

Bassoon, a Novel Zinc-finger CAG/Glutamine-repeat Protein Selectively Localized at the Active Zone of Presynaptic Nerve Terminals

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Abstract. The molecular architecture of the cytomatrix of presynaptic nerve terminals is poorly understood. Here we show that Bassoon, a novel protein of >400,000 M_r , is a new component of the presynaptic cytoskeleton. The murine *bassoon* gene maps to chromosome 9F. A comparison with the corresponding rat cDNA identified 10 exons within its protein-coding region. The Bassoon protein is predicted to contain two double-zinc fingers, several coiled-coil domains, and a stretch of polyglutamines (24 and 11 residues in rat and mouse, respectively). In some human proteins, e.g., Huntingtin, abnormal amplification of such polyglutamine regions causes late-onset neurodegeneration. Bassoon is highly enriched in synaptic protein preparations. In cultured hippocampal neurons, Bassoon colo-

calizes with the synaptic vesicle protein synaptophysin and Piccolo, a presynaptic cytomatrix component. At the ultrastructural level, Bassoon is detected in axon terminals of hippocampal neurons where it is highly concentrated in the vicinity of the active zone. Immunogold labeling of synaptosomes revealed that Bassoon is associated with material interspersed between clear synaptic vesicles, and biochemical studies suggest a tight association with cytoskeletal structures. These data indicate that Bassoon is a strong candidate to be involved in cytomatrix organization at the site of neurotransmitter release.

Key words: trinucleotide repeats • mouse *bassoon* gene • presynaptic terminals • rat brain • synapses

CHEMICAL synapses are sites of cell–cell contact between neurons mediating interneuronal communication. Both the presynaptic terminal and the postsynaptic compartment comprise a highly specialized cytoskeleton underlying the synaptic membranes (Burns and Augustine, 1995). This cortical cytoskeleton, together with cell adhesion molecules and components of the extracellular matrix, act to keep pre- and postsynaptic compartments in register (Hall and Sanes, 1993; Burns and Augustine, 1995; Garner and Kindler, 1996). At the postsynaptic side, an electron-dense meshwork of fine filaments, the

postsynaptic density (PSD)¹, underlies the membrane, and is thought to anchor and cluster neurotransmitter receptors. Molecules involved in this function include rapsyn/43K protein at the cholinergic neuromuscular junction (Froehner, 1991), gephyrin at glycinergic synapses, and SAP90/PSD-95, chapsyn-110/PSD-93, and SAP102 at glutamatergic central synapses (Garner and Kindler, 1996; Kirsch et al., 1996; Kennedy, 1997).

The presynaptic nerve terminal is the principal site of regulated neurotransmitter release. The region of the presynaptic plasmalemma over which synaptic vesicles dock, fuse, and release neurotransmitter is called the active zone (Landis et al., 1988). Typically, several hundred synaptic vesicles are localized in the vicinity of the active zone (Burns and Augustine, 1995). Although a number of pro-

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1. *Abbreviations used in this paper:* aa, amino acid; LIM, *lin-11/ISL-1/mec-3*-like; MAGUK, membrane-associated guanylate kinase homologue; PSD, postsynaptic density.

teins that are involved in synaptic vesicle fusion and endocytosis have been identified and characterized (Südhof, 1995; De Camilli and Takei, 1996), the cellular mechanisms restricting synaptic vesicle fusion to the active zone remain unclear. It is reasonable to assume that the cytomatrix at the active zone is intimately involved in determining the sites of synaptic vesicle fusion.

To date, only few cytomatrix proteins have been identified that may play a role in this process. One candidate protein is synapsin I, which has been reported to link synaptic vesicles to the presynaptic cytoskeleton (Landis et al., 1988; Hirokawa et al., 1989). Further candidates are members of the family of membrane-associated guanylate kinase homologues (MAGUKs), the Rab3 effector protein Rim, and the presynaptic cytomatrix component Piccolo. MAGUKs, including synapse-associated proteins SAP90/PSD-95, SAP97, and chapsyn-110/PSD-93, are found in distinct presynaptic terminals, and bind and cluster presynaptic ion channels in vitro (Kistner et al., 1993; Kim et al., 1995; Müller et al., 1995; Kim et al., 1996). In addition, presynaptic MAGUK expression appears to be essential for the proper assembly of the neuromuscular synapse in *Drosophila* (Budnik et al., 1996; Thomas et al., 1997a; Thomas et al., 1997b). However, to date no specific function in synaptic vesicle docking and fusion could be assigned to MAGUKs. Rim is a large presynaptic zinc-finger protein that interacts with Rab3 in its GTP (but not GDP)-bound form and, when transfected into PC12 cells, enhances regulated exocytosis in an Rab3-dependent manner (Wang et al., 1997). Piccolo, a recently identified 420-kD cytoskeleton-associated protein, has been detected primarily within presynaptic nerve terminals of asymmetric type I synapses (Cases-Langhoff et al., 1996), but to date its function is unknown. Both Rim and Piccolo are highly enriched in synaptic junctional protein preparations (Cases-Langhoff et al., 1996; Wang et al., 1997).

In this study we have identified a novel protein that is found in the presynaptic compartments of rat brain synapses. It appears exquisitely localized to the area just proximal to the active zone. We refer to this protein as Bassoon, a novel member of the ensemble of presynaptic proteins that are involved in orchestrating events at the nerve terminal. Bassoon has two double-zinc finger domains known to be involved in protein-protein interactions (Sanchez-Garcia, 1994), three presumptive coiled-coil regions, and a stretch of 11 (mouse)-24 (rat) glutamine residues, most of them encoded by CAG repeats. This latter feature is shared, for example, with Huntington or the ataxins (for review see Reddy and Housman, 1997). Abnormal expansion of the CAG/glutamine repeats in these genes/proteins is involved in a number of human genetic disorders, including Huntington's disease or autosomal dominant cerebellar ataxias, that are characterized by late onset of degeneration of particular groups of neurons (Reddy and Housman, 1997).

Materials and Methods

Cloning and Sequence Analysis of Rat Bassoon cDNA and Mouse *bassoon* Gene

The cDNA clone *sap7f* was isolated from a λ gt11 expression library with polyclonal antibodies generated against a rat brain synaptic junction preparation as described previously (Kistner et al., 1993; Langnaese et al., 1996).

Overlapping cDNA clones were obtained by several rounds of screening of λ gt10 (CLONTECH Laboratories, Inc., Palo Alto, CA) and λ ZAP II (Stratagene, La Jolla, CA) adult rat brain cDNA libraries with the 32 P-labeled *sap7f* cDNA or mouse genomic clones. Parts of the mouse genomic Bassoon DNA were isolated by screening a 129 SVJ mouse genomic λ FIXII library (Stratagene) with rat Bassoon probes. Deoxyoligonucleotides were derived from exon 4 sequences (5'-TGTTTTAGGAGTCCCAGGAGGCA-3'; 5'-TGAAGCAGAAAGGGCCACAGGGG-3'), and were used to identify P1 phages containing the *bassoon* gene by PCR (129 SVJ mouse genomic P1 library; Genome Systems Inc., St. Louis, MO). Exon-containing fragments were identified with rat Bassoon cDNA probes on Southern blots, isolated from agarose gels, and subcloned into pBluescript (Stratagene). Hybridization to λ -phage bound to Hybond N filters was carried out at 65°C in Rapid-hyb buffer (Amersham Corp., Arlington Heights, IL) as described by the supplier. Sequencing of the cDNA clones subcloned into pBluescript vectors was performed using the fluorescent dye dideoxy termination method in combination with an automated DNA sequencer (Applied Biosystems, Inc., Foster City, CA). Sequences were analyzed with the GCG program package (Genetics Computer Group, Inc., Madison, WI).

Antibody Production

The cDNA insert of *sap7f* (733 bp) was subcloned into the unique EcoRI site of the bacterial expression vector pGEX- λ IT (Pharmacia Biotech Sverige, Uppsala, Sweden). A 75-kD glutathione S-transferase (GST)-Bassoon fusion protein was expressed in *Escherichia coli* XL Blue and purified on glutathione-sepharose 4B as described by the manufacturer (Pharmacia Biotech Sverige). The fusion protein was used to generate Bassoon antibodies in mice and rabbits. The IgG fraction of rabbit antisera was isolated using GammaBind Plus Sepharose™ (Pharmacia Biotech Sverige) following the instructions of the manufacturer. The monoclonal antibody mab7f was produced by the University of Alabama at Birmingham hybridoma facilities. Antibodies against Piccolo were generated as described previously (Cases-Langhoff et al., 1996). Rabbit antiserum against synapsin I was provided by Dr. M. Mäder, Göttingen, Germany. Monoclonal antibody against synaptophysin was purchased from Boehringer Mannheim (Mannheim, Germany).

RNA Preparation, Northern Analysis, and In Situ Hybridization

Isolation of total RNA from several rat tissues as well as Northern blotting and hybridization with 32 P-labeled *sap7f* cDNA probes was performed as described (Langnaese et al., 1997).

In situ hybridization experiments were performed as described previously (Langnaese et al., 1997) with a 40-mer antisense oligonucleotide derived from the Bassoon cDNA (5'-ACAGCGGTGTCTCTTCTCC-CAAGTTGTCTTCTCGGCGC-3'). Identical results were obtained with three independent oligonucleotides. Controls including competition with 100-fold excess of unlabeled oligonucleotide, RNAase treatment of sections before hybridization or hybridization with sense probe did not yield any specific signal (see Fig. 1 C). Hybridization signals were visualized with a Fujix BAS 3000 Bio Imager (Fuji Photo Film Co., Ltd., Tokyo, Japan).

Chromosomal Localization of the Mouse *bassoon* Gene

For mapping the mouse *bassoon* gene, fluorescence in situ hybridization (FISH) on mouse metaphase chromosomes was performed using standard protocols (Lichter and Cremer, 1992). A mixture of four biotin-labeled genomic clones in pBluescript containing in total 46 kb of the mouse *bassoon* gene were hybridized to metaphase preparations from mouse embryonic fibroblast cultures. Signals were detected and amplified using biotin-conjugated anti-avidin antibody (5 μ g/ml) and fluorescein-avidin (5 μ g/ml). The slides were counterstained with Vecta-Shield/DAPI (Vector Labs, Inc., Burlingame, CA). Signal detection and imaging were achieved using a DMRB/E photomicroscope (Leica Mikrosysteme GmbH, Bensheim, Germany) and the Cytovision system (Applied Imaging, Santa Clara, CA). Mouse chromosomes were identified by inverted DAPI banding.

Isolation of Subcellular Protein Fractions and Immunoblot Analysis

Tissue fractionation was carried out essentially as described by Carlin et al. (1980) with some modifications: brains of 30-d-old rats were homogenized in homogenization buffer (5 mM Hepes, pH 7.4; 320 mM sucrose)

containing a protease inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany); cell debris and nuclei were removed by 1,000 g centrifugation. The supernatant was spun for 20 min at 13,000 g, resulting in supernatant S2 and pellet P2 (crude membrane fraction). S2 was centrifuged at 100,000 g for 1 h, and the resulting supernatant was taken as cytoplasmic fraction (S100). The P2 pellet was further fractionated by centrifugation in a sucrose step gradient as described by Carlin et al. (1980). For isolation of the synaptic junctional proteins (PSD fraction), the synaptosomal fraction of the first gradient was diluted with 320 mM sucrose (60 ml/10g wet tissue) and an equal volume of 1% Triton X-100, 320 mM sucrose, and 12 mM Tris-HCl, pH 8.1. The suspension was kept on ice for 15 min, and was centrifuged for 30 min at 32,800 g. The pellet was resuspended in 320 mM sucrose, 1 mM NaHCO₃ (6 ml/10 g wet tissue), and an equal volume of 1% Triton X-100. 320 mM sucrose was added, and synaptic junctional proteins were pelleted by a 2-h centrifugation at 201,800 g. All steps were carried out at 4°C.

Extraction experiments of P2 pellets with various agents as specified in Table 1 were performed in the following way: P2 pellets were resuspended in homogenization buffer, aliquoted into six samples (200 µg protein each), and centrifuged at 15,000 g for 20 min. Each pellet was then resuspended in 0.5 ml of one of the extraction buffers, incubated for 15 min at 4°C by gentle shaking, and centrifuged again for 15 min at 100,000 g. The resulting pellets were washed in homogenization buffer and dissolved in 80 µl gel-loading buffer (Laemmli, 1970). The supernatants were precipitated with trichloroacetic acid, and the resulting pellets were dissolved in 80 µl loading buffer. For SDS-PAGE, 20 µl/lane of each fraction were loaded. Proteins were separated on 5–20% polyacrylamide gels under fully reducing conditions, and were transferred onto nitrocellulose. For immunodetection, Western blots were incubated overnight with primary antibody and processed using the ECL detection system (Amersham Buchler, Braunschweig, Germany).

Preparation and Immunofluorescence Microscopy of Primary Hippocampal Cultures

Hippocampal cultures were prepared and grown on coverslips as described by Goslin and Banker (1991), washed in PBS (0.9% NaCl; 100 mM sodium phosphate buffer, pH 7.4), fixed with methanol at -20°C for 15 min, and blocked with 5% (vol/vol) FCS in PBS for 30 min. For immunofluorescence double-labeling experiments, cultures were incubated overnight with rabbit or mouse anti-Bassoon antibodies (1:250 dilution) and either a monoclonal antibody against synaptophysin (1:10 dilution; Boehringer Mannheim) or a polyclonal anti-Piccolo antiserum (1:1,000 dilution; Cases-Langhoff et al., 1996) at 4°C in 5% FCS in PBS. After three washes in PBS, coverslips were incubated overnight at 4°C with goat anti-mouse and anti-rabbit IgG antibodies conjugated with either fluorescein, Cy3, or Cy2 (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Secondary antibodies were diluted 1:100 in 5% FCS in PBS. Photographs were taken using an Aristoplan photomicroscope (Leitz, Wetzlar, Germany).

Immunohistochemistry of Tissue Sections

30-d-old male rats were used for immunohistochemical studies. Tissue preparation for microscopic analysis was done as described (Richter et al., 1996). Sections were incubated for 2 d at room temperature with mab7f Bassoon antibody (1:5,000 dilution) in combination with rabbit antiserum against synapsin I (1:500 dilution). After three washes with PBS, application of secondary antibodies coupled to Cy3 or Cy2 followed for 1 h at room temperature. Secondary antibodies were diluted 1:250 when coupled to Cy3, and 1:100 when coupled to Cy2. Both possible combinations of secondary antibodies, i.e., anti-rabbit Cy2/anti-mouse Cy3 and anti-rabbit Cy3/anti-mouse Cy2, were used to exclude fluorescent dye effects and yielded identical results. Analysis was done by confocal microscopy (TCS4D; Leica Mikrosysteme GmbH) and scans in several consecutive layers were saved as single images.

Immunoelectron Microscopy

30-d-old male rats were used for immunohistochemical studies. Tissue preparation for electron microscopic analysis was done as described by Richter et al. (1996). To test for nonspecific immunolabeling, sections were incubated exactly as described above, but in the absence of the first antibody, with preimmune rabbit serum or with an antibody solution that was preincubated with the fusion protein (2.3 mg/ml). In no case was any nonspecific immunoreactivity observed.

Localization of Bassoon in isolated synaptic structures with gold-conjugated antibodies was done using a modified protocol of De Camilli et al. (1983b). In brief, cortex and cerebellum from P30 rat brain were homoge-

nized in homogenization buffer (0.25 M sucrose, 25 mM KCl, 5 mM MgCl₂, 2 mM EGTA in 10 mM phosphate buffer, pH 7.4) and centrifuged for 10 min at 1,000 g to remove nuclei and cell debris. The supernatant (5 ml) was mixed with 35 ml fixation buffer (3% paraformaldehyde, 0.1% glutardialdehyde in 5 mM phosphate buffer), kept on ice for 30 min, and spun at 13,000 g for 45 min. The resulting pellet was rehomogenized in 0.6 ml of 5 mM phosphate buffer (pH 7.4), mixed with an equal volume of prewarmed 2% agarose in 5 mM phosphate buffer, and gently poured into coverslip frames. The agarose blocks were cut into 60-µm slices using a vibratome (Leica Inc., Deerfield, IL) and incubated for 30 min at room temperature in PBS containing 5% BSA and 0.1% CWF skin gelatin (Aurion) to block nonspecific binding. After this preincubation, sections were incubated overnight with rabbit anti-Bassoon antibody diluted 1:100 in PBS containing 0.1% BSA-C (Aurion, Wageningen, The Netherlands) and 0.2% sodium azide at room temperature. The sections were rinsed in BSA-C/PBS (3 × 10 min) and incubated with 50-fold diluted anti-rabbit IgG gold conjugate (5 nm; Sigma Chemical Co., St. Louis, MO) in BSA-C/PBS for 4 h. After extensive washing in PBS, sections were postfixed in 2% glutardialdehyde in PBS (15 min), and in 1% osmium tetroxide in PBS for 1 h. Further preparation for electron microscopic analysis was performed as described by Richter et al. (1996). The ultrathin sections were examined with a Leo912 electron microscope (Leo Elektronenmikroskopie GmbH, Oberkochen, Germany) and imaged with a Megascan 2K CCD camera (Gatan, Inc., Pleasanton, CA) using the digital micrograph Gatan 2.5 software.

Results

Identification of Bassoon

In search for new components of central nervous system synapses, we have used rabbit antisera against brain synaptic junctional protein preparations to screen a rat brain cDNA expression library (Garner et al., 1993; Kistner et al., 1993; Langnaese et al., 1996). One of the isolated clones, sap7f, contained a 733-nucleotide-long cDNA insert with a continuous open reading frame; its sequence did not resemble that of any known protein. Northern hybridization of sap7f cDNA probes to RNA preparations from 30-d-old rats revealed a band at ~13 kb in the brain, but not in liver, heart, skeletal muscle, or C6 glioma cells (Fig. 1 A), nor in testis, kidney, spleen, or thymus (data not shown). To determine the transcript distribution in the brain, *in situ* hybridization experiments were performed with a ³⁵S-labeled antisense oligonucleotide probe. As shown in Fig. 1 B, sap7f transcripts are widely expressed in the rat brain with highest levels in the cerebellum, the hippocampal formation, the piriform cortex, and the cerebral cortex. Application of 100-fold excess of unlabeled oligonucleotide specifically blocked hybridization signals (Fig. 1 C).

Starting with sap7f cDNA as probe, a set of overlapping clones spanning the entire protein coding region and parts of the untranslated regions of the corresponding mRNA was isolated from rat brain cDNA libraries (Fig. 2 A). Moreover, recombinant λ- and P1-phages were isolated containing the murine *bassoon* gene, and its exon-intron organization was determined by comparing mouse genomic DNA and rat cDNA. The gene has at least 13 exons, 10 of which harbor the entire open reading frame for Bassoon (Fig. 2 B). The positions of these introns with respect to the protein sequence are indicated in Fig. 3 A. Interestingly, approximately half of the cDNA sequence is contained in the large exon 5 (6.6 kb). The gene displays a rather compact structure in the region of exons 4–11 containing only introns <2 kb. In contrast, exons 1, 2, and 3 are spaced by larger introns. The 5' end of the gene has not yet been identified. Using FISH, the *bassoon* gene has been mapped to mouse chromosome 9F (data not shown).

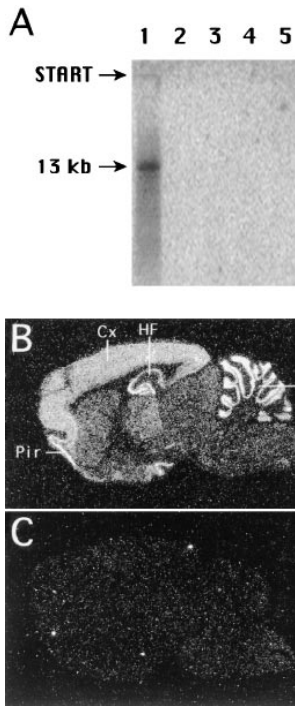


Figure 1. Bassoon transcripts in rat brain. (A) Northern analysis. A Nylon filter containing 20 μ g total RNA from brain (lane 1), liver (lane 2), heart (lane 3), and skeletal muscle (lane 4) of 30-d-old rats and from C6 glioma cells (lane 5) was hybridized with 32 P-labeled sap7f cDNA insert. (B and C) In situ hybridization. Sagittal sections of rat brain were hybridized with a 40-mer 35 S-labeled oligonucleotide probe in the absence (B) or presence (C) of 100-fold excess of unlabeled oligonucleotide. Cb, cerebellum; Cx, cerebral cortex; HF, hippocampal formation; Pir, piriform cortex.

As deduced from the nucleotide sequences, the encoded protein, hereafter referred to as Bassoon, consists of 3938 and 3942 amino acid (aa) residues in rat and mouse, respectively (Fig. 3 A), and has a calculated M_r of \sim 420 kD. The overall sequence identity of the two proteins is 96%. The putative initiation site for translation in rat and mouse (CCACCAUGG) favorably coincide with the consensus

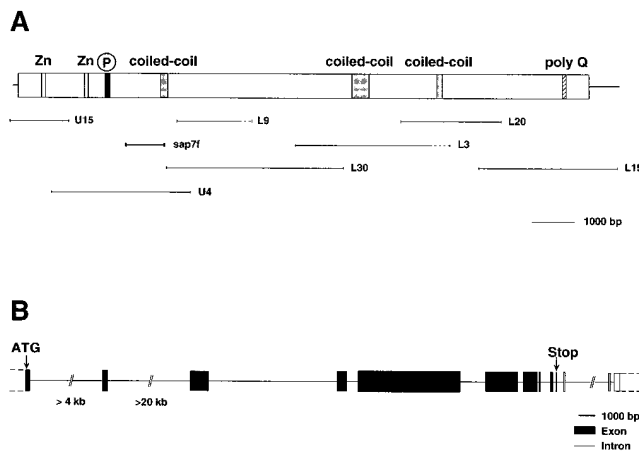


Figure 2. Structure of Bassoon cDNA and gene. (A) Physical map of the rat Bassoon cDNA. Protein-coding region is boxed. Predicted coiled-coil domains are indicated in gray. The extension of analyzed cDNA clones is indicated. Recombined or intron-containing regions are represented by a broken line. (B) Exon-intron organization of the murine *bassoon* gene. Protein coding region is indicated by filled, 3'-untranslated region by open boxes. Note, intron positions with respect to the open reading frame are indicated in Fig. 3 A. Zn, double zinc-finger motifs; P, heptad repeats, potential phosphorylation sites for proline-directed kinases; poly Q, poly-glutamine stretch.

motif described for vertebrates (Kozak, 1987). In rat cDNA the putative start codon is preceded by an in-frame stop codon. Comparison of nucleotide and deduced amino acid sequences to public databases showed that Bassoon has no significant overall similarity to any known protein. However, computer analysis of the predicted amino acid sequence revealed that Bassoon harbors two zinc-finger domains (aas 162–223 and 457–518 in rat), each with two zinc-finger motifs. The two Bassoon motifs share a higher degree of sequence identity with each other (47%) than with the zinc fingers of any other protein included in public databases (Fig. 3 B). The most closely related double zinc fingers are those of rabphilin, the rabphilin-related protein Noc2, and the Rab3a-interacting molecule Rim. Between aas 568 and 588 of the rat sequence, a region of three consecutive heptad repeats occurs. The corresponding region of murine Bassoon contains two additional repeats of this motif (consensus sequence: K-A-S-P-Q-A/T-A/T/K). Proximal to the COOH terminus (rat aa 3775–3799), a stretch of 24 consecutive glutamine residues is predicted (Fig. 3 A). Nineteen of these glutamines—14 consecutive ones—are encoded by CAG triplets. Interestingly, the number of glutamines in murine Bassoon (11 residues; Fig. 3 A) differs from that in rat. A computer-assisted examination of secondary structure of Bassoon predicts long α -helical regions. In particular for three regions high probabilities for coiled-coil structures are predicted (Figs. 2 A and 3 A).

To characterize the protein encoded by the Bassoon transcript, rabbit polyclonal antisera and a mouse monoclonal antibody, mab7f, were generated against a recombinant fusion protein containing the sap7f-encoded polypeptide fragment. On immunoblots of rat brain membrane fractions, these antisera detect two major protein bands of $>$ 400 and 350 kD (Fig. 4). In addition, a number of smaller immunoreactive bands are detectable. We assume that mature Bassoon migrates at $>$ 400 kD, while the other protein bands are proteolytic degradation products. This assumption is based on several observations: (a) the apparent molecular mass of $>$ 400 kD favorably coincides with the calculated M_r of \sim 420,000; (b) Two rabbit antisera and mab7f recognize a similar protein pattern on immunoblots (not shown), and yield identical results in immunohistochemistry (see below); (c) Both the number of bands $<$ 400 kD and their relative intensity as compared with that of the largest polypeptide varied from preparation to preparation (data not shown). (d) Northern analyses using probes derived from different regions of the Bassoon cDNA did not detect additional Bassoon transcripts in the adult brain, suggesting that the smaller polypeptides are not products from alternatively processed transcripts.

Bassoon is a Synaptic Protein

As the sap7f cDNA was isolated using antibodies against synaptic junctional protein preparations, we sought to examine whether Bassoon actually copurifies with these preparations, and how its subcellular distribution compares to that of other synaptic proteins. These include the presynaptic cytomatrix component Piccolo (Cases-Langhoff et al., 1996), the PSD protein SAP102 (Müller et al., 1996), the integral synaptic vesicle protein synaptophysin (Wiedenmann and Franke, 1985), and the vesicle- and cy-

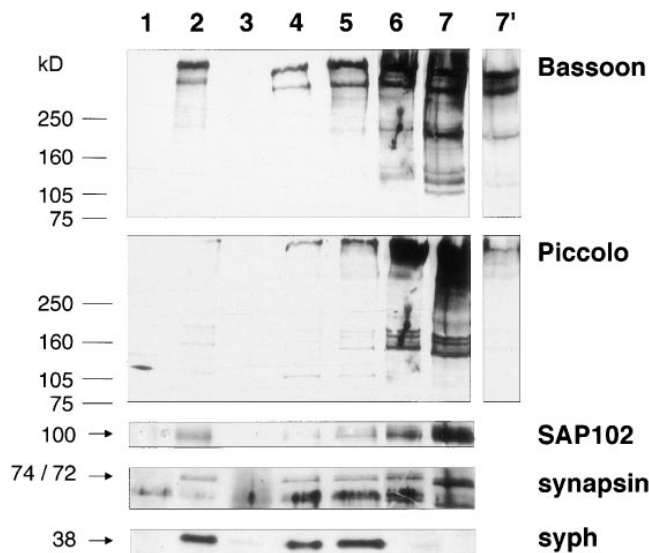


Figure 4. Bassoon is highly enriched in synaptic junctional protein preparations. Synaptic proteins were prepared according to Carlin et al. (1980). Western blots (15 μ g protein per lane) of the soluble protein fraction (lane 1), the crude membrane fraction P2 (lane 2), the myelin fraction (lane 3), the light membranes fraction (lane 4), the synaptosomal fraction (lane 5), detergent-extracted synaptosomes (lane 6; i.e., One Triton; Kennedy, 1997) and the twice triton-extracted PSD fraction (lanes 7 and 7'; Two Triton; Kennedy, 1997) were probed with mab7f using a chemiluminescent detection system (lane 7' represents a short exposure of lane 7). Sizes of marker proteins are indicated in kD. Two major protein bands of 420 and 350 kD and several putative proteolytic cleavage products are enriched in the PSD fraction. For comparison, Western blots were reprobed with antibodies against Piccolo, SAP102, synapsin I, and synaptophysin (syph). Note that a major degradation product of synapsin is also detected by the synapsin antiserum (Sikorski et al., 1991).

(Cases-Langhoff et al., 1996), is essentially co-distributed with Bassoon in processes of hippocampal neurons (Fig. 5, C and D). These observations support the view that Bassoon is a synaptic protein.

We next determined the distribution of Bassoon as compared with synapsin I (Fig. 6) and synaptophysin (not shown, data are similar to that of synapsin) in rat brain sections by immunofluorescence microscopy. As an example, the hippocampal CA3 region was analyzed. At an intermediate magnification, synapsin and Bassoon appear largely codistributed as indicated by the emergence of yellow mixed color of the two fluorescent dyes (Fig. 6, A–C; Cy2, Bassoon; Cy3 synapsin). At a high magnification the two antigens do not colocalize. Bassoon immunoreactivity appears as distinct little spots adjacent to unstained dendrites, whereas synapsin displays a more homogeneous distribution within synaptic terminals (Fig. 6, D–K). A similar differential localization within presynapses has been observed for Piccolo vs synaptophysin (Cases-Langhoff et al., 1996).

Bassoon is Associated with Presynaptic Structures of Hippocampal Synapses

The subsynaptic localization of Bassoon was further explored by immunoelectron microscopy on ultrathin sec-

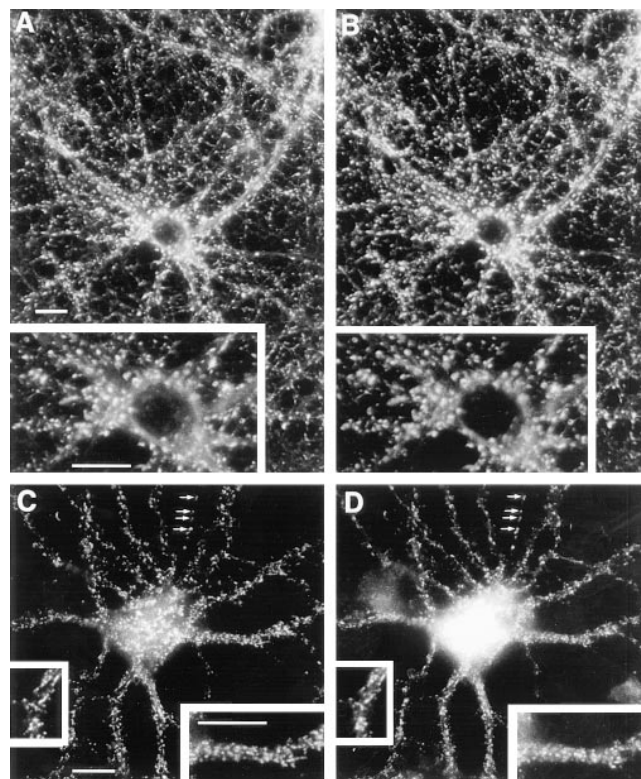


Figure 5. Synaptic localization of Bassoon, synaptophysin, and Piccolo immunoreactivities in mature cultures of hippocampal neurons (21 d in vitro). Double images of hippocampal neurons fluorescently labeled with polyclonal rabbit (A) or mouse (C) antibodies against Bassoon, a monoclonal antibody against synaptophysin (B), or a polyclonal anti-Piccolo antiserum from rabbit (D). Secondary goat anti-rabbit IgG and anti-mouse antibodies were coupled to fluorescein (A), Cy3 (B and C), and Cy2 (D). Insets are close-ups of areas shown in the larger panels. Bassoon colocalizes with the presynaptic proteins synaptophysin and Piccolo (see arrows in C and D). Bars, 10 μ m.

tions from various regions of 30-d-old rat brain. In all preparations, Bassoon immunoreactivity was detected with both polyclonal antibodies and monoclonal antibody mab7f exclusively in presynaptic nerve terminals (Fig. 7). Generally the immunoreaction product is highly concentrated at sites of synaptic contact. For example, in excitatory mossy fiber terminals in the *stratum lucidum* of the hippocampal CA3 region, which make multiple contacts to postsynaptic neurons, Bassoon immunoreactivity is largely restricted to regions at the presynaptic membrane opposite to PSDs (Fig. 7 A). Figs. 7 B and C give examples of the distribution of Bassoon immunoreactivity at shaft and spine synapses in the *stratum lucidum* and the *stratum moleculare* of CA3, respectively. Again, immunoreactivity is very strong at the synaptic contact site, and at least the shaft synapses contain synaptic vesicles not surrounded by immunoreaction product.

To confirm further the presynaptic localization of Bassoon we have applied immunogold electron microscopy to isolated synaptosomes. No specific labeling of synaptic structures was observed when the first antibody was omitted (Fig. 7 F), or when a rabbit antibody against the extracellular

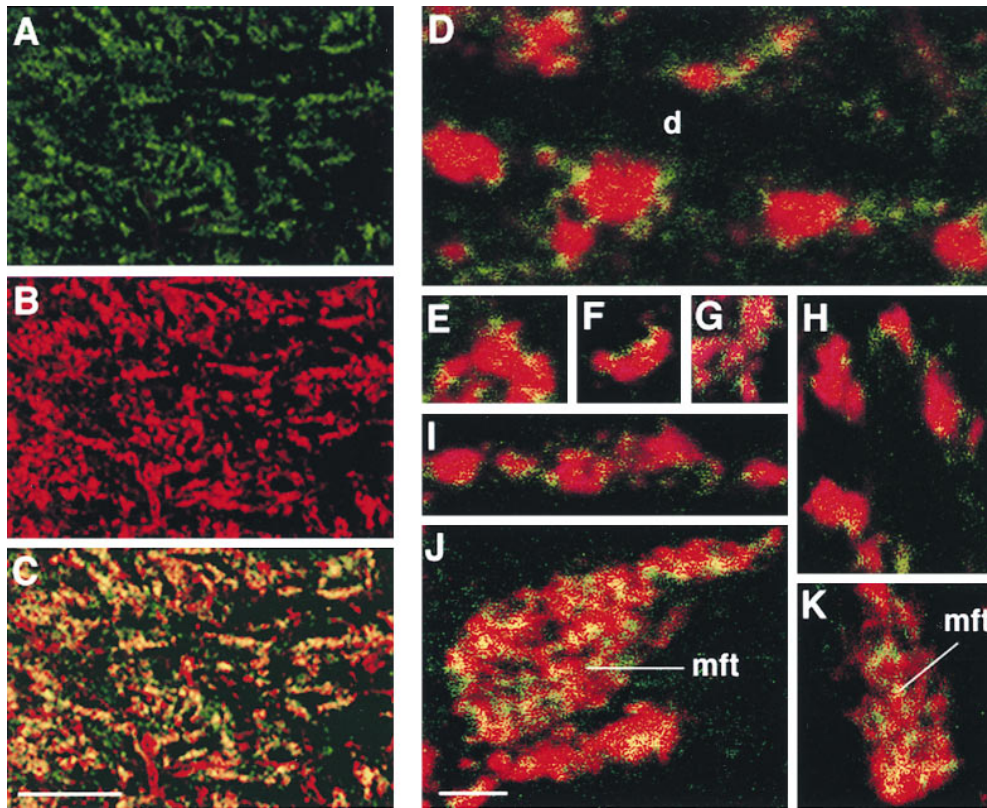


Figure 6. Comparison of the distribution of Bassoon and synapsin I immunoreactivities at synapses of the hippocampal CA3 region. Confocal images of the distribution of Bassoon (A) and synapsin I (B). At intermediate magnification the two fluorescent labels appear largely codistributed (C). High magnification images suggest a more restricted localization of Bassoon as compared with synapsin in examples of individual synaptic structures (D–K), including shaft and spine synapses (D–I) as well as mossy fiber terminals (mft, J and K). Dendrites (d) are unstained. Bassoon and synapsin immunoreactivities were detected with Cy2-coupled goat anti-mouse and Cy3-coupled goat anti-rabbit antibodies, respectively. Bars: (A–C) 20 μm ; (D–K) 2 μm .

matrix protein brevican (Seidenbecher et al., 1995) was used (data not shown). As shown in Fig. 7 D, gold particles are restricted to the presynaptic element, and are interspersed between clear synaptic vesicles. In the presynaptic compartment, the distribution appears nonhomogeneous with the highest concentration of particles in the vicinity of the electron-dense material at the synaptic contact site, although particles were barely found directly at the presynaptic membrane. It has to be noted that in using preembedding immunogold labeling, the nonhomogeneous distribution of Bassoon in nerve terminals is not as obvious as when it is revealed by immunoperoxidase staining in situ (Figs. 7 A–C). This fact may be due to the labeling method where gold-labeled antibodies can merely enter synaptosomes that are open for some time during incubation, and thus may have lost a fraction of their reserve pool vesicles. In this context it is interesting that synaptic structures were observed that have lost the plasma membrane around the presynaptic element during preparation (Fig. 7 E). In these structures clear synaptic vesicles remain embedded in a network of amorphous material labeled with gold particles.

To assess the nature of Bassoon interaction with the presynaptic element, biochemical extraction studies were performed with various agents on the brain P2 crude membrane fraction. Neither high-salt conditions, nonionic detergents like Triton X-100 or Octylglucoside, nor zwitter ionic detergents such as CHAPS, are able to solubilize significant amounts of Bassoon (Table I). In contrast, combinations of CHAPS and high salt result in a partial solubilization of the protein. Combinations of nonionic detergents and high salt also lead to partial solubilization of Bassoon (data not shown); however, under these conditions enhanced proteo-

lysis is observed to hinder the reliable interpretation of the results. One molar Tris-HCl that partly solubilizes spectrin from the cortical cytoskeleton (Hayes et al., 1991) does not release Bassoon. On the other hand, alkaline conditions that typically bring peripheral membrane proteins into solution also solubilize Bassoon, as do the chaotropic salt potassium rhodanite, urea, or the ionic detergent SDS. These results indicate that Bassoon is not an integral membrane protein, but tightly interacts with preparations of the membrane-associated cortical cytoskeleton.

Discussion

Bassoon is a novel protein expressed in brain. It contains only few predictable structural features, including two zinc-finger and three coiled-coil domains, and harbors a stretch of polyglutamines encoded by CAG repeats. This later feature makes the human *BASSOON* gene a candidate for an association with late-onset neurodegenerative diseases caused by expansion of CAG repeats (Reddy and Housman, 1997). Bassoon copurifies with synaptic junctional protein preparations (PSD fraction)² and is detected

2. It should be noted that Bassoon is a presynaptic protein that is highly enriched in the so-called PSD protein fraction. It has been shown previously that presynaptic proteins do occur in this fraction (Langnaese et al., 1996). Another well-documented example of this kind is the presynaptic cytomatrix component Piccolo (Cases-Langhoff et al., 1996). In a recent publication Ziff (1997) provides electron microscopic evidence for the appearance of presynaptic dense projections in the PSD preparation. This indicates that, though the PSD fraction indeed contains primarily postsynaptic proteins (Kennedy, 1997), the name of the fraction may be misleading. Therefore, we refer to this fraction as synaptic junctional protein preparation.

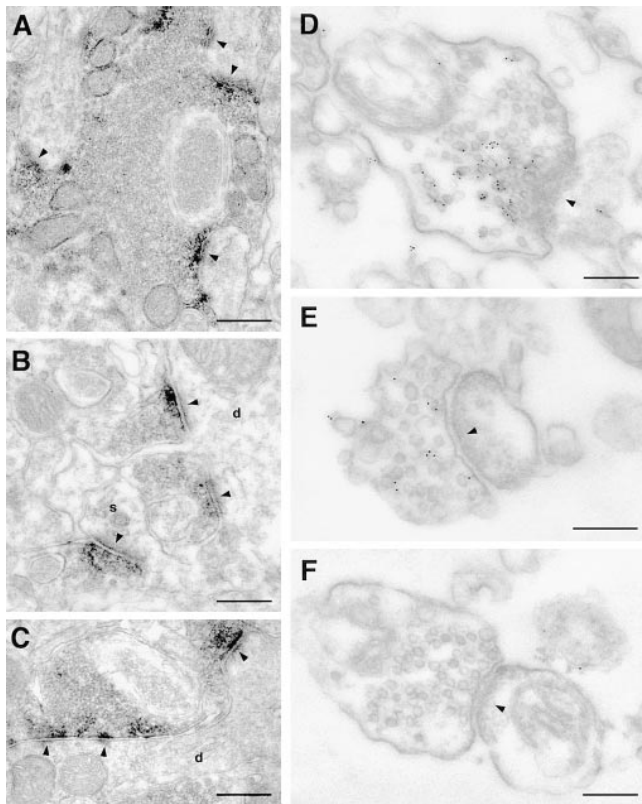


Figure 7. Ultrastructural localization of Bassoon in presynaptic terminals. Electron micrographs of ultrathin sections of rat brain (A–C) and agarose-embedded synaptosomal preparations (D–F) are shown. Analyses were performed with mab7f (A–C) and rabbit polyclonal antiserum against Bassoon (D–E). (A) Excitatory mossy fiber bouton in the *stratum lucidum* of the hippocampal CA3 region. The presynaptic terminal is densely filled with synaptic vesicles. The peroxidase reaction product is highly concentrated at regions opposite to PSDs (arrowheads). Spiny dendritic processes of pyramidal cells are unstained. (B) Immunopositive axon expansions or terminals contacting a dendritic shaft (d) and spine (s) in the *stratum lucidum* of CA3. (C) Bassoon immunoreactivity in shaft synapse of the molecular layer of CA3 with apparently separate active zones. Immunoreactivity appears most intense at sites facing PSDs. Bars: (A) 0.5 μm ; (B) 0.2 μm ; (C) 0.4 μm . (D–F) Ultrastructural localization of Bassoon in nerve terminals by immunogold electron microscopy. Rabbit antibodies were visualized by anti-rabbit IgG coupled with 5 nm gold. Gold particles are highly enriched in presynaptic elements. Bassoon appears not uniformly distributed between all synaptic vesicles (D). Gold labeling of amorphous material between vesicles is preserved, even if the plasma membrane around the presynaptic element is missing (E). Upon incubation without first antibody only very few gold particles are found scattered throughout the preparation (F). PSDs are indicated by arrowheads. Bars (D–F), 0.2 μm .

in presynaptic nerve terminals of a variety of synapses. Within nerve terminals, Bassoon immunoreactivity is found concentrated in patches underneath the plasma membrane at regions located opposite to PSDs. This particular localization together with its biochemical characteristics is consistent with the hypothesis that Bassoon is tightly associated with or an integral component of the presynaptic cytomatrix at the site of neurotransmitter release.

Table I. Association of Bassoon with Rat Brain Membranes

Conditions for extraction of P2 crude membrane fraction*	Solubilization of Bassoon immunoreactivity
5 mM Hepes, pH 7.4; 320 mM sucrose	–
25 mM Tris-HCl, pH 8.0 [‡]	–
1 M Tris-HCl, pH 8.0	–
0.5 M NaCl	–
1 M NaCl	–
100 mM Na ₂ CO ₃ ; pH 11.5 (no Tris-HCl)	++
1% Triton X-100	± [§]
1% <i>n</i> -Octyl- β -D-glucopyranoside	–
1% CHAPS	± [§]
1% CHAPS; 1 M NaCl	+
1% SDS	++
8 M urea	+
3 M potassium rhodanite	++

*For preparation of fraction P2 and details of extraction see Materials and Methods. [‡]If not stated otherwise, all further extractions were done in the presence of 25 mM Tris-HCl, pH 8.0. [§]Occasionally extraction of some Bassoon immunoreactivity in the range of 100–200 kD is observed. It is not clear, however, whether this extraction is due to real solubilization or proteolytic release of soluble fragments of Bassoon.

Lessons from the Primary Structure of Bassoon

Bassoon is a very large polypeptide consisting of 3938 (rat) and 3942 (mouse) amino acid residues that do not belong to any known protein family. Nonetheless, Bassoon contains a number of sequence motifs that allow some speculation concerning its functions. Two double zinc-finger motifs are located in the amino-terminal part of Bassoon. Multiple classes of zinc finger-containing proteins have been described that are involved either in protein–nucleic acid or protein–protein interactions. The Bassoon zinc-finger motifs show some structural features related to LIM (*lin-11/ISL-1/mec-3*-like) domains, a steadily growing family of structural motifs involved in protein–protein interactions (Sánchez-García, 1994). Typically, LIM domains appear as double zinc fingers with a finger loop size of 17 ± 1 residues and a spacing of two amino acid residues between the two fingers. With sizes of 16 (first loop) and 14 amino acids (second loop), the putative zinc fingers in Bassoon almost match this LIM motif size. The spacing between the two finger entities is four instead of two residues. Although Bassoon lacks a conserved coordinating histidine in the first finger structure as well as some other characteristic amino acid residues characteristic for LIM domain proteins, the structural features described above suggest a role for the zinc finger motif in protein–protein interactions. This protein–protein interaction is underscored by the fact that the Bassoon zinc-finger motifs show highest similarity to the zinc fingers of rabphilin (Shirataki et al., 1993), a protein known to interact with and regulate the activity of the synaptic vesicle-associated small GTPase Rab3 (for review see Südhof, 1997). Interestingly, Rim, another potential regulator of Rab3, also interacts with its target via a zinc-finger domain (Wang et al., 1997). The sizes of the zinc-finger loops of rabphilin, its relative Noc2 (Kotake et al., 1997), and Rim differ even more from those of typical LIM-type domains than the lengths of Bassoon zinc fingers. However, all four proteins share the four-amino acid residue spacing between the two finger structures (see Fig. 3 B). Thus, the zinc fingers of Bassoon may interact with vesi-

cle-associated proteins in the presynapse, and may potentially be involved in regulating the synaptic vesicle cycle.

Superhelix-forming coiled-coil domains are another class of structural entities involved in inter- or intramolecular protein-protein interactions (Lupas, 1996). By computer analysis, Bassoon is predicted to have three coiled-coil-forming domains of various lengths that may play a role in the interaction of Bassoon with other presynaptic proteins. Another remarkable feature is the region of heptad repeats (K-A-S-P-Q-A/T-X) that varies in length between rat (three copies) and mouse (five copies) Bassoon. The repeats may serve as phosphorylation sites for proline-directed protein kinases, including stress-activated and mitogen-activated protein kinases (for review see Cohen, 1997). Proline-directed protein kinases are known to phosphorylate and thereby regulate a number of cellular substrates, including cytoskeletal components such as neurofilament proteins in response to various kinds of extracellular signals (Cohen, 1997; Giasson and Mushynski, 1997).

Intriguingly, proximal to the COOH-terminal end of Bassoon is a polyglutamine region encoded by multiple CAG codons. Also, this polyglutamine stretch is variable in length between the two species. The presence of expanded CAG repeats in a number of genes has been described, and has been directly implicated in dominantly inherited neurodegenerative disorders characterized by anticipation (for review see Reddy and Housman, 1997; Ross, 1997). These disorders include Huntington's disease caused by CAG expansion in the *HD* gene; spinobulbar muscular atrophy where the androgen receptor gene is affected; dentatorubral-pallidoluysian atrophy, and several types of spinocerebellar ataxias (Reddy and Housman, 1997; Ross, 1997). At present it is unclear whether the CAG repeats in the *BASSOON* gene are associated with any neurodegenerative disorder. Mapping of the mouse *bassoon* gene to chromosome 9F did not immediately suggest a candidate gene, but clearly will facilitate the characterization and mapping of the human *BASSOON* gene—an important first step in assessing its involvement in debilitating diseases.

Bassoon is Concentrated at the Presynaptic Active Zone

Bassoon exhibits a widespread synaptic distribution throughout the adult rat brain. High levels of Bassoon transcripts are observed in several brain regions including the hippocampus and the cerebellum. We have analyzed several types of synapses in these two brain regions for the distribution of Bassoon at the ultra-structural level. The most striking feature is the restricted distribution of Bassoon immunoreactivity within presynaptic terminals. This is most obvious in the large mossy fiber boutons in the *stratum lucidum* of the hippocampal CA3 region. These large fusiform expansions, filled with synaptic vesicles and mitochondria, are studded with excitatory synapses (Llinás and Walton, 1990; Amaral and Witter, 1994). Bassoon immunoreactivity is unevenly localized within these nerve terminals, and appears to be concentrated at regions of the presynaptic bouton that are juxtaposed to the PSD. This restricted localization contrasts with the much-wider distribution of other presynaptic proteins such as synapsin I (De Camilli et al., 1983a), synaptophysin (Wiedenmann

and Franke, 1985; Kagotani et al., 1991), syntaxin (Garcia et al., 1995), and SNAP-25 (Garcia et al., 1995), and suggests a role for Bassoon in events occurring near or at the active zone. Immunogold localization studies on isolated synaptic structures support this view. Bassoon is found interspersed between a subpopulation of synaptic vesicles proximal to the synaptic cleft. However, no or very few Bassoon molecules appear to be associated directly with the presynaptic membrane facing the synaptic junction.

Electron microscopic studies of the presynapse have revealed the presence of a fine filamentous network anchored to the junctional plasmalemma (Landis et al., 1988; Hirokawa et al., 1989). In synaptic junction preparations, frequently vesicle-containing presynaptic structures that are not wrapped by a plasma membrane are observed. We assume that these structures represent the presynaptic cytomatrix that sticks to the junctional membrane as a gel-like amorphous network. Bassoon molecules are included in this cytomatrix.

In a recent study we described another presynaptic protein called Piccolo (Cases-Langhoff et al., 1996). This 420-kD protein is also found in a wide variety of presynaptic terminals throughout rat brain. This observation, as well as the colocalization of Piccolo and Bassoon in cultured hippocampal neurons shown here, implies that both proteins can occur in an overlapping, if not identical, set of synapses. Interestingly, the subsynaptic localization of Piccolo in asymmetric type I synapses, e.g., in hippocampal mossy fiber terminals, is virtually identical to that described here for Bassoon, suggesting that both proteins are concentrated near the active zone of the same synapse.

Immunogold localization studies presented here and in a previous paper (Cases-Langhoff et al., 1996) raise the question as to whether Bassoon and Piccolo may directly interact with synaptic vesicles. Immunoreactivity of both proteins is found in the light membrane fraction (see Fig. 4) which includes a major fraction of synaptic vesicles. Preliminary experiments suggest that Bassoon is present in crude conventional vesicle preparations as described by Huttner et al. (1983). There is, however, no enrichment of Bassoon immunoreactivity in this fraction, and at present we cannot exclude that Bassoon partitions into this fraction as a contamination (Sanmartí-Vila, unpublished observation). In any case, association with detergent-insoluble cytomatrix is much stronger than with detergent-soluble membrane fractions.

Possible Functional Implications

The restricted localization of Bassoon and Piccolo suggests that they serve specific functions at synaptic junctions. Both proteins are components of the presynaptic cytomatrix. As such they may play a role in the structural and functional organization of the synaptic vesicle cycle, i.e., the release of neurotransmitter by calcium-triggered exocytosis, the endocytotic retrieval of vesicles and the refilling with neurotransmitter (for review of the synaptic vesicle cycle see Südhof, 1995; De Camilli and Takei, 1996).

Synapsins are presynaptic proteins thought to anchor synaptic vesicles to actin filaments (Hirokawa et al., 1989). The exact localization of synapsin-associated synaptic ves-

icles within the presynaptic terminal is still a matter of debate (compare Pieribone et al., 1995; Rosahl et al., 1995). One hypothesis implies that synapsin I is associated with the reserve pool of vesicles that are localized in a zone distal from the transmitter release site, whereas vesicles in the proximal zone are devoid of synapsin I (Pieribone et al., 1995; Takei et al., 1995). We have shown that within the presynaptic terminal, Bassoon and Piccolo are concentrated adjacent to the synaptic cleft. This distribution is complementary to that proposed for synapsin I. Adopting the abovementioned hypothesis, Bassoon and Piccolo may be associated with synaptic vesicles of the release pool, and thus exert their functions (e.g., in synaptic vesicle cycling) in a compartment spatially distinct from that of synapsin I action.

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