# Identification of Protein IT of the Intestinal Cytoskeleton as a Novel Type I Cytokeratin with Unusual Properties and Expression Patterns

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*Abstract.* A major cytoskeletal polypeptide ($M_r$ ~46,000; protein IT) of human intestinal epithelium was characterized by biochemical and immunological methods. The polypeptide, which was identified as a specific and genuine mRNA product by translation in vitro, reacted, in immunoblotting after SDS-PAGE, only with one of numerous cytokeratin (CK) antisera tested but with none of many monoclonal CK antibodies. In vitro, it formed heterotypic complexes with the type II CK 8, as shown by blot binding assays and gel electrophoresis in 4 M urea, and these complexes assembled into intermediate filaments (IFs) under appropriate conditions. A chymotrypsin-resistant $M_r$ ~38,000 core fragment of protein IT could be obtained from cytoskeletal IFs, indicating its inclusion in a coiled coil. Antibodies raised against protein IT decorated typical CK fibril arrays in normal and transformed intestinal cells. Four proteolytic peptide fragments obtained from purified polypeptide IT exhibited significant amino acid sequence homology with corresponding regions of coils I and II of the rod domain of several other type I CKs. Immunocytochemically, the protein was specifically detected as a prominent component of intestinal and gastric foveolar epithelium, urothelial umbrella cells, and Merkel cells of epidermis. Sparse positive epithelial cells were noted in the thymus, bronchus, gall bladder, and prostate gland. The expression of protein IT was generally maintained in primary and metastatic colorectal carcinomas as well as in cell cultures derived therefrom. A corresponding protein was also found in several other mammalian species. We conclude that polypeptide IT is an integral IF component which is related, though somewhat distantly, to type I CKs, and, therefore, we propose to add it to the human CK catalogue as CK 20.

In epithelial cells, a major element of the cytoskeleton is represented by IFs formed by the cytokeratins (CKs),[1] the largest and most complex group within the multigene family of intermediate-sized filament (IF) proteins (16, 17, 21, 24, 28–31, 65, 83–86). Besides the 10 different CKs that are typical of hair- and nail-forming cells ("trichocytes") but also occur in certain papillary appendages of the tongue and some thymic reticulum cells (also called "hard α-keratins"; e.g., 9, 41), 19 different CK polypeptides have so far been distinguished in human epithelial cells that are expressed in different combinations in the various pathways of epithelial differentiation during development and in epithelial tumors (24, 31, 65, 66, 74, 83–86). These epithelial CKs, also referred to as "soft keratins", can be generally divided into two subfamilies, the type I and the type II CKs (9, 12, 29–31, 73–75, 82–86). In mammals, all type I CKs identified so far have turned out to be more acidic than the members of the type II subfamily (21, 24, 65, 82) but this difference of charge is obviously not a necessary principle as shown from recent analyses of fish CKs (61). The basic principle of CK filament formation seems to be the requirement of two chains of each type I and type II CK polypeptides to constitute the heterotypic tetramer as the predominant, if not exclusive building unit, both in vivo and in vitro (2, 9, 12, 25, 33, 39, 40, 73–75, 83, 85, 91).

While in stratified epithelia complex sets of CK "pairs" (for term see 19, 85) have been found which are typical of certain cell types in these tissues (24, 65, 66, 74, 85, 86), mainly four CK polypeptides have so far been identified in the various kinds of single-layered ("simple") epithelia, i.e., the two type I CKs 18 and 19 and the two type II polypeptides 7 and 8 (19–22, 24, 65, 66, 73, 82, 86). Basically, CK expression in simple epithelial cells can be reduced to three major patterns: 8:18; 8:18, 19; 7, 8:18, 19 (for the rarely occurring pattern 7, 8:18, see 10, 87). In the cells of the small intestine and colon of higher vertebrates, one of the most studied tissues, the three CKs 8, 18, and 19 have consistently been found (22, 65, 66, 71, 92). In addition, however, we had consistently noticed, in our preparations of intestinal cytoskele-

1. *Abbreviations used in this paper*: CK, cytokeratin; IF, intermediate-sized filament.

tons, a high salt buffer- and detergent-resistant polypeptide of $M_r$ ~46,000 and an isoelectric point (in 9.5 M urea) of pH ~6.1 which we designated "protein IT" because of its striking occurrence in intestinal mucosa (36, 63, 65, 67). Subsequently, we have found this protein also as a consistent component of the IF cytoskeleton of Merkel cell tumors of the skin but not in other neuroendocrine tumors (64). Although this polypeptide showed some similar biochemical properties as the known CKs we could not decide on its possible cytokeratinous nature because it did not react with a large number of established CK antibodies, nor with antibodies broadly cross-reacting between diverse IF proteins such as that described by Pruss et al. (72), and because we were unable to formally exclude a proteolytic derivation from CK 8. Therefore, we have characterized this protein in greater detail by purification and IF reconstitution, partial amino acid sequencing, in vitro translation of mRNAs, and with specific antibodies. We now present evidence that protein IT represents a hitherto unrecognized type I CK present in several kinds of cells of the simple epithelium type.

## Materials and Methods

### Tissues and Cells, and Preparation of Cytoskeletons

In most biochemical studies, cytoskeletal preparations of human intestinal mucosa were used. Fresh human duodenum obtained immediately after duodeno-pancreatectomy (performed for removal of pancreatic carcinomas) was cut open, rubbed off gently with cellucotton, and mucosal villi were scraped off with controlled manual pressure using a spatula. The tissue material thus obtained was immediately frozen in liquid nitrogen and stored at −80°C. In some experiments, specimens from the jejunum, ileum, or colon were used, but these contained lesser amounts of protein IT. From the scraped tissue material, cytoskeletal preparations were made (1) by homogenization with a Polytron homogenizer (Kinematica, Luzern, Switzerland), and extraction for up to 20 min with 5 vol of 10 mM Tris-HCl buffer (pH 7.2) containing 1.5 M KCl, 0.5% Triton X-100, 5 mM EDTA, 0.4 mM PMSF, followed by centrifugation (10 min, 13,000 *g*) and two washes by resuspension in 5 vol of 10 mM Tris-HCl (pH 7.2) containing 5 mM EDTA and 0.4 mM PMSF, using a Dounce homogenizer (Kontes Glass Co., Vineland, NJ), and recentrifugation. All steps were carried out on crushed ice or in a cold room of ~4°C. The resulting cytoskeletal pellet was stored at −80°C.

For comparisons, various other normal and malignant human tissues were obtained during surgery or, in a few cases, from autopsies. Pig tissues were obtained at a local slaughterhouse, rat and mouse tissues from healthy laboratory animals. Tissue samples were immediately frozen in isopentane precooled in liquid nitrogen to ~−140°C or directly in liquid nitrogen. Of all tissues used, aliquot samples were routinely processed for fixation and paraffin embedding, followed by pathohistological evaluation. Furthermore, the identity of the tissues used was assured by hematoxylin and eosin staining of cryostat sections consecutive to those used for the experiments.

Athymic ("nude") mouse transplantation tumors CX-1, CXF-87 (passage 10), and CXF-112 derived from human adenocarcinomas of the colon (CX-1 is derived from HT-29 cells; see below) were obtained from the Tumor Bank of the German Cancer Research Center (Heidelberg, FRG), excised, and immediately frozen in liquid nitrogen (see reference 66).

The following established colorectal carcinoma-derived cell culture lines from the American Type Culture Collection (ATCC), Rockville, MD, were grown essentially as recommended: HT-29 (ATCC HTB 38; see reference 14); Caco-2 (ATCC HTB 37; 14); LoVo (ATCC CCL 229; 11); SW 1116 (ATCC CCL 233); DLD-1 (ATCC CCL 221); and COLO 320 DM (ATCC CCL 220). Cell cultures of the human bladder carcinoma cell line RT-4 (67, 79) and the human hepatocellular carcinoma cell line Hep-G2 (13) were also used. Cells of the human mammary carcinoma cell line MCF-7 were grown as described (see reference 65). In some experiments, cultured cells were metabolically labeled with [$^{35}$S]methionine (20). Cytoskeletal fractions of cultured cells were prepared essentially as described (1).

### Isolation and In Vitro Translation of RNA

Total cellular RNA and poly(A)$^+$-RNA were prepared from nude mouse transplantation tumors and HT-29 cell cultures and translated in vitro, using a reticulocyte lysate system as described (48–50). In some experiments, the translation products were immunoprecipitated, using guinea pig antibodies against either protein IT or both proteins IT and CK 18 that had been coupled, at 1:200 dilution, to protein A–Sepharose beads (Pharmacia, Uppsala, Sweden), by incubation of the beads with translation assay solution for 1 h at room temperature, followed by centrifugation of the beads and several washes. Total proteins of the translation assays or proteins immunoprecipitated therefrom were analyzed by two-dimensional gel electrophoresis and autoradiography.

### Gel Electrophoresis

SDS-PAGE and two-dimensional gel electrophoresis, using either IEF or NEPHG electrophoresis were as described (1). Second-dimension SDS-PAGE was usually performed at elevated ionic strength as described by Achtstätter et al. (1); in some experiments, the usual low salt buffer system was used.

In some experiments, ~20-μm-thick cryostat sections of intestinal mucosa were collected in the frozen state and rapidly immersed into boiling SDS-PAGE sample buffer (see also 22). After cooling and centrifugation, the proteins present in the supernatant were precipitated by addition of cold acetone (9 vol; −20°C), kept at −20°C overnight and, after centrifugation and drying, subjected to two-dimensional gel electrophoresis.

Two-dimensional gel electrophoresis under nondissociating conditions in the presence of 4 M urea was carried out as described (73) using tube gels (0.3 cm diam; 1 mA per tube) and gel system No. 11, except that 4 M urea was used instead of 10 M. Subsequently, the tube gels were subjected to second-dimension SDS-PAGE using the elevated salt-buffer system. Separations of type I and type II CKs with increasing urea concentrations ("melting") have been described (25, 39).

Proteins were visualized by staining with Coomassie brilliant blue R 250 and, in labeling experiments, with subsequent processing for fluoroautoradiography using Amplify™ (Amersham-Buchler, Braunschweig, FRG). When the protein loading was low, a silver staining method was used (for references see 1). Proteins separated by preparative SDS-PAGE were stained with 4 M sodium acetate (1).

### Preparation of Protein IT

Protein IT was isolated from cytoskeletal material from duodenal mucosa by preparative gel electrophoresis (SDS-PAGE) using the elevated salt buffer system (1). After cutting out the band containing protein IT, protein elution, and vacuum dialysis, a second preparative SDS-PAGE with the low salt buffer system was performed, and practically pure protein IT was isolated from the appropriate gel band.

Alternatively, protein IT was enriched from duodenal cytoskeletal material by chromatographic procedures (1, 39, 40). Briefly, the material was first subjected to DEAE-cellulose anion exchange chromatography in the presence of 8 M urea, using a linear gradient from 0 to 100 mM guanidinium hydrochloride for elution (1). Fractions of the first absorbance (280 nm) peak (at ~29 mM guanidinium hydrochloride) contained mainly CK 8. The first two peaks (38 and 41 mM) of a broad group of several poorly separated peaks contained protein IT but also CK 18 in approximately equal amounts. The proportion of protein IT decreased in the last peaks of this group. The last peak (at 53 mM guanidinium hydrochloride) contained mainly CK 19. The combined fractions of the peaks at 38 and 41 mM guanidinium hydrochloride were applied directly to reverse-phase HPLC using a 4.6 × 250 mm Hi-Pore RP 304 column (BioRad Laboratories, Richmond, CA). The proteins were eluted in a acetonitrile gradient (33–50% in 0.1% TFA) (1). After two small peaks containing pure CK 8, the main peak eluting at ~44% acetonitrile contained almost pure protein IT, with occasional trace contaminations of CK 8, its degradation products, or CK 18, as recognized by SDS-PAGE and immunoblotting.

### Immunoblotting and Antibodies

Immunoblotting of proteins separated by SDS-PAGE or two-dimensional gel electrophoresis was as described (1), usually without incubation in the 4 M urea-containing "protein refolding buffer."

As primary antibodies, a series of established monoclonal antibodies against CKs were used. For most of these antibodies (CAM 5.2; $K_G$ 8.13;

$K_s$pan 1-8.136; $K_s$ 8.1.42; $K_s$ 18.174; $K_s$ 18.27.IV; CK-2; $K_s$ 19.1; $K_s$ 19.2.105; KL 1), the CK polypeptide specificities, references, and sources have been listed in previous articles (10, 15, 36, 41, 47, 59, 67). In addition, the following murine monoclonal antibodies were also applied: antibody AE 1 recognizing several type I CKs (86; kindly provided by Dr. T.-T. Sun, New York University, New York); antibody KA 4 against CKs 14, 15, 16 and 19 (70; available from Triton Biosciences Inc., Alameda, CA); antibody PKK 1 reacting mainly with simple epithelial CKs (43; from Lab-Systems, Helsinki, Finland); antibody DAKO-CK1, corresponding to clone LP 34, a broad spectrum CK antibody (54; obtained from DAKOPATTS, Hamburg, FRG); the CK 18-specific antibodies CK-1, CK-3 and CK-4 (as CK-2; antibodies CK-1 and CK-3 were kindly provided by Dr. M. Osborn, Max-Planck-Institute for Biophysical Chemistry, Göttingen, FRG; CK-4 is available from Boehringer Mannheim GmbH, Mannheim, FRG) as well as RGE 53 (76). In addition, the CK 19-specific antibodies BA 16 and BA 17 were used (4; kindly provided by Dr. J. Bartek, Research Institute of Clinical and Experimental Oncology, Brno, Czechoslovakia). In other experiments, guinea pig antisera raised against bovine muzzle epidermal prekeratins (cf. 17, 18, 21, 22, 64), which react with many type I CKs, were used; in some experiments, such antisera were affinity purified on nitrocellulose paper strips containing CKs 14-17 as described later. Moreover, we used the monoclonal antibody IFA recognizing a common antigenic determinant of many though not all IF proteins (72; hybridoma cells obtained from ATCC; for reactivity see also 5, 8).

Immunoreactive bands and spots were visualized by incubation with peroxidase-conjugated rabbit antibodies against mouse or guinea pig immunoglobulins (DAKOPATTS), which was followed, after washing (see reference 1), by staining with a DAB solution in PBS containing $H_2O_2$ and 0.015% nickel-II-sulfate. Alternatively, $^{125}$I-labeled protein A was used instead of secondary antibodies, and the reaction was monitored by autoradiography (1). Total proteins present on the nitrocellulose filters used for blot transfer were stained with Ponceau-S red (Sigma Chemie, Munich, FRG; 1) or India ink (37). "Dot blot" assays with monoclonal antibodies, including lu-5 (89), were performed exactly as described (26).

### *Tryptic Peptide Map Analysis*

For peptide mapping, individual polypeptides separated by two-dimensional gel electrophoresis were excised, radiolabeled with [$^{125}$I]iodine, digested with trypsin and separated by combined electrophoresis and chromatography (for references see 65, 82).

### *Binding Assays and Proteolytic Cleavage Experiments*

Nitrocellulose blot-binding assays with radioiodinated purified rat CK polypeptide 8 (A) or human CK 18 were performed essentially as described (40, 59, 61).

Proteolytic digestion with chymotrypsin of cytoskeletal material containing intact IFs was also performed according to Hatzfeld et al. (40). Typical digestion patterns were obtained using enzyme-to-substrate ratios of 6.6-9.0:1,000 (wt/wt) and digestion times between 30 and 50 min at room temperature.

### *In Vitro Reconstitution of Heterotypic CK Complexes and IFs*

CKs 8, 18, and protein IT purified from duodenal mucosa cytoskeletons by chromatography, or native cytoskeletal material from duodenal mucosa, were dissolved in a 10 mM Tris-HCl buffer (pH 8.0) containing 2.5 mM DTT and 9.5 M urea. Insoluble material was pelleted at 13,000 *g*, and the supernatants were dialyzed at room temperature, either alone or in near-stoichiometric mixtures, against the same buffer but containing only 4 M urea. Aliquots of the resulting solution were cleared by brief low speed centrifugation and the supernatant processed for electrophoresis under nondissociating conditions in the presence of 4 M urea (see above).

For the in vitro reconstitution of CK filaments (see reference 39), purified CK 8 and protein IT were dissolved at 1 mg/ml in 10 mM Tris-HCl buffer (pH 8.0) containing 9.5 M urea and 2.5 mM DTT and dialyzed, alone or in an ~1:1 mixture, against 4 M urea containing 2.5 mM DTT and 10 mM Tris-HCl (pH 7.6) for 1 h and then for 2 h against 10 mM Tris-HCl (pH 7.6), 2.5 mM DTT ("floating membrane filters"; VS 0.025; Millipore Continental Water Systems, Bedford, MA). The resulting material was subjected to negative staining and examined by electron microscopy (39, 73).

### *Amino Acid Sequence Analysis*

Cytoskeletal proteins from duodenal mucosa were separated by SDS-PAGE using the precautions described by Walsh et al. (90). The proteins were stained for 30 min in 0.5% Coomassie brilliant blue R 250 in $H_2O$ under gentle shaking, then destained in $H_2O$. The protein bands were excised and stored at −20°C in a buffer containing 50 mM Tris-acetate (pH 8.5), 0.2% SDS, 1 mM DTT, 10% glycerol. Protein IT was electroeluted for 20 h in an elution device essentially as described by Hunkapiller et al. (45), and precipitated with cold (−20°C) acetone (9:1 [vol/vol]). Protein IT (30–40 μg) was then dissolved in 100 μl of a buffer containing 10 M urea, 100 mM Tris-HCl (pH 8.0), 5 mM DTT, 1 mM EDTA under gentle stirring for 2–4 h. The 10 M urea solution was diluted with 100 μl of buffer containing 100 mM Tris-HCl (pH 8.0), and 0.05% NP-40 (Fluka AG, Buchs, Switzerland). Digestion was started by the addition of 5 μg *Staphylococcus aureus* (V8) protease (ICN Biomedicals, Inc., Costa Mesa, CA) and carried out overnight at room temperature under shaking. For HPLC separation of peptides, a microbore HPLC system (model 130 A; Applied Biosystems, Foster City, CA) was used. The digest was acidified with 30 μl of 10% TFA in $H_2O$, loaded on a Vydac $C_{18}$ column (2.1 × 150 mm; The Separations Group, Hesperia, CA). The peptides were eluted at a flowrate of 150 μl/min and with the following acetonitrile gradient: buffer A: 0.1% TFA; buffer B: 80% $CH_3CN$, 0.085% TFA; gradient: 0–25 (20 min)–25 (10 min)–35 (15 min)–35 (10 min)–45 (10 min)–100% (25 min). Peptide-containing peaks were collected and directly applied to a filter pretreated with BioBrene (Applied Biosystems) and sequenced, using a pulse-liquid protein sequencer (model 477A; Applied Biosystems) with an on-line phenylthiohydantoin analyzer (model 120A, Applied Biosystems).

### *Preparation of Specific Antibodies Against Protein IT*

A guinea pig was immunized with protein IT enriched by preparative gel electrophoresis (only one electrophoresis step; see above) essentially as described by Franke et al. (19). The antiserum contained antibodies against protein IT as well as some antibodies to CK 18 (64). Antibodies against protein IT were isolated from the serum by cross-absorption of either the serum (diluted 1:40 in PBS containing 1% BSA and 0.1% sodium azide), or the Ig fraction from this serum (obtained by ammonium sulfate precipitation) using nitrocellulose strips containing purified CKs 8, 18, and 19 from human MCF-7 cells (alternatively, total MCF-7 cytoskeletal proteins were used in some experiments). Cross-absorption was performed by repeated incubations with the nitrocellulose strips that were intermittently regenerated by incubation first in 3 M KSCN (in PBS) and then in PBS. In some experiments, the affinity-purification step on nitrocellulose paper strips containing SDS-PAGE-separated protein IT (see reference 52) was used and here bound antibodies were eluted from the strips with 3 M KSCN (in PBS) and vacuum-dialyzed against PBS.

### *Immunocytochemistry*

Methods used for immunofluorescence, immunoperoxidase and immunoelectron microscopy of cryostat tissue sections and cultured cells were as described (3, 16–19, 22, 23, 26, 47, 49, 67).

## *Results*

### *Gel Electrophoresis and Immunoblotting*

Cytoskeletal proteins prepared from human intestinal mucosa consistently revealed, on two-dimensional gel electrophoresis (Fig. 1, *a* and *b*), the three major CKs 8, 18, and 19, together with a fourth major polypeptide spot previously designated IT (65). With an $M_r$ value of ~46,000 and a series of isoelectric variants, this component was positioned between CKs 8 and 18 (Fig. 1, *a* and *b*). Its major, most basic isoelectric variant had an isoelectric pH value (in 9.5 M urea) of ~6.1 and was slightly more acidic than the nonphosphorylated variant of CK 8. The next more acidic variant isoelectric at pH 5.8 was usually present in somewhat lower amounts (Fig. 1 *a*). A further, minor and more acidic variant was de-

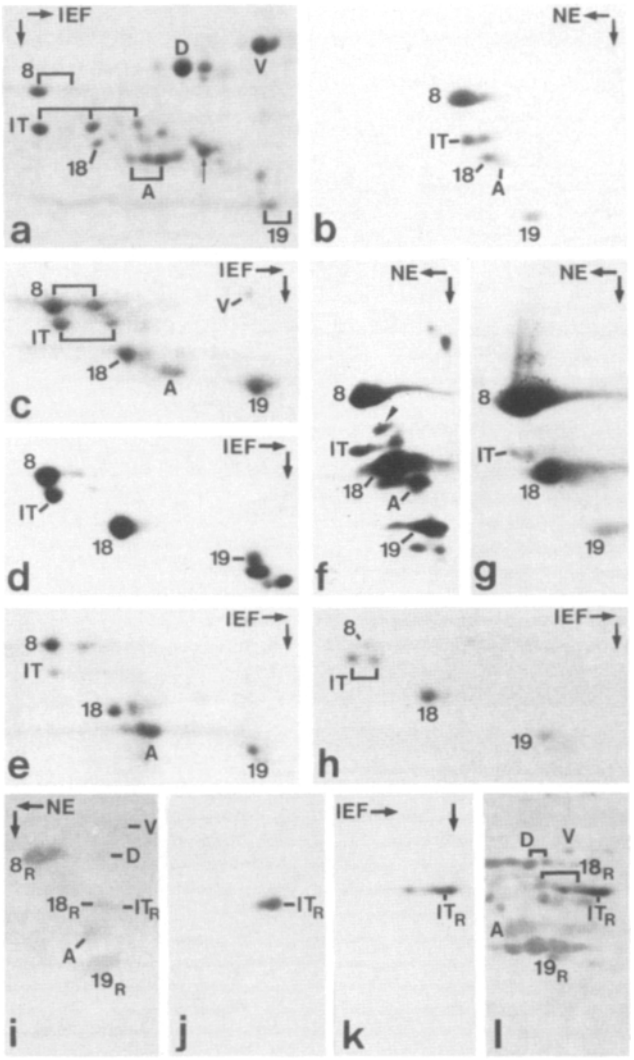


*Figure 1.* Identification of protein IT in normal and malignant intestinal epithelial cells (*a–h*, human; *i–l*, rat) after two-dimensional gel electrophoresis of cytoskeletal proteins using protein staining (*a–c*, *f*, *i*, and *l*), immunoblotting (*j* and *k*), in vivo labeling (*g*), and labeling during in vitro translation of mRNA (*d*, *e*, and *h*). IEF or NE (NEPHG electrophoresis) indicate the first dimension of separation (higher pH values are to the left); vertical arrows denote the direction of the second dimension SDS-PAGE, using either the elevated (*a*, *b*, *f*, *g*, *i*, and *j*) or the low (*c–e*, *h*, *k*, *l*) salt buffer system. Cytokeratins are designated by Arabic numerals (65). The thin arrow in *a* designates an unidentified nonkeratinous component; the arrowhead in *f* a degradation product of CK 8 (see reference 81); *A*, actin; *V*, vimentin; *D*, desmin from *lamina propria* and smooth muscle cells included in some preparations. (*a* and *b*) Electrophoresis of cytoskeletal proteins from human jejunal (*a*) and duodenal (*b*) mucosa (Coomassie blue staining), showing prominent amounts of protein IT. (*c* and *d*) Coelectrophoresis of cytoskeletal proteins from CX-1 colon carcinoma (*c*, Coomassie blue staining) and [$^{35}$S]methionine-labeled translation products immunoprecipitated after in vitro translation of the corresponding poly-A(+)-mRNA (*d*, fluoroautoradiograph). (*e*) Similar experiment as in (*c* and *d*), showing total (i.e., nonimmunoprecipitated) translation products from total CXF-87 RNA (fluoroautoradiograph). Note the identification of the major (basic) variant of polypeptide IT as a genuine translation product (*d* and *e*). (*f* and *g*) Cytoskeletal proteins of HT-29 cells (*f*, silver staining; *g*, fluoroautoradiograph of [$^{35}$S]methionine-labeled proteins). (*h*) Translation products immunoprecipitated after in vitro translation of total RNA from HT-29 cells (fluoroautoradiograph). Note the presence of an additional, more basic variant of IT protein. (*i–l*) Electrophoresis of cytoskeletal proteins of rat small intestinal mucosa and corresponding immunoblot experiments. Total blot-transferred proteins are shown in *i* (Ponceau S red staining) and *l* (India ink staining subsequent to immunoblot). The corresponding immunoblots, using guinea pig antibodies against human IT protein and visualization of bound secondary antibodies by immunoperoxidase staining (*j* and *k* corresponding to *i* and *l*, respectively) reveal a single positive protein ($IT_R$), which is slightly more acidic than the rat orthologue to CK 18 (CK $18_R$).

tected in some preparations but not in all. Patterns of tryptic peptide maps were essentially identical for these three isoelectric variants and lacked similarity with those of any of the established IF proteins such as CKs 8, 18, and 19, vimentin and desmin (not shown; see references 64–66, 82). This suggested that polypeptide IT was not merely a proteolytic product of other cytoskeletal proteins such as CK 8 which is known to be particularly vulnerable to such degradations (81).

Protein IT was present at the highest amounts relative to other cytoskeletal proteins in preparations of villi from small intestine, notably duodenum and jejunum, where it occurred in amounts only slightly lower than those of CK 8 and exceeding those of both CKs 18 and 19 (Fig. 1, *a* and *b*). Its proportion was lower in preparations from mucosa of the ileum and the colon which, in turn, contained more CK 19 (not shown; e.g., Fig. 2 *b* of reference 65). Polypeptide IT was also identified among the total duodenal mucosa proteins that had been instantly denatured by immersion of frozen sections into boiling SDS-PAGE sample buffer (data not shown).

Protein IT was also a major cytoskeletal protein in human colonic adenocarcinomas grown in nude mice (Fig. 1 *c*). When total RNA or poly-A$^+$-RNA from such tumors was translated in vitro and the resulting products immunoprecipitated by antisera against protein IT (see below) and analyzed by two-dimensional gel electrophoresis, the polypeptide IT spot was clearly identified (Fig. 1, *c* and *d*). This showed that protein IT is a genuine polypeptide encoded by a distinct mRNA. Remarkably, however, the amount of mRNA encoding polypeptide IT was lower than those of mRNAs encoding CKs 8, 18, and 19, as it appeared only as a minor translation product before enrichment by immunoprecipitation (Fig. 1 *e*).

Protein IT was also consistently found in cytoskeletal preparations from cultured human colon carcinoma cells of cell line HT-29 (Fig. 1 *f*; see also Fig. 2 *k* of reference 65). In such cells, however, an additional, more basic isoelectric variant (pH ~6.25) was often seen (cf. Fig. 1 *h*). When HT-29 cells were incubated with medium containing [$^{35}$S]methionine, polypeptide IT was significantly labeled but to a lesser extent than CKs 8 and 18 (e.g., Fig. 1 *g*). In vitro translation of mRNA from HT-29 cells also showed the presence of mRNA encoding polypeptide IT among the HT-29 mRNAs (Fig. 1 *h*).

A cytoskeletal protein with similar preparative and gel electrophoretic properties as human protein IT, which also

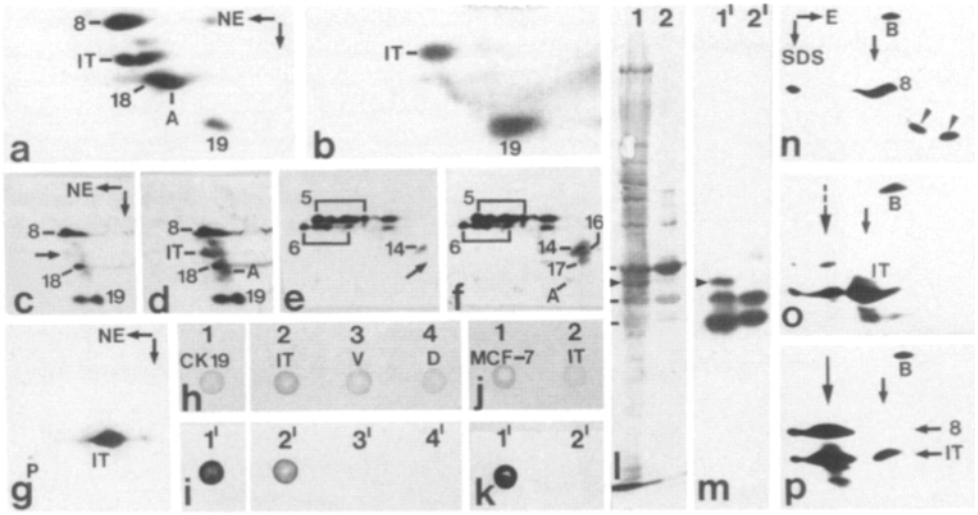


*Figure 2.* Characterization of human protein IT by immunoblotting (*a–k*), "blot-binding" assays (*l* and *m*), and native electrophoresis (*n–p*). (*a* and *b*) Two-dimensional gel electrophoresis of duodenal cytoskeletal proteins (*a*, Coomassie blue staining) and corresponding immunoblot after reaction with guinea pig antibodies against bovine snout CKs that had been affinity purified on CKs 14–17 (*b*, autoradiograph after secondary $^{125}$I-protein A binding). Note that these type I CK-specific antibodies react both with CK 19 and protein IT (*b*). (*c–f*) Immunoblot results of cytoskeletal proteins from duodenal mucosa (*c* and *d*) and hair follicle root sheaths (*e* and *f*) after separation by two-dimensional gel electrophoresis, transfer to nitrocellulose, and reaction with IFA antibody (*c* and *e*, immunoblot reactions, peroxidase staining; *d* and *f*, subsequent India ink staining). Note that this antibody is negative not only with protein IT (*arrow* in *c*; *d*) but also with CKs 16 and 17 (*arrow* in *e*; *f*), whereas CKs 8, 18, and 19 (*c*) and CKs 5, 6, and 14 (*e*) are positive. (*g–k*) Dot-blot assays (*h* and *j*, Ponceau S staining; *i* and *k*, corresponding immunoreaction, peroxidase staining) with antibodies lu-5 (*i*) and $K_s$ 18.174 (*k*), showing the positive reaction of electrophoretically purified protein IT (purity is demonstrated by gel electrophoresis in *g*; *P*, 3-phosphoglycerokinase from yeast, added for reference) with CK antibody lu-5 (dots *2* and *2'* in *h* and *i*; positive control: dots *1* and *1'*, purified CK 19; negative controls: dots *3* and *3'*, purified vimentin, and dots *4* and *4'*, purified desmin; see reference 26). In contrast, the CK 18-antibody $K_s$ 18.174 is negative with protein IT (dots *2* and *2'* in *j* and *k*; positive control: dots *1* and *1'*, showing total cytoskeletal proteins from MCF-7 cells). (*l* and *m*) Nitrocellulose blot-binding assay, using $^{125}$I-labeled CK 8 (A) from rat liver. The blot of SDS-PAGE-separated cytoskeletal proteins from human small intestinal mucosa (lane *1*) and MCF-7 cells (lane *2*) is shown by India ink staining in *l*; *m* shows the corresponding autoradiograph after incubation with labeled CK A in 4 M urea-containing buffer (40). The bars denote, from top to bottom, CKs 8, 18, and 19; the arrowheads designate protein IT. Note the positive reaction not only of CKs 18 and 19 but also of protein IT (*m*, *arrowhead*), indicating heterotypic complex formation. (*n* and *o*) Electrophoresis under nondissociating conditions in the presence of 4 M urea (*E*; from *left to right*), followed by SDS-PAGE (*SDS*) in the second dimension, of purified CK 8 (*n*), protein IT (*o*), and the complex formed by an approximately equimolar mixture of CK 8 and protein IT (*p*; silver staining). Note that in the mixture both proteins migrate in *E* to an identical position (*large arrow* in *p*), corresponding to a slowly migrating heterotypic complex, as compared to the faster migration of each of the individual polypeptides (*small arrows* in *n* and *o*). Minor polypeptide spots probably represent degradation products of CK 8 (*n*, *arrowheads*) or protein IT (*o*, *bottom spots*) or both (*p*). The hatched arrow in *o* designates a trace of contamination of CK 8 included in a complex with protein IT; the small vertical arrow in *p* points to some protein IT not included in the complex. *B*, BSA added for reference. For further designations, see Fig. 1.

reacted with the antibodies specific for the human protein (see below, Fig. 4, *d* and *e*), was found in intestinal mucosa of the rat (Fig. 1, *i–l*). Relative to the rat ortholog of human CK 18 (*18$_R$* in Fig. 1, *i–l*; component D; see reference 19, 20, 22), the IT protein ortholog (*IT$_R$*) was more acidic (pH ~5.35 in the system used) and migrated slightly faster in SDS-PAGE, corresponding to $M_r$ ~46,000 (Fig. 1, *i–l*).

A series of immunoblotting experiments was performed in order to assess the relationship of protein IT to CKs. Only one guinea pig antiserum raised against the type I CKs VIa,b from bovine muzzle epidermis, which in immunoblotting reacted with most human type I CKs (including CKs 10, 11, 13, 14, 16, 17, and 19, but not 18) but with none of the type II CKs (including CKs 4–8) showed a significant and strong reaction with protein IT (see also 64). After affinity purification on nitrocellulose paper strips containing CKs 14–17 from human hair follicles, the remaining antibodies were still positive for polypeptide IT in immunoblotting experiments using duodenal cytoskeletal proteins (Fig. 2, *a* and *b*). While this result suggested, but did not prove, a true cross-reactivity (i.e. very similar epitope[s]) between type I CKs and polypeptide IT, a large series of established monoclonal antibodies against various CKs of type I and type II did not react with protein IT. These negative antibodies included not only several broad spectrum CK antibodies ($K_G$ 8.13, $K_s$pan 1-8.136, KL 1, LP 34, PKK 1) but also the monoclonal antibodies AE 1 and KA 4, both known to react with several type I CKs, and monospecific antibodies to CKs 8 ($K_s$ 8.1.42 and CAM 5.2), 18 (e.g., CK-1, CK-2, CK-3, CK-4, RGE 53, $K_s$ 18.174, and $K_s$ 18.27.IV), 19 ($K_s$ 19.1, $K_s$ 19.2.105, BA 16, and BA 17), 13 (1C7, 2D7; see reference 88), and to an epitope common to 13, 14, and 16 ($K_s$ 13.1; see reference 67).

Monoclonal antibody IFA (72) whose epitope is located at the COOH-terminal end of the α-helical rod domain that is common to most IF proteins (34, 60), strongly reacted, in immunoblots of duodenal cytoskeletal proteins, with CKs 8, 18, and 19 but was negative with protein IT (Fig. 2, *c* and *d*). One should note, however, that certain established type I CKs such as components 16 and 17 also did not react with this antibody under our experimental conditions (Fig. 2, *e* and *f*; see also reference 8).

The widely used monoclonal antibody lu-5 has been shown to react, in dot-blot assays, with a wide range of type I and type II CKs but not with other IF proteins (26),

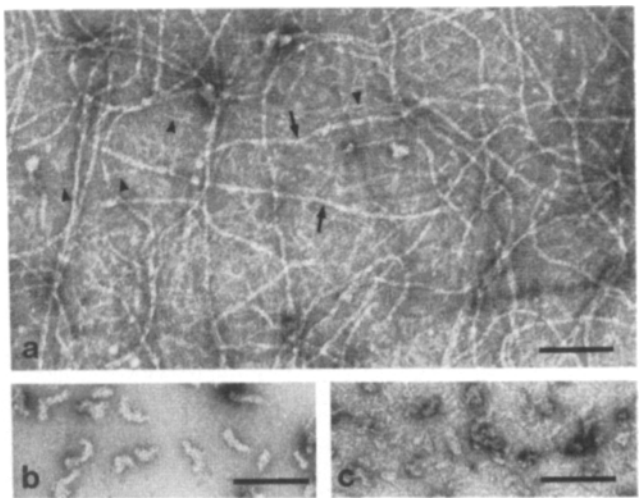


*Figure 3.* Electron micrographs showing typical IFs (*arrows*) reconstituted from an equimolar mixture of purified human CK 8 and protein IT (*a*, negative staining). Note also some loosely arranged protofilaments in the background (*arrowheads*). In control experiments with only CK 8 (*b*) or protein IT (*c*) coarse rodlike aggregates are seen but no IFs. Bars, 0.1 μm.

whereas it was negative in immunoblots of SDS-PAGE-separated CKs. Therefore, we purified protein IT by two-step preparative gel electrophoresis, to avoid any contamination with CKs 8 or 18 (for purity, see Fig. 2 *g*), and applied the protein partially refolded in buffer containing 4 M urea to the same dot-blot assay. As shown in Fig. 2, *h* and *i*, antibody lu-5 reacts with protein IT just like with other CKs, but not with vimentin and desmin (see reference 26). In a control dot-blot assay with antibody $K_s$ 18.174, cytoskeletal proteins containing CK 18 were strongly positive whereas protein IT was negative (Fig. 2, *j* and *k*), which also showed that no contaminating CK 18 was present in our IT protein preparations.

### *Molecular Assembly Studies*

Hatzfeld et al. (40) have established an in vitro binding assay which allows to identify and distinguish type I and type II CKs by the heterotypic binding of a radiolabeled solubilized CK to a blot-immobilized CK (for examples also see references 59, 61). When gel electrophoretically separated, cytoskeletal proteins from small intestinal mucosa were transferred to nitrocellulose paper (Fig. 2 *l*) and incubated with purified, $^{125}$I-labeled rat CK 8 (A; see references 19, 20, 40), positive reactions indicating specific binding were observed not only for CKs 18 and 19, as expected, but also for polypeptide IT (Fig. 2 *m*). Although in such tests the reactivity of protein IT was somewhat weaker than those of CKs 18 and 19, it was clearly specific as all the other proteins present, including CK 8 (Fig. 2 *l*), were completely negative (Fig. 2 *m*). Corresponding results were obtained in similar assays, using two-dimensional gel electrophoresis (data not shown). In the complementary assay with radioiodinated CK 18, only CK 8 was reactive but not the other proteins, including the type I CKs 18 and 19 and protein IT (data not shown). Thus, in this assay, protein IT behaved like a type I CK.

We then tested whether polypeptide IT was able to form correct heterotypic complexes with type II CKs in solution. The original approach for the analysis of the formation of heterotypic CK complexes and their melting behavior, which implies two-dimensional gel electrophoresis at different urea concentrations and is based on differences of isoelectric points between the individual CKs (25), was not feasible in the case of polypeptide IT as its isoelectric point at 9.5 M urea is very similar to that of its putative complex partner, CK 8 (Fig. 1, *a–h*). Alternatively, we therefore performed "native complex electrophoresis" of a near-stoichiometric

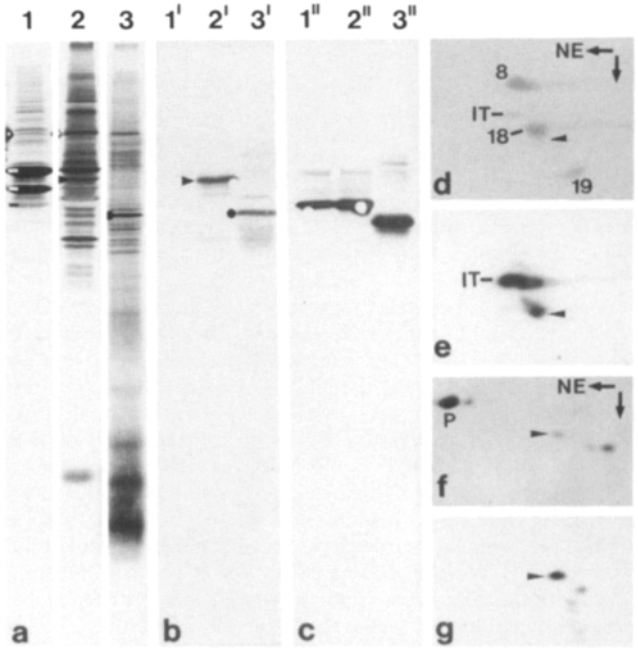


*Figure 4.* Gel electrophoresis of polypeptide fragments produced by chymotryptic digestion of human duodenal cytoskeletal material (*a* and *f*), combined with immunoblotting using guinea pig antibodies against protein IT (*b* and *g*; antibody characterization: *d* and *e*) and a murine CK 19 antibody (*c*). (*a*) SDS-PAGE (15% acrylamide; silver staining) of proteins of intact cytoskeletal material from MCF-7 cells (lane *1*; bars, from *top to bottom*, denote CKs 8, 18, and 19; the latter appears underrepresented in the photograph due to its yellow color) and from duodenal mucosa (lane *2*; crude cytoskeletal preparation; *arrowhead* designates protein IT; *small white dots* denote the position of BSA), in comparison with chymotryptic digestion products derived from duodenal mucosal cytoskeletons (lane *3*). (*b* and *c*) Corresponding immunoblots (peroxidase staining) using antibodies against protein IT (*b*) and, for comparison, antibody $K_s$ 19.2.105 against CK 19 (*c*). Note the specific reaction of the former antibodies only with protein IT (*b*, lane *2'*, *arrowhead*) and primarily with one major chymotryptic fragment (*dot* in *b*, lane *3'*; corresponding to the *dot* in *a*, lane *3*; $M_r$ ~38,000) that is slightly larger than the chymotryptic rod fragment of CK 19 (*c*, lane *3"*). (*d–g*) Two-dimensional gel electrophoresis of HT-29 cytoskeletal proteins (*d*, Ponceau S red staining after transfer to nitrocellulose) and of chymotryptic digestion products from duodenal mucosal cytoskeletons (*f*, showing several spots of polypeptides more acidic than actin; Coomassie blue staining) and corresponding immunoblots (peroxidase staining) using antibodies against protein IT (*e* and *g*). Note that these antibodies specifically react with protein IT (*e*), a minor degradation product derived therefrom (*arrowheads* in *e* and *d*) and one major distinct $M_r$ ~38,000 chymotryptic fragment (*f* and *g*, *arrowheads*). For further designations see legends to Figs. 1 and 2.

| | |
|---|---|
| IT-A | EKMFMQNLNDXLASYL |
| ck 14 | VGSEKVTMQNLNDRLASYLDKV |
| ck 16 | VGSEKVTMQNLNDRLASYLDKV |
| ck 15 | SGNEKITMQNLNDRLASYLDKV |
| ck 19 | AGNEKLTMQNLNDRLASYLDKV |
| ck 13 | TGNEKITMQNLNDRLASYLEKV |
| ck 10 | SGNEKVTMQNLNDRLASYLDKV |
| ck 18 | IQNEKETMQSLNDRLASYLDKV |

| | |
|---|---|
| IT-B | EVQIKQWYETNAPRAG....RDYSAYYRQIE |
| ck 14 | EVKIRDWYQRQRP.AEI...KDYSAYFKTIE |
| ck 16 | EVKIRDWYQRQRP.SEI...KDYSPYFKTIE |
| ck 15 | EVKIHDWYQKQTP.ASP..ECDYSQYFKTIE |
| ck 19 | EVKIRDWYQKQGP.GPS...RDYSHYYTTIQ |
| ck 13 | EVKIRDWHLKQSP.ASP..ERDYSPYYKTIE |
| ck 10 | EGKIKEWYEKHGN.SHQGEPRDYSKYYKTID |
| ck 18 | ESKIREHHEKKGP...QV..RDWSHYFKTIE |

| | |
|---|---|
| IT-C | EVNAAPQLNLGVIMNE |
| ck 14 | VNVEMDAAPGVDLSRILNEMRD |
| ck 16 | VNVEMDAAPGVDLSRILNEMRD |
| ck 15 | VNVEMDAAPGVDLTRILAEMRE |
| ck 19 | VSVEVDSAPGTDLAKILSDMRS |
| ck 13 | VNVEMDATPGIDLTRVLAEMRE |
| ck 10 | VNVEMNAAPGVDLTQLLNNMRS |
| ck 18 | LTVEVDAPKSQDLS1IMADIRA |

| | |
|---|---|
| IT-D | EKELQSKLSVKATQL |
| ck 14 | QNLEIELQSQLSMKAS.LENS |
| ck 16 | QGLEIELQSQLSMKAS.LENS |
| ck 15 | QELEIELQSQLSMKAG.LENS |
| ck 19 | QGLEIELQSQLSMKAA.LEDT |
| ck 13 | QGLEIELQSQLSMKAG.LENT |
| ck 10 | QALEIELQSQLALKQS.LEAS |
| ck 18 | QSLEIRLDRMRNLKAS.LENS |

*Figure 5.* Partial amino acid sequences of human protein IT fragments obtained by digestion with *Staphylococcus* V8 protease and separation by reverse-phase HPLC: IT-A, IT-B, fragments with high homology to coil Ia of type I CKs; IT-C and IT-D, fragments with high homology to coil II. These sequences are compared with the corresponding sequences of various human type I CKs: CK 14 (60), CK 16 (77, 80), CK 15 (57), CK 19 (3), CK 13 (53, 62), CK 10 (78, 93; see reference 51), CK 18 (56). Residues identical in polypeptide IT and at least one of the other CKs are in bold letters. Dots denote omissions introduced to optimize alignment.

mixture of CK 8 and protein IT at 4 M urea, i.e., under non-dissociating conditions. CK 8 and protein IT, purified first by DEAE cellulose chromatography and then by reverse-phase HPLC, when applied separately, migrated slightly slower than the reference protein BSA (*B*, Fig. 2, *n* and *o*), suggesting that they were present as monomers or homodimers under these conditions. However, when CK 8 and protein IT were applied in a near stoichiometric mixture, both polypeptides migrated much more slowly in the first dimension, with a retardation compatible with the interpretation of a heterotetrameric complex (Fig. 2 *p*). When a mixture of CKs 8 and 18 was applied in a parallel experiment, they migrated together in first-dimension electrophoresis, and similarly to the mixture of CK 8 and protein IT (not shown). Since CKs 8 and 18 have been shown, by independent methods, to be present as a heterotetramer complex in 4 M urea (40, 73–75), the present data suggest that the complex of CK 8 and protein IT is also a heterotetramer. When similar experiments were performed with native cytoskeletal proteins from duodenal mucosa (i.e., total cytoskeletal material solubilized in 9.5 M urea-containing buffer and dialyzed against 4 M urea), CKs 8, 18, 19 and protein IT all migrated to a position corresponding to that of the authentic complex of CKs 8 and 18.

To examine whether mixtures of CK 8 and protein IT can also assemble into IFs, in vitro reconstitution experiments were performed using the same protein preparations as for native gel electrophoresis (Fig. 2, *n* and *o*), followed by dialysis against a 10 mM Tris-HCl buffer, and electron microscopy. In such tests, not only arrays of 2–5 nm protofilaments were observed but also many long ~10 nm filaments with typical IF appearance (Fig. 3 *a*; see reference 39). Only short rod-like or irregular structures but practically no filaments were observed in control experiments using CK 8 or protein IT alone (Fig. 3, *b* and *c*).

A typical feature of IF proteins, when integrated in subunit complexes or IFs, is the existence of a relatively protease-resistant core fragment of approximately $M_r$ 38,000, largely corresponding to the $\alpha$-helical rod domain forming coiled coils (see references 9, 35, 40, 83, 91). Therefore, we examined whether fragments produced from native CK IFs from intestinal mucosa by limited chymotryptic digestion also included a fragment derived from protein IT. When cytoskeletal material from duodenal villi (Fig. 4 *a*, lane *2*; lane *1* shows as a control MCF-7 cell cytoskeletons) was subjected to limited chymotryptic cleavage, several new bands appeared in the $M_r$ range of 35,000–40,000 (Fig. 4 *a*, lane *3*). These polypeptides were characterized in immunoblot experiments using guinea pig antibodies specific for protein IT

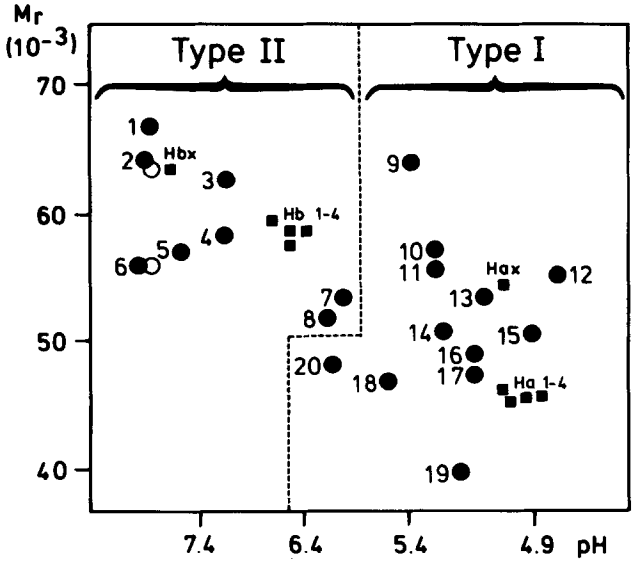


*Figure 6.* Schematic representation of the catalogue of human cytokeratins, supplemented by CK 20 (protein IT). The epithelial (CK 1–CK 20) and trichocytic (*Ha 1-4, Hax, Hb 1-4, Hbx*; see reference 41) CK polypeptides are shown as closed circles and squares, arranged according to their migration in two-dimensional gel electrophoresis: abscissa, isoelectric pH in the presence of 9.5 M urea; ordinate, $M_r$ value in SDS-PAGE. Open circles indicate the existence of a second kind of CK 2 (one typically expressed in epidermis, the other in gingiva and hard palate epithelium) and possibly also CK 6 (for references see 31). Note that CK 20 is the most basic type I cytokeratin.

(for specificity of these antibodies and their lack of cross-reactivity with CKs 8, 18, and 19 see Fig. 4, *a* and *b*, lanes *1*, *2*, *1'*, *2'*, *d*, and *e*). These antibodies did also not react, in immunoblotting, with any other CK or cytoskeletal protein present in a variety of human cells and tissues (including epidermis, hair follicle root sheaths, MCF-7 and HeLa cells; not shown) and recognized only protein IT, not only among the (intact) cytoskeletal proteins from duodenum (Fig. 4, *a* and *b*, lanes *2*, and *2'*) but also among total cellular proteins of colonic carcinomas (not shown).

In the chymotryptic digests, these antibodies specifically reacted with one major band, corresponding to a $M_r$ of ~38,000 (Fig. 4, *a* and *b*, lanes *3*, and *3'*, *solid circles*). A monoclonal antibody against CK 19 used for comparison recognized its antigen among the intact proteins (Fig. 4, *a* and *c*, lanes *1*, *2*, *1''*, and *2''*) as well as among the products of chymotryptic digestion of IFs (Fig. 4, *a* and *c*, lanes *3*, and *3''*). Correspondingly, monoclonal antibodies against CK 18 recognized, among the chymotryptic digestion products, a polypeptide doublet slightly larger than the fragment from polypeptide IT ($M_r$ ~40,000; not shown) and antibody K$_s$pan 1–8.136 reacted with intact CK 8 and, in the digest, with several bands in the $M_r$ ranges of 35,000–38,000 and 20,000–23,000 (not shown; see reference 40). When the chymotryptic digestion products were separated by two-dimensional gel electrophoresis and immunoblotted, again one major polypeptide spot was recognized by the protein IT antibodies (Fig. 4, *f* and *g*). Tryptic peptide map comparison of this immunoreactive $M_r$ ~38,000 product of the chymotryptic reaction with the intact polypeptide of $M_r$ ~46,000 showed very similar patterns, supporting our interpretation of the derivation of this fragment from IT.

### *Partial Amino Acid Sequences of Peptide Fragments*

For determinations of amino acid sequences, cytoskeletal proteins of duodenal mucosa were separated by SDS-PAGE. The band containing polypeptide IT was excised from the gels and the protein electroeluted and subjected to digestion with staphylococcal V8 protease. Peptide fragments were separated by microbore reverse phase HPLC and sequenced by Edman degradation.

Amino acid sequence comparison (Fig. 5) showed that the fragment IT-A had a high homology to the beginning of coil Ia of type I CKs. In a comparison with CKs 10, 13, 14, 15, 16, 18, and 19, a total of 13 out of 16 IT-A residues were identical to those in the majority of the type I CKs, whereas two residues were different in protein IT from all the other CKs. Fragment IT-B could be aligned to a region close to the end of coil Ia, but here the differences from the other type I CKs were much greater: out of the 27 residues determined, 9 were different in protein IT and they did not occur in any of the type I CKs.

The fragments IT-C and IT-D were highly homologous to two coil II regions of the type I CK subfamily. Also in these two fragments, several residues were shared with all the type I CKs but several unique amino acid exchanges distinguished polypeptide IT from all the type I CKs. In fragment IT-C these are: leucine (position 8), asparagine (position 9), glycine (position 11), and valine (position 12). In fragment IT-D there are four such differences: lysine (positions 2 and 7), valine (position 10), and threonine (position 13), and in position 14 there is an additional glutamine in protein IT (Fig. 5).

These sequence homologies provide direct proof that protein IT belongs to the type I subfamily of CKs, and it is therefore included in the catalogue of human CKs as CK 20, especially as it can obviously complement and replace CKs 18 and 19 in intestinal epithelia. Fig. 6 represents an accordingly completed catalogue of human CKs on the basis of analyses by two-dimensional gel electrophoresis.

### *Immunolocalization of Cytokeratin 20 in Cells and Tissues*

The guinea pig antibodies specific for CK 20 (protein IT) allowed its immunolocalization in human cells. In all cases, the immunocytochemical results were in agreement with our biochemical data concerning the presence of CK 20 (for the urothelium, see also 67). However, understandably, CK 20 was not detected biochemically in those tissues in which only few CK 20-positive cells were seen immunocytochemically.

Immunofluorescence microscopy on cell cultures of the human colon carcinoma-derived line HT-29 showed extended cytoplasmic staining, sometimes with recognizable fibrillar pattern (Fig. 7 *a*), which varied considerably in intensity even among adjacent cells. In cells of the line LoVo, also derived from a human colonic carcinoma, a very distinct fibrillar immunofluorescence was seen in many cells, including mitotic ones (Fig. 7, *b* and *c*): in a typical, exponentially growing culture, ~20% of the cells were positive and these tended to be enriched in the periphery of the cell colonies (Fig. 7, *b* and *c*). In some cells, the immunofluorescence appeared, partly or totally, in the form of small "dots" (Fig. 7, *d* and *e*), reminiscent of the spheroidal CK aggregates described in mitotic cells of various origins, colon carcinomas included (7, 23, 32, 44, 55), but also in interphase cells of some lower vertebrates (see reference 47). Variable subpopulations of cells immunostained with CK 20 antibodies were also consistently observed in the colorectal carcinoma lines DLD-1, SW 1116, and Caco-2, whereas in our cell cultures of line COLO 320 DM positive cells were only occasionally encountered. Likewise, a considerable proportion of CK 20-positive cells were seen in cell cultures of the bladder carcinoma line RT-4 and in a small proportion of positive cells occurred in the hepatocellular carcinoma line Hep-G2. MCF-7 mammary carcinoma cells as well as a number of other human nonintestinal cells examined were completely negative with the CK 20 antibodies. In immunoelectron microscopy of HT-29 cells, these antibodies decorated typical IF structures (not shown).

A large spectrum of different normal human tissues, as well as some rat tissues, were examined for the presence of CK 20, using immunofluorescence and immunoperoxidase microscopy. In all positive cells, the staining was confined to the cytoplasm and often fibrillar structures could be resolved (Fig. 8). Results are presented in Fig. 8.

In human small intestine (Fig. 8 *a*), strong and uniform staining for CK 20 was detected in both major kinds of epithelial cells, enterocytes and goblet cells, in regions lining the villi. In the crypts, the staining appeared more patchy (see also reference 63). Some small and slender, but lumen-reaching "undifferentiated" cells at the bases of the crypts were also positive whereas adjacent cells were negative (Fig. 8 *a*, inset). The colonic mucosa exhibited an essentially similar pattern as the small intestine, with a rather uniform staining of the surface epithelium and the necks of the crypts

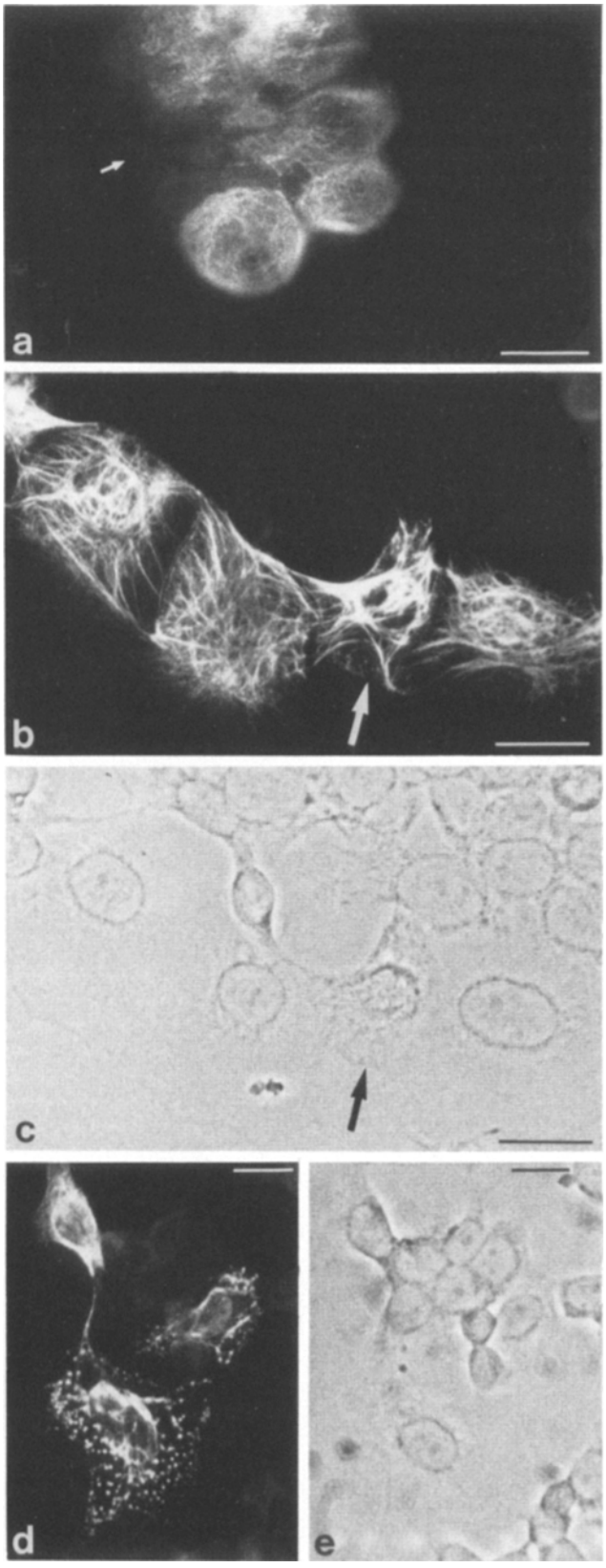


*Figure 7.* Immunofluorescence microscopy of cultured cell lines derived from human colonic carcinomas, using guinea pig antibodies specific for CK 20 (protein IT). (*a*) HT-29 cells, showing a finely fibrillar reaction. Note that some cells in this colony are negative (one is denoted by an arrow). (*b–e*) LoVo cells (*b* and *d*, immunofluorescence; *c* and *e*, corresponding phase-contrast micrographs), exhibiting a prominent CK-type fibrillar network (*b*) also in a mitotic cell (*arrows* in *b* and *c*), whereas in some cells the CK 20 label appears in small dots (*d*). Again, note the presence of negative cells next to positive ones (cf. *b* and *d* with *c* and *e*). In contrast, antibody $K_s$ 18.174 against CK 18 decorated all cells present in this culture (not shown). Bars, 20 μm.

contrasting the heterogeneous staining of the cells located at the bodies and the bases of the crypts (Fig. 8 *b*).

In rat colonic mucosa, again the most differentiated surface epithelium was uniformly positive, whereas the enterocytes of deeper crypts were only weakly immunostained or not at all (Fig. 8 *c*). In these crypts, however, we observed scattered, single, dendritic cells, often situated between the enterocytes and the basal lamina, which were conspicuously strongly positive for CK 20 (Fig. 8 *c*, *arrow*) and might be endocrine in nature.

Zonal differences were also apparent in human stomach mucosa (Fig. 8, *d* and *e*). The foveolar epithelium was strongly and uniformly positive for CK 20 (Fig. 8 *d*). The specific glands of the corpus mucosa (not shown) as well as most mucous cells of the pyloric glands (Fig. 8 *e*, *bottom*) remained unstained. However, a variable number of single CK 20-positive epithelial cells was observed in the upper zone of the pyloric glands (Fig. 8 *e*). A considerable proportion of these single CK 20-positive cells represents endocrine cells as shown by double immunolabeling with an antibody against chromogranin (Moll, R., and W. W. Franke, unpublished data).

Normal human liver and pancreas were completely negative. In contrast, in rat liver and pancreas the epithelium of the biliary and pancreatic ducts was strongly immunostained whereas the hepatocytes and the pancreatic acinar and islet cells were negative (not shown). The significance of these species differences remains unclear. In human gall bladder mucosa, only very few epithelial cells were positive. Human lung was almost entirely negative for CK 20, with the exception of extremely scarce CK 20-positive columnar cells present in some samples of bronchial epithelium (not shown). In human thymus, a small proportion of the reticulum cells, mostly in the thymic medulla, was stained for CK 20.

In human and rat urothelium, CK 20 immunostaining was found mostly in superficial ("umbrella") cells, exhibiting marked cell heterogeneity of reaction (Fig. 8 *f*). In human prostate gland, a very small subpopulation of glandular cells was positive (not shown). In fetal and adult human skin, the only cells positively stained for CK 20 were the Merkel cells (Fig. 8 *g*; see reference 64). All other epithelia examined, including the epithelia of human salivary glands (Fig. 8 *h*), mammary gland, human and rat kidney parenchyme, and human female genital tract were negative for CK 20, as were all nonepithelial tissues.

Synthesis of CK 20 was also observed in colon carcinomas, primary tumors as well as metastases (a peritoneal metastasis is shown in Fig. 8 *i*; 63) and other carcinomas which arise from CK 20-producing epithelia (63, 64, 67). A detailed study of the distribution of CK 20 in human tumors and possible diagnostic implications will be presented elsewhere (Moll, R., and W. W. Franke, manuscript in preparation).

A CK 20-related polypeptide was also found in the duo-

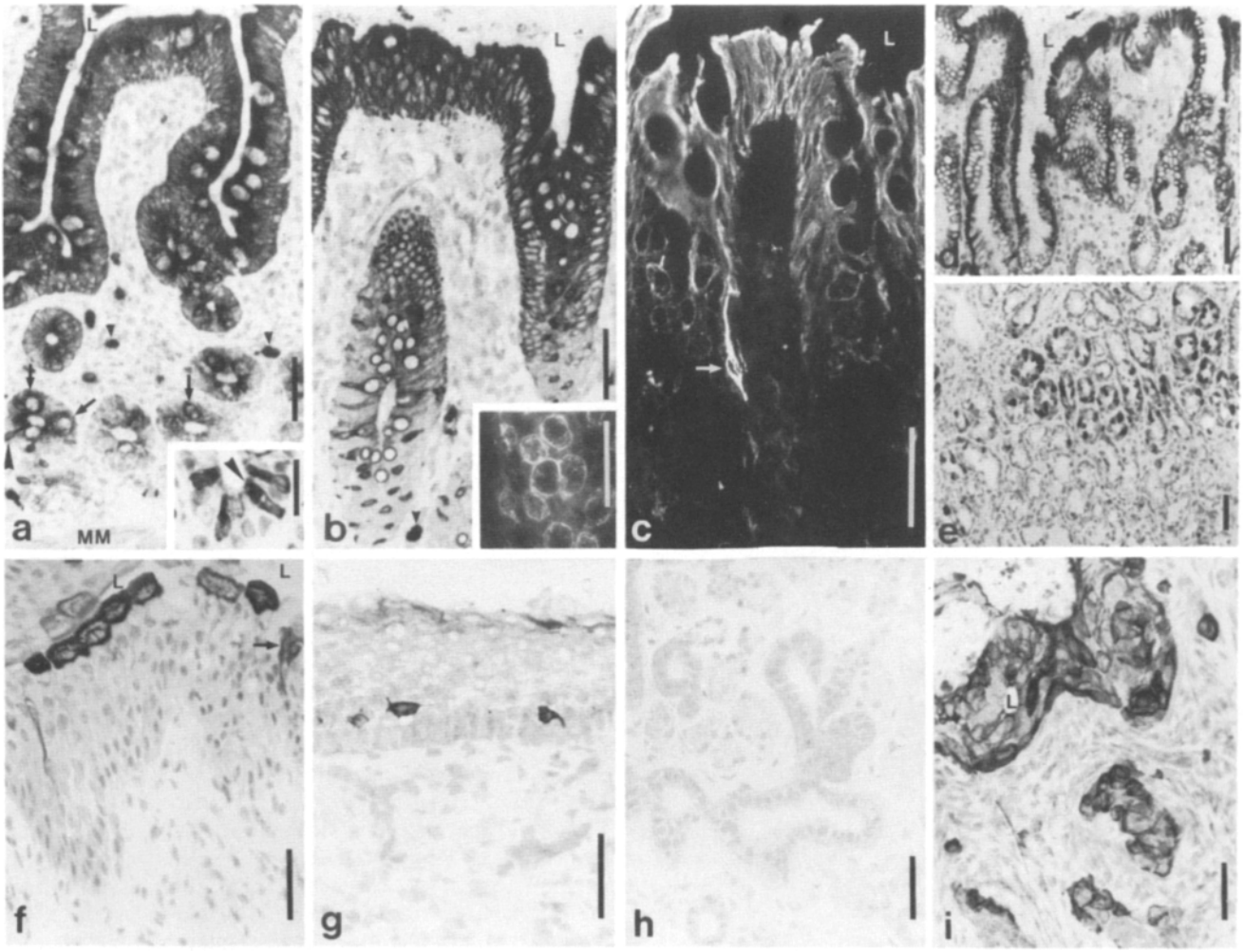


*Figure 8.* Immunocytochemical detection of CK 20 (protein IT) in various human (*a*, *b*, and *d–i*) and rat (*c*) tissues, using specific guinea pig antibodies in immunoperoxidase (*a*, *b*, and *d–i*) and immunofluorescence (*c*, *inset* in *b*) microscopy on acetone-fixed cryostat sections. (*a*) Duodenal mucosa, showing uniform staining of epithelial intestinal cells along the villi (*top*), which is particularly strong in the apical zones of enterocytes, and heterogeneous staining in the crypts (*bottom* and *inset*). The subpopulation of positive crypt cells includes slender undifferentiated (*large arrowheads*) and goblet cells (*arrows*). *MM*, *muscularis mucosae*. Staining of some infiltrating leukocytes in the *lamina propria* is due to the presence of endogenous peroxidase (*small arrowheads*, *a* and *b*). (*b*) In colonic mucosa, the surface epithelium is uniformly positive for CK 20 while the crypt epithelium shows lesser, heterogeneous staining. Note the conspicuous basketlike staining pattern of the goblet cells (*inset*) that is similar to the patterns previously described for reactions with antibodies to other CKs (e.g., 18, 22, 24). (*c*) Rat colon also shows predominant staining of the surface epithelium. Note one strongly stained dendritic cell within the crypt epithelium (*arrow*). (*d* and *e*) Human stomach mucosa (pyloric region) is characterized by extended positive staining of the foveolar epithelium (*d*), as well as staining of scattered epithelial cells in the upper glandular zone (*e*). (*f*) In the urothelium (ureter), a proportion of umbrella cells is moderately or strongly decorated by the CK 20 antibodies. Note weaker staining of a few intermediate cells of the urothelium (*arrow*). (*g*) Specific decoration of dendritic Merkel cells in human fetal (14 wk) plantar epidermis in parabasal position. (*h*) Negative reaction of human submandibular gland. (*i*) Moderately to poorly differentiated adenocarcinoma of the colon (abdominal wall metastasis) revealing heterogeneous, mosaic-like staining of tumor cells for CK 20. *L*, lumen. Bars: 50 μm; insets, 20 μm.

denal epithelium of the pig, by both immunoblotting of cytoskeletal proteins and immunofluorescence microscopy (not shown). Interestingly, in the mouse, only small intestinal mucosa showed some scarce and scattered epithelial cells reactive with the CK 20 antibodies, which were mainly located in the crypts, whereas other murine tissues (liver, urinary bladder, endometrium, and tongue) were completely negative for CK 20 (not shown).

## Discussion

Our results show that a polypeptide of $M_r$ ~46,000 from human intestinal mucosa, which is enriched in IF cytoskeletons together with CKs 8, 18, and 19 and which we have previously designated protein IT (65), is a genuine translation product and a member of the type I CK subfamily. The identification of this polypeptide as a CK has been extraordinarily difficult as it did not cross-react with any of the numerous available monoclonal CK antibodies, including the monoclonal IFA antibody (72) that is widely used as a general IF probe. However, we are also aware of the fact that this antibody, which recognizes a rod epitope near the end of coil II common to many IF proteins (see references 34, 59), does react well with all type II CKs but not, or only very weakly, with several type I CKs, including human CKs 16 and 17 (this study; see also references 5, 8). Our partial

amino acid sequence data may provide an explanation of these reactions. On the one hand, the obvious homologies to other type I CKs identify this polypeptide as a member of this IF protein subfamily; on the other hand, CK 20 differs in a remarkably high number of amino acid positions from all the other sequenced type I CKs. This should be expected to result in the inactivation of a number of epitopes specific for, or common to, other members of this family.

While CK 20 differs from other type I CKs by its exceptionally high isoelectric point (see, however, the broad range of isoelectric values of both type I and type II CKs in fish; 61) it functions as a typical type I CK in several ways: (*a*) It forms heterotypic complexes with type II CKs; (*b*) it integrates with CK 8 into IF rod structures in a similar way as CKs 18 and 19 do; (*c*) it is part of the general cytoplasmic IF fibril system typical for desmosome-connected epithelial cells; and (*d*) it can show similar transient changes of organization from IF to spheroidal aggregate structure, as reported for other CKs (see references 7, 23, 32, 44, 55). Moreover, it is apparently conserved during mammalian evolution as an equivalent, probably orthologous polypeptide occurs in several other species.

In contrast to the other simple epithelial CKs 8, 18, and sometimes also 19 which may be expressed in certain nonepithelial cells (for references see 3, 15, 27, 47), CK 20 seems to be strictly epithelium specific. Both our biochemical analyses and the more sensitive immunocytochemical studies have revealed a unique and unusually restricted tissue distribution spectrum of CK 20 that seems to occur only in certain cells of some simple and a few "complex" epithelia. A limited tissue distribution restricted to a subset of simple glandular epithelia and to transitional epithelium has been described for a putative CK-IF-associated epitope recognized by a certain monoclonal antibody (69) but this antigen is clearly different from CK 20. The immunocytochemical observations made with CK 20 antibodies probably reflect true synthesis because of the correlation between immunostaining intensities and the protein amounts found on gel electrophoresis (for the urothelium, see also 67).

CK 20 is usually coexpressed with simple epithelial CKs, most commonly with CKs 8, 18, and 19, as in foveolar epithelium of the stomach mucosa, in intestinal epithelium, in colon carcinomas and cell lines derived therefrom, and in Merkel cells and neuroendocrine tumors of the skin (for the latter see also 42, 64). In these cell types, CK 20 is obviously present in heterotypic complexes with the type II CK 8 in which it seems to "compete" with the other type I CKs 18 and 19. Correspondingly, CK 20 is found in large amounts in tissues and cells in which these last are relatively low, and vice versa. In extreme cases, certain cells can produce CKs 8 and 20 as their by far predominant CK "pair" (examples include some specimens of duodenal epithelial cells and Merkel cell tumors; this study and reference 64). In these cells, the CK IFs present must be constituted almost entirely by heterotetrameric complexes of CKs 8 and 20. Other CK 20-expressing epithelia such as the urothelium (umbrella cells) contain, in addition to CK 8, the type II CK 7, and here it is clear that the cells expressing CK 20 also synthesize CKs 7, 8, 18, and 19 since all these can be localized uniformly in the same umbrella cells (see reference 67). The reticulum cells of the thymus represent the only, partly and potentially, CK 20-expressing cell type that is capable of differentiation to stratified epithelial features, including several "stratification-related" CKs (66) and the ability to form the epidermoid Hassall bodies. It remains to be studied by double-label immunofluorescence microscopy whether some of the sparse thymic reticulum cells producing CK 20 also synthesize stratification-related CKs.

In both human and rat tissues, most of the CK 20-containing cells are simple epithelial cells with a polar organization, including the umbrella cells of the urothelium. Clearly, in both major types of intestinal adluminal cells CK 20 is also enriched, together with the other CKs, in the tonofilaments of the "subapical skeletal disk" (18, 22; for review see 68). Some kind of polarity is also seen in Merkel cells. The only exception here appears to be represented by the thymic reticulum cells. However, there is no obvious correlation with a specific cellular function of these polar epithelial cells since CK 20 is not only found in diverse secretory cells, particularly mucus-secreting ones (goblet cells, gastric foveolar cells), but also in the absorptive cells of the gastrointestinal tract and in protective surface-lining cells such as urothelial cells. Moreover, several of these tissues such as the mucosa of the small and large intestine, the stomach and the urothelium, display histological cell-to-cell heterogeneity of CK 20 expression with a conspicuous predominance of CK 20-rich cells in the luminally most exposed regions of the mucosa. Perhaps, CK 20-containing IFs and tonofilaments make a special contribution to the mechanical stability of these surface-lining tissues. In more general terms, this pattern of occurrence of CK 20 may be interpreted in correlation with the process of terminal differentiation in intestinal and urothelial cells, comparable with the expression of "suprabasal" CKs in stratified squamous epithelia (for examples see references 28, 31, 46, 83, 85). This would explain, for example, why in the intestinal epithelium only a few of the undifferentiated cryptal cells (for a recent review see 38) but nearly all differentiated enterocytes and goblet cells are strongly positive for CK 20. Clearly, however, the synthesis of CK 20 is not restricted to postmitotic, terminally differentiating cells as it has also been detected in cultured cells in mitosis (Fig. 7, *b* and *c*) and in carcinomas (see also reference 63).

Obviously, the synthesis of CK 20 can be continued during fundamental changes of cellular characteristics such as malignant transformation, progression, and metastasis of carcinomas, and during in vitro culturing. For example, the formation of CK 20-containing IFs is maintained in neuroendocrine (Merkel cell) tumors (64) of the skin and in urothelium-derived carcinomas (see reference 67), including poorly differentiated grade 3 tumors (thus, at least in this kind of tumor the correlation of CK 20 expression with the level of differentiation as discussed above does not seem to hold). Similarly, CK 20 synthesis is a stable feature in adenocarcinomas of the colon (this study and 63), and in various colon carcinoma cell lines such as HT-29 and Caco-2. This maintenance of expression makes CK 20 a valuable cell typing marker for tracing certain cell lineages during development and for the differential diagnosis of primary and metastatic carcinomas, particularly for tumors of the gastrointestinal tract and the bladder. Hence, immunocytochemistry with CK 20 antibodies, especially in combination with antibodies to CKs 19 and 7 (for examples see 4, 6, 10, 67, 71, 87, 92), opens new possibilities in the differentiation of the broad group of simple epithelium-derived carcinomas. It will also be interesting to compare the advent of CK 20 in

endodermal differentiation during embryogenesis and its presence in tumors in direct comparison with other intestine-type cytoskeletal marker proteins such as villin (for review see 58).

An unexpected and conspicuous feature of the distribution of CK 20 is the frequency of cell heterogeneity (i.e., the pronounced tendency to appear in mosaic-like patterns of positive and negative cells), even in tissues in which this protein is generally prominent as in the intestinal epithelium. While certain IF proteins such as CKs 8 and 18 are characterized by their uniform expression throughout simple epithelia (for references see Introduction), "patchy" heterogeneities have repeatedly been noted for other CKs (for examples see references 4, 6, 67, 88). The appearance patterns of CK 20-positive cells in these tissues as well as in some of the CK 20-positive tumors seem to be at random, and the cells positive for CK 20 are otherwise morphologically indistinguishable from those negative for this protein. Of course, more detailed and special studies will be needed to decide whether the individual cells positive for CK 20 differ positionally and/or functionally from the negative cells or whether CK 20 expression in these tissues is merely due to stochastic "leakiness" of expression and without crucial functional importance, as recently described for the expression of CK 18 in a subpopulation of cultures of transformed nonepithelial cells (49). Hopefully, cloned nucleic acid probes for CK 20, which so far have been astonishingly refractory to our cDNA cloning attempts, will help in understanding the principles of the regulation of this unusual cytoskeletal component.

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