Change of Cytokeratin Filament Organization
during the Cell Cycle: Selective Masking of an
Immunologic Determinant in Interphase PtK₂ Cells

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ABSTRACT The organization of intermediate-sized filaments (IF) of the cytokeratin type was studied
in cultures of PtK₂ cells in which typical IF structures are maintained during mitosis, using a
monoclonal antibody (KG 8.13). This antibody reacts, in immunoblotting experiments, with the larger
of the two major cytokeratin polypeptides present in these cells but, using standard immunofluores-
cence 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 PtK₂ cells.

Intermediate-sized filaments (IF)¹ of vertebrates are cytoplas-
mic 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 aggrega-
tion (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 inter-
preted 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 obser-
vations 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 poly-
meric 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 phys-
iodologically regulated. In the present study, we describe obser-
vations 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 (PtK₂) cells.
These observations have been made possible by the use of a
monoclonal antibody, K₉ 8.13 (23), which recognizes a cy-
tokeratin determinant, the exposure of which is modulated
during the cell cycle.
MATERIALS AND METHODS

Cells: PtK2 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_{G8} 8.13 (IgG_{s}) has recently been described in detail (23). Conventionally prepared antibodies (IgG fractions of 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 PtK2 cells (3, 6, 18, 20, 24, 25). Guinea pig antibodies to vimentin have also been described (6, 24). To control the specificity of the second antibodies used in their reaction with IF of PtK2 cells, we also used other monoclonal antibodies to cytokeratins (20).

Immunofluorescence Microscopy: PtK2 cells grown on glass coverslips (at various densities from 1 d after plating to confluence) 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°C 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 in 5% in 0.1 M Tris-HCl buffer (pH 7.2) containing 140 mM NaCl and 0.1% 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 MgCl2, prior to direct application of first antibody. (d) The same as in (c), except with fixation in -20°C methanol and -20°C acetone before application of first antibody. (e) The same as in (c), except with additional wash (5 min) in PBS. (f) As done in (e), except methanol and acetone at 4°C. (g) The same as in (f), except incubation in methanol and acetone were both at room temperature. (h) After treatment with buffer containing Trition 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 MgCl2, prior to direct application of first antibody. (i) 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).

(j) The same as in (f), except 1% Nonidet P-40 was used instead of Triton X-100. (k) 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. (l) 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 antimouse 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 OsO4 fixation, and processed for ultrathin sectioning as described (28).

Gel Electrophoresis and Detection of Antigens: Polypeptides of whole PtK2 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_{G8} 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 PtK2 cells, two major cytokeratin polypeptides have been identified, in addition to vimentin (3, 29, 31). One has an apparent M, 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_{G8} 8.13 reacts only with the larger cytokeratin (Fig. 1).

Immunofluorescence microscopy of PtK2 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 PtK2 cells this meshwork is altered but fibrillar structures are still well discerned, in agreement with previous reports (12, 19, 20). By contrast, antibody K_{G8} 8.13 does not stain interphase PtK2 cells but reacts only with mitotic stages (Fig. 2). This specific reaction in mitotic PtK2 cells only is illustrated by double immunofluorescence microscopy in Fig. 3, a-d which also demonstrate that this reaction is not restricted to prophase-telephases 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_{G8} 8.13 with cytokeratin structures of permitotic but not interphase PtK2 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 PtK2 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 PtK2 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_{G8} 8.13 followed by reaction with 125'I-labeled protein A (lane 2, autoradiography). 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.
with methanol and acetone as described under methods
buffer containing Triton X-100 or Nonidet P-40 and then
cytokeratin filaments to murine immunoglobulins. Therefore,
we examined various preparative conditions which, in inter-
phase cells, might unmask the determinant recognized by K6
monoclonal antibodies demonstrates the accessibility of the
PtK2 cells suggest that this reaction is the result of the masking
PtK2 cells with various other conventional and
monoclonal antibodies demonstrates the accessibility of the
cytokeratin filaments to murine immunoglobulins. Therefore,
examined various preparative conditions which, in inter-
phase cells, might unmask the determinant recognized by K6
8.13. It has been found that when cells are extracted first with
buffer containing Triton X-100 or Nonidet P-40 and then
methanol and acetone as described under methods f l
above significant fibrillar staining with antibody K6 8.13 is
seen in interphase PtK2 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
PtK2 cells treated in this way with guinea pig antibodies and
with murine monoclonal antibody K6 8.13 shows that the
fibrillar structures stained with antibody K6 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 unmasking effect. This unmasking of the determinant
recognized by antibody K6 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 K6 8.13 has also
been achieved by brief treatments with 2 M urea and trypsin.
Electron microscopic examination has confirmed that anti-
body K6 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 myo-
tubes. However, direct proof by experimental unmasking of
this determinant has not been presented for this case. Our
observations in PtK2 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 inter-
phase 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., refer-
ces 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 positive masking of the type described here, immunoblot-
ting 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 K6 8.13 reacts with a determinant of a group of
cytokeratin polypeptides present in diverse mammalian spe-
cies (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 PtK2 cells during mitosis but not during most of the
interphase indicates that the organization of cytokeratin fila-
ments is not constant but goes through systematic and dy-
namic changes, both when the cell enters mitosis and when it
re-establishes its interphase architecture. PtK cells maintain
intermediate-sized filament structures during mitosis, al-
though changes in their display have been noticed by electron

**Figure 2** Immunofluorescence microscopy of mitoses-rich PtK2

cell cultures after staining with murine monoclonal antibody K6
8.13. Note that antibody K6 8.13 reacts exclusively with mitotic
and early postmitotic cells. Nuclei of unstained cells are indicated by
N. Bar, 40 μm. × 820.
FIGURE 3 Double label immunofluorescence microscopy of PtK2 cells, using guinea pig antibodies against cytokeratin (a and c) same as in Fig. 2, a–c) and murine antibody KG 8.13 (b and d). Note that antibody KG 8.13 reacts only with filaments of mitotic cells (a and b) and postmitotic cells of early G1 phase (c and d). Bars, 30 μm. × 900.
FIGURE 4 Immunofluorescence microscopy of PtK2 cells treated sequentially with Triton X-100 as well as methanol and acetone using monoclonal antibody Kc 8.13 (a-c). Note that cytokeratin filaments positively stained with antibody Kc 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 PtK2 cells are not identical to those of interphase PtK2 cells and can be clearly distinguished by monoclonal antibody Kc 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 nonfilbrillar aggregates (20–22).

Our experiments do not allow us to decide whether the masking of the Kc 8.13 determinant in cytokeratin filaments of PtK2 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 Kc 8.13 determinant of PtK2 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.

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