Site specificity in the interactions of synapsin 1 with tubulin

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Synapsin 1 is one of a family of phosphoproteins located on small synaptic vesicles (SSV) in the presynaptic terminal, and probably plays a critical role in the process of neuronal exocytosis by providing regulated linkages between SSV and the cytoskeleton. Two forms of synapsin 1 are produced from a single gene by differential mRNA splicing: 1a, 706 amino acid residues, and 1b, 670 residues. Synapsin 1 has two structural domains, a globular *N*-terminal head domain and an elongated tail domain. Electron microscopy of nerve terminals *in situ* and reconstitution studies *in vitro* indicates that synapsin 1 can interact with microtubules, microfilaments and brain spectrin. *In vitro*, synapsin 1 can bundle microtubules. This could either occur by synapsin 1 being at least bivalent for microtubules, or by univalent synapsin 1 molecules aggregating to form complexes that are more than univalent. To resolve this question, we have taken the approach of preparing defined fragments of synapsin 1 from each structural domain and analysing them for tubulin-binding activity. Our results show that there are tubulin-binding sites in both head and tail domains. We conclude that synapsin 1 monomers should be able to cross-link microtubules.

INTRODUCTION

The synapsins are a family of proteins located on small synaptic vesicles (SSV) (Sudhof *et al.*, 1989). They were originally identified through studies of brain protein phosphorylation by cyclic AMP-dependent protein kinase (Ueda & Greengard, 1977). They are bound to the surface of SSV through interactions with both lipid and protein components of the vesicle (Benfenati *et al.*, 1989a). Two different genes encode the synapsins, giving rise to synapsin 1 and synapsin 2 (Sudhof *et al.*, 1989). Synapsin 1 in bovine brain has two forms, 1a (706 amino acid residues, 74472 Da) and 1b (670 residues, 70396 Da). They are derived from the same gene by alternative mRNA splicing of exons encoding the C-terminus of the protein.

Two major structural domains exist in the synapsins (Landis, 1988; Hirokawa *et al.*, 1989). Synapsins 1 and 2 have a conserved globular head as the *N*-terminal domain (residues 1–421). A variable tail, which differs between synapsins 1 and 2, makes up the remainder of the molecule (Sudhof *et al.*, 1989). The head domain of synapsin 1 is conveniently isolated by digestion of the molecule with *Clostridium* collagenase (specificity Pro-Xaa+Gly-Pro) (Ueda & Greengard, 1977), since the tail contains several sites for this enzyme, and so is extensively degraded.

One view of the role of synapsin 1 in nerve terminals has been summarized in several reviews (for example Baines, 1987; Greengard, 1987; Linstedt & Kelly, 1987; Kelly, 1988; Hirokawa *et al.*, 1989). In this view, synapsin 1 provides regulated linkages between SSV and the cytoskeleton. These linkages allow SSV to be organized both in the presynaptic cytoplasm and at the area on the presynaptic plasma membrane that constitutes the active zone for exocytosis. On depolarization, synapsin 1 is phosphorylated as a result of the activation of protein kinases. Its interactions with both cytoskeleton and SSV are thereby weakened, thus releasing SSV either for direct exocytosis or for movement towards the presynaptic plasma membrane for a subsequent round of exocytosis.

The cytoskeletal proteins that interact with synapsin 1 include brain spectrin, microfilaments (via actin), microtubules (via tubulin) and neurofilaments (via the 68 kDa subunit) (Baines & Bennett, 1985, 1986; Bahler & Greengard, 1987; Steiner *et al.*,

1987a; Landis *et al.*, 1988; Hirokawa *et al.*, 1989). In the case of each of the last three, synapsin 1 causes the formation of bundles. Each of these activities has been confirmed by electron microscopy of nerves *in situ*, although it is likely that interaction with neurofilaments only occurs in axons during the transport of synapsin 1 to nerve terminals from its site of synthesis in or near the neuronal cell body.

The interaction of synapsin 1 with microtubules places it in the category of a 'membrane-bound microtubule-associated protein' (MAP). It co-isolates with microtubules through cycles of polymerization and depolymerization from whole-brain extracts or with microtubules prepared from purified tubulin (Baines & Bennett, 1986; Goldenring *et al.*, 1986). At saturation, one tubulin dimer binds one synapsin 1. In principle, bundling could occur through synapsin 1 being multivalent for microtubules, or through univalent synapsin 1 molecules coming together to form complexes that are bivalent or more for microtubules. Evidence for each has been put forward. On the basis of cross-linking with short-range cross-linkers, Font & Aubert-Foucher (1989) concluded that synapsin 1 could dimerize through interactions of head domains. If each tail domain contained a microtubule-binding site, the dimer should be bivalent. On the other hand, three separate electron-microscopic studies of rotary-shadowed purified synapsin 1 showed it to be monomeric, at least in dilute solution (Steiner *et al.*, 1987b; Landis *et al.*, 1988; Hirokawa *et al.*, 1989). Hirokawa *et al.* (1989) examined synapsin 1–microtubule bundles adsorbed on mica; their images of the bundles provided support for both views. In some cases, synapsin 1 molecules appeared bound to microtubules by both head and tail domains; elsewhere microtubules were joined by the tails of synapsin 1 dimers.

A prediction from these diverse observations would be that it should be possible to define biochemically distinct tubulin-binding sites in both the head and tail domains. Definition of these sites would be important in understanding regulation of the activities of synapsin 1. Separate protein kinase sites exist in the head (cyclic AMP-dependent protein kinase and Ca²⁺/calmodulin-dependent protein kinase 1) and in the tail (Ca²⁺/calmodulin-dependent protein kinase 2) (Czernic *et al.*, 1987). In principle, each kinase could separately regulate its neighbouring

Abbreviations used: PEME, 0.1 M-Pipes/1 mM-EGTA/1 mM-MgCl₂/0.1 mM-EDTA, pH 6.8; Dip-F, di-isopropyl phosphorofluoridate; SSV, small synaptic vesicles; MAP, microtubule-associated protein; IC₅₀, concentration causing 50% inhibition.

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site. In addition, calmodulin can bind directly to the head domain of synapsin 1 (Okabe & Sobue, 1987; Hayes *et al.*, 1991).

In the present paper we provide evidence for biochemically distinct tubulin-binding sites in the head and the tail domains. We have generated proteolytic fragments of bovine synapsin 1 and defined their origins in head and tail regions. Fragments from both head and tail bind tubulin; we conclude that head and tail contain independent tubulin-binding sites.

MATERIALS AND METHODS

Materials

Bull brains were obtained from a local slaughterhouse and were processed for storage at -80°C as described previously (Bennett *et al.*, 1985). Sheep brains were processed within 30 min of slaughter for the preparation of microtubule protein. Sheep brains were preferred for the preparation of microtubules, since they were obtainable much more rapidly after slaughter. Phosphocellulose P11 was obtained from Whatman. ^{125}I -labelled Bolton–Hunter reagent was from ICN. Proteolytic enzymes and di-isopropyl phosphorofluoridate (Dip-F) were obtained from Sigma Chemical Co.

Preparation of proteins

Synapsin 1 was purified from bovine brain as described previously (Bennett *et al.*, 1985), and concentrations were determined spectrophotometrically (Ueda & Greengard, 1977). Microtubule protein (HP2) was prepared from sheep brain and stored at -80°C (Dentler *et al.*, 1975). Tubulin was purified from microtubule protein, after further depolymerization and centrifugation at 4°C (CS3), by phosphocellulose chromatography (Williams & Detrich, 1979). Purified tubulin was stored frozen in small portions in liquid N_2 until use.

Proteolytic digestion of synapsin 1

Synapsin 1 was digested with collagenase as described by Ueda & Greengard (1977); in these experiments *Clostridium histolyticum* collagenase, specificity -Pro-Xaa-Gly-Pro-, was used. For glutamate-specific cleavage, synapsin 1 or isolated collagenase head fragment in $50\text{ mM-NH}_4\text{HCO}_3$ (pH 7.8)/ 2 mM-EDTA was digested for 45–90 min at 37°C with *Staphylococcus aureus* V8 proteinase at an enzyme/substrate ratio 1:20 (Drapeau, 1976). Digestion was stopped by addition of Dip-F to a final concentration of 1 mM . Chymotryptic digestion was carried out in $10\text{ mM-sodium phosphate}$ (pH 7.4)/ 1 mM-EDTA for 5–10 min at 37°C at an enzyme/substrate ratio 1:100. The reaction was stopped by the addition of Dip-F to a final concentration of 1 mM .

Anti-(synapsin 1) antibody

An anti-(synapsin 1) antibody was prepared by inoculation of New Zealand White rabbits with intact bovine synapsin 1, by using a 3-week inoculation schedule, with Freund's complete adjuvant for the first injection, and incomplete adjuvant for four subsequent injections; $100\text{ }\mu\text{g}$ of protein was used per injection. The resulting serum was used at a dilution of 1:500 or 1:1000 in immunoblotting. It was judged to be specific for synapsin 1, since it only reacted with bands of apparent molecular mass 75 kDa and 73 kDa on blots of brain homogenates; these bands comigrated on gels with authentic synapsin 1 (results not shown). Pre-immune serum showed no such reaction. In immunoperoxidase staining of sections of cerebellum, it gave a pattern of labelling consistent with specificity for synapsin 1 (results not shown).

An antibody specific for the 20 kDa chymotryptic fragment of synapsin 1 was affinity-purified from this serum. A chymotryptic

digest was resolved on an SDS/11–18% polyacrylamide gel and transferred to nitrocellulose paper. The paper was stained with Ponceau S and the 20 kDa band was cut out. This was then used for the affinity purification of anti-(20 kDa fragment) antibody from whole anti-(synapsin 1) serum by the method of Olmsted (1981).

Gel electrophoresis and immunoblotting

The method of Laemmli (1970) was used for SDS/PAGE on 12.5% or 11–18% gradient gels. The method of Rayner & Baines (1989) was used for immunoblotting.

Sequencing of synapsin 1 fragments

The Immobilon blot method was used (Matsudaira, 1989). Peptides in *S. aureus* V8 proteinase or chymotryptic digests were resolved on SDS/12.5% polyacrylamide gels and transferred to Immobilon P (Millipore). Blots were stained with Coomassie Blue and bands cut out. These were subjected to automated gas-phase N-terminal sequencing by JayTee Biosciences (University of Kent Research and Development Centre).

Isolation of the synapsin 1 head domain by calmodulin affinity chromatography

The method of Hayes *et al.* (1991) was used. A collagenase digest was prepared from 0.7 mg of synapsin 1. The digest was enriched in the 48 kDa head fragment and was dialysed against buffer A [$500\text{ mM-NaCl}/20\text{ mM-Hepes}$ (pH 7.4)/ $0.2\text{ mM-CaCl}_2/0.2\text{ mM-dithiothreitol}$]. Non-diffusible peptides were applied to a 2 ml column of calmodulin–Sepharose (equilibrated in buffer A) and washed with 10 column vol. of buffer A. Bound protein was eluted from the column with buffer B (buffer A with 5 mM-EGTA , but no CaCl_2). The 48 kDa peptide comprised 90% of the Coomassie Blue-detectable material, with other peptides of 43 kDa and 52 kDa being minor contaminants. These peptides both react with anti-(synapsin 1) sera and are likely to be other digestion products.

Pelleting assays

Pelleting of synapsin 1 or its fragments with microtubules was carried out by the method of Baines & Bennett (1986). Microtubules were polymerized from phosphocellulose-purified tubulin using taxol. Synapsin 1 fragments ($400\text{ }\mu\text{g/ml}$) in 0.1 M-Pipes (pH 6.8)/ $1\text{ mM-EGTA}/1\text{ mM-MgCl}_2/0.1\text{ mM-EDTA}$ (PEME) were mixed with microtubules ($100\text{ }\mu\text{g/ml}$) and incubated at 37°C for 45 min. Mixtures were centrifuged in a Beckman Airfuge at 100000 g for 15 min, and pellets and supernatants were analysed by SDS/PAGE.

Electron microscopy

Mixtures of synapsin 1 or its fragments and taxol-stabilized microtubules were prepared as described above. Processing for negative staining and electron microscopy was by standard procedures (Baines & Bennett, 1986).

^{125}I -tubulin blot overlay

A modification of the method of Davis & Bennett (1984) was used. Purified tubulin dimer (1.5 mg/ml in PEME/ 0.1 mM-GTP) was labelled with ^{125}I -labelled Bolton–Hunter reagent to give a specific radioactivity of $156\text{ }\mu\text{Ci/mg}$. Nitrocellulose replicas of SDS/polyacrylamide gels were prepared and incubated in two changes of blot buffer [$0.15\text{ M-NaCl}/10\text{ mM-sodium phosphate}$ (pH 7.4)/ $1\text{ mM-EDTA}/0.2\%$ Triton X-100/ $1\text{ mM-NaN}_3/4\%$ (w/v) BSA] for 15 min each to block the residual protein binding of the nitrocellulose. The blots were placed in fresh blot buffer with ^{125}I -tubulin, which was diluted to give a final concentration of 10 nM (relative to dimer) and $0.5\text{--}1\text{ }\mu\text{Ci/ml}$. The blots were

shaken gently overnight at 4 °C, washed three times in blot buffer with BSA and washed twice in blot buffer without BSA. Bound tubulin was detected by autoradiography for 1–4 h. As controls, parallel blots were incubated in the presence of a 50-fold excess of unlabelled tubulin to ensure that binding was saturable and not an artifact of the labelling procedure. Blots were quantified by densitometry, ensuring that the signal measured was within the linear range of the film response. In further control experiments, we have found that MAP 2 resolved on 5–15% gels binds labelled tubulin saturably (results not shown). ¹²⁵I-tubulin itself does not bind to tubulin on the blot, since the labelled tubulin is rendered non-polymerizable by the blot buffer, which contains EDTA and no added nucleotide.

Computer analysis of protein sequences

The University of Wisconsin Genetics Computer Group package was made available by the Science and Engineering Research Council through SEQNET. Analysis used the programs COMPARE, DOTPLOT, BESTFIT, PEPTIDEMAP and PEPTIDE-SORT.

RESULTS

Preparation of synapsin 1 fragments

In order to assess the sites of interaction between synapsin 1 and microtubules, it was necessary to have available defined fragments of synapsin 1 that might contain those activities. Ueda & Greengard (1977) showed that the globular head domain could be conveniently dissected out by the use of collagenase. This allows direct examination of whether the head can bind microtubules. From the sequence of synapsin 1 this fragment should represent residues 1–453 (predicted polypeptide chain molecular mass 48 842 Da) (Fig. 1, lane 4). However, methods for isolation of the tail are less well defined. Inspection of the sequence of bovine synapsin 1b revealed that the tail domain has no glutamic acid residues beyond residue 409; synapsin 1a has a glutamic acid residue at 675. It should be possible to use *S. aureus* V8 proteinase to isolate the tail as a limit fragment of about 27 kDa from both species [predicted polypeptide chain molecular mass: 1a (residues 409–675) 27 004 Da; 1b (residues 409–670) 26 339 Da] as well as producing several head-derived fragments. In the tail region there are few tyrosine, phenylalanine or tryptophan residues, the major cleavage sites for chymotrypsin. Although there are other possible residues for cleavage, including several methionine and leucine residues, it should be possible to produce intermediate-sized fragments from the tail with this enzyme.

Analysis of the time course of *S. aureus* V8 proteinase digestion showed that a 27 kDa fragment did indeed occur (Fig. 1, lane 6). Other fragments, of 22–23 kDa, 15–17 kDa and 10–12 kDa, were also prominent during digestion. None of these represented limit fragments under our digestion conditions; this may be because other proteinases are common contaminants of *S. aureus* V8 proteinase preparations (Drapeau, 1976). Chymotryptic digestion produced fragments of 14–20 kDa after 10 min (Fig. 1, lane 7), which were degraded further on longer digestion. Collagenase digestion produced a 48 kDa fragment, as expected (Fig. 1, lane 4).

To determine the origin of these fragments, and in particular whether any of them came from the tail, several approaches were taken. The major 27 kDa V8 proteinase fragment was subjected to sequence analysis. The four *N*-terminal residues were Leu-Val-Val-Asn. These residues align uniquely with residues 409–412 in synapsins 1a and b. This confirms that the 27 kDa fragment represents the tail region and the last few residues of the head. Although two different fragments (from synapsins 1a and 1b)

were predicted, they were not resolved on the SDS/polyacrylamide gel. We also attempted, unsuccessfully, to sequence the 23 kDa and 12 kDa V8-proteinase fragments and the 20 kDa chymotryptic fragment.

To establish which V8 proteinase fragments were derived from the head, the 48 kDa head fragment was purified from a collagenase digest by calmodulin affinity chromatography. Digestion of this with V8 proteinase gave the 23 kDa, 17 kDa and 12 kDa fragments, but not the 27 kDa (Fig. 2). This confirms the location of the 27 kDa fragment in the tail, since it must be susceptible to collagenase cleavage, and shows that the 23 kDa, 17 kDa and 12 kDa fragments are head-derived. Since neither of

Fig. 1. SDS/PAGE of proteolytic fragments of synapsin 1

The Figure shows SDS/11–18% gradient-PAGE gels of synapsin 1 and its fragments (Coomassie Blue stain). Numbers on the right-hand side of lanes show apparent molecular masses (in kDa). Lanes 1 and 2 show highly purified bovine brain synapsin 1 at loadings of 0.5 µg and 12 µg; lane 3, collagenase alone (lines indicate polypeptides in the collagenase sample); lane 4, collagenase digest of synapsin 1 (the head domain is the 48 kDa fragment marked 48); lane 5, V8 proteinase alone; lane 6, V8 proteinase digest; lane 7, chymotryptic digest (at this loading no chymotrypsin can be seen on the gel).

Fig. 2. Digestion of the isolated head domain with V8 proteinase

The head domain of synapsin 1 was isolated from a collagenase digest by calmodulin affinity chromatography as described in the Materials and methods section. The isolated head appears as a 48 kDa band on SDS/11–18%-gradient-PAGE (lane 1). The head was digested with V8 proteinase, and the digest was analysed by SDS/PAGE (lane 2). The position of the proteinase is marked (V8). Fragments of 23 kDa, 17 kDa and 12 kDa are produced from the isolated head.

Fig. 3. Reactivity of the antibody to the 20 kDa chymotryptic fragment

The Figure shows immunoblots with either affinity-purified antibody to the 20 kDa chymotryptic fragment (lanes 1, 2, 4 and 6) or anti-synapsin 1 antibody (lanes 3 and 5). Numbers on the left-hand side show apparent molecular masses (in kDa). Lane 1, synapsin 1; lane 2, chymotryptic digest; lanes 3 and 4, collagenase digest; lanes 5 and 6, V8 proteinase digest. Note that the antibody to the 20 kDa chymotryptic fragment cross-reacts with the 18 kDa and 16 kDa fragments in the chymotryptic digest (lane 2), the 27 kDa fragment in the V8 proteinase digest (lane 6) and with intact synapsin 1 (lane 1). The antibody does not react with any of several head-derived fragments, i.e. the 48 kDa collagenase fragment (lane 4) or the 23 or 17 kDa V8 proteinase fragments (lane 6). However, the whole anti-synapsin serum reacts with each (lanes 3 and 5).

the 23 kDa or 17 kDa fragment contains collagenase sites, they cannot overlap with the 27 kDa fragment.

To establish the location of the 20 kDa chymotryptic fragment, an affinity-purified antibody to this fragment was prepared (see the Materials and methods section). This antibody reacted with the 20 kDa chymotryptic fragment as well as 18 kDa and 16 kDa chymotryptic fragments. When tested on the V8 proteinase digest, it reacted selectively with the 27 kDa tail fragment. The antibody did not react with the 48 kDa collagenase head fragment. The 20 kDa, 18 kDa and 16 kDa chymotryptic fragments are therefore likely to be tail-derived (Fig. 3). However, it is possible that they overlap with a region of the head that does not have epitopes recognized by the anti-(20 kDa fragment) antibody.

Synapsin 1 also has a well-characterized site for cyclic AMP-dependent protein kinase in Ser-9. By using ^{32}P labelling, this was found previously (Hayes *et al.*, 1991) to reside in 5 kDa and 12 kDa V8 proteinase fragments, which are likely to represent residues 1–52 and 1–121, on the basis of a comparison of the apparent size of the fragment with the synapsin 1 sequence. Thus the 23 kDa and 17 kDa V8 proteinase head fragments, which are not phosphorylated by this enzyme, are not likely to contain the *N*-terminus.

Pelleting of synapsin 1 fragments with microtubules

To analyse which of the various fragments may interact with tubulin, we employed pelleting with taxol-stabilized microtubules prepared from phosphocellulose-purified tubulin. Mixtures of digests and microtubules were pelleted in an Airfuge, and pellets and supernatants were analysed by SDS/PAGE (Fig. 4). The 48 kDa collagenase head fragment was found to co-sediment with microtubules, in agreement with the results reported by Aubert-Foucher & Font (1990) (results not shown). Fig. 4(c) shows that V8 proteinase fragments of 27 kDa (from the tail), 23 kDa and 17 kDa (both from the head) co-sediment with microtubules. In chymotryptic digests (Fig. 4b), the 20 kDa, 18 kDa and 16 kDa tail-derived fragments all co-sediment. To ensure specificity of pelleting, all fragment mixtures were centrifuged before mixing with microtubules. Parallel sedimentations without added microtubules showed that none of the fragments pelleted significantly without added tubulin (results not shown). In summary, these results indicate that fragments from both head and tail can bind to microtubules.

Do the head and tail regions contain independent tubulin-binding sites?

Although the pelleting shows that fragments derived from both head and tail domains can co-sediment with microtubules, it does not prove that there are independent binding sites in head and tail. It is conceivable that the fragments may aggregate and bring down fragments that do not in themselves contain tubulin-binding sites.

To address this, we took advantage of the blot-binding method of Davis & Bennett (1984). In this method, fragments are separated by SDS/PAGE and transferred to nitrocellulose. Individual fragments are then probed for tubulin-binding activity by incubating the blot with ^{125}I -tubulin. Fig. 5 shows the results

Fig. 4. Co-sedimentation of synapsin 1 and synapsin 1 fragments with taxol-stabilized microtubules

Synapsin 1 or proteolytic digests were incubated with taxol-stabilized microtubules (100 $\mu\text{g}/\text{ml}$) at 37 °C for 45 min. The mixtures were centrifuged at 100 000 *g* for 15 min, and samples of pellets and supernatants were analysed by SDS/11–18 % gradient-PAGE; the numbers by the side of the gels show apparent molecular masses (in kDa). (a) Left-hand lane, phosphocellulose-purified tubulin; centre lane (S), synapsin 1 plus microtubules (supernatant); right-hand lane (P), synapsin 1 plus microtubules (pellet). (b) Results of pelleting a 5 min chymotryptic digest (left-hand three lanes) and a 10 min chymotryptic digest (right-hand three lanes) with microtubules: lanes S and P in each case show supernatants and pellets; the unlabelled lanes show composition of the digests. (c) Results of pelleting a V8 proteinase digest with tubulin: left-hand lane, V8 proteinase digest; centre lane (S), V8 proteinase digest plus microtubules (supernatant); right-hand lane (P), V8 proteinase digest plus microtubules (pellet). Note that intact synapsin 1 is recovered in the microtubule pellet. V8 proteinase fragments from the head (23 kDa and 17 kDa) also pellet with tubulin, as do the V8 proteinase tail fragment (27 kDa) and the chymotryptic tail fragments (20 kDa, 18 kDa and 16 kDa). Some of the major fragments show no binding (e.g. the 14 kDa chymotryptic fragment and the 12 kDa V8 proteinase fragment).

Fig. 5. Interaction of ^{125}I -tubulin with synapsin 1 or its fragments on gel blots

Bovine brain tubulin was radiolabelled with ^{125}I -labelled Bolton-Hunter reagent. (a), (b) and (c) show replicate gels. (a) and (d) were stained with Coomassie Blue and (c), (d), (e) and (f) were transferred to nitrocellulose. Numbers on each side show apparent molecular masses (in kDa). Blots were probed with ^{125}I -tubulin. In (c) and (f) a 50-fold excess of unlabelled tubulin was added to compete with specific binding of ^{125}I -tubulin. All blots contained 4% BSA and 0.2% Triton X-100 to eliminate non-specific binding. Lanes 1, V8 proteinase digest; lanes 2, V8 proteinase; lanes 3, collagenase digest; lanes 4, collagenase; lanes 5, synapsin 1; lanes 6, microtubule protein. (d), (e) and (f) show chymotryptic digest. Note the specific binding of ^{125}I -tubulin to synapsin 1 and the 48 kDa, 27 kDa and 23 kDa fragments in (b). A 17 kDa V8 proteinase fragment is also labelled. In (e), 20 kDa, 18 kDa and 16 kDa fragments are specifically labelled. In the microtubule protein, tau protein and MAP 1 light chains are labelled. Under the conditions used (non-polymerized ^{125}I -tubulin at 4 °C), no labelling of tubulin is observed.

of such an experiment. Intact synapsin 1 binds ^{125}I -tubulin strongly (lane 5). The collagenase 48 kDa head fragment binds ^{125}I -tubulin, as does a 57 kDa fragment in the same preparation (lane 3). The 27 kDa (tail), 23 kDa and 17 kDa (head) V8 proteinase fragments also bind (lane 1), as do 20 kDa, 18 kDa and 16 kDa (tail) chymotryptic fragments (Fig. 5e). In each case the binding is judged to be specific, since it can be inhibited by addition of unlabelled tubulin to the assay. The IC_{50} for inhibition in this assay is 84 ± 10 nM for the 27 kDa V8 proteinase fragment and 140 ± 10 nM for the 23 kDa V8 proteinase fragment (Fig. 6). Although it is not possible to extrapolate from these IC_{50} values to the association constant for each reaction, these results indicate that the tail may bind tubulin more strongly than the head. Note also in this experiment that the specificity of the blot binding is confirmed by (1) the lack of binding of the labelled tubulin to the proteolytic enzymes present and (2) the binding of labelled tubulin to tau proteins (broad band at 60–80 kDa) and MAP 1 light chains (doublet at approx. 30 kDa) in lane 6, which contains microtubule protein. No binding is seen with the high-molecular-mass MAPs, because they do not transfer efficiently from the 11–18% gel. These results confirm that the fragments defined as microtubule-binding in pelleting experiments each contain tubulin-binding sites.

Does proteolytic digestion of synapsin 1 abolish microtubule-bundling activity?

An important question relating to the microtubule-bundling activity of synapsin 1 is whether the bundling comes about through synapsin 1 being multivalent for microtubules, or through univalent synapsin 1 molecules coming together to form complexes that are bivalent or more for microtubules. If the latter, then the collagenase-produced head should retain bundling activity, since Font & Aubert-Foucher (1989) have shown that the head is the putative site of oligomerization. Furthermore, since large head fragments are retained in V8 proteinase digests (23 kDa and 17 kDa), it is possible that these should bundle too.

On the other hand, if bundling requires binding sites in both head and tail, the isolated head, or the separated head and tail, should not bundle.

To address this, we examined the ability of synapsin 1 and its fragments to bundle microtubules, using negative-stain electron microscopy. Taxol-stabilized microtubules (Fig. 7a) showed no tendency to form bundles on their own. Intact synapsin 1 (200 $\mu\text{g}/\text{ml}$) produced substantial bundles when incubated with taxol-stabilized microtubules (100 $\mu\text{g}/\text{ml}$) (Fig. 7b). On the other hand, when collagenase, chymotryptic or V8 proteinase fragments at an equivalent concentration were incubated with taxol-stabilized microtubules, no bundling was found (Figs. 7c–7e). It seems likely that bundling requires intact synapsin, and that head or tail fragments alone are not competent to bundle.

Synapsin 1 does not contain regions of sequence clearly identifiable as microtubule-binding sites

In their analysis of the protein sequence of rat synapsin 1 deduced from the sequence of cDNA, McCaffery & De Gennaro (1986) noted three apparent imperfect repeats in the tail region. A comparison of these repeats with the microtubule-binding repeats of MAP 2 and tau protein showed a moderate level of similarity (Baines, 1989). Subsequently, a corrected version of the rat sequence as well as the bovine and human sequences have been published (Sudhof *et al.* 1989; Sudhof, 1990). Now that the head appears to have microtubule-binding sites and the rat sequence has been corrected, we examined whether there is any evidence for the presence of sequences resembling microtubule-binding sites in the head or tail, using a broader comparison.

The sequences of bovine synapsin 1 (Sudhof *et al.*, 1989) and bovine tau protein (Himmler, 1989) were compared by dot-matrix analysis, using windows of 18 residues and stringencies between 9 and 15 (results not shown). The resulting dot plots did not support the existence of specific sequences resembling the tubulin-binding sites of tau protein in head or tail. For the tail, the similarities previously noted appear to be a consequence of the overall amino acid composition of the regions concerned, rather than any specific sequence similarity. Likewise, we could detect no significant resemblance to the repeat Lys-Lys-Glu-Glu motif in the microtubule-binding domains of MAP 1b (Noble *et al.*, 1989).

DISCUSSION

Although synapsin 1 was first detected some 17 years ago, its precise role *in vivo* is still a matter for debate. It is localized over synaptic vesicles and seems to be implicated in the process of exocytosis. Within the head domain there is a region with surfactant properties; this has suggested the possibility that part of its function *in vivo* might be as a fusogen promoting the fusion of synaptic vesicles with the presynaptic plasma membrane (Benfenati *et al.* 1989b). In addition, *in situ* it seems to form a bridge between synaptic vesicles and various other structures in the presynaptic terminus (Landis *et al.*, 1988; Hirokawa *et al.*, 1989), including cytoskeletal structures. Hirokawa *et al.* (1989) suggested that close to the junction between the synaptic terminus and the end of the axon body, synapsin 1 would link SSV to microtubules, and microtubules to each other. Electron microscopy of mixtures reconstituted *in vitro* provided evidence for two modes of microtubule cross-linking by synapsin 1. In the first mode, the tail would be bound to one microtubule, while the head interacted with another. In the second mode, a dimeric synapsin 1 molecule could be formed by head-to-head interaction. One tail would link to one microtubule, the other to another microtubule. Electron microscopy therefore suggests that there should be binding sites in the head and tail of synapsin 1 that would be biochemically distinct.

Fig. 6. Inhibition of binding of ^{125}I -tubulin to V8-proteinase fragments by increasing concentrations of unlabelled tubulin

V8 proteinase-digested synapsin 1 was resolved by SDS/PAGE and transferred to nitrocellulose paper. ^{125}I -tubulin was incubated with the blots as described in the Materials and methods section but in the presence of increasing amounts of unlabelled tubulin. Relative amounts of ^{125}I -tubulin bound to 27 kDa and 23 kDa fragments on the blots were measured by densitometry of corresponding autoradiographs, ensuring linearity of film response. Results are expressed as a percentage of the amount bound in the absence of added unlabelled tubulin. IC_{50} values of 84 ± 10 nM and 140 ± 10 nM are deduced for the 27 kDa and 23 kDa fragments respectively.

In this paper, using synapsin 1 fragments we have shown that both head-derived and tail-derived fragments bind microtubules. Aubert-Foucher & Font (1990) dissected synapsin 1 with trypsin, and, although they found evidence for a tubulin-binding site in the head, they could detect no binding activity in the tail. They therefore suggested that microtubule bundling requires dimeric synapsin, since synapsin 1 monomers would be univalent. In this scheme there would be no role for the tail. A prediction from this is that synapsin 1 after digestion with collagenase (i.e. the 48 kDa head domain) would still be competent to bundle microtubules. However, using electron microscopy, we have found that neither the intact head nor smaller head-derived fragments can bundle microtubules. Our data show that microtubule bundling requires both head and tail domains.

Aubert-Foucher & Font (1990) found a tubulin-binding tryptic fragment of 31 kDa which contained the site for cyclic AMP-dependent phosphorylation (Ser-9). It is not possible to assign a precise C-terminus to this fragment, particularly since synapsin 1 is very rich in potential tryptic cleavage sites. On the basis of its size, it should extend to somewhere between residues 281 (predicted molecular mass of Arg-7/Leu-8-Lys-281 is 28900 Da) and 318 (predicted molecular mass of residues 8–318 is 33121 Da), allowing for error on estimate of size based on SDS/PAGE. We have noted that the V8 proteinase 12 kDa fragment which contains the site for cyclic AMP-dependent phosphorylation (residues 1–121) does not bind tubulin. It seems reasonable to postulate that the head site for binding tubulin probably lies between residues 122 and 318. The minimum sequence requirements for binding to tubulin are not likely to be established until a molecular-genetic analysis of the site(s) can be carried out.

In contrast with Aubert-Foucher & Font (1990), we have found direct evidence for interaction of a site or sites in the tail region of synapsin 1 with tubulin. In both blot-binding and pelleting, tail-derived fragments from both V8 proteinase and chymotryptic digests bind tubulin. Indeed, it is possible that the tail-derived fragments bind tubulin more avidly than do the head-derived fragments; however, confirmation of this point must await purification of each set of fragments and measurement of precise affinities.

Our observation that the tail region binds tubulin is the third ligand-binding activity described in that region. Interactions with proteins of the synaptic vesicle (Benfenati *et al.*, 1989a) and actin filaments (Bahler *et al.*, 1989) have also been described. It may be that there are three distinct ligand-binding sites in the tail, but this need not be the case. Correas *et al.* (1990) showed that a tubulin-binding repeat of MAP 2 and tau protein was also competent to bind actin. Furthermore, no instance of a synaptic vesicle being bridged to either a microtubule or a microfilament by a single tail was detected by electron microscopy (Hirokawa *et al.*, 1989). Although this could be a steric effect, it will be interesting to test whether, like MAP 2 and tau protein, there may be mutual actin- and microtubule-binding sites, and whether indeed synaptic vesicles can bind at that site.

Fig. 7. Electron microscopy of mixtures of taxol-stabilized microtubules and synapsin 1 or its fragments

The Figure shows negatively stained taxol-stabilized microtubules alone (a) or in the presence of (b) intact synapsin I, (c) chymotryptic digest, (d) V8 proteinase digest or (e) collagenase digest. Scale bar, $0.1 \mu\text{m}$.

In summary, we have defined biochemically distinct tubulin-binding sites in both head and tail domains. This is consistent with the observations by Hirokawa *et al.* (1989) that microtubules can be cross-linked either by monomeric synapsin I (binding via head and tail domains) or by dimeric synapsin I (binding via two separate tail domains).

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