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Association of microtubules and intermediate filaments in chicken gizzard cells as detected by double immunofluorescence

(smooth muscle cells/cytoskeleton/desmin/tubulin)

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ABSTRACT **By double indirect immunofluorescence, using guinea pig and rabbit antibodies to tubulin and to desmin, we have simultaneously labeled microtubules and intermediate filaments in cultured chicken embryo gizzard cells. At the resolution of the light microscope there was extensive but not complete superposition of the labeling patterns for the two filamentous structures within cells in interphase and an essentially complete dissociation of the two labeling patterns in cells in mitosis. These results indicate that there is an extensive association of microtubules and intermediate filaments in these interphase muscle cells and suggest that this association is regulated metabolically.**

Three major classes of chemically distinct fibrillar structures have been recognized in the cytoplasm of eukaryotic nonmuscle cells: microfilaments, intermediate filaments, and microtubules. Whereas both the microfilaments and the microtubules are apparently composed of closely similar or identical subunits from cell to cell in an organism, intermediate filaments (defined by their diameters, ≈10 nm) are of several types, each with chemically and antigenically distinctive subunits (for review, see ref. 1). By electron microscopic (cf. refs. 2 and 3) and single-fluorophore immunofluorescence (e.g., refs. 4–8) studies, the overall distributions of intermediate filaments in cultured cells have generally been found to be unrelated to the distributions of microfilament structures. On the other hand, there are several indications that intermediate filaments and microtubules are somehow functionally associated in different types of cells and that to some degree their distributions may be correlated within interphase cells (see *Discussion*).

Of particular relevance to the present studies, in some published electron micrographs of cross sections of cultured fibroblast cell processes (9), intermediate filaments have been observed to form fairly regular cylindrical arrays around individual microtubules, and in longitudinal thin sections of fibroblasts and epithelioid cells (10–12) occasionally there has been observed an extended parallel alignment of an individual intermediate filament close to a microtubule (figure 6*b* in ref. 10 and figure 6*d* in ref. 11). In most fields in such longitudinal sections, however, there was little indication of an extended close association of the two structures. Therefore, it is still uncertain whether an association does exist between intermediate filaments and microtubules, let alone what its nature and extent might be. It also must be recognized that the different types of intermediate filaments (cf. ref. 1) may show different associative properties toward microtubules.

Immunofluorescence labeling of microtubules (13–15) or intermediate filaments (4–8) in different individual cells has revealed the typical fibrillar distribution of each of the two structures but, by their nature, these observations have been unable to establish any correlation between these distributions. In this paper, we report simultaneous double immunofluorescence observations of microtubules and desmin-containing intermediate filaments in embryonic chicken gizzard cells. The results show that, at the level of resolution of the light microscope, there is an extensive but not complete superposition of the filamentous distributions of the labels for tubulin and desmin during interphase, suggesting that extensive association of the two filaments does indeed exist in these cells.

MATERIALS AND METHODS

Antigens and Antibody Preparations. Tubulin from 12- to 13-day chicken embryo brains was purified by two cycles of assembly/disassembly as described (16, 17). The preparation consisted of tubulin and small amounts (1–2% as estimated by densitometry of Coomassie-stained $NaDodSO_4$/polyacrylamide gels) of high molecular weight microtubule-associated proteins. Desmin, the name given (18, 19) to the major protein subunit of muscle cell intermediate filaments, was purified from chicken gizzard smooth muscle essentially as described (19). However, prior to the preparative gel electrophoresis we included two steps of column chromatography: (*i*) DEAE-cellulose (DE-52, Whatman) chromatography was performed in 20 mM Tris acetate, pH 6.8/8 M urea; elution was carried out with a linear KCl gradient (0–300 mM). (*ii*) Hydroxylapatite (HTP, Bio-Rad) chromatography was carried out in 50 mM K phosphate, pH 7.0/8 M urea; elution was with a linear K phosphate gradient (50–300 mM). The desmin-containing fraction (not shown) was subsequently purified by preparative polyacrylamide gel electrophoresis, eluted from the gel, and used for immunization. All purification steps were carried out at 0–4°C in the presence of 0.2 mM phenylmethylsulfonyl fluoride (Sigma) to minimize proteolytic breakdown of desmin.

Antibodies to tubulin and desmin were induced in rabbits and guinea pigs by lymph node or intradermal injection, respectively, of 100 μg of each antigen in complete Freund's adjuvant followed by two similar intradermal injections at 2-week intervals. Affinity-purified antibodies were prepared by using the antigens immobilized on glutaraldehyde-activated Ultrogel AcA22 (20). The two antibody preparations did not show any crossreactivity by double immunodiffusion; nevertheless, they were further passed through the heterologous immunoadsorbents to eliminate any possible minor crossreactivity.

Goat antibodies against rabbit and guinea pig IgG were prepared and affinity-purified on the respective immunoadsorbent. Each of these preparations was rendered monospecific

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Abbreviations: Fl, fluorescein isothiocyanate; Rh, rhodamine-lissamine sulfonyl chloride.

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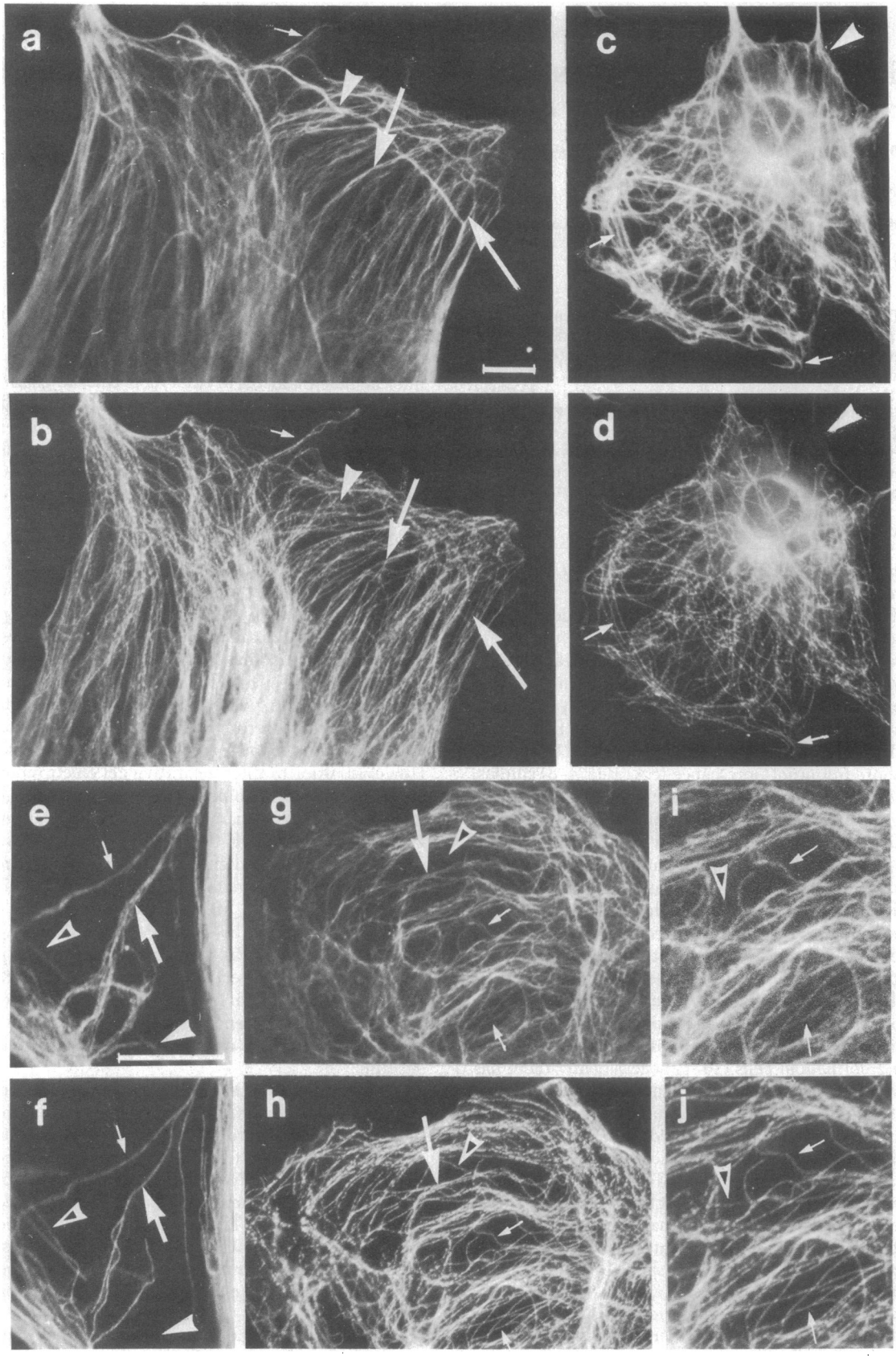

FIG. 1. Indirect double immunofluorescent labeling of chicken embryo gizzard cells for desmin (*a*, *c*, *e*, *g*, and *i*) and tubulin (*b*, *d*, *f*, *h*, and *j*, respectively). For the pairs *a*/*b* and *e*/*f*, the mixture of primary antibodies consisted of rabbit antitubulin and guinea pig antidesmin; for the pairs *c*/*d*, *g*/*h*, and *i*/*j* (higher magnifications of *g* and *h*), the mixture was of guinea pig antitubulin and rabbit antidesmin antibodies. In each case, the treatment with the primary antibodies was followed by treatment with a mixture of cross-adsorbed Rh-conjugated goat antibodies to rabbit IgG and Fl-conjugated goat antibodies to guinea pig IgG. The small arrows indicate examples of pairs of thinly labeled microtubules and thinly labeled desmin filaments that are superimposed. The large arrows point to more broadly labeled microtubule bundles and, superimposed,

by cross absorption on the heterologous immunoadsorbent. The two goat antibody preparations were conjugated to either fluorescein isothiocyanate (Fl) or to rhodamine-lissamine sulfonyl chloride (Rh) as described (21). Conjugates with a fluorophore-to-protein molar ratio of 3–4 were separated on DEAE-cellulose columns and used for the immunocytochemical labeling.

Immunofluorescence. Embryonic chicken gizzard cells, grown on glass coverslips, were fixed with 3% formaldehyde and made permeable by brief treatment with Triton X-100 as described (22). The immunolabeling was performed by incubation of the treated cells with a mixture of the rabbit and guinea pig primary antibodies; the cells were then washed and incubated with the mixture of the Fl- and Rh-labeled goat anti-IgG secondary antibody preparations. In control experiments the specific antibodies to either of the antigens were replaced with the corresponding nonimmune IgG and the mixture of secondary antibody reagents was then added as above. The stained cells were observed by using a Zeiss Photoscope III with filter settings for rhodamine and fluorescein fluorescence used at the same focal setting for each pair.

RESULTS

Cultured embryonic chicken gizzard cells were simultaneously labeled for both desmin and tubulin by indirect double immunofluorescence by using as primary antibodies either a mixture of rabbit antitubulin and guinea pig antidesmin or rabbit antidesmin and guinea pig antitubulin. Labeling of desmin and of tubulin in the corresponding cell showed a remarkable degree of similarity of the distributions of the two fibrillar structures (Fig. 1). Closely similar results were obtained with either mixture of the two primary antibodies (compare the pairs Fig. 1 *a*/*b* or *e*/*f* with pairs Fig. 1 *c*/*d* or *g*/*h*). However, as noted by Lazarides (23), a significant proportion of these cells (20–30%) were not immunolabeled for desmin but essentially 100% were labeled for tubulin.

Certain features of the paired distributions shown in Fig. 1 may be noted. There were desmin-labeled fibrillar structures that exhibited no correspondingly distributed labeling for tubulin (for examples, see solid arrowheads) as well as tubulin-labeled structures that showed no labeling for desmin (open arrowheads). The remaining, and major, fraction of the fluorescent structures showed superposition of the two labels. These structures were of two types: intensely labeled desmin-containing structures (that were probably due to bundles of intermediate filaments) with superimposed tubulin labeling, and intensely labeled bundles of tubulin-stained structures with superimposed desmin labeling (paired large arrows). Possibly most interesting for our purposes (see *Discussion*), there were many lightly labeled desmin-containing structures that showed superimposed light labeling for tubulin (for examples, see paired small arrows).

In control experiments for the specificity of the double immunolabeling, shown in Fig. 2, one of the two primary antibodies was replaced with its corresponding normal IgG. When the fixed and permeabilized cells were treated first with guinea pig antidesmin and normal rabbit IgG and then with a mixture of Rh-conjugated goat anti-guinea pig IgG and Fl-conjugated goat anti-rabbit IgG, only Rh fluorescence was observed. Similarly, exclusively Fl fluorescence was observed when the primary mixture contained guinea pig normal IgG and rabbit antitubulin antibodies.

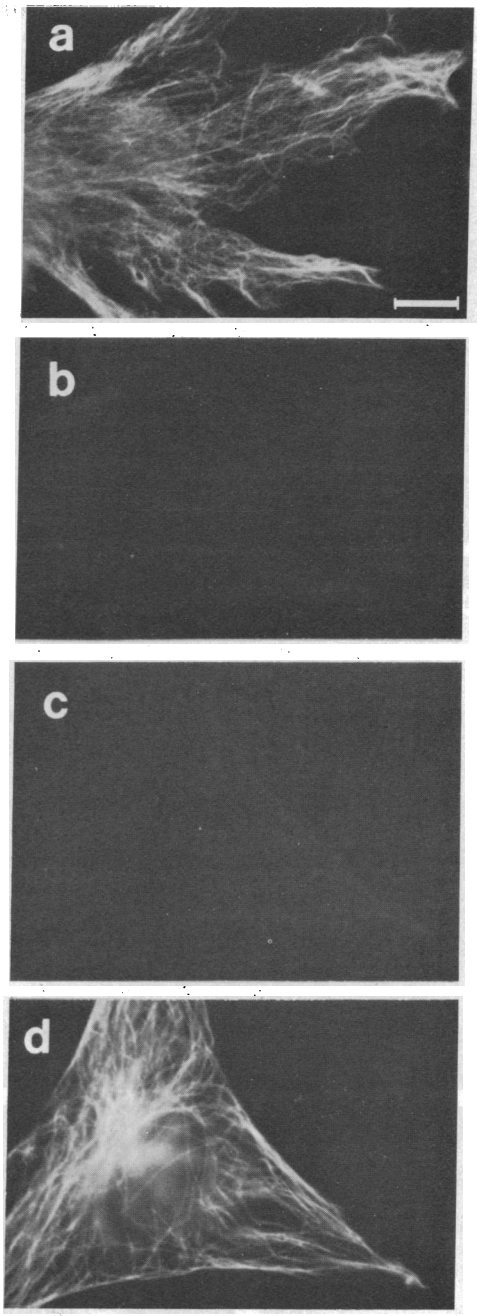

FIG. 2. Specificity controls for the desmin/tubulin double immunofluorescent labeling. (*a* and *b*) Primary mixture contained guinea pig antibodies to desmin and normal rabbit IgG, followed by the same mixture of secondary reagents, Rh-conjugated goat antibodies to guinea pig IgG and Fl-conjugated goat antibodies to rabbit IgG, that was used in the experiments of Fig. 1. The Rh and Fl fluorescence patterns are shown in *a* and *b*, respectively. Only the Rh labeling for desmin was observed. (*c* and *d*) Primary mixture consisted of rabbit antibodies to tubulin and normal guinea pig IgG, followed by the same mixture of secondary fluorescent antibody reagents used in *a* and *b*. Rh labeling (*c*) was not seen; only Fl labeling of tubulin (*d*) was observed. (Bar in *a* denotes 10 μm.)

Interphase cells treated with vinblastine (Fig. 3 *a* and *b*) or Colcemid (not shown) to disaggregate their microtubules exhibited the expected disorganized tubulin labeling as well as

more thickly labeled desmin filaments. The solid arrowheads designate structures that are desmin-labeled but not tubulin-labeled; the open arrowheads indicate tubulin-labeled structures that are not desmin-labeled. (Bar in *a* is 10 μm and represents the magnification of *a*, *b*, *c*, *d*, *g*, and *h*; bar in *e* is 10 μm and represents the magnification in *e*, *f*, *i*, and *j*.)

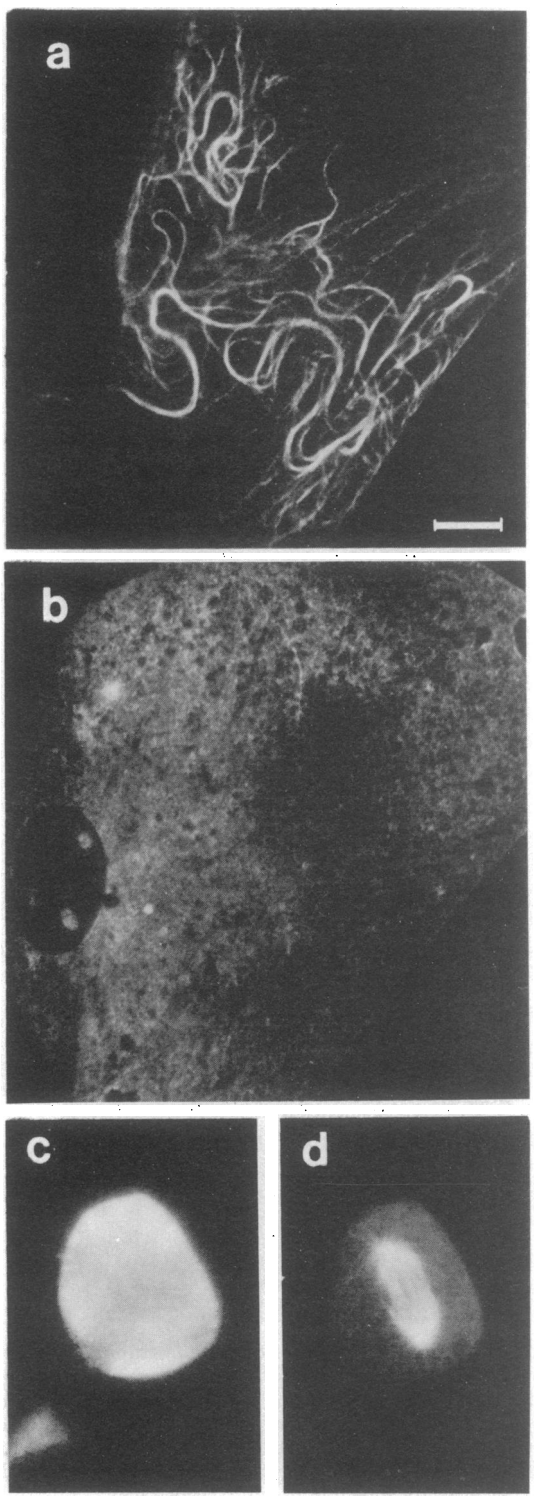

FIG. 3. (*a* and *b*) Effect of vinblastine on the immunolabeling patterns for desmin (*a*) and tubulin (*b*). Chicken embryo gizzard cells were treated with vinblastine (1 μg/ml) for 2 hr and then fixed and doubly immunolabeled as in Fig. 1 *a* and *b*. (*c* and *d*) Occasional cells in the population of the chicken embryo gizzard cell culture undergoing mitosis at the time of double immunolabeling for desmin (*c*) and tubulin (*d*) as in Fig. 1 *a* and *b*.

the highly condensed fibrous labeling for desmin previously reported (23, 24). Cells in mitosis exhibited a clearly different distribution of desmin labeling (Fig. 3*c*) and spindle-associated tubulin labeling (Fig. 3*d*).

DISCUSSION

The large degree of superposition of the immunofluorescent patterns observed with desmin and tubulin antibodies in these interphase chicken gizzard cells makes it important to emphasize that the immunolabeling of each antigen was indeed monospecific. This specificity was demonstrated as follows. (*i*) The results were closely similar with either rabbit or guinea pig primary antibodies to each of the two antigens. (*ii*) Some of the cells (20–30%) showed no labeling for desmin (not shown) but were labeled for tubulin. (*iii*) The superposition of the two labeling patterns was not complete in those cells that were doubly labeled. Certain fibrillar arrays were immunolabeled for one but not the other antigen (arrowheads in Fig. 1). (*iv*) The control experiments in Fig. 2 showed a complete segregation of the labeling reactions for the two antigens. (*v*) Interphase cells treated with vinblastine (Fig. 3 *a* and *b*) or cells in mitosis (Fig. 3 *c* and *d*) showed entirely uncorrelated distributions for the two antigens. It is therefore clear that the two antigens were indeed independently immunolabeled.

The extensive superposition of the desmin and tubulin patterns is to be contrasted with the results of similar simultaneous double-fluorescent labeling experiments carried out with actin (or myosin) and tubulin in fibroblasts and other cells (17, 22, 25) or with actin and desmin in the chicken gizzard cells (unpublished results). In these cases, no correlations were observed between the distributions of microfilaments and microtubules or microfilaments and intermediate filaments.

The substantial but not complete overlapping of the desmin and tubulin patterns suggests that some kind of extensive association exists between the intermediate filaments and microtubules in these interphase cells. The fact (Fig. 3 *a* and *b*) that the disruption of microtubules by vinblastine results in aggregation and rearrangement of the intermediate filaments, confirming earlier observations (2, 7, 23, 24, 26), agrees with the view that the distributions of intermediate filaments and microtubules are dependent at least to some extent. An important corollary of these results which is not widely appreciated at the present time is that changes in cellular properties induced by microtubule-dissociating drugs such as vinblastine or colchicine are not necessarily ascribable to the direct involvement of microtubules in the control of these properties but may instead reflect the involvement of intermediate filaments, whose distribution may be grossly altered when microtubules are disrupted.

A number of earlier studies have suggested that the distributions of intermediate filaments and microtubules are somehow correlated in interphase cells. In elongated processes of fully spread fibroblasts in culture, intermediate filaments and microtubules have been observed to lie interspersed with one another in generally parallel arrays in the direction of the long axis of the process (10). Occasionally, an individual intermediate filament and a microtubule have been observed in close parallel register in such processes (10–12). Apparent morphological relationships between intermediate filaments and microtubules have also been seen in nerve axons (27, 28) and in the nucleifilled channels within the syncytia formed after certain viral infections of cultured cells (29). Our results indicate that in chicken gizzard cells there is indeed a remarkably intensive association between intermediate filaments and microtubules.

At the level of resolution of the light microscope, the association between intermediate filaments and microtubules that we have observed could result from either of two types of mechanisms, noninteractive or interactive. In the former, only physical or steric interactions between the two structures would

be involved. For example, a cylindrical array of intermediate filaments might form a channel within which one or more microtubules might be confined, perhaps because the polymerization of tubulin was favored within such channels. No chemical binding between the two types of filaments would exist. By interactive mechanisms we mean that some kind of chemical binding is involved, either directly between the two types of filaments or indirectly through some third molecular species. Our immunofluorescence results cannot discriminate between an interactive or noninteractive association of intermediate filaments and microtubules. This is especially the case when the superposition of immunolabeling patterns was between thickly labeled strands of both types of filamentous structures (large arrows, Fig. 1); whether these represent separate and adjacent bundles or individually interspersed filaments of both types cannot be determined at this resolution.

On the other hand, in many instances (such as those noted with small arrows in Fig. 1), thinly labeled strands of microtubules appeared to be superimposed for distances of several micrometers over thinly labeled strands of intermediate filaments. Such thinly labeled strands of microtubules have been shown by coordinated immunofluorescent and electron microscopic observations of detergent-extracted cells (30) to represent single microtubules. It is therefore conceivable that, in such instances, single microtubules exist in extended parallel alignment with a few (and perhaps only one) intermediate filaments. If such were the case, it would imply that an interactive mechanism was involved in the association. However, it would require coordinated immunofluorescent and electron microscopic observations of the kind carried out by Osborn *et al.* (30) to determine whether such individual filament alignments did occur.

Whatever the mechanism that accounts for the extensive superposition of the two immunolabeled distributions, it is apparently under the metabolic control of the smooth muscle cell. This is first suggested by the fact that the superposition is not at all complete. Labeled microtubules are seen without associated intermediate filaments and vice versa. If we assume that each type of structure is intrinsically homogeneous, these findings therefore suggest that the association between them is in a dynamic steady state. The dynamic reversibility of the association is strongly indicated by the essentially complete lack of correlation of the immunolabeling patterns for desmin and tubulin in cells undergoing mitosis (Fig. 3 *c* and *d*). The gross reorganization of various cytoskeletal elements that occurs during mitosis must include a disruption of the association of intermediate filaments and microtubules, a conclusion that is in accordance with other types of observations (31, 32).

The intermediate filaments studied in this paper are of a specific type containing desmin found in smooth, cardiac, and striated muscle. Whether the several distinctive intermediate filaments found in other types of cells, which can be distinguished by specific antibodies as well as by other criteria (for review, see ref. 1), can also become associated with microtubules under appropriate conditions could be determined by separate double immunofluorescence experiments of the kind reported in this paper.

In earlier studies from this laboratory (17) a close association of microtubules and mitochondria in several different cell types was demonstrated by double immunofluorescence observations. It was suggested on the basis of these and earlier studies (27, 28) that this association might be of the interactive type, either directly between microtubules and mitochondria or indirectly, through other components. In view of the extensive association of microtubules and at least one type of intermediate filament reported in this paper, an association of mitochondria with either of these two structures would appear to be equally possible. To distinguish between these two possibilities, specific immunolabeling of all three components in the same cell might be carried out, with the objective of detecting associations of mitochondria with those strands of intermediate filaments or of microtubules that were not associated with the other.

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