Isolation and Characterization of Desmosome-associated Tonofilaments from Rat Intestinal Brush Border

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Abstract

Epithelial cells of the small intestine, like those of other internal organs, contain intermediate-sized filaments immunologically related to epidermal prekeratin which are especially concentrated in the cell apex. Brush-border fractions were isolated from rat small intestine, and apical tonofilaments attached to desmosomal plaques and terminal web residues were prepared therefrom by extraction in high salt (1.5 M KCl) buffer and Triton X-100. The structure of these filaments was indistinguishable from that of epidermal tonofilaments and, as with epidermal prekeratin, filaments could be reconstituted from solubilized, denatured intestinal tonofilament protein. On SDS polyacrylamide gel electrophoresis of proteins of the extracted desmosome-tonofilament fractions, a number of typical brush-border proteins were absent or reduced, and enrichment of three major polypeptides of Mr 55,000, 48,000, and 40,000 was noted. On two-dimensional gel electrophoresis, the three enriched major polypeptides usually appeared as pairs of isoelectric variants, and the two smaller components (Mr 48,000 and 40,000) were relatively acidic (isoelectric pH values of 5.40 and below), compared to the Mr 55,000 protein which focused at pH values higher than 6.4. The tonofilament proteins were shown to be immunologically related to epidermal prekeratin by immunoreplica and blotting techniques using antibodies to bovine epidermal prekeratins. Similar major polypeptides were found in desmosome-attached tonofilaments from small intestine of mouse and cow. However, comparisons with epidermal tissues of cow and rat showed that all major polypeptides of intestinal tonofilaments were different from the major prekeratin polypeptides of epidermal tonofilaments. The results present the first analysis of a defined fraction of tonofilaments from a nonepidermal cell. The data indicate that structurally identical tonofilaments can be formed, in different types of cells, by different polypeptides of the cytokeratin family of proteins and that tonofilaments of various epithelia display tissue-specific patterns of their protein subunits.

Most epithelial cells of vertebrates contain proteins related to epidermal prekeratin ("cytokeratins"; reference 1) which form a meshwork of bundles of intermediate-sized filaments ("tonofilaments") extending through the cytoplasm and which are often attached to the desmosomes of the surface membrane (1-7; for earlier references, see 8-12). This meshwork of desmosome-attached tonofilaments is characterized by its resistance to extraction in buffers of low and high salt concentrations as well as nonionic detergents (1, 6, 11-14). Taking advantage of this insolubility has allowed desmosome-tonofilament complexes to be isolated and purified from epidermal tissue (11).

In many epithelial cells with a highly polar architecture, e.g., intestinal epithelium showing abundant microvilli at the apical cell surfaces, such cytokeratin filaments are locally enriched in a special subapical zone (5, 7, 15, 16) and form a "subapical skeletal disk" (5) demarcated by the apical ring of spot desmosomes (8, 9). To date, tonofilament-rich cytoskeletons have been prepared only from whole tissues or cells (1, 6, 11, 13, 14, 16-18). Moreover, most of this work has been done with keratinocytes which are abundant in filaments containing special prekeratin characteristic of keratinocyte differentiation (1, 3, 10, 11, 13, 17, 18). We have studied the polypeptide composition of a defined tonofilament fraction of nonepidermal cells, the desmosome-attached tonofilaments. In particular, we have examined the cytokeratin polypeptides present in such a topologically defined cell fraction of tonofilaments and com-
pared the composition of the apical desmosome-associated tonofilaments with that of similar tonofilaments of other epithelial cells.

For this we have chosen the "brush borders" because of the relative ease with which these apical cell portions can be isolated from intestinal epithelium, and in view of the general interest and progress in the elucidation of the molecular organization of the contractile and cytoskeletal elements of this structure (19-36). In the present study we describe a procedure by which complexes of apical tonofilaments and residual desmosomal plaques can be isolated as a brush-border subfraction resistant to treatment with high salt buffers and nonionic detergents. These structures show a relatively simple protein composition, with a predominance of cytokeratin polypeptides which, however, are different from those present in epidermal tonofilaments.

**MATERIALS AND METHODS**

**Animals and Tissues**

Male Sprague-Dawley rats (250-300 g body weight) were starved for 18 h and killed by cervical dislocation. The small intestine was removed, divided into parts of ~30 cm in length, and thoroughly rinsed with cold (4°C) phosphate-buffered saline (PBS; 155 mM NaCl, 10 mM sodium phosphate buffer, pH 7.4) supplemented with 3 mM Na$_3$SO$_4$.

Similarly, preparations of small intestine from adult mice (NMRI strain) were made. Small intestine of adult Holstein cows (two pieces of ~30 cm in length from the proximal part of the jejunum) was freshly obtained at a local slaughter-house and rinsed several times with ice-cold PBS containing 3 mM Na$_3$SO$_4$ and 0.1 mM phenylmethanesulfonyl fluoride (PMSF). The tissue was incubated in this medium for ~30 min at 0°C and further processed for the isolation of brush borders.

Brush-muzzle epidermis enriched in stratum spinosum tissue was obtained as described (1, 11) and similarly small slices of "soft" epidermis were excised from the lip region of adult rats.

**Isolation Procedures**

Isolation of intestinal brush borders and microvilli followed the protocols used by previous authors (19-22, 37) with some modifications (38): The rinsed intestinal tube pieces were filled with buffer A (96 mM NaCl, 8 mM KH$_2$PO$_4$, 5.6 mM Na$_3$HPO$_4$, 1.5 mM KCl, 10 mM EDTA, 0.1 mM diethyldithioctetandel, 0.1 mM PMSF, and 1 mM e-amino caproic acid, pH 6.8), the ends were tied with thread, and the intestines were immersed and incubated in 0.3 M sucrose for 15 min at 0°C. The epithelial cell layer was released from the lamina propria by gentle rubbing of the intestines, and the cells were harvested by flushing the lumen with buffer A followed by centrifugation at 300 g for 10 min. The cells were suspended in 300 ml of buffer B (4 mM EDTA, 1 mM EGTA, 10 mM imidazole, PMSF, diethyldithiioctetandel, and e-amino caproic acid (as above), pH 7.3), sedimented at 800 g for 5 min, and resuspended in 40% sucrose in buffer C with a loosely fitting Dounce homogenizer ( Kontes Co., Vineland, N. J.). The crude brush-border suspension was layered on top of 50 and 65% (wt/vol) sucrose (in buffer C), respectively, and centrifuged for 1 h at 30,000 g. Purified brush borders were harvested by aspiration from the 50/65% sucrose boundary, diluted with buffer C, and collected by centrifugation at 1,500 g for 15 min.

Microvilli were prepared from purified brush borders by rigid homogenization in buffer C using the rotating blade homogenizer (see above) at full speed (10 cycles for 10 s each). This suspension was once more diluted with buffer C and centrifuged at 1,500 g for 10 min. The supernate was saved and the pellet again treated with the homogenizer, followed by centrifugation at 1,500 g for 10 min. The resulting pellet was discarded and the supernate (combined with that saved from the previous step) was centrifuged for 15 min at 30,000 g. This pellet was resuspended in a small volume of buffer C with a Potter-Elvejem glass-Teflon homogenizer, centrifuged at 3,500 g for 5 min, and the resulting supernate was used as purified microvillar fraction.

Cytoskeletal preparations highly enriched in desmosome-associated tonofilaments were prepared from purified brush borders by mixing 1 vol of brush-border suspension in buffer C with 3 vol of 2.0 M KCl, 0.2 M NaCl, 1.08 Triton X-100, 10 mM Tris-HCl buffer (pH 7.4), protease inhibitors and dithioerythritol as above, and incubation for 30 min at 4°C. The residual cytoskeletal elements were pelleted at 50,000 g for 20 min. The pellets then were either used directly or extracted once more in 1.0 M KCl, 0.1 M NaCl, 0.5% Triton X-100, 10 mM Tris-HCl buffer (pH 7.4), and pelleted again. All high salt-treated fractions were then washed three times with PBS (supplemented with protease inhibitors and dithioerythritol) by resuspension and centrifugation. The washed cytoskeletal residues were stored at ~70°C as tightly packed pellets or were used directly for electrophoretic and electron microscope analyses.

For analysis of total cell proteins, the intestinal epithelium was gently scraped from the luminal surface, directly deep-frozen at the temperature of liquid nitrogen, and stored at ~70°C. Cytoskeletal fractions from freshly made epithelial cell dispersions or deep-frozen tissue homogenates were prepared as described above for brush borders.

Desmosome-tonofilament complexes from bovine muzzle and rat lip epidermis were isolated as described (1, 11) using the following modification: The slices of epidermal tissue were homogenized in "pH 9 water" (11) for 3 min in a Polytron homogenizer (Fa. Kinematica, Lucerne, Switzerland) at setting 3. The "MDT-fraction" (11) was resuspended by homogenization (as above) in 10 mM Tris-HCl (pH 7.4) containing 1 M KCl and 1% Triton X-100 and magnetic stirring for 30 min in the coldroom. Material pelleted after another centrifugation (~5,000 g, 15 min) was washed once by resuspension in 10 mM Tris-HCl (pH 7.4) and pelleted again.

**Reconstitution of Brush-border Tonofilaments**

Cytoskeletal preparations from purified rat intestinal brush borders washed with PBS in the presence of PMSF and dithioerythritol (see Materials and Methods) were homogenized in 8 M urea buffered with 40 mM Tris-HCl (pH 7.2) containing 25 mM 2-mercaptoethanol, incubated with stirring for 2 h at room temperature, and then centrifuged at 200,000 g for 2 h at 20°C. The supernate was dialyzed for 24 h at room temperature against 50 mM Tris-HCl (pH 7.6), 10 mM 2-mercaptoethanol, with three changes of buffer, and for another 4 h against 50 mM Tris-maleate buffer (pH 5.5). By lowering the pH, the formerly clear solution of cytoskeletal elements became turbid. Centrifugation at 120,000 g for 2 h at 4°C resulted in a pellet consisting of reconstituted tonofilaments which were processed for chemical and morphological analyses as described below.

Alternatively (cf. 39), the material solubilized in buffer containing 8 M urea was first dialyzed overnight against 4 M guanidine hydrochloride (in 50 mM Tris-HCl, pH 9) containing 25 mM 2-mercaptoethanol, incubated with stirring for 2 h at room temperature, and then centrifuged at 200,000 g for 2 h at 20°C. The supernate was dialyzed against 50 mM Tris-maleate buffer (pH 5.5) containing the same amount of mercaptoethanol and 2 mM MgCl$_2$, and finally against the pH 5.5 buffer as described above.

**Gel Electrophoresis**

One-dimensional polyacrylamide gel electrophoresis with SDS was performed as described (1, 40), at different acrylamide concentrations, with either 0.1% or 1% SDS in the electrode buffer (16). For two-dimensional gel electrophoresis (41, 42), ampholine buffers for optimal isoelectric focusing of polypeptides in the pH range 4.0-7.0 were used. For isoelectric focusing the material was directly solubilized either in the specific lysing buffer or by one of the following procedures: (a) The samples were ground at ~70°C in a precooled porcelain mortar in the presence of 1 vol of a slurry of arencus quartz that had been equilibrated with PBS containing 1% SDS and 5% 2-mercaptoethanol and cooled to the same temperature. The homogenization mixture was transferred into small plastic vials and immediately incubated at ~100°C for 10 min. After cooling to room temperature, lysis buffer was added (at a ratio of 9:1 vol/vol in the case of the O'Farrell procedure), the slurry was mixed by whisking, and the quartz and insoluble material were pelleted at ~10,000 g for 5 min. The supernate was used for gel electrophoresis. (b) The modification described by Kelly and Cotman (43) was used. (c) The sample was solubilized by boiling for 7 min in 100 μl of 10 mM sodium-potassium-phosphate buffer (pH 7.5) containing 5% SDS and 1% 2-mercaptoethanol (cf. 40). After cooling to room temperature, the solution was cleared by centrifugation for 10 min at ~10,000 g, and the supernate was mixed with 9 vol of acetic acid. Protein was allowed to precipitate in the cold (~20°C), and the pelleted precipitate was washed once with ~20°C cold aceton-water (9:1, vol/vol) mixture and a second time in cold 96% acetic, each time by resuspension and centrifugation. The final pellet was dried under N$_2$ and kept dry until solution in lysis buffer (41) was used for isoelectric focusing.

Reference proteins used in co-electrophoresis are mentioned in the text and the legends to the figures.
Antibodies

The preparation and characterization of guinea pig antisera against total prekeratin and against individual prekeratin polypeptides from bovine hoof stratum corneum as well as the IgG fractions and monospecific antibodies obtained from these antisera have been described (1-3, 16, 44). In addition, we have used guinea pig antibodies made against total purified prekeratin and individual electrophoretically separated polypeptides from desmosome-attached tonofilaments of bovine muzzle (16, 44). Antibody preparations that reacted with several, though not with all prekeratin polypeptides present in this fraction (Fig. 1) as well as antibodies that reacted only with one epidermal prekeratin polypeptide (for details see reference 44) were used. All antibody preparations used in this study showed strong staining of cytokeratin fibrils of intestinal epithelial cells of rat (Fig. 2 a and b), mouse and cow (not shown).

Immunological Detection of Polypeptides in Agarose Overlays and on Nitrocellulose Paper

Polypeptides that had been separated by one- or two-dimensional gel electrophoresis were transferred (45) to nitrocellulose paper sheets by blotting for 15 h at room temperature. The blots were further processed by a modification of the method of Towbin et al. (46): The sheets were soaked in 1% bovine serum albumin (BSA) in PBS for 12 h at room temperature, rinsed three times with PBS, and incubated, with gentle shaking, for 1 h at room temperature with the specific solution of guinea pig antibodies diluted 1:100 with PBS containing 2% BSA. Excess antibodies were washed off first with PBS (1 h with five changes of buffer), then once with 0.5 M NaCl phosphate-buffered to pH 7.4, and finally twice with PBS. The paper sheets were then incubated for 2 h at room temperature with ¹²⁵I-labeled protein A (specific radioactivity 30 mCi/mg) diluted with PBS containing 2% BSA to give a total radioactivity of 0.5 μCi/sheet. The sheets were washed first with 0.5% Triton X-100 in PBS for 1 h at room temperature, with five changes of medium, then with 0.5 M NaCl-containing buffer (see above), and finally once with PBS. The blot papers were thoroughly dried between sheets of filter paper at 60°C for 15 min. The blots were exposed to a Kodak X-Omat R film for 2 d at -70°C.

Immunoreplicas of polyacrylamide gels using agarose gel overlays were made as described (44, 47, 48).

Immunofluorescence Microscopy

Indirect immunofluorescence microscopy on frozen tissue sections and on isolated intestinal epithelial cells and brush border fractions (for preparation see reference 5) using guinea pig antibodies and fluorescein-coupled rabbit antibodies against guinea pig globulins was as described (e.g., 1, 44, 49, 50). Preimmune sera and antibodies to a number of other proteins were used for controls (e.g., 1, 2, 5, 44).

Electron Microscopy

Samples were fixed and processed for electron microscope examination of ultrathin sections as mentioned (5). Isolated tonofilaments were used directly or after reconstitution (see above) for negative staining with uranyl acetate or phosphotungstic acid (11, 14).

RESULTS

Isolated Brush Borders and Desmosome-Tonofilament Complexes

The brush-border fractions obtained from rat small intestine consisted of relatively pure apical portions from absorptive
cells, comparable in purity to preparations described in the literature from intestine of rat (21, 51), mouse (52), guinea pig (37), and chicken (19, 20, 22, 31; for biochemical criteria of purity see also reference 38). The preservation of the typical brush-border structures such as microvilli, terminal web, and associated apico-lateral plasma membrane, including zonula adhaerens junctions, was as good as that described for similar preparations by other authors (19–22, 37, 51), and the ultrastructure of the desmosomes and tonofilaments was indistinguishable from that described in intact cells (e.g., 5, 9, 21, 28, 53). We found, however, that special precautions had to be taken to provide the maintenance of association of desmosomes and tonofilaments with the terminal web. In particular, the intensity of homogenization of the detached mucosal cells was critical and had to be carefully controlled. When the homogenization was prolonged, the apical desmosomes and the associated tonofilament tangles tended to break off from the terminal web–zona adhaerens complex. Examination of morphological and gel electrophoretic data published in the literature showed that most of the preparations described apparently included only very small amounts of desmosomes and tonofilaments (e.g., 20–25, 51).

The conditions for optimal recovery of desmosomes and tonofilaments in brush-border fractions had to be experimentally determined for the different species; for example, the desmosome-tonofilament material was much more readily lost from border fractions in chicken than in rat. The maintained association of the tonofilament material with the isolated brush borders was also demonstrable by immunofluorescence microscopy using antibodies to prekeratin (Fig. 2; cf. reference 5). These antibodies did not stain the microvilli and the upper region of the terminal web but strongly decorated fibrillar material associated with the most basal regions of the isolated brush borders (Fig. 2). Controls using actin antibodies showed normal staining of microvilli and terminal web (not shown here; for description of antibodies see reference 44) as described by other authors (e.g., 23, 26, 31, 32), and antibodies to α-actinin reacted only with the terminal web region, as expected (23, 26, 31–33).

Extraction of isolated brush borders with Triton X-100 and high salt buffers resulted not only in “demembranation” (cf. 20) but also in the removal of microvillar cores and much of the terminal web material, leaving structures with a “tumbleweed-like” organization consisting of residual desmosome plaques, a dense meshwork of apical tonofilaments, and residual terminal web structures, especially around microvillar rootlets (Fig. 3). The purity of this fraction and the typical flexuous (“wavy”) arrays of tonofilaments in such brush-border residues extracted with high salt–Triton solutions are shown in Figs. 3 and 4. The arrangement of the tonofilaments was largely at random with only little tendency to fasciate, except for filament regions approaching the desmosome plaques (Fig. 5). At higher magnification, these filaments (7–9 nm in outer diameter) revealed an unstained, probably hollow core of ~3 nm (Fig. 4), similar to what has been described for desmosome-attached tonofilaments of intact cells (e.g., 5, 9, 11, 54). The tonofilaments showed intimate association with two structures that were resistant to the vigorous extractions applied: (a) the microvillar rootlets of the terminal web region (Figs. 3 and 4), and (b) the desmosomal plaques of the apical ring of maculae adhaerentes characteristic of these epithelial cells (Fig. 5; for cell anatomy see references 5, 8, 9, 28). Usually these desmosomal plaques appeared as “hemidesmosomal” residues, apparently resulting from the rupture of the desmosome structures in the plane of the midline during the preparation (Fig. 5 a and b). Partial disintegration of the hemidesmosomal plaque structures into subunit fragments (Fig. 5 c) was also often observed (cf. 11). Maintained symmetrical junctional residues containing desmosomal remnants from two adjacent epithelial cells were only rarely seen in such fractions (e.g., Fig. 5 d).

Residues of plaque portions of the zonula adhaerens were also occasionally identified, usually in the immediate vicinity of the desmosomal plaque residues.

Reconstitution of Filament Structures from Denatured Proteins of Brush-border Tonofilaments

A large proportion, though not all, of the protein material contained in isolated brush-border tonofilament–desmosome complexes could be solubilized in high concentrations of urea or guanidinium hydrochloride (see Materials and Methods). As described for epidermal prekeratin dissolved in denaturing concentrations of urea (13, 17, 39, 55, 56), filamentous structures were formed from such solutions upon removal of the urea by dialysis (Fig. 6). In contrast to the high regularity of diameters of reconstituted filaments of epidermal prekeratin treated in parallel (39), reconstituted filament structures from brush-border tonofilaments were much more variable in thickness and often showed ends fraying out in thinner “subfilaments” (Fig. 6, arrows). In addition to filament structures, these preparations of proteins, allowed to renature during removal of urea, consistently contained dense aggregates of granular material (Fig. 6) of a yet unidentified nature.

Gel Electrophoretic Analysis of Tonofilament Proteins

When polypeptides of brush-border fractions from rat small intestine were compared, on SDS polyacrylamide gel electrophoresis, (a) with those of high salt-extracted desmosome tonofilament fractions and (b) with purified microvilli subfractions, striking differences of protein composition were found (Fig. 7 a and b). Desmosome-tonofilament complexes obtained after after one extraction with high salt buffer were practically devoid of typical brush border membrane polypeptides in the range of apparent molecular weights of 130,000–160,000 which include the microvillar sucrase-isomaltase complex (Fig. 7 a, brackets in slots 3 and 6). Residual desmosome-tonofilament fractions were also devoid of other microvillar proteins such as the polypeptide of M, 95,000 (“villin,” 25; cf. 29, 31, 36) and the M, 68,000 component (“fimbrin,” 30; cf. 29, 36). Alpha-actinin which is believed to be primarily, if not exclusively, located at the terminal web–zona adhaerens transition (23, 26, 27, 31) was also absent in these extracted fractions. Two brush-border polypeptides larger than myosin (Fig. 7 a, slots 3 and 7), which were enriched in microvillar subfractions, were also greatly reduced in the desmosome-tonofilament fraction. By contrast, three other proteins, i.e., actin, the “M, 110,000 protein,” and a component comigrating with myosin (Fig. 7 a–c; cf. 22–24, 32, 36), were retained in the cytoskeletal residues in considerable quantities, although their proportion relative to the total protein present was greatly reduced. The most striking observation, however, was the enrichment in the tonofilament fraction of three polypeptide bands of apparent M, 55,000 (component A), 48,000 (component D), and 40,000

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FIGURE 3  Electron micrograph of a section through pelleted desmosome-associated tonofilament complexes isolated from brush borders of rat small intestine, showing the preservation of structures and the purity of the fraction. The fraction shown here has been extracted with high salt buffer only once. Note the predominance of tonofilaments, residues of desmosomes (D) and of terminal web material, including microvillar bases. Bar, 1 μm. × 25,000.
which were only minor polypeptides, if detectable at all, in the microvilli isolated from the same brush-border fraction (Fig. 7a, slots 3-6). These three polypeptides (A, D, and M, 40,000) were tentatively identified as tonofilament cytokeratins, i.e., proteins of prekeratin-like nature (see below).

Further extraction of the desmosome-tonofilament fraction with high salt buffer (see Materials and Methods) resulted only in a slight further increase of these three bands (components A, D, and M, 40,000), together with some retained M, 110,000 polypeptide and a concomitant relative decrease of residual actin and myosin (Fig. 7b). Direct co-electrophoresis with reference proteins on SDS polyacrylamide gels, showed component A to have a similar mobility as desmin, component Vla (M, ~55,000; reference 16) of bovine muzzle prekeratin, and glutamate dehydrogenase. The electrophoretic mobility of component D was similar to that of polypeptide VII of bovine muzzle prekeratin (Fig. 7a), although, on higher resolution gels containing 4% SDS, it appeared to migrate clearly faster than component VII of epidermal prekeratin (compare Fig. 7b). The smallest component had an electrophoretic mobility higher than that of actin and comigrated with aldolase (M, 40,000). All three components, strongly enriched in desmosome-tonofilament fractions, were not only observed in high salt-extracted brush-border fractions but also in cytoskeletal residues prepared directly by extraction of whole intestinal epithelial cells with high salt buffers containing Triton X-100 (data not shown). This indicates that the polypeptide components detected were present in the intact cells and not derived by proteolytic breakdown during the isolation.

On two-dimensional gel electrophoresis of the polypeptide contained in the fraction of desmosome-attached tonofilaments, the same major components were identified (Fig. 8): Component A appeared after isoelectric focusing at a slightly higher pH than co-electrophoresed bovine serum albumin, with two distinct components (apparent pH values of ~6.40 and 6.44). Component D was much more acidic and and focused at a slightly lower pH than α-actin, also showing two distinct isoelectric variants (approximate isoelectric pH values: 5.39 and 5.36). The relative intensity of component D on two-dimensional gel electrophoresis was somewhat variable in different experiments but was generally greater when the cytoskeletal residue was solubilized according to procedure c described in Materials and Methods. The third major component of apparent M, 40,000 was almost isoelectric to component D, and the more acidic variant of this pair of polypeptides appeared at variable intensities in different preparations. The appearance of cytokeratin polypeptides in two or more distinct isoelectric variants may reflect different degrees of phosphorylation and is characteristic not only for cytokeratins of various cultured cells (these authors, unpublished data; for keratinocytes see also reference 17) and rat liver (16) but also for proteins of other types of intermediate-sized filaments (for review see 12). The residual actin observed in such high salt-extracted desmosome-tonofilament fractions was exclusively of

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**Figure 4**
Electron micrograph of the fraction introduced in Fig. 3, showing the ultrastructure of the extracted apical tonofilaments and their association with microvillar bases (arrowheads). Note the electron-transparent cores of tonofilaments (e.g., inset, lower left and upper right, denoted by bracket and arrowhead). Bars, 0.1 μm. x 133,000. Inset, x 300,000.
FIGURE 5  Electron micrograph of pelleted desmosome-tonofilament complexes obtained after extraction of isolated brush borders with high salt buffers and Triton X-100, showing the abundance of residual desmosomal plaques (arrows in a; D in b and d) and their association with tonofilaments which in their attachment regions often exhibit a tendency to form bundles (seen in b at higher magnification). A partly disrupted desmosome plaque is shown in c; bars denote plaque fragments. A preserved symmetrical desmosome residue is presented in d. Bars, 1 μm (a) and 0.2 μm (b-d). (a) × 35,000; (b) × 90,000; (c) × 70,000; (d) × 70,000.
the nonmuscle $\beta$- and $\gamma$-type (Fig. 8), in agreement with Bretscher and Weber (22). A number of smaller cytoskeletal polypeptides appeared, at variable intensities, in positions almost corresponding to a diagonal line between components A and actin (Fig. 8) but the significance of these polypeptides was hard to assess: their artificial origin by proteolytic degradation, during isolation or in the urea-containing lysis buffer (41), cannot be excluded (for a similar series of degradation products from another cytoskeletal protein, vimentin, see references 12, 57, and 58).

Comparison of the two-dimensional gel electrophoretic pattern of the major polypeptides of the desmosome-tonofilament fraction (Fig. 8) with those of other cytoskeletal proteins also showed that this fraction did not contain detectable amounts of other intermediate filament proteins, in particular vimentin and desmin (compare Fig. 8 with two-dimensional gel electrophoretic separations of mammalian desmin and vimentin published in references 57–59). Nonidentity of major tonofilament proteins with vimentin and desmin was also established by co-electrophoresis in two-dimensional separations (data not shown).

**Immunologic Identification of Prekeratin-like Proteins**

Polypeptides of desmosome-tonofilament fractions from rat intestinal brush border separated by one- and two-dimensional gel electrophoresis were examined by reaction with antibodies to prekeratin(s) from bovine muzzle using immunoreplica and blotting techniques (see Materials and Methods). The three major polypeptides (components A and D and the $M_r$ 40,000 polypeptide) were identified as related to epidermal prekeratin by reaction with prekeratin antibodies. A typical example of a blot of brush-border proteins separated by one-dimensional gel electrophoresis after exposure to a specific prekeratin antibody preparation is presented in Fig. 7c (slot 3). With this antibody preparation only component A showed strong binding of antibodies. When antisera to epidermal prekeratins that showed
FIGURE 7 (a–c) SDS polyacrylamide gel electrophoresis showing major polypeptides of brush-border fractions from rat small intestine and subfractions derived therefrom. Fig. 7 a presents (7.5% acrylamide gel containing 0.1% SDS): slot 1, reference proteins (from top to bottom: phosphorylase a, bovine serum albumin, actin, and chymotrypsinogen); slot 2, prekeratin from bovine muzzle (components I, III, IV, VI, and VII are separated under these conditions); slot 3, total brush-border fraction; slots 4 and 5, different loadings of fraction of desmosome-attached tonofilaments obtained after one extraction with high salt buffer and Triton X-100; slot 6, microvillar fraction derived from brush-border fraction shown in slot 3; slot 7, material obtained after extraction of the 10-fold protein amount of isolated microvilli as shown in slot 6 with high salt buffer and Triton X-100 as described for preparation of desmosome-attached tonofilaments (material shown in slots 4 and 9). Dots (slot 1) denote the component that may correspond to the additional band described a, , , and . The small arrow points to a consistently observed minor component which has not entered the focusing gel, probably because it has not been solubilized. IEF, direction of isoelectric focusing; SDS, direction of second-dimension gel electrophoresis in the presence of SDS.

Comparison of Major Polypeptides of Intestinal Tonofilaments with Those of Epidermal Tonofilaments

Although prekeratins from epidermal desmosome-attached tonofilaments cross-react immunologically with major proteins of desmosome-tonofilament fractions from intestinal brush border, the conditions used, this antibody has shown strong binding only to cytokeratin component A of brush-border tonofilaments. Other components of insoluble brush-border proteins were also identified as related to prekeratins. For example, a blot of desmosome-tonofilament proteins separated by two-dimensional gel electrophoresis and exposed to the same broadly cross-reacting antibody preparation shown in Fig. 1 allowed positive identification of both component A and the Mr 40,000 polypeptide as related to epidermal prekeratins (Fig. 9). In addition, some minor components only weakly stained with Coomassie Blue were recognized as prekeratin-like polypeptides by their intense binding of prekeratin antibodies. These prekeratin-like polypeptides may include certain proteolytically derived minor components. Interestingly, the same prekeratin antibodies to bovine muzzle prekeratin that did not react with epidermal prekeratin component VII (Fig. 1) also did not react with the similarly sized and similarly charged, though not identical, intestinal tonofilament component D (Figs. 7c and 9). Several other prekeratin antibodies, however, did react with component D of intestinal tonofilament fractions (data not shown here).

**FIGURE 8** Two-dimensional gel electrophoresis of desmosome-associated tonofilaments of brush-border fractions from rat intestine without (inset) and with a-actin from rabbit skeletal muscle added as internal reference protein. The pH values have been determined in isoelectric focusing gels performed in parallel. In addition, isoelectric pH values and molecular weights have been determined by co-electrophoresis of reference proteins, including BSA, desmin, vimentin, tropomyosin (not shown). Components A and D are denoted by brackets, the lower brackets indicate the position of the two isoelectric variants of the Mr 40,000 protein. Acts in are denoted a, b, γ. The small arrow points to a consistently observed minor component that may correspond to the additional band described on one-dimensional gel electrophoresis in slot 7 of Fig. 7b. Horizontal bars denote material of component A, actin, and Mr 40,000 protein which has not entered the focusing gel, probably because it has not been solubilized. IEF, direction of isoelectric focusing; SDS, direction of second-dimension gel electrophoresis in the presence of SDS.
FIGURE 9 Autoradiofluorogram of an immunological reaction of polypeptides of the desmosome-tonofilament fraction from intestinal brush border separated by two-dimensional gel electrophoresis, transferred by blotting onto nitrocellulose paper and treated with antibodies to bovine muzzle prekeratin which show cross-reactivation between various epidermal prekeratins (same serum as described in Fig. 1), and 125 I-protein A. Labeling of major components identified by comparison with the blotting paper stained with Coomassie Blue is as presented in Fig. 8. In addition, the position of actin is demarcated by the horizontal bars; the arrowhead denotes a spot showing an especially intense reaction of a minor component with the prekeratin antibodies. Note absence of reaction with component D.

Discussion

The procedure described here allows the isolation of a topologically defined subfraction of a type of intermediate-sized filaments, i.e., tonofilaments, from a nonepidermal cell, e.g., the brush border of intestinal cells. Similar procedures may be used to isolate high salt-insoluble cytoskeletal elements from other epithelial cells and subcellular fractions. The material isolated consists almost exclusively of tangles of tonofilaments, many of which are connected to residual structures of the desmosomal plaques and of the terminal web and are enriched in cytokeratins. We have not yet been able to identify possible constituent proteins of the desmosomal plaque proper, similar to the relatively large polypeptides described for isolated desmosomes from bovine muzzle epidermis by Skerrow and Maltsev (10, 60, 61). The relationship of the proteins found in high salt-extracted apical tonofilaments from intestinal cells to the ethylenediamine-extractable protein associated with tonofilaments of rat epidermis (62) also remains an open question.

The reason for the unusually high resistance of some actin, myosin, and the “Mr 110,000 protein” to the extraction in high salt buffers and Triton X-100 (see also reference 31) is not understood (for extraction of most brush-border actin and myosin in high salt buffer see references 24 and 30). Similar preparative resistance of actin to high salt treatment has been reported in several other cells (e.g., 1, 57, 58, 63). Our present observation in extracted brush borders of an intimate association of the tonofilaments of the “subapical skeletal disk” with both the desmosomes and the terminal web residues, in particular the bases of the microvillar rootlets, is compatible with the
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10. Motolyu, A. G. 1975. Desmosomes, filaments, and keratohyalin granules in the rat liver (for immunological reactivity see reference 64). However, the data suggest that the major polypeptides identified in this fraction are constitutive subunits of tonofilaments. Clearly, polypeptides of intestinal tonofilaments are related, by immunological and biochemical criteria, to prekeratin from epidermal tissue (for references see Introduction) and to wool keratin (for immunological cross-reactivity see reference 66). However, they differ, as well, from epidermal prekeratins by sizes and electrical charges (this study). On the other hand, the pattern of intestinal tonofilament polypeptides shows closer similarities to cytokeratins from other internal epithelial organs: For example, polypeptides similar to components A and D of intestinal tonofilaments have also been found in high salt-extracted cytoskeletons from rat and mouse liver (16) and rat and bovine urothelium (S. Winter, E.-D. Jarasch, D. Schiller, and W. W. Franke, unpublished data). This might suggest that the cytokeratins of epithelial cells of internal organs are more related to each other than to epidermal prekeratins. Yet, the relationship of the various cytokeratins is even more complicated since significant differences of cytokeratin polypeptide composition can also be found among various internal organs (this authors, manuscript in preparation), and different prekeratin polypeptides are expressed even in different layers of skin and in epidermal keratinocytes grown in culture (1, 11, 17, 65-69). We conclude that the different filaments, which are indistinguishable in different epithelial cells, can be formed by completely different sets of proteins of the cytokeratin family. These compositional differences apparently do not result in profound structural and, probably also, functional differences, in agreement with observations that the different combinations of polypeptides of epidermal prekeratin and cytokeratins of epithelial cells can reconstitute, after denaturation, tonofilament-like structures in vitro (e.g., 1, 13, 17, 39, 55, 56).

It is conceivable that only a portion common to all the different polypeptides of the cytokeratin family, probably the core region that is enriched in α-helical conformation and relatively resistant to trypsin treatment, is critical for the establishment and maintenance of the tonofilament structure (13, 70-72). The biological meaning of the different polypeptide composition of the same filament structure in different cells and tissues is unclear. Interestingly, the diversity of the cytokeratins present in different epithelia is somewhat reminiscent of the diversity of actins that occur in different lines of myogenic differentiation (73). In a detailed and systematic comparison of the various cytokeratins expressed in different types of epithelia, we are currently examining whether the specific patterns of cytokeratin polypeptides of different vertebrates are related to various lines of epithelial differentiations during embryogenesis.
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