The Brush Border Cytoskeleton Is Not Static: In Vivo Turnover of Proteins

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ABSTRACT The shape and stability of intestinal epithelial cell microvilli are maintained by a cytoskeletal core composed of a bundle of actin filaments with several associated proteins. The core filaments are intimately associated with the overlying plasma membrane, in which there occur rapid turnover of proteins and constant incorporation of new membrane. Previous work has shown that starvation or inhibition of protein synthesis results in modulation of microvillar length, which indicates that there may be cytoskeletal protein turnover. We demonstrate herein, by means of in vivo pulse labeling with radioactive amino acids, that turnover of brush border cytoskeletal proteins occurs in mature absorptive cells. Turnover of cytoskeletal proteins appears to be quite slow relative to membrane protein turnover, which suggests that the turnover of these two microvillar compartments is not coupled. We thus conclude that cytoskeletal protein turnover may be a factor used to maintain normal length and stability of microvilli and that the cytoskeleton cannot be considered a static structure.

The cytoskeleton of non-muscle cells has been implicated as the agent responsible for cell shape maintenance and cell motility. The actomyosin-based shape changes in non-muscle cells are modeled on movements of smooth and skeletal muscles and provide force for such events as cytokinesis, capping of receptors on lymphocytes, and intestinal brush border contraction. Other actin-based shape changes are regulated by assembly, disassembly, or rearrangements of the cytoskeleton in such diverse structures as stress fibers in fibroblasts (1) and filopodia of sea urchin coelomocytes (2). Another possible dynamic aspect of the cytoskeleton, protein turnover, has received little attention.

The brush border of intestinal epithelial cells has a well-defined and stereotyped cytoskeleton. The shape and stability of brush border microvilli are maintained by a cytoskeletal core of actin filaments bundled by the proteins villin (3–6) and fimbrin (7, 8); this bundle is cross-linked to the membrane by spirally arranged cross-filaments (9), probably composed of a 110,000-M_r polypeptide (10) associated with calmodulin (11, 12). The microvillar cores associate with the terminal web filament system, which includes myosin (13, 14) and TW 260/240, a fodrin-like protein (15–17), among other proteins. In recent years several reports on brush border motility have been published (18–22), thereby extending our knowledge of this system. While many details remain to be elucidated, it is now generally accepted that contraction of the brush border is generated by a terminal web adherens-zone circumferential ring, which contains both actin and myosin (20).

While much is known about the structure and motility of this cytoskeleton, very little is known about the factors and mechanisms that regulate microvillar length or how the seemingly static cytoskeleton interacts with the overlying membrane. It has been demonstrated that the brush border membrane is quite dynamic in terms of protein turnover, with the half-lives of both enzymes (23, 24) and glycoproteins (25) being <1 h. Whether microvillar cytoskeleton protein turnover occurs and, if so, whether it is coupled with such membrane turnover is not known. In the presence of such turnover, the fact that microvilli of a single brush border and those from each animal's duodenum are of about the same length (14) indicates that some form of length regulation must be occurring. In vitro experiments suggest that microvillus length might be regulated by the actin monomer concentration and/or by the presence of filament bundling proteins (26). The observation of LeCount and Grey (27) that blocking protein synthesis in chicks caused a transient shortening of intestinal microvilli indicates that levels of core filament protein synthesis and degradation may play some role in regulating microvillar length.

The present study demonstrates, by means of in vivo pulse labeling with radioactive amino acids, that synthesis and degradation of major brush border cytoskeletal proteins occur

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in mature absorptive cells. Therefore, these results suggest that the cytoskeleton is quite dynamic. Levels of protein synthesis may therefore be a factor for maintaining normal length and stability of microvilli in vivo by regulating the intracellular concentration of actin and other microvillar core proteins.

MATERIALS AND METHODS

Isotope Administration

In the single-label experiments, 250 μCi of [^{35}S]methionine (1,435 Ci/mmol; all isotopes were obtained from Amersham Corp., Arlington Heights, Il) in saline was injected into the proximal loop of the duodenum of adult chickens according to the methods described by Omdahl et al. (28), with the modification that the chickens were anesthetized with halothane (0.75–1.5% Fluorothane; Ayerst Laboratories, New York) instead of with ether. The isotope was chased with a 1,000-fold amount of unlabeled methionine 1 h after isotope administration. The body wall and incision was sutured and the animals were given free access to food and water. Demembranated brush border cytoskeletons were isolated as described previously (9) from the proximal loop only of chickens that had received isotope 6, 12, 18, and 24 h earlier. The single-label experiments were performed with Rhode Island Red chickens, which resulted in less pure preparations of brush borders (Fig. 2). The same trends were obtained with experiments performed with White Leghorn chickens, which were used in all other experiments because they yielded much purer brush border cytoskeletons (Fig. 3). The major contaminants of these preparations are nuclei. The vast majority of membrane proteins are removed by the Triton X-100 treatment.

In the double-label experiment, based on that described by Arias et al. (29), 10 μCi of [^{14}C]leucine (330 mCi/mmol) was injected into the duodenal loop of each of six halothane-anesthetized chickens 10.5 h before brush border preparation. The animals were kept under anesthesia for 1 h, at which time the isotope was chased by injection of a 1,000-fold excess concentration of unlabeled leucine. The incision was sutured and the animals were given free access to food and water. 5 h before brush border preparation the [^{14}C]leucine injected chickens were reanesthetized and 50 μCi of [^{3}H]leucine (1 Ci/mmol) was injected into the duodenal loop and then chased with unlabeled leucine 1 h later as described above. As controls, both isotopes were injected simultaneously into each of four chickens either 10.5 or 5 h before brush border preparation and chased 1 h later as described above.

Gel Electrophoresis and Determination of Radioactivity: Gel electrophoresis using 5–20% polyacrylamide SDS 1.5-mm-thick gradient slab gels was performed according to Laemmli (30) with the [^{35}S]-methionine-labeled material. The gel was stained with Coomassie Blue, photographed, prepared for fluorography with PPO according to Bonner and Laskey (31), and exposed at −70°C for 38 d. In the double-isotope experiments, 4 mg of solubilized brush border cytoskeletal proteins were separated on 1.5-mm-thick 5–20% polyacrylamide SDS gradient preparative gels prepared with DATD (*N,N'*diallyltartardiamide; Bio-Rad Laboratories, Richmond, CA) substituted for *Bis*(*N,N'*methylene-*bis*-acrylamide). Three separate gels were run from each brush border preparation. Individual bands were sliced from each stained gel, thoroughly destained, and dissolved in 3% periodic acid overnight at room temperature. Proteins were then precipitated with 10% trichloroacetic acid, and the precipitates were collected by centrifugation at 9,500 *g* for 20 min. Pellets were washed twice in H_2O, transferred to Aquasol (New England Nuclear, Boston, MA), and disintegrations per minute (dpm) were determined for each band in a liquid scintillation counter (Minirak; LKB Instruments, Inc., Gaithersburg, MD) programmed to detect ^{3}H and ^{14}C simultaneously. Mean values were then compared; in the case of the experimentals, 18 values (six chickens with three gels of each) were used to determine means. Ratios of ^{3}H to ^{14}C were analyzed statistically by means of the Student's *t* test or by analysis of variance. One advantage of the double-isotope method in turnover studies is that results do not depend upon total recovery of protein from the gels since the significant information is in the ratio of ^{3}H to ^{14}C counts incorporated into the same protein. As disintegrations per minute were relatively low (30–280 dpm above a background of <10), each sample was counted for 60 min.

RESULTS

Cells Studied

One concern in studying these cells is that intestinal absorptive cells are part of a constantly renewing cell population with an average cell life of ~48 h (32). Nevertheless, we are convinced for several reasons that intraluminal administration of the isotopes and proper choice of the time course of isotope administration minimize or eliminate possible influences by differentiating cells arising from the mitotic cell population. First, Alpers (23) has documented that protein synthesis is ongoing in all mature absorptive cells along the length of the villus and has shown (33) that intraluminally administered amino acids are preferentially taken up and incorporated into protein by villus absorptive cells relative to crypt cells, which were found not to readily take up intraluminally administered amino acids. Second, the cell isolation method preferentially isolates villus cells from the upper two-thirds of the villus, a finding documented by light microscopy of the isolated epithelium, which showed no contamination by crypts. As a control to the method itself, the route of isotope administration (intraluminal injections) was found to yield a true pulse label of the proteins showing only low levels of radioactivity in the free amino acid pool 1 h after isotope administration (data not shown); this finding is in agreement with similar studies by Alpers (33). By contrast, intraperitoneal injections were found not to produce a pulse label (data not shown). Finally, great care was taken to ensure that the animals had free access to food and water (which they were observed to use), since intestinal microvilli have been reported to shorten in fasted animals (34).

Time Course

Preparations of brush border cytoskeletal proteins 6, 12, 18, and 24 h after administration of [^{35}S]methionine show a peak of incorporation at 6 h, a considerable decline by 12 h, a slight increase by 18 h, and a low at 24 h (Fig. 1). Fluorography of polyacrylamide gels from this experiment clearly indicates that all cytoskeletal proteins are synthesized, incorporated into the brush border, and are degraded or removed over time in a continuous manner (Fig. 2). The second peak of incorporation at 18 h, detected by total counts or by fluorography, is probably due to the low amount of detectable de novo assembly of brush border cytoskeletons in differentiating cells, originating from the mitotic population, which have migrated up the villus in 18 h to become absorptive cells.

Relative Rate of Turnover

Relative turnover rates of the different cytoskeletal proteins were determined by the double-isotope technique of Arias et al. (29). Calculations of absolute protein half-lives based on measurements of decay of radioactivity have been shown to be inexact because of reutilization of label (35); therefore, we limited our studies to determinations of relative turnover rates.

Based on the results from the [^{35}S]methionine experiment,

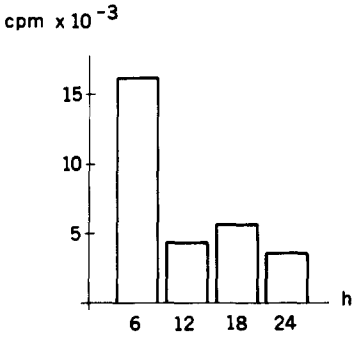

FIGURE 1 This figure shows the incorporation of [^{35}S]-methionine into total brush-border cytoskeletal protein in a pulse-chase experiment. Counts per minute for 250 μg of cytoskeletal protein from animals pulsed 6, 12, 18, and 24 h prior to brush-border cytoskeleton isolation are shown.

the double-isotope experiment was designed in a way to keep possible interference by proteins synthesized by differentiating cells minimized. Therefore, isotopes were injected intraluminally at most 10.5 h prior to the preparation of brush borders. The results (Table I) demonstrate that all cytoskeletal proteins, including actin, turn over. An analysis of variance test shows that the variance between control means is not significantly higher than expected for a homogeneous population (P = 0.05). On the other hand, an analysis of variance test comparing the experimental means with control means demonstrates that they are clearly not from the same population (P = 0.05). A t test comparing each protein from the experimentals with its corresponding control shows that all experimentals are significantly different from their controls (P = 0.02 for fimbrin; P = 0.01 for all others). The two proteins thought to be membrane associated, 110,000 and TW 260/240, turn over at a significantly higher rate (P = 0.05) than actin (Table I). Among the experimentals, no difference in turnover rate could be detected between myosin, villin, fimbrin, and actin as determined by an analysis of variance (P = 0.05).

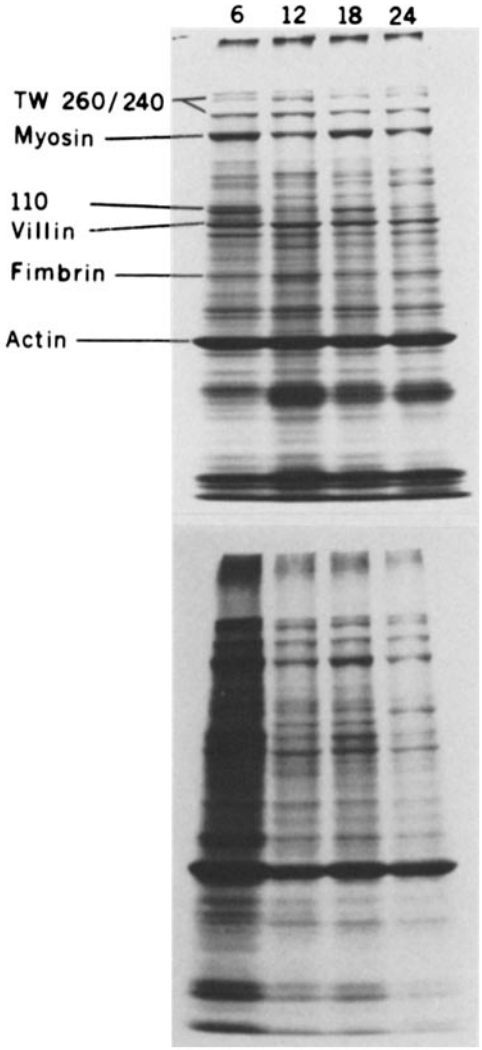

FIGURE 2 (*a*) SDS polyacrylamide gel stained with Coomassie Blue loaded with identical amounts of cytoskeletal proteins prepared 6, 12, 18, and 24 h after [^{35}S]methionine injection. *110*, 110-Kdalton protein. (*b*) Fluorograph of the same gel developed after 38 d of exposure, showing time course of incorporation of label into all major cytoskeletal proteins.

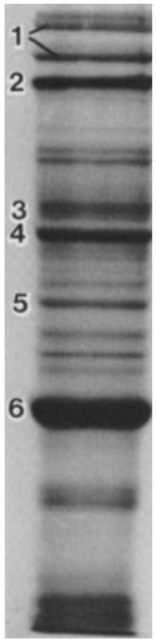

FIGURE 3 A representative gel of the brush-border cytoskeletal preparation used in the double-isotope experiments. Note relatively few contaminating bands compared with gels in Fig. 2*a*. *1*, TW 260/240; *2*, myosin; *3*, 110-Kdalton protein; *4*, villin; *5*, fimbrin; *6*, actin.

TABLE I

*$^{3}H/^{14}C$ Ratios of Major Cytoskeletal Proteins**

	(5/10.5)		Controls (5 h)	
	x	s	x	s
TW 260/240	5.5811	(1.2497)	3.4370	(0.2312)
Myosin	5.2738	(1.2894)	3.2992	(0.3667)
110,000 protein	5.6244	(1.1253)	3.3624	(0.3667)
Villin	5.2894	(0.8688)	3.3413	(0.1883)
Fimbrin	5.2297	(1.3489)	3.3313	(0.2384)
Actin	4.6102	(0.7312)	3.2482	(0.1385)

* Ratios shown represent mean values (x) of six separate experiments (four for control values); s = standard deviation.

DISCUSSION

While the membranes of many cell types, including that of the intestinal brush border, have been shown to be very dynamic in terms of protein turnover, the underlying cytoskeleton, which is responsible for determining the shape and mobility of the surface, has generally been thought of as being rather static. The results presented in this study clearly demonstrate the synthesis and incorporation of specific cytoskeletal proteins into a well-defined cytoskeleton followed by their removal. The proteins found in the microvillar core (actin, fimbrin, and villin) turn over at the same relative rate and therefore apparently in a coordinate fashion. The membrane-associated microvillus cross-filament protein (110,000) and the terminal web protein, TW 260/240, turn over at a faster rate than actin. Similar pulse chase experiments have demonstrated synthesis and degradation of brush border membrane proteins in fully differentiated intestinal absorptive cells (23–25, 36, 37). Actin and myosin have been shown to turn over coordinately in tissue culture cells (38), although the exact location or assembly state of the proteins was unknown. In addition, actin, tropomyosin, and troponin have been shown to turn over asynchronously in muscle cells in vivo (39). Therefore, turnover of cytoskeletal proteins may be a general process common to all cells.

There may be several physiological purposes of brush bor-

der cytoskeleton protein turnover. Although there is a continuous renewal and turnover of the brush border plasma membrane, including incorporation of intrinsic glycoproteins and enzymes (23–25, 37) mediated by fusion of Golgi vesicles with the plasma membrane, it is unlikely that cytoskeletal turnover is directly coupled with membrane turnover. Membrane protein turnover appears to be extremely rapid, of the order of <1 h (24, 25), whereas the results of our single-label experiments indicate that cytoskeletal protein turnover is of the order of many hours. Studies on contractile protein turnover in muscle also indicate the slowness of turnover of such proteins (38) relative to that of membrane proteins. However, the finding that the cross-filament protein of the microvillus core that links the actin bundle to the membrane turns over at a higher rate than the other core proteins indicates that there may be some interaction of this protein with the membrane. Only through a direct comparison of relative rates of turnover of membrane proteins with that of the cross-filament protein can evidence of coupling of turnover of these two compartments be related. However, our results do indicate that protein turnover is a feature of the cytoskeleton and not just the overlying membrane.

Another possible implication of turnover of actin and other microvillar core proteins may be microvillar length regulation. Physiological and experimental studies of intestinal cells and microvillar length support the contention that cytoskeletal protein turnover may play a role in regulation of microvillus length. First, our results indicate that turnover of microvillar cytoskeletal proteins occurs even though microvilli do not appear to grow in length (14), while other experiments show that inhibiting protein synthesis will induce microvillar shortening (27). Second, physiological studies have determined that: (*a*) vitamin D administration, which causes an increase in Ca^{++} transport by absorptive cells among other metabolic effects (40), induces increased synthesis of actin by absorptive cells (41, 42); and (*b*) fasting of animals causes a shortening of brush border microvilli (34). Therefore, the microvillar cytoskeleton is not a static but a dynamic structure.

In another cellular cytoskeletal system, the miocrotubule, it has been suggested that the size of the monomer pool of tubulin regulates the amount of assembled microtubules (43) and that the amount of monomeric tubulin regulates tubulin synthesis (44, 45). Recently, Cleveland and Havercroft (46) have presented evidence suggesting that the level of regulation is not transcriptional as previously thought. The physiological and experimental observations described above, coupled with our results, suggest that the ratios of the pool of unincorporated protein to that in the over-turning cytoskeleton may regulate microvillus length in a manner similar to the way microtubule length is regulated. Whether it is the pool of actin monomers or that of another core protein that might regulate filament length is unknown at this time. The existence of such a length-regulating mechanism may provide the cell a means of responding to the physiological conditions of the animal.

Recently, Blikstad et al. (47) have reported on the synthesis and assembly of spectrin during avian erythropoiesis. They suggest a scheme whereby the amount of β-spectrin synthesized and incorporated into the cytoskeleton regulates the rate of assembly of stoichiometric amounts of the spectrin complex since α-spectrin is found in great excess in the cell. Whether the level of control in the brush border cytoskeleton for assembly is transcriptional as in most eukaryotic genes studied thus far, post-transcriptional as in the tubulin system, or post-translational as spectrin assembly in erythropoiesis, is not known at this time.

The existence of protein turnover in the brush border cytoskeleton also raises questions about the site of assembly of newly synthesized proteins into this cytoskeleton. Actin monomer addition occurs at both ends of the microvillar core filament bundle in vitro, with a strong bias for addition at the membrane-associated end (26). The addition of newly synthesized actin to the brush border cytoskeleton demonstrated here may be an indication of the occurrence of in vivo treadmilling of actin, a process heretofore demonstrated only in vitro (48–51). This scenario is likely because newly synthesized actin monomers are probably added to one end of the filament bundle and removed (or degraded) at the other end of the bundle. If addition occurs at the ends of filaments, then it is also unlikely that the filaments are capped by capping proteins, which have been shown to block monomer addition to actin filaments in vitro (for review see reference 52). However, the exact mechanism, rate, site of actin assembly, and site of degradation in vivo are presently not known.

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