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Process formation in Sf9 cells induced by the expression of a microtubule-associated protein 2C-like construct

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ABSTRACT **To understand the roles of various microtubule-associated proteins (MAPs) in the development of axons and dendrites, we have expressed individual neuronal MAPs in normally rounded Sf9 host cells. We previously reported that expression of tau protein in these cells results in the elaboration of long processes containing dense bundles of microtubules (MTs). These bundles generally terminate in the hillock region of the cell body, and almost all of the MTs within the bundles are oriented with their plus ends distal to the cell body. Here we report the expression of a construct that approximates the MAP2C sequence and also induces the elaboration of processes with dense bundles of predominantly plus-end-distal MTs. Whereas tau generally results in a single process, there is a significantly greater tendency for the MAP2C-like construct to induce multiple processes. In contrast to the tau processes, the MT bundle in these processes extends far into the cell body. This latter observation suggests that MAP2C and tau have different effects on MT assembly and/or transport events in the cell. Although both of these MAPs can organize MTs that are competent to participate in process formation, the detailed organization of MTs induced by each of the two constructs is distinctive, and these differences may be relevant to axonal and dendritic differentiation.**

Microtubules (MTs) are key architectural elements that serve as the substrate for bidirectional transport of cytoplasmic organelles. In the axon, MTs are tightly packed and are all oriented with their plus ends distal to the cell body (1, 2), whereas dendritic MTs are spaced farther apart and are oriented in both directions (2, 3). These differences in polarity orientation may have profound implications with regard to the growth properties of neurites and their capacity to accommodate the transport of different types of cytoplasmic organelles. What are the mechanisms by which axons and dendrites develop different types of MT arrays and what roles do these arrays play in generating the unique shapes of these two types of processes? The various microtubule-associated proteins (MAPs) in axons and dendrites, particularly the fact that axons are highly enriched in the MAP tau, whereas dendrites are highly enriched in MAP2 (4–7), may contribute to these distinct morphologies. Several studies now point to a role for tau protein in the generation of some features of an axonal morphology (8–11). We have utilized baculoviral vectors in Sf9 cells to analyze the cellular consequences of MAP expression. After infection with vectors carrying certain neuronal MAPs, the normally rounded Sf9 ovarian cells undergo a cytoskeletal reorganization and morphological changes that primarily involve process formation. The expression of tau in these cells caused them to extend long slender processes that contain dense bundles of MTs (8). The spacing between MTs was about half that observed in axons, but similar to the arrangement in the axon, almost all of the MTs in the Sf9 cell processes were aligned with their plus ends distal (9).

MAP2 is encoded by a single gene (12) that undergoes developmentally regulated alternative splicing (13). Early in rat postnatal brain development, 1372 aa are inserted within the projection domain of MAP2. Thus MAP2 shifts in its apparent molecular mass on SDS/PAGE gels from ≈70 kDa in the juvenile form (MAP2C) to ≈280 kDa in the mature form. The juvenile form of MAP2 is present throughout the neuron and the mature form of MAP2 is restricted to the somatodendritic compartment (13). Thus MAP2C and mature MAP2 may play different roles in regulating MT organization. Here we report the effects of expressing a construct that closely resembles MAP2C on MT organization and process formation.

METHODS

Spodoptera frugiperda (Sf9) cells were obtained from the American Type Culture Collection (ATCC #CRL 1711) and were used to propagate wild-type and recombinant baculoviruses. Sf9 cells were grown in suspension or as a monolayer at 27°C in Grace's medium (GIBCO) supplemented with 10% (vol/vol) fetal bovine serum. The baculovirus transfer vector, pBlueBac, engineered by Invitrogen (San Diego) to include β-galactosidase as a marker, was used to transfer a construct called FS (14) into the genome of *Autographa californica* multiple nuclear polyhedrosis virus by homologous recombination. FS approximates the MAP2C sequence, the only difference being in the exact sites of the internal deletion. Rat MAP2C lacks aa 147–1519; the corresponding deletion in mouse FS is aa 228–1621 (14). The transfer vector, pBlueBac-FS, has the FS coding sequence inserted into the unique *Nhe* I cloning site of the pBlueBac transfer vector.

The pBlueBac-FS transfer plasmid was cotransfected into Sf9 cells with a modified form of the baculovirus, *Autographa californica* nuclear polyhedrosis virus, designated AcRP6-SC, using the Lipofectin procedure (GIBCO). AcRP6-SC was linearized by digestion with endonuclease *Bsu*36I (15). Viral DNA (1 μg) and transfer vector plasmid DNA (5 μg) were used to transfect the Sf9 cells. After 48 h at 27°C, the transfection supernatant was purified by a standard plaque assay for occlusion-body-negative recombinant viral plaques and by limiting dilution (16). The presence of the FS insert in the recombinant virus was confirmed by PCR using extracellular virus from infected Sf9 cells lysed in detergent and proteinase K and incubated at 60°C for 1 h. The PCR primers were located within the MAP2C sequence. The recombinant virus was amplified in Sf9 cells to yield titers of 10^8 to 10^9 plaque-forming units/ml. For comparisons with tau-induced

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Abbreviations: MAP, microtubule-associated protein; MT, microtubule.

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processes, a construct containing the three-repeat tau isoform cloned in baculovirus was used (8).

To confirm that the Sf9 cells expressed FS, antibody AP18, which reacts with MAP2C (17), was used to label both the cells and the recombinant protein on immunoblots. Morphological analysis was performed on an IBAS image analysis system linked to a charge-coupled device camera. The association of MAP2C with the cytoskeleton was assessed by extracting the cells in 0.2% Triton X-100 for 1 min prior to fixation. FS protein was prepared by sonicating the cells, adjusting the low-speed supernatant to 0.75 M NaCl, and boiling for 4 min. This supernatant was dialyzed, gel-electrophoresed, and analyzed on an immunoblot.

Standard transmission electron microscopy and MT polarity determinations were performed as described (2, 9, 18). For the "hooking" procedure, the cells were lysed in a MT assembly buffer (9) in the presence of exogenous brain tubulin. The exogenous tubulin adds onto the existing MTs in the form of lateral protofilament sheets that, when viewed in cross-section, reveal the polarity orientation of the MTs. The curvature of the hook in a clockwise direction indicates that the plus end of the MT is directed toward the observer, and a counterclockwise hook indicates the opposite. The sample number for each ultrastructural feature analyzed was 10.

RESULTS

Expression of FS in Sf9 Cells. After infection with the recombinant baculovirus containing FS, Sf9 cells extended processes (Fig. 1*A*) that were reactive with the MAP2C antibody AP18 (Fig. 1*D*). Control cells including noninfected cells, wild-type infected cells, and cells expressing other gene products did not extend processes (8). With a multiplicity of infection of 5, the onset of FS expression was detectable 24 h after the addition of virus to the medium, a time that was coincident with process formation. By 48 h, nearly 60% of the cells had processes and upward of 80% were reactive with AP18. Only antibody-positive cells had processes. At 72 h

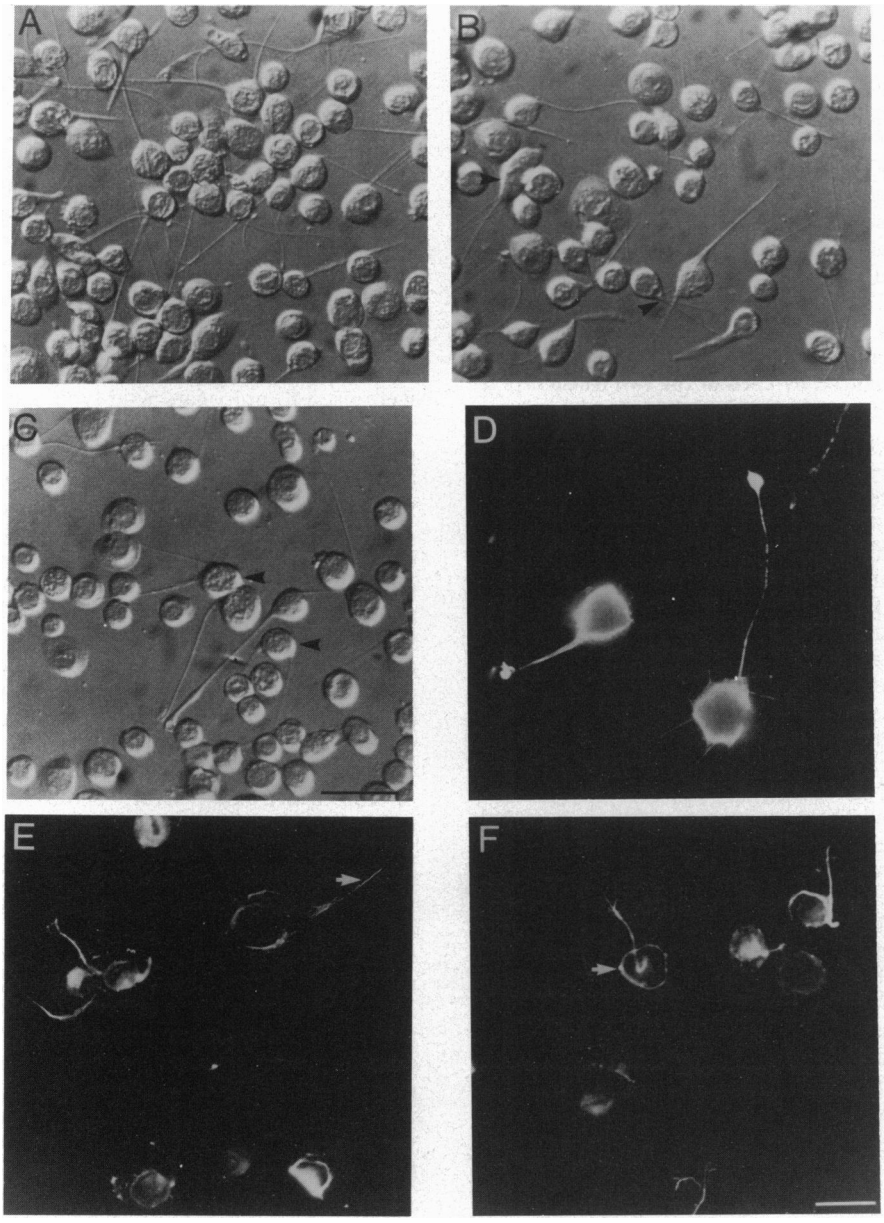

FIG. 1. (*A–C*) Process formation in Sf9 cells expressing FS. Arrowheads indicate cells with multiple processes. (Bar = 40 μm for *A–C*.) (*D*) AP18 labeling of Sf9 cell expressing FS with multiple processes. (*E* and *F*) AP18 labeling of Sf9 cells expressing FS after Triton X-100 extraction. Arrow shows label in a process (*E*) and around the rim of the cell (*F*). (Bar = 20 μm for *D–F*.)

when cell death became apparent, the protein was most abundant. After heat treatment, FS in the Sf9 cells was the major detectable protein by Coomassie blue staining (data not shown). Its molecular mass was similar to that of MAP2C (≈70 kDa) and it migrated as several tightly spaced bands, suggesting that FS underwent posttranslational molecular mass shifts in the Sf9 cell (Fig. 2). Control Sf9 cells and cells infected with the wild-type virus alone did not react with MAP2 antibodies. The association of FS protein with the cytoskeleton in the Sf9 cells was demonstrated by retention of AP18 immunoreactivity after extraction with Triton X-100. Interestingly, Triton-resistant immunoreactivity was observed not only in processes but also often in a ring-like pattern around the cell (Fig. 1 *E* and *F*). The ring-like pattern was only rarely observed in the tau-expressing Sf9 cells.

In comparison to tau-expressing Sf9 cells, FS expression resulted in a significantly greater tendency to induce multiple processes (Fig. 1 *B–D*). To document these differences, we measured the number of processes per cell, the summed lengths of the processes per cell, and the mean process length per cell (Table 1). The total summed lengths of the processes per cell did not significantly differ between FS and tau; however, the mean length of each process was shorter for the processes induced by FS. Therefore, total process length was conserved and the increase in the total number of processes was compensated for by a decrease in their mean length. Among those cells with multiple processes, there was no tendency toward any type of asymmetry, such as that observed in many neuronal cells that have a single process significantly longer than the others. Both tau- and FS-expressing cells often had a swelling at the most distal end of the process (Fig. 1*D*). There was no significant difference in the caliber of either type of process (data not shown). To control for the possibility that differences may occur in the expression of FS and tau, both constructs utilized the same polyhedrin promoter, the multiplicity of infection was the same for both viruses, and equal numbers of cells gave bands of apparently identical intensity on Western blots.

Ultrastructural Observations. Ultrastructurally there were both similarities and differences between cells induced to express FS protein or tau. Processes induced by either of the two MAPs contained dense bundles of MTs with a packing density far greater than observed in neuronal processes. Figs. 3 and 4 show longitudinal and cross-sections, respectively, through cells induced to express FS protein. The wall-to-wall spacing between neighboring MTs in the FS-induced processes was 22 ± 4 nm (mean ± SD), only slightly greater than that measured for tau (20 ± 6 nm for the four-repeat isoform and 21 ± 4 nm for the three-repeat isoform). The principal difference between cells induced to express each MAP was the appearance and length of the MT bundle in the region of the cell body. As in tau-transfected cells (9) and like axonal and dendritic MT bundles (19), none of the MT bundles in the cells induced to express FS protein were continuous with any discernable MT nucleating structure such as the centrosome. However, in tau-transfected cells, the cell body contained several short MT bundles that were not associated with process outgrowth, whereas in FS-transfected cells, virtually all of the MT bundles appeared to be associated with process formation. Moreover, with tau, the MT bundle generally stopped abruptly or occasionally splayed out before terminating within the first few micrometers of the hillock region. In contrast, with FS, the MT bundle extended well into the cell body, typically traversing at least half its diameter (>10 μm; see Fig. 3*a*). In many cases, what appeared to be a single MT bundle extended from the tip of one process, through the entire cell body, to the tip of a second colinear process on the other side of the cell body (Fig. 3 *c* and *d*). Cross-sectional analyses through the cell body indicated that this arrangement was actually the result of two MT bundles, one in each process, overlapping within the cell body (Fig. 4*c*). Cross-sectional analyses through the processes revealed decreasing numbers of MTs with increasing distances from the cell body (data not shown). No evidence was obtained for free minus ends of MTs along the processes, as was the case for tau.

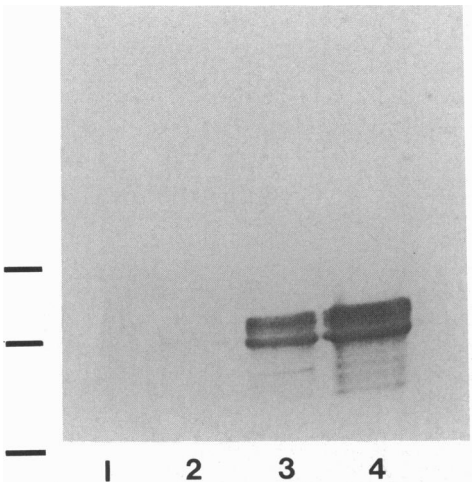

FIG. 2. FS-enriched fraction was prepared from Sf9 cells at 24 h (lane 2), 48 h (lane 3), and 72 h (lane 4) after infection and analyzed on an immunoblot with MAP2C antibody AP18. Lane 1 shows uninfected Sf9 cells, demonstrating the absence of antibody reactivity with any endogenous protein. Molecular mass standards are (top to bottom) phosphorylase *b* (111 kDa), bovine serum albumin (68 kDa), and ovalbumin (43 kDa).

Microtubule Polarity Studies. As was the case in tau-induced processes, the close spacing of MTs in the FS-induced processes was not optimal for hook formation or interpretation. The frequency of hooking (53 ± 30%) was generally lower than for axons or dendrites, and the tight spacing between MTs often resulted in contact between hooks from neighboring MTs, rendering their curvature ambiguous. Nevertheless, as was the case with tau, our data show a clear pattern. The hooks were predominantly clockwise as viewed from the tip of the process looking back toward the cell body, indicating a predominance of plus-end-distal MTs. For 10 processes examined, there were a total of 132 clockwise hooks, 13 counterclockwise hooks, 33 ambiguous hooks, and 376 unhooked MTs. The proportion of clockwise hooks was 89 ± 8%, virtually identical to that reported for tau (9). Due to uncertainty in the hooking data, the precise fraction of plus-end-distal MTs was unclear based on these samples alone. In general, a proportion of commonly hooked MTs >90% is considered uniform, although the proportion in the axon is generally higher, usually exceeding 95% (2, 18). Based on all of these considerations, we conclude that FS-induced processes, like tau-induced processes, contain MTs of uniform or nearly uniform polarity orientation with their plus ends distal to the cell body.

DISCUSSION

These studies address the mechanisms by which neuronal processes form and maintain their unique shapes. Expression of either tau or FS protein in normally rounded Sf9 cells causes them to elaborate long slender processes that contain dense bundles of MTs. Clearly, the assembly or enhanced stabilization of MT polymer is an important feature of this response. However, the drug taxol, which specifically enhances MT assembly and stabilization, did not induce process outgrowth (8), demonstrating that these effects alone cannot account for the capacity of tau or FS to induce process outgrowth. Rather, it appears that MTs must organize in

Table 1. Comparison of tau- and FS-induced processes

Construct	No. of cells				Total process length, μm	Process length, μm
	1 process	2 processes	3 processes	>3 processes		
FS	20.67 ± 2.60	11.67 ± 2.03	6.6 ± 1.86	11.0 ± 1.53	129.22 ± 6.30	57.44 ± 2.62
T	35.67 ± 1.45	9.6 ± 1.45	2.0 ± 0.58	2.33 ± 0.88	122.37 ± 6.45	84.59 ± 4.22
	($P < 0.0005$)	($P < 0.10$)	($P < 0.025$)	($P < 0.0005$)	($P < 0.10$)	($P < 0.0005$)

FS refers to the MAP2C-like construct and T is tau. For each determination three sets of 50 cells were counted. All values are the mean ± SEM. The *P* values are for FS- vs. T-induced processes.

conjunction with specific molecules such as tau or MAP2C-like molecules to induce process outgrowth. Caution must be exercised in likening FS to MAP2 isoforms; nevertheless, both molecules have identical repeated sequences in the MT-binding domain and induce MT bundles when transfected into several different cell lines (14). The segment of deleted amino acids contains several threonine or serine residues immediately on the N-terminal side of a proline. These putative phosphorylation sites for MAP kinases may regulate the affinity of MAP2C for the MTs (20). Recent observations with rat MAP2C expression in Sf9 cells also resulted in multiple process formation (N.L., C. C. Garner, and K.S.K., unpublished observations).

The functional differences among MAPs are unknown. For MAP2 and tau, these differences may be subtle since they share homologous MT-binding domains (21). Ultrastructurally, both MAPs induced the formation of densely packed almost paracrystalline bundles of MTs that are significantly tighter than observed in either axons or dendrites. They differed in that tau-expressing Sf9 cells often had free MT bundles in the cell body and the MT bundles associated with processes originated at the hillock. In contrast, FS-expressing cells lacked free MT bundles in the cell body, but those bundles associated with processes extended deep into the cell body. The MT organization also differed with regard to the numbers of MTs in each type of process. In tau-induced processes, the number of MTs found at a series of cross-sections within a process varied upward and downward as a function of length, an organization that suggested MTs in such processes had free minus ends. In contrast, FS-induced processes had decreasing numbers of MTs as a function of length and lacked MTs with free minus ends.

Among the possible explanations for these different ultrastructural appearances is the differential regulation of subunit addition at the plus and minus ends. Although plus-end addition is favored, MTs assembled with FS protein may, in comparison to MTs assembled with tau, have an enhanced capacity for minus-end addition. A second possibility is tau-containing MTs and FS-containing MTs differ in their transport properties. The MTs in processes containing MTs with free minus ends, as observed in tau-expressing cells, might be moving with their plus ends leading, whereas MTs in processes induced by FS protein containing bundles that penetrate well inside the cell body might be moving with their minus ends leading (i.e., back toward the cell body). This directionality may arise by differential engagement of an as yet unidentified slow-transport motor by each of the two MAPs. Unfortunately, information concerning the possibility of retrograde cytoskeletal transport is lacking.

Since the polarity orientation of the MTs in the FS-induced or tau-induced processes was aligned with the plus end distal to the cell body it is unlikely that polarity orientation of MTs in axons and dendrites is solely due to these MAPs. This conclusion is not surprising in that minor neurites that give

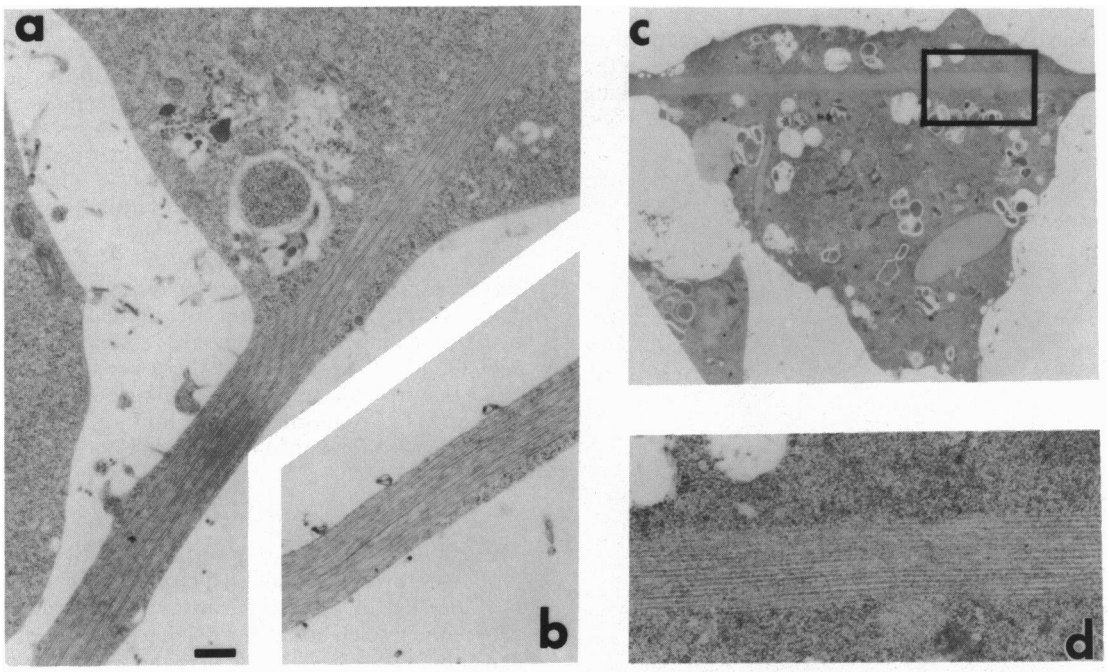

FIG. 3. Transmission electron micrographs of longitudinal sections through Sf9 cells infected with recombinant baculovirus and expressing FS. (*a*) Region of the cell body continuous with a process. MTs are present as a dense tightly packed bundle that is continuous between the process and the cell body. The MT bundle is continuous for several micrometers into the cell body and remains tightly packed. (*b*) Process farther from the cell body. (*c*) Another cell body in which two processes emerged from opposite sides of the cell body. The two processes are colinear, and the MT bundles of the two processes meet within the cell body, forming one continuous bundle. (*d*) Higher magnification image of the boxed portion of *c*, clearly identifying the filaments in the bundles as MTs. (Bars: *a* and *b*, 0.5 μm; *c*, 1.5 μm; *d*, 0.33 μm.)

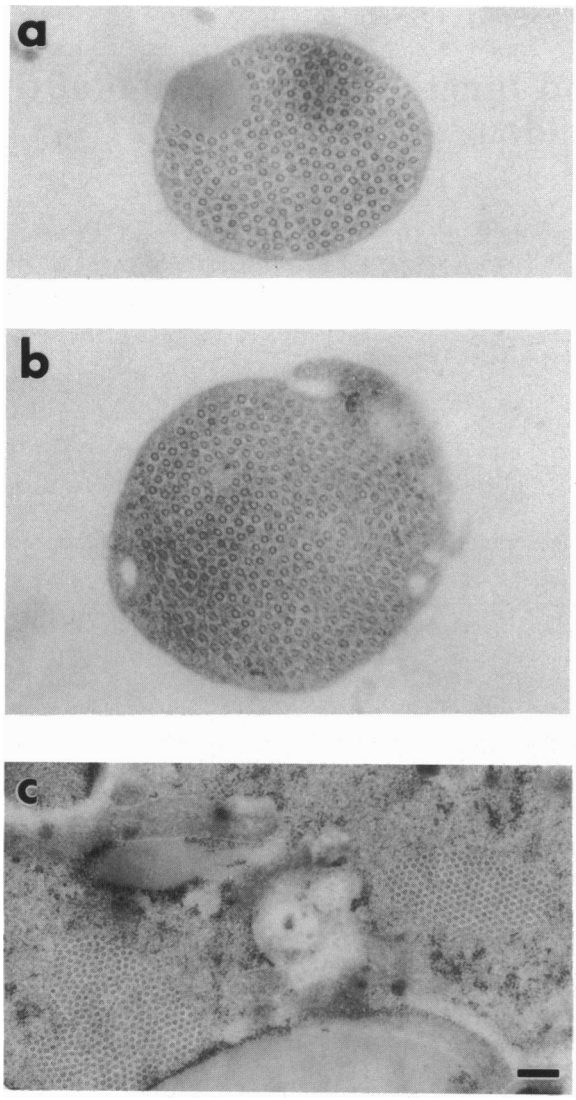

FIG. 4. Cross-sections of FS-induced processes. These cross-sections are taken from regions of a cell similar in morphology to that shown in Fig. 3c, with two colinear processes. (a and b) Two regions of one of the processes, roughly 15 μm apart. The MT number decreases with distance from cell body. (c) Region within the cell body. Two MT bundles are apparent, suggesting that the MT bundle in the cell body represents the overlap of two distinct bundles, one from each of the two processes. (Bars: a and b, 150 μm; c, 300 μm.)

rise to both axons and dendrites contain both of these MAPs (6, 22) and also contain exclusively plus-end-distal MTs (18). The distal end of mature dendrites, where MAP2 is also present, contains aligned MTs with the plus end distal (2). Furthermore, by far the most typical organization of MTs across cell types is uniform plus ends toward the plasmalemma (for example, see ref. 23). Uniform polarity may represent a default arrangement of MTs and dendritic differentiation requires that a subpopulation of MTs reverse their orientation.

There was a significant tendency for FS to induce multiple processes, whereas tau usually elaborated a solitary process in infected Sf9 cells (Table 1). Tau induced the formation of multiple bundles of MTs in the cell body (9), but apparently only one of these bundles is capable of deforming the plasmalemma sufficiently to form a process. The capacity of FS to form multiple processes may be due to an inherent property of the MTs induced by this protein or due to an interactive role of FS protein with other cell structures, such as the cortical actin cytoskeleton where it could create more sites for process emergence. A role for MAP2 in multiple process formation has been suggested in studies using MAP2 antisense oligonucleotides in cerebellar macroneurons, a treatment that prevented the consolidation of their lammelopodia into minor neurites (24). It seems reasonable that the mechanisms by which different MAPs affect multiple process outgrowth may also help regulate the branching of processes, a phenomenon occasionally observed with FS. The distinct cellular functions of different MAPs may be related to the tendency of neurons to generate a single axon and multiple dendrites.

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