Cancer-associated Cleavage of Cytokeratin 8/18 Heterotypic Complexes Exposes a Neoepitope in Human Adenocarcinomas*

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The intermediate filament network in simple glandular epithelial cells predominantly consists of heterotypic complexes of cytokeratin 8 (K8) and cytokeratin 18 (K18). In contrast to other cytokeratins, K8 and K18 are persistently expressed during malignant transformation, but changes in cell morphology are accompanied by alterations in the intermediate filament network. To study molecular changes, K8 and K18 were purified from surgically removed colon cancer and normal epithelia tissues. Western blotting and amino acid sequencing revealed the presence of abundant K8 and K18 fragments, truncated at the N terminus, from cancerous, but not normal, epithelial cells. The fragmentation pattern indicates proteolysis mediated by several enzymes, including trypsin-like enzymes. The cancer-associated forms of K8 and COU-1 are specifically recognized by the human antibody, COU-1, cloned from the B cells of a cancer patient. We demonstrate that COU-1 recognizes a unique conformational epitope presented only by a complex between K8 and K18. The epitope is revealed after proteolytic removal of the head domain of either K8 or K18. A large panel of recombinant K8 and K18 fragments, deleted N- or C-terminally, allowed for the localization of the COU-1 epitope to the N-terminal part of the rod domains. Using surface plasmon resonance, the affinity of COU-1 for this epitope was determined to be \(10^9\) M\(^{-1}\), i.e. more than 2 orders of magnitude higher than for intact heterotypic K8/K18 complexes. The cellular distribution of truncated K8/K18 heterotypic complexes in viable adenocarcinomas cells was probed using COU-1 showing small fibrillar structures distinct from those of intact K8/K18 complexes. Previously we demonstrated the binding and subsequent internalization of recombinant Fab COU-1 to live human liver cells. We have thus characterized a cancer neoepitope recognized by the humoral immune system. The results have biological as well as clinical implications.

The 10 nm intermediate filaments (IF)† provide resilience in response to mechanical stress by forming a stable network attached to specific desmosome cell-cell contacts (1). IF can be classified into groups expressed in higher eukaryotes in a tissue-specific and cell type-restricted pattern (2, 3). In epithelial cells, IFs consist of stoichiometrically equal amounts of type I (smaller and acidic) and type II (larger, neutral, or basic) cytokeratin polypeptides, which form strongly interacting heterodimers (4–6). Cytokeratin 8 (K8) and cytokeratin 18 (K18), type I and II, respectively, are the major components of IFs of simple or single layer epithelia, such as those of the intestine, the liver, and the breast ducts, although cytokeratin 19 (K19) is also present and forms heterodimers with K8 (2, 5). The assembly of IF involves several association steps where the α-helical rod domain of the cytokeratin molecules plays the central role (7–9). The head and the tail domains are not thought to be part of the filamentous backbone but to protrude laterally and contribute to profilament and IF packing, and to IF interaction with other cellular components (10–12). Cotransfection of head-deleted K8 and K18 resulted in formation of a dispersed non-fibrillar pattern, whereas cotransfection of a combination of one headless plus one intact cytokeratin resulted in formation of cytoplasmic granules or fibrils (12). More detailed analysis showed that only short and irregular IF was generated when K8 and K18 were N-terminally truncated by deleting the first 66 amino acids of K8 and K18, whereas only tetromers were generated when a major part of the H1 domain was removed (13).

During cell transformation and tumor development, the cell type specificity of K8 and K18 is conserved, making them useful as clinical histopathological markers (2, 14, 15). Interestingly, it has been found that oncogenes, which activate the Ras signal transduction pathway, stimulate K18 gene expression through transcription factors, such as members of the ETD and AP-1 families, including jun and fos (16–18). The continuous and sometimes even increased expression of K8 and K18 in tumor cells, while many other proteins, including other cytokeratins, are lost, may be the result of constitutive activation of the Ras pathway. Supporting the notion that K8 and K18 play a role in the tumorigenic phenotype, increased expression of K8 and K18 has been found at the invasive front of some tumors (14). In addition, an association between K8 and K18 expression and increased invasiveness and metastatic properties through special interaction with the extracellular environment has been observed (19–21).

mAb, monoclonal antibody; FCS, fetal calf serum; AP, alkaline phosphatase; QFF, Q-Sepharose fast flow; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; PVDF, polyvinylidene difluoride membranes; FITC, fluorescein isothiocyanate; GST, glutathione S-transferase.

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†The abbreviations used are: IF, intermediate filaments; K8, cytokeratin 8; K18, cytokeratin 18; HmAb, human monoclonal antibody;
Previous work from our group and others (22–25) has indicated that K8 and K18 may be modified in cancer cells and used as immunodiagnostic markers and therapeutic targets for adenocarcinomas. In the present study, we detail the composition of K8/K18 filaments in viable cancer cells.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—The human monoclonal antibody (HmAb) COU-1 was produced and purified as described (26). In brief, COU-1 is secreted by the hybridoma cell line, B9165, derived by fusing human lymphoblastoid cells (WI-L2-729-HF2) with lymphocytes from mesenteric lymph nodes of nude mice, a colon cancer patient (27). The cell was grown in protein-free medium, and COU-1 was purified from the culture supernatant by affinity chromatography on Sepharose-coupled monoclonal antibody (mAb) against human μ-chain (HB57, ATCC, Manassas, VA) and further purified by gel permeation chromatography. IgM purified from normal human serum (Cappel, Cochranville, PA) was used as a control. Murine mAbs, M20 directed against normal K8, CY-90 directed against normal K18, and A63-B2A2 directed against normal K19, were obtained from Sigma.

**ELISA**—ELISA wells (Costar, Cambridge, MA) were coated overnight at 4 °C with fractions from the purification of cytokeratin or with different recombinant K8/K18 complexes (5 μg/ml) in PBS, pH 7.4. The wells were blocked with PBS and incubated with 3% (w/v) bovine serum albumin in PBS for 1 h at 37 °C, and incubated with COU-1 for 2 h at 37 °C. Plates were washed 10× with PBS, 0.05% Tween 20 (PBS/Tween), and bound antibody was detected with alkaline phosphatase (AP)-labeled goat anti-human κ-chain (Sigma, 1/1000) in PBS/Tween. Bound antibody was visualized with para-nitrophenyl phosphate (Sigma) at 1 mg/ml, 1 mM MgCl₂, 100 mM NaCl, pH 9.6, and read at 405 nm.

**Cell Culture**—The human breast adenocarcinoma cell line MCF7 (ATCC) was maintained in minimum Eagle’s medium (Invitrogen), supplemented with 10% FCS, non-essential amino acids, 1 mM sodium pyruvate, 1 mM HEPES buffer, 100 units of penicillin/ml, 100 mg of streptomycin/ml, and 2 mM l-glutamine. The human colon adenocarcinoma cell line HCT-15 was grown in Super Broth medium, supplemented with 20 mM MgCl₂ and 50 mg of carbenicillin/ml, at 37 °C until A₅₇₀ reached 0.6. Protein expression was then induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (Sigma) and 4 μM CAMP, and the cultures were allowed to grow for an additional 3 h at 30 °C. The bacteria were pelleted at 4,000 × g for 15 min at 4 °C. For SDS-PAGE, the pellet was resuspended in sample buffer and sonicated 5 × 10 s before electrophoresis. For purification of recombinant K8 or K18, the pellet of a 400-ml culture, grown and purified K8 or K18 proteins were expressed and purified as described (26). In brief, COU-1 is secreted by E. coli DH5a harboring plasmids encoding a panel of K8 and K18 proteins were analyzed. The panel consisted of cDNA encoding the full-length and several N-terminal and C-termi-}

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HCl, 1 M NaCl, pH 2.0, and remained active for 10 measurements. The association and dissociation rate constants, $k_a$ and $k_{off}$, were determined from a series of measurements, as described previously (31). Association and dissociation constants were deduced from the kinetic data using the Bioevaluation program version 3.1 (Biacore Inc.).

**Confocal Laser Scanning Microscopy**—Cells were seeded into Lab-Tek chamber slides (Nalge Nunc, Naperville, IL) and allowed to grow and adhere to the glass slides for 48 h at 37 °C, 5% CO$_2$. Cells were fixed with ice-cold 96% ethanol for 5 min, washed 3× with PBS, and blocked with 10% normal goat serum in PBS for 1 h at room temperature. COU-1 (5 μg/ml in PBS) together with either mouse anti-K8 antibody (1/1000 in PBS) or mouse anti-K18 antibody (1/1000 in PBS) were added to the cells and incubated overnight at 4 °C. After washing with PBS, the cells were incubated with FITC-labeled goat anti-human α-chain and Texas Red-labeled goat anti-mouse IgG antibody (1/2000 in PBS) for 1 h at room temperature in the dark. The cells were washed with PBS 3× for 5 min, and the slides were mounted with anti-fading reagent in PBS/glycerol (Slow Fade™, Molecular Probes, Eugene, OR). Results were analyzed using an MRC-1024 confocal laser scanning microscope (Bio-Rad) attached to a Zeiss Axiovert 100TV. As a control, all experiments were also performed omitting the primary antibody or including species and isotype-matched control immunoglobulin instead of the primary antibody. In addition, differential interference contrast images of the analyzed cells were obtained.

**RESULTS**

**Purification of Cytokeratins from Colon Cancer Tissue and Normal Colon Epithelium.**—To study the composition of the K8/K18 filaments in normal and malignant glandular epithelia and to determine the basis for the adenocarcinoma-specific binding of HmAb COU-1, total cytokeratin was separately purified from fresh tissue of surgically removed colon cancer tissue and normal colon epithelia using the procedure described under “Experimental Procedures.” Following separation by QFF anion-exchange chromatography, the fractions containing COU-1 reactivity were found in the first and second peak of the gradient (fractions 41–48) as determined by ELISA (Fig. 1A). Western blot analysis demonstrated reactivity of COU-1 with three main bands in the same fractions (Fig. 1B). These three bands represented only a portion of the proteins with molecular masses in the 41–46 kDa range found in these fractions, as revealed by Coomassie Blue staining of the SDS-separated gels (Fig. 1C). Western blot analysis and Coomassie Blue staining of cytokeratin purified from colon cancers of four patients revealed a similar pattern of protein bands, reactive and non-reactive with HmAb COU-1.

Cytokeratin was also isolated from normal colon epithelia obtained from three individuals using the same purification procedure to compare the nature of the K8/K18 in colon cancer versus normal colon tissues. Tissue lysates and purified cytokeratin preparations (QFF eluate) from the two sources were examined by Western blotting used a panel of K8-, K18- and K19-specific antibodies. When approximately equal amounts of cytokeratin from colon and the normal epithelia were analyzed, protein bands (in the 41–46 kDa range) of equal intensity were observed following staining with the murine K18 antibody (CY-90), which recognizes a linear epitope in the C-terminal part of K18 (Fig. 2). In contrast, when the same preparations were stained with COU-1, protein bands (in the 41–46 kDa range) were only observed in the cytokeratin preparations from the colon cancer tissue and not from the normal colon epithelia (Fig. 2).

**Amino Acid Sequencing Revealed N-terminal Truncated Fragments of Both K8 and K18 from Viable Cancer Cells**—To determine the nature of the different K8/K18-like proteins...
found in the colon cancer tissues, purified cytokeratin preparations from primary colon cancer tissues of three patients were individually separated on large 14% polyacrylamide gels, the proteins blotted, and Coomassie Blue-stained. In addition, a cytokeratin preparation purified from the human colon adenocarcinoma cell line colo137 was also separated, the proteins blotted, and Coomassie Blue-stained. Fig. 3A shows a typical blot of a colon cancer tissue sample, displaying 10 bands.

Strips of the blot were incubated with either mAb M20 (K8), CY-90 (K18), or COU-1. At this increased separation, 5 bands showed COU-1 reactivity. Additional bands, not stained with COU-1, were stained either with the anti-K8 mAb, the anti-K18 mAb, or both (Fig. 3A). All 10 bands were N-terminal sequenced, revealing that the bands, except one, corresponded to different forms of K8, K18, and K19 (Fig. 3B). The additional band was identified as migration inhibitory factor-related protein 8 (MRP8, also known as calretinin), a calcium-binding protein that has been suggested to bind cytokeratins (32). Most of the bands represented N-terminally truncated K8 or K18, with the identified amino acid sequence starting at residues 23–76. The N-terminal truncations of K8 corresponded to residues 23, 40, 66, and 76, whereas the truncations of K18 corresponded to residues 50 and 68. Significantly, the K8 and K18 truncations were found at the same residues in three different
primary colon cancers and the human colon adenocarcinoma cell line colo137, indicating that the truncations were caused by specific proteases. Analysis of the sequences surrounding the cleavage sites suggests that at least two proteases are responsible for the cleavage, including one trypsin-like protease. The bands recognized by COU-1 were the N-terminally truncated K8 and K18. Interestingly, not all the N-terminally truncated K8 and K18 proteins were recognized by COU-1. For example, no COU-1 binding was observed to N-terminally truncated K8 protein where the first 22 amino acid was missing nor did the antibody react with intact K8 or K18. The latter two were identified by staining with K8 and K18 antibody, respectively (bands 1 and 2 in Fig. 3A), but not by N-terminal sequencing, because they were N-terminally blocked (K18 contains an acetylated serine at its N terminus).

Mapping the COU-1 Epitope Using Complexes of Recombinant K8 and K18 Fragments—To detail the nature of the epitope recognized by COU-1, it was mapped using a panel of recombinant intact as well as N- and C-terminally deleted K8 and K18 polypeptides expressed as GST fusion proteins (Fig. 4). Initially, the panels of K8 and K18 fragments were separated by SDS-PAGE and blotted onto PVDF membranes. To ensure that equal amounts of K8 and K18 polypeptide were loaded on the gels, gels run in parallel were stained with Coomassie Blue, and blots were stained with an anti-GST antibody (Figs. 5A and 6A). Subsequent analyses of Western blots with COU-1 surprisingly showed that COU-1 did not bind to any of the individual K8 or K18 fragments nor to the intact K8 or K18 molecules (Figs. 5B and 6B). In each experiment, MCF7 cell lysate was also included as a positive control, demonstrating bands at molecular masses of 41–46 kDa. Due to the control experiments, we could conclude that the lack of signal with COU1 was not due to insufficient amounts of K8 or K18 loaded on the gel or incomplete transfer of proteins. In addition, the panels of K8 or K18 fragments were recognized by conventional mAbs anti-K8 and anti-K18, respectively. The anti-K8 reacted strongly with K18-(1–356), K18-(1–385), and intact K18, but not with K18-(1–312), indicating that its epitope was located in the region 312–356 (Fig. 5C). We next examined the binding of COU-1 to heterotypic complexes of K8 and K18,
which were formed by incubating Western blots of the panel of K18 fragments with intact purified K8 (Fig. 5 D). COU-1 bound strongly to complexes formed between intact K8 and K18-(1–213) through K18-(1–385), only weakly to intact K8/K18-(1–187) and intact K8/intact K18, and no binding was seen to intact K8/K18-(1–72) and intact K8/K18-(1–124) (Fig. 5 D). In addition, a PVDF membrane containing the SDS-PAGE-separated K18 fragments was incubated with purified intact K8 prior to staining with COU-1 (D).

Because the N-terminal sequencing demonstrated that both K8 and K18 in colon cancer were truncated, we wanted to study the binding of COU-1 to K8/K18 heterotypic complexes where both K8 and K18 were truncated. In parallel, Western blots containing the C-terminally deleted fragments surrounding the COU-1 epitope on K18 were incubated with K8 fragments surrounding the K8 part of the COU-1 epitope or intact K8. As shown in Fig. 7, A strong binding of COU-1 was observed to K8-(1–213)/K8-(1–129). In contrast, the epitope recognized by COU-1 is not, or only minimally, exposed on K8-(1–124)/intact K8 or K8-(1–124)/K18-(1–233) (Fig. 7, B and C). We also reversed the setup described above such that Western blots of the C-terminal deleted fragments of K8 were incubated with the fragments of K18 surrounding the COU-1 epitope. As shown in Fig. 7, D–F, the epitope recognized by COU-1 was equally exposed when K8-(1–129) was complexed with K18-(1–124), K18-(1–187), or K18-(1–213). No COU-1 binding was observed to any of the heterotypic complexes containing K8-(1–85) or K18-(1–72). Taken together, these results confirm that the epitope recognized by COU-1 involves the K8 region 85–129 and the K18 region 72–124. As shown in Figs. 4 and 8, this region involves the C-terminal part of the N-terminal head domain and the N-terminal part of the first helical domain, 1A, of the α-helical rod domain of both K8 and K18. The results further demonstrate that this epitope is poorly exposed on heterotypic complexes of intact K8 and K18, even when intact K8 is complexed with K18-(1–124). As depicted in Fig. 8, the COU-1 epitope is revealed when the first domain, A1, of the α-helical rod is not in its normal coiled-coil structure. This can be caused by truncation removing essential contact points for the existing association, leaving the COU-1 binding region of the K8/K18 complex in an unfolded state.

COU-1 binding to a panel of heterotypic complexes consisting of N-terminally deleted K8 and K18, missing the first 129 amino acids or more (Fig. 4), combined with intact K8 and K18 were also tested using the heterotypic Western blot assay.
HeLa cells infected with adenovirus and from uninfected HeLa cells were purified and separated by SDS-PAGE. Coomassie Blue staining of the gel demonstrated an additional band with a molecular mass of 41 kDa in the adenovirus-infected HeLa cell preparation, corresponding to cleaved K18. Incubation of Western blots of the adenovirus-infected HeLa cells with anti-K8 or anti-K18 mAbs resulted in staining only with the anti-K8 or anti-K18 mAbs but not with COU-1 (data not shown), indicating that the cytokeratin fragments found in the adenocarcinomas were not a result of adenovirus infection.

K8 and K18 Polypeptides Associate in Vitro during Western Blot Processing—The results above indicate that the COU-1 epitope is presented only by heterotypic K8/K18 complexes and not by individual K8 and K18 molecules. This appears to conflict with the observed COU-1 binding to cytokeratin on Western blots of SDS-separated cancer cell lysates where the K8/K18 complexes would be expected to remain dissociated. A possible explanation was that during the incubation steps, part of the different cytokeratins dissociate from the membrane and subsequently attach to and form stable heterotypic complexes with their complementary cytokeratin still bound to the membrane. To examine this hypothesis, half of a Western blot of colon cancer Colo137 cell lysate was fixed with ethanol before incubation with the antibodies, whereas the other half was processed as usual without fixation. Staining was observed with mAb anti-K18 on both the fixed and the unfixed blots, whereas staining with COU-1 was observed only on the unfixed blot. Our earlier immunohistochemical studies showed that ethanol fixation of tissue sections had no effect on the COU-1 antigen. To confirm that the COU-1 epitope was not affected by the ethanol treatment, we simulated the staining of Western blots by dot blots of the cancer lysate with or without fixation and found staining with COU-1 on both. The conclusion from this series of experiments is that indeed formation of cytokeratin heterodimers takes place during the processing of the Western blot and that such heterodimer formation by partially truncated cytokeratin is required for the formation of the COU-1 epitope.

Affinity of COU-1 for Truncated and Intact Heterotypic K8/K18 Complexes—COU-1 binding to the different recombinant heterotypic K8/K18 complexes was estimated by ELISA. Purified recombinant fragments of K8 or intact K8 were mixed with purified recombinant fragments of K18 or intact K18 in a molar ratio of 1:1 in urea. The samples were then dialyzed against PBS to allow the formation of the heterotypic complex and coated at 5 μg/ml on ELISA plates. Intact K8 was combined with K18-(1–124), K18-(1–187), K18-(1–213), and intact K18. In addition, intact K18 was combined with K8-(1–65), K8-(1–85), K8-(1–129), and K8-(1–233). In accordance with the results from Western blot analysis, COU-1 bound with various intensity to all the complexes, except to K8-(1–65)/intact K18 and intact K8-(1–85)/intact K18. Fig. 10 shows the titration of COU-1 on three of the heterotypic complexes, demonstrating significantly stronger binding to the fragmented K8/K18 than to intact K8/K18 complexes.

We extended this inquiry by measuring the kinetic parameters for the binding of COU-1 to different recombinant heterotypic K8/K18 complexes by real time biospecific interaction analysis using the surface plasmon resonance technique. COU-1 exhibited high affinity binding to the heterotypic complexes of K8-(1–124)/intact K18 and K8-(1–124)/K18-(1–124). The kinetic parameters for K8-(1–124)/intact K18 were \( k_{\text{on}} = 1.7 \times 10^5 \text{M}^{-1} \text{s}^{-1}, k_{\text{off}} = 1.2 \times 10^{-4} \text{s}^{-1} \), with derived association (\( K_a \)) and dissociation constants (\( K_d \)) of 1.4 \times 10^5 \text{M}^{-1} and 7.1 \times 10^{-10} \text{M}. The binding of COU-1 to K8-(1–124)/K18-(1–124) was slightly lower with \( k_{\text{on}} = 2.8 \times 10^5 \text{M}^{-1} \text{s}^{-1}, k_{\text{off}} = 3 \times 10^{-4} \text{s}^{-1} \).

FIG. 7. Generation of the COU-1 epitope. PVDF membrane blots containing SDS-PAGE-separated C-terminally deleted K18 or K8 fragments were incubated with different purified C-terminally deleted K8 or K18 fragments to allow the formation of K8/K18 complexes prior to staining with COU-1. A–C, the blots with recombinant K18 fragments deleted C-terminally in the proximity to the COU-1 epitope, K18-(1–72), K18-(1–124), K18-(1–187), and intact K18 were incubated with two different K8 fragments deleted C-terminally in the proximity to the COU-1 epitope, K8-(1–129) (A), K8-(1–233) (B), or intact K8 (C). The epitope recognized by COU-1 is absent or only minimally exposed on K18-(1–124)/intact K8, K18-(1–124)/K8-(1–233), or intact K8/intact K8. In contrast, strong binding of COU-1 was observed to K18-(1–124)/K8-(1–129). D–F, in the reverse setup, blots with K8 fragments C-terminally deleted in proximity to the COU-1 epitope K8-(1–85), K8-(1–129), K8-(1–233), and intact K8 were incubated with the fragments of K18 deleted in the proximity to the COU-1 epitope K18-(1–124) (D), K18-(1–187) (E), and K18-(1–213) (F). The epitope recognized by COU-1 was equally exposed when K8 fragment 1–129 was complexed with K18-(1–124), K18-(1–187), or K18-(1–213). No COU-1 binding was observed to any heterotypic complexes containing K8-(1–85) or K18-(1–72). However, in contrast to the results with the murine K8 and K18 mAbs, no COU-1 binding was observed to any of these N-terminally deleted heterotypic K8/K18 complexes, indicating that the COU-1 epitope was located within the N-terminal 129 residues (data not shown). Because the N-terminal sequencing data and the recombinant mapping data indicated that the COU-1 epitope were well exposed when the first 65 amino acids of K8 and the first 49 amino acids of K18 were missing (Fig. 8), we generated two additional N-terminally deleted fragments, K8-(66–483) and K18-(50–430) as GST fusion proteins. As shown in Fig. 9, significantly stronger COU-1 binding was observed to K8-(66–483)/K18-(50–430) and K8-(66–483)/intact K18 than to intact K8/K8-(50–430) or intact K8/intact K18. Similar COU-1 binding was achieved with C- and N-terminally truncated K8/18 fragments when the GST was removed from the fusion proteins by thrombin cleavage (data not shown).

The COU-1 Epitope Is Not Exposed following K18 Cleavage by Adenovirus Protease—The adenovirus L3 23-kDa protease promotes specific cleaving of the N-terminal domain of K18, while leaving K8 intact in adenovirus-infected HeLa cells (33, 34). This cleavage results in the removal of region 1–73 of the head domain of K18 and the disassembly of the cytokeratin network into spheroid globules. Cytokeratin K8/K18 from
In contrast, COU-1 exhibited an 100-fold lower binding to intact K8/intact K18 with $k_{on} = 9.1 \times 10^3 \text{ M}^{-1} \text{s}^{-1}$, $k_{off} = 5.0 \times 10^{-5} \text{ s}^{-1}$ and $K_a$ and $K_d$ of $1.8 \times 10^7 \text{ M}^{-1}$ and $5.5 \times 10^{-8} \text{ M}$, respectively.

Probing the Cellular Distribution of Truncated Heterotypic K8/K18 Complexes Using Confocal Microscopy—To evaluate the cellular distribution of normal K8 and K18 compared with truncated K8/K18 heterotypic complexes, breast and colon cancer cell lines (MCF-7 and colo137) were costained with COU-1 and either mAb anti-K8 or anti-K18 and analyzed by high resolution confocal microscopy (Figs. 11 and 12). The mela-
noma cell line M21, known not to contain K8, K18, or K19, was included as control. Anti-K8 and anti-K18 both stained long fibers of IFs forming complex interconnecting networks in both adenocarcinoma cell lines. The fibers emanate from a perinuclear ring, from which the filaments appear to connect to the nuclear surface and extend throughout the cytoplasm, terminating at the plasma membrane. In contrast, COU-1 exhibited a speckled pattern in the two adenocarcinoma cell lines, with staining of short filament fragments and rod-like particles, indicative of fragmented IF. No staining with any of the three antibodies was observed in the melanoma cell line.

Examination of the staining pattern of MCF7 cells within

FIG. 11. Subcellular distribution of N-terminally truncated K8/K18 recognized by COU-1 and intact K18 recognized by a murine anti-K18 antibody. Ethanol-fixed MCF7 breast cancer cells were incubated with COU-1 (A and E) and anti-K18 (B and F). Bound COU-1 was detected with FITC-goat anti-human κ-chain antibody (green) and bound anti-K18 with Texas Red goat anti-mouse IgG antibody (red). Differential interference contrast images (D and H) were included to visualize the composition of the cells. Partial colocalization, as visualized by yellow in the merged images (C and G), was observed between the two antibodies. N-terminally truncated K8/K18 complexes identified by COU-1 were predominantly found in newly formed, proliferating cancer cells (arrows), whereas stable K18 structures were present in equal quantities in all cells (arrowheads).

FIG. 12. Subcellular distribution of N-terminal-truncated K8/K18 recognized by COU-1 and K18 recognized by a murine anti-K18 antibody in MCF7 breast cancer cells. Cells were processed and stained by COU-1 (A and E) and anti-K18 (B and F) as described in Fig. 11. Differential interference contrast images (D and H) were included to visualize the composition of the cells. Whereas whole intermediate filaments were stained with anti-K18 (arrowheads), COU-1 (arrows) only stained short fibrils and globular structures. Some colocalization of the two antibodies, as visualized by yellow in the merged images (C and G), was observed.
cell clusters revealed that only the peripheral, newly formed, proliferating cells were strongly positive for COU-1, whereas all cells were stained with K8 and K18 mAbs (Fig. 11). Within the proliferating cells of a cluster, COU-1 staining was most prominent at the cell surface facing away from the cluster. In contrast, anti-K8 and anti-K18 stained the intermediate filamentous network throughout the cells. The speckled COU-1 staining was seen in close association to the intact intermediate filament network, as determined by overlay of images stained with COU-1 and anti-K8 or anti-K18 (Fig. 12).

**DISCUSSION**

In this study, we investigated the composition of cytokeratin heterotypic complexes found in malignant and normal glandular epithelia. N-terminally truncated forms of K8/K18 complexes were identified only in cancerous epithelia, whereas intact K8/K18 complexes were observed in both normal and cancerous simple glandular epithelia. Breakdown products of K8 and K18 in carcinomas have been observed by others (22, 35–37), but a detailed biochemical analysis of these breakdown products and their possible functional role in cancer cells has not been undertaken. Our finding that K8/K18 is cleaved in the N-terminal part of the two proteins at identical sites in cancers from different patients suggests that specific proteases are involved. We have found only one other study where K8 or K18 fragments from cancers were analyzed by N-terminal sequencing. That study identified truncated K8 missing the first 75 amino acids in a lung cancer, i.e., a truncation identical to one of those we observed (36). Examination of the amino acid sequences at the cleavage sites on K8 and K18 revealed some homology. The cleavage sites on amino acids 22 and 40 on K8, and at amino acid 50 on K18, all contained the (S/F/V)XSR ↓X(S/V) consensus sequence, suggesting that the enzyme responsible for these cleavages is a trypsin-like protease. Analysis of the amino acid sequences in the vicinity of the cleavage sites revealed one other site on K8 that had the same general sequence (amino acid 32, GSR ↓ I) but was not cleaved. This suggests that the amino acids at P3 or P1′ positions of the substrate are also influencing the recognition by this protease. We were unable to identify a consensus sequence (TAV ↓ T, SPL ↓ V, and TG1 ↓ A) at the three remaining cleavage sites on K8 and K18. A protease that requires less stringent recognition conditions or several different proteases may be responsible for these cleavages. A possible candidate may be elastase, which accepts valine, leucine, and isoleucine in the P1 position. The possibility that these K8 and K18 fragments were generated during the purification of cytokeratin from the tissue samples cannot be excluded, but several observations indicate that this is unlikely. Degradation of cytokeratins by Ca2+-activated proteases, which on two-dimensional gel electrophoresis appears as a typical “staircase” pattern, has been described when nucleases were used for cytokeratin purification (37). However, nucleases were not used in our purification, and a mixture of five enzyme inhibitors was present at all times. Moreover, cytokeratin fragments were not observed following purification of cytokeratin from normal colon epithelia using identical purification conditions. Furthermore, COU-1, which only recognizes the truncated form of K8/K18, as discussed below, detects its epitope in cancerous but not in normal epithelia when tissue samples are minimally handled and immediately fixed.

In contrast to the earlier views, the maintenance of the cytokeratin network in epithelial cells is now known to be a dynamic process involving constant restructuring by assembly and disassembly of intermediate bundles (38). Microinjection of biotin-labeled cytokeratin or transfection with fluorescence-labeled cytokeratin has demonstrated an inward-directed flow of diffuse material at the cell periphery moving in the form of dots and thin filaments toward the deeper cytoplasm, where it coalesces with other filaments and filament bundles (39). Although this process occurs in both normal and malignant epithelial cells, our results and those of others (40) indicate the presence of a second degradation pathway specific for cancer cells. This pathway may be ubiquitin-dependent.

A second finding of this study was that a human antibody, COU-1, cloned from a tumor-draining lymph node of a colon cancer patient, specifically recognizes the N-terminal truncated form of K8 and K18 when the two cytokeratins form a heterotypic complex. Previous analyses (26, 41) of COU-1 reactivity were interpreted to indicate selective reaction with K8. The COU-1 epitope, in addition to being localized intracellularly, is also found on the cell surface of cancer cells (25). COU-1 as well as recombinant Fab COU-1 were internalized upon binding to the cells. Also other studies (24, 42, 42–44) have found modified K8 and K18 on the surface of cells and shown that they may act as putative receptors or cofactors to cellular receptors. Modified K8 exposed on the surface of breast cancer cells were thus found to bind plasminogen and tissue plasminogen activator (24, 42, 44), whereas K18 exposed on the surface of hepatocytes might act as a receptor for thrombin-antithrombin complexes (43).

Increased levels of intact and fragmented K8/K18, initially identified as tissue-polypeptide antigen, have been reported in sera of cancer patients, and several clinical studies have found increased levels of tissue-polypeptide antigen in various cancers, including colon, breast, and prostate (45–47). Furthermore, a kit (CYFRA 21-1, Roche Diagnostics) is currently being marketed in some parts of Europe for specific detection of a lung cancer-associated K19 fragment (48, 49). The assays used for these measurements have employed antibodies that recognize both the intact and the fragmented forms of K8, K18, and K19. It seems likely that the use of an antibody, such as COU-1, that specifically recognizes the cancer-associated cleaved K8/K18 would increase the specificity of such assays. It has been assumed that the circulating cytokeratin fragments were a result of tumor cell necroses, but intact and fragmented K8 and K18 are released by non-necrotic adenocarcinoma cell lines into the tissue culture media (22, 50). This suggest that K8/K18 are proteolytically cleaved in viable cancer cells and that active release of fragmented K8/K18 occurs, although a pathway for the export of these cytoplasmic proteins has not been identified. In agreement with this we found labeling of viable cells by COU-1. Costaining of cancer cells with COU-1 and conventional anti-K8 and anti-K18 demonstrated that COU-1 stained various fibrillar and spheroid structures closely associated with the CK8/CK18 network. Similar abnormal cytokeratin staining pattern was found by Bader et al. (12) when cytokeratin-devoid mouse 3T3-L1 cells were stably or transiently transfected with cDNA encoding K8 or K18 without the N-terminal head domain. The head and the tail domains are not thought to be part of the filamentous backbone but to protrude laterally and contribute to profilament and IF packing and to interact with other cellular components (10–12) Cytokeratins lacking the head and tail domains are capable of coiled-coil and higher order lateral interactions but are deficient in filament elongation (13, 51, 52). Thus, the headless K8 and K18 formed varying proportions of fibrillar and granular structures, including dispersed granules.

Proteolytic cleavage of K18 in association with apoptosis has been reported (55). However, the cleavage sites for the apoptotic proteases, caspase-3, -6, and -7, are located in the conserved L1–2 linker and in C-terminal tail domain and are quite distant to the N-terminal cleavage sites we have found in viable tumor tissue. Recently, an antibody (M30) was reported to
recognize a neoepitope only exposed in apoptotic cancer cells and not viable or necrotic cells (54). This neoepitope is exposed when the C-terminal tail domain was liberated after cleavage by caspase-3, -6, or -7 into 22-, 21-, and 19-kDa fragments. The cleavage sites observed in colon cancer cells are also different from the one reported for adenovirus-infected HeLa cells, where the 73 most N-terminal amino acids of K18 were removed (33, 34). Surprisingly, no COU-1 binding to cleaved K8/K18 heterotypic complexes from infected HeLa cells was observed, whereas COU-1 bound K8/K18 complexes where the 67 most N-terminal amino acids of K18 were removed. This suggests that additional removal of 6 amino acids may cause conformational changes that prevent COU-1 from binding. Finally, changes in the K8/K18 heterotypic network have been observed in Mallory bodies, which are characteristic cytoplasmic inclusions in hepatocytes associated with alcoholic hepatitis and other chronic hepatocellular injuries including cholestasis and various metabolic disturbances (55–57). The Mallory bodies consist predominantly of K8 and variable amounts of K18 assembled in a nonfilamentous manner as well as keratin components, such as the Mø120-1 antigen and ubiquitin. A murine mAb K54-5 that specifically recognizes a neoepitope as a result of these K8/K18 conformational changes has been reported (58). This epitope was present on both individual native K8 and K18 and only available for antibody binding when K8 and K18 were dissociated and thus seems to be distinct from to the COU-1 epitope.

In conclusion, our biochemical analyses show that a unique conformational neoepitope is generated in viable adenocarcinoma cells, but not in normal epithelia cells, upon specific proteolytic cleavage of the K8/K18 complex. The epitope is specifically recognized by a human monoclonal antibody COU-1 isolated from a cancer patient, which is one of the few human antibodies that have made it into clinical use, and showed successful imaging of tumors in colon cancer patients. The identification of the unique epitope elucidates the pathogenic selectivity of the antibody. An increasing body of evidence indicates that K8/K18 is intimately associated with cell migration and invasiveness, and fragmentation of K8/K18 may influence these processes. The missing N-terminal head domain of K8/K18 contains several important phosphorylation sites, including Ser-33 and Ser-52 on K18, which has been associated with filament reorganization and distribution and important for K18 binding to 14-3-3 proteins (59, 60). The biological implications of the N-terminal cleavage of K8/K18 and the identification of proteases involved in this process warrant further study.

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