

## Assembly of Keratin onto PtK<sub>1</sub> Cytoskeletons: Evidence for an Intermediate Filament Organizing Center

BARRY S. ECKERT, ROGER A. DALEY, and LINDA M. PARYSEK

*Department of Anatomical Sciences, School of Medicine, State University of New York at Buffalo, Buffalo, New York 14214*

**ABSTRACT** Purified keratin, solubilized in 8 M of urea, was added to Triton X-100-extracted PtK<sub>1</sub> cells in 5 mM PIPES buffer. The buffer conditions induced assembly of keratin filaments which appear to associate with nuclei of extracted cells. These keratin fibers extend beyond the original margin of the cells and frequently form bridges between adjacent cells. Electron microscopy shows that keratin filaments associate closely with the surface of the nucleus. We suggest that the site of association between keratin and the nucleus may represent an intermediate filament organizing center.

The cytoskeleton is made up of a complex network of microtubules, intermediate filaments, and microfilaments. In the case of microtubules, a focal point from which the cytoplasmic array is assembled and/or organized has been described and named the "microtubule organizing center" (MTOC) by Pickett-Heaps (9). This center has been shown to contain centrioles in animal cells (2) but appears quite amorphous in plant cells (9) and slime molds (10). The role of the MTOC in nucleating the assembly of cytoplasmic microtubules has been established by immunofluorescent labeling of microtubules in cells recovering from cold treatments (3). In these experiments, microtubules appear to grow from a single point near the nucleus. A close correlation between the MTOC and the spindle pole, suggested by the presence of centrioles in both structures, has also been established. Such an organizing center specific for growth and/or organization of other cytoskeletal components has not been established.

Recent work in our laboratory (4, 5) has shown that disruption of the keratin network of living epithelial cells by microinjection of antibody specific for keratin results in formation of a juxtannuclear filament cap. This observation led to the suggestion that this cap may indicate the presence of an organizing center (6). Here we present further evidence for the presence of an organizing center for intermediate filaments.

### MATERIALS AND METHODS

#### *Cell Culture*

Rat kangaroo kidney epithelial cells (PtK<sub>1</sub>) were grown in Ham's F-12 media supplemented with 10% fetal calf serum. For experiments, cells were grown on 18-mm square cover slips. When cells were to be embedded for thin-section electron microscopy, cover slips were first coated with MS-122 fluorocarbon dry release agent (Miller-Stevenson Chemical Co., Danbury, Conn.) to facilitate separation of the glass from the embedding media. For whole-mount electron microscopy, cells were grown on Formvar- and carbon-coated gold grids.

#### *Keratin Preparation*

Keratin was isolated from calf hoof as described by Steinert et al. (11), and was stored lyophilized. Keratin was dissolved in 8 M of urea, 10 mM Tris, pH 9.0, and stored at 4°C in solution for up to a week before use. The solution was clarified at 10,000 g for 20 min and the protein concentration determined by absorbance at 280 nm.

#### *Antibody Preparation*

Details of preparation and characterization of rabbit anti-keratin have been described elsewhere (6). Antibody specific for centrioles was a generous gift from Sari Brenner (1).

#### *Keratin Assembly Experiments*

PtK<sub>1</sub> cells were rinsed with phosphate-buffered saline (PBS) and extracted for 3 min with 0.2% Triton X-100, 100 mM PIPES (pH 6.94), 1 mM EGTA, 10 mM *n*-tosyl-L-arginine methyl ester (TAME). Cytoskeletons were then placed in 1 ml of 5 mM PIPES (pH 6.94), an aliquot of keratin in 8 M of urea was added, and the preparations were incubated for 30 min at 37°C. The concentration of keratin in 8 M of urea was kept high so that not >100 µl of this solution was added. A threshold concentration was determined by adding various dilutions of keratin. In most experiments, keratin concentration was adjusted so that the final concentration in the preparation was 0.3–0.4 mg/ml. Depression of the pH and dilution of the urea by buffer resulted in assembly of keratin. Some preparations were pretreated with a 1:20 dilution of anti-keratin for 30 min at 37°C and rinsed with PIPES. The keratin was then added as described above. Keratin was also added as assembled filaments. Keratin solution was dialysed overnight against 5 mM PIPES, pH 6.94. The resulting filament suspension was substituted for the keratin solution in the above procedure.

#### *Immunofluorescence*

Cover slips were fixed for 5 min in acetone, rehydrated in PBS, and treated with primary antibody for 45 min at 37°C. Rhodamine-conjugated goat anti-rabbit IgG (N. L. Cappel Laboratories, Inc., Cochranville, Pa.) or fluorescein-conjugated goat anti-human IgG (Miles Laboratories, Inc., Research Products Div., Elkhart, Ind.) was added to rinsed cover slips which were incubated as before. Cover slips were mounted in Elvanol (DuPont Instruments, S & P Div., Wilmington, Del.) and observed on a Zeiss Ultraphot IIIB equipped with epifluorescence optics. Micrographs were taken on Kodak Plus-X film and developed in D19.

## Electron Microscopy

For thin sectioning, cover slips were fixed for 30 min in 2% glutaraldehyde in 100 mM sodium cacodylate (pH 7.2), rinsed, and postfixed for 30 min in 1% osmium tetroxide, dehydrated in ethanol, and embedded in Epon/Araldite. Sections were stained with uranyl acetate and lead citrate and examined on a JEOL 100-B electron microscope. Whole-mounts were fixed with glutaraldehyde as above, dehydrated, and dried by the critical point method. Samples were shadowed with carbon. Whole-mounts were examined at 1,000 kV on the JEOL 1000 high voltage electron microscope at the University of Colorado.

## RESULTS

Anti-keratin immunofluorescence of extracted cells after keratin reassembly reveals a straight fiber with one end associated with the nucleus and the other end extending beyond the original cell margin (Fig. 1). In all cases, the point of association of keratin with the nucleus was near its upper surface. For this reason, the cytoskeletal network of the cell is out of the plane of focus and does not appear in Figs. 1, 2, and 3. Appearance of this fiber requires a threshold keratin concentration of 0.2 mg/ml. Treatment of Triton X-100-extracted cells with anti-keratin before addition of exogenous keratin tended to reduce the number of cytoskeletal fibers around the nucleus. This treatment had no apparent effect upon assembly of exogenous keratin onto cytoskeletons. Observation of several cells that were close together (cultures were ~80% confluent) revealed a close correlation between position of the assembled keratin fiber and location of neighboring cells (Fig. 2). The keratin fiber extended in a straight line from one nucleus to another. Under these conditions, nuclei often showed two or three sites of keratin fiber association and virtually all cells showed at least one connection with a neighbor. Cells did not show large numbers of sites, even when higher keratin concentrations were used. Mitotic cells were apparently dislodged by the extraction process and were not observed. If preassembled keratin filaments were added, fibers were still observed between all neighboring nuclei but these were usually not straight (Fig. 3). Control preparations, in which 8 M of urea solution lacking keratin was used, show normal keratin networks and no assembled keratin fiber.

Electron microscopy of whole-mount preparations showed the straight fiber observed in the light microscope (Fig. 4). This preparation was not treated with antibody before or after keratin assembly. These whole-mount preparations revealed little detail of the association between the exogenous keratin

fiber and the nucleus although the normal appearance of the cytoskeleton can be seen. It is clear, however, that the straight keratin fiber tends to associate with the nucleus and passes by cytoskeletal fibers that are nearby. To better visualize this region of association, ultrathin sections were prepared. In these sections, the straight keratin fiber is clearly visible and can be followed to the nucleus (Fig. 5). No unusual morphology is seen where the keratin fiber lies close to the nucleus. However, there appears to be an association between the keratin bundle and nuclear material (Fig. 5, single arrows). Some sections, such as that in Fig. 5, indicate that association between the straight keratin fiber and the nucleus may occur at more than one site (shown by double arrows).

Specimens were prepared for immunofluorescence using anti-centriole antibody to determine whether the centriole was related to keratin assembly. As is shown in Fig. 6, the centriole is not found near the site of apparent keratin fiber association with the nucleus. Observation of numerous preparations demonstrates that the position of the centriole appears to be independent of this site. This observation is supported by electron microscope examination of numerous sectioned preparations which revealed that the keratin fibers were always associated with the nucleus and not with the centriole.

## DISCUSSION

The work presented here suggests that exogenous keratin, added under conditions that induce filament assembly, displays a tendency to associate with a site on or near the nucleus. Although the PtK<sub>1</sub> cytoskeletons appear to contain the normal network of fibers, the assembled keratin does not associate with these elements. Filaments that assemble form a bundle which follows a relatively straight course beyond the original cell boundaries. Since this occurs with or without antibody pretreatment, formation of the bundle is not the result of antibody cross-linking. The observation that relatively few of these keratin bundles form on any single cytoskeleton indicates that there is a specific association with one or two structures. Nonspecific attachment of keratin to the Triton X-100-extracted nucleus would be expected to yield multiple fibers radiating outward, resulting in an aster appearance.

It is particularly interesting that, when two or more cells are close together, the keratin fiber assembles in a straight line between them. It is unclear whether this fiber forms by unidirectional growth from one nucleus to the other or by growth of

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FIGURE 1 An immunofluorescence micrograph of a PtK<sub>1</sub> cytoskeleton, prepared after addition of exogenous keratin. A long fiber, which labels with anti-keratin, is seen with one end at the nucleus (N). The periphery of the cell, as it existed *in vivo*, is indicated by arrows.  $\times 800$ .

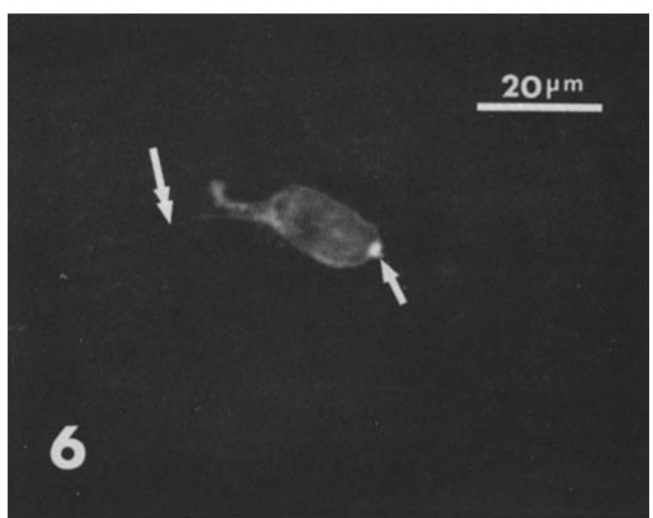
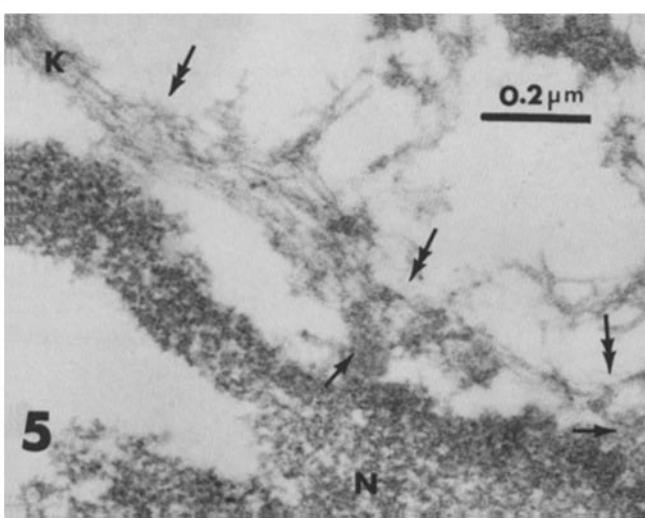
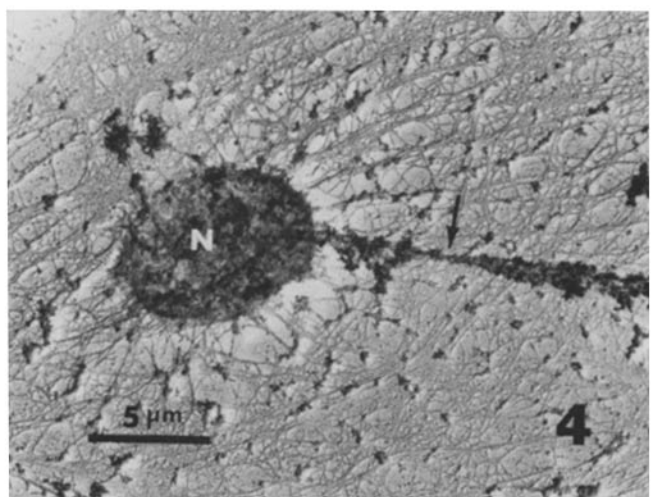
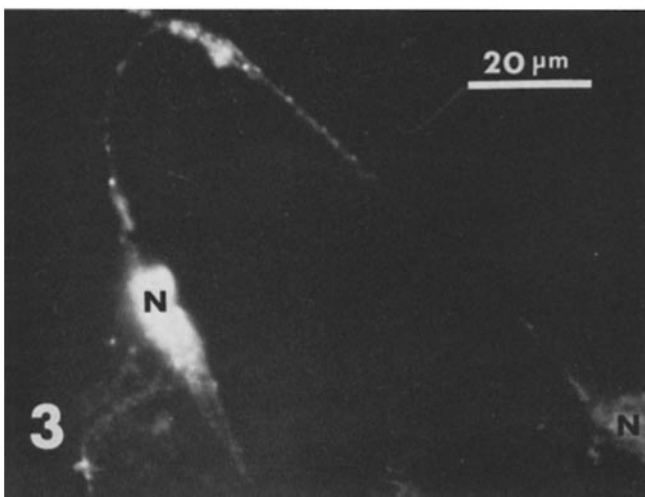
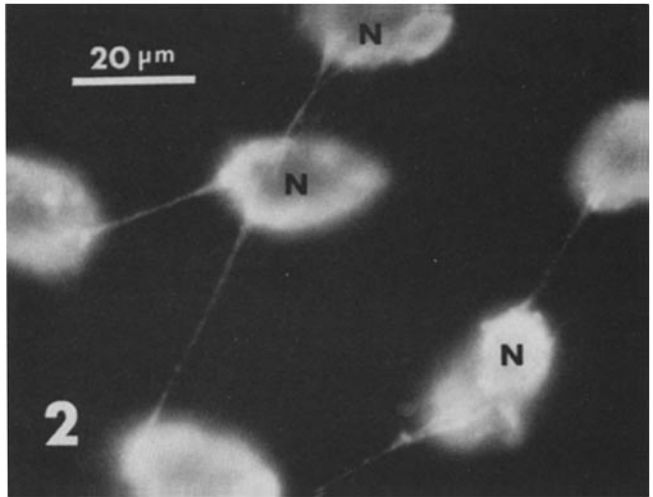
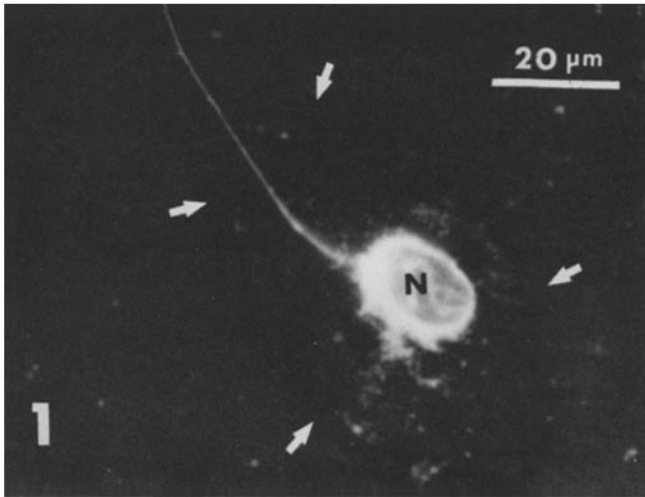
FIGURE 2 A preparation similar to that in Fig. 1 showing a region where numerous cells are in contact. Keratin has assembled as straight fibers connecting nuclei (N).  $\times 800$ .

FIGURE 3 An immunofluorescence micrograph of a preparation similar to those in Figs. 1 and 2, except that preformed keratin filaments were added. The connection between nuclei (N) does not follow a straight course.  $\times 800$ .

FIGURE 4 A high-voltage electron micrograph of a whole PtK<sub>1</sub> cytoskeleton to which exogenous keratin was added. The straight keratin fiber (arrow) is visible, with one end close to the nucleus (N) and extending through the cell.  $\times 2,400$ .

FIGURE 5 An ultrathin section of a PtK<sub>1</sub> cytoskeleton to which exogenous keratin was added. The straight keratin bundle (K) appears to associate closely with the nucleus (N) at three points (double arrows). Single arrows indicate apparent association between keratin filaments and nuclear material.  $\times 75,000$ .

FIGURE 6 An immunofluorescence micrograph similar to Figs. 1 and 2, except that antibody which stains centrioles was used. The assembled keratin fiber is faintly visible (double arrow) due to nonspecific fluorescence. The position of the brightly staining centriole is indicated by the single arrow.  $\times 800$ .



bundles from each nucleus which meet end-to-end. Addition of preformed keratin filaments to extracted cells results in formation of keratin fibers of varying length which run between nuclei. In this case, fibers frequently do not follow a straight path. This suggests that filaments which are longer than necessary have been randomly fit between neighboring nuclei. Thus, it would seem that, under conditions favoring assembly, formation of the fiber between nuclei is controlled in some way such that fibers of correct length and direction are produced.

The observations discussed above indicate that assembly of exogenous keratin may be influenced by its association with a structure near the nucleus. For this reason, the site of association may be more than a simple attachment point; it may represent an intermediate filament organizing center (IFOC). A relation of this structure to cellular organization is further suggested by the close correlation between the position of neighboring cells and the location of assembled keratin. Since keratin filaments (tonofilaments) have been shown to insert into desmosomes (7), the position of the IFOC may be influenced by these structures. Thus, the IFOC-tonofilament-desmosome complex may represent a channel to convey information about cellular contacts.

The composition of the IFOC is still unclear although it apparently is neither extracted by Triton X-100 nor affected by anti-keratin treatment. Our study suggests that the centriole is an organelle independent of the IFOC. Although the possibility exists that some filaments may run between the IFOC and the centriole, an association between these structures was not indicated by the electron microscope data. Observations of negatively stained or thin-sectioned IFOC preparations have revealed little in the way of distinctive morphology.

It has recently been shown by Goldman et al. (8, and personal communication) that keratin filaments of transformed mouse epidermal, or PAM, cells closely approach nuclear pores. These authors have suggested that nuclear pores may serve as anchoring points for these filaments on the entire nuclear envelope. Our data fit this suggestion, particularly since our thin sections of the IFOC region often suggest numerous association points with the nucleus. If keratin filaments anchor

to nuclear pores, the IFOC may be composed of one or more nuclear pores as well as additional components. These additional elements may provide organization to the numerous filaments anchored at the pores. Further work is underway to examine both location and components of the IFOC.

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