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Molecular Characterization and Spatial Distribution of SAP97, a Novel Presynaptic Protein Homologous to SAP90 and the *Drosophila* Discs-Large Tumor Suppressor Protein

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Synapses are highly specialized sites of cell–cell contact involved in signal transfer. The molecular mechanisms modulating the assembly and stability of synapses are unknown. We previously reported the identification of a 90 kDa synapse-associated protein, SAP90, that is localized at the presynaptic termini of inhibitory GABAergic synapses. SAP90 is a mosaic protein composed of three 90 amino acid residue repeats, an SH3 domain and a region homologous to guanylate kinases. SAP90 shares domain specific homology with a family of proteins involved in the assembly and possibly stability of sites of cell contact. These include the product of the lethal(1) discs-large-1 (dlgA) tumor suppressor gene and the zonula occludens proteins ZO-1, ZO-2. The further characterization of cDNA clones encoding components of synaptic junctions has lead to the identification of a 97 kDa protein, called SAP97, that exhibits a strong overall sequence similarity to SAP90. The present study was undertaken to determine the spatial distribution of SAP97, and to reveal further clues to the possible roles of these proteins in synapses. Light and immunoelectron microscopic analysis of the rat hippocampal formation revealed that SAP97 is localized in the presynaptic nerve termini of excitatory synapses. In other brain regions, SAP97 is found in and along bundles of unmyelinated axons. SAP97 is not restricted to the CNS, but is also present at the basal lateral membrane between a variety of epithelial cells. In cultured T84 cells, it is restricted to the cytoplasmic surface of the plasma membranes between adjacent cells, but not at the edges of cells lacking cell–cell contact suggesting a role for SAP97 in cell adhesion. These data suggest that members of the SAP90/SAP97 subfamily may be involved in the site specific assembly, stability or functions of membrane specialization at sites of cell–cell contact.

[Key words: synapses, guanylate kinase, SH3 domain, unmyelinated axons, epithelial cell junctions, cell adhesion]

Synapses are asymmetric structures composed of a presynaptic terminal involved in neurotransmitter (Nt) release and a postsynaptic transmitter reception apparatus (Hall and Sanes, 1993). At the ultrastructural level, the inner surface of the presynaptic terminal is composed of an electron dense thickening (Gray, 1961) that is contiguous with a cytoplasmic matrix of 10 nm filaments that fill the bouton. Nt containing vesicles are connected to this network via fine filamentous bridges (Landis, 1988). To understand the dynamic events taking place in this active zone of the nerve terminal requires the molecular and functional characterization of its individual components. Based primarily on studies of the neuromuscular junction (Hall and Sanes, 1993), it is clear that many of the structural and extracellular matrix proteins associated with synaptic junctions are involved in the aggregation of receptors and channels, the maintenance of the presynaptic and postsynaptic membranes in close proximity, and the anchoring and transport of synaptic vesicles to the presynaptic membrane (Landis, 1988; Hall and Sanes, 1993). However, little is known concerning the means by which these structural components mediate the site specific assembly of various synapses or regulate the alterations in synapse morphology associated with changes in synaptic efficacy (Trifaró and Vitale, 1993).

Utilizing primarily biochemical approaches, a number of prominent cytoskeletal and regulatory proteins, that copurify with CNS synaptic junctional preparations, have been identified. These include actin, myosin, tubulin, MAP2, fodrin, dystrophin, calmodulin, Ca^{2+}/calmodulin-dependent protein kinase II, cAMP-dependent protein kinase, and protein kinase C (see Walsh and Kuruc, 1992; Garner et al., 1993). However, none of these proteins are synapse specific suggesting there are additional proteins that may be involved in the site specific assembly of synapses.

Through the utilization of molecular approaches, we and others have been able to identify novel groups of proteins, some of which are associated specifically with synapses (see Garner et al., 1993). One recently identified synapse associated protein,

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SAP90, has been localized in the presynaptic nerve terminals of inhibitory GABAergic synapses in rat cerebellum (Kistner et al., 1993) as well as in inhibitory synapses in the hippocampus (Kistner et al., unpublished observations). Biochemical studies have demonstrated that SAP90 is not associated with synaptic vesicles but is tightly associated with synaptic junctions presumably via the cytoskeletal matrix (Kistner et al., 1993). SAP90 and an identical protein, PSD-95 which has been isolated and characterized from postsynaptic density preparation, are structurally related to the product of the *Drosophila* lethal(1) discs-large-1 (dlg) tumor suppressor gene, *dlg*-A (Cho et al., 1992; Kistner et al., 1993). The product of the *dlg*-A gene has been localized to septate junctions between epithelial cells of the imaginal disks during larval development (Woods and Bryant, 1991). Both SAP90/PSD-95 and *dlg*-A possess a carboxyl-terminal domain that are similar to the yeast guanylate kinase suggesting that they may be involved in the metabolism of guanine nucleotides (Woods and Bryant, 1991; Cho et al., 1992; Kistner et al., 1993). Since homozygous mutants in *dlg*-A affect both the formation of septate junctions and the proliferative state of these cells (Woods and Bryant, 1989), it has been suggested that *dlg*-A is part of a signal transduction mechanism that is involved in regulating the assembly or stability of septate junctions (Woods and Bryant, 1991).

The isolation of SAP90/PSD-95 from rat brain clearly demonstrates that mammals express at least one protein that is related to *dlg*-A. However, since dlg-A and SAP90/PSD-95 are not identical in structure and do not overlap in their tissue and subcellular distributions, it is not clear if they are functionally equivalent though these data do suggest that mammals probably express additional variants of SAP90/PSD-95. Three proteins ZO-1, ZO-2, and p55 were recently shown to share a number of structural similarities with *dlg*-A and SAP90/PSD-95, yet are clearly more distantly related proteins (see Fig. 1*C*) (Ruff et al., 1991; Willott et al., 1993; Jesaitis and Goodenough, 1994). Each was found associated with a different membrane specialization, for example, ZO-1 and ZO-2 with the tight junctions between epithelial cells (Stevenson et al., 1986; Jesaitis and Goodenough, 1994) and p55 with the cortical membrane cytoskeleton of erythrocytes (Ruff et al., 1991). One question that arises is whether the structural diversity observed among SAP90/PSD-95, *dlg*-A, ZO-1, ZO-2, and p55 is somehow correlated to functional and perhaps morphological differences between their respective membrane specializations. To address this question, we sought to identify and determine the structure and spatial distribution of addition members of this protein family in the CNS.

In this report, we present the nucleotide and deduced amino acid sequence of a 97 kDa protein (SAP97) whose primary structure is closely related to SAP90. Antibodies generated against unique protein sequences in SAP97 have been used to localize it in presynaptic terminals of excitatory synapses in the hippocampus, along unmyelinated axons and at sites of contact between epithelial cells. This distribution suggests a role for SAP97 in cell adhesion.

Materials and Methods

cDNA cloning, sequencing, and analysis. Clone 31f was isolated from a λgtll expression library with a polyclonal antibody generated against a rat brain synaptic junction preparation as previously described for clone 2d encoding SAP90 (Kistner et al., 1993). The clones 31f3, 31f3A, 31f5 were isolated with the cDNA insert from clone 31f by screening a λZAPII cDNA library prepared from adult rat cerebellum (kindly provided by O. Pongs, Hamburg). The hybridization of phage bound to Hybond N filters was performed as described by the manufacturer (Amersham). The cDNAs from positive λ clones were isolated using the *in vivo* excision method according to the manufacturer's instructions (Stratagene) and sequenced on both strands using the T7-DNA polymerase system (Pharmacia). Protein and nucleic acid sequence analysis were performed using DNASTAR programs (Madison, WI).

RNA isolation and Northern blotting. Total RNA was isolated from various tissues by a guanidinium isothiocyanate procedure (Chomczynski and Sacchi, 1987). Total RNA separated on 1.2% formaldehyde agarose gels was blotted onto Genescreen Plus (New England Nuclear) and hybridized with α-^{32}P-labeled cDNA probes as described previously (Kistner et al., 1993) or with oligonucleotide probes labeled by kinasing with γ-^{32}P-ATP (Sambrook et al., 1989).

Preparation and purification of antibodies. Polyclonal antisera against SAP97 were generated by injecting fusion proteins created between glutathione-S-transferase and the first 163 amino acid residues into mice or rabbits. DNA fragments were isolated from cDNA clones after amplification using the polymerase chain reaction (PCR) according to the manufacturer's instructions (Stratagene) with the following oligonucleotide pair GGGGATCCATGCCGGTCCGGAAG and CCGAATTCATTTGCCTTATGGG. Amplified DNA-fragments were cut with BamHI and EcoRI, purified and subsequently subcloned into the pGEX-2T vector (Pharmacia). The glutathione-S-transferase fusion-protein (GST-163) was purified with glutathione-sepharose 4B as described by the manufacturer (Pharmacia). Mice or rabbits were initially immunized with 0.5 ml of a 1:1 emulsion of 1 mg/ml purified fusion protein in Freund's complete adjuvant and subsequently with two additional injections after 14 and 28 d in Freund's incomplete adjuvant. The mouse and rabbit antibodies were purified from the antisera using the GST-163 fusion protein immobilized on nitrocellulose filters (Schleicher and Schüll) as described previously (Kistner et al., 1993). These antibodies were found to be indistinguishable in their specificity for either neuronal or epithelial tissues.

Expression of SAP97 in E. coli. A DNA fragment containing the complete open reading frame of SAP97 was isolated from a full length cDNA clone 31f.2 (not shown) after polymerase chain reaction amplification according to the manufacturer's instructions (Stratagene) with the following oligonucleotide pair GGGGATCCATGCCGGTCCGGAAG and CCGAATTCTTTTTCTTTTGCTGG. Amplified DNA was cut with BamHI and EcoRI and the fragments purified. The 500 bp BamHI/EcoRI fragment was first subcloned into the pRK174 vector (Studier et al., 1990). Subsequently, the 2.5 kbp BamHI/BamHI fragment was subcloned into the BamHI site in the pRK174 vector containing the 500 bp BamHI/EcoRI fragment. Positive clones were transformed into the *E.coli* strain BL21 and the expression of SAP97 was induced with IPTG as described (Studier et al., 1990). The cells were harvested by centrifugation at 5000 × *g*, resuspended in the same volume of SDS-PAGE loading buffer and boiled for 5 min.

Protein isolqtion and Western blotting. The isolation of cytosolic and membrane fractions from homogenates of various tissues was performed as described previously (Kistner et al., 1993). The extraction of brain membranes with 100 mM DTT, 2 M $MgCl_2$, 1% Triton × 100, 3% NP40, 1% CHAPs, 100 mM Na_2CO_3 pH 11.5, 6 M guanidine HCl, or 1 M Tris was performed as follows. Postnatal day 30 rat brains were homogenized at 0.5 gm/ml and spun at 500 × *g* to remove nuclei and cell debris at 4°C. The membranes were then pelleted at 100,000 × *g*, resuspended in homogenization buffer, aliquoted into seven samples, and respun at 100,000 × *g*. Each pellet was then resuspended in 2 volumes of one of the eight extraction buffers, incubated at room temperature for 30 min and once again spun at 100,000 × *g*. The pellets were dissolved in 1× SDS sample buffer, whereas the supernatants were first dialyzed for 1 hr at 4°C against 10 volumes phosphate-buffered saline (PBS) before adding 10× SDS sample buffer. Tris extraction was performed according to Hayes et al. (1991). Protein preparations were separated on 10% SDS polyacrylamide gels, transferred to nitrocellulose (Schleicher und Schüll), blocked, and incubated with purified primary antibody as previously described (Kistner et al., 1993). Blots were subsequently incubated with alkaline phosphatase-conjugated goat anti-mouse or anti-rabbit antibodies (Dianova, Hamburg) diluted 1:1000 in PBS, washed as described above and the bound secondary antibody was visualized by incubating with 33 μl nitroblue tetrazolium (50 mg/ml in 70% dimethylformamide) and 16.5 μl of 5-bromo-4-chloro-3-indolyl-phosphate (50 mg/ml in dimethylformamide) in 10 ml of alka-

line phosphatase buffer (100 mM Tris HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$).

Immunohistochemistry. The perfusion, sectioning and staining of postnatal day 30 rat brain, as well as their visualization both at the light and electron microscopic levels, was performed as previously described (Kistner et al., 1993) utilizing the affinity purified mouse mAb-163 antibody followed by peroxidase conjugated rabbit anti-mouse antibodies (Dianova). Longitudinal and cross section of the choroid plexus were stained with the affinity purified mouse mAb-163 antibody and visualized with a fluorescein conjugated rabbit anti-mouse antibody (Dianova). Eight micrometer sections of small intestine were taken and double stained with the purified rabbit rAb-163 antibody and rhodamine conjugated phalloidin (Sigma). The rAb-163 antibody was visualized with a fluorescein conjugated goat anti-rabbit antibody (Dianova).

The T84 cell line (Dharmsathaphorn et al., 1984) were grown on Permanox slides (Nunc) in a 1:1 mixture of Ham's F12 medium and Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum for 7 d with media changes every second day. The cells were washed in PBS at room temperature (RT), fixed with 4% paraformaldehyde in PBS for 20 min, and permeabilized with 0.5% Triton X-100, 4% paraformaldehyde in PBS for 4 min. The cells were stained with the purified rabbit rAb-163 antibody overnight at 4°C, washed in PBS and incubated with a fluorescein conjugated goat anti-rabbit antibody (Dianova) and visualized on a Zeiss Axiovert microscope with appropriate filters.

In situ *hybridization. In situ* hybridization was performed essentially as described by Burgin et al. (1989). In brief rat brains were removed, embedded in Tissue-Tek and immediately frozen at −70°C. Frozen sections were cut at 14 μm, collected on gelatin subbed slides and dried on an 80°C hot plate. The sections were fixed in 4% paraformaldehyde in PBS for 5 min, washed three times with PBS for 5 min, dehydrated in ethanol, and air dried. The sections were incubated for 2 hr in 50 μl of hybridization solution (10 mM HEPES pH 7.5, 600 mM NaCl, 100 mM DTT, 1 mM EDTA, 50% deionized formamide, 10% dextran sulfate, 1 × Denhardt's, 100 μg/ml salmon sperm DNA, 100 μg/ml yeast RNA) at 37°C and hybridized overnight at 37°C in a humidified chamber with 10,000 cpm/μl hybridization solution. The oligonucleotides (antisense, GTACCATTAACATAAGTTGGTGTCTCCAAGCTGTCTGTGT; sense, TGGGCTTCACGATCGCAGGTGGCACTGAGACAACCCG-CA) were labeled by tailing with terminal transferase (Pharmacia) and α-^{35}S-dATP (Amersham). The sections were rinsed in 1 × SSC, washed 10 min at room temperature in 1 × SSC, 30 min at 37°C in 1 × SSC, 30 min at 55°C in 2 × SSC, and 10 min at room temperature in 2 × SSC. The slides were dehydrated in ethanol and exposed to Amersham β-max film for 3–7 d. The film was processed according to the manufacturer's recommendations (Amersham). The slides were further processed by dipping into 1:1 Ilford K-5 emulsion, air dried, and exposed for 1–2 weeks. Sections were observed and photographed under dark-field optics with a Zeiss Axiovert photo microscope.

Results

Molecular characterization of SAP97

In an approach designed to isolate cDNAs encoding proteins associated with synapses (Garner et al., 1993), clone 31f was isolated from a λgtll expression library with a polyclonal antibody generated against a rat brain synaptic junction preparation as previously described for clone 2d encoding SAP90 (Kistner et al., 1993). The protein encoded by clone 31f was characterized by isolating a series of overlapping cDNAs and sequencing them from both strands (Fig. 1*A,B*). The encoded protein contains 878 amino acid residues with a calculated M_r of 97,000. The proposed initiation codon for this protein is preceded by a potential ribosome initiation sequences, described by Kozak (1989) and by several upstream in-frame stop codons (Fig. 1*B*). One clone 31f10 was found to contain a 99 nucleotide insertion situated at nucleotide #933. This insertion most likely represents an alternative splicing event, since splice site consensus sequences are not found at the junctions and the reading frame is not disrupted suggesting the existence of at least one additional isoform of SAP97 (Fig. 1*B*).

An alignment of the deduced amino acid sequence from the 31f cDNAs with that deduced for SAP90 reveals a 53% overall sequence identity between these two proteins (Fig. 1*B*). When aligned to yield the highest degree of identity, the amino terminal sequences of both proteins differ in length and exhibit no obvious sequence similarity, while the remainder of each molecule can be separated into five regions each sharing between 69 and 92% sequence identity (Fig. 1*B*). Based on its clear familial relationship with SAP90 and its association with synaptic junction, we have designated the 97 kDa protein encoded by clones overlapping with 31f as synapse associated protein 97 (SAP97). Hydropathy plot of the deduced amino acid sequence from SAP97 revealed that it is a hydrophilic protein lacking a leader sequence that could perform as a translocation signal to the endoplasmatic reticulum or stretches of hydrophobic residues that could anchor it in membranes.

As observed previously for SAP90 (Kistner et al., 1993), SAP97 was found to share 43% overall sequence identity to the protein product of the *Drosophila* gene, *dlg*-A (diagramed in Fig. 1*C*; Woods and Bryant, 1991). These identities are clustered in the five regions highly conserved between SAP90 and SAP97 (Fig. 1; Kistner et al., 1993). The first region of high sequence similarity is a stretch of 90 amino acid residues that is repeated in the second and third regions of sequence similarity (amino acid residues 64–390 of SAP90 and 191–509 of SAP97). When aligned, the homologous repeats of SAP90 and SAP97 are more than 86% identical, whereas heterologous repeats from each protein display only up to 38% identity with the highest sequence conservation between repeats 1 and 2 (not shown). The fourth region of similarity (amino acid residues 435–493 and 554–612, respectively) contains 69% identical amino acid residues (Fig. 1*B*) and conforms to a consensus sequence for a src homology 3 (SH3) domain (Musacchio et al., 1992). The fifth region present in the last 205 amino acid residues of both proteins shares 33% identity and 77% similarity to the yeast guanylate kinase (YGK) (Fig. 1*B*) (Berger et al., 1989). This enzyme catalyzes the phosphorylation of GMP at the expense of ATP according to the reaction $Mg^{2+}ATP + GMP = ADP + Mg^{2+} + GDP$, where Mg^{2+} is required for catalysis (Stehle and Schulz, 1992). Although direct evidence for GK activity is lacking, a majority of the residues in the YGK that have been shown to be involved in the binding of GMP and Mg^{2+} (Stehle and Schulz, 1992) are either identical or conservatively substituted in SAP97, SAP90, *dlg*-A and p55 (Fig. 1*C*; Woods and Bryant, 1991; Bryant and Woods, 1992; Cho et al., 1992; Kistner et al., 1993). For example, Arg38, Arg41, Glu44, Tyr50, and Tyr78 involved in binding the phosphoryl group in GMP in the YGK are conserved in SAP97: Arg722, Arg725, Glu728, Tyr734, and Tyr763. Also conserved is Asp98 (Asp783 in SAP97) that binds Mg^{2+} and in the YGK and adenylate kinases and are crucial for catalysis (Stehle and Schulz, 1992), as well as Glu69 (Glu754 in SAP97) that is involved in distinguishing between guanine and adenine and a conservatively replaced residue involved in binding the guanine ring Ser80 (Thr765 in SAP97). In contrast, the placement of those residues necessary for ATP binding are not generally conserved or are deleted except in p55 (Bryant and Woods, 1992). These include a glycine-rich loop near the N-terminus of YGK (residues 8–15) (residues 695–699 in SAP97) as well as Arg131, Arg135, Arg146 that together form the giant anion hole that accommodates the β-phosphate group of ATP as well as Asn168 that binds the N7 of ATP. While this might imply that these domains in SAP97, SAP90, and *dlg*-A have evolved a novel function, recent studies on the nucleotide

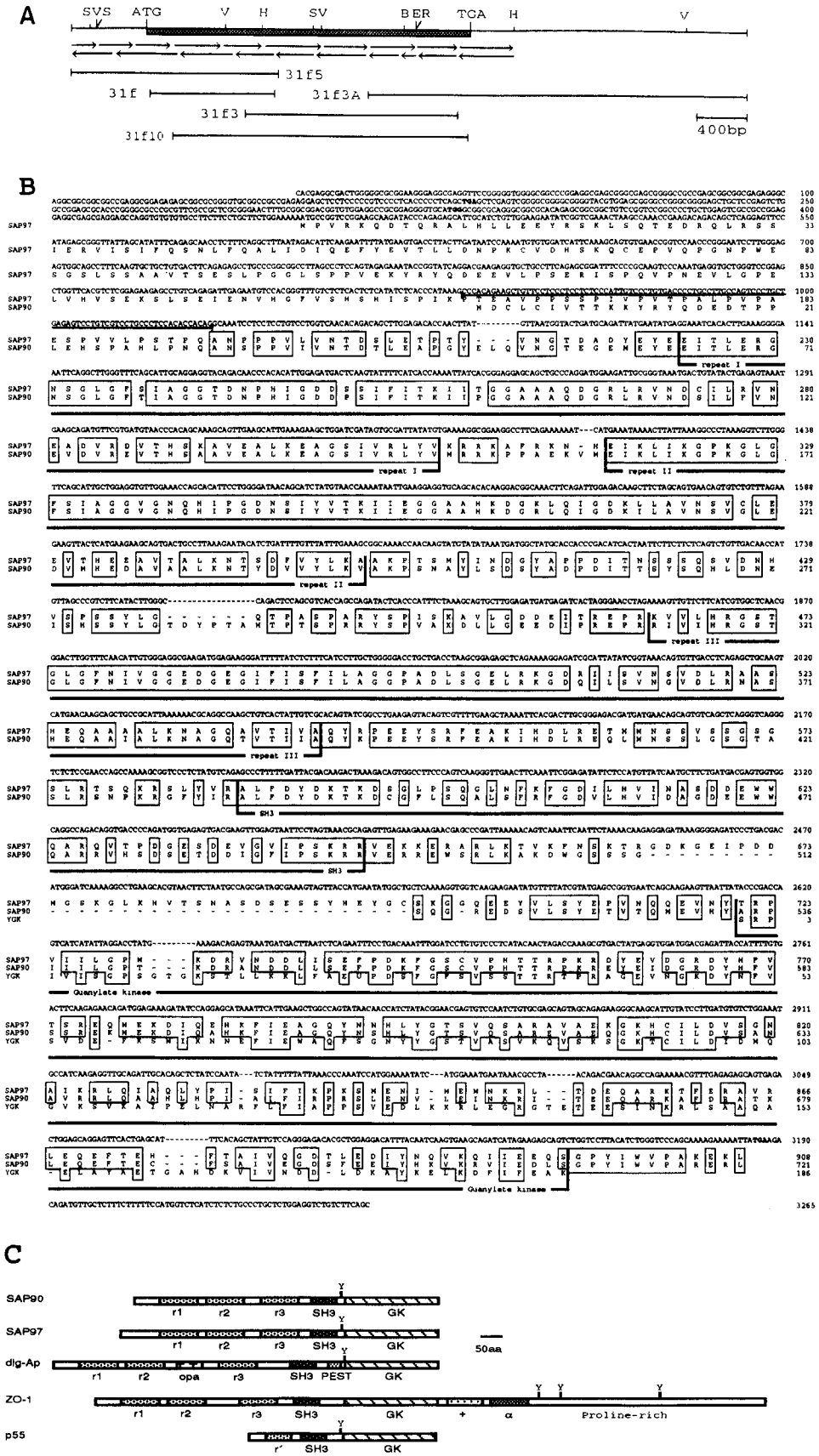

Figure 1. Primary structure of SAP97. *A*, Composite restriction map, clone organization, and sequencing strategy for cDNA clones encoding SAP97. The coding region is represented by a *stippled box*, and the initiation and stop codons are indicated by their three letter codes. *Arrows* indicate the length and direction of sequencing. Restriction sites are designated as follows: *B*, BamHI; *ER*, EcoRV; *H*, HindIII; *V*, PvuI; *S*, Sst I. *B*, Nucleotide sequence of SAP97; deduced amino acid sequence of SAP97 in single letter code aligned with that deduced for SAP90 and the yeast guanylate kinase (YGK). The in-frame stop codons upstream to the start codon are shown in *boldface letters*. Identical amino acids found conserved between SAP97 and SAP90 as well as the guanylate kinase have been *boxed*. The three 90 amino acid residue repeats, the SH3 domain, and the guanylate kinase domain are *double-underlined*. The cDNA clone 31f10 contains an insertion of 99 nucleotides beginning at position 933 has been *underlined*. *C*, Schematic comparison of the structural domains within SAP97, SAP90, *dlg*-A, ZO-1, and p55 proteins. The 90 amino acid residue repeat domains are labeled (*r1–r3*) and the partial repeat in P55 (*r'*). In addition the SH3 and the guanylate kinase (*GK*) domains conserved in all five proteins are labeled. Unique to *dlg*-A is an opa repeat and a PEST sequences (Woods and Bryant, 1991), while ZO-1 contains an additional carboxyl-terminal region including an acidic domain (+), alternatively spliced domains (α,β_1,β_2) and proline-rich motifs that could be potential SH3 domains binding sites. *Y* indicates the position of tyrosine phosphorylation consensus sequences representing potential SH2 binding sites.

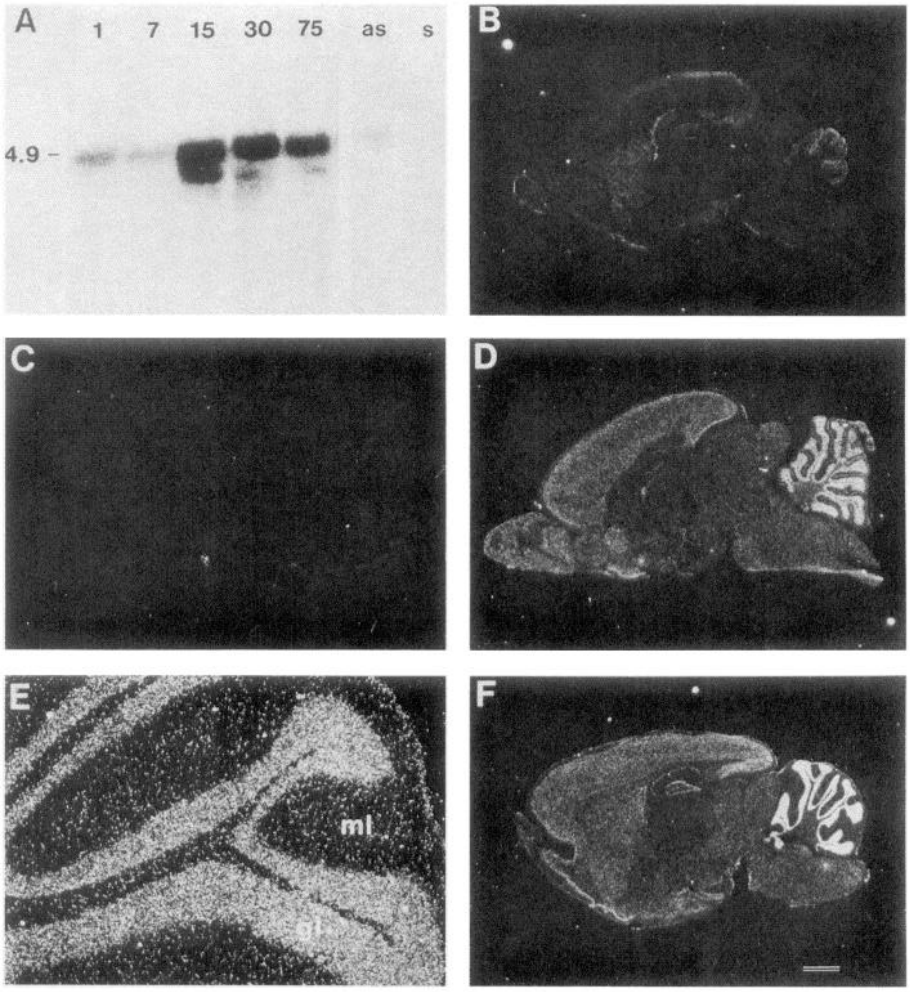

Figure 2. Temporal and spatial expression of SAP97. *A*, A developmental Northern blot of 10 μg of total RNA from P1, P7, P15, P30, and P75 rat brain probed with the ^{32}P-labeled cDNA insert from clone 31f. This probed hybridizes with a 4.9 and a 4.4 kb mRNAs. Lanes *as* and *s* are Northern blots from P30 rat brain probed with the ^{32}P-labeled antisense (*as*) and sense (*s*) oligonucleotides. Only the antisense oligonucleotide hybridizes to an RNA with the same molecular weight as that seen by the 31f cDNA fragment. *B–F*, *In situ* hybridization of P5 (*B*), P15 (*C, D*), and P30 (*E, F*) sagittal rat brain sections probed either with the antisense oligonucleotide (*B, D–F*) or the sense oligonucleotide (*C*). *E*, A close up of P30 cerebellum demonstrating that the mRNAs encoding SAP97 isoforms are expressed in the granular cell layer and not in Purkinje cells or interneurons in the molecular layer.

binding capacity of a bacterial expressed lymphocyte members of this family (Lue et al., 1993) as well as SAP90 (Kistner et al., unpublished observations) indicate that these proteins retain the capacity to bind GMP, GDP, ATP, ADP, but not GTP *in vitro*. This suggests that these proteins do indeed encode guanylate kinases and perhaps have acquired a novel adenosine di- and triphosphate binding site.

Temporal and spatial expression of the SAP97 transcripts

A developmental Northern blot of total RNA from postnatal days 1–75 (P1–P75) rat brain hybridized with the 31f cDNA reveals that the different alternatively spliced isoforms of SAP97 are encoded by at least two mRNAs, of 4.9 and 4.4 kb. The larger mRNA, already present in the first postnatal week, reaches peak levels, that are maintained even in mature brain, by P15 (Fig. 2*A*). In contrast the 4.4 kb mRNA appears abruptly around P15 declining in amount as the brain matures (Fig. 2*A*). *In situ* hybridization of sagittal brain sections with oligonucleotide probes permitted a detailed examination of the temporal and spatial distribution of the SAP97 mRNAs in developing rat brain (Fig 2*B–F*). The antisense probe, tested on Northern blots of P30 rat total RNA, was found to hybridize with a 4.9 kb mRNA of the same molecular weight as that seen by the 31f cDNA (Fig. 2*A*). In contrast, there was no hybridization signal with the sense probe on Northern blots (Fig. 2*A*) or in tissue sections (Fig. 2*C*). In P5 rat brain only a weak hybridization signal was observed with the antisense probe (Fig. 2*B*), though by P15 a pronounced increase in the expression levels of the SAP97 mRNA are seen in the cerebellum, cerebral cortex and olfactory bulb. Virtually no signal could be detected in thalamic brain regions nor in the inferior and superior colliculus (Fig. 2*D*). In P30 rat brain, the overall distribution pattern of the SAP97 mRNA is very similar to that at P15 with an even higher relative level of expression in the cerebellum (Fig. 2*F*). A close-up of P30 cerebellum shows that the silver grains are found in the granule cell layer (Fig. 2*E*). Silver grains were not found associated with Purkinje cells, interneurons in the molecular layer nor in oligodendrocytes in the white matter tracts (details not shown).

Spatial distribution of SAP97 in rat brain

To investigate the subcellular distribution of the proteins encoded by these mRNAs, both mouse and rabbit antibodies were generated against the unique amino terminal sequences in SAP97 (amino acid residues 1–163) (Fig. 1*B*) fused to glutathione-S-transferase (GST-163). On Western blots of rat brain membranes, the affinity purified mouse (mAb-163) and rabbit (rAb-163) antibodies specifically recognized a protein doublet at 140 kDa (Fig. 3). Since the observed molecular weight of SAP97 is larger than that calculated from its deduced amino acid sequence (97 kDa), we expressed the coding region of SAP97 in *E. coli* and found that the bacterial protein migrates with the same mobility as the higher of the two 140 kDa brain protein bands (Fig. 3). At present, it is not clear what causes SAP97 to

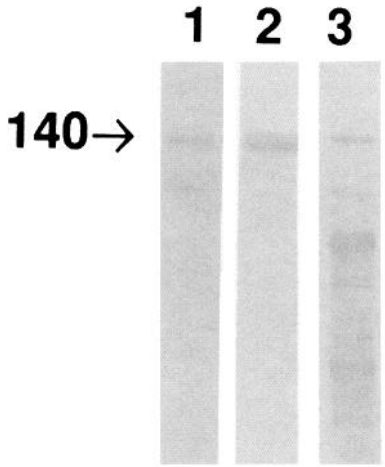

Figure 3. Western blot analysis of SAP97. Western blots of protein extracts from P30 rat brain (*lanes 1* and *2*) as well as a protein extract from *E. coli* expressing recombinant SAP97 (*lane 3*) were stained with the purified antisera either from mouse (mAb-163) (*lane 1*) or rabbit (rAb-163) (*lane 2* and *3*). Recombinant SAP97, expressed in *E. coli*, runs with a mobility very similar to the upper of the 140 kDa bands expressed in rat brain. Additional bands in *lane 3* are due to premature termination of recombinant SAP97 expressed in *E. coli*. The 90 kDa band in *lane 1* is proteolytic breakdown product of the 140 kDa protein.

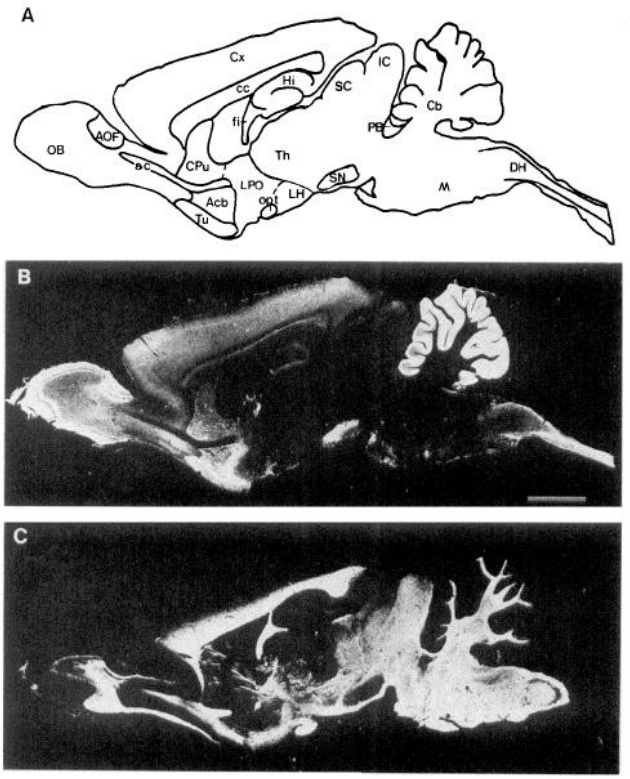

Figure 4. Spatial distribution of SAP97 and myelin basic protein (MBP) in P30 rat brain. Sagittal sections of P30 rat brain have been immunoperoxidase stained either with the affinity purified mouse mAb-163 antibody (*B*) or with a mouse monoclonal antibody against MBP (*C*). Relevant brain regions have been marked on a sketch of a sagittal view of an adult rat brain (*A*). Strong immunoreactivity with the mAb-163 antibody is seen in the cerebellum (*Cb*), the olfactory bulb (*OB*), the dorsal horn of the spinal cord (*DH*), in the olfactory tubercle (*Tu*), and in the accumbens (*Acb*) as well as in the substantia nigra (*SN*), in the hippocampus (*hi*), in the corpus callosum (*cc*) and in the cortex (*Cx*). Low amount of staining is seen in the caudate putamen (*CPu*). Unstained is the thalamus (*Th*), the lateral preoptic area (*LPo*), the medulla (*M*), the optic chiasm (*opt*), the anterior commissure (*ac*), and the superior colliculus (*SC*) and inferior colliculus (*IC*). *AOF*, Accessory olfactory formation; *fi*, fimbria hippocampus; *LH*, lateral hypothalamic area; *PB*, parabrachial nucleus. A comparison of the brain section stained with mAb-163 (*B*) and anti-MBP (*C*) reveals a complementary staining pattern of these two antibodies. In a number of brain regions, SAP97 is found in axon-rich regions like the molecular layer of the cerebellum (see also Fig. 6), the olfactory nerve and glomeruli layer in the olfactory bulb. However, it is not found along myelinated axons, for example, the white matter of the cerebellum (see detail Fig. 6), the anterior commissure (see detail Fig. 7), the dorsal hippocampal commissure (see detail Fig. 7).

exhibit its anomalous behavior on SDS polyacrylamide gels, nor whether the lower 139 kDa band is due to proteolytic breakdown or posttranslational modifications, or is the product of an alternatively spliced mRNA. A 90 kDa immunoreactive band that sometimes appears either with the mouse (Fig. 3) or rabbit antibodies (see Fig. 8*B*) could be shown to be a proteolytic degradation product of the larger 140 kDa protein (data not shown).

In sagittal sections of P30 rat brain, the affinity purified mouse mAb-163 and rabbit rAb-163 antibodies were used to examine the spatial distribution of SAP97. Both antibodies were found to exhibit indistinguishable staining patterns in brain sections demonstrating that both antibodies have similar if not identical specificity. For internal consistency the mouse mAb-163 antibodies were used for these brain studies revealing that SAP97 displays a comparable distribution as observed for its mRNA (compare Figs. 2*F*, 4*B*). For example, SAP97 immunoreactivity can be found in a variety of brain regions such as the olfactory bulb, cerebral cortex, hippocampus, and spinal cord.

Presynaptic localization of SAP97 in rat hippocampus

Given the presynaptic localization of SAP90 (Kistner et al., 1993) and the appearance of SAP97 in a number of areas of the rat brain with high synaptic density, we examined by light and electron microscopy whether SAP97 was also present in synapses. Light microscopy studies of P30 sagittal rat brain sections with mAb-163 showed a strong immunoreactivity in the outer two-thirds of the molecular layer of the dentate gyrus (Fig. 5*A,G*). These layers are innervated by fiber tracts from the perforant pathway originating from layers two and three from the entorhinal cortex. The inner layer which is more weakly stained receives input from the ipsi- and contralateral hilus (Bayer, 1985). Electron microscopic studies of both the inner third and outer two-thirds reveals the presence of immunoreactive product in presynaptic nerve endings (Fig. 5*H*). In each examined case, these terminals were found situated on dendritic spines and possessed clear circular shaped vesicles. While the exact identity of these synapses still needs to be determined, these features are characteristics of excitatory synapses in this region (Laatsch and Cowan, 1966).

In all regions of the hippocampus, the strongest staining with mAb-163 is observed on both sides of the pyramidal cells in the area of the stratum oriens and radiatum (Fig. 5*A,C,E*). In the CA1 region (Fig. 5*C*), again the reaction product is found in presynaptic structures associated with dendritic spines (Fig. 5*D*). Although in the CA3 region (Fig. 5*E*), SAP97 is also found in clearly definable presynaptic nerve terminals, its highest concentration is found within axon fibers situated next to the cell body layers (Fig. 5*F*). Mossy fiber tracts and their terminals are unstained.

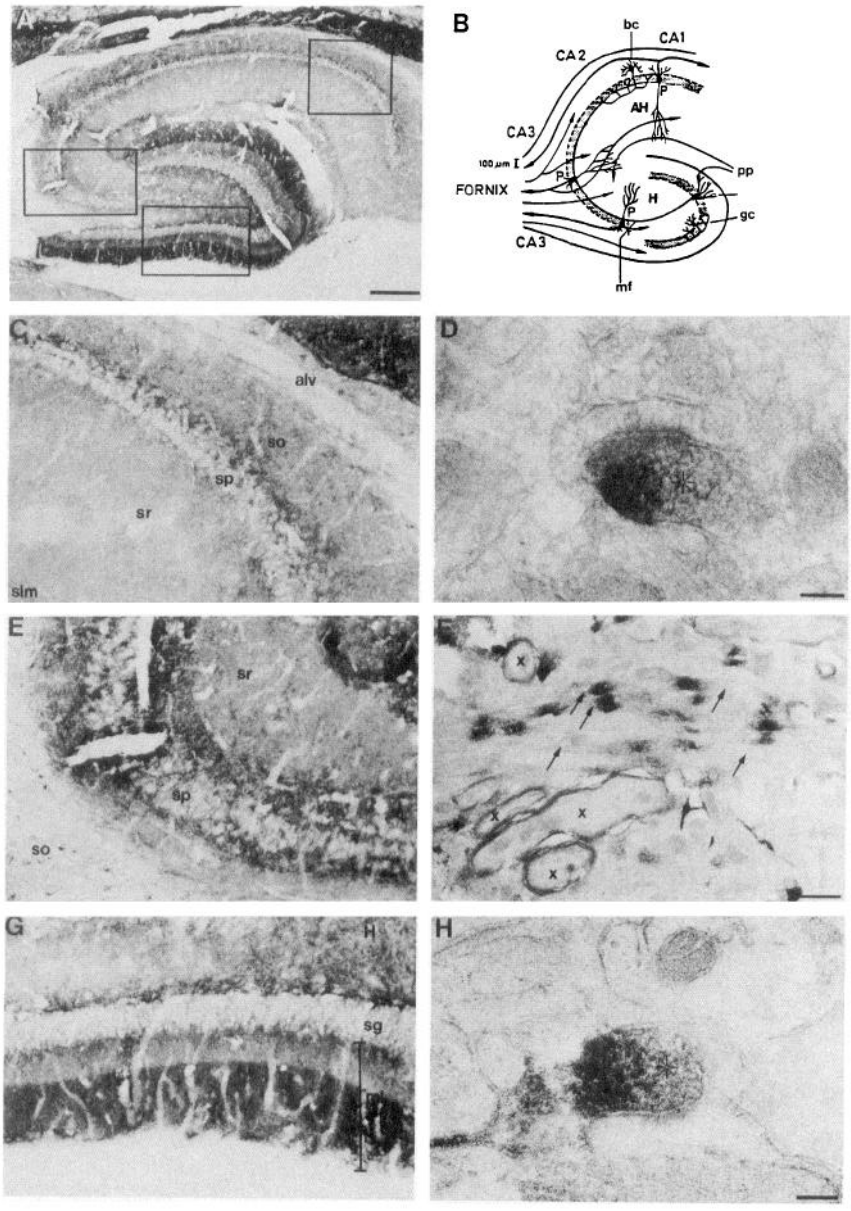

Figure 5. Localization of SAP97 in P30 rat hippocampus. Sagittal sections from P30 rat hippocampus were immunoperoxidase stained with the affinity purified mAb-163 antibody (*A*). *Boxed regions in A* are shown in higher magnification in *C, E,* and *G* with corresponding electron micrographs from each region shown in *D, F,* and *H*, respectively. *B*, Shows a sketch of a sagittal view of the hippocampus. The mAb-163 antibody is seen to strongly stain the outer two-thirds and to a lesser extend the inner third of the molecular layer of the dentate gyrus (*A, G*). Prominent staining is also seen in the hilus and in proximal regions of the stratum oriens and radiatum adjacent to the pyramidal cell body layer in the CA3 (*E, F*) and CA1 (*C, D*) regions of Ammon's horn. At the electron microscopic level the immunoperoxidase reaction product in the dentate gyrus (*H*) and CA1 (*D*) is seen in presynaptic terminals (*star*) associated with asymmetric synapses. In the CA3 region (*F*), the staining of distinct regions along unmyelinated axons (*arrows*) are seen, yet myelinated axons (*x*) are not stained. *AH*, Ammon's horn; *alv*, alveus; *bc*, basket cells; *H*, hilus; *ml*, molecular layer; *gc*, granule cells; *mf*, mossy fibers; *P*, pyramidal cells; *pp*, perforant pathway; *so*, stratum oriens; *sr*, stratum radiatum; *sg*, substantia gelatinosa; *slm*, stratum laculosum moleculare; *sp*, substantia pyramidale. Scale bars: *A*, 0.3 mm; *D*, 0.2 µm; *F*, 1.2 µm; *H*, 0.2 µm.

Subcellular localization of SAP97 in rat cerebellum

In P30 cerebellar sections, the mouse mAb-163 antibody exhibits a very strong staining throughout the cerebellar molecular layer (Fig. 6*A*). The granule cell layer is more weakly stained and no obvious staining of white matter is observed. Note that virtually all staining with mAb-163 can be completely blocked with the GST-163 fusion protein (Fig. 6*B*). A closer examination of semithin sections with (not shown) and without (Fig. 6*C*) counterstaining with toluidine blue permitted the identification of all major cell types by morphological criteria. Immunoperoxidase reaction product was not observed in the cell bodies of any of the major cell types (Fig. 6*C*). Immunoreactivity in the molecular layer appears as small dots, distributed more or less evenly in the neuropil between cell somata, dendrites, and vessels as well as along axon fibers transversing the Purkinje cell layer (Fig. 6*C*). At the electron microscopic level immunostaining in the molecular layer is restricted largely to the cross-sections of parallel fibers and to a lesser extent with presynaptic terminals. Postsynaptic structures and other elements remained unstained (Fig. 6*D*). Reaction product in the granule cell layer appears not to be associated with granule cell dendrites but with granule and golgi cell axons; however, this will require further investigations. Together with the data on the spatial expression pattern of SAP97 mRNA (Fig. 2) these immunohistochemical data demonstrate that in the cerebellum the SAP97 mRNA is expressed in granule cells, and the encoded protein is primarily subcompartmentalized within axons and to a lesser extent the excitatory nerve terminals of this subpopulation of cerebellar neurons.

SAP97 is associated with unmyelinated axons

An examination of mAb-163 staining in other brain regions, in particular the olfactory bulb, spinal cord, and corpus callosum, reveals high levels of SAP97 in a variety of axon tracts throughout the brain (Figs. 4*B*, 7). A comparison of the mAb-163 staining pattern with that of a monoclonal antibody against myelin basic protein (MBP) demonstrates that several of these fiber tracts, including the molecular layer of the cerebellum and ol-

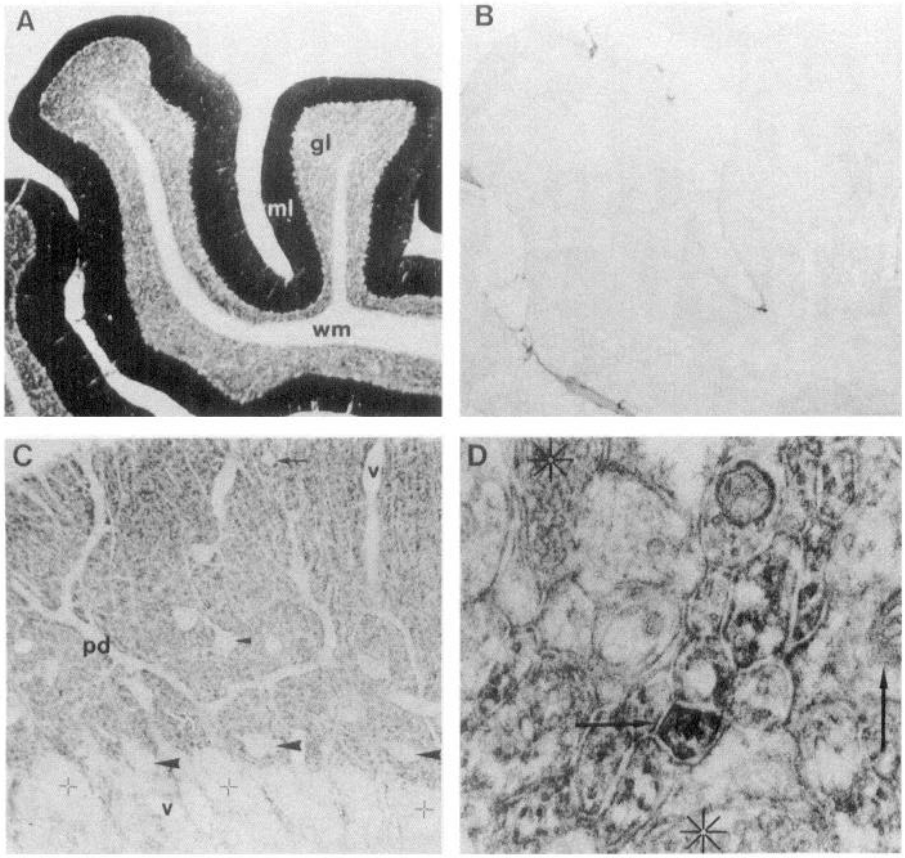

Figure 6. Localization of SAP97 in P30 rat cerebellum. Sagittal sections from P30 rat brain were immunoperoxidase stained either with the affinity purified mouse mAb-163 antibody (*A, C, D*) or with the mAb-163 serum preabsorbed with glutathione-S-transferase-SAP97 fusion protein (GST-163) (*B*). The mAb-163 antibody is seen to stain the molecular layer and to a lesser extent the granule cell layer (*A, C*), whereas the white matter is not stained (*A*). In semithin sections (*C*) immunoreactivity appears as small dots in the neuropil between cells and their dendrites. Most major cell types including Purkinje (+), Golgi (not labeled), basket (*broad arrowheads*), stellate cells (*small arrowheads*), and oligodendroglia (*small arrow*) can be identified based on morphological criteria and are immunonegative. At the electron microscopic level (*D*), the staining is restricted largely to the cross sections of parallel fibers (*arrowheads*) and to a lesser extent synapses (*) and other elements remain unstained.

factory fiber bundles, are unmyelinated (Fig. 4*B,C*), whereas in tracts that are predominantly myelinated such as the cerebellar white matter, the fimbria of the hippocampus and the dorsal hippocampal commissure, SAP97 is absent or found in low levels.

To examine the association of SAP97 with unmyelinated axons in more detail, we determined the distribution of SAP97 at both light and EM levels in two additional brain regions, the dorsal horn of the spinal cord and the corpus callosum, which have mixed populations of myelinated and unmyelinated axons. In the dorsal horn of the spinal cord there is a prominent SAP97 staining of the outer layers (Fig. 7*D*) where unmyelinated axons (Fig. 7*E*) of C fibers coming from the nociceptors are found (Grant, 1985). Immunoelectron microscopic analysis revealed that while myelinated fibers are present, SAP97 is restricted to the unmyelinated axons (Fig. 7*F*). Similarly, in the corpus callosum where immunostaining with antibodies against SAP97 and MBP are observed (Fig. 7*A,B*) by immunoelectron microscopy, SAP97 is restricted to the unmyelinated fibers (Fig. 7*C*).

SAP97 is expressed in non-neuronal tissues

To address whether SAP97 is brain-specific as previously observed for SAP90/PSD-95 (Cho et al., 1992; Kistner et al., 1993), a variety of rat tissues were examined for the presence of the SAP97 and its mRNAs. Northern blotting revealed the presence of a 4.9 kb mRNA that hybridized with the 31f cDNA in heart, muscle, liver, lung and testis with the same relative mobility as that seen in P30 rat brain (Fig. 8*A*). Moreover, we also observed a 140 kDa immunoreactive protein in Western blots of the brain, heart, muscle, and liver tissue incubated with the rabbit affinity purified rAb-163 antibody (Fig. 8*B*) indicating that SAP97 is broadly expressed. Although the mouse mAb-163 antibody exhibited a high background staining of most non-neural tissue, the rabbit rAb-163 antibody produced a sharp restricted staining pattern. Thus utilizing the rAb-163 antibody, SAP97 was localized in the small intestine in the distal, older segments (Haffen et al., 1986) of the intestinal villi (Fig. 9*D*). The smooth muscle layer, submucosa, muscularis mucosa, lamina propria were found to lack SAP97, while the lateral membranes between epithelial cells in the crypt and proximal segments of the villi are lightly stained (Fig. 9*D–F*). A closer examination of the epithelial cells in the distal segments of the villi and the crypt stained with rAb-163 (Fig. 9*E,F*) revealed that the distribution of SAP97 somewhat overlapped with actin at the junctions between the columnar epithelial cells (Fig. 9*B,C*). However, in contrast to the high concentration of actin at the apical membrane of the epithelia in the villus (Fig. 9*B,C*), SAP97 levels were much higher at the basal side of these cells (Fig. 9*E,F*). To verify that the basal lateral distribution of SAP97 between epithelial cells was not related to the rabbit antibody, both the rabbit (not shown) and mouse antibodies (Fig. 9*H,I*) were used to stain epithelial cells of the choroid plexus. Here, SAP97 was also found along the basal lateral membranes between these epithelial cells (Fig. 9*H,I*).

The distribution of SAP97 observed between epithelial cells in various tissues was also examined in cultured cell lines. In the epithelial cell line T84, the rAb-163 antibody detected a 140 kDa immunoreactive protein band by Western blotting (Fig. 8*B*, lane 9), as well as the junctions between these cells grown in monolayers (Fig. 9*G*). Edges of cells not in contact with other cells are not stained (Fig. 9*G*) nor was the plasma membrane of isolated or dividing cells (not shown). Though low levels of SAP97 could be detected in the fibroblast cell line NIH 3T3 by

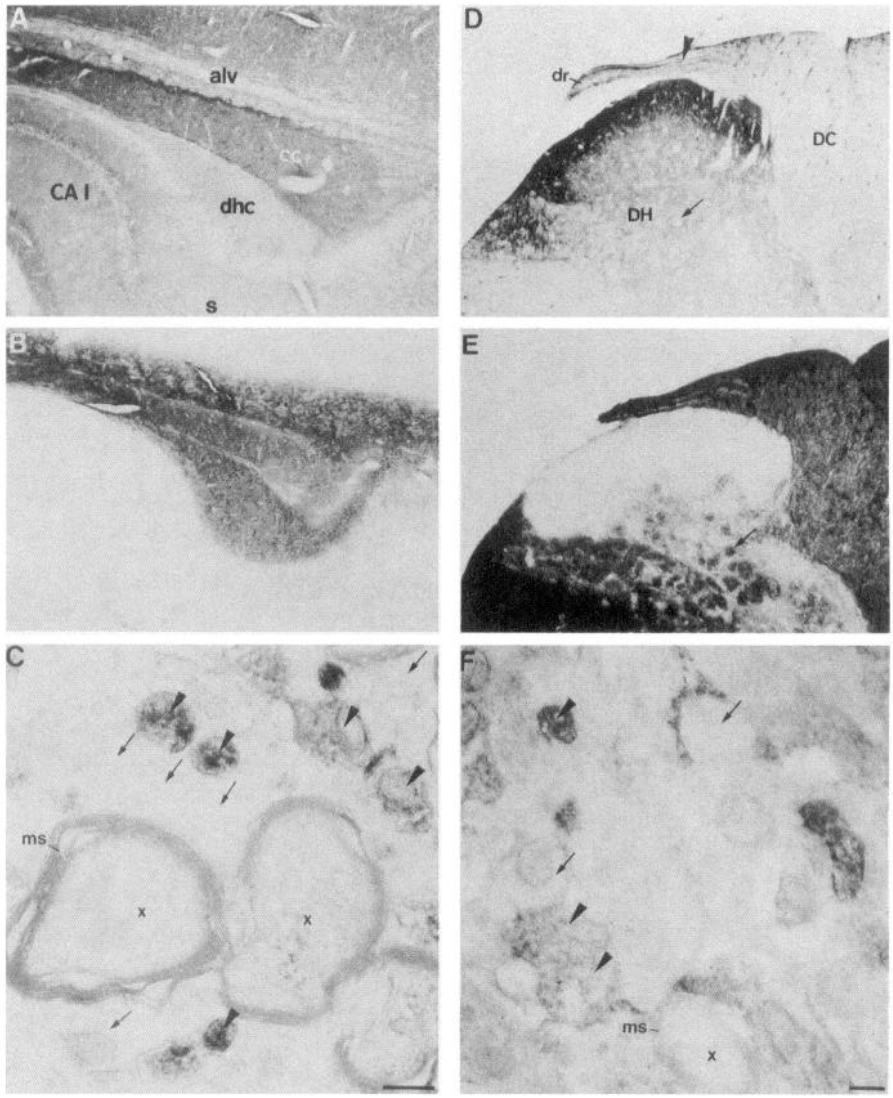

Figure 7. SAP97 in unmyelinated axons: light and electron microscopic comparison of the distribution of SAP97 and myelin basic protein (MBP) in corpus callosum and in spinal cord. Sagittal sections from P30 rat corpus callosum (*A–C*) and spinal cord (*D–F*) were immunoperoxidase stained with the affinity purified mouse mAb-163 antibody (*A, C, D, F*) or with a monoclonal antibody against MBP (*B, E*). Although at the light level SAP97 was found to codistribute with MBP in the corpus callosum (*cc*), to a lesser extent in the dorsal hippocampal commissure (*dhc*) but not the subiculum (*s*) or hippocampus (see *A* and *B*), in electron microscopic studies of the corpus callosum (*C*), SAP97 was restricted to a subset of unmyelinated axons (*arrowheads*, stained; *arrows*, unstained unmyelinated axons) and is absent from the myelin sheath (*ms*) and myelinated axons (*x*). Similarly in the spinal cord, SAP97 is localized in some fibers in the dorsal root spinal nerve (*dr*) and in the outer layers of the dorsal horn (*DH*), with a little staining in the inner layers. This contrasts with MBP (*E*) which is concentrated in the dorsal column (*DC*), lateral and ventral white matter and bundles of myelinated axons in the dorsal horn (*arrow*). Electron micrograph of dorsal horn (*F*) reveals the association of SAP97 with a subset of unmyelinated axons (*arrowheads*). Scale bars, 0.25 μm.

Western blotting, the contact sites between adjacent cells were not stained (data not shown).

SAP97 is tightly associated to the plasma membrane

Since the deduced amino acid sequence of SAP97 contains no transmembrane domains, yet it is often found colocalized with F-actin at the plasma membrane, we sought to determine the nature of its association with membranes. In brain, liver, heart, and muscle homogenates spun to separate soluble from membrane associated protein, the 140 kDa immunoreactive SAP97 bands were found in both fractions suggesting that there are cytosolic and membrane associated forms of SAP97 (Fig. 8*B*). Further extraction of the insoluble protein fractions from brain membranes with a variety of conditions revealed that the membrane form of SAP97 can only be released with 6 M guanidinium, HCl or 1% SDS (Fig. 8*C*). It could not be removed with alkaline treatment, 1.5 M NaCl, nor 1% Triton × 100 (Fig. 8*C*), suggesting that it is not a peripheral membrane protein. A possible association with the membrane cytoskeleton was tested by extracting brain membranes with 1 M Tris and 0.1% Triton X-100, known to release actin, ankyrin and spectrin (Hayes et al., 1991). Approximately one third of the membrane associated SAP97 could be released (Fig. 8*C*) in contrast to about 70% of the spectrin and actin (not shown). These experiments indicate that the tight association of SAP97 with the membrane is not necessarily through a direct interaction with actin, ankyrin, or spectrin. These characteristic are analogous to those observed for SAP90/PSD-95, *dlg*-A and p55 (Ruff et al., 1991; Woods and Bryant, 1991; Kistner et al., 1993). In the case of p55, its binding to the erythrocyte plasma membrane is thought to be mediated both by its palmitoylation of cysteine residues (Ruff et al., 1991) and its binding to band 4.1 and glycophorin C (Alloisio et al., 1993).

Discussion

In this study, we presented the molecular characterization and spatial distribution of SAP97, a novel isoform of the presynaptic protein SAP90, in rat brain and non-neuronal tissue. An alignment of the deduced amino acid sequence of SAP97 and SAP90 revealed that these two proteins share 70% identical amino acid residues concentrated in three domains: a carboxyl terminal domain that shares sequence similarities to the yeast, *E. coli* and bovine guanylate kinases (Berger et al, 1989; Gentry et al., 1990; Gaidarov et al., 1993), a 60 amino acid long motif resembling a src homology 3 (SH3) domain and three 90 amino acid residue repeats in their amino terminal sequences that are present

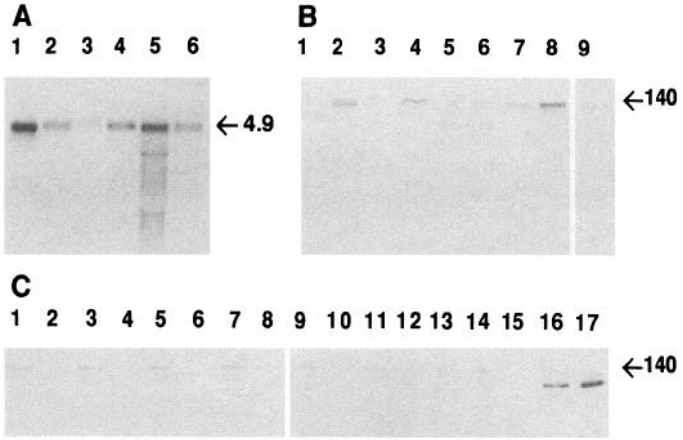

Figure 8. Tissue distribution and extraction behavior of SAP97. *A*, Northern blot of total RNA prepared from P30 brain, muscle, liver, heart, lung, testis (*lanes 1–6*, respectively), hybridized with labeled insert from clone 31f, reveals the presence of a 4.9 kb mRNA in each of these tissues. *B*, Immunoblot containing 25 μg of protein extract from P30 brain (*lanes 1* and *2*), heart (*lanes 3* and *4*), muscle (*lanes 5* and *6*), and liver (*lanes 7* and *8*) incubated with the purified rabbit rAb-163 antibody. *Lanes 1, 3, 5,* and *7* contain the 100,000 × *g* supernatant fractions, whereas *lanes 2, 4, 6,* and *8* the membrane pellet fractions. Variations in banding pattern at 140 kDa may represent the expression of slightly different isoforms of SAP97 in these tissues. Bands below 120 kDa are most likely degradation products. *Lane 9* is an immunoblot of 25 μg of a total protein extract from cultured T84 cells stained with rAb-163. *C*, Extraction characteristic of SAP97 from brain membranes (*lane 1*). *Even-numbered lanes* contain extractable protein, while *odd-numbered lanes* contain protein that remained associated with the membranes after centrifugation. Membranes were extracted with 100 mM DTT (*lanes 2* and *3*), 2 M MgCl2 (*lanes 4* and *5*), 1% Triton X-100 (*lanes 6* and *7*), 3% NP40 (*lanes 8* and *9*), 1% CHAPS (*lanes 10* and *11*), 100 mM Na_2CO_3, pH 11.5 (*lanes 12* and *13*), 6 M guanidine HCl (*lanes 14* and *15*), or with 1 M Tris pH 8.0 (*lane 16* and *17*). SAP97 can only be extracted with 6 M guanidine hydrochloride (*lane 14*) and in part with 1 M Tris (*lane 16*). The 90 kDa band is due to proteolysis of the 140 kDa band during the course of the experiment, and the increased staining in *lanes 16* and *17* is due to more protein being loaded from the extraction experiment with 1 M Tris.

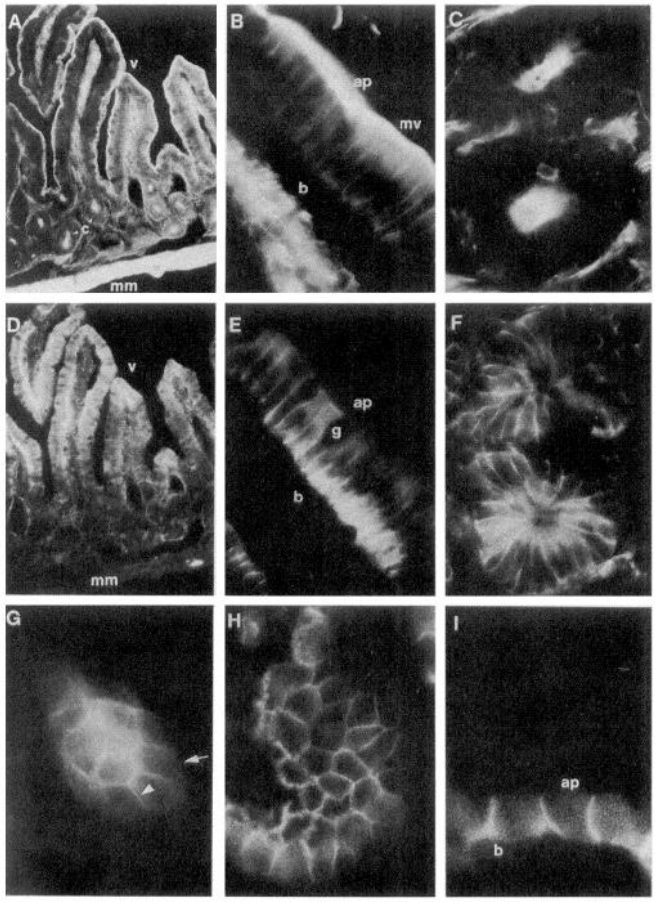

Figure 9. Immunocytochemical localization of SAP97 in non-neuronal tissues. Sections of small intestine (*A–F*) were double stained with the purified rabbit rAb-163 antibody (*D–F*) as well as with phalloidin (*A–C*). The core of the villi (*v*), the crypt (*c*), the terminal web (*tw*) of the brush border epithelial cells, as well as the muscularis mucosae (*mm*) are actin-rich and stained with phalloidin (*A–C*). The brush border epithelial cells along the more distal segments of the villi are intensely stained with rAb-163 (*D*). Less but clear staining is also seen between the epithelial cells in the crypt (*F*). In contrast to the high levels of F-actin at the apical (*ap*) end of the epithelial cells (*B, C*), SAP97 primarily exhibits a basal lateral (*b*) membrane distribution (*E, F*). Globlet cells (*g*) appear not to be stained with rAb-163 (*E*). Brain sections retaining segments of the choroid plexus were stained with the mouse mAb-163 antibody (*H, I*). In both longitudinal (*H*) and cross-sections (*I*), SAP97 is localized at the lateral plasma membrane between epithelial cells of the choroid plexus, again with a more basal lateral (*b*) distribution. SAP97 is also found at the junctions (*arrowhead*) between cultured T84 epithelial cells stained with the rAb-163 antibody, but not at the edges of cells lacking neighbors (*arrow*, *G*) nor in dividing cells or single cells (not shown). *A* and *D*, 400×; *B, C,* and *E–I*, 1000×.

in a number of membrane cytoskeletal associated proteins. The results will be discussed in terms of (1) the spatial distribution of SAP97 in excitatory presynaptic nerve terminals, along unmyelinated axons and the elaborate membrane cytoskeleton associated with these neuronal structures, (2) the relationship of SAP97 to the product of the *Drosophila* tumor suppressor gene *dlg*-A (Woods and Bryant, 1991) and the zonula occludens proteins ZO-1 and ZO-2 (Willott et al., 1993; Jesaitis and Goodenough, 1994) and their presence at sites of cell–cell contact between epithelial cells; and (3) the putative function of the structural domains in SAP97 and their relationship to analogous domains found in other proteins.

SAP97 in the CNS

Antibodies generated against the unique amino terminal sequences in SAP97 were used to determine its spatial distribution in postnatal day 30 rat brain sections. Immunoperoxidase stained hippocampal sections examined by electron microscopy (EM) revealed that SAP97 in the CA1, CA3 areas of the hippocampus and in the dentate gyrus can be localized in presynaptic nerve terminals (Fig. 5) a finding similar to that observed for SAP90 (Kistner et al., unpublished observations). However while SAP90 is found in inhibitory nerve terminals (Kistner et al., 1993, unpublished observations), SAP97 appears to be localized in the nerve terminals of excitatory synapses (Fig. 5). In both cases the peroxidase reaction product was observed to fill the nerve terminals. Biochemical analysis indicates that in these nerve terminals neither protein is associated with synaptic vesicles but with the cortical cytoskeleton associated with the synaptic junctions (Kistner et al., 1993, unpublished observations). At present it is not clear whether there is a functional significance to the presence of SAP90 in inhibitory and SAP97 in excitatory synapses, or whether it only represents their cell-type-specific expression. In either case the distribution pattern of SAP97 is more complex. For example, whereas SAP90 is re-

stricted to nerve terminals, SAP97 exhibits three different patterns in neurons: (1) in the hippocampal pyramidal cells it is restricted to nerve terminals; (2) in cerebellar granule cells a disproportionate amount of this protein was found in and along their unmyelinated axons, rather than in their synaptic boutons; and (3) in pyramidal cells situated in the entorhinal cortex that innervate the apical dendrites of granule cells in the dentate gyrus, SAP97 was found in both their unmyelinated axons and synaptic terminals. At present, a molecular explanation for this varied subcellular distribution pattern of SAP97 is not known. Thus far, two SAP97 variants have been identified that appear to arise via alternative splicing (Fig. 1; Wenzel, unpublished observations). However, it is not yet clear whether one variant is localized in synapses and another along the unmyelinated axons. In addition, it should be noted that SAP97 is not expressed in all neurons that are either unmyelinated or release excitatory neurotransmitters from their nerve terminal, for example, superior and inferior colliculus and granule cells in the dentate gyrus.

An important question raised by these results is why should unmyelinated axons and for the most part presynaptic nerve terminals possess a related but distinct member of the SAP90 family. One possible explanation is that while these cellular domains are functionally distinct, they both possess thick submembranous actin-spectrin cytoskeleton or "undercoat" (Landis, 1988). Such undercoats are found in association with a variety of membrane specializations in neurons, including dendritic spines (Landis, 1988), the axon initial segment (Palay and Palay-Chen, 1974), nodes of Ranvier in myelinated axons (Ellisman, 1988), along the length of unmyelinated axons (Weiss, 1990) and synaptic junctions (Landis, 1988; Hall and Sanes, 1993). In each case the membrane cytoskeleton of these undercoats is thought to work in conjunction with cell adhesion molecules such as cadherins and integrins to regulate the spatial distribution of ion channels and receptors creating functionally distinct membrane domains (Bennett and Gilligan, 1993). The specificity of these different membrane specialization is thought to lie in their complement of cytoskeletal proteins (Bennett, 1993; Bennett and Gilligan, 1993). For example, at the node of Ranvier a specific isoform of ankyrin ($ankyrin_{node}$) is involved in restricting the mobility of voltage-dependent sodium channels and the Na^+/K^+ATPase (Kordeli et al., 1990; Bennett and Gilligan, 1993). This isoform is also restricted to the initial segment of myelinated axons (Kordeli et al., 1990), while another, $ankyrin_R$, is confined to the plasma membrane of the cell body and dendrites (Kunimoto et al., 1991). One alternatively spliced variant of a third isoform, $ankyrin_B$ (440 kDa), is targeted to unmyelinated axons and excluded from axons following myelination (Kordeli et al., 1990). Similarly, isoforms of other undercoat associated proteins such as spectrin, band 4.1 and adducin are spatially segregated not only in neurons but other polarized cells such as epithelial cells (for review, see Bennett and Gilligan, 1993). These observations suggest that the uniqueness of each membrane specialization is established through basic principles whereby structurally similar but functional distinct molecules are assembled. As with the SAP90 family of related proteins, functional diversity is accomplished through multiple genes for each component and by alternative splicing of their pre-mRNAs (Bennett and Gilligan, 1993).

SAP97 in non-neuronal tissues

In contrast to the brain specific expression of SAP90, Western and Northern analysis revealed that SAP97 is also found in non-neuronal tissues. Immunofluorescence microscopy of sections from small intestine and choroid plexus demonstrated that SAP97 is highly concentrated in the distal more mature segments of the villi, where it is localized at the basal lateral membrane between the epithelial cells. Strong immunofluorescent staining of plasma membrane is seen at sites of cell–cell contact between cells of the human cell line T84 in culture. Plasma membranes in T84 cells not in contact with other cells are not stained indicating that SAP97 is functionally associated with sites of cell–cell contact between epithelial cells. At present, it is not clear whether SAP97 is involved in the initial events of cell–cell contact or rather in the stabilization of these adhesive sites. In the intestine, epithelial cells are continuously generated in the crypt region, from where they are pushed up in a side-by-side juxtaposition to the top of the villus and then shed (Haffen et al., 1986). Relatively speaking, lesser amounts of SAP97 are found in the crypt and proximal segments of the villi compared to the intense staining of older more mature distal segments of the villi suggesting a role for SAP97 at more mature junctions. This is consistent with the late onset of expression of SAP97 in nervous tissue.

Three other proteins structurally related to SAP97 have also been found at specialized junctions between epithelial cells: the product of the *Drosophila* tumor suppressor gene *dlg*-A and the zonula occludens proteins ZO-1 and ZO-2 (Woods and Bryant, 1991; Willott et al., 1993; Jesaitis and Goodenough, 1994). *Dlg*-A exhibits a similar overall domain structure to SAP97, however also possesses opa repeats and a PEST sequence (Fig. 1*C*). ZO-1 is less similar to SAP97 containing the three 90 amino acid repeats and an SH3 domain; however, its guanylate kinase domain has been extensively modified and there is a large carboxyl-terminal extension containing both acidic and proline-rich segments (Willott et al., 1993). From the limited sequence data on ZO-2, it exhibits a similar structural organization to ZO-1 (Jesaitis and Goodenough, 1994). *Dlg*-A is localized in the larvae of *Drosophila* in the septate junctions of the epithelial cells of imaginal disks. In homozygous mutants of *dlg*-A these junctions do not form (Woods and Bryant, 1989) resulting in a disorganized actin associated cytoskeleton, a loss of cell–cell adhesion and a proliferation of these epithelial cells into solid tumors (Woods and Bryant, 1989). These data suggest that *dlg*-A plays a role in regulating cell growth. Based on the presence of its putative guanylate kinase, an intriguing possibility has been suggested that these altered morphological characteristics involve a guanine nucleotide-mediated signal transduction pathway initiated from the septate junctions (Woods and Bryant, 1991). The second protein ZO-1 was initially described as the main tight junction protein in epithelial cells (Stevenson et al., 1986). Further experiments have shown that ZO-1 is also found in endothelial cells and the highly specialized epithelial junctions of Sertoli cells and glomerular epithelial cells (Balda et al., 1993). It has also been colocalized with cadherin in nonepithelial cells (Willott et al., 1993), can bind spectrin tetramers (Itoh et al., 1991) and may be involved in propagating cytoskeletal assembly signals subsequent to cadherin-mediated cell contact (Anderson et al., 1993). ZO-2 appears to be restricted to tight junctions in a number of epithelia (Jesaitis and Goodenough, 1994). In contrast to *dlg*-A, ZO-1, and ZO-2, SAP97 in epithelial cells has not been localized to one specific junctional complex, such as tight, adherent or desmosomal junctions, but was found all along their basal lateral membranes. Its restricted appearance, at the lateral membrane, to sites of cell–cell contact in T84 cells

in culture indicate that, as with ZO-1, it may be involved in the mechanisms of cell adhesion between epithelial cells. By analogy, we would propose that SAP90 and SAP97 in nerve terminals are involved in the formation and stabilization of synaptic junctions and that SAP97 may serve an additional role in the fasciculation of tracts of unmyelinated axons in the cerebellum, corpus callosum, olfactory nerve, and spinal cord. Clearly, future studies will be focused on resolving these possibilities.

Functional domains of SAP97

In the amino terminal domain of SAP97 are three tandem 90 amino acid residue repeats whose sequences are highly conserved in SAP90/PSD-95 and in the product of the *Drosophila* tumor suppressor gene *dlg*-A (Fig. 1*C*; Cho et al., 1992; Kistner et al., 1993). The spacing between these repeats in SAP90 and SAP97 have been maintained, whereas in *dlg*-A they are separated by the insertion of additional amino acid residues (30–300 residues) (Kistner et al., 1993). Alternatively spliced variants thus far identified in SAP90 and SAP97 flank the repeat containing regions (Fig. 1; Wenzel et al., unpublished observations). These repeats are not restricted to this family of putative guanylate kinases. For example, three copies of the repeat have been identified in the zonula occludens proteins ZO-1 (Willott et al., 1993), one so far in ZO-2 (Jesaitis and Goodenough, 1994), five copies in a protein tyrosine phosphatase PTP (Walton and Dixon, 1993), and a partial repeat in a human erythrocyte membrane protein p55 (Bryant and Woods, 1992) (Fig. 1*C*). There are two intriguing possible functions for these repeats. One is that they are involved in the tight binding of these proteins to the cortical cytoskeleton and the second is as a possible structural role. Since these sequences are nearly identical between SAP90 and SAP97, we would also predict that divergent sequences surrounding the repeats are perhaps responsible for the different sorting properties of these molecules. Interestingly, it is exactly in these neighboring sequences that the alternatively spliced variants of SAP90 and SAP97 have been found (Fig. 1; Müller and Garner, unpublished observations).

Also present in SAP97 and other members of this super family of related proteins is a 60 amino acid residue motif that is homologous with the src homology 3 (SH3) domains. SH3 domains have been identified in a variety of membrane cytoskeletal and signaling proteins associated with the Ras-guanine nucleotide signal transduction pathway that regulates the growth and differentiation of cells (McCormick, 1993). While the function of the SH3 domain in SAP97 is not known, the SH3 domains in at least two other proteins have been shown to be sites of protein–protein interaction. For example, the SH3 domains in Grb2, an adaptor protein (Olivier et al., 1993), binds proline-rich sequences in Sos1 (Rozakis-Adcock et al., 1993). Sos1 (Son of Sevenless) is a guanine-nucleotide-releasing protein that interact directly with Ras resulting in the activation of the Ras-dependent kinase cascade (McCormick 1993). A second example is c-abl, a non-receptor tyrosine kinase, whose SH3 domain binds a member of the rho family of GTPase activating proteins (Cicchetti et al., 1992). These SH3 containing proteins regulate the activity of these small GTP-binding proteins by controlling the ratio of bound GTP to GDP (Rozakis-Adcock et al., 1993). These data suggest that in SAP90, SAP97, *dlg*-A, ZO-1, ZO-2, and p55, their SH3 domains are also sites of protein–protein interaction that may be involve in either regulating the activities of these proteins or acting together with the repeats in targeting each to specific membrane specialization. Should SAP90, SAP97, *dlg*-A, and p55 actually encode authentic guanylate kinases, the SH3 could be a good candidate in modulating the activity of these enzymes as has been observed for the SH3 domain in c-src which acts as a negative regulator of its tyrosine kinase (Kato et al., 1986).

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