads indicate EGFP-BsnC. (a) Western blotting (WB). (b) Protein staining. CBB, Coomassie brilliant blue. (c) Various constructs within the central region (CAST-2). (d) The minimum BsnBD of CAST. The results are representative of three independent experiments.

were detected and, from their molecular mass, they were thought to be Bassoon, Munc13-1, and RIM1 (Fig. 1 B, b). Because the band with the highest molecular mass seemed to be Piccolo, we cut out the band and analyzed it by mass spectrometry. We obtained four partial peptides; aa 579–597, ALGGDLAAAIPSSPQPTPK; aa 2642–2656, SASIPIPEP-LALDR; aa 4576–4591, VSLQQSPLVMSSVVEK; and aa 4708–4720, SLNPEWNQTVIYK, which showed 100% identity to the internal aa sequence of rat Piccolo (unpublished data). These results suggest that Piccolo is also a component of the CAST-dependent molecular complex.

Up to now, the role of the SNARE complex in neurotransmitter release has been the most extensively studied of such complexes, and much information has been accumulated. The major components, VAMP2, SNAP-25, and syntaxin form a tight molecular complex at the site of neurotransmitter release (Söllner et al., 1993; Jahn and Südhof 1999). This molecular complex is thought to be a minimum machinery for the regulated exocytosis (Weber et al., 1998). The SNARE complex has been shown to be SDS-resistant in unboiled samples (Hayashi et al., 1994; Pellegrini et al.,

1995; Taubenblatt et al., 1999). Thus, we directly solubilized the SM3 fraction in SDS with or without boiling and then subjected the samples to SDS-PAGE, followed by Western blotting. The protein bands of SNAP-25 and syntaxin were shifted to higher molecular mass in the unboiled sample but not in the boiled sample (Fig. 1 C; Hayashi et al., 1994; Pellegrini et al., 1995; Taubenblatt et al., 1999). On the other hand, none of the CAZ proteins showed a similar shift in their mobility under the same conditions (Fig. 1 C). These results suggest that CAZ proteins form a large protein complex tightly associated with the presynaptic active zone, but one that is not SDS-resistant. It is noted that the protein levels of RIM1, Munc13-1, and Bassoon appear to be lower in the boiled samples. At present, what the significance is of this is unclear, but it may be due to the heat sensitivity of RIM1, Munc13-1, and Bassoon, which could result in the loss of the protein levels.

Direct binding of Bassoon and Piccolo to CAST

We examined whether Bassoon directly binds to CAST. For this purpose, first, we constructed the EGFP-tagged COOH- and NH₂-terminal regions of Bassoon (EGFP-BsnC, aa 2356–3926 and EGFP-BsnN, aa 1–2269) and examined whether full-length Myc-CAST binds them in HEK293 cells. Myc-CAST was coimmunoprecipitated with EGFP-BsnC (Fig. 2 A), but not EGFP-BsnN (unpublished data), indicating that CAST binds the COOH-terminal region of Bassoon. Next, we performed pull-down assays using various GST fusion proteins of CAST (Fig. 2 B). EGFP-BsnC bound to GST-CAST-2 (Fig. 2 C, a and b). The minimum Bassoon-binding domain (BsnBD) of CAST was the central region (aa 441–600; GST-CAST-2D; Fig. 2 C, c and d).

We further examined the CAST-binding domain (CasBD) of Bassoon. For this purpose, we performed pull-down assays using GST-CAST-2A (Fig. 2 Cc) and various EGFP-tagged Bassoon constructs (Fig. 3 A). EGFP-BsnC1 (aa 2505–3355) and -BsnC4 (aa 2356–3555), but not EGFP-BsnC2 (aa 2356–2855) or -BsnC3 (aa 3456–3926), bound to GST-CAST-2A (Fig. 3 B, a). A GST-Bsn construct, containing the third coiled-coil domain (aa 2933–2995; CasBD), bound to EGFP-CAST (Fig. 3 B, b). These results indicate that this direct binding of CAST and Bassoon is mediated through the regions containing the second coiled-coil domain of CAST and the third coiled-coil domain of Bassoon. Consistently, EGFP-BsnCΔCasBD did not bind GST-CAST-2D (Fig. 3 C, a), and EGFP-CAST-ΔBsnBD did not bind GST-BsnCasBD (Fig. 3 C, b). It has been thought that Bassoon is anchored to the presynaptic plasma membrane through its COOH-terminal region, and that its NH₂-terminal region extends into the synaptic bouton (Garner et al., 2000). CAST localizes at the CAZ very close to the presynaptic plasma membrane (Ohtsuka et al., 2002). Our present result that CAST directly binds the COOH-terminal region of Bassoon is consistent with these earlier observations.

A homology search revealed that the CasBD of Bassoon (aa 2933–2995) is highly homologous to the corresponding region of Piccolo (unpublished data). Thus, we examined whether CAST directly binds Piccolo. We first performed the pull-down assay using GST-CAST-2A (Fig. 2 C, c).

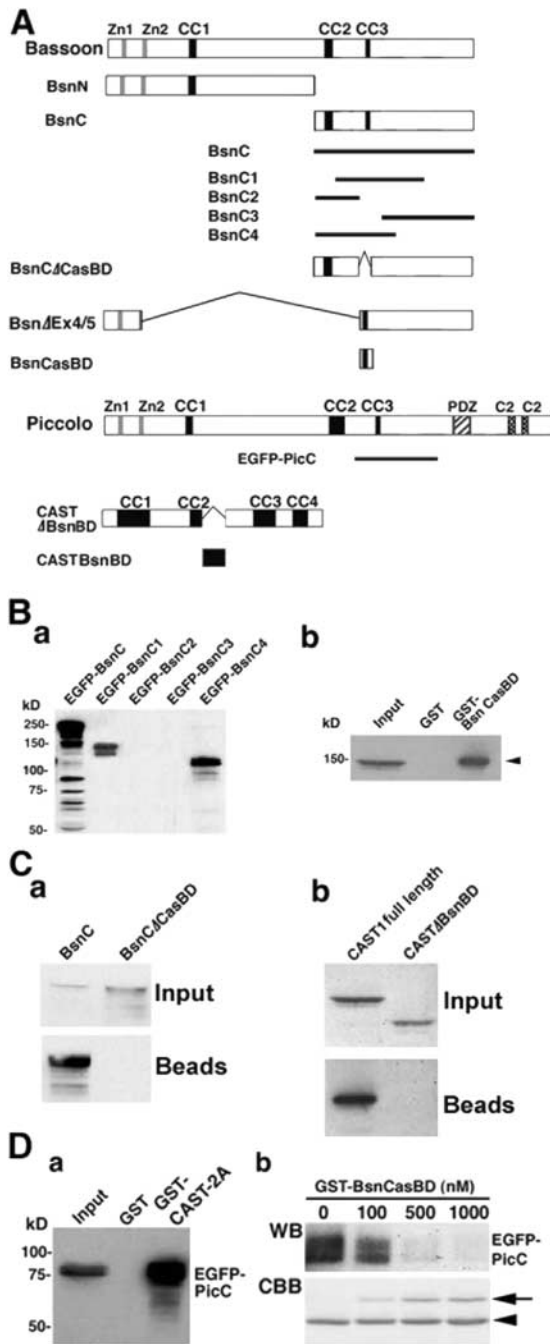


Figure 3. CAST-binding domain of Bassoon. (A) Various GFP- and GST-tagged constructs of Bassoon, Piccolo, and CAST. Zn, zinc finger; CC, coiled-coil domain. (B) The CasBD of Bassoon. The extract of HEK293 cells expressing various constructs of Bassoon and CAST were incubated with the glutathione-Sepharose beads containing GST-CAST-2A and GST-BsnCasBD, respectively. Proteins that bound to the beads were analyzed by Western blotting using anti-GFP Ab. (a) Pull-down assay using GST-CAST-2A. (b) Pull-down assay using GST-BsnCasBD. Arrowhead indicates EGFP-CAST. (C) Binding assay of Bsn Δ CasBD and CAST Δ BsnBD. Extracts of HEK293 cells expressing EGFP-Bsn Δ CasBD and EGFP-CAST Δ -BsnBD were incubated with glutathione-Sepharose beads containing GST-CAST-2D and GST-BsnCasBD, respectively. Proteins that bound to the beads were analyzed by Western blotting using anti-GFP Ab. (a) Pull-down assay using GST-CAST-2D. (b) Pull-down assay using GST-BsnCasBD. Neither the deletion constructs bound CAST or Bassoon. The results are representative of three independent

EGFP-PicC (aa 3601–3960; Fig. 3 A) bound to GST-CAST-2A, but not to GST alone (Fig. 3 D). Next, we examined the mode of the binding of Bassoon and Piccolo to CAST. For this we performed the pull-down assay using Hexahistidine (His)-tagged CAST-2A. The binding of Piccolo to CAST was inhibited with an excess amount of GST-BsnCasBD (Fig. 3 D, b), indicating that the binding of Piccolo and Bassoon to CAST was competitive. These results indicate that CAST directly binds not only Bassoon but also Piccolo both in vitro and in vivo. However, it is likely that CAST binds just one of either Bassoon or Piccolo in vivo because the same binding site of CAST is shared by these two molecules.

We further examined the binding of CAST and Bassoon in a heterologous expression system. As to Piccolo, its full-length cDNA was not available to us. Full-length Myc-CAST or EGFP-Bassoon was expressed in HEK 293 cells. Myc-CAST formed large immunoreactive structures in HEK293 cells as described previously (Fig. 4 A, a; Ohtsuka et al., 2002). Similarly, EGFP-Bassoon formed large immunoreactive structures in HEK293 cells (Fig. 4 A, b). It is intriguing that heterologously expressed CAST was largely insoluble with the treatment by Triton X-100 (Ohtsuka et al., 2002), whereas heterologously expressed Bassoon was soluble (Fig. 4 B, a and b). When both Myc-CAST and EGFP-Bassoon were expressed, CAST formed the immunoreactive structures again and Bassoon colocalized with CAST at the structures (Fig. 4 A, c). Moreover, in this case, EGFP-Bassoon and Myc-CAST were both recovered in the Triton X-100-insoluble fraction (Fig. 4 B, c). This pattern was similar to the case of heterologously expressed RIM1 in HEK293 cells (Ohtsuka et al., 2002). These results provide another line of evidence for the direct binding of CAST and Bassoon.

A ternary complex formation of CAST, RIM1, and Bassoon

Because CAST has different binding sites for RIM1 and Bassoon (Fig. 2; Ohtsuka et al., 2002), we then attempted to examine whether CAST could form a ternary complex with RIM1 and Bassoon. For this, we transfected each full-length expression plasmid of CAST, RIM1, or Bassoon into HEK293 cells, extracted each protein, and mixed them in various combinations, followed by immunoprecipitation using the anti-GFP Ab for Bassoon, the anti-Myc Ab for CAST, or the anti-HA Ab for RIM1. First, when Bassoon

experiments. Input contains 15% of the extract used for the assay. (D) Binding of Piccolo to CAST. (a) Binding of Piccolo to the central region of CAST. The extract of HEK293 cells expressing EGFP-PicC was incubated with the glutathione-Sepharose beads containing GST-CAST-2A or GST alone. Proteins that bound to the beads were analyzed by Western blotting using the anti-GFP-Ab. (b) Competitive binding of Piccolo and Bassoon to CAST. The extract of HEK293 cells expressing EGFP-PicC was incubated with the beads containing His-CAST-2A in the presence of GST-BsnCasBD at the indicated concentration. WB, Western blotting; CBB, Coomassie brilliant blue. Arrow and arrowhead indicate GST-BsnCasBD and His-CAST-2A, respectively. The binding of Piccolo to CAST was inhibited with the excess amount of GST-BsnCasBD. GST alone had no effect on the binding (unpublished data).

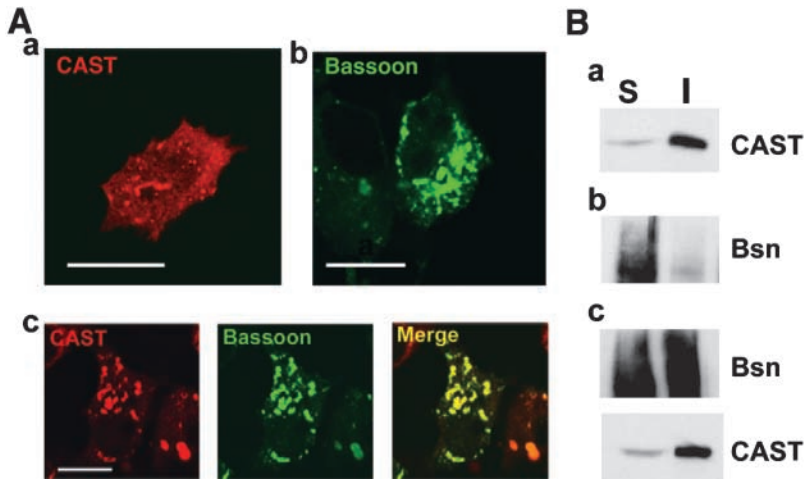


Figure 4. Colocalization of CAST and Bassoon in the heterologous expression system.

(A) Colocalization of CAST and Bassoon at the large immunoreactive structures. HEK293 cells were transfected with pCIneo-Myc-CAST and/or pEGFP-Bassoon. Cells were fixed at 48 h after transfection. CAST and Bassoon were visualized by staining using anti-Myc Ab and Cy3-conjugated secondary Ab, and by direct fluorescence of EGFP, respectively. (a) Cells expressing Myc-CAST alone. (b) Cells expressing EGFP-Bassoon alone. (c) Cells expressing both Myc-CAST and EGFP-Bassoon. Bars, 10 μ m. (B) Recruitment of Bassoon to the Triton X-100-insoluble fraction. Cells expressing Myc-CAST alone, EGFP-Bassoon alone, or both were lysed and centrifuged to collect the Triton X-100-soluble and -insoluble fractions. Comparable amounts of each fraction were analyzed by Western blotting using the anti-Myc and anti-GFP Abs. (a) Myc-CAST alone. (b) EGFP-Bassoon alone. (c) Both Myc-CAST and EGFP-Bassoon. S, soluble fraction; I, insoluble fraction. The results are representative of three independent experiments.

was immunoprecipitated, CAST was coimmunoprecipitated in the presence or absence of RIM1, whereas RIM1 was coimmunoprecipitated only in the presence of CAST (Fig. 5 A). Second, when CAST was immunoprecipitated, both RIM1 and Bassoon were coimmunoprecipitated independently of each other (Fig. 5 B). Third, when RIM1 was immunoprecipitated, CAST was coimmunoprecipitated in the presence or absence of Bassoon, whereas Bassoon was coimmunoprecipitated only in the presence of CAST (Fig. 5 C). These results, together with the above observations, indicate that CAST forms a ternary complex with RIM1 and Bassoon.

Mechanism of localization of CAST and Bassoon in neurons

We examined the mechanism of localization of CAST and Bassoon at synapses in primary cultured rat hippocampal neurons. We first expressed EGFP-CAST Δ BsnBD, which could not bind Bassoon (Fig. 3 C, b), and it colocalized with Bassoon (Fig. 6 a) and synaptophysin (unpublished data), suggesting that CAST has the potency to localize at synapses without the BsnBD. Next, we expressed various EGFP-tagged constructs of Bassoon. The full-length protein colocalized with CAST and synaptophysin (unpublished data). EGFP-BsnN was diffusely distributed in the axon (Fig. 6 b). A recent report has clearly revealed that the NH₂-terminal region of Bassoon (aa 1–609), containing a consensus motif for N-myristoylation (MGNEASLEG), shows punctate signals in the axon, which colocalize with synaptophysin (Dresbach et al., 2003). Although we could not detect such punctate signals in the case of our GFP-BsnN construct, it might be simply due to the deficiency of the N-myristoylation, because GFP is tagged at the NH₂ terminus of our Bassoon construct. Alternatively, it might be due to the overexpression of the construct. On the other hand, EGFP-BsnC and EGFP-Bsn Δ Ex4/5, lacking exons 4 and 5, colocalized with CAST (Fig. 6, c and d) and synaptophysin (unpublished data). In the latter case, a Bassoon mutant, lacking exons 4 and 5, has been produced in Bassoon knockout mice, and the endogenous mutant protein

is also shown to be tightly associated with the CAZ (Altrock et al., 2003). Thus, the data for our EGFP-Bsn Δ Ex4/5 construct was consistent with this reported observation. Interestingly, EGFP-Bsn Δ CasBD, which could not bind to CAST (Fig. 3

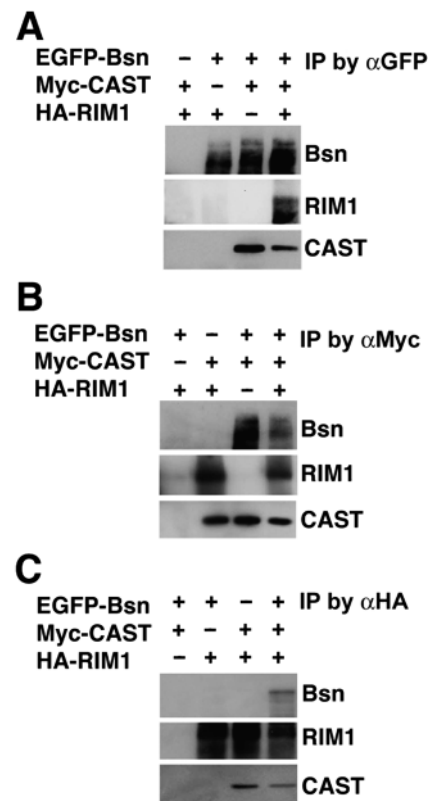


Figure 5. Ternary complex formation of CAST, RIM1 and Bassoon.

Each expression plasmid of Myc-CAST, HA-RIM1, or EGFP-Bassoon was transfected into HEK293 cells. Each protein was extracted and mixed in the indicated combinations, followed by immunoprecipitation using the anti-GFP, anti-Myc, or anti-HA Ab. Immunoprecipitates were then analyzed by Western blotting using the indicated Abs. IP, immunoprecipitation. The results are representative of three independent experiments.

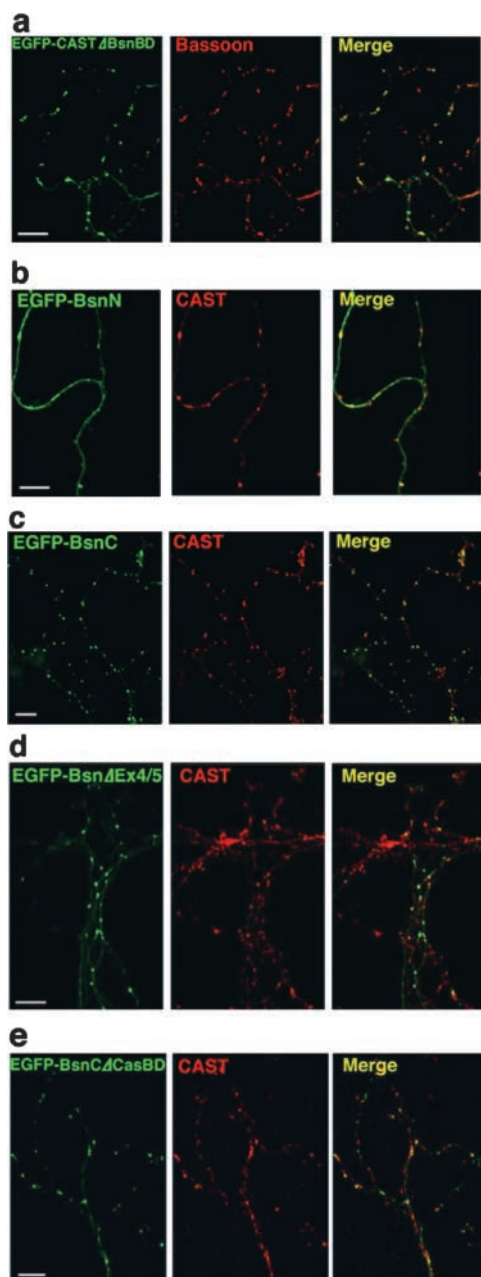


Figure 6. Localization of the CAST and Bassoon constructs at synapses. Neurons at 7 or 8 d of culture were transfected with the indicated expression vectors, and stained with anti-Bassoon or anti-CAST Abs at 10–12 d of culture. EGFP signals were detected by FITC channel. The blue color for Cy5 is changed to red. Bars, 10 μ m.

C, a), also colocalized with CAST (Fig. 6 e) and synaptophysin (unpublished data). Together, these results suggest that Bassoon localizes at synapses through at least two regions: the NH₂ terminus (aa 1–609) and the COOH-terminal region excluding the CasBD.

Implication of the binding of RIM1 and Bassoon to CAST in neurotransmitter release

In the last set of experiments, we examined whether the binding of RIM1 and Bassoon to CAST is involved in neurotransmitter release.

Table I. mRNA expression of CAZ and exocytotic proteins

	SCGNs	Rat brain
CAST	+	+
RIM1	+	+
Bassoon	+	+
Piccolo	+	+
Rab3A	+	+
Syntaxin1A	+	+

mRNA expressions were determined by RT-PCR. Presence (+) of mRNA expressions are shown.

For this purpose, we used cultured rat superior cervical ganglion (SCG) neurons, because peptides or proteins can be readily introduced into their relatively large presynaptic cell bodies by microinjection; and the injected materials can rapidly diffuse to nerve terminals forming synapses with adjacent neurons, allowing the effects of the stimulated release of acetylcholine to be accurately monitored by recording the excitatory post-synaptic potentials (EPSPs) evoked by action potentials in the presynaptic neurons (Mochida et al., 1998).

Before undertaking the electrophysiological assays, we first examined expression and localization of CAZ proteins such as CAST, RIM1, and Bassoon in cultured SCG neurons. RT-PCR assay revealed that CAZ proteins including CAST and several synaptic proteins were expressed at the mRNA level (Table I). We further examined the localization of CAST, RIM1, and Bassoon using their Abs. CAST showed dotty signals around cell bodies, which colocalized with those of Bassoon, RIM1, and synaptophysin (Fig. 7). These results suggest that the CAZ proteins localize at synapses of cultured SCG neurons.

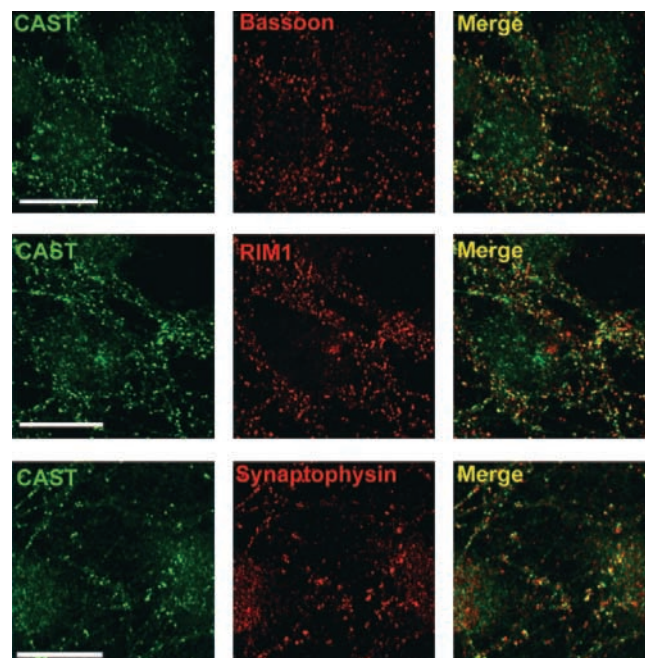


Figure 7. Localization of CAST, RIM1, and Bassoon at synapses in cultured SCG neurons. Rat SCG neurons at 5–6 wk in culture were doubly stained using anti-CAST and anti-Bassoon, anti-RIM1 or antisynaptophysin Abs. Bars, 30 μ m. The results are representative of three independent experiments.

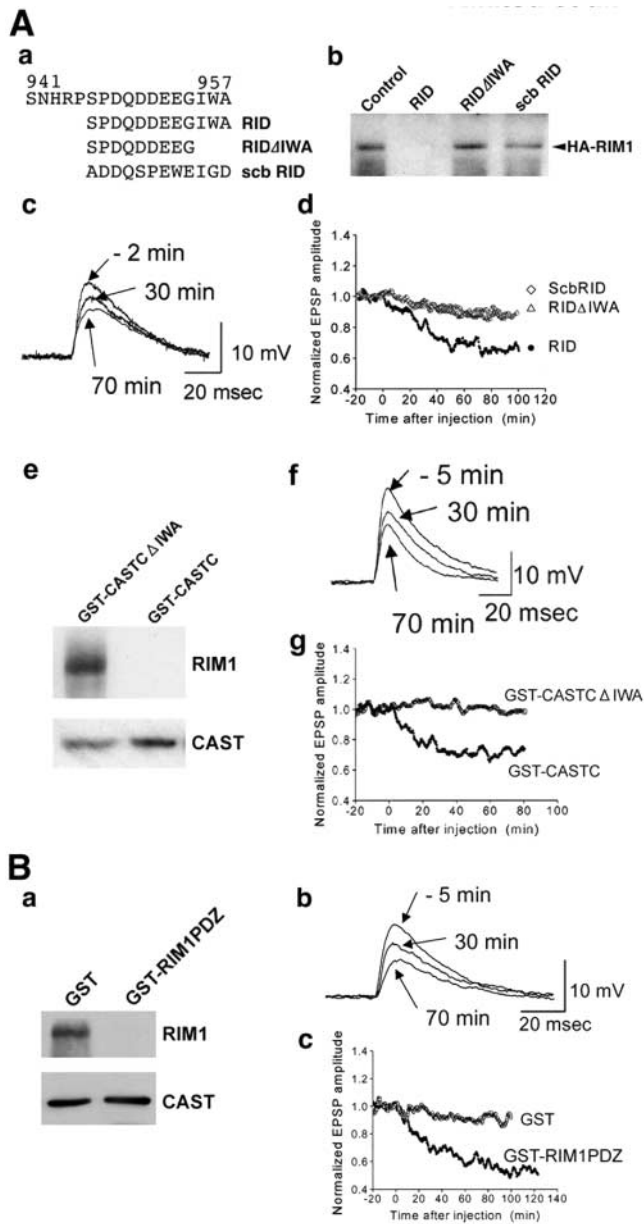


Figure 8. Effect of the binding of CAST and RIM1 on synaptic transmission. (A) Effects of the COOH-terminal regions of CAST on synaptic transmission. (a) Sequences of the CAST peptides. RID, RIM1-interacting domain; scb RID, scrambled RID. (b) Effects of the peptides (5 μ M each) on the binding of HA-RIM1 to immobilized GST-CAST-4. The binding was inhibited by RID, but not by RID Δ IWA or scb RID. (c and d) Effects of the CAST peptides (1 mM each in the injection pipette) on synaptic transmission. Presynaptic neurons were stimulated every 20 s CAST peptides were introduced into presynaptic neurons at $t = 0$. EPSPs from representative experiments with the injection are illustrated in c. Normalized and averaged EPSP amplitudes are plotted from five experiments with RID, RID Δ IWA, or scb RID peptide in d. (e) Effects of the COOH-terminal regions of CAST (5 μ M each) on the binding of CAST and RIM1. Immunoprecipitation assays of Myc-CAST and HA-RIM1 were performed in the presence of GST-CAST Δ IWA or GST-CASTC, followed by Western blotting using the anti-Myc and anti-HA Abs. GST-CASTC inhibited the binding but GST-CASTC Δ IWA did not. (f and g) Effects of the recombinant CAST proteins (150 μ M each in the injection) on synaptic transmission. Presynaptic neurons were stimulated every 20 s. Recombinant CAST proteins were introduced into presynaptic neurons at $t = 0$. Normalized and averaged EPSP

To test the binding of RIM1 and CAST is involved in neurotransmitter release, we then prepared the RIM1-interacting domain (RID) peptide, and the RID Δ IWA and scrambled RID peptides as the controls (Fig. 8 A, a). The last three aa (IWA) are critical for the binding of CAST and RIM1 (Ohtsuka et al., 2002). In-vitro binding assays revealed that RID, but not RID Δ IWA or scrambled RID, inhibited the binding of RIM1 to CAST (Fig. 8 A, b). We then microinjected these peptides into presynaptic SCG neurons. RID inhibited synaptic transmission (Fig. 8 A, c and d), and at 70 min after injection of RID, the EPSP amplitude was reduced by $-33 \pm 9.1\%$ ($n = 5$). In contrast, neither RID Δ IWA nor scrambled RID produced any significant decrease in the EPSP amplitude ($P = 0.025$, 0.023 unpaired t test at 70 min after the injection of RID versus RID Δ IWA and RID versus scrambled RID, respectively). To confirm these results, we prepared two GST fusion proteins containing the COOH-terminal aa of CAST; GST-CASTC Δ IWA (aa 938–954) and GST-CASTC (aa 938–957). As with the RID peptide, GST-CASTC inhibited the binding of RIM1 and CAST but GST-CASTC Δ IWA did not (Fig. 8 A, e). We then microinjected these recombinant proteins into presynaptic SCG neurons. GST-CASTC inhibited synaptic transmission (Fig. 8 A, f and g); and at 70 min after the injection of GST-CASTC, the EPSP amplitude was reduced by $-26 \pm 5.8\%$ ($n = 5$). In contrast, GST-CASTC Δ IWA produced no significant decrease in the EPSP amplitude ($P = 0.0042$, unpaired t test at 70 min after the injection of GST-CASTC versus GST-CASTC Δ IWA). To further examine the involvement of the binding of CAST and RIM1 in neurotransmitter release, we prepared GST-RIM1 PDZ, which inhibited the binding of RIM1 and CAST (Fig. 8 B, a). Microinjected GST-RIM1 PDZ significantly impaired synaptic transmission in SCG neurons but GST alone did not (Fig. 8 B, b and c). At 70 min after the injection of GST-RIM1 PDZ, the EPSP amplitude was reduced by $-34 \pm 4.2\%$ ($n = 5$; $P = 0.0081$, unpaired t test at 70 min after the injection of GST-RIM1 PDZ versus GST alone). These results indicate that CAST dynamically binds RIM1, and that this dynamic binding is involved in neurotransmitter release.

To test the binding of Bassoon and CAST is involved in neurotransmitter release, we attempted to use GST-CAST-2D containing the BsnBD (Fig. 2 C, c) that disrupted the binding in vitro (Fig. 9 A, a), because we lacked an effective peptide that disrupts the binding of Bassoon to CAST in

amplitudes are plotted from five experiments with GST-CASTC or GST-CASTC Δ IWA in f. (B) Effect of the PDZ domain of RIM1 on synaptic transmission. (a) Effect of the GST fusion protein containing the PDZ domain on the binding of CAST and RIM1. Immunoprecipitation assays of Myc-CAST and HA-RIM1 were performed in the presence of GST alone or GST-RIM1 PDZ (5 μ M each), followed by Western blotting using the anti-Myc and anti-HA Abs. GST-RIM1 PDZ inhibited the binding but GST alone did not. (b and c) Effect of GST-RIM1 PDZ on synaptic transmission. Presynaptic neurons were stimulated every 20 s GST alone or GST-RIM1 PDZ (170 μ M each) were introduced into presynaptic neurons at $t = 0$. EPSPs from representative experiments with the injection are illustrated in b. Normalized and averaged EPSP amplitudes are plotted from five experiments with GST-RIM1 PDZ or GST alone in c.

in vitro (unpublished data). Microinjection of GST-CAST-2D into presynaptic SCG neurons significantly inhibited synaptic transmission (Fig. 9 A, b and c); and at 70 min after injection, the EPSP amplitude was reduced by $-35 \pm 2.8\%$ ($n = 7$). As a control, the carrier solution showed no significant effect ($P < 0.0012$, at 70 min after injection of GST-CAST-2D versus the carrier solution). Next, we prepared GST-Bsn-CasBD (Fig. 3 A), which inhibited the binding of CAST and Bassoon (Fig. 9 B, a). Then, we microinjected GST-Bsn-CasBD into presynaptic SCG neurons. GST-Bsn-CasBD significantly inhibited synaptic transmission (Fig. 9 B, b and c). At 70 min after injection of GST-Bsn-CasBD, the EPSP amplitude was reduced by $-30 \pm 6.9\%$ ($n = 5$). In contrast, heat-denatured GST-Bsn-CasBD, as a control, produced no significant decrease in the EPSP amplitude ($P = 0.0075$, unpaired t test at 70 min after injection of GST-Bsn-CasBD versus heat-denatured GST-Bsn-CasBD). These results indicate that the binding of CAST and Bassoon is also involved in neurotransmitter release.

Discussion

CAST-dependent large molecular complex at the CAZ

Here, we have demonstrated that the CAZ proteins thus far known form a large molecular complex in vivo, including CAST, RIM1, Munc13-1, Bassoon, and Piccolo. Interestingly, this CAST-dependent molecular complex is SDS-nonresistant and is not as stable as the so-called SNARE complex, although it is tightly associated with the CAZ. Therefore, we think that the CAZ proteins disassemble at once on treatment with 1% SDS at the early step of immunoprecipitation from the PSD fraction, and then reassemble to form the proper molecular complex upon dilution with Triton X-100 (Fig. 1 B). Although such large proteins may often be the cause of nonspecific binding, we assume, in accord with the following lines of evidence, that this CAST-dependent complex formation is specific: first, these proteins well colocalize at the CAZ (Ohtsuka et al., 2002); second, the binding of CAST and RIM1 is highly specific (the last three aa [IWA] of CAST are essential for its binding RIM1 [Ohtsuka et al., 2002]); and third, the binding of CAST and Bassoon is also specific. We have demonstrated here that the central region of CAST directly binds the region containing the third coiled-coil domain of Bassoon: and fourth, electrophysiological studies show that these bindings via the domains are implicated in synaptic transmission.

Moreover, we have shown that Bassoon and Piccolo are simultaneously coimmunoprecipitated with CAST. Because the binding of Bassoon and Piccolo to CAST is competitive, the result suggests that the distinct complexes, Bassoon-CAST-RIM1-Munc13-1 and Piccolo-CAST-RIM1-Munc13-1, may exist at synapses. Alternatively, these two distinct complexes may be further associated with each other through CAST, because we have already obtained the result, by immunoprecipitation assay using HEK293 cells, that CAST forms a homo-oligomer (Deguchi-Tawarada et al., 2004). Probably, the large molecular complex formed by these CAZ proteins is the molecular basis for the presynaptic active zone characterized as the slightly electron-dense region. However, considering the diversity of synapses, we can

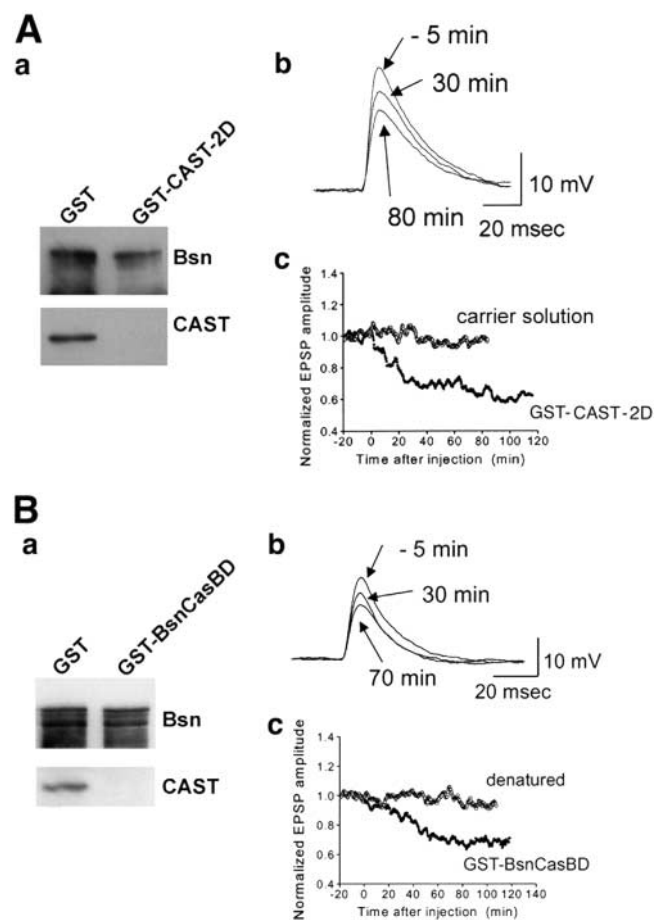


Figure 9. Effect of the binding of CAST and Bassoon on synaptic transmission. (A) Effect of the BsnBD of CAST on synaptic transmission. (a) Effect of GST-CAST-2D (BsnBD) on the binding of CAST and Bassoon. Immunoprecipitation assay of Myc-CAST and EGFP-Bassoon was performed in the presence of GST alone or GST-CAST-2D (5 μ M each), followed by Western blotting using the anti-Myc and anti-GFP Abs. GST-CAST-2D inhibited the binding but GST alone did not. (b and c) Effect of GST-CAST-2D (150 μ M) on synaptic transmission. The presynaptic neuron was stimulated every 20 s. The recombinant CAST protein was introduced into the presynaptic neuron at $t = 0$. EPSPs from the representative experiments with the injection are illustrated in (b). Normalized and averaged EPSP amplitudes are plotted from seven experiments with GST-CAST2D or the carrier solution alone in (c). (B) Effect of the CasBD of Bassoon on synaptic transmission. (a) Effect of GST-BsnCasBD on the binding of CAST and Bassoon. Immunoprecipitation assay of Myc-CAST and EGFP-Bassoon was performed in the presence of GST alone or GST-BsnCasBD (5 μ M each), followed by Western blotting using the anti-Myc and anti-GFP Abs. GST-BsnCasBD inhibited the binding but GST alone did not. (b and c) Effect of GST-BsnCasBD (150 μ M) on synaptic transmission. The presynaptic neuron was stimulated every 20 s. The recombinant Bassoon protein was introduced into the presynaptic neuron at $t = 0$. EPSPs from the representative experiments with the injection are illustrated in b. Normalized and averaged EPSP amplitudes are plotted from five experiments with GST-BsnCasBD or denatured (boiled) GST-Bsn-CasBD in c.

easily imagine that there are more CAZ-specific proteins at the presynaptic active zone. Identifying new CAZ proteins and the step by step analysis of their protein-protein interactions should provide insights into the different structures of the active zones of various synapses.

Mechanisms of localization of CAST and Bassoon

Although these CAZ proteins, including CAST, RIM1, Munc13-1, Bassoon, and Piccolo, are specifically and tightly associated with the CAZ, their mode of localization at the CAZ is still unclear, being rather more complicated than envisaged. For example, the binding of RIM1 and Munc13-1 was the first to show the direct binding among CAZ proteins that plays a crucial role in priming of synaptic vesicles (Betz et al., 2001). It was originally thought that the binding did not affect the individual synaptic localization of RIM1 or Munc13-1 (Betz et al., 2001). However, recent work on RIM1 knockout mice shows that, among the synaptic proteins examined, which included other RIM1-binding proteins, only the expression of Munc13-1 is reduced by ~60% (Schoch et al., 2002), suggesting that RIM1 may affect synaptic localization and/or expression of Munc13-1 by directly binding Munc13-1. More recently, interesting progress on ascertaining the localization mechanism of CAZ proteins has been made by constructing and expressing various Bassoon mutants in cultured hippocampal neurons (Dresbach et al., 2003). The region (aa 1692–3263) containing the second and third coiled-coil domains of Bassoon is shown to be essential for its association with the CAZ; findings that are consistent with our results (Fig. 6). Together with our present and the other group's observations (Dresbach et al., 2003), however, it is likely that the second and third coiled-coiled domains of Bassoon are not essential for its association with the CAZ and thus the targeting signals of Bassoon to the CAZ may reside in the aa 1692–2087, aa 2565–2714, and/or aa 3015–3263 regions of Bassoon excluding these coiled-coil domains. This might also mean that Bassoon can localize to the CAZ independently of CAST, because CAST directly binds the region containing the third coiled-coil domain of Bassoon. Conversely, our present result could suggest that CAST can localize to the CAZ independently of Bassoon (Fig. 6). However, because CAST and Bassoon have been shown to form the large immunoreactive structures (Fig. 4; Ohtsuka et al., 2002; Dresbach et al., 2003), there is the possibility that they form oligomers through presumably their coiled-coil domains. In fact, at least CAST forms a homo-oligomer (Deguchi-Tawarada et al., 2004), although the precise mode of the oligomerization is unclear. Thus, we cannot rule out the possibility that synaptic localization of EGFP-CAST Δ BsnBD and EGFP-Bsn Δ CasBD might simply be due to their oligomerization with endogenous CAST and Bassoon, respectively. In this regard, the determination of precise domains for the oligomerization of CAST and Bassoon should shed new light on the complicated localization mechanisms of these CAZ proteins.

RIM1- and Bassoon-dependent roles of CAST in neurotransmitter release

It has been shown that the direct binding of RIM1 and Munc13-1 is involved in the priming of synaptic vesicles (Betz et al., 2001) and that the localization of RIM1 at the CAZ appears to be CAST dependent (Ohtsuka et al., 2002). Thus, it is likely that the inhibition of the binding of RIM1 to CAST might affect the RIM1–Munc13-1 pathway, presumably by the mislocalization of RIM1 at the active zone, resulting in a reduction of neurotransmitter release. More-

over, we have demonstrated here that the dynamic binding of Bassoon and CAST is also involved in neurotransmitter release. Although it is unclear whether the inhibitory effects on synaptic transmission by CAST and Bassoon are due to mislocalization of CAST and/or Bassoon at the active zone, at least the disruption of the binding of CAST and Bassoon might affect the function of Bassoon required for normal neurotransmitter release (Altrock et al., 2003). A functional linkage between RIM1 and Bassoon has not been determined, but, by forming the ternary complex with RIM1 and Bassoon, CAST may play a role as a “platform” on which the signaling pathways of Bassoon and RIM1 could be molecularly coupled.

At this time we cannot exclude the possibility that the inhibitory effect is possibly due to the disruption of the binding of Piccolo to CAST, because Bassoon and Piccolo share the same binding site in CAST. Bassoon and Piccolo are structurally similar, but Piccolo has additional PDZ and C2 domains (Wang et al., 1999; Fenster et al., 2000), implying that Piccolo is involved in similar and/or distinct signaling pathways from Bassoon in neurotransmitter release. Among the CAZ proteins so far identified, knockout mice of RIM1, Munc13-1, and Bassoon have been reported and characterized, which are phenotypically and physiologically different (Augustin et al., 1999; Castillo et al., 2002; Schoch et al., 2002; Altrock et al., 2003). Comparison of Bassoon mutant mice with Piccolo and CAST mutant mice, and the analysis of double and triple mutants should be of great help in understanding the distinct functions of the different CAZ proteins at both molecular and whole animal levels.

In conclusion, many CAZ proteins form a dynamic multi-complex, presumably through CAST, at the CAZ and that this CAST-dependent molecular complex is involved in neurotransmitter release. Neurotransmitter release requires formation of the SNARE complex, which is regulated by many modulators (Jahn and Südhof, 1999). Although the exact sequential steps of the dynamic protein–protein interactions in neurotransmitter release are currently unknown, RIM1 appears to be downstream of Rab3 (Wang et al., 1997), and Munc13-1 appears to be downstream of RIM1 and is implicated in the priming of synaptic vesicles by directly binding to syntaxin (Brose et al., 2000). Genetic studies from *C. elegans* support this notion. Overexpression of the open form of syntaxin, which can form the SNARE complex, suppresses the phenotype of UNC13 or RIM/UNC10 mutant animals (Koushika et al., 2001; Richmond et al., 2001). Therefore, it is likely that the CAZ proteins in the multicomplex are physically and functionally associated with the SNARE complex and regulate neurotransmitter release, although our biochemical analysis does not yet reveal this molecular linkage. Therefore, further studies are necessary for better understandings of the molecular mechanisms that spatially and temporally regulate the coupling of the CAZ protein complex and the neurotransmitter release machinery.

Materials and methods

Constructs

Prokaryotic and eukaryotic expression vectors were constructed in pEGFPC1 (CLONTECH Laboratories), pRSET (Invitrogen), and pGEX (Amersham Biosciences) using standard molecular biological methods. The Bassoon cDNA

fragments with various lengths were obtained by RT-PCR from rat brain and connected to construct full-length cDNA, which was used as a template for the Bassoon constructs except for those of BsnC for which a human cDNA clone (KIAA0434) was used. pEGFP-CASTΔBsnBD was constructed by connecting the NH₂-terminal (aa 1–440) and COOH-terminal (aa 601–957) regions. pEGFP-BsnCΔCasBD was constructed by connecting the NH₂-terminal (aa 2356–2936) and COOH-terminal (aa 2995–3926) regions. pEGFP-BsnΔEx4/5 was constructed by connecting the NH₂-terminal (aa 1–504) and COOH-terminal (aa 2890–3938) regions (Altrock et al., 2003). Other constructs for CAST and RIM1 were prepared as described previously (Ohtsuka et al., 2002). The GST- and His-tagged fusion proteins were purified according to the manufacturer's protocols. The RIM1 and Piccolo cDNAs were supplied by S. Seino (Chiba University, Chiba, Japan).

Abs

Rabbit antiserum specific for CAST was raised against GST-CAST (aa 107–138) and was affinity purified with GST fusion protein covalently coupled to CNBr-activated Sepharose (Amersham Biosciences). This Ab specifically recognized CAST but not a related protein (Nakata et al., 1999; Monier et al., 2002; Wang et al., 2002; Deguchi-Tawarada et al., 2004). Anti-HA, anti-Myc, anti-GFP (Roche), anti-Munc13-1 (Synaptic Systems GmbH), anti-VAMP2, anti-Bassoon (StressGen Biotechnologies), anti-Rab3, anti-N-cadherin, anti-Munc18, anti-RIM1 (Transduction Laboratories), anti-SNAP-25, anti-CASK, antisynaptophysin (CHEMICON International, Inc.), antisyntaxin (WAKO), and anti-β-catenin (Sigma-Aldrich) Abs were purchased from commercial sources. Cy5-conjugated anti-mouse and -rabbit IgGs (Jackson ImmunoResearch Laboratories) were used as secondary Abs to detect primary Abs in immunofluorescence microscopy of primary cultures.

Immunoprecipitation from the SM3 and PSD fraction

To obtain the SM3 and PSD fractions, subcellular fractionation of rat brain was prepared as described previously (Cohen et al., 1977; Ohtsuka et al., 2002). In brief, the synaptosomal membrane fraction was centrifuged at 48,200 g at 4°C and purified on a set of sucrose density gradients consisting of 0.85, 1.0, and 1.2 M sucrose. The band between 1.0 and 1.2 M sucrose was collected and used as the SM3 fraction. This fraction contains the pre- and post-synaptic membranes, plus PSDs and docked vesicles. The SM3 fraction was treated with 1% Triton X-100 and further centrifuged at 48,200 g at 4°C. The resultant pellet was used as the PSD fraction.

For immunoprecipitation from the SM3 fraction, proteins were extracted from the SM3 fraction (500 μg of protein) in 500 μl of 0.1% SDS in a Triton X-100 lysis buffer (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 1% wt/vol Triton X-100, and a protease inhibitor cocktail [Roche]) with gentle rotation at 4°C for 1 h. The sample was centrifuged at 37,500 g at 4°C to obtain the supernatant, which was further incubated with 50 μl of protein A-Sepharose beads for preclearing at 4°C for 1 h. The mixture was centrifuged at 3,000 rpm at 4°C for 5 min to obtain the supernatant as the extract of the SM3 fraction. 5 μg of rabbit nonimmune IgG, the anti-CAST, anti-Myc, or anti-Bassoon Ab was added to the extract and the mixture was gently rotated at 4°C for 2 h. After adding 20 μl of protein A-Sepharose beads, the sample was further rotated at 4°C for 2 h. After extensively washing the beads with the extract buffer five times, 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 subjected to SDS-PAGE, followed by Western blotting using various Abs for the indicated proteins.

For immunoprecipitation from the PSD fraction, proteins were extracted from the PSD fraction (1 mg of protein) with 1% SDS, followed by dilution with 5 vol of the Triton X-100 lysis buffer. Immunoprecipitation was done as described above using a wash buffer, 0.2% SDS in the Triton X-100 lysis buffer, followed by Western blotting and silver staining (Silver Quest Kit; Invitrogen).

Pull-down assay

Pull-down assays were prepared as described previously (Ohtsuka et al., 2002). In brief, HEK293 cells expressing the indicated expression vectors 10-cm dishes were collected and proteins were extracted with 1.0 ml of Triton X-100 lysis buffer at 4°C for 30 min. The sample was centrifuged at 15,000 rpm at 4°C for 20 min to collect the supernatant. 500 μl of the supernatant was incubated with 20 μl of glutathione-Sepharose beads containing the indicated GST fusion proteins (~1 μg of protein each) at 4°C for 1 h. After the beads were extensively washed with the Triton X-100 lysis buffer, the bound proteins were eluted by boiling the beads in the SDS sample buffer for 5 min. The samples were subjected to SDS-PAGE,

followed by protein staining with Coomassie brilliant blue and Western blotting. To examine the effects of COOH-terminal peptides of CAST, pull-down assays were prepared as described in the presence of each peptide (5 μM).

Expression of CAST and Bassoon in HEK293 cells

HEK293 cells on 13-mm-diam cover glasses were transfected with the indicated expression vectors by LipofectAMINE2000 (Invitrogen). After 48 h of transfection, the cells were fixed and stained, followed by immunofluorescence microscopic analysis (Ohtsuka et al., 2002).

For biochemical assay, HEK293 cells transfected with each expression vector in a 10-cm dish were collected and proteins were extracted from the cells with 1 ml of the Triton X-100 lysis buffer. The sample was centrifuged at 10,000 g at 4°C for 20 min to obtain the supernatant and pellet fractions, which were used as the Triton X-100-soluble and -insoluble fractions, respectively.

Immunoprecipitation from HEK293 cells

Immunoprecipitations using HEK293 cell lysates were performed as described previously (Ohtsuka et al., 2002). In brief, each expression plasmid was transfected in HEK293 cells, and proteins were extracted with the Triton X-100 lysis buffer and mixed in various combinations. After incubation for 2 h, 1.0 μg of the indicated Ab was added to the sample, followed by incubation at 4°C for 1 h. And 20 μl of protein A-Sepharose beads was added to the mixture and the sample was further incubated for 1 h. The beads were extensively washed with the Triton X-100 lysis buffer and the bound proteins were eluted by boiling them in the SDS sample buffer. The samples were then analyzed by Western blotting.

For analysis of ternary complex formation of CAST, RIM1, and Bassoon, immunoprecipitation was performed as follows. Each expression plasmid of Myc-CAST, HA-RIM1, or EGFP-Bassoon was transfected into HEK293 cells, and the protein was extracted with the Triton X-100 lysis buffer and mixed in various combinations. After incubation at 4°C 1 h, immunoprecipitation was performed as described in the previous paragraph using 1.5 μg of the anti-GFP, anti-Myc, or anti-HA Ab. The samples were then analyzed by Western blotting.

Rat hippocampal neuron culture, transfection, and immunocytochemistry

Primary cultures of hippocampal neurons were prepared as described previously (Bito et al., 1996). Cells were transfected at 7 or 8 d of culture by a lipid-mediated gene transfer method using an Effectene Kit (QIAGEN). Immunostaining was performed at 9 or 12 d of culture.

The transfected cells were fixed with 2% PFA and 4% sucrose in PBS, pH 7.4, for 20 min at RT. After washing with PBS, cells were permeabilized with 0.25% Triton X-100 in PBS for 15 min. Nonspecific binding was blocked with 25% Block Ace (Dainippon Pharmaceutical) containing 0.25% Triton X-100 for 2 h. Cells were incubated with primary Abs diluted in 2.5% Block Ace and 0.25% Triton X-100 in PBS for 2 h, followed by secondary Abs. Stained cells were observed with a confocal laser microscopy using a 63× oil immersion objective lens (model LSM510; Carl Zeiss Microimaging, Inc). Collected data were exported as 8-bit TIFF files and processed using Adobe Photoshop® 5.5.

SCG neurons culture, immunocytochemistry, and electrophysiological recordings

Culture of SCG neurons was prepared as described previously (Mochida et al., 1994). For immunocytochemistry of CAST, Bassoon, RIM1 and synaptophysin, SCG neurons cultured for 5–6 wk were fixed and stained as described above for hippocampal neurons.

EPSP recording and injection of the recombinant proteins or peptides were performed as described previously (Mochida et al., 1994, 1998). Recombinant proteins or peptides were dissolved in injection solution (10 mM Hepes, pH 7.4, 136 mM potassium acetate, and 5 mM Mg²⁺-ATP) and introduced into the presynaptic cell body by diffusion from a glass pipette (17–20 MΩ tip resistance). 5% fast green FCF (Sigma-Aldrich) as the carrier solution was introduced in the pipette solution to confirm entry in to the presynaptic cell body. The injection pipette was removed 3–4 min after starting injection. Neurons were superfused with modified Krebs' solution containing 5 mM Ca²⁺. Collected electrophysiological data using software written by the late L. Tauc (Centre National de la Recherche Scientifique, Gif-sur-Yvette, France) were analyzed with Origin (Microcal Software Inc.). The peak amplitudes of EPSP were averaged. The resultant values were smoothed by eight-point moving average algorithm and plotted against recording time with t = 0 indicating the presynaptic injection.

Other procedures

Mass spectrometry was performed as described previously (Ohtsuka et al., 2002). Figures were produced using Adobe Photoshop® 5.5.

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