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REORGANIZATION OF ARRAYS OF PREKERATIN FILAMENTS DURING MITOSIS

Immunofluorescence Microscopy with Multiclonal and Monoclonal Prekeratin Antibodies

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SUMMARY

Indirect immunofluorescent labelling of different epithelial cell lines for intermediate filaments of the prekeratin type revealed prominent changes in the organization of prekeratin during mitosis. In three out of four cell lines tested (Henle-407, A-431 and HeLa cells) the filamentous prekeratin networks disappeared at the initiation of mitosis and the immunofluorescent labelling was concentrated in small cytoplasmic bodies. This observation was obtained with both polyspecific rabbit anti-bovine prekeratin antibodies and with monospecific antibodies produced by mouse hybridomas. In a fourth cell line, PtK_2, prekeratin filaments were retained throughout mitosis, mainly in the mitotic poles, whereas the central areas of the cells were apparently devoid of filaments. The addition of colchicine to the different cultured cells induced alterations in the organization of prekeratin filaments which were usually manifested by the formation of thicker filament bundles. It did not induce the formation of the prekeratin–cytoplasmic bodies in interphase cells. However, upon prolonged incubation in the presence of colchicine, there was an increase in the number of mitotically arrested cells and a parallel increase in the number of cells containing prekeratin cytoplasmic bodies. It is thus proposed that the state of organization of prekeratin in these cells is cell-cycle-dependent and may be modulated to permit radical shape changes as those occurring during mitosis.

It is now generally accepted that many of the dynamic processes in eukaryotic cells are mediated and controlled by the coordinated action of several types of cytoplasmic fibrillar systems including microfilaments, intermediate filaments and microtubules [1–5]. A wealth of information has accumulated in recent years on the involvement of these filament networks, especially microfilaments and microtubules, in a variety of cellular activities, such as cell locomotion, membrane dynamics, endocytosis and cell division. The detailed molecular basis for the involvement of cytoskeletal systems in these processes is, however, still largely unclear.

The least characterized of the three major classes of cytoskeletal filaments mentioned above are the intermediate (10 nm) filaments (for review see [4]). These filaments are abundant constituents of most eukaryotic cells, although their component proteins greatly differ from one cell type to the other. Thus, it was possible to define at least five major types of intermediate filaments including muscle type intermediate filaments which consist of desmin or skeletin [6, 7], vimentin filaments of mesenchymal origin [8], neurofilaments from nerve cells [9, 10], glial filaments [9, 11] and prekeratin filaments of epithelial cells [12–14]. One of the common features of most inter-

mediate filaments is their low solubility in aqueous buffers. They are resistant to high salt or detergent extractions and dissociate only at very low pH or in the presence of concentrated urea solutions [6, 7, 15]. These physical and chemical stabilities contribute, most probably, to the 'skeletal' properties of intermediate filaments. It is not clear, however, how the rigid cytoskeletal framework is modified during processes that involve radical shape changes such as locomotion, spreading and, especially, mitosis.

It was thus of interest to study the fate of the various types of intermediate filaments during mitosis. A report by Blose [16] indicated that vimentin-type intermediate filaments are retained during mitosis in vascular endothelial cells. In these cells vimentin filaments are organized in a tightly packed perinuclear ring which apparently divides and migrates into the two daughter cells. Desmin filaments in chicken gizzard cells, on the other hand, dissociate transiently during mitosis and reassemble after the two daughter cells separate and respread [17]. In a recent study [18] it has been found that in PtK_2 cells (epithelioid line) both prekeratin and vimentin filaments are largely retained throughout mitosis, though the two systems exhibit an independent distribution in the cells. It could not be evaluated from this study whether partial depolymerization of the filaments occurred during the mitotic process [18].

In the present study we have investigated the fate of intermediate filaments of the prekeratin type in four different epithelial cell lines during mitosis. The distribution of prekeratin filaments was visualized by immunofluorescence microscopy using both multispecific rabbit antibodies against purified bovine hoof prekeratin and monoclonal mouse antibodies. In three of these cell lines (HeLa, epithelial carcinoma A-431 and human intestinal epithelial cell line, Henle-407) we found that during mitosis the cells become rounded up, the well organized prekeratin filaments which are typical for the cell during interphase disappear and the label becomes concentrated in discrete cytoplasmic bodies. In a fourth cell line (PtK_2) the dissociation of the prekeratin network was at most partial, in agreement with Aubin et al. [18].

Shortly after mitosis was completed a filamentous prekeratin network reformed in the two daughter cells. We propose that the state of prekeratin filaments in the various cell types studied is under the control of a cell cycle-dependent regulatory system that can induce rapid disorganization (partial or complete) and reassembly of prekeratin filaments in vivo.

MATERIALS AND METHODS

Cells

All cell lines were maintained in culture in Dulbecco's Modified Eagle medium (DMEM, Gibco) containing 10% fetal calf serum (FCS), at 37°C in a humid atmosphere of 95% air and 5% CO_2. HeLa cells were obtained from Dr R. Lotan, epidermal carcinoma cells A 431 were originally developed by Dr G. Todaro. Intestinal epithelial cells Henle-407 (H-407) were from the American Tissue Culture Collection (CCl_6). PtK_2 cells were kindly supplied by Drs E. Schmid and W. Franke. Myeloma P3-NS1/1-Ag4-1 (NS1) were kindly provided by Dr C. Milstein.

Antigens

Bovine prekeratin was extracted from the hooves of fetal calf following published procedures [14]. The material extracted with urea was centrifuged for 2.5 h at 200000 g and then subjected to two cycles of polymerization–depolymerization as follows: The extract containing (~5 mg/ml protein in 8 M urea) was dialysed against 2 mM Tris-HCl buffer, 10 mM β-mercaptoethanol, 10^{-4} M PMSF, pH 7.6, and then subjected to one cycle of freezing and thawing. The aggregate formed was collected by centrifugation (16000 g, 15 min) and redissolved in 8 M urea+25 mM β-mercaptoethanol, pH 9.0. This procedure was repeated twice and the final product consisted of pure prekeratin showing a 6-band pattern on SDS–polyacrylamide gels with mol. wt in the range of 43000–

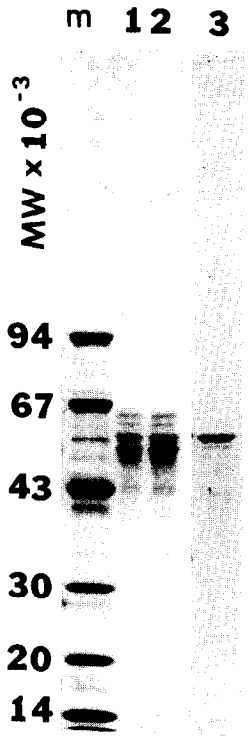

Fig. 1. SDS polyacrylamide gel electrophoresis of bovine-hoof reconstituted prekeratin. *m*, Markers: Phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and α-lactalbumin (from Pharmacia, Sweden). *1, 2*, Reconstituted bovine hoof prekeratin, 5 μg and 20 μg, respectively; *3*, pure vimentin from human foreskin fibroblasts.

67000 (fig. 1). The differences between the relative amounts of the various prekeratin components described here and those described earlier [14] may stem from the fact that the bovine hoof used here was of fetal origin.

Immunochemical procedures

Antibodies to the reconstituted prekeratin preparation were raised in rabbits. The antigen (500 μg/rabbit), emulsified in complete Freund's adjuvant (CFA) was injected intradermally and booster injections were given 3 and 5 weeks later. Antibody titer was evaluated by solid phase radioimmunoassay [19] using urea-solubilized antigen and by immunofluorescent labeling of various types of cells, as will be described.

For the isolation of pure antibodies by affinity chromatography prekeratin was coupled to glutaraldehyde-activated ultrogel, AcA_{22} [20]. Prekeratin in 8 M urea was dialysed against 1% SDS in 0.1 M Na phosphate buffer, pH 7.0, diluted 1 : 5 with the phosphate buffer and coupled to the activated gel (500 μg protein/ml of settled gel).

Monoclonal antibodies to prekeratin were prepared essentially as delineated in [21, 22]. In order to obtain hybridomas producing anti-prekeratin monoclonal antibodies, BALB/c mice were injected subcutaneously and intradermally with 25 μg of bovine prekeratin (dialysed against 2 mM Tris and emulsified in CFA) followed by a booster injection 2 weeks later. Mice that developed anti-prekeratin antibodies were injected intraperitoneally with 600 μg of the antigen and 3 days later their spleen cells were fused with the NS1 cells at 5 : 1 ratio using 41% polyethylene glycol 1500 as fusing agent [21, 22]. Hybrid cells growing in HAT selective medium were further selected according to their ability to secrete specific anti-prekeratin antibodies as tested by the microplate radio immunoassay [19]. Several independent hybrid cultures producing anti-prekeratin antibodies were cloned and recloned in agar and the isolated clones were grown in ascites form in (BALB/c×DBA/2)F1 mice. The specificity of the monoclonal anti-prekeratin antibodies was verified by radioimmunoassay, immunofluorescent labelling of cells and labelling of gel-purified individual prekeratin components. The IgG fraction of the ascites fluids prepared by ammonium sulphate precipitation was used here.

Goat-antibodies to rabbit IgG were coupled to rhodamine-lissamine sulphonylchloride as described [23]. Fluorescein isothiocyanate (FITC)-labelled goat IgG against mouse IgG were purchased from Miles-Yeda (Israel) and further purified on a mouse IgG immunoadsorbent. The fraction containing an average of three fluorophores per IgG molecule was isolated by DEAE cellulose chromatography [23]. Immunofluorescent labelling was carried out on cells fixed and permeabilized with methanol and acetone at −20°C. Fluorescent microscopy was carried out with a Zeiss photoscope III or IM35 equipped with filter sets for selective observation of fluorescein and rhodamine.

RESULTS

The distribution of prekeratin in epithelial cells during interphase

Sparsely plated monolayer cultures of four different epithelial cell lines were fixed with cold methanol–acetone and immunolabelled indirectly for prekeratin. This was done using affinity purified antibodies to bovine hoof reconstituted prekeratin filaments and rhodamine-labelled goat anti-rabbit IgG, or, alternatively, with the purified IgG of monoclonal antibodies to prekeratin, followed by fluorescent goat anti-mouse IgG. The labelling patterns observed in interphase cells (fig. 2) consisted of an elaborate

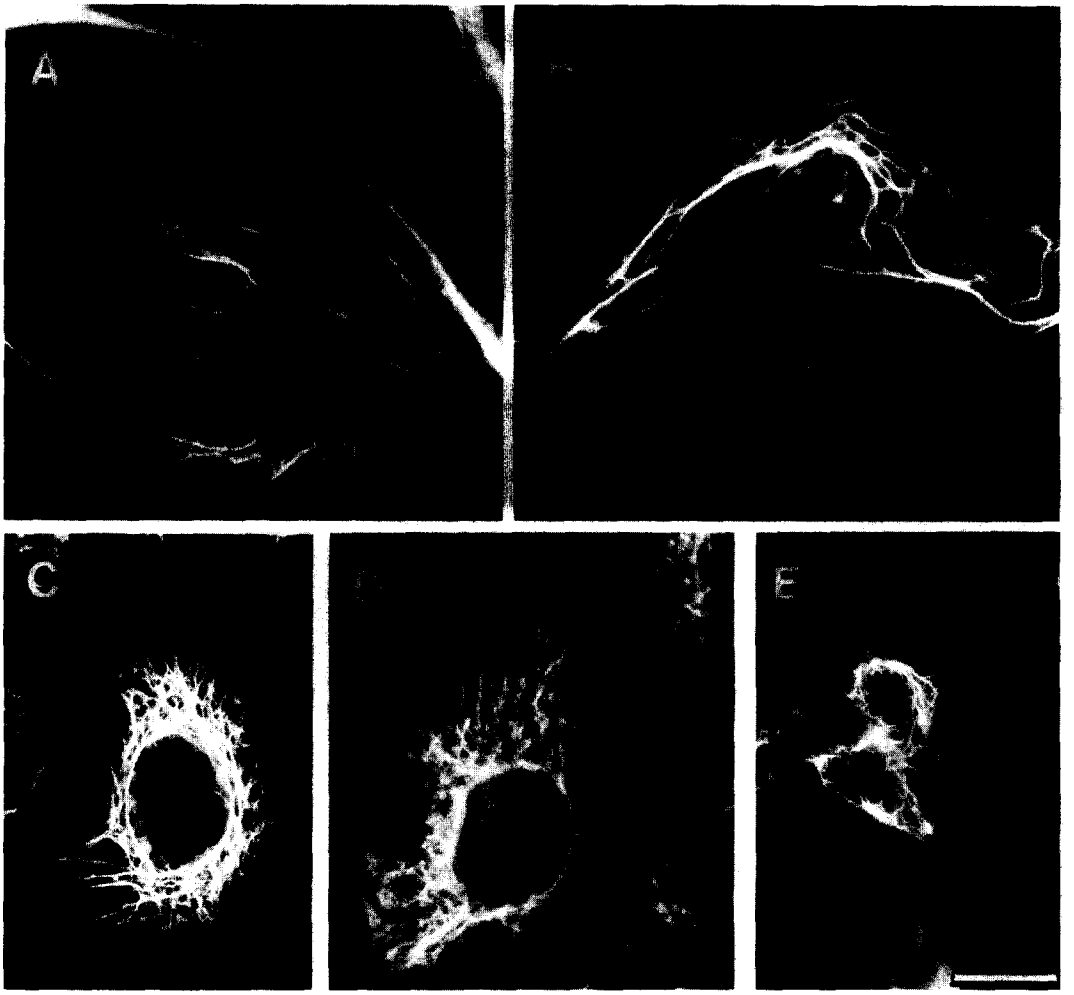

Fig. 2. Immunofluorescent labelling of prekeratin filaments in cells during interphase. (*A*) Epidermal carcinoma A-431 cells labelled with rabbit anti-prekeratin and rhodamine-labelled goat anti-rabbit IgG. (*B*) PtK_2 cells labelled as in (*A*). (*C*) Intestine epithelium–Henle 407 (H-407) line, labelled with rabbit antibodies as in (*A*). (*D*) H-407 cells labelled with mouse monoclonal anti-prekeratin antibodies and fluorescein-labelled goat anti-mouse IgG. Note the similarity of the labelling patterns obtained with the two antibody reagents in (*C*) and (*D*). (*E*) HeLa cells immunolabelled for prekeratin as in (*A*). Bar, 10 μm.

wavy network of filaments that were most abundant in the perinuclear area and extended towards the cell periphery. The different cell types tested exhibited a wide range of spreading potentials, A-431 being the most extensively spread out, and HeLa cells, the least spread.

There were, however, notable differences in the organization of prekeratin filaments in the different cells, HeLa cells (fig. 2*E*) and human intestinal epithelial line H-407 (fig. 2*C*) contained fine networks of prekeratin filaments throughout the cytoplasm. Epidermal carcinoma A-431 cells exhibited both fine filament network as well as numerous thick bundles (fig. 2*A*). In

PtK_2 cells extensive labelling was associated with thick bundles of prekeratin filaments (fig. 2*B*) as previously described [13].

The results shown above were obtained by immunolabelling with affinity purified multispecific rabbit antibodies. When monospecific hybridoma antibodies to bovine prekeratin were used instead of these antibodies essentially identical results were obtained. The cell shown in fig. 2*D* (human intestinal epithelium H-407) was immunolabelled with the monoclonal anti-prekeratin antibodies. The labelling pattern is very similar to that obtained for the same cell type using the rabbit multispecific antibodies (fig. 2*G*). A detailed characterization of the specificity of these and several other monoclonal lines will be given elsewhere.

Redistribution of prekeratin filaments during mitosis

Screening of the monolayer cultures of the four types of cells revealed a significant number of mitotic cells. The mitotic cells were usually rounded up (except for PtK_2 which retained a well spread morphology) and the nuclear membrane was no longer visible. The chromosomes, at various stages of condensation and segregation, could be detected in the cells (see phase photomicrographs in fig. 3). The proportions of mitotic cells varied from one cell type to the other. In cultures of H-407, HeLa and PtK_2, 2–5% of the cells in the monolayer were mitotic, whereas in A-431 the values observed were lower (ca 1%), possibly because the mitotic cells detached easily during manipulation of the culture.

When immunolabelled with purified rabbit antibodies to bovine hoof reconstituted prekeratin and rhodamine-goat anti-rabbit IgG, significant alterations were detected in the organization of prekeratin. The least affected were PtK_2 cells (fig. 3*A–D*) in which dense filament bundles were retained throughout mitosis, though these filaments accumulated mainly near the mitotic poles of the cells and were largely missing from the cell center. The examples shown here are of late anaphase (fig. 3*A, B*) and metaphase (fig. 3*C, D*). These observations are in good agreement with the recent study of Aubin et al. [18]. A much more dramatic change in prekeratin distribution was found with the other three cell lines. Mitotic HeLa cells, immunolabeled for prekeratin, exhibit almost no filamentous labelling. The fluorescent staining (fig. 3*F, H*) was partly diffused throughout the cytoplasm and was mainly associated with numerous cytoplasmic bodies. This pattern was prominent throughout the various stages of mitosis, from prophase to late anaphase or telophase, as shown in fig. 3*H, F*.

The same type of pattern was observed also in H-407 (fig. 3*I–L*) and A-431 cells (fig. 3*O–P*). Often the prekeratin-labelled cytoplasmic bodies were concentrated during late anaphase and telophase between the segregating chromosomes in the center of the dividing cell, as shown in fig. 3*N*.

As mentioned above, the immunogen used throughout these studies consisted of several polypeptides that participate in the reformation of prekeratin filaments. It was thus of great importance to us to determine whether the same prekeratin components constitute both the cytoplasmic filaments and the cytoplasmic bodies during mitosis. The labelling results obtained with the monoclonal antibodies (fig. 3*I, J*) indicated that the same antigenic determinant is indeed localized both on the filament networks (fig. 2*D*) of H-407 cells and on the mitotic cytoplasmic bodies.

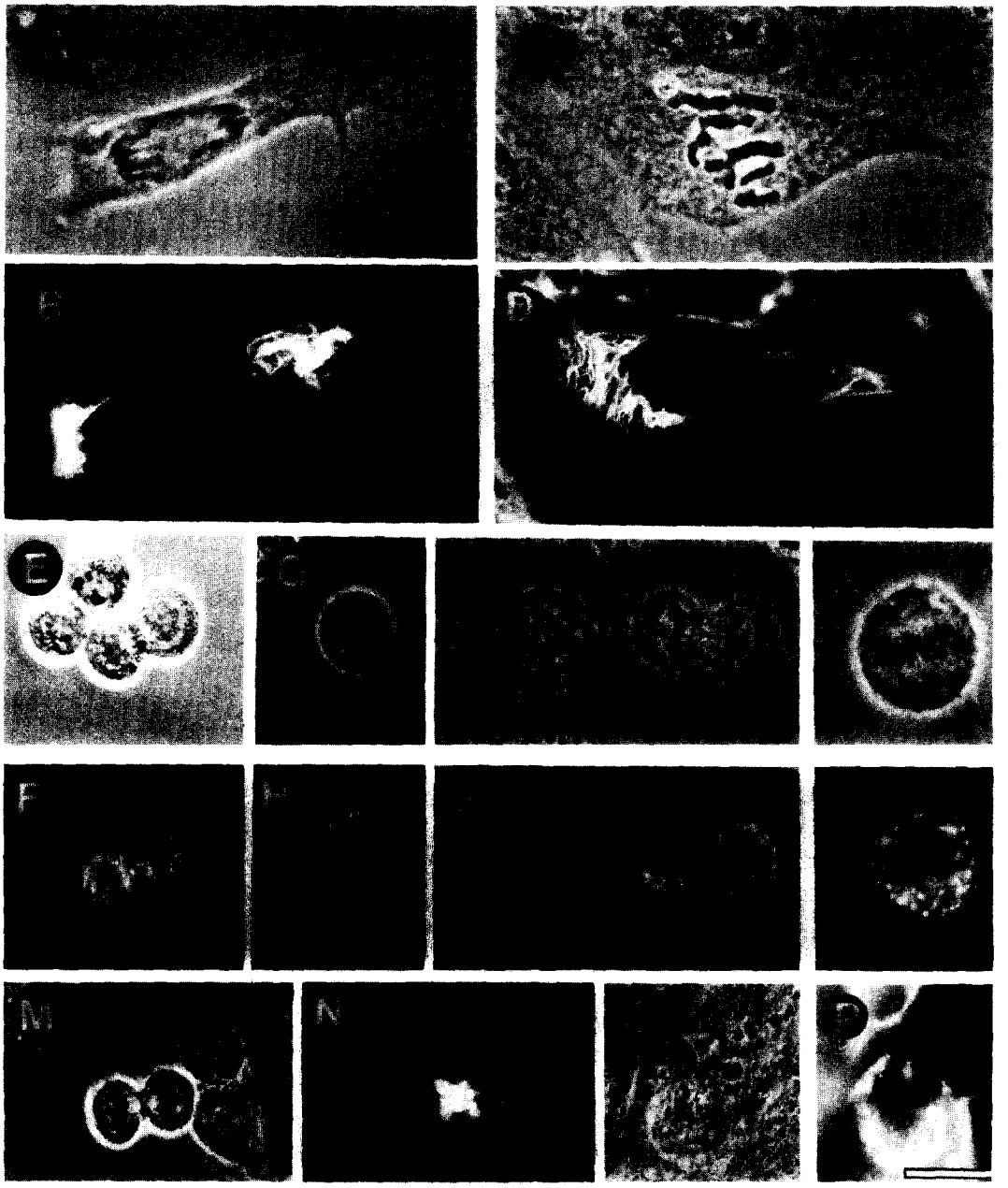

Fig. 3. The distribution of prekeratin in mitotic cells. (*A*) Phase photomicrograph of a PtK_2 cell fixed during anaphase. (*B*) The same cell immunolabelled for prekeratin with rabbit antibodies and rhodamine-goat anti-rabbit IgG. (*C*) Phase photomicrograph of a PtK_2 cell fixed during metaphase. (*D*) Immunofluorescence labelling of the same cell with rabbit anti-prekeratin antibodies as in (*B*). (*E, G*) Phase photomicrographs of HeLa cells fixed during late anaphase or telophase (*E*) and prophase (*G*). (*F, H*) The same cells as in (*E*) and (*G*) respectively, immunolabelled for prekeratin as in (*B*). Note the intracellular prekeratin-containing cytoplasmic bodies in the mitotic cells and their absence from non-mitotic cells (doublet in upper left corner of (*F*)). (*I, K*) Phase photomicrograph of H-407 cells fixed during late anaphase–telophase (*I*) and metaphase (*K*). (*J, L*) The same cells as in (*I*) and (*K*), immunolabelled for prekeratin, using monoclonal mouse anti-prekeratin antibodies (*J*) and rabbit multispecific antibodies (*L*). The prekeratin-containing

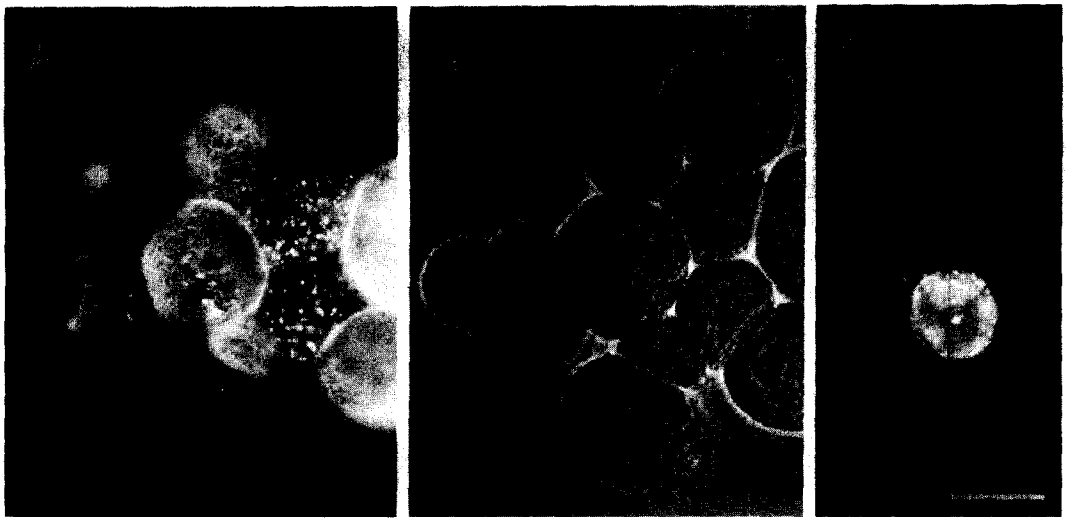

Fig. 4. Effect of colchicine (0.1 μg/ml 4 h) on the organization of prekeratin in mitotic cells. (*A*, *B*) Immunofluorescence labelling for prekeratin (*A*), and phase photomicrograph (*B*) of colchicine-treated HeLa cells. Note the overall rounding up of the cells, the retention of filaments in non-mitotic cells and the appearance of cytoplasmic bodies in mitotic cells only. (*C*) Immunofluorescence labelling of colchicine-treated H-407 cells for prekeratin. Bar, 10 μm.

Effect of colchicine on organization of prekeratin filaments during mitosis

In view of the possibility that the disassembly of cytoplasmic microtubules which occurs in mitotic cells might affect the integrity of prekeratin filaments (see Discussion) we have tested the effect of colchicine treatment on the organization of prekeratin filaments. Cultures of A-431, H-407 and HeLa cells were treated for various periods (up to 8 h) with colchicine and the cells were then fixed and immunolabelled for prekeratin.

Interphase cells retained their filamentous prekeratin network, though notable changes occurred in the distributions of these filaments, in line with previous described results [13]. H-407 and HeLa cells became progressively rounded up and their fine cytoplasmic filaments apparently aggregated into thicker bundles. In A-431 cells the same treatment brought about aggregation of prekeratin arrays around the nucleus (not shown). It should be mentioned, however, that the colchicine treatment was sufficient to induce rapid deterioration of microtubular structure.

Cells that were arrested during mitosis by the drug were identified by phase microscopy. These cells assumed a rounded shape and the nuclear membrane and nucleoli were not visible (fig. 4). Upon increase in incubation time with colchicine a higher percentage of the cells reached mitosis. In a culture of HeLa cells for example, up to 40% of the cells reached mitosis after 5 h of incubation with the drug. Longer incubation times induced also progressive

cytoplasmic bodies are detected only in mitotic cells, whereas neighbouring cells fixed during interphase retain filamentous labelling patterns (*J*). (*M*, *N*) Phase and immunofluorescence labelling (with rabbit antibodies to prekeratin) of HeLa cells during telophase. The cytoplasmic bodies are frequently localized in the vicinity of the cleavage furrow. (*O*, *P*) Phase photomicrograph and immunofluorescence labelling of mitotic A-431 cell with rabbit anti-prekeratin antibodies. The mitotic cell is attached on top of well spread cells and displays many prekeratin-containing cytoplasmic bodies. Bar, 10 μm.

changes of filaments in interphase cells and were therefore avoided.

Immunofluorescence analysis of colchicine-treated cells (tested with HeLa and H-407) indicated that prekeratin-containing cytoplasmic bodies were formed in essentially all the cells arrested in mitosis and in those cells only. It appears that the increase in number of cells containing the prekeratin cytoplasmic bodies after prolonged colchicine treatment is not a direct result of the drug treatment, nor is it due to dissociation of microtubules *per se* and therefore supports the idea that the state of organization of prekeratin filaments is controlled by a cell-cycled dependent regulatory system.

DISCUSSION

The extensive studies on the organization of the various cytoplasmic filaments and their possible functions point to two major roles of these fibrillary systems; the first is the cytoskeletal role, namely the formation of stable cellular networks that may stabilize cell shape, strengthen intracellular and cell–substrate attachments and resist mechanical perturbations. The other is the cytodynamic role which is probably essential for cellular activities such as cell locomotion, control of membrane dynamics, mitosis, etc. These two functional properties of the cytoskeleton may often be conflicting and probably require certain control mechanisms that render it more stable or more versatile and deformable, according to the specific physiological state of the cell. A mechanism for such transitions has not yet been defined. Nevertheless, significant differences were noted between the cytoskeletal organization in motile and sessile cells, and even between the central areas of the cell and the peripheral lamellipodia and ruffles which are involved in active-membrane movements [24–26]. During mitosis, fibroblasts for example, have been shown to lose the well organized stress fibers, round up and develop an actin–myosin–α-actinin-containing contractile ring [27–29]. The microtubule network dissociates at the initiation of mitosis and assembles to form the mitotic spindle.

Unlike the microtubule and microfilament systems, on which much physicochemical data is available regarding monomer–polymer transitions, the intermediate filaments (especially prekeratin) are highly insoluble in vitro under physiological and near-physiological conditions. In order to solubilize prekeratin filaments one has to use either high urea concentration or acidic pH. Since prekeratin filaments form elaborate networks in the cytoplasm of many epithelial cells, it seemed important to determine the fate of these filaments during mitosis. The existing information on the organization of intermediate filaments of the various classes during mitosis is still limited. It has been shown for cultured vascular endothelial cells, that the perinuclear intermediate filaments (vimentin type) are retained throughout mitosis and actually divide into two rings that segregate into the two daughter cells [16]. In chicken smooth muscle (gizzard) cells we have found that the organized intermediate filaments which are partially associated with microtubules during interphase disappear and desmin labelling is found mainly in a diffuse pattern [17].

The present study indicates that prominent changes occur during mitosis in the organization of prekeratin filaments in three different types of epithelial cell lines, namely A-431, HeLa and H-407. These changes included the disappearance of well organized filaments, an increase in diffuse labelling and the appearance of prekeratin-

containing cytoplasmic bodies. These modifications were transient and after mitosis was completed the two rounded up daughter cells developed a fibrillar prekeratin network and respread on the substrate. In many ways these apparently 'physiological' cytoplasmic bodies resembled the pathological Mallory bodies that occur in alcoholic individuals, and griseofulvin-fed rats [30–32]. The ethilogical and biochemical relationships between the two are, however, still unclear.

A point of major concern here was the specificity of the immunochemical reagents. The antibodies prepared in rabbits were directed against whole, reconstituted prekeratin filaments and were affinity purified on the same antigen. Therefore it could contain antibodies towards several or all the prekeratin components as well as against minor proteins that co-purify with this antigen. To overcome this difficulty we have also used here monoclonal antibodies prepared in mice. The monoclonal antibodies used in this study cross-reacted with several prekeratin components but did not bind to smooth muscle or fibroblastic cells. The general advantage of using this type of immunochemical monoclonal reagent is its defined and restricted specificity to a single determinant on the antigen [33]. For us this was taken as an indication that the filaments which we observe during interphase and the cytoplasmic bodies of the mitotic cells do indeed carry identical or cross-reactive antigenic site(s).

An exception to the phenomenon described above was the epithelial line PtK_2. These cells display networks of bundles of prekeratin filaments with variable thickness (usually much thicker than those of the other cells tested). During mitosis the network was apparently partially dissociated, though significant amounts of filament bundles were retained throughout mitosis, mainly close to the mitotic poles of the dividing cells as previously shown by Aubin et al. [18]. Cytoplasmic bodies as described for the other cells were not observed. It should be pointed out, however, that the central area of the cells in which the contractile ring was expected to form, was usually devoid of prekeratin filaments which otherwise might have interfered with cytokinesis. We have no direct explanation as to the reason for the differences between PtK_2 cells and the other three lines tested with respect to prekeratin reorganization during mitosis. One possibility which we consider, however, is that the relatively thick prekeratin bundles in PtK_2 have a high stability and thus remain more intact through mitosis. It should also be pointed out that PtK_2 cells remain flattened and attached to the substrate during mitosis, unlike the other three lines studied, which assume a rounded-up shape. The temporal and causal relationships between prekeratin disorganization and rounding up during mitosis are, however, still unclear.

What is the molecular basis for the transitions in prekeratin organization during mitosis? One possibility considered was that the disassembly of microtubules that occurs at the initiation of mitosis induces such changes. This possibility was raised since it has been shown for several types of intermediate filaments that they may be partially associated with microtubules and that microtubule-dissociating drugs also have a profound effect on the organization of the intermediate filaments [17, 34, 35]. We therefore treated the various cells that form typical prekeratin-containing cytoplasmic bodies (H-407, HeLa, A-431) with colchicine for various periods. It was initially verified by antitubulin labelling that dissociation of microtubules was essential-

ly complete within 15–30 min of treatment. Nevertheless, the increase in percentage of cells containing the prekeratin cytoplasmic bodies was much slower and these structures were found only in cells that were arrested during mitosis, as detected by phase microscpy. Essentially all and only these cells contained the typical dotted labelling with anti-prekeratin antibodies. The percentage of mitotic cells increased with the length of the incubation period, and after about 5 h of incubation reached about 40% for HeLa cells and somewhat less for H-407.

Although the detailed molecular mechanism responsible for the transient reorganization of prekeratin during mitosis is not yet understood, the present study indicates that a cell cycle-dependent regulatory mechanism does exist and that it may modulate the state of organization of cellular intermediate filaments under various physiological states such as cell division.

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