

Isolation and Initial Characterization of the Mammalian Midbody

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ABSTRACT Midbodies were isolated from synchronized cultures of Chinese hamster ovary (CHO) cells and their protein composition was studied by means of SDS PAGE. Gels of the midbodies included alpha and beta tubulins as major bands (~30% of the total protein) and ~35 other bands, none of which constituted >3.5% of the total protein. Extraction of the isolated midbodies with Sarkosyl NL-30 solubilized the midbody microtubules but left the central, dense matrix zone of the midbody intact. A protein doublet of ~115,000 mol wt was retained preferentially by the particulate fraction containing the matrix zones, indicating it to be a component of the matrix. The 115,000 mol wt doublet was also present in gels of isolated mitotic spindles from CHO cells. The overall protein composition of the isolated spindles was very similar to that of the isolated midbodies.

An understanding of the mechanism(s) of mitosis requires knowledge of the molecular components of the spindle and of their interactions. Compositional analysis of isolated spindles should provide a means for detecting spindle proteins, particularly any proteins that may be unique to the spindle. Identification of spindle proteins from isolated spindles has, however, proven difficult. Electrophoretic (3, 11, 19, 24, 25, 27) and other biochemical (11, 16, 22, 24, 29) analyses of isolated spindles have been complicated by the fact that there has been no way to distinguish bona fide spindle proteins, other than tubulin, from nonspecific contaminants. This problem has been somewhat overcome for the identification of spindle microtubule-associated proteins (MAPS) by cycling microtubule protein from mitotic sources through cycles of polymerization-depolymerization (2, 19). Also, comparative two-dimensional gel electrophoresis of mitotic cell extracts and isolated spindles has recently been used to obtain preliminary identification of what may be a spindle-specific protein (30).

To look for novel mitotic proteins we have taken advantage of special characteristics of the mammalian midbody. The midbody is formed from bundles of spindle microtubules that span the mitotic interzone at the end of anaphase. Such bundles consist primarily of two groups of microtubules. Each group extends into the equator from one side and stops there, the ends of its microtubules overlapping with those of the group entering the equator from the opposite side (12, 13). A dense matrix substance coats the microtubules in the zone of overlap. Furrowing pushes these microtubule bundles into a compact grouping, forming the midbody (7, 20). That the midbody has

a functional role in cell division is not certain, but previous work has indicated that after cessation of active furrowing it may serve to maintain the division of cytoplasm established by the furrow (17, 18).

The spatial distribution of the midbody matrix before late anaphase, when it is first detected by electron microscopy, is not known. Regardless, the matrix is a spindle-associated substance that appears to have the interesting property of binding microtubules together. Further, the matrix is a particularly attractive candidate for study, since in the midbody it is present as a relatively large and easily recognized structure that can be distinguished from spindle microtubules; this is a unique feature among spindle components, and of special advantage for investigating composition.

One attempt to characterize midbody composition has been published (8). We report here our efforts to isolate and characterize midbodies from Chinese hamster ovary (CHO) cells, and to identify matrix components.

MATERIALS AND METHODS

Cell Growth and Synchronization

Monolayer cultures of CHO cells were maintained in Ham's F-12 medium supplemented with 5% fetal bovine serum. Synchronization was achieved using successive thymidine and nocodazole blocks after the method of Zieve et al. (31). Mitotically-arrested cells were allowed to accumulate in nocodazole for 5–6 h, at which time they were harvested by gently shaking the culture flasks. Cells obtained in this manner were washed once with fresh medium and resuspended without nocodazole at a concentration of $2-3 \times 10^6$ cells per milliliter. Cells were maintained in suspension at 37°C by gentle stirring and were allowed to progress

through mitosis. By 25 min into the recovery period over 90% of the arrested mitotics had furrowed and formed midbodies.

Midbody Isolation

The procedure for midbody isolation was developed from the method used by Chu and Siskin to isolate spindles from HeLa cells (3). As soon as the vast majority of cells had completed furrowing, the cells were pelleted at 200 g for 3 min and gently resuspended in 25 vol of a hypotonic swelling solution consisting of 1 M hexylene glycol (2-methyl-2,4-pentandiol), 20 μ M MgCl₂, and 2 mM piperazine-*N,N'*-bis(2-ethane sulfonic acid) (PIPES), pH 7.2 at room temperature. Cells were immediately pelleted at 200 g for 3 min and vigorously resuspended in 50 vol of a lysing solution consisting of 1 M hexylene glycol, 1 mM EGTA, 1% Nonidet P-40, 2 mM PIPES, pH 7.2 at 37°C. Disruption of the cells and release of midbodies was completed by vigorous vortexing for 30 sec in this solution. Midbodies released by vortexing were stabilized by chilling on ice and by adding to the lysate 0.3 vol of cold 1 M hexylene glycol, 50 mM 2-(*N*-morpholino)ethane sulfonic acid (MES), pH 6.3, to lower pH. The lysate was centrifuged at 250 g for 10 min to remove large debris. The supernatant from this spin was layered over a cushion of 40% glycerol (weight per volume) in 50 mM MES, pH 6.3, and centrifuged for 20 min at 2,800 g to pellet midbodies. This pellet was resuspended in 50 mM MES, pH 6.3, and centrifuged again through 40% glycerol. After a final wash in MES buffer, midbodies were either prepared for electrophoresis or used in extraction experiments.

Midbodies used for extraction were given a second wash with the 50 mM MES buffer and then resuspended in this buffer. Sarkosyl NL-30 (1% wt/vol in 50 mM MES, pH 6.3; gift of Ciba-Geigy Corp., Pharmaceuticals Div., Summit, NJ) was added with mild vortexing to a final concentration of 0.2–0.6%. Extraction was allowed to proceed on ice and was monitored with phase contrast microscopy. When extraction was judged complete the preparations were centrifuged at 3,700 g for 15 min, the supernatant removed, and the pellet washed once with 50 mM MES buffer. Proteins in the supernatant were concentrated by precipitation with 10 vol of cold acetone, followed by centrifugation at 3,000 g for 15 min.

Mitotic spindles were prepared by the same procedure as midbodies except that the cells were harvested after 10 min recovery from nocodazole; vortexing was limited to 5–10 sec, and the final wash contained 10% glycerol.

Electron Microscopy

Samples of isolated midbodies or spindles were centrifuged onto Formvar-coated grids (10 min, 2,500 g), and then positively stained with 0.5% aqueous uranyl acetate. In some instances samples were fixed with 2% glutaraldehyde before centrifugation. This variation produced no observed changes in morphology.

Electrophoresis

Electrophoresis was performed using the discontinuous buffer system of Laemmli (9) on 7.5% acrylamide slab gels. Gels were stained with Coomassie Blue. For densitometry, slabs were dried between dialysis membranes, using heat and vacuum. Gels so prepared were scanned at 600 nm, and peaks of interest were cut out and weighed to quantitate data. The approximate molecular weights of protein bands were determined by comparing their migration with the migration of standards of known molecular weights.

RESULTS

Morphology of the Isolated Midbodies

The final product of the isolation procedure is shown in Fig. 1. Individual isolated midbodies were readily recognized with phase contrast optics. Each midbody displayed a dark matrix zone at the approximate center of a less dense shaft corresponding to the midbody microtubules. Some variation in size and shape among the midbodies was observed, reflecting differences in the stage of cytokinesis at the time of isolation (18). Typically, midbodies had a diameter of $\sim 1 \mu$ m at the matrix zone, and an overall length of 3 to 5 μ m. Examination of the midbodies with polarization optics showed that the birefringence of the microtubule bundle was preserved through isola-

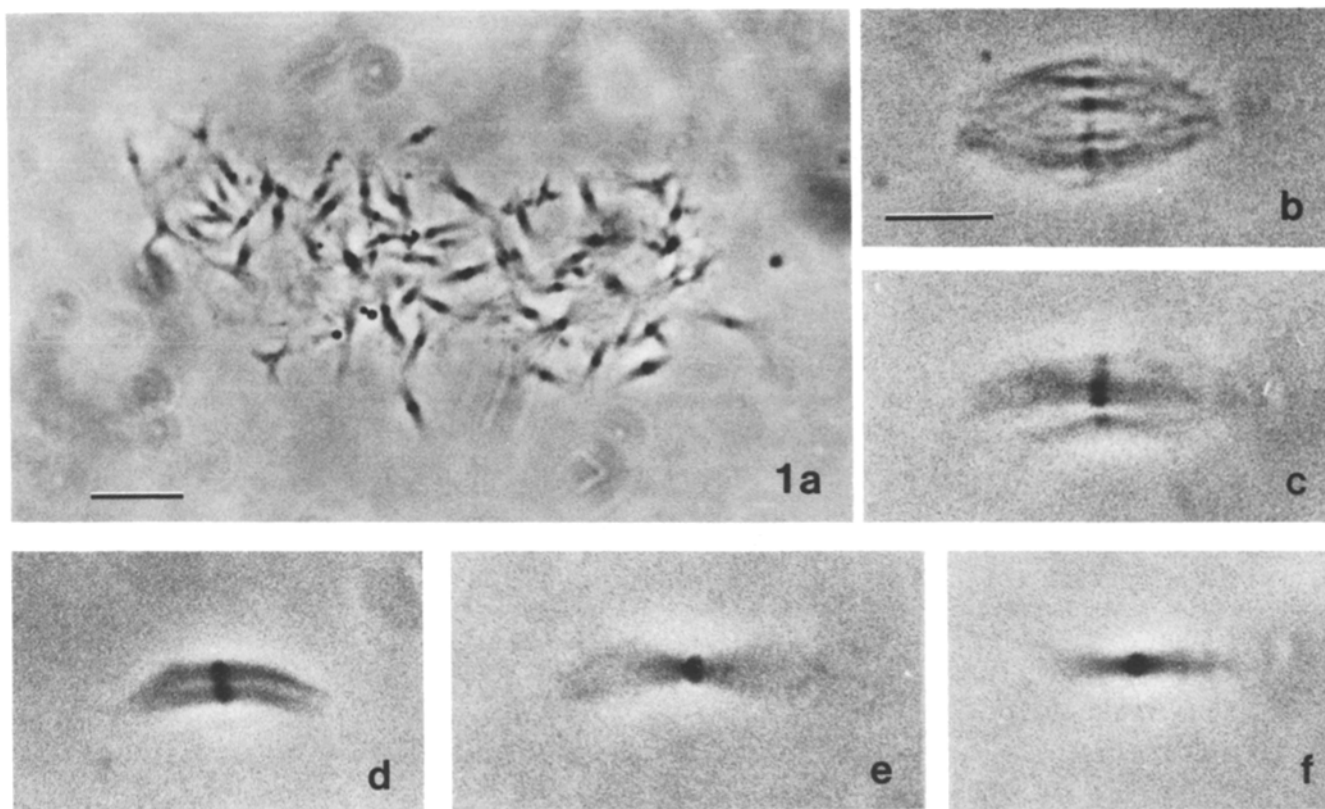


FIGURE 1 Isolated midbodies. (a) A field of midbodies isolated from synchronized CHO cells. Bar, 10 μ m. \times 1,200. (b–f) Various stages in midbody formation isolated from L cells. Similar stages were obtained from CHO cells. (b) Telophase spindle; (c, d) clusters of interzonal microtubules obtained at different stages of furrowing; (e, f) midbodies as seen immediately at the end of furrowing and 10–20 min after furrowing, respectively. Bar, 5 μ m. \times 2,800.

tion. The amounts of particulate and fibrous debris accompanying the midbodies through the isolation procedure were small. When cells were harvested at earlier times in their recovery from nocodazole, various stages of midbody formation were obtained. Examples of these stages are seen in Fig. 1 *b-f*.

Whole midbodies examined by electron microscopy displayed the same range of shapes seen by light microscopy (Fig. 2). Distortion and clumping of the microtubules caused by drying of the grids, coupled with the dense staining of the midbodies, made recognition of individual microtubules difficult. Individual tubules or small clusters of microtubules could be seen at the ends of the microtubule bundle, where tubules tended to splay apart from each other. The appearance of these microtubules indicated the presence of adherent cytoplasmic material, forming a source of contamination not removed by centrifugation.

Protein Composition of the Isolated Midbodies

The SDS polyacrylamide gels of two different preparations of midbodies are shown in Fig. 3. Lanes *B* and *C* each contain all of the material obtained from midbody preparations that

started with $\sim 10^8$ mitotic cells. Two major bands were present in the midbody preparations. These bands were present in approximately equimolar quantities and comigrated with the alpha and beta subunits of porcine neurotubulin (lane *A*). Given the large numbers of microtubules characteristic of midbodies, these bands certainly represent alpha and beta tubulins. Together they constituted $\sim 30\%$ of the total protein.

In addition to tubulin about 35 bands of different intensities were present. None of these bands constituted $>3.5\%$ of the total protein. For purposes of reference the more prominent bands have been numbered from 1 to 10 on Fig. 3. A band was assigned a number if it constituted 2% or more of the total protein, with the exception of component 9. Component 9 made up $<1\%$ of the total protein, but is referenced because it has a mobility corresponding to that of actin. We feel that the relatively low amount of a protein at the position of actin reflects the effectiveness of the isolation procedure in separating midbodies from other cell components. Component 7, a doublet of $\sim 70,000$ mol wt showed identical electrophoretic mobility to a doublet present in twice-cycled neurotubules, and so may represent tau protein (21). Components 3 and 4, when optimally resolved, were seen to consist of closely spaced

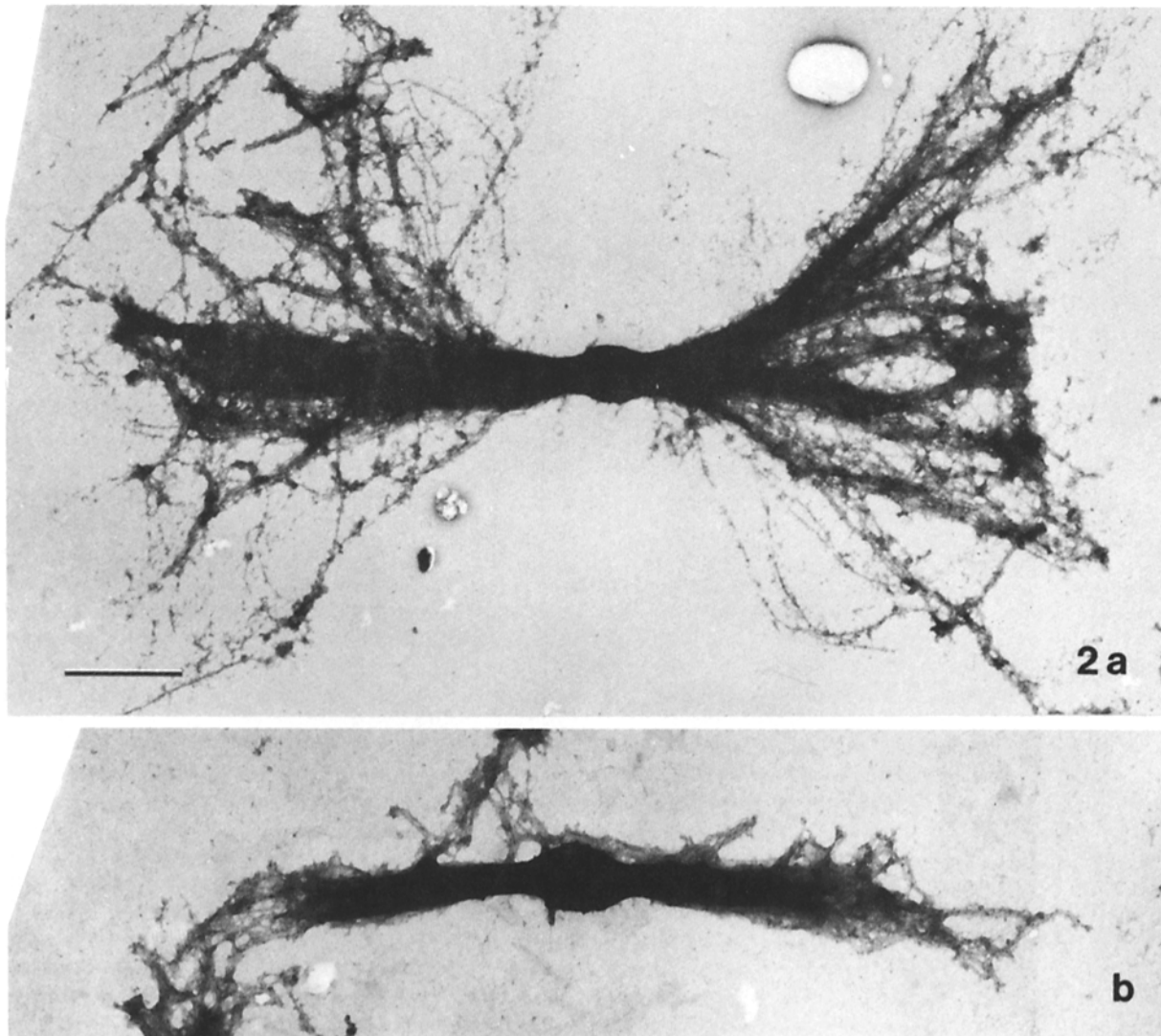


FIGURE 2 Whole mounts of isolated midbodies as seen with the electron microscope. Shown are a midbody that was isolated shortly after the end of furrowing (*a*), and one isolated at a stage ~ 10 min later (*b*). Bar, $2 \mu\text{m}$. $\times 7,900$.

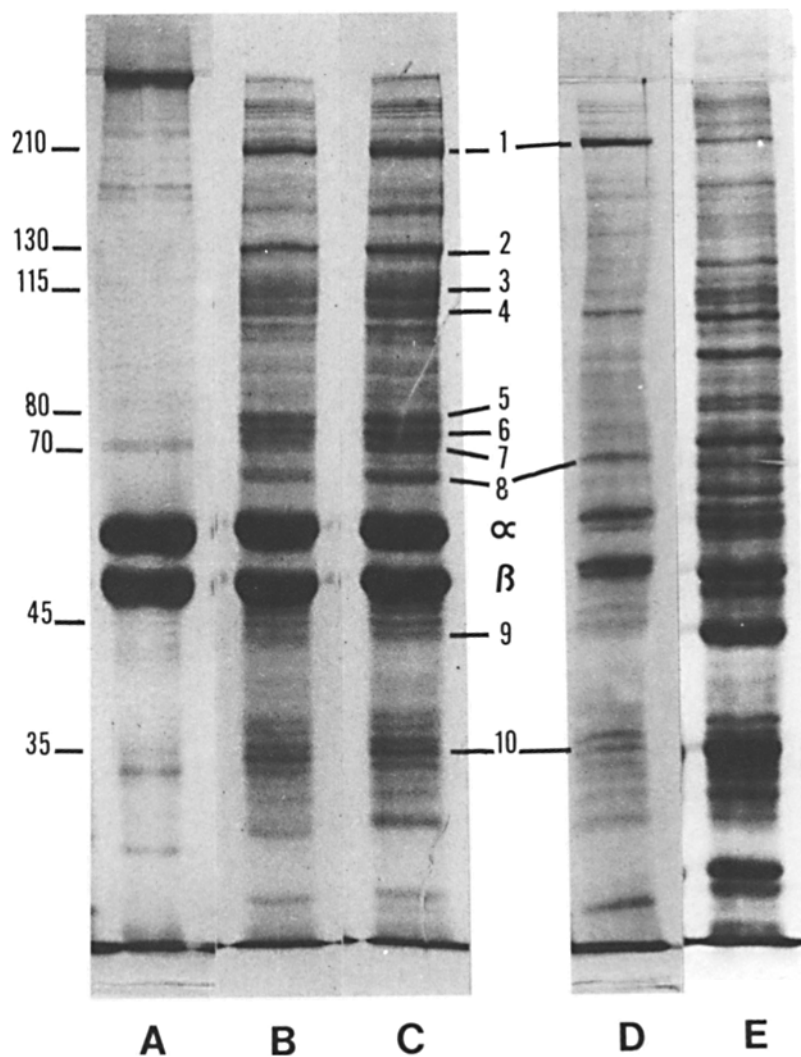


FIGURE 3 SDS polyacrylamide gels of isolated midbodies. Scale at the left indicates approximate molecular weights $\times 10^3$. Lane A, twice-cycled porcine neurotubules; lanes B and C, total protein obtained in two separate midbody isolations; lane D, small, particulate debris pelleted from the lysate after removal of midbodies; lane E, protein composition of the lysate after removal of midbodies and the material of lane D. The numbers between lanes C and D are to reference specific protein bands. The origin of lane A aligns with those of the other lanes; the diffuse staining above the conspicuous high molecular weight band at the top of the gel is in the stacking gel. Positioning of specific bands in lane D was slightly altered due to distortion of the gel on drying. Correspondence of the mobilities of the bands in lane D to those in the other lanes was confirmed by comparison with other samples on the same gel.

doublet bands. Isolated midbodies contained very little material that comigrated with the group of high molecular weight MAPs characteristic of neurotubules (Fig. 3).

By comparing the electrophoretic patterns of midbodies with those of fractions lacking midbodies it has been possible to identify components that are likely to be contaminants of our preparations. Small, particulate debris from the disrupted cells remained in the original lysate after midbodies were pelleted. This material was pelleted by centrifugation at 12,000 to 25,000 g for 15 min. Fig. 3 (lane D) shows an SDS gel of this particulate material; prominent bands were present at the positions of components 1, 8, and 10 as well as those of alpha and beta tubulins and several of the unnumbered components from isolated midbodies. As determined from the work on detergent-extracted midbodies, described below, the conditions used to pellet intact midbodies from the lysate would also be sufficient to pellet any matrix zones from which most microtubules had been sheared away. It is thus unlikely that components 1, 8, or 10, which constitute prominent bands in the particulate debris, are midbody proteins.

Detergent Extraction of Isolated Midbodies

To identify proteins of the midbody matrix, agents were sought that would selectively solubilize isolated midbodies. Previous work, using cells grown on cover slips and lysed into

solutions containing different protein denaturants, showed that the matrix zone is stable under conditions that extract the midbody microtubules (J. M. Mullins, unpublished results). After being subjected to the isolation procedure, however, the microtubule bundle of the midbody is considerably stabilized; it is not appreciably extracted by incubation with 2 M NaCl, 2 M KI, or 4 M urea. Selective solubilization was achieved, however, by extracting midbodies with ionic detergents. Sarkosyl NL-30, used at a concentration of 0.4%, gave the best results of several detergents tested.

Upon exposure to 0.4% Sarkosyl at 0°C the microtubule bundle of the midbody immediately began to grow paler, as seen with phase contrast optics. After 30 min to 1 h of extraction the microtubule bundle was no longer detectable in the phase microscope, but the phase-dense matrix zone remained visible. The selectivity of this extraction was not perfect in that the matrix zones tended to lose contrast very gradually during the extraction and, in some cases, appeared to become slightly swollen. It is thus likely that some solubilization of matrix material also took place.

Extracted midbodies were pelleted onto support grids, positively stained with uranyl acetate, and examined with the electron microscope (Fig. 4). The pelletable structures remaining after extraction consisted mainly of the matrix zones from the midbody equator. Some amorphous material remained

attached to the matrix zone. The amount of the amorphous residue varied from specimen to specimen within a given preparation. The characteristic shape and dimensions of the matrix zone, however, were generally retained throughout the extraction procedure.

Protein composition of the pellet and supernatant fractions resulting from Sarkosyl extraction of midbodies is illustrated in Fig. 5. Lanes A-C and D-F include all the material obtained from two separate extraction experiments. In each case the supernatant (~0.5 ml) was removed in two steps, with the intent of removing the upper portion of the supernatant without disturbing the small pellet containing the matrix zones. Fig. 5 compares the results from a less extensively extracted preparation (lanes A-C, 30 min in Sarkosyl) with results from a more extensively extracted preparation (Lanes D-F, 50 min in Sarkosyl). In both experiments alpha and beta tubulins were almost completely extracted. The extent to which the prominent nontubulin bands referenced in Fig. 3 were extracted varied. In the less extensively extracted midbodies (A-C), prominent bands were retained in gels of the pelleted material at the positions of components 3-7. Of these components 3 and 4 were present almost exclusively in the pelleted material, whereas 5-7 were partially extracted and constituted prominent bands also in the supernatant fractions. The pellet from the 50-min extraction also contained prominent bands at the positions of components 3-7. Comparison of the pelleted and superna-

tant fractions showed that components 5-7 were extensively solubilized by Sarkosyl. Components 3 and 4 were also partially extracted, but, of the two, component 3 appeared to be retained preferentially in the pelleted material.

Comparison of different lanes on slab gels by densitometry is inexact due to differences in widths among lanes. As a means of comparing the relative amounts of components 3-7 that were extracted by Sarkosyl, however, scans of lanes D-F were made and the total weight of peaks from the supernatant compared with that of the pellet fraction for components 3-7. By this means ~60% of component 3, 40% of component 4, and from 20 to 30% of components 5-7 were found to be retained by the pellet. These figures are not exact, but establish the relative degree of extraction for the major bands of the pellet. From the above observations we conclude that component 3, a doublet of 115,000 mol wt, is a constituent of the midbody matrix, but components 5-7 are probably not.

Morphology and Composition of Isolated Spindles

We were interested to see which of the proteins characteristic of the isolated midbodies would also be found in preparations of mitotic spindles. Of particular interest was the question of whether component 3, which is implicated as a matrix component, would be present in spindles before late anaphase,

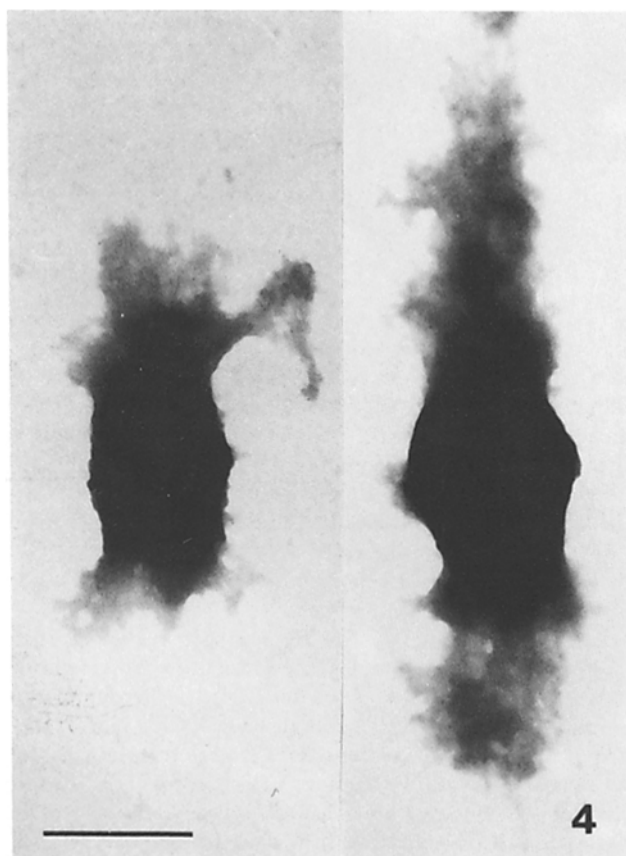


FIGURE 4 Whole mounts of two midbodies after extraction with 0.4% Sarkosyl NL-30. Matrix zones are intact, with varying amounts of amorphous material remaining from the microtubule bundle. Bar, 1 μ m. \times 19,600.

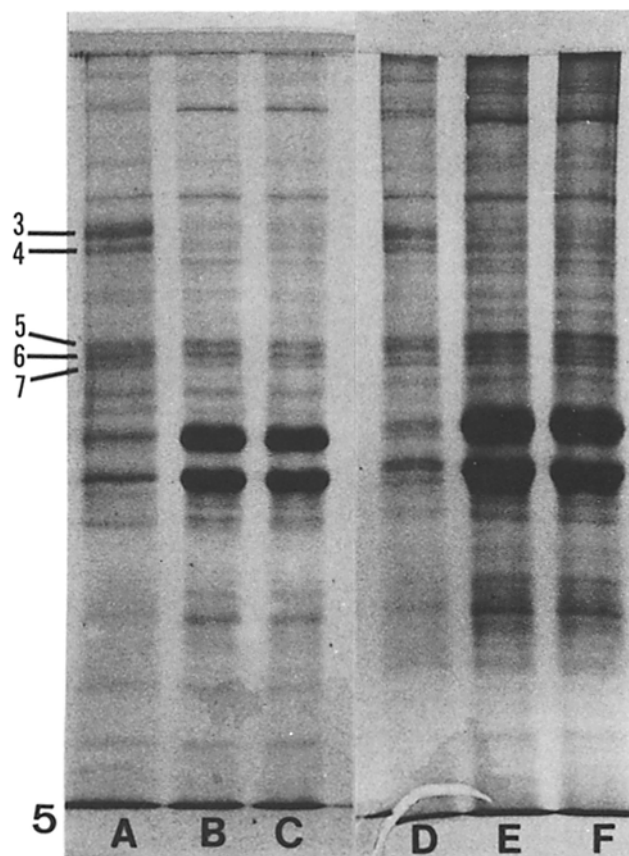


FIGURE 5 SDS polyacrylamide gels of the total material obtained from two different Sarkosyl-extracted populations of midbodies. Lanes A-C show the results from a 30-min extraction, and lanes D-F from a 50 min extraction. Lanes A and D are the pelleted materials, lanes B and E are the upper portions, and lanes C and F are the lower portions of the supernatants. Reference numbers to the left of lane A are the same as those of Fig. 3.

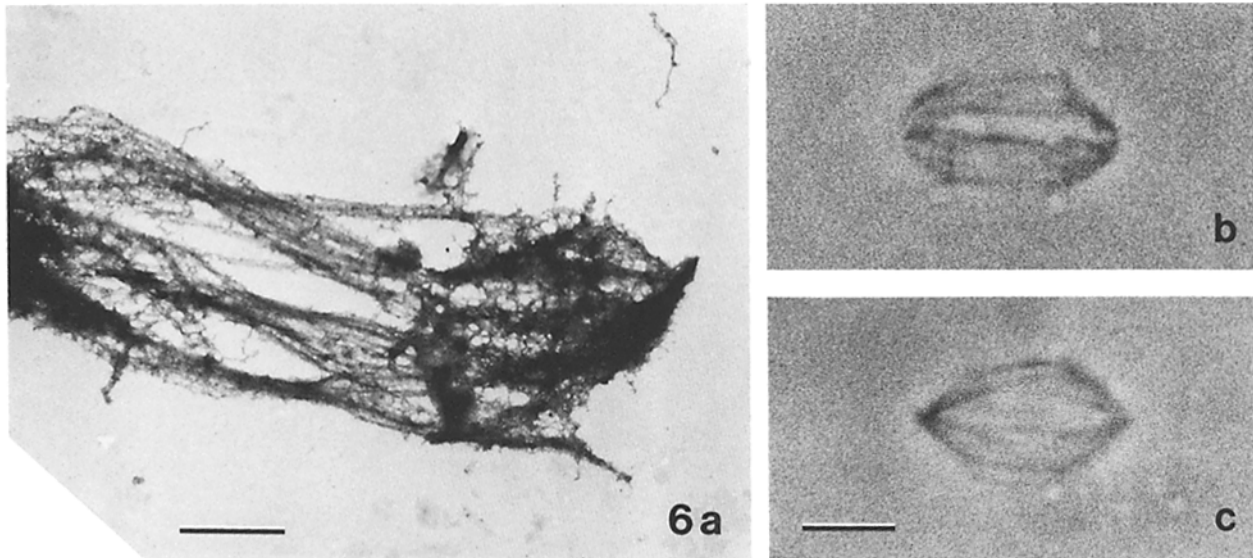


FIGURE 6 Isolated CHO spindles. (a) Whole mount of what was judged to be an anaphase spindle, seen with the electron microscope. Bar, 2 μm . \times 6,600. (b, c) Isolated spindles seen with phase contrast. Bar, 5 μm . \times 2,400.

when matrix is first identified by electron microscopy. Accordingly, the midbody isolation procedure was modified to allow isolation of spindles as outlined in Materials and Methods. Spindles were obtained from populations of cells at a time when most cells were in prometaphase to metaphase stages. Examples of the isolated spindles obtained are shown in Fig. 6. Chromosomes became dispersed in the isolation media, and so were lost from the spindles, leaving only phase-dense spindle fibers as structures recognizable by light microscopy. Whole mounts of isolated spindles viewed by electron microscopy were seen to consist primarily of bundles of spindle microtubule. As with isolated midbodies, the spindle microtubules were associated with what appeared to be adhering materials that probably constituted a source of cytoplasmic contamination. From the appearance of what were judged to be anaphase spindles, as seen in Fig. 6a, both continuous and kinetochore microtubules were present in the isolated spindles.

Fig. 7 provides a side-by-side comparison of SDS gels of isolated spindle and midbody preparations. Qualitatively the two preparations were very similar in protein composition. Alpha and beta tubulins accounted for $\sim 35\%$ of the total protein of the spindle isolates. Component 3, for which we obtained preliminary identification as matrix protein, was present in the spindles, as was component 4. Differences between electrophoretic patterns of midbodies and spindles included the virtual absence of component 6 and greatly reduced prominence of component 5 in the spindles; bands at positions corresponding to molecular weights of $\sim 90,000$ and $85,000$ (arrows, Fig. 7) were enhanced in the spindles compared with the midbodies.

DISCUSSION

Methods used in this work have allowed the preparation of small but usable quantities of midbodies and spindles from mammalian cells. The electrophoretic profiles of these isolates are distinctly different from those of the particulate and soluble fractions produced during the isolation procedure; further, tubulin constitutes a large fraction of the total protein of the isolates. These facts, combined with the relatively clean appearance of the isolates as seen by light and electron micros-

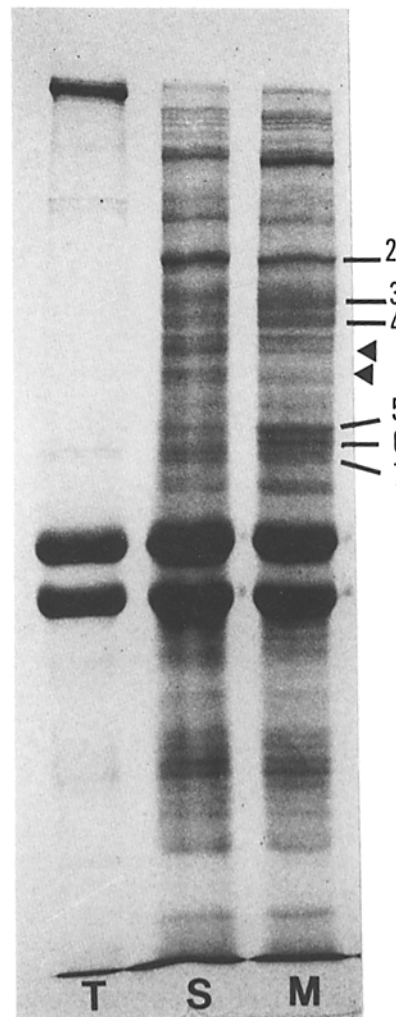


FIGURE 7. SDS polyacrylamide gel comparing electrophoretic profiles of twice-cycled porcine neurotubules (T), isolated CHO spindles (S), and isolated CHO midbodies (M). Reference numbers correspond to those of Fig. 3. Arrows indicate two bands enhanced in spindle preparations compared to midbody preparations.

copy, indicate that many of the electrophoretic bands should represent actual midbody or spindle proteins rather than non-specific contaminants.

By a combination of selective extraction and electrophoresis we have identified a 115,000 mol wt doublet as a protein component of the midbody matrix. The presence of this protein in gels of preanaphase spindles indicates that it should be considered a spindle component as well as a component of the midbody.

Our results contrast with those from the only other published work on the protein composition of the midbody. From two-dimensional gels of L cell midbody preparations Krystal et al. (8) reported a putative midbody protein of 42,000 mol wt. This protein was distinguished from actin by differences in isoelectric point. On our one-dimensional gels this protein should lie at the approximate position of actin, or component 9 of Fig. 3. However, component 9 constitutes <1% of the total protein in our preparations, and is not a matrix protein as judged by the results of Sarkosyl extraction. That the amount of actin relative to the amount of tubulin was high in the L cell preparations suggests a contamination of the midbodies by cytoplasmic debris, making assignment of the 42,000 mol wt protein to the midbody problematical.

Qualitatively the protein composition of the isolated midbodies and spindles is very similar. This is not entirely surprising, since the midbody is formed from elements of the continuous spindle. It is of interest that the 115,000 mol wt protein is present in preparations of prometaphase-metaphase spindles, because matrix has not been detected in the spindle by electron microscopy until late anaphase. Some redistribution of the matrix must take place in the spindle during anaphase to concentrate the matrix at the equator.

Of the prominent nontubulin bands in the midbody and spindle gels, only the 115,000 mol wt doublet can be called a spindle protein with any certainty. Because tau proteins have been localized in mammalian spindles and midbodies by immunofluorescence (4, 10), however, it is a reasonable assumption that component 7, a doublet with mobility identical to that of a 70,000 mol wt doublet from neurotubules, is tau. Those bands that are prominent in only spindle or midbody gels will merit further attention, as they may reflect compositional differences among the different categories of spindle microtubules.

A role for the midbody matrix in mitosis is not known, but information from several electron microscope studies suggests some interesting possibilities. Midbody microtubules are considerably more resistant to disruption by chemical or physical agents than are other spindle microtubules (26). Such enhanced stability may result from the association of the midbody tubules with the dense matrix or other MAPs. The midbody matrix may also be significant for the maintenance of spindle form. Results from quantitative electron microscopy of mammalian cells (1, 13, 14) and from recent data on the polarities of spindle microtubules (5, 6, 27) are consistent with the idea that the spindle is composed of two half-spindles that slide actively apart during anaphase elongation (12, 13). The meticulous studies of diatom mitosis done by Pickett-Heaps and his colleagues (15, 23, 28) support this concept. The diatom-continuous spindle resembles a midbody in its design. It consists of two groups of microtubules that associate in a zone of overlap at the equator. A dense matrix coats the microtubules at the overlap. As the continuous spindle elongates during anaphase, the zone of overlap concomitantly shortens; the simplest explanation for this is that the two half-spindles are sliding apart. Of particular interest for this discussion is that the dense matrix remains associated specifically with the zone of overlap, becoming more concentrated as the overlap shortens. The matrix

appears to maintain the close association between the microtubules of the two halves of the continuous spindle.

The metazoan spindle is organized differently from the diatom spindle, but both produce the same basic mitotic movements. It is reasonable to assume that the metazoan spindle operates in a similar manner, but is simply less compactly organized. Matrix may thus serve as the glue that maintains the two half-spindles in association with each other, not becoming apparent in electron micrographs until late anaphase, when it has become sufficiently concentrated at the spindle equator. This idea is supported by the observation that in HeLa cells matrix spans a progressively shorter length along bundles of interzonal tubules during successively later stages of anaphase (13). Also supportive is the presence of the 115,000 mol wt matrix protein in preanaphase spindles.

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