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Mitochondria are associated with microtubules and not with intermediate filaments in cultured fibroblasts

(triple immunofluorescent labeling/vimentin/tubulin/cytochrome *c* oxidase)

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ABSTRACT **Triple-immunofluorescence experiments with antibodies to cytochrome *c* oxidase, tubulin, and vimentin have been used to immunolabel the mitochondria, microtubules, and intermediate filaments inside the same cultured fibroblasts. In particular, fibroblasts were immunolabeled after they had either been transformed by infection with Rous sarcoma virus or given long-term treatment with cycloheximide. These treatments induced redistribution of the intermediate filaments into a perinuclear arrangement, segregated away from the microtubules, which remained extended to the cell periphery. In such cells, many labeled mitochondria were observed to be codistributed with the peripherally located microtubules. From these results, we infer that an association, probably involving some type of chemical linkage(s), between mitochondria and microtubules exists in these cells that is independent of the intermediate filaments.**

One set of functions that has been ascribed to microtubules in interphase cells is the regulation of the location and movement of various intracellular organelles (cf. refs. 1–10), including the mitochondria. Such functions might be mediated by one or more kinds of molecular interactions between microtubules and the several organelles. The most compelling evidence for some such interaction between microtubules and mitochondria has been obtained from electron micrographs of neuronal axons of insects (9), in which what appear to be cross-bridges extending from microtubules to proximal mitochondria have been observed. Axons are, however, specialized structures in which intracellular transport mechanisms are especially highly developed, and it is therefore a question whether similar interactions between microtubules and mitochondria occur more generally in nonneuronal cells. In particular, in amorphous cells without a high degree of organization of the cytoskeleton, it may be difficult to observe cross-bridges between microtubules and certain membrane-bound organelles by electron microscopy even though indirect evidence for the existence of linkages is strong (1). In such cases, the technique of immunofluorescence, although its resolution is much lower than that of electron microscopy, may nevertheless have advantages in giving an overview of the distributions of two different intracellular components in the same cell. If these distributions are closely parallel, evidence for a possible interaction between the two components is thereby provided.

In a previous study from our laboratory (11), immunofluorescence experiments were carried out involving the simultaneous immunolabeling of microtubules (with antibodies to tubulin) and of mitochondria (with antibodies to cytochrome *c* oxidase) in a variety of cell types in culture. At the resolution of the light microscope, there was a remarkable degree of superposition of individual mitochondria on cytoplasmic microtubules in the periphery of the well-spread cells. It was suggested, therefore, that some kind of specific linkage, either direct or indirect, existed between the mitochondria and microtubules in these cells. It was recognized explicitly in this study, however, that the association could be indirect; for example, if the microtubules were linked to, and extensively codistributed with, intermediate filaments and the mitochondria were also linked to intermediate filaments, then, at the resolution of the immunofluorescence technique, in double-labeling experiments the mitochondria would appear to be associated with the microtubules. More recently, evidence has been obtained by double-immunofluorescence experiments that microtubules are indeed extensively codistributed with either desmin-type intermediate filaments in smooth muscle cells (12) or vimentin-type intermediate filaments in normal fibroblasts (13), making it difficult from our prior observations (11) to determine whether the mitochondria are associated primarily with microtubules or intermediate filaments.

We have therefore carried out simultaneous immunolabeling experiments in cells that had been subjected to conditions that produce marked segregation of the intermediate filaments from the microtubules in the periphery of fibroblasts and have inquired whether or not the mitochondria remain codistributed with the microtubules under these circumstances. One such condition is the transformation of normal fibroblasts by Rous sarcoma virus (ref. 13, see also ref. 14) and another involves the long-term treatment of normal fibroblasts with cycloheximide and other inhibitors of protein synthesis (15). In both conditions, cells can be found whose microtubules are in a fibrillar array extending out to the cell periphery, whereas the vimentin intermediate filaments are largely retracted to a perinuclear distribution. Under these circumstances, the mitochondria in the cell periphery are observed to be codistributed with the microtubules, suggesting that the two structures may indeed be linked to one another.

MATERIALS AND METHODS

The preparation and affinity purification of monospecific rabbit antibodies to chicken brain tubulin (11) and of guinea pig antibodies to vimentin from baby hamster kidney cells (13) have been described. The rabbit antiserum directed against beef heart cytochrome *c* oxidase was the gift of Efraim Racker and was used as in our previous study (11). The preparation and crossadsorption of affinity-purified goat antibodies to rabbit IgG and of goat antibodies of guinea pig IgG, their conjugation to fluorescein and rhodamine fluorophores, and the procedures used in the immunolabeling of the cells were carried out as reported (11, 12). Because the immunolabeled mitochondria can easily be distinguished from the filamentous microtubules, it

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is possible to label them with the same fluorophore and then use the second fluorophore to immunolabel the intermediate filaments, thus permitting the triple immunolabeling of mitochondria, microtubules, and intermediate filaments in the same cell. Accordingly, the primary rabbit antibodies to tubulin (6 μg/ml), rabbit antiserum to cytochrome *c* oxidase (70 μg of protein/ml), and guinea pig antibodies to vimentin (20 μg/ml) were mixed and applied to the formaldehyde-fixed Triton-permeabilized cells (11), which were then treated with a mixture of fluorescein-conjugated goat antibodies to rabbit IgG and rhodamine-conjugated goat antibodies to guinea pig IgG.

The NRK cells infected with the temperature-sensitive mutant (LA23) of Rous sarcoma virus (the gift of Peter K. Vogt) and the WI38 cells were used in our previous study (13). The cells were grown on glass coverslips for 2 days or longer; for some experiments, cycloheximide (20 μg/ml) was added to the medium 24 hr (15) before fixation and immunolabeling.

RESULTS

LA23-NRK cells grown at 39°C exhibit the normal phenotype. They are well-spread cells with a well-developed cytoskeleton. In such interphase cells, there is a strong relationship between the distribution of mitochondria (discrete elongated bodies) and of microtubules out to the cell periphery (Fig. 1*A*). This is also the case with the WI38 cells (Fig. 2*A*). Furthermore, in these two types of cells, the distribution of vimentin intermediate filaments (Figs. 1*B* and 2*C*, respectively) shows a substantial degree of correspondence with the distribution of microtubules in the same cell. These two sets of observations with fibroblasts exhibiting the normal phenotype make it difficult to decide whether the distribution of mitochondria is more closely coordinated with the microtubules or the intermediate filaments in these cells, although it is not infrequently possible to find a mitochondrion (Fig. 2*A*, arrow) superimposed on a microtubule strand where there does not appear to be a corresponding labeled strand of intermediate filament (Fig. 2*C*).

LA23-NRK cells grown at 33°C exhibit the transformed phenotype and, in such cells, in contrast to the situation with cells grown at 39°C, the microtubules and intermediate filaments no longer are codistributed (13). Most of the transformed cells are rounded-up but, in the minority of cells that are relatively flat, the microtubules (Fig. 1*C*) extend out to the cell periphery although the vimentin intermediate filaments are retracted into a perinuclear distribution (Fig. 1*D*). In such cells, the mitochondria show an extensive codistribution with the microtubules in the cell periphery (Fig. 1*C*, arrow) where the labeling for intermediate filaments is absent (Fig. 1*D*, corresponding arrow).

In a variety of cells, long-term cycloheximide treatment results in collapse of the vimentin intermediate filaments into a perinuclear distribution (15). This is also the case for the LA23-NRK cells grown at 39°C (normal phenotype) (Fig. 1*F*) and the WI38 cells (Fig. 2*D*). In such treated cells, the microtubules remain extended to the cell periphery (Figs. 1*E* and 2*B*) and the mitochondria appear to be codistributed with, and in favorable fields coincident with, strands of microtubules, in regions of the cells that show no labeling for vimentin intermediate filaments (Figs. 1*F* and 2*D*, respectively).

DISCUSSION

We have earlier reported (11) that double-immunofluorescent labeling for cytochrome *c* oxidase and tubulin in a variety of cultured cells showed that the labeled mitochondria were distributed along and coincident with the labeled microtubules at the level of resolution of the light microscope. On the other hand, there was no correlation between the distributions of the mitochondria and the F-actin microfilaments in these cells. We have now carried out triple-immunofluorescent labeling of fibroblasts for vimentin with one fluorophore, as well as for cytochrome *c* oxidase and tubulin with a second fluorophore and observed the simultaneous distributions of intermediate filaments, mitochondria, and microtubules in the same cells. In particular, under two quite different sets of conditions that induced marked segregation of the intermediate filaments from the microtubules, mitochondria were found to be codistributed with the microtubules in the cell periphery in the apparent absence of vimentin intermediate filaments. The conclusion is made that an absence of detectable vimentin immunolabeling implies a profound depletion, if not physical absence, of intermediate filaments from the region of the cell under examination. This is based first on the evidence that vimentin-containing intermediate filaments are by far the predominant type of intermediate filaments in fibroblasts (16) and second on the inference that even single vimentin intermediate filaments are visible in immunofluorescence. The latter inference is drawn by extrapolating to intermediate filaments the observations made by Osborn *et al.* (17) with microtubules, which showed directly that *individual* microtubules can be detected by immunofluorescent labeling. Despite the fact that a single filament is well below the resolution of the light microscope, the immunofluorescent beam emanating from it after indirect immunolabeling is readily detectable. With this inference that single vimentin intermediate filaments are also detectable in immunofluorescence, our results suggest, therefore, that mitochondria are specifically associated with microtubules, independently of the intermediate filaments, in these cells.

What is the nature of this association between mitochondria and microtubules? Given the low resolution of the immunofluorescence technique, one possibility is that the association does not involve linkage between the mitochondrion and a microtubule but only proximity of the two—e.g., the mitochondria and other large organelles might simply be confined within cytoplasmic channels that are formed by loosely organized and more-or-less parallel arrays of cytoskeletal filamentous elements. However, the vimentin intermediate filaments in these cells were much more numerous than the microtubules (cf., for example, Fig. 1 *A* and *B*). If the mitochondria were simply entrapped in channels formed by microtubules and intermediate filaments (18), then on a probabilistic basis they should have been associated more frequently with intermediate filaments than with microtubules in our immunofluorescent observations. On the contrary, however, in cells in which both filament types extended out toward the cell periphery, labeled mitochondria were seen at a low but significant frequency to be associated with strands of isolated microtubules where there were no labeled intermediate filaments (cf. arrow, Fig. 2*A*) but not the reverse; that is, we did not observe labeled mitochondria associated with isolated strands of intermediate filaments where no labeled microtubules were present. Nor did the retraction of the intermediate filaments from the cell periphery lead to any gross alteration in either the numbers of mitochondria in the cell periphery or in their coincident distribution with strands of microtubules (cf., for example, Fig. 2 *A* and *B*).

It seems to us more reasonable, therefore, to suggest that our results reflect some type of specific chemical linkage between mitochondria and microtubules in these cells. These linkages might be relatively few in number for any mitochondrion–microtubule pair and, for this and other reasons (1), might be difficult to detect in thin-section electron microscopy of such cells. Such putative linkages may be related to the cross-

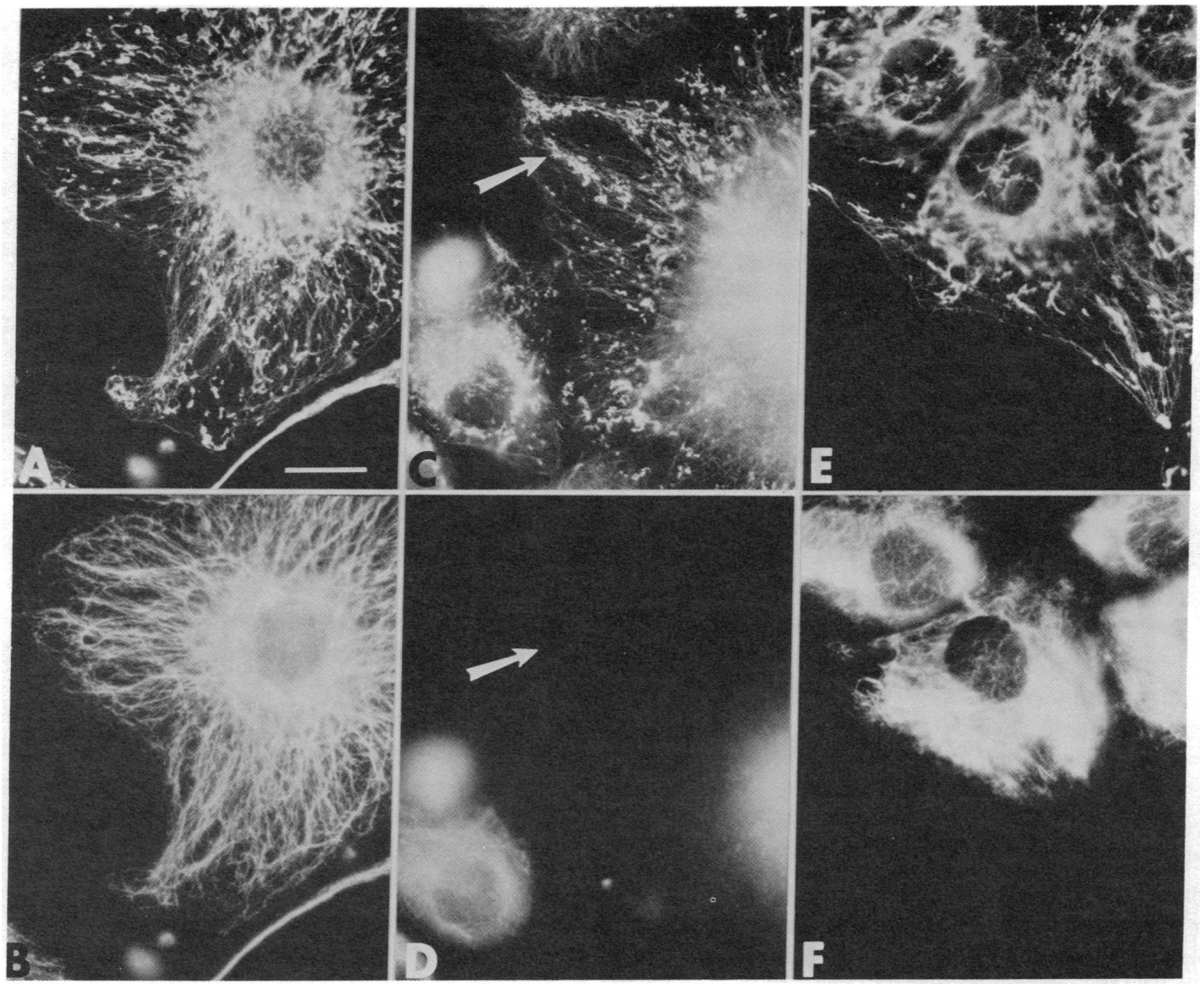

FIG. 1. NRK cells infected with a temperature-sensitive mutant (LA23) of Rous sarcoma virus. (*A* and *B*) Cells grown at 39°C, exhibiting the normal phenotype. (*C* and *D*) Cells grown at 33°C, exhibiting the transformed phenotype. (*E* and *F*) Cells grown at 39°C, after 24 hr of treatment with cycloheximide at 20 μg/ml. (*A*, *C*, and *E*) Double indirect immunolabeling with fluorescein–antibody conjugates of cytochrome *c* oxidase [labeling mitochondria (the short rod-like elements, arrow in *C*)] and of tubulin (labeling microtubules) simultaneously. (*B*, *D*, and *F*) Immunolabeling with rhodamine–antibody conjugates of vimentin (labeling intermediate filaments) in the cells shown in *A*, *C*, and *E*, respectively. The arrow in *D* indicates the peripheral region of the cell in *C* from which the vimentin intermediate filaments have retracted on transformation, as indicated by the complete absence of immunolabeling for vimentin. The coordinate distribution of the mitochondria with microtubules can be observed in each case.

bridges that have been observed between mitochondria and microtubules in electron micrographs of insect neuronal axons (9). The components involved in such axonal cross-bridges are unknown. Clearly, further progress in this area requires that any such putative linkage factor(s) be identified and characterized biochemically.

The existence of chemical linkages between mitochondria and microtubules could explain a number of observations regarding the locations and movements of mitochondria inside cultured fibroblastic cells. For example, Wang and Goldman (18) observed that, during the early spreading of baby hamster kidney cells on a substratum, mitochondria and other intracellular organelles (seen under phase-contrast optics) were absent from the cell periphery at times when, according to electron microscopic observations, microtubules and intermediate filaments were confined to a perinuclear distribution in the cell. After longer times of spreading, however, when microtubules and intermediate filaments were observed to have invaded the cell periphery, correspondingly many mitochondria and other organelles were found located there. Clearly, these observations could be explained if chemical linkages existed between mitochondria and microtubules [as well as other linkages between microtubules and intermediate filaments (12, 13)] in these cells.

The saltatory movements of mitochondria and other organelles in fibroblasts have also been studied in some detail (18). The stop-and-go character of these movements may be associated with the making and breaking of linkages of mitochondria to microtubules. The problem of saltation is a complex one, however, and deserves more extended molecular analysis than is appropriate here (cf. ref. 19).

Finally, it is of interest to view our results in relationship to similar immunofluorescence experiments suggesting that the cytoplasmic enzyme creatine phosphokinase is associated with the intermediate filaments inside cultured nonmuscle cells (20). The mitochondria, which supply ATP to the cytoplasm, and creatine phosphokinase, which catalyzes the formation of ATP from creatine phosphate and ADP, are two of the important components of energy production in these cells. The suggestions that these components are associated with two different cytoskeletal elements, elements that may themselves interact with one another under appropriate conditions (12, 13), are clearly relevant to the distribution and flux of chemical energy inside these cells.

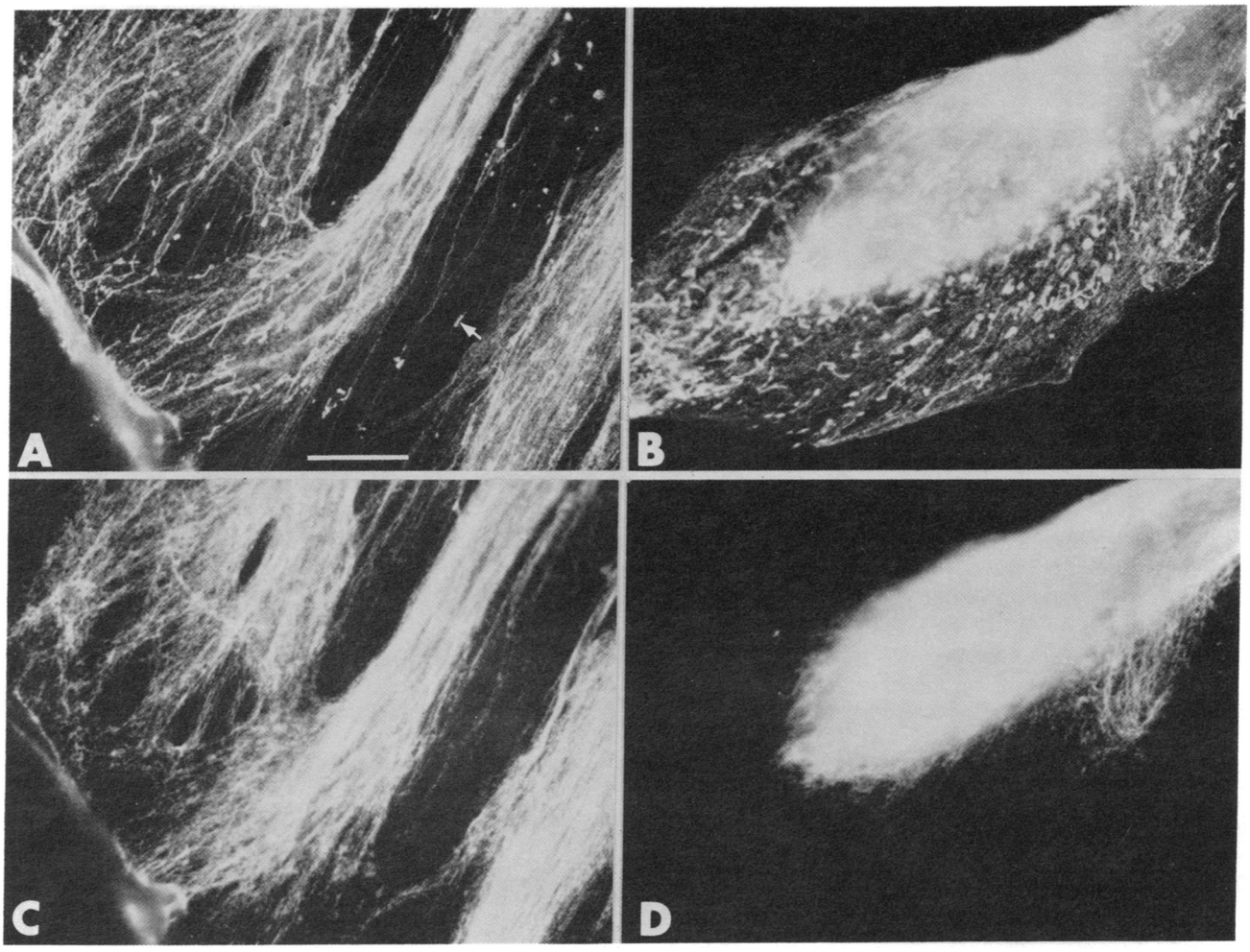

FIG. 2. WI38 human fibroblasts. (*A* and *C*) Grown in normal media at 39°C. (*B* and *D*) Grown at 39°C after 24 hr of treatment with cycloheximide at 20 μg/ml. (*A* and *B*) Simultaneous immunolabeling of mitochondria and microtubules. (*C* and *D*) Immunolabeling of intermediate filaments in the corresponding cells, as in Fig. 1. The arrow in *A* indicates a mitochondrion superimposed on a thinly labeled microtubule where there is no labeled intermediate filament in *C*. The mitochondria remain codistributed with the microtubules in *B* when the intermediate filaments are retracted from the cell periphery in *D*.

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