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# Membrane Association of Presynaptic Cytomatrix Protein Bassoon

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**Components of the specialized cytomatrix at active zones of presynaptic nerve terminals are thought to be involved in organizing synaptic events such as immobilisation or translocation of synaptic vesicles and assembling active zone components. The 420-kDa non-transmembrane protein Bassoon is a specific component of the presynaptic cytomatrix that shares features with both cytoskeleton-associated and peripheral-membrane proteins. Using immunogold electron microscopy we show here that synapse associated Bassoon is distributed in a subregion of active zones. Using a biochemical assay we show that a fraction of Bassoon is membrane associated. Electron microscopy performed on the same biochemical fraction further revealed that Bassoon is associated with vesicular structures. Together these data suggest that at least a fraction of Bassoon is associated with a membraneous compartment in neurons.** 

***Key Words:*** **synapse; active zone; neurotransmitter release; immunogold EM.**

Neurotransmitter release from synaptic terminals is restricted to specialized areas of the presynaptic plasma membrane, the active zones, where synaptic vesicles (SVs) dock and fuse (1). While the mechanisms restricting transmitter release to active zones are unknown, a specialization of the cortical cytoskeleton, the presynaptic cytomatrix or CAZ (cytomatrix at active zones) is thought to play a role in active zone assembly and function (2). Recently we identified a 420 kDa-protein termed Bassoon as a component of the CAZ. Bassoon is found both at conventional excitatory and inhibitory synapses throughout the brain and at a subset of retinal ribbon synapses (3–6). In nerve terminals of cultured neurons Bassoon colocalizes with SV marker proteins and Piccolo/Aczonin, a CAZ-protein structurally related to Bassoon (7–9), prior to the clustering of glutamate receptors, suggesting a role in active zone assembly (10, 11). In adult brain, the majority of Bassoon is firmly anchored in the CAZ as indicated by its enrichment in detergent-resistant preparations of synaptic junctions. However, a fraction of Bassoon is detected in the light membrane fraction that includes SVs (3). An important step towards elucidating the function of Bassoon thus is finding out whether it is a purely cytoskeleton associated protein, or whether it indeed can bind membranes and what is the nature of these membranes.

Abbreviations used: BSA, bovine serum albumin; CAZ, cytomatrix at active zones; EM, electron microscopy; PBS, phosphate buffered saline; SV, synaptic vesicle.

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## MATERIALS AND METHODS

*Cryo-immunogold labelling of tissue sections.* The experimental protocol for the cryo-immunogold technique by modifying a method from Tokuyasu (12) has been described in detail recently (6). Briefly, cryo-sections of hippocampus and neurohypophysis derived from adult Wistar rats were immunolabeled and stained according to a modified method from Griffiths (13). The following solutions were applied subsequently: 1. 50 mM glycine in phosphate buffered saline (PBS: 137 mM NaCl, 3 mM KCl, 15 mM $Na^+/K^+$-phosphate buffer, pH 7.4; for 10 min); 2. 5% bovine serum albumin (BSA) in PBS (15 min); 3. primary antibody in PBS containing 1% BSA (2 h); 4. PBS buffer (3 × 5 min); 5. gold-conjugated secondary antibody (2 h); 6. PBS buffer (6 × 3 min); 7. 2.5% glutaraldehyde in PBS (10 min) for postfixation; 8. distilled water (4 × 2 min). Sections were then floated on drops of 1.5% methyl cellulose, containing 2% uranyl acetate (10 min on ice). They were examined in a Zeiss EM 902 electron microscope operated at 80 kV. Antibodies were: Monoclonal antibody against synaptobrevin II (1:50, clone 69.1; Synaptic Systems, Göttingen, Germany); polyclonal antibody against Bassoon (1:20; tom Dieck *et al.*, 1998). Secondary antibodies (10 nm immunogold-



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conjugated goat anti-rabbit IgG or goat anti-mouse IgG) were obtained from British BioCell International (Cardiff, UK) and used at a dilution of 1:20.

*Subcellular fractionation.* Fractions were prepared according to Huttner *et al.* (14). Fraction LP2, representing a crude vesicle fraction, was subjected to floatation analysis. The floatation assay was performed as described in Hsu *et al.* (15) with minor modifications. Briefly, 300 mg total protein were resuspended in 300 $\mu$l gradient buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM dithiothreitol) including 55% sucrose with or without 1% Triton X-100 and homogenized with a 25-gauge needle. After incubating 20 min on ice, samples were centrifuged for 10 min at 4°C at 5000 rpm in a table top centrifuge to remove air bubbles which would hinder placement of the suspension under the gradient. The suspension was placed under a 25–52.5% sucrose gradient using a $\mu$l-syringe. After centrifugation at 100000 g for 16 h in a SW55 rotor (Beckman T7) the gradient was fractionated into 200 $\mu$l portions. Fractions were analysed by Western blotting and immunodetection as described (3).

*Immunogold analysis of subcellular fractions.* For immunogold analysis, pellet LP2 obtained from 3 P30 rat cortices was resuspended in 5 ml fixation buffer (3% paraformaldehyde, 0.1% glutaraldehyde in 5 mM phosphate buffer pH 7.4) for 1 h and spun at 13000 g for 45 min. The resulting pellet was rehomogenized in 0.2 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. Cutting of agarose blocks and further processing of sections were performed as described (3). Antibodies applied were a mixture of rabbit anti-Bassoon (3; 1:50) and mouse anti-synaptophysin (1:100; DAKO, Hamburg, Germany) and a mixture of anti-rabbit IgG gold conjugate (5 nm) and anti-mouse IgG gold conjugate (10 nm; Sigma).

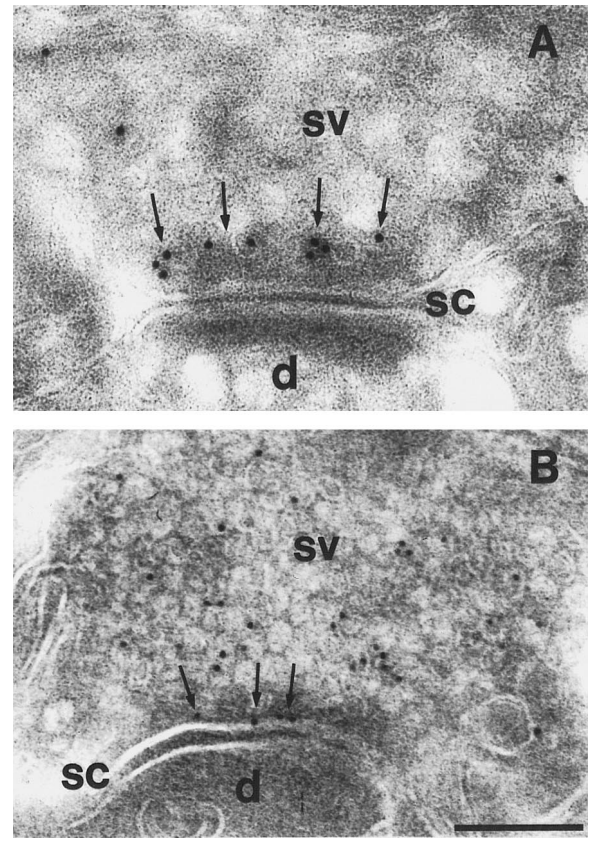


**FIG. 1.** Immunolabeling of synaptic contacts in the rat hippocampal CA3 subfield using the cryo-immunogold technique. (A) On immunolabeling for Bassoon, gold particles are concentrated close to but at a distance from the active zone (small arrows). In addition, scarce labeling of synaptic vesicles is observed. (B) The anti-synaptobrevin II antibody labels synaptic vesicles. Gold particles are also found in immediate apposition to the plasma membrane at the active zone (small arrows), presumably representing synaptobrevin II bound to its plasma membrane receptors (23). d, dendrite; sc, synaptic cleft; sv, synaptic vesicles. Bar = 200 nm.

## RESULTS

In presynaptic nerve terminals, Bassoon is found highly enriched at neurotransmitter release sites (3). Here, we have used immunogold EM to determine the localization of Bassoon at hippocampal synapses in detail. The analysis revealed a striking enrichment of Bassoon immunoreactivity interspersed between SVs that are situated in the immediate vicinity of the active zone plasma membrane (Fig. 1A). Gold particles were consistently located at a distance (between one and three SV diameters) from the active zone plasma membrane. In contrast, immunoreactivity for the SV transmembrane protein synaptobrevin II was found throughout SV aereas including the aerea of docked SVs (Fig. 1B). Occasionally, Bassoon immunoreactivity was found in extrasynaptic locations, where it typically appeared to be associated with vesicular structures (Fig. 1A).

To investigate whether Bassoon or fractions of Bassoon may be associated with membranes we employed a floatation assay, in which membranes and membrane associated proteins float up when placed under a sucrose gradient, while soluble proteins and proteinaceous aggregates remain at the bottom (15, 16). The major fraction of Bassoon associated with pellet P2 of classical synaptic junction preparations (17, 3) does not float under these conditions, presumably due to association of Bassoon mainly with the protein rich synaptic cytomatrix (data not shown). Therefore we used as starting material LP2 fractions, which contain synaptic vesicles and other membranes released from lysed synaptosomes as well as non-synaptosomal organelles (14). This fraction contained about 10–20% of total Bassoon of adult rat brain (data not shown). In the assay, Bassoon immunoreactivity clearly peaked in fractions taken from the top of the gradient, indicating that Bassoon was associated with membranes (Fig. 2A). Accordingly, Bassoon immunoreactivity largely codistributed with immunoreactivity for the SV transmembrane protein synaptophysin in the upper fractions of the gradient. However, the peak of Bassoon immunoreactivity was shifted, typically by one fraction, from that of synaptophysin, raising the possibility that Bassoon or a fraction of Bassoon was associated with membranes distinct from SVs. Moreover, unlike Bassoon, synaptophysin produced a second peak closer

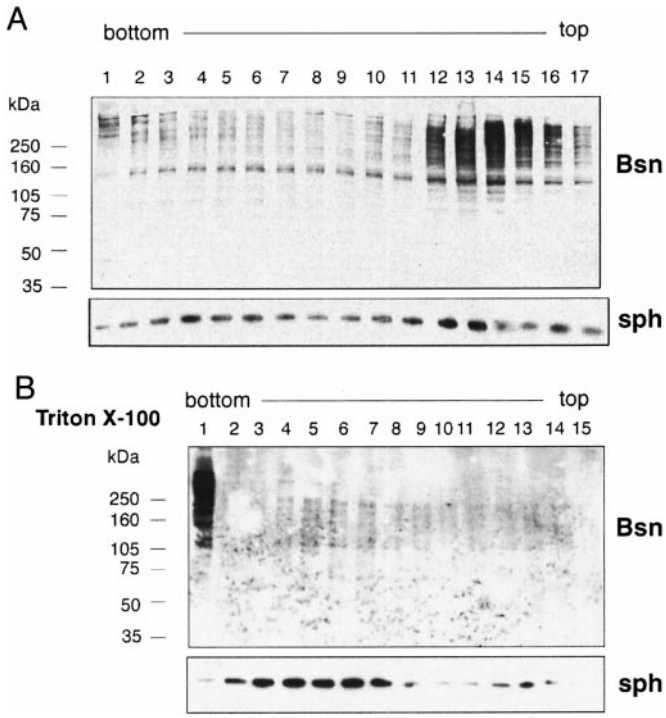


**FIG. 2.** Western blot analysis of samples taken from a floatation assay, in which membranes and associated proteins float up from the bottom of a sucrose gradient. (A) Bassoon (Bsn) is highly enriched in samples taken from the top of the gradient, indicating that it is bound to membranes. The SV transmembrane protein synaptophysin (sph) is enriched in the same region of the gradient, but has an additional peak and a wider distribution within the gradient. (B) When assayed upon solubilisation of membranes, Bassoon does not float into the gradient, while synaptophysin preferentially migrates into fractions near the bottom.

to the bottom of the gradient. This biochemical behavior is identical to that described for synaptobrevin and may in fact be characteristic for SV transmembrane proteins (16). Solubilization of membranes with Triton X-100 prior to assaying resulted in loss of Bassoon immunoreactivity from floating fractions and its redistribution to the bottom of the gradient (Fig. 2B). This result is consistent with Bassoon being released as a monomeric protein or protein complex upon extraction of membranes. Synaptophysin immunoreactivity also redistributed from the top of the gradient, but did not enrich in the pellet, presumably through incorporation into Triton micelles. To further corroborate these data we analysed LP2 fractions by EM. Immunogold labelling revealed that Bassoon was associated with two types of structures, i.e. either vesicular membranes (Figs. 3A–3C) or amorphous entities (Figs. 3D and 3E), whereas synaptophysin was only found associated with vesicles. Typically, Bassoon positive amorphous material was itself found associated with vesicles (Figs. 3D and 3E). Double positive vesicles (Fig. 3C) were rare (less than 5% of synaptophysin positive vesicles).

## DISCUSSION

In this study we show that a fraction of Bassoon is associated with rat brain membranes and provide immunogold EM data on the ultrastructural localization of Bassoon in nerve terminals. The characteristic localization of Bassoon at a regular distance from the active zone plasma membrane is reminiscent of its localization at retinal synapses (5), and raises the possibility that Bassoon in general performs a function specifically related to the proximal pool of SVs situated between docked SVs and reserve pool SVs. Several layers of SVs in addition to docked SVs remain associated with active zones upon perturbation of the reserve pool in lamprey giant synapses (18, 19), and CAZ-proteins like Bassoon may serve to anchor these vesicles. However, given the large size of Bassoon, additional interactions with both the plasma membrane and more distally located SVs are well conceivable.

An important finding of our study is that a fraction of Bassoon associates with membranes, because this argues against the possibility that Bassoon is a purely cytoskeleton associated protein. We have previously detected Bassoon immunoreactivity in light membrane fractions obtained by density gradient centrifugation, but due to the limitations of this method could not exclude the possibility that Bassoon partitioned into this fraction as a purely proteinaceous contamination (3). The results of our floatation assay prove that a fraction of Bassoon is bound to membranes. A possible mechanism for membrane association might be N-myristoylation, for which Bassoon contains a consensus sequence (3).

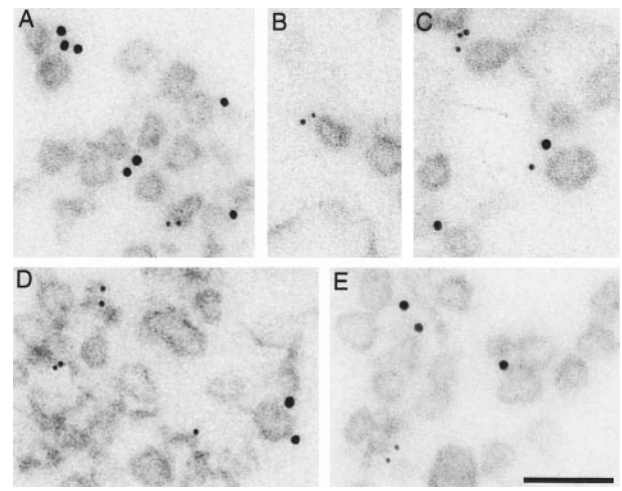


**FIG. 3.** Double-immunolabeling of crude vesicle fraction LP2. Fractions were fixed and double immunolabeled for Bassoon (small gold particles) and synaptophysin (large gold particles). Bassoon was found associated with vesicles (A–C) and amorphous material (D, E), while synaptophysin was only associated with vesicles (A–E). Bar = 100 nm.

Bassoon was previously found to be associated with amorphous material interspersed between SVs in isolated synaptosomes (3). The present data would be consistent with a subset of vesicles carrying Bassoon and amorphous material representing remnants of the cytomatrix. However, the majority of Bassoon is firmly anchored in the CAZ in a detergent resistent fashion (20, 3) and does not float in the membrane association assay. Thus, protein found to float may include Bassoon localized outside the CAZ. This fraction of Bassoon may be associated with SVs, as suggested by our in situ data, or with organelles distinct from SVs. In keeping with the latter possibility, the peak of Bassoon immunoreactivity in floating fractions was shifted as compared to the peak of synaptophysin. Recent data suggest that synaptogenesis involves co-transport of active zone transmembrane proteins and SV components via transport vesicles (21). In support of this notion, in embryonic brain the majority of Bassoon is bound to vesicles, and these vesicles also carry Piccolo, but not synaptophysin, suggesting that they represent a novel class of transport vesicles (22). Hence, the fraction of Bassoon we find to be membrane-associated, might represent Bassoon en route to synapses, where it might be involved in turnover of CAZ-associated Bassoon.

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