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Intermediate filaments in monkey kidney TC7 cells: Focal centers and interrelationship with other cytoskeletal systems

(keratins/vimentin/microtubules/cytochalasin B/microfilaments)

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ABSTRACT **Two-dimensional gel electrophoresis of intermediate-sized filament-enriched cytoskeletons of epithelial monkey kidney TC7 cells has shown that they are composed of at least two keratins (isoelectric focusing 36, M_r = 48,500; IEF 46, M_r = 43,500; HeLa protein catalogue number) and vimentin. Indirect immunofluorescence as well as immunoelectron microscopy using antibodies directed against specific polypeptides sometimes revealed a discontinuous staining of keratin-containing filaments. Indirect immunofluorescence analysis of cells stained with keratin or vimentin antibodies also revealed a bright perinuclear staining in 58% of the cells in interphase. Of particular interest were focal centers from which filaments radiated. Double-label immunofluorescence using tubulin and keratin antibodies showed that these centers codistributed with focal arrays of microtubules (most likely centrosomes) in interphase cells but were not colocalized with centrioles in mitosis or, in many cases, with the microtubule organizing centers seen after release from nocodazole treatment. Treatment of TC7 cells with demecolcine (10 μg/ml, 20 hr) resulted in a drastic rearrangement of the keratin and vimentin filaments. Likewise, treatment with cytochalasin B (10 μg/ml, 1 hr) produced a star-like arrangement of the keratin and vimentin filaments and, in most cases, these codistributed with patches of actin. The results provide evidence for the interaction of intermediate filaments (keratins and vimentin) with both microtubules and microfilaments.**

Intermediate-sized filaments (7–11 Å in diameter) are ubiquitous elements (for references, see refs. 1 and 2) that have been implicated in various intracellular roles, including nuclear anchorage (3, 4), organelle interactions (5–12), and gene expression (13); however, the molecular mechanisms underlying these functions remain largely unknown. To date, much emphasis has been placed on determining their subunit composition (for references, see refs. 1, 2 and 14), with less emphasis on their structural and functional relationships with other cytoskeletal systems. In the present study we analyzed the composition and distribution of intermediate-sized filaments in epithelial monkey kidney TC7 cells. The results, including data on cells treated with either demecolcine or cytochalasin B, provide evidence for the interaction of intermediate filaments (keratins and vimentin) with both microtubules and microfilaments.

MATERIALS AND METHODS

Cells. African green monkey kidney TC7 cells (a gift of A. Graessmann) were grown as monolayer cultures in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics (penicillin, 100 international units/ml; streptomycin, 50 μg/ml).

The procedures for labeling cells with [^{35}S]methionine (15, 16), two-dimensional gel electrophoresis (17, 18), preparation of low-salt- and high-salt-extracted cytoskeletons (4), and indirect immunofluorescence (10) have been described in detail elsewhere. The vimentin antibody was a gift from S. Blose.

Immunoelectron Microscopy. Immunoelectron microscopy was performed on unfixed intermediate filament-enriched cytoskeletons on electron microscope grids (4) using the indirect immunogold staining procedure according to De Mey *et al.* (19). After antibody treatment, cytoskeletons were negatively stained with aqueous uranyl acetate (4).

RESULTS

Intermediate-Sized Filament Proteins of TC7 Cells. Fig. 1 shows a region of a two-dimensional gel fluorograph [isoelectric focusing (IEF)] of proteins from asynchronous TC7 cells labeled for 20 hr with [^{35}S]methionine. The positions of the intermediate-sized filament proteins [keratins: IEF 36 (M_r = 48,500) and 46 (M_r = 43,500); vimentin: IEF 26; HeLa protein catalogue number] (20–22) as well as of α- and β-tubulin and actin are indicated as reference. The authenticity of the intermediate filament proteins has been confirmed by two-dimensional gel electrophoretic analysis of [^{35}S]methionine-labeled cytoskeletons enriched in intermediate filaments (Fig. 1*B*) (4) and of immunoprecipitates of total TC7 proteins reacted with a broad-specificity keratin antibody (Fig. 1*C*) (23).

Intermediate Filaments of TC7 Cells: Perinuclear Bodies and Their Relationship with Microtubule Organizing Centers (MOC). Immunofluorescence staining of methanol/acetone-fixed TC7 cells with a mouse polyclonal antibody raised against HeLa keratin IEF 46 (24) revealed a discontinuous staining of filaments (Figs. 2*a* and *b*) as well as the presence of strongly fluorescent perinuclear bodies (arrows in Fig. 2*a*; such bodies were seen in about 58% of the cells in interphase) similar to those described by Borenfreund *et al.* (25) in cultured hepatoma cells and by Hormia *et al.* (26) in endothelial cells. Immunoelectron microscopy using the immunogold staining procedure (19) revealed that the discontinuous staining was not due to the fragmentation of the filament system but rather due to a real discontinuous distribution of label along segments of the filament net (Fig. 2*c*). The same antibody stained filaments in a continuous fashion in transformed human amnion cells (AMA cells) (24), as determined both by immunoelectron microscopy (small particles in Fig. 2*d*) and immunofluorescence microscopy (Fig. 2*e*). A discontinuous staining of TC7 cells was also observed with the broad-specificity keratin antibody (not shown). Staining of TC7 cells with vimentin antibodies revealed a continuous

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Abbreviations: IEF, isoelectric focusing; MOC, microtubule organizing centers.

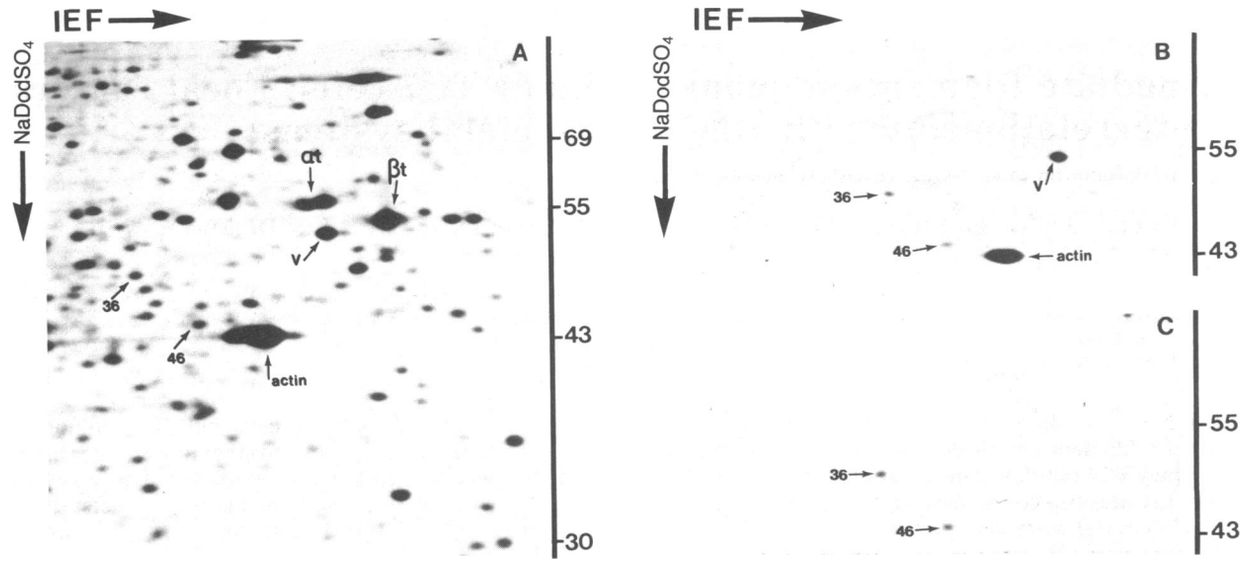

FIG. 1. Intermediate-sized filament proteins of TC7 cells. Two-dimensional gel electrophoresis (IEF). Only the relevant parts of the gels are shown. (*A*) [^{35}S]Methionine-labeled proteins from TC7 cells labeled for 20 hr. (*B*) [^{35}S]Methionine-labeled proteins from TC7 cells extracted with 0.5% Triton X-100 in Pipes buffer (4), followed by extraction with buffers of low and high ionic strength (4). (*C*) [^{35}S]Methionine-labeled proteins from TC7 cells immunoprecipitated with a broad-specificity keratin antibody (23). Essentially the same results were obtained with the keratin IEF 46 antibody. The spot on the upper corner in *C* corresponds to a common contaminant of immunoprecipitates. Molecular weights are indicated as $M_r \times 10^{-3}$.

staining of a subset of filaments, as judged by immunoelectron microscopy (not shown).

The perinuclear bodies could be shown, by analysis of enucleated cells (cytoplasts; antikeratin staining; Fig. 2*f*), to be cytoplasmic structures. In many cases filaments were seen radiating from the bodies (Fig. 2*g*), (25, 27, 28). Normally, one body per cell was observed, although in some cases two or three focal centers were seen, and these were often interconnected by filaments. The perinuclear bodies also stained brightly with vimentin antibodies (arrows in Fig. 2*h*).

Methanol/acetone-treated TC7 cells reacted with tubulin antibodies also revealed strong perinuclear staining sites in some of the interphase cells (arrow in Fig. 2*i*) and, in most cases, these codistributed with the focal arrays of intermediate filaments, as judged by double-label immunofluorescence (Fig. 2*j*, tubulin staining; Fig. 2*k*, keratin staining). However, similar analysis of mitotic cells showed that the centrioles (Fig. 2*l*) do not codistribute with the focal arrays (Fig. 2*m*; seen in only 20% of the mitotic cells). Also, after nocodazole treatment (10 μg/ml, 20 hr; refs. 29–30) and recovery (3 min), the MOC (31) and the intermediate filament focal centers usually had different cellular locations (Fig. 2 *n* and *o*; arrows indicate the positions of the intermediate filament focal centers), although, in some cases, they were in close proximity (Fig. 2*p* and *q*) or were coincident (not shown).

By double-immunofluorescence microscopy using rhodamine-labeled phalloidin (Fig. 2*r*) and keratin IEF 31 antibody (Fig. 2*s*) (24), the perinuclear bodies were found to be negative for actin. The IEF 31 antibody (crossreacting partially with TC7 keratins; see ref. 24 for crossreactivity of this antibody) was used thus to obtain a weak staining of the focal centers.

Demecolcine Induced a Redistribution of the Keratin- and Vimentin-Containing Filaments. Treatment of TC7 cells with demecolcine (20 hr, 37°C) resulted in the disassembly of microtubules (results not shown) and a drastic redistribution of the keratin (Fig. 2*t*) and vimentin (not shown) filaments. Fewer discontinuously stained filaments were also observed towards the cell periphery. The demecolcine treatment also affected the focal centers (arrow in Fig. 2*t*), which were then observed in only about 19% of the cells. A partial, although less dramatic, effect of antimitotic drugs on keratin-containing filaments has also been observed in PTK2 cells (32).

Cytochalasin B Affects the Organization of Intermediate-Sized Filaments and Microfilaments. Treatment of TC7 cells with cytochalasin B (10 μg/ml, 1 hr, 37°C) followed by methanol/acetone fixation revealed a star-like arrangement of the keratin (arrows in Fig. 3*a*) and vimentin filaments (not shown) similar to that recently reported in mouse KLN 205 carcinoma cells treated simultaneously with colchicine and cytochalasin D (33). The effect of cytochalasin B on intermediate filaments in TC7 cells could be observed already after

FIG. 2 (*on next page*). Fluorescence and immunoelectron microscopy of TC7 cytoskeletons. Except where indicated, the cells first were treated with methanol (4 min at −20°C) and then treated with acetone (as for methanol) prior to incubation with the antibody. (*a* and *b*) TC7 cells reacted with HeLa keratin IEF 46 antibody; (*c* and *d*) immunoelectron microscopy of intermediate filament-enriched cytoskeletons of TC7 cells (*c*) and AMA cells (*d*), both with the keratin IEF 46 antibody (small gold particles). Note discontinuous label in TC7 cells and continuous label in AMA cells. The large gold particles in *d* arise from a second label for vimentin (suboptimal concentration of second antibody) and should be ignored. (*e*) AMA cells reacted with IEF 46 antibody; (*f*) TC7 cytoplast reacted with IEF 46 antibody; (*g*) TC7 cells reacted with HeLa keratin IEF 31 antibody; (*h*) TC7 cells reacted with vimentin antibodies; (*i*) TC7 cells reacted with tubulin antibodies; (*j* and *k*) double-immunofluorescence of interphase TC7 cells reacted with tubulin (*j*) and broad-specificity keratin antibody (*k*); (*l* and *m*) double-immunofluorescence of mitotic TC7 cells reacted with tubulin (*l*) and broad-specificity keratin antibody (*m*); (*n–q*) double-immunofluorescence of nocodazole-treated TC7 cells (10 μg/ml; 20 hr, 3-min recovery), fixed and reacted with tubulin (*n* and *p*) and the broad-specificity keratin antibody (*o* and *q*); (*r* and *s*) double-immunofluorescence of acetone-fixed TC7 cells reacted with rhodamine-labeled phalloidin (*r*) and keratin IEF 31 antibody (*s*); (*t*) TC7 cells treated with demecolcine (10 μg/ml; 20 hr, 37°C) and reacted with the broad-specificity keratin antibody. (*a*, *g*, and *j–q* ×380; *b*, ×600; *c*, ×40,000; *d*, ×55,000; *e*, *h*, and *t*, × 280; *i*, *r*, and *s*, ×240.) In *j–s*, arrows indicate equivalent points in the micrographs.

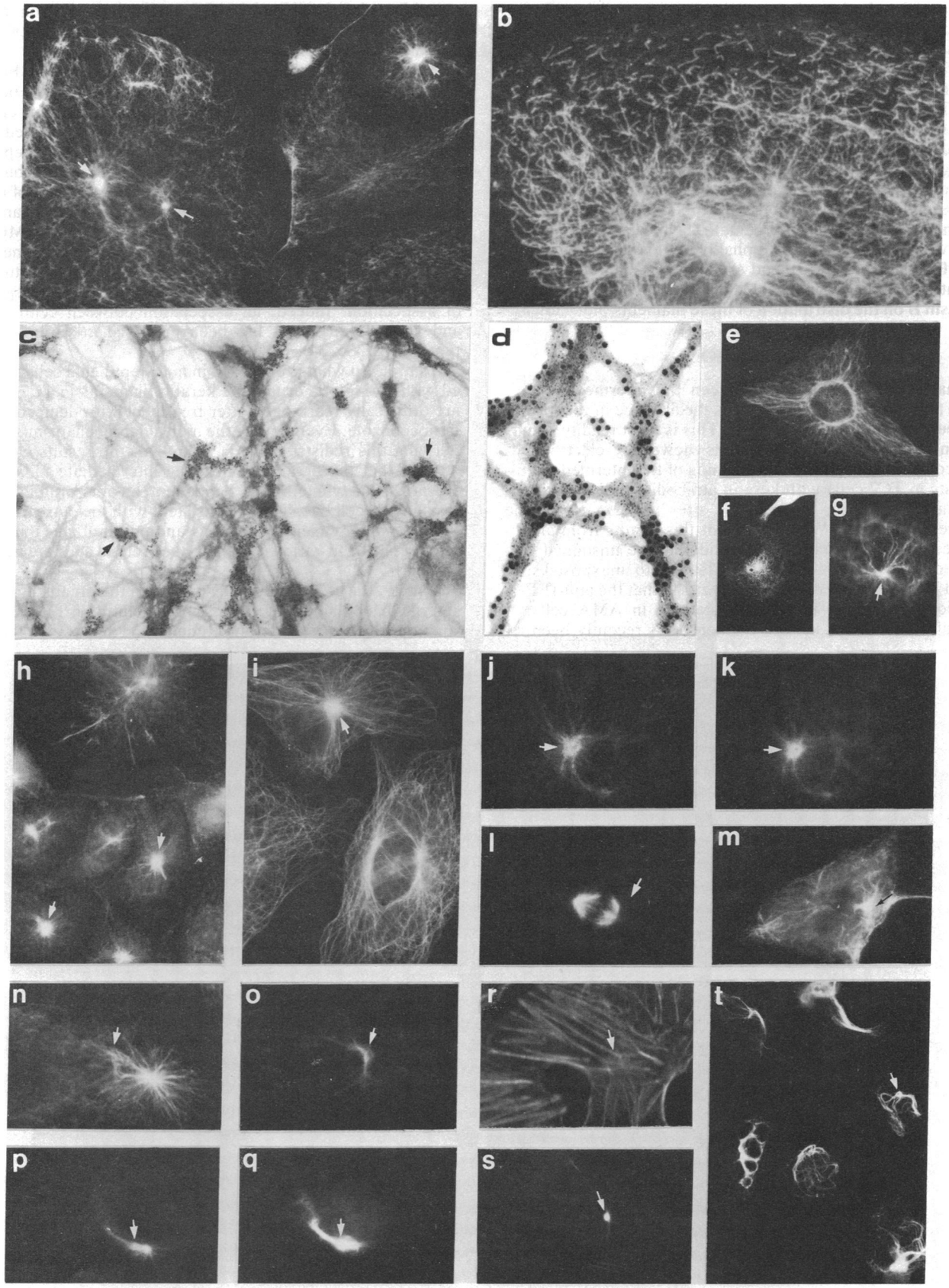

FIG. 2. (*Legend appears at the bottom of the preceding page.*)

20 min of incubation; the experiments reported here have been carried out after 60 min of treatment. In parallel experiments, no significant effect of cytochalasin B was observed with PTK2 cells (Fig. 3*f*; keratin staining). Double-label immunofluorescence of cytochalasin B-treated TC7 cells (acetone fixation) with the keratin IEF 31 antibody (Fig. 3*b*) and rhodamine-labeled phalloidin (Fig. 3*c*) showed that many of the keratin star-like aggregates codistributed with the actin aggregates (arrows in Fig. 3*b* and *c*). In many cells we observed a diffuse fibrillar region surrounding the focal centers that reacted with both the keratin antibody (Fig. 3*d*) and rhodamine-labeled phalloidin (Fig. 3*e*). The focal centers did not stain with rhodamine-labeled phalloidin, even after cytochalasin B treatment (Fig. 3*d* and *e*). Similar studies using tubulin antibodies have not revealed any dramatic effect of cytochalasin B on the distribution of these filaments (not shown).

DISCUSSION

A characteristic difference between the intermediate filament and microtubule networks is the absence in the former of freely exposed filaments ends. This is most readily apparent in whole-mount cytoskeletons viewed by electron microscopy. The discontinuous staining of the intermediate filaments in TC7 cells with keratin antibodies indicates a segmental distribution of antigenic sites along parts of the filament networks. Whether or not this results from an absence of certain keratin polypeptides in the unstained sections or a structural conformation leading to unexposed sites is not known. However, it is noteworthy that the anti-IEF 46 antibody showed a continuous staining in AMA cells. A granular staining of keratin filaments has recently been reported in a clonal cell line (BMGE + HM) selected from bovine mammary gland epithelial cell cultures that lacks vimentin and desmin filaments (34).

The presence of perinuclear bodies that may contain both keratins and vimentin is of interest as these cytoplasmic bodies may correspond to intermediate filament organizing centers (25, 27, 28). These centers have not been observed in many other cultured cell lines and therefore may not represent typical organizing centers as in the case of microtubules (31). In interphase, they codistribute with focal arrays of microtubules (most likely, centrosomes) but were separate from the centrioles in mitosis and (in many cases) the MOC induced after brief recovery from nocodazole treatment. These results suggest that these are independent structures (see also refs. 26 and 27), although there may be some degree of association. Indeed, an association between centrioles and vimentin filaments has been proposed in various cell types (6, 25, 35–37).

Contrary to what is observed in many epithelial cells treated with antimitotic drugs, the keratin filaments in TC7 cells are drastically rearranged after treatment with demecolcine or nocodazole. Even though the molecular mechanism(s) underlying this redistribution is unknown, the results clearly suggest that the organization of the intermediate filaments (keratins and vimentin) is dependent on microtubules, at least in this cell line. This is consistent with the close association between vimentin filaments and microtubules that has been reported in various cell lines (see ref. 38 and references therein).

Finally, the effect of cytochalasin B on the organization of intermediate filaments (keratins and vimentin) in TC7 cells is an important observation. Recently, Knapp *et al.* (33)

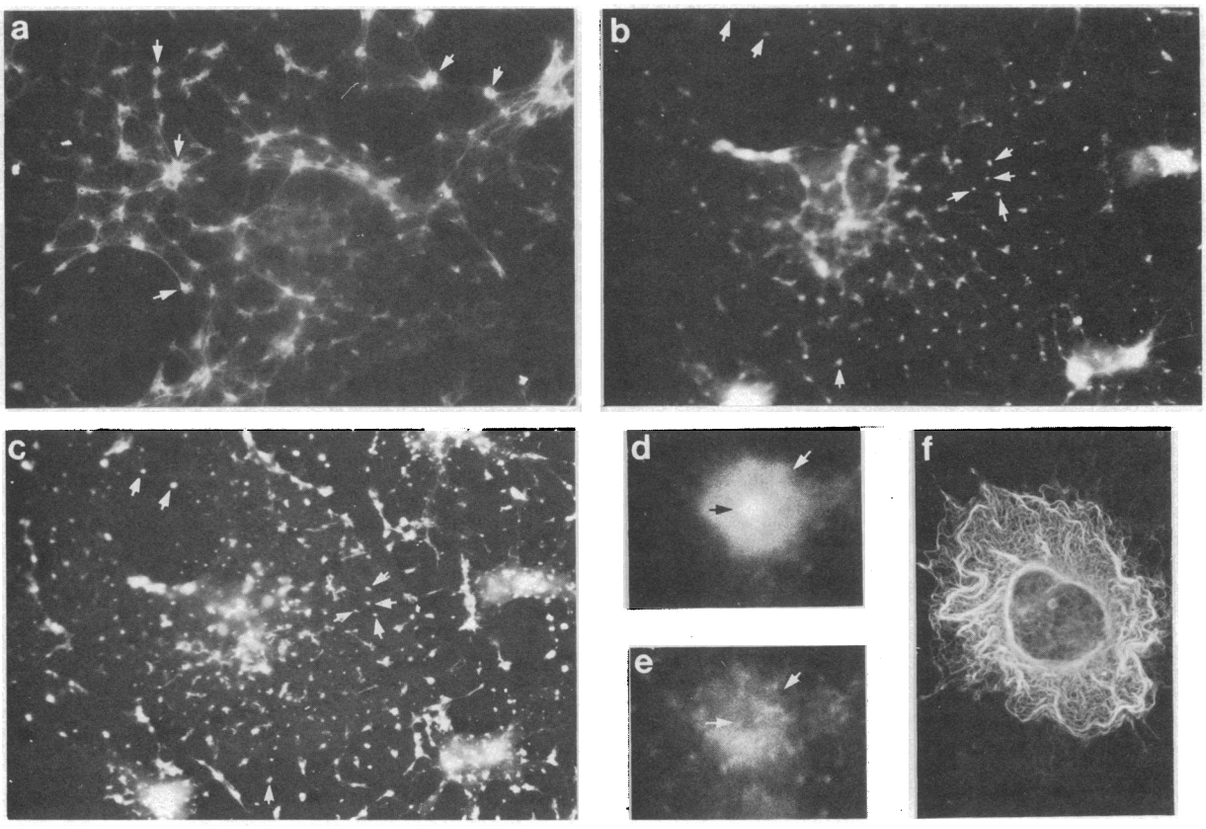

FIG. 3. Fluorescence microscopy of cytochalasin B-treated TC7 cells. Cells were treated for 60 min at 37°C with 10 μg of cytochalasin B per ml prior to fixation. (*a*) Methanol/acetone-treated TC7 cells reacted with a broad-specificity keratin antibody; (*b–e*) double-immunofluorescence of acetone-fixed TC7 cells reacted with keratin IEF 31 antibody (*b* and *d*) followed by reaction with rhodamine-labeled phalloidin (*c* and *e*). Arrows indicate equivalent points in the micrographs. (*f*) Methanol/acetone-treatment PTK2 cells reacted with the broad-specificity keratin antibody. (*a–c* and *f*, ×380; *d* and *e*, ×3,000.)

showed that treatment of KLN 205 carcinoma cells with colchicine together with cytochalasin D converted the keratin cytoskeleton into a series of star-like structures, which they suggested are maintained by multiple membrane attachment sites. In our studies (using only cytochalasin B), we have further shown that many of the star-like structures codistribute with actin patches, a fact that would suggest that intermediate filaments may interact with microfilaments at least in certain regions of the cell.

In summary, our studies point towards a close relationship (structural or functional or both) between the various cytoskeletal systems in TC7 cells and add support to the notion that cytoskeletal interactions may be different in various cell types.

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