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

(xytokeleton/vimentin/double immunofluorescence)

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ABSTRACT Double immunofluorescent labeling experiments for lysosomes and either microtubules or vimentin intermediate filaments in cultured well-spread fibroblasts show a remarkable degree of superposition of the lysosomes and the microtubules. Under two different sets of conditions where the microtubules and intermediate filaments are well segregated from one another, the lysosomes remain codistributed with the microtubules. It is suggested that this specific association of lysosomes with microtubules reflects some type(s) of linkage(s) between them and that such linkages may play an important role in the location and intracellular transport of lysosomes inside cells.

Microtubules (MT) and intermediate filaments (IF) have been increasingly recognized as playing important roles in regulating the location and movement of intracellular organelles (cf. ref. 1). In this article, we are specifically concerned about the relationships between lysosomes and cytoskeletal elements. Previous studies have suggested an involvement of MT or IF or both in lysosome activities and functions. For example, the salutary movements of lysosomes and other organelles have been correlated with MT integrity (2, 3), the intracellular distribution of lysosomes in mouse macrophages has been correlated with the degree of MT and IF extension into the cytoplasm (4), and the secretion of lysosomal enzymes in polymorphonuclear leukocytes was shown to be affected by the colchicine-induced disruption of MT (5). These effects, however, only indirectly suggest an association of lysosomes with cytoskeletal elements, and more direct evidence concerning a possible physical association is desirable.

In principle, electron microscopy is the technique of choice for the detection and characterization of any physical linkages between specific organelles and cytoskeletal filaments. For cells with an amorphous internal structure, however, transmission electron microscopy of random ultrathin sections may be of only limited use for this purpose, because it may be difficult to find linkage elements in any given field of a section. Under these circumstances, double immunofluorescence light microscopy may yield significant information about organelle–filament associations. Despite the low resolution of the method, it may reveal distinct spatial correlations of a specific immunolabeled organelle with a particular type of immunolabeled cytoskeletal filament throughout an entire cell. If other filament types show no such spatial correlations with the organelle in question, the suggestion is that a specific association exists between the organelle and the spatially correlated filament. We have used this method previously to provide evidence for an association of mitochondria with MT (6, 7) in a variety of cultured cells. In this paper, similar double, indirect immunofluorescence experiments have indicated a strong spatial correlation of immunolabeled lysosomes with MT in cultured fibroblasts, but not with vimentin IF, suggesting that some kind(s) of linkages exist between lysosomes and MT in these cells.

MATERIALS AND METHODS

Antibody Preparations. Antisera to lysosomes (8) were raised in rabbits to a membrane fraction of lysosomes isolated from rat liver, and anti-lysosome antibodies were purified by extensive absorption of the sera with other cellular fractions (9). The anti-lysosome antibodies reacted with primary and secondary lysosomes, according to immunoperoxidase labeling in electron microscopy, and occasionally with some Golgi stacks and the plasma membrane (8). The antigen recognized by these antibodies appears to be a 100,000-dalton protein, according to immunolabeling procedures. The guinea pig antibodies to chick brain tubulin and vimentin isolated from baby hamster kidney cells, were affinity-purified monospecific antibodies previously characterized (10). Affinity-purified and cross-absorbed goat antibodies to rabbit IgG and to guinea pig IgG and their conjugation to fluorescein and rhodamine fluorophores were as described (10).

Cells and Immunofluorescence Labeling. All experiments were carried out with normal rat kidney (NRK) cells infected with a temperature-sensitive mutant (LA23) of Rous sarcoma virus. The infected cells were from stocks kindly supplied by P. K. Vogt of the University of Southern California. LA23-infected NRK cells were grown on coverslips either at 39°C, where they exhibit the normal phenotype, or at 33°C, where they are transformed. In some experiments, cycloheximide (20 μg/ml) was added to the medium 15 hr before the end of the growth period; in others, nocodazole (10 μg/ml) was added 6 hr before. After a minimum of 2 days of growth, the cells were fixed for 20 min with 3% formaldehyde and then permeabilized by a 5-min exposure to 0.2% Triton X-100. Immunolabeling was carried out as described (7, 10). In some of the experiments, in order to visualize more clearly the spatial relationships of lysosomes and MT, optical leak-through of the rhodamine immunofluorescence for tubulin into the fluorescein immunofluorescence for lysosomes was deliberately generated. This was achieved by appropriately increasing the fluorescence intensity of tubulin labeling and by longer photographic exposures, so as to produce the desired effects. This required calibration with each different batch of primary and secondary antibodies used. Immunolabeling was observed by epifluorescence with a Zeiss Photomicroscope-III instrument with the filters CZ487710 for fluorescein immunofluorescence and CZ487714 for rhodamine.

Abbreviations: MT, microtubules; IF, intermediate filaments; NRK cells, normal rat kidney cells.

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RESULTS

In LA23-infected NRK cells grown at 39°C and exhibiting the normal phenotype, double immunolabeling for lysosomes (Fig. 1A) and for microtubules (Fig. 1B) in the same cells revealed a high concentration of lysosomes in the vicinity of the microtubule-organizing center (arrows) near the cell nucleus. The exposure in Fig. 1A does not allow clear visualization of the many lysomes out in the cell periphery, which are shown in Fig. 1C. In this field, the immunolabeled lysosomes are the bright dots of fluorescein fluorescence, and the background filamentous labeling is due to the optical leak-through of the rhodamine immunolabeled microtubules that also were recorded separately in Fig. 1D through the rhodamine optical filter. More than 80% of the labeled lysosomes in the cell periphery were superimposed on labeled MT, and this number was increased under different photographic conditions that revealed other labeled MT not apparent in Fig. 1C.

In such well-spread cells exhibiting the normal phenotype, it is known that MT and IF show substantial codistributions in double immunofluorescence experiments. Therefore, in order to discriminate whether the lysosome association was primarily with MT or IF, two sets of conditions were used that result in an intracellular dissociation of the two filament types (7). One of these conditions involved growth of the LA23-infected NRK cells at 33°C, at which temperature the cells exhibited the transformed phenotype, and the vimentin IF were retracted around the nucleus (Fig. 2D), while the MT remained extended to the cell periphery (Fig. 2B). The labeled lysosomes in such cells with retracted IF remained distributed out in the cell periphery (Fig. 2C), to a large extent superimposed on the labeled MT (Fig. 2A and B). The second condition involved growth of the cells at 39°C in the presence of cycloheximide, which is known to cause a retraction of the vimentin IF around the nucleus (Fig. 2H). Under these circumstances, the lysosomes were still found in relatively normal amounts out in the cell periphery (Fig. 2G), where they were largely superimposed on MT (Fig. 2E and F).

In the presence of nocodazole in LA23-infected NRK cells

![Images of Fig. 1](attachment:images.png)

**Fig. 1.** LA23-infected NRK cells grown at 39°C (normal phenotype). Double indirect immunofluorescent labeling for lysosomes (bright dots in A and C) and MT (B and D). Treatment with a mixture of rabbit anti-lysosome antibodies (40 μg/ml, see text) and affinity-purified guinea pig anti-tubulin antibodies was followed by treatment with a mixture of cross-adsorbed rhodamine- or fluorescein-conjugated affinity-purified goat antibodies to rabbit and guinea pig IgG. (A and D) Rhodamine labeling. (B and C) Fluorescein labeling. Note the high concentration of lysomes (arrow in A) in the vicinity of the MT-organizing center (arrow in B). Out in the cell periphery, most of the lysosomes (C) are closely associated with individual MT (C and D). The background filamentous labeling in C is due to the rhodamine fluorescence of labeled MT that was allowed to leak through the fluorescein filter, producing an effect of superimposing the two patterns. The longer time of exposure of C compared to D allows the visualization of some additional labeled MT. All fields are at the same magnification. (Bar in B = 20 μm.)
FIG. 2. (Legend appears at the bottom of the next page.)
grown at 39°C, the MT were disrupted and vimentin IF were condensed to a perinuclear distribution, as has been observed by others in similar systems (cf. ref. 13). In such nocodazole-treated cells, the lysosomes appeared to be aggregated to some extent (large bright spots in Fig. 3A) but otherwise to be distributed randomly in the cytoplasm and showed no spatial correlation with the collapsed IF (Fig. 3B). In particular, there was no longer a concentration of lysosomes near the nucleus, as observed in Fig. 1A, in these cells in which the microtubule-organizing center was disrupted.

**DISCUSSION**

We have provided evidence by double immunofluorescence light microscopy that lysosomes are to a remarkable extent codistributed with MT inside cultured fibroblasts. The antilyosome antibodies used in this study (8) did not discriminate between primary and secondary lysosomes, so that this conclusion presumably applies to both types. The codistribution of lysosomes with MT is manifested in several circumstances.

(i) A concentration of lysosomes is spatially coincident with the concentration of MT that exists in the vicinity of the microtubule-organizing center (Fig. 1 A and B).

(ii) A large fraction of the lysosomes that are located out in the periphery of the cell are closely associated with the dispersed and convoluted MT that are also present there (Fig. 1 C and D).

(iii) Because the interpretation of this last observation is complicated by the fact that MT and vimentin IF are codistributed with one another in LA23-infected NRK cells grown at 39°C (10), it is especially interesting that under two quite different sets of conditions where the vimentin IF are retracted to a perinuclear distribution away from the MT, a large number of lysosomes remain out in the cell periphery codistributed with the MT still located there. This is true for LA23-infected NRK cells grown at 33°C exhibiting the transformed phenotype (Fig. 2 A–D) and for the cells grown at 39°C in the presence of cycloheximide exhibiting the normal phenotype (Fig. 2 E–H).

(iv) When the MT are disrupted with nocodazole and the IF become aggregated into perinuclear bundles (Fig. 3B), the lysosomes appear to be randomly dispersed (14) and no longer tethered (Fig. 3A).

These results indicate that lysosomes are extensively associated with MT but not with vimentin IF inside fibroblasts. Because there is much evidence that the distribution of actin microfilaments is not coordinated with either MT or IF in these cells (cf. refs. 15 and 16), no significantly spatial correlation of lysosomes with microfilaments is indicated. Therefore, our results are not consistent with the suggestion of Moore et al. (17) that there is an association of lysosomes with microfilaments, as studied with polymorphonuclear leukocytes. The specificity and extent of the lysosome-MT codistribution in the fibroblasts suggest that some kind or kinds of direct or indirect linkages exist between lysosomes and MT in these interphase cells that involve most of the lysosomes at any given time. Such linkages could play important roles in lysosomal activities, including the saltatory move-

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**Fig. 2** (on preceding page). LA23-infected NRK cells grown at 33°C (transformed phenotype) (A–D) and grown at 39°C (normal phenotype) (E–H) after 15 hr of treatment with cycloheximide at 20 μg/ml. Double, indirect immunofluorescent labeling for lysosomes (bright spots in A and E) and MT (B and F) or for lysosomes (C and G) and vimentin IF (D and H). In the pairs A/B and E/F, lysosomes are fluorescein labeled and MT are rhodamine labeled. The rhodamine fluorescence for tubulin was allowed to leak through the fluorescein filter in A and E. In the pairs C/D and G/H, lysosomes are rhodamine labeled. For IF labeling (D and H), affinity-purified guinea pig antibodies to vimentin were used, followed by fluorescein-conjugated affinity-purified goat antibodies to guinea pig IgG. Note in each case the coordinate distribution of lysosomes (A and E) and MT (A/B and E/F). Both are concentrated in a perinuclear area, and lysosomes can be seen out in the cell periphery superimposed on individual MT (small arrows, for example). In this region (large arrows in C/D and G/H) lysosomes are not associated with IF, which have retracted around the nucleus as indicated by the absence of vimentin labeling. Cell edges are indicated by arrowheads in E and G. All fields are at the same magnification. (Bar in D = 20 μm.)

**Fig. 3**. LA23-infected NRK cells grown at 39°C (normal phenotype). Double indirect immunofluorescent labeling for lysosomes (A) and vimentin IF (B) after 6 hr of treatment with nocodazole at 10 μg/ml. For rhodamine labeling of lysosomes and simultaneous fluorescein labeling of IF, see Figs. 1 and 2, respectively, for procedures. Note that lysosomes are randomly distributed in the cytoplasm, to some extent apparently in patches (large bright dots in A), but show no spatial correlation with the IF collapsed near the nucleus (B). MT are completely disrupted under these conditions (not shown). (C) Same field in Nomarski optics. Cell edges are indicated by arrowheads. (Bar = 20 μm.)
ments of lysosomes inside cells that have been shown to stop upon disruption of microtubules (2, 3) and the secretory functions of lysosomes, which also appear to depend upon the integrity of microtubules (5).

The evidence presented here for a preferential association of lysosomes with MT does not exclude the possibility that, in other cells and under other metabolic conditions, lysosomes may associate with IF. By means of immunofluorescence observations similar to those presented here, we earlier demonstrated (6, 7) an association of mitochondria with MT and not with IF in cultured cells. However, although there is evidence from several studies that mitochondrial-MT associations are preferred in certain systems (1, 7, 18, 19), there is also evidence for mitochondrial-IF interactions in other cases (20–23).

The present results make a specific contribution to the growing body of evidence that most, if not all, intracellular organelles may form direct or indirect linkages to one or more cytoskeletal filaments in most eukaryotic cells. In structurally well-organized nerve axons, regularly arrayed linkages between MT or IF and various membrane-bounded organelles have been observed by transmission electron microscopy (20, 24, 25), although specific reference to linkages to primary or secondary lysosomes has not often been made in these studies. It also is not evident that special cases such as the nerve axon are of general relevance; the axon is specialized for the long-distance transport of membrane-bounded organelles, and may exhibit cytoskeletal-organellar connections for the purpose that are not necessarily characteristic of other cells. However, the immunofluorescence observations of lysosome-MT association reported in this paper and similar evidence for mitochondrion-MT (6, 7, 18) and for Golgi apparatus-MT (26) associations in amorphous cultured cells as well as electron microscopic evidence for associations of nuclei with IF in striated muscle (22, 23, 27, 28), of nuclei with MT/IF in other cells (29), and of secretory granules with MT in cultured pancreatic beta cells (30) are among the kinds of evidence for the existence of a wide range of organelle-cytoskeletal filament interactions in eukaryotic cells, which very likely serve to locate these organelles and to direct their movements inside cells.

The molecular bases for such organelle-cytoskeletal interactions are only poorly understood. Whether different membrane-bound organelles that interact with a particular cytoskeletal filament do so via a common recognition mechanism or via specific mechanisms for different organelles is not clear. In cases where MT are involved, the linkage elements may include the high molecular weight MT-associated proteins (MAPs) (31, 32), which project from microtubules (33) and which appear also to associate with IF (34–36). In vitro evidence implicating MT-associated proteins has been reported in the case of MT-secretory granule interactions (37), but further investigations along these lines are required with a variety of organelle-cytoskeletal filament systems.

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