Keratin Expression in Rat Intestinal Crypt and Villus Cells

ANALYSIS WITH A PANEL OF MONOCLONAL ANTIBODIES*

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Seven monoclonal antibodies were prepared against cytoskeletal components of rat intestinal brush borders. In the following paper (Chandler, J. S., Calnek, D., and Quaroni, A., J. Biol. Chem. 266, 11932–11938), three of them were shown to be specific for, respectively, keratin 8 (RK4), keratin 19 (RK7), and a newly identified type I keratin (keratin 21) (RK5). With these antibodies we have investigated the changes in keratin gene expression accompanying intestinal cell differentiation. Keratin 21 was detected exclusively in differentiated villus cells and in goblet, enteroendocrine, and Paneth cells in the crypts; in the proliferative crypt cells keratin 19 was predominant. Analysis of keratins expressed by cultured rat crypt cells (IEC cells) confirmed the absence of keratin 21 in undifferentiated intestinal cells. Changes in keratin's expression similar to those observed with cell differentiation in the adult intestinal mucosa were also demonstrated during early fetal intestinal development: the stratified epithelium present at 15–16 days of gestation contained predominantly keratin 19 with only a small amount of keratin 8; keratin 21 was first detected at 18–19 days of gestation, concomitant with the appearance of a well formed brush border and an apical cytoplasmic terminal web. These results suggest that keratin tonofilaments may play a role in the morphological and structural alterations accompanying intestinal cell differentiation in vivo.

The epithelium of the small intestine is characterized by compartmentalization of the proliferative cells in the crypts of Lieberkühn and of the functional absorptive cells covering the villi; specialized differentiated cells (goblet, enteroendocrine, and Paneth cells) are also present in the crypts (1). Each villus is surrounded by several crypts, and as the newly differentiated epithelial cells migrate from the upper region of the crypts to the villus tips, they acquire ultrastructural characteristics and a complement of brush border enzymes which easily distinguish them from the proliferative crypt cells (1). A typical feature of the absorptive villus cells is the presence of a well developed brush border at their luminal aspect, composed of numerous slender microvilli with a core of parallel actin microfilaments (2–6), and a complex network of cytoplasmic filaments, called the terminal web, in the apical cytoplasm (5, 7). This portion of the intestinal cells can be isolated with ease in amounts large enough to allow a detailed biochemical analysis of its major constituent proteins and has been therefore a favorite model system for the study of the organization of actin filaments and associated proteins (3, 4, 6, 8, 10, 11). Many of them have been well characterized, and their localization either in the microvillus core (actin, villin, fimbrin, 110 K-kDa protein, and calmodulin) or in the terminal web (myosin, tropomyosin, actin, vinculin, filamin, TW 260/240, gelsolin, and caldesmon) has been established by immunohistochemical techniques at the light and electronmicroscopic level (4–6, 7, 10–12). Marked ultrastructural differences exist between proliferative crypt cells and absorptive villus cells. While the luminal aspect of the crypt cells is also covered by numerous microvilli, these are shorter, wider, more irregular in shape and distribution than those of the villus cells (1, 13). In addition, no well developed terminal web is present in the undifferentiated crypt cells; instead, the bundles of actin microfilaments comprising the core of the microvilli penetrate into the cell cytoplasm to a depth of 3–5 μm, and cellular organelles are present in the upper portion of the apical cytoplasm (13, 14). Recent studies (14–16) have demonstrated that actin, villin, myosin, tropomyosin, and spectrin are already concentrated in the luminal cytoplasm of the stem cells, present at the bottom of the crypts and displaying relatively few and short microvilli. Thus, brush border formation may involve reorganization of existing cytoskeletal proteins induced by an as yet unidentified cellular component. The terminal web becomes organized as a structure detectable at the ultrastructural level somewhat later, as the newly differentiated cells reach the top of the crypts.

Like most other epithelial cells of vertebrates (17–28), the cells covering the intestinal villi, and in the crypts, are known to contain relatively large amounts of proteins immunologically related to epidermal prekeratin, which form a complex cytoplasmic network of intermediate filaments (5, 8, 17). These filaments extend apically from the many spot desmosomes that join the lateral plasma membranes of adjacent epithelial cells (5, 7–9). Dense networks of keratin tonofilaments are also present in the terminal web, and when visualized by quick-freeze, deep-etch rotary replication they were found to connect with the rootlets of the microvilli cores via thin filaments of unknown composition (5), suggesting that they may play a key role in the organization of the terminal web and of the microvilli.

In the epidermis, and other stratified squamous epithelia, terminal differentiation is marked by major alterations in keratin gene expression (19, 24, 29–32). To investigate the possibility that changes in the composition of keratin intermediate filaments might also play a role in the organization of the apical cytoplasm during differentiation of the intestinal cells, we have examined in detail the molecular composition

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of intestinal cytokeratins in crypt and villus cells. In this and the following article (33) we have demonstrated that marked changes in keratin composition take place as the intestinal cells migrate from the base of the crypts to the villus tips and during fetal intestinal maturation, and identified a new keratin exclusively expressed by differentiated intestinal cells.

EXPERIMENTAL PROCEDURES

Materials—Commercial sources of materials were as follows: Balb/c mice (15–17 g) and Sprague-Dawley rats (CD strain, 100–170 g), either sex (Charles River Breeding Laboratories Inc., Wilmington, MA); for collection of fetal intestines, rats were bred in our facilities; Dulbecco's modified Eagle's medium with 4.5 g/liter glucose, fetal bovine serum, irradiated, and penicillin-streptomycin mixture (M. A. Bioproducts, Walkersville, MD); Tris, 4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid (HEPES),1 phenylmethylsulfonyl fluoride (PMSF), aprotinin, leupeptin, and antipain (Sigma); affinity-purified goat-anti-mouse IgG (H+L), F(ab') fragment (fluorescein isothiocyanate-conjugated), and mouse immunoglobulin subtype identification kit (Boehringer Mannheim); &C-labeled molecular weight markers (cardiac anhydride, bovine serum albumin, phosphorylase b, globulins, myosin) (Du Pont-New England Nuclear); acrylamide, bis-acrylamide, nonidet p-40, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), sodium dodecyl sulfate-polycrylamide gel electrophoresis; NEPHGE, nondenaturing polyacrylamide gel electrophoresis; NEPHGE, nonequilibrium pH gradient electrophoresis. Methods used to purify total cellular RNA from intestinal tissues and cultured intestinal gel cells (IEC cells), and characterization of the cDNA probes specific for keratins 8, 19, and 21 used in these studies are included in the following article (33). The mouse Endo B (mouse keratin 18 homolog) cDNA (43) was obtained as an insert in a pUC9 plasmid from Dr. Robert G. Oshima (La Jolla Cancer Research Foundation, La Jolla, CA). An EcoRI restriction fragment of approximately 1000 base pairs (43) was isolated and used as a keratin 18 probe. A defensin (44) cDNA fragment (CB587) used as a control in some experiments was obtained from Dr. Charles Bevins (The Children's Hospital of Philadelphia). All cDNA probes were labeled and used as described in the following paper (11924), p. 1.6% of pH 5-8 ampholines were used in most cases); gels containing 0.1% SDS as previously described (38). At the end of the electrophoresis, gels were stained for protein with Coomassie Blue R or silver stain by the method of Blum et al. (39). Alternatively, proteins were transferred to nitrocellulose membranes for immunoblotting (see below). Two-dimensional slab-gel electrophoresis was performed as described by O'Farrell (40) with some modifications; the samples were thawed, freeze dried, and then directly solubilized in lysis buffer. Ammonia buffers for optimum isoelectric focusing of polyepitides in the pH range 4.0–7.0 were used. Alternatively, nonequilibrium pH gradient electrophoresis (41) was used in the first dimension; samples were run in the presence of 8 M urea and 2% LKB amphiolines (Pharmacia-LKB Biotechnology Inc.) (0.4%) of pH 3.5–10 + 1.6% of pH 5–8 amphiolines were used in most cases); gels were run at 400 V in 4% acrylamide tubular gels for 4 h. Immunoblotting—Proteins from SDS gels or two-dimensional gels were immunoblotted (four changes, 200 ml each over a 1-h period) in 50 mM Tris-HCl, pH 7.4, + 0.1% SDS, and then transferred to nitrocellulose membranes as described (42), but using a carbonate blot buffer (10 mM NaHCO3, 3 mM Na2CO3, pH 9.5, in 20% MeOH) in a Bio-Rad Trans-Blot cell. Transfer was at 60 V for 90 min. Membranes were blocked overnight in PBS containing 3% bovine serum albumin and 0.05% NaNO3, and then incubated with monoclonal antibodies (straight hybridoma-conditioned media or ascites fluids diluted 1:100 in PBS containing 0.2% bovine serum albumin) or antiserum (diluted 1:50 in PBS) for 2 h at room temperature. After washing in PBS the membranes were incubated with alkaline phosphatase-conjugated goat-anti-mouse IgG (Bio-Rad Laboratories) or rabbit-anti-mouse IgG (Promega Biotec, Madison, WI) diluted 1:5000 in PBS containing 0.2% bovine serum albumin, further washed in PBS, followed by a 5-min wash in 5 mM MgCl2, 100 mM NaCl, 20 mM Tris-HCl, pH 9.5 (alkaline phosphatase substrate buffer), and finally incubated in the same buffer with freshly prepared nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) substrate for alkaline phosphatase detection (GIBCO-BRL Life Technologies, Gaithersburg, MD). Finally, the blots were rinsed with water, photographed, and air-dried.

Purification and Analysis of Intestinal RNAs by Northern Blotting—Methods used to purify total cellular RNA from intestinal tissues and cultured intestinal gel cells (IEC cells), and characterization of the cDNA probes specific for keratins 8, 19, and 21 used in these studies are included in the following article (33). The abbreviations used are: HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (UT); diethiothreitol; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate buffered saline; ETA, [ethylen- bis (sulfosuccinimidyldiacylate); SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; NEPHGE, nondenaturing polyacrylamide gel electrophoresis.

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Preparation of Monoclonal Antibodies—Monoclonal antibodies to small intestinal brush border cytoskeleton, purified from adult rat villus cells as described by Bretscher and Weber (4), were prepared as previously described (37, 45). The presence of keratin tonofilaments in the detergent-extracted fractions used for immunization was confirmed by immunofluorescence staining with a mouse monoclonal antibody to PKK1 cytokeratin (Labsystems, Chicago, IL) and a polyclonal antiserum prepared to keratin from fetal bovine hoof (Miles Sci., Naperville, IL). Balb/c mice were immunized with subcutaneous injections of 200 µg of protein in 100 µl of PBS, mixed with 100 µl of complete (primary immunizations) or incomplete (booster injections) Freund’s adjuvant. 3 days after the last injection spleen cells were obtained and fused with NSI myeloma cells (46). Hybridomas were selected with HAT medium (hypoxanthine, amipuretin, and thymidine containing) (47) in the presence of mitomycin-C-treated 3T3 cells. Double-cloned hybridomas were selected with HAT medium (hypoxanthine, amipuretin, and thymidine containing) (47) in the presence of mitomycin-C-treated 3T3 cells. Double-cloned hybridomas were used for immunoglobulin subtype determination and large scale antibody production in ascites form. The monoclonal antibodies were purified from the ascites fluids by affinity chromatography on a protein A-Sepharose 4B column (48).

RESULTS
Preparation and Characterization of Monoclonal Antibodies to Rat Intestinal Cytokeratins—Hybridoma cultures obtained from three independent fusions were tested for antibody production by an enzyme-linked immunoabsorbent assay (45) employing brush border cytoskeleton as target antigen, followed by immunofluorescence staining of small intestinal frozen sections. Cultures of interest were cloned twice by dilution plating in the presence of mitomycin-C-treated 3T3 cells. Double-cloned hybridomas were used for immunoglobulin subtype determination and large scale antibody production in ascites form (45). All monoclonal antibodies were found to be of the IgG1 subtype, and were purified from the ascites fluids by affinity chromatography on a protein A-Sepharose 4B column (48).

Antigen specificity of the RK1–7 antibodies was determined by Western blotting, employing desmosome-associated tonofilaments purified from rat intestinal brush borders as described by Franke et al. (18). In addition to some residual actin, three major polypeptides, of apparent M, 52,000, 46,000, and 40,000, shown by Franke et al. (18) to be immunologically and biochemically related to epidermal cytokeratin, were detected by SDS-PAGE (Fig. 1, lane 2). They were stained by antibodies to rat keratins 8 + 18 (Fig. 1, lane 3) and to PKK1 cytokeratin (Fig. 1, lane 4). In preliminary experiments, the RK1–7 antibodies were all found to recognize polypeptides in the same M, range (see for example Fig. 1, lane 5).

Using total cytoskeletal preparations obtained from villus cells, the RK1-7 antibodies demonstrated distinct, although partially overlapping, polypeptide specificities (Fig. 2). Similar results were obtained with tonofilaments reconstituted (9) from cytoskeletal preparations of intestinal brush borders or isolated crypt and villus cells, solubilized in a buffer containing 8 M urea and 25 mM mercaptoethanol and then dialyzed against a low pH buffer (data not shown). However, a very poor yield of reconstituted tonofilaments (15–25% of solubilized protein recovered) was consistently found with solubilized cytoskeletal preparations from crypt cells. Corresponding protein recoveries with the other two cytoskeletal preparations were in the range of 50 to 75%.

Three monoclonal antibodies, showing selectivity for the M, 52,000 (RK4), the M, 48,000 + 46,000 (RK5), and the M, 40,000 (RK7) components (Fig. 2), were selected for further investigation. Evidence in support of their specificity for, respectively, keratins 8, 21, and 19 is presented in the following article (33).

Immunofluorescence staining of different rat tissues demonstrates that the RK1–7 antibodies were in all cases specific for epithelial cell components, but with distinct patterns of cellular reactivity (Table I). In rat skin, only the sebaceous and sweat glands were stained (with RK1, RK4, RK7) suggesting that none of the antibodies we have produced recognize epidermal keratins. Antibody RK4-decorated intracellular filamentous present in most simple epithelia of internal organs examined, a finding consistent with its specificity for keratin 8 (49). Antibody RK5 demonstrated a very limited cell and tissue reactivity outside the intestinal tract, recognizing only proximal tubular cells in the kidney ar a few cells at the top of the foveolar epithelium in the stomach. The specificity of antibody RK7 (Table I) was consistent with the previously reported distribution of keratin 19 in human cells and tissues (49). All the nonepithelial tissues examined (spinal cord, skeletal and heart muscle, connective tissues in various
organs) were negative with the RK1–7 antibodies.

Keratin Expression in Intestinal Crypt and Villus Cells—Indirect immunofluorescence staining of rat small and large intestines with the RK1–7 antibodies showed positive reaction only in the epithelial cells; staining was particularly intense in correspondence with the apical and basal regions of the cells’ cytoplasm (Fig. 3). With the exception of RK3 and RK5, the other antibodies produced equivalent staining patterns: all epithelial cell types present in the intestinal mucosa, including goblet, Paneth and endocrine cells, were stained with similar intensity. Representative staining patterns obtained with antibodies RK4 and RK7 are presented in Fig. 3. In contrast, RK5 (and RK3 which, however, produced a weak fluorescence and was not used in further studies) reacted exclusively with differentiated intestinal cells: the absorptive and goblet cells present on the villi were stained most intensely (Fig. 3b), but Paneth, goblet and enteroendocrine cells in the crypts were also positive (Fig. 3e). The proliferative crypt cells were either weakly stained or completely negative (compare Fig. 3e with the corresponding pattern of crypt cell staining obtained with antibody RK4, Fig. 3d). Interestingly, while both RK4 and RK5 appeared to stain predominantly the entire periphery (luminal, basal, and lateral aspects) of the absorptive villus cells (Fig. 3, a and b), the same cells stained with RK7 showed in addition a fibrillar pattern clearly extending to the central regions of the cells’ cytoplasm (see arrows, Fig. 3c). In contrast, in the crypts RK4 and RK7 produced similar or identical staining patterns, with an intense reaction only in the apical cytoplasm of the epithelial cells (Fig. 3, d and f). This suggests that cytokeratin tonofilaments of different composition may coexist in the absorptive villus cells, perhaps including filaments composed of keratin 19 alone, or with a partner different from keratin 8. Keratin 19 is unique in that it lacks the carboxyl-terminal non-α-helical segment found in all other keratins (50, 51), and has been suggested to form homopolymers and participate in interactions with other cytoplasmic components (50). In vitro reconstitution experiments seem however to exclude the possibility that it is capable of forming typical intermediate filaments without a Type II cytokeratin partner (61).

Further analysis of keratins composition and distribution in intestinal cells at different stages of differentiation was performed by two-dimensional slab gel electrophoresis and Western blotting. For these studies, villus and crypt cells were separately released from the proximal jejunum, where Paneth cells are least abundant (1), by the method of Weiser (34).
Pools of villus and crypt cells were separately extracted with a Triton X-100-containing buffer, followed by three extractions with a high salt (1.5 M KCl) solution. The remaining insoluble proteins were analyzed by either isoelectric focusing (IEF) or nonequilibrium pH gradient electrophoresis (NEPHGE) in the first dimension. The two techniques yielded similar results, and only those obtained by the latter procedure will be presented and discussed in the following. Marked differences in the polypeptide patterns observed with villus and crypt cell fractions were readily apparent (Fig. 4, a and b). Blotting with a mixture of all RK1-7 antibodies (Fig. 4, c and d) demonstrates the keratin nature of most spots observed in silver-stained gels, the major exception being actin. The use of antibodies RK4, RK5, and RK7 individually allowed the identification of keratin 8-, 19-, and 21-related polypeptides, respectively (only the result obtained with RK5 is shown in Fig. 4e). It should be noted, however, that the staining intensity of individual spots observed in Western blots was often not representative of their actual relative abundance as determined by silver staining (compare, for example Figs. 4b and 4d).

In villus cell fractions (Fig. 4a), keratin 8 (present as two major isoelectric variants) was the most abundant (approximately twice the amount of keratin 19, based on densitometric scanning of protein-stained gels), and keratin 21 was identified on Western blots with antibody RK5 as two spots differing both in M, and pI (Fig. 4c). This was in contrast with the situation in fetal intestines, where only the higher M, keratin 21 spot was detected (see below). In crypt cell fractions, keratin 19 far surpassed keratin 8 in abundance (Fig. 4b), and keratin 21 was either absent or produced very faint staining (which did not reproduce photographically in Fig. 4f) with antibody RK5. Two other major spots were detected in silver-stained gels (labeled x and y in Fig. 4b), but their keratin nature could not be conclusively determined since they reacted only with a mixture of all monoclonals, and RK4, RK5, and RK7 did not individually recognize them.

**Analysis of Keratins Expressed in IEC Cells**—Monolayer cultures of intestinal crypt-like cells (IEC cells) have been established by various groups, including ours (52, 53), from fetal or newborn rats and shown to lack most markers typical of differentiated enterocytes. Immunofluorescence staining with antibodies RK1-7 demonstrates significant differences in reactivity when compared to the intestinal epithelium in vivo. Only RK1 and RK4 produced strong cytoplasmic staining typical of keratin-type intermediate filaments (data not shown). All other antibodies were either negative or produced very weak fluorescence. Analysis of cytoskeletal preparations obtained from confluent IEC-17 cells by Western blotting of two-dimensional gels (Fig. 5) demonstrates the presence of keratin 8 alone, and antibodies RK5 and RK7, specific for keratins 21 and 19, respectively, were negative. Additional polypeptide spots were visible in silver-stained gels, but they did not react with any of the monoclonal antibodies we have produced. Northern blot analysis of total cellular RNA purified from IEC-17 cells (Fig. 6) confirms and extends the above findings. Only the rat keratin 8 probe (33) produced a distinct signal; no mRNA band could be detected with probes specific for keratins 19, 21, or 18 (43). A cDNA probe for defensin/cryptidin, a corticotatin-related peptide which accumulates to high levels in adult intestinal crypt epithelium (44), was used as a positive control in these studies (Fig. 6). These results demonstrate the absence of keratin 21 in proliferative
intestinal cells, and suggest that additional keratin(s), not detected by the available antibodies and cDNA probes, is (are) expressed in cultured intestinal cells.

Analysis of Keratins Expressed at Different Stages of Fetal Intestinal Development—Cytoskeletal fractions obtained from the entire fetal intestines were resolved by two-dimensional slab gel electrophoresis (NEPHGE followed by SDS-PAGE), and gels were either processed for silver staining or analyzed by immunoblotting with a mixture of all RK1–7 antibodies or with antibodies RK4, RK5, and RK7 used separately. The results obtained are summarized in Fig. 7, where representative protein and keratin patterns obtained from fetal intestines at 16, 18, and 20 days of gestation are presented. At 16 days of gestation, only keratin 19 could be clearly identified on silver-stained gels (Fig. 7a); immunoblotting with a mixture of all RK1–7 antibodies (Fig. 7b) or individually with RK4 and RK7 (not shown) confirmed the presence of keratin 19 and also detected a relatively small amount of keratin 8, while keratin 21 was totally absent (Fig. 7c). This pattern of keratin polypeptides was therefore very similar to that observed with crypt cell fractions from adult intestine (Fig. 4b). At a period of gestation marking the beginning of the process of brush border morphogenesis and intestinal cell differentiation (18–19 dg), a small amount of keratin 21 was first detected immunologically as two isoelectric variants of apparent M, 48,000 (Fig. 7, e and f); a progressive increase in the relative amount of keratin 8 was also observed. A more marked change in the keratin pattern was observed at 20 dg, when formation of the intestinal villi and expression of several brush border enzymes were clearly established over the entire length of the small intestine. The relative amounts of both keratins 8 (Fig. 7, g and h) and 21 (Fig. 7, i–l) increased markedly, compared to keratin 19. Coincident with these changes the complexity of keratin patterns on both the silver-stained gels (Fig. 7g) and immunoblots (Fig. 7h) increased dramatically. These results are suggestive of extensive post-translational processing of keratins generating several isoelectric variants and/or proteolytic degradation products, particularly evident with antibodies RK4 and RK7.

DISCUSSION

Both biochemical and immunohistochemical observations indicate that the seven monoclonal antibodies RK1–7 we have produced are all specific for proteins belonging to the keratin group of intermediate filaments. Thus: (a) they stained only epithelial cells in a variety of rat tissues and organs (Fig. 3 and Table I); (b) the antigens for which they are specific were resistant to extraction with nonionic detergents and buffers of both low and high salt concentration; (c) the reactivity of all antibodies was preserved in filaments reconstituted from tonofilament-desmosome complexes and total intestinal cell cytoskeletons solubilized in high concentrations of urea; (d) they recognized major polypeptide components of desmosome-enriched tonofilaments obtained from intestinal brush borders, whose keratin nature has been previously demonstrated by Franke and coworkers (8, 9); (e) the M, range of the polypeptides recognized by these antibodies (40,000–53,000) is typical of cytokeratins associated with “simple” epithelia (20, 23, 27, 30, 49). Conclusive evidence for the
keratin specific of the three antibodies we have selected for further studies, RK4, RK5, and RK7, is presented in the following paper (33).

Keratin tonofilaments constitute the most heterogeneous class of intermediate filaments and their composition has been shown to vary considerably depending on epithelial cell type, stage of epithelial differentiation, proliferative activity of the cells, and period of embryonic development (17, 21, 23, 27, 28, 30, 49, 54–57). In human tissues at least 30 different keratin gene products have been identified: 10 (“hard α-keratins”) typical of trichocytes (hair and nail keratins) and the other 20 (“soft α-keratins”) expressed in various human epithelia in a cell type-specific pattern. Also, homoologous keratins have been identified in other mammalian species (17, 19, 21, 23, 27, 30, 49). The latter group of keratins has been further subdivided into two classes, type I (smaller and acidic, including keratins 9–20), and type II (larger and neutral or basic, including keratins 1–8). Individual members of each class are selectively expressed in epithelial cells, and appear to form filaments only as heteropolymers of tetrameric subunits containing two type I and two type II polypeptides (19, 23, 27, 30, 55, 58).

Sequence homologies within each class of “soft” keratins is very high, and it is not therefore surprising that both polyclonal and monoclonal antibodies prepared to keratins often display a widespread cross-reactivity (17, 20, 25–27, 31). This was the case for our antibodies RK1, RK2, and RK6 when they were used in Western blots (Fig. 2, lanes 1, 2, and 6; two-dimensional gels not shown); each of them appeared to react with more than one keratin present in intestinal cytoskeletal fractions, possibly including keratins 18 and 20. They were therefore used mainly in combination with the remaining monoclonals we have produced to obtain as complete as possible a pattern of keratin polypeptides in the cytoskeletal fractions examined (Fig. 2, lane 8; Fig. 4, c and d). Complex polypeptide patterns on two-dimensional gels were also obtained with RK4 and RK7, specific for keratins 8 and 19, respectively (33). This could reflect extensive posttranslational processing of individual keratin polypeptides, like limited proteolysis to which keratin 8 is known to be particularly vulnerable (59), and extent of phosphorylation (21, 60, 61). It is also important to note that the intensity of the individual spots on Western blots often did not reflect their relative abundance (based on protein staining of parallel gels), contributing to the complexity of the patterns observed with each antibody (RK4 in particular) on Western blots.

In simple and transitional epithelia, cytokeratins 7, 8, 18, and 19 are typically expressed (49). The ones present in human (21, 62–64) and rat (9) small and large intestine have been previously investigated: in addition to the expected keratins 8, 18, and 19, a new type I member (formerly known as IT) now designated keratin 29) has been reported (65). Northern blot analysis has also shown that the intestine, together with stomach and uterus, is a major site of keratin 19 expression (66). The results obtained in our study are generally in agreement with the above findings: as identified with antibodies RK4 and RK7, respectively, keratins 8 and 19 were clearly the major tonofilament components of the cytoskeleton isolated from whole intestine or villus cell fractions (Fig. 2, lanes 4 and 7; Fig. 4a). We could not identify with certainty keratin 18 on Western blots, due to the lack of a monospecific antibody, but its mRNA could be readily detected on Northern blots (Fig. 6), although it appeared to be much less abundant than that of the other intestinal keratins we have analyzed.

Our antibody RK5 allowed the identification and characterization (33) of a new type I keratin, which we have termed “keratin 21” (33), with a highly restricted expression, limited to differentiated cells in small and large intestine (Fig. 2 and Table I), epithelial cells of the proximal kidney tubuli, and perhaps a few cells at the top of the foveolar epithelium in the stomach. This pattern of distribution is very similar to that of the recently described M. 46,000 humaa keratin 20 (65), but based on all available information it is not possible to conclude with certainty whether they are homologous gene products. Although the human keratin 20 appeared to be much more basic than keratin 21 in two-dimensional gels (65), the presumed rat homolog recognized by the same antisem in two-dimensional Western blots (65) appeared to have a pI similar to that of keratin 21 (Fig. 4e). However, our antibody RK5 recognized two bands on SDS-PAGE (Fig. 1, lane 5; Fig. 2, lane 5) and two spots on two-dimensional Western blots (Fig. 4e). Based on the developmental studies (Fig. 7) demonstrating the presence of only the higher M, component before birth, and the complete amino acid sequence derived from cDNA sequencing (33) we tentatively concluded that the apparent M, of keratin 21 on SDS-PAGE is 48,000, and the lower M, component represents the product of posttranslational modifications. Differences between keratins 20 and 21 were also apparent in terms of cell and tissue distribution: (a) our antibody RK5 stained only a few cells at the top of the stomach mucosa, in contrast with the much more extensive distribution in this organ reported for keratin 20 (including mucous cells of the pyloric glands and the entire foveolar epithelium); (b) no keratin 20 could be detected in the rat or human kidney parenchyma (65); (c) many human colon tumor cell lines (HT-29, LoVo, DLD-1, SW1116, Caco-2) have been reported to express significant levels of keratin 20 (65); in contrast, Northern blot analysis of total cellular RNAs using a keratin 21-specific cDNA probe revealed a positive signal only in SW1116 and HT-29 cells: LoVo and Caco-2 cells were completely negative. These discrepancies, and the limited sequence comparisons possible at the present time, discussed in the following papers (33), seem to exclude that keratins 20 and 21 are homologous gene products, but a positive resolution of this issue will have to await cloning and sequencing of a significant portion of the keratins 20 and 21 cDNA.

Immunohistochemical studies (Fig. 3) and Western blot analysis (Fig. 4) of cytoskeletal proteins obtained from isolated cell fractions have demonstrated marked differences in cytokeratin polypeptides expressed by proliferative crypt cells and differentiated intestinal epithelial cells. In villus cells, keratins 8 and 19 were the major components, with an approximate ratio of 2:1 (based on protein staining). Keratin 21 could be detected in significant amounts only in villus cell fractions; a weak spot was sometimes observed with cytoskeletal preparations from crypt cells, but was probably due to the presence of a minor population of differentiated (goblet, Paneth, enteroendocrine) cells also in the crypts, which were stained with antibody RK5 (Fig. 3e). In villus cell cytoskeletons, at least two other major polypeptides were observed in two-dimensional gels (labeled a and b in Fig. 4a): they were stained with the total mixture of RK1–7 antibodies (Fig. 4c), but due to the lack of monospecific antibodies they could not be identified with certainty (at least one of them may have been keratin 18). In cytoskeletal preparations from crypt cells, only a small amount of keratin 8 was observed (Fig. 4b), presumably distributed among all cells in the crypts (Fig. 3d), and the only major keratin which could be identified with our antibodies was keratin 19. This is in accordance with the

2 D. Calnek, unpublished observations.
previously observed prevalence of this keratin in other undifferentiated, rapidly proliferating, cell types (50). Two other polypeptides (labeled x and y in Fig. 46), more acidic than keratin 19, were observed in two-dimensional gels obtained with cytoskeletal preparations from crypt cells, and they were stained with a mixture of RK1-7 antibodies, but not with RK4, RK5, or RK7 alone (Fig. 4, b and d). They may represent still uncharacterized keratins, isoelastie variants of keratin 18, or even keratin 20. However, their rather acidic pl would be inconsistent with their belonging to the type II keratin family, raising the question of whether in crypt cells tonofilaments may exist made exclusively of type I keratin(s) (50).

The changes in intestinal keratin expression observed during late embryonic development (Fig. 7) were similar, in many respects, to those seen during crypt-to-villus differentiation in adult rat and provided further evidence for a specific role of keratin 21 in differentiated enterocytes. Starting at the earliest time of gestation examined (15 days, data not shown), and until the period of maturation (18-19 days of gestation) characterized by formation of the intestinal villi, immunoblotting analysis revealed that the keratin 19 was the major intermediate filament component of the intestinal epithelial cells (Fig. 7, a and b). This keratin pattern was quite similar to that observed in adult crypt cells, with the possible exception of the two additional polypeptides recognized by a mixture of all RK1-7 antibodies and labeled “x and y” in Fig. 46. Only a very small amount of keratin 8 (undetectable on silver-stained gels, see Fig. 7, a and d) could be observed by immunoblotting (Fig. 7, b and e). Although we could not directly identify keratin 18 on immunobots, no major polypeptide spot was observed in its expected position (8, 9) on silver-stained two-dimensional gels, or after incubation with a mixture of all RK1-7 antibodies. Brush border morphogenesis and formation of a well organized terminal web at 18-19 days of gestation (1, 67, 68) was accompanied by marked changes in the keratins pattern (Fig. 7, g and i), including the appearance of keratin 21 and a marked increase in the relative abundance of keratin 8. At the same time in the rat, or comparable periods of development in chicks and mice, other important cytoskeletal proteins (such as villin, brush border myosin I, spectrin, caldesmon, TW 260/240) have been found to appear, or undergo significant changes in cellular distribution (69-74).

The findings obtained with cytoskeletal preparations from cultured intestinal crypt cells (IEC cells) were surprising. While typical keratin filaments could be observed by immuno-fluorescence staining with the RK1 and RK4 antibodies (data not shown), only keratin 8 could be identified with certainty in two-dimensional gels (Fig. 5). No significant amounts of keratins 19 or 21 could be detected. These results were confirmed by Northern blot analysis of total cellular RNA extracted from IEC-17 cells (Fig. 6), which in addition revealed the absence of keratin 18 mRNA. Here again one must suspect the presence in these cells of at least one unidentified keratin, possibly belonging in this case to the type I class, but distinct from the keratins 18 and 19 typically observed in simple epithelia.

At present, one can only speculate about the possible significance of these changes in keratin expression with respect to the structure and function of the intestinal epithelial cells, since to date it has proven difficult to attribute specific functions to individual members of the keratin family. The presence of large amounts of cytotkeratin tonofilaments in the apical region of the absorptive villus cells (6, 7-9) is compatible with a structural role in the organization, and possibly the function, of the terminal web and of the contractile apparatus of the brush border. Residual actin and myosin have been found to resist extraction with detergents, high and low salt solutions, during preparation of desmosome-attached tonofilaments (9, and this study), which may indicate an interaction with keratin filaments. In situ, the intestinal tonofilaments network has been shown to be associated with both desmosomes and the bases of the microvillar rootlets (5, 7). It has been suggested that at least in some keratins the carboxyl-terminal domains protrude outside the body of the polymerized filament, and may be involved in specific interactions with other cellular components (75, 76). If indeed the apical tonofilaments are involved in the organization and function of the terminal web, the observed differences in keratins composition between crypt and villus cells may be, at least in part, responsible for the absence of this intracellular structure in the proliferative crypt cells (1, 14, 15). While extensive keratin networks may be necessary to maintain the functional integrity of differentiated intestinal cells, they may prove deleterious for rapidly proliferating cells, where evidence has been obtained for marked alterations in keratin filaments organization during the prereplicative phase of growth (60, 61).

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