Published October 1, 1983

Change of Cytokeratin Filament Organization during the Cell Cycle: Selective Masking of an Immunologic Determinant in Interphase PtK_2 Cells

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ABSTRACT The organization of intermediate-sized filaments (IF) of the cytokeratin type was studied in cultures of PtK_2 cells in which typical IF structures are maintained during mitosis, using a monoclonal antibody (K_G 8.13). This antibody reacts, in immunoblotting experiments, with the larger of the two major cytokeratin polypeptides present in these cells but, using standard immunofluorescence microscopy procedures, does not react with the cytokeratin filaments abundant in interphase cells, in striking contrast to various antisera and other monoclonal cytokeratin antibodies. In the same cell cultures, however, the antibody does react with cytokeratin filaments of mitotic and early postmitotic cells. The specific reaction with cytokeratin filaments of mitotic cells only is due to the exposure of the specific immunologic determinant in mitosis and its masking in interphase cells. Treatment of interphase cells with both Triton X-100 as well as with methanol and acetone alters the cytokeratin filaments and allows them to react with this monoclonal antibody. A similar unmasking was noted after treatment with buffer containing 2 M urea or low concentrations of trypsin. We conclude that the organization of cytokeratin, albeit still arranged in typical IF, is altered during mitosis of PtK_2 cells.

Intermediate-sized filaments (IF)[1] of vertebrates are cytoplasmic structures that are notoriously stable, both mechanically and chemically, and resist extractions in buffers of a broad range of ionic strengths and pH values (for reviews, see references 1–4). Different types of IF have been distinguished by subunit polypeptide composition and by immunological criteria and their specific expression has been related to routes of cell differentiation (1–3, 5–7). Yet they all share some common principles of morphology and homologies of amino acid sequence (1–4, 8–10). In spite of their remarkable stability, IF may undergo redistribution of filaments as well as rearrangements of subunit proteins in living cells. It has been described that arrays of vimentin filaments are reorganized during mitosis (11–15) and drug-induced perinuclear aggregation (11, 16–17) and similar observations have been made for cytokeratin IF in mitosis of some epithelial cells (6, 12, 18, 19). In most cases such re-distributions have been interpreted as altered distributions of intact IF that do not involve intrafilamentous changes such as disassembly and re-assembly of IF subunits, in agreement with electron microscopic observations of normal-looking IF in all stages of mitosis of various cultured cells of mesenchymal (11, 13, 15) and epithelial (19) origin. By contrast, certain epithelial cells exhibit a drastic, transient change of IF organization during mitosis in which IF are unravelled into different, yet still insoluble and polymeric structures that aggregate into variously sized, spheroidal masses containing cytokeratin (20–22). These observations suggest that the structural state of at least certain IF is physiologically regulated. In the present study, we describe observations indicating that systematic, though less conspicuous changes of cytokeratin IF organization also occur in mitotic cells which, at the electron microscopic level, maintain typical IF morphology such as cultured rat kangaroo (PtK_2) cells. These observations have been made possible by the use of a monoclonal antibody, K_G 8.13 (23), which recognizes a cytokeratin determinant, the exposure of which is modulated during the cell cycle.

[1] *Abbreviations used in this paper:* IF, intermediate-sized filaments; PtK_2, cultured rat kangaroo cells.

THE JOURNAL OF CELL BIOLOGY · VOLUME 97 OCTOBER 1983 1255–1260

Published October 1, 1983

MATERIALS AND METHODS

Cells: PtK_2 cells were grown as previously described (19). For enrichment of mitotic stages some cell cultures were treated with 10^{-6} M colcemid for 12–24 h (19).

Antibodies: The monoclonal murine antibody K_G 8.13 (IgG_2) has recently been described in detail (23). Conventionally prepared antibodies (IgG fractions or affinity-purified) obtained from guinea pigs and rabbits immunized with epidermal prekeratins from bovine muzzle have previously been described, including their reaction with IF of PtK_2 cells (3, 6, 18, 20, 24, 25). Guinea pig antibodies to vimentin have also been described (6, 24). To control the stainability of cytokeratin IF in all phases of the cell cycle of PtK_2 cells, we also used other monoclonal antibodies to cytokeratins (20).

Immunofluorescence Microscopy: PtK_2 cells grown on glass coverslips to various densities (from 1 d after plating to confluency) were rinsed with phosphate-buffered saline at room temperature. The standard fixation procedure included dipping for 5 min in −20°C methanol and then for 1 min in −20° acetone, followed by air-drying. Variations of this procedure included the following:

(*a*) Extended (2 or 5 min) or repeated (6 × 1 min) incubations in acetone. (*b*) Use of ethanol instead of methanol. (*c*) Rinsing in PBS, followed by incubation for 5 min in 10 mM Tris-HCl buffer (pH 7.2) containing 140 mM NaCl and 0, 1, or 5 mM $MgCl_2$ (cf. reference 26) before application of methanol and acetone. (*d*) The same as *c*, except methanol and acetone were at 0°C or at room temperature. (*e*) Rinsed cells incubated in Tris-buffer containing 140 mM NaCl and 1% Triton X-100 (with or without 1 mM $MgCl_2$) for various periods of time (1, 2, 3, 4, 5, and 10 min), followed by an additional wash (5 min) in Tris-buffer, prior to application of first antibody (cf. reference 26). (*f*) The same as in *e*, except with fixation in −20°C methanol and −20°C acetone before application of first antibody. (*g*) The same as *e*, except with additional wash (5 min) in PBS. (*h*) As done in *f*, except methanol and acetone were at 4°C. (*i*) The same as in *f*, except incubation in methanol and acetone were both at room temperature. (*j*) After treatment with buffer containing Triton X-100 (as above) cells were incubated in high salt buffer (10 mM Tris-HCl, 140 mM NaCl, 1.5 M KCl, pH 7.2) for 5 min or 30 min, then rinsed in PBS containing 1 mM $MgCl_2$, prior to direct application of first antibody. (*k*) The same as in *j*, except after rinsing in PBS the specimens were incubated in methanol and acetone of various temperatures (−20°C, 4°C, room temperature). (*l*) The same as in *f*, except 1% Nonidet P-40 was used instead of Triton X-100. (*m*) After standard treatment with methanol and acetone cells were dipped into PBS containing 2 M urea (ultrapure) for a few seconds, followed by washes in PBS. (*n*) Cells treated with methanol and acetone were dipped into PBS containing trypsin (~20 μg/ml), followed by washes in PBS.

Incubation with the respective first antibody was for 45 min at room temperature, followed by several washes in PBS, and incubation with second antibody. In the case of the murine antibodies second antibodies were either fluorescein- or rhodamine-labeled rabbit anti-mouse IgG (freshly prepared or purchased from Miles-Yeda, Rehovot, Israel) or rhodamine-labeled goat antibodies to mouse (Cappel Laboratories, Cochranville, PA). When guinea pig antibodies were used in the first place they were visualized with rhodamine- or fluorescein-conjugated goat or rabbit antibodies to guinea pig IgG (freshly prepared or purchased from Miles-Yeda or Cappel Laboratories). After incubation with the specific second antibodies specimens were rinsed twice in PBS, air-dried and mounted in Mowiol (Hoechst, Frankfurt, Federal Republic of Germany).

Photomicrographs were taken with a Zeiss photomicroscope III (Zeiss, Oberkochen, Federal Republic of Germany). For double label immunofluorescence, both the murine and the guinea pig antibodies were applied at the same time (cf. reference 27). Controls for specificity of the second antibodies used in double immunofluorescence were routinely included.

Electron Microscopy: Cells grown on cover slips were fixed and processed for electron microscopy of ultrathin sections as previously described (19). For immunoelectron microscopy cells were treated in the specific way used for immunofluorescence microscopy, then were incubated with the first antibody for 1 h at room temperature. After washing three times with PBS, goat antibodies to mouse IgG that had been coupled to 5 nm colloidal gold particles (Janssen Chemicals, Beerse, Belgium) were added in PBS and incubated for 2 h. After three washes with PBS, specimens were fixed with 2.5% glutaraldehyde for 15 min, followed by OsO_4-fixation, and processed for ultrathin sectioning as described (28).

Gel Electrophoresis and Detection of Antigens: Polypeptides of whole PtK_2 cells and cytoskeletons made therefrom (29) were separated by gel electrophoresis, transferred on nitrocellulose paper sheets and examined by the immunoblotting technique as previously described (23).

RESULTS

The monoclonal antibody K_G 8.13 recognizes, in epithelia of man and cow, a determinant present in almost all members of the "basic subfamily" of cytokeratin polypeptides as well as in cytokeratin D (No. 18 of the human catalog; cf. reference 30) of man and cow (23). In cytoskeletons of PtK_2 cells, two major cytokeratin polypeptides have been identified, in addition to vimentin (3, 29, 31). One has an apparent M_r value of 54,000 and is related to cytokeratin "A" of other species, the other is more acidic, has an electrophoretic mobility similar to that of actin and appears to be related to component "D" of higher mammalian species (3, 23, 29). Of these, antibody K_G 8.13 reacts only with the larger cytokeratin (Fig. 1).

Immunofluorescence microscopy of PtK_2 cells in interphase with conventional cytokeratin antibodies of rabbits guinea pigs reveals an intricate meshwork of wavy cytokeratin fibrils characteristic of this cell type (not shown; cf. references 3, 6, 7, 12, 18, 20, 31, 32). The same fibrillar meshwork is also seen with various monoclonal murine antibodies (not shown; cf. references 20, 32, 33). In mitotic PtK_2 cells this meshwork is altered but fibrillar structures are still well discerned, in agreement with previous reports (12, 19, 20). By contrast, antibody K_G 8.13 does not stain interphase PtK_2 cells but reacts only with mitotic stages (Fig. 2). This specific reaction in mitotic PtK_2 cells only is illustrated by double immunofluorescence microscopy in Fig. 3, *a–d* which also demonstrate that this reaction is not restricted to prophase-telophase stages of mitosis (Fig. 3, *a* and *b*), but extends to postmitotic stages of early G1 phase of both daughter cells (Fig. 3, *c* and *d*). This specific reaction of antibody K_G 8.13 with cytokeratin structures of perimitotic but not interphase PtK_2 cells is not restricted to mitoses that have rounded off but is also seen in mitotic cells that have remained flat, thus demonstrating that it is related to the mitosis as such but not the morphological shape change. It is further evident that this change in antigenic reaction with PtK_2 mitoses is also seen in thin cytoplasmic projections as they occur in some normal mitoses and, more frequently, in mitotic stages arrested by treatment with colcemid (data not shown).

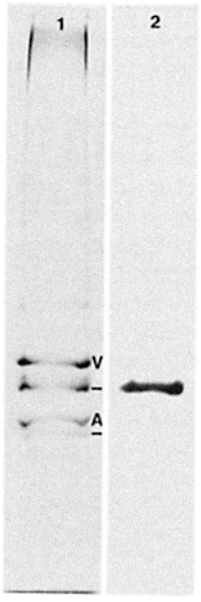

FIGURE 1 Cytoskeletal polypeptides obtained from PtK_2 cells after extraction with Triton X-100 and high salt buffer have been separated by SDS PAGE and stained with Coomassie Blue (lane *1*) or blotted on nitrocellulose paper and allowed to react with monoclonal antibody K_G 8.13 followed by reaction with ^{125}I-labeled protein A (lane *2*, autoradiofluorography). The two horizontal bars denote the two cytokeratins 1 (*upper*) and 2 (*lower*). *V*, vimentin; *A*, actin. Note that only cytokeratin 1 reacts with the antibody.

Published October 1, 1983

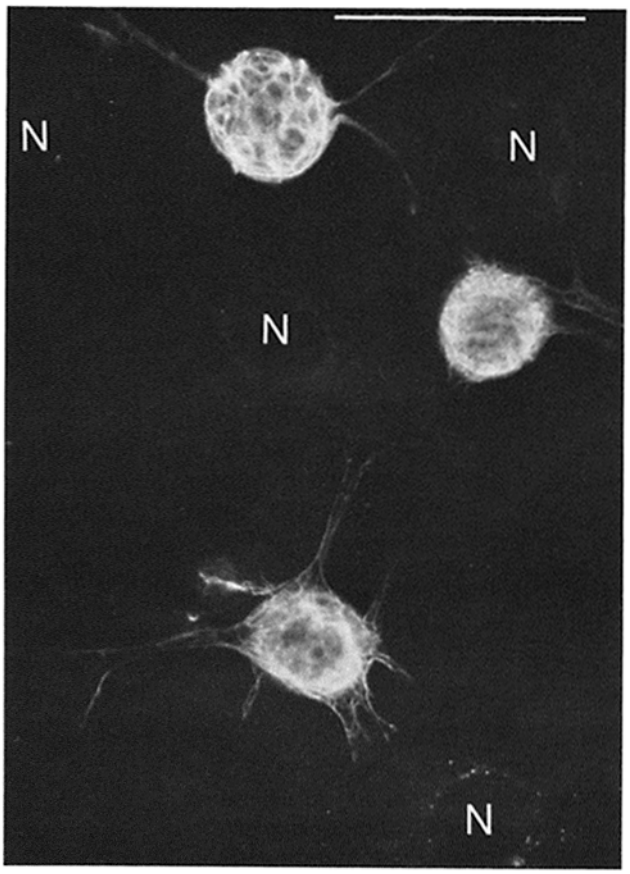

FIGURE 2 Immunofluorescence microscopy of mitoses-rich PtK_2 cell cultures after staining with murine monoclonal antibody K_G 8.13. Note that antibody K_G 8.13 reacts exclusively with mitotic and early postmitotic cells. Nuclei of unstained cells are indicated by *N*. Bar, 40 μm. × 820.

These observations of a specific reaction of antibody K_G 8.13 with cytokeratin filaments of mitotic but not interphase PtK_2 cells suggest that this reaction is the result of the masking of the determinant recognized in denatured cytokeratin (Fig. 1) during interphase. The strong staining of cytokeratin fibrils in interphase PtK_2 cells with various other conventional and monoclonal antibodies demonstrates the accessibility of the cytokeratin filaments to murine immunoglobulins. Therefore, we examined various preparative conditions which, in interphase cells, might unmask the determinant recognized by K_G 8.13. It has been found that when cells are extracted first with buffer containing Triton X-100 or Nonidet P-40 and then with methanol and acetone as described under methods *f–l* above significant fibrillar staining with antibody K_G 8.13 is seen in interphase PtK_2 cells (Fig. 4, *a–c*). Positive reaction is seen in all cells although the distinctiveness of the fibrillar fluorescence is often variable from cell to cell in the same culture. Double label immunofluorescence microscopy of PtK_2 cells treated in this way with guinea pig antibodies and with murine monoclonal antibody K_G 8.13 shows that the fibrillar structures stained with antibody K_G 8.13 are identical to those stained by cytokeratin antibodies that are positive also with cells processed according to the standard procedure. Inclusion of millimolar concentrations of Mg^{2+} or Ca^{2+} in the various buffers has not resulted in significant differences of this umasking effect. This unmasking of the determinant recognized by antibody K_G 8.13 has been most effective and uniform when both methanol and acetone were used at 4°C or at room temperature rather than at −20°C. Unmasking of the determinant recognized by antibody K_G 8.13 has also been achieved by brief treatments with 2 M urea and trypsin.

Electron microscopic examination has confirmed that antibody K_G 8.13 specifically binds to bundles of cytokeratin filaments. Positive decoration on IF bundles is seen in mitotic cells as well as in interphase cells treated with both the detergent and the organic solvents (data not shown).

DISCUSSION

Our results present the case of a specific cell cycle-dependent re-arrangement of IF structures that is recognized by the selective masking and unmasking of a specific immunologic determinant in cytokeratin filaments. Selective masking has been discussed by Lazarides et al. (34) as a likely explanation of an unexpected reaction of another type of IF, desmin, with a monoclonal antibody (D76) that reacts with desmin only in later stages of myotube differentiation but not in early myotubes. However, direct proof by experimental unmasking of this determinant has not been presented for this case. Our observations in PtK_2 cells demonstrate cell cycle-dependent masking of an IF determinant by experimental unmasking. This occurrence of a selective and complete masking of a determinant in an IF system should be taken as a reason for concern and caution in interpreting negative results obtained in immunolocalization studies, especially when monoclonal antibodies are used. The phenomenon described here may also explain unexpected heterogeneities of reactivity in interphase cells of the same culture (for examples, see references 21, 33, 35, 36). It may also be relevant for some observations in frozen tissue sections, and failures to certain monoclonal or conventionally obtained antibodies to react with fibrils of some epithelial cells but not with those of others (e.g., references 6, 7, 25, 32, 33, 37, 38) may reflect differential masking rather than differences of expression. Woodcock-Mitchell et al. (39) have presented an example of a monoclonal antibody that does not bind to its cytokeratin determinant in suprabasal layers of epidermis and have suggested that this might be due to selective masking in situ. We propose to include, as controls for possible masking of the type described here, immunoblotting tests on proteins from the specific cell colonies or layers under question as well as treatments with unmasking reagents as they have been successful in this study.

Antibody K_G 8.13 reacts with a determinant of a group of cytokeratin polypeptides present in diverse mammalian species (23). Interestingly, this determinant is differently exposed during interphase in cultured cells from various species. Whether this different reactivity of the same determinant in different cells reflects functional differences or only tolerance of changes in an unimportant region of this molecule is not known.

The accessibility of the cytokeratin determinant recognized in PtK_2 cells during mitosis but not during most of the interphase indicates that the organization of cytokeratin filaments is not constant but goes through systematic and dynamic changes, both when the cell enters mitosis and when it re-establishes its interphase architecture. PtK cells maintain intermediate-sized filament structures during mitosis, although changes in their display have been noticed by electron

Published October 1, 1983

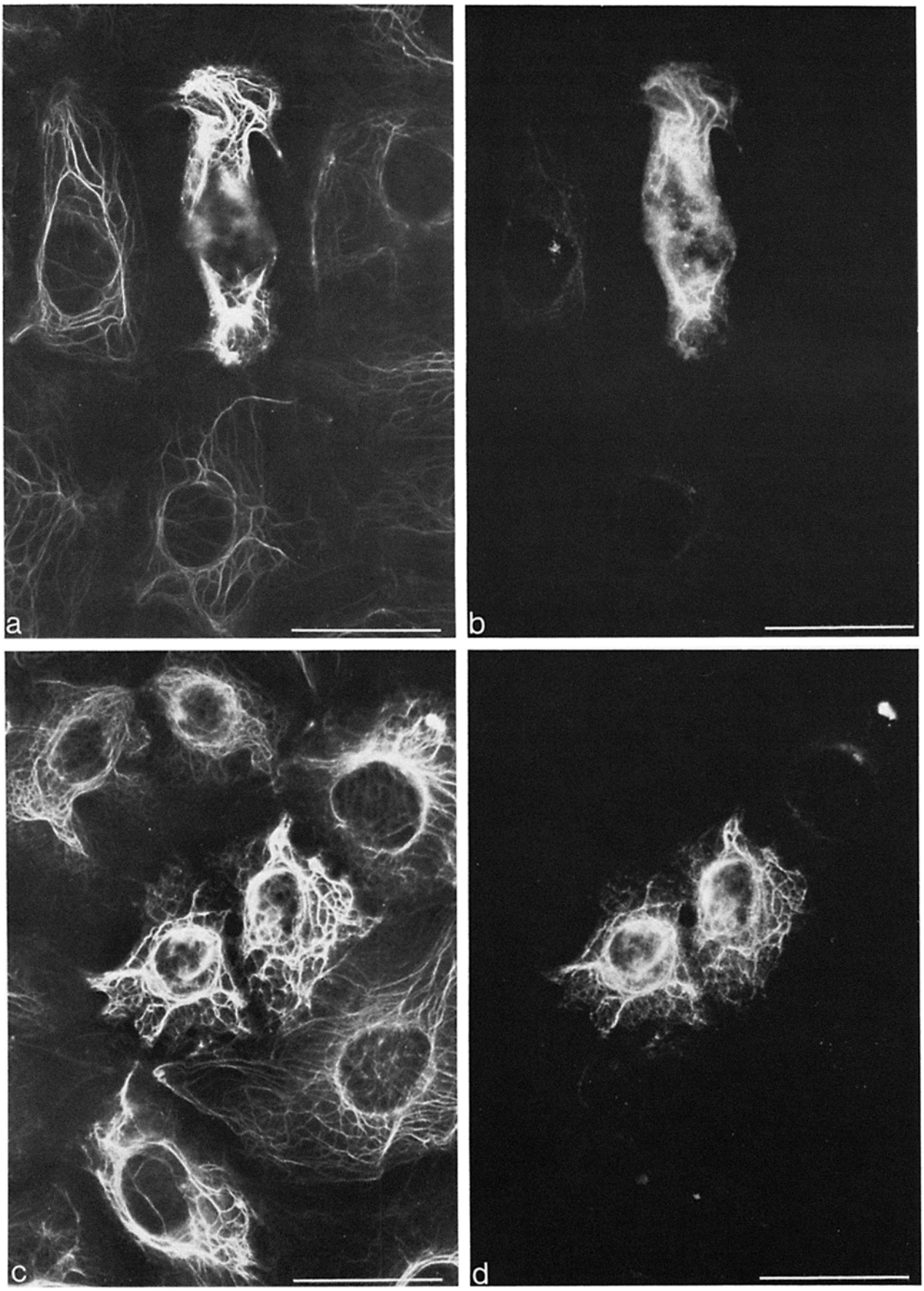

FIGURE 3 Double label immunofluorescence microscopy of PtK_2 cells, using guinea pig antibodies against cytokeratin (*a* and *c*; same as in Fig. 2, *a–c*) and murine antibody K_C 8.13 (*b* and *d*). Note that antibody K_C 8.13 reacts only with filaments of mitotic cells (*a* and *b*) and postmitotic cells of early 61 phase. (*c* and *d*). Bars, 30 μm. × 900.

Published October 1, 1983

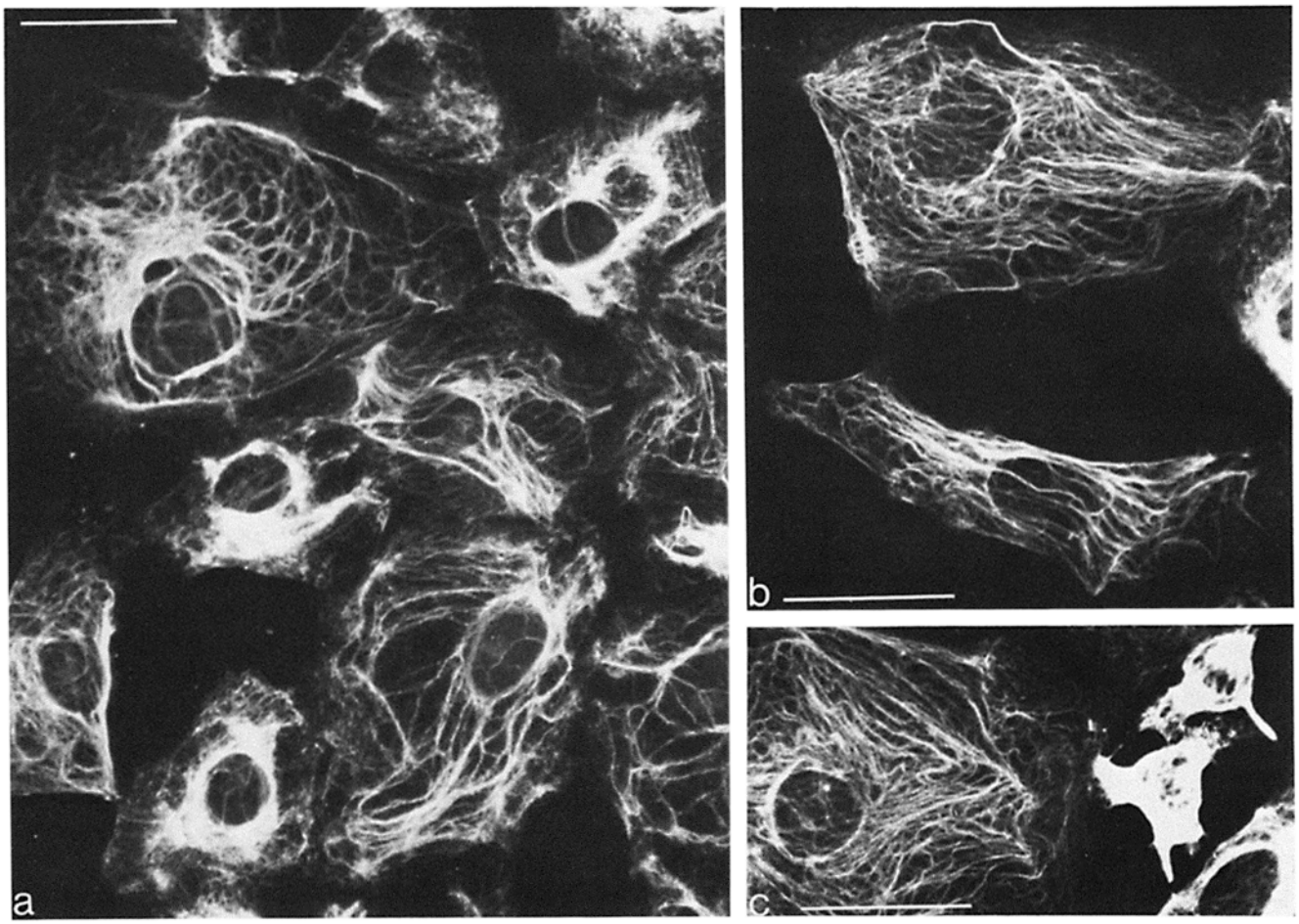

FIGURE 4 Immunofluorescence microscopy of PtK_2 cells treated sequentially with Triton X-100 as well as methanol and acetone using monoclonal antibody K_G 8.13 (*a–c*). Note that cytokeratin filaments positively stained with antibody K_G 8.13 are recognized in cells extracted in this way (*a*, survey; *b*, a large and a small cell; *c*, an interphase cell, in the left, and two mitotic cells in the upper right). Bars, 30 μm. (*a*) × 700; (*b*) × 750; (*c*) × 750.

microscopy (19) as well as immunofluorescence microscopy (14, 18), different from many other epithelial cells in which cytokeratin filaments are transformed into spheroidal aggregates of non-IF structures (20–22). Now our observations that cytokeratin filaments of mitotic PtK_2 cells are not identical to those of interphase PtK_2 cells and can be clearly distinguished by monoclonal antibody K_G 8.13 suggest that perimitotic changes of IF organization may be much more common, albeit not necessarily as dramatic as in those cells which transiently transform their cytokeratin filaments into nonfibrillar aggregates (20–22).

Our experiments do not allow us to decide whether the masking of the K_G 8.13 determinant in cytokeratin filaments of PtK_2 interphase cells is due (*i*) to the specific association with a noncytokeratin protein or (*ii*) to an intrinsic change in the arrangement of the cytokeratin polypeptides. Treatment with Triton X-100 or Nonidet P-40 does not detectably change the electron microscopic appearance of these filaments (cytokeratins are even capable of reconstituting intermediate-sized filaments in vitro in the presence of 1% Triton X-100, data not shown). The various treatments used for unmasking could "loosen" the specific polypeptide arrangement within IF or extract a masking component. It is also possible that the perimitotic changes in the K_G 8.13 determinant of PtK_2 cells are related to modifications of cytokeratins (for reports of increased phosphorylated of vimentin in mitosis, see references 40, 41). The observed changes in IF organization may have, beyond the structural aspect, functional importance: the surface pattern of IF which, in the living cell, provide a large area for potential structure-bound reactions may not be constant and inactive but may be involved in the regulation of other cellular activities.

We thank Caecilia Kuhn and Klaus Mähler for expert technical help.

The work has been supported in parts by the Deutsche Forschungsgemeinschaft (grant Fr 308/15).

Received for publication 20 April 1983, and in revised form 28 June 1983.

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