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Calcium control of the intestinal microvillus cytoskeleton: Its implications for the regulation of microfilament organizations

(actin/villin/calmodulin/membranes/trifluoperazine)

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ABSTRACT **The microvillus core-filament bundle from intestinal epithelial cells is a highly ordered structure containing actin and four major associated proteins. Two of these, villin and calmodulin, bind calcium ions ($K_d \approx 10^{-6}$ M) in the physiologically important range. Because ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid is present throughout the purification and the isolated cores contain levels of calcium substoichiometric to calmodulin, the protein is bound in the structure without calcium saturation. 10-[3-(4-Methyl-1-piperazinyl)propyl]-2-trifluoromethylphenothiazine, a calmodulin-specific drug, removes the protein from the cores without visibly affecting their ultrastructure. Calmodulin-depleted cores rebind exogenously supplied brain calmodulin. Although the core filaments are stable when the calcium level is less than 10^{-7} M, they disassemble when it is greater than 10^{-6} M. This appears to be due to the calcium-sensitive allosteric transition of villin from an F-actin bundling protein to an F-actin severing protein. The actions of the two calcium-binding proteins, villin and calmodulin, are discussed in terms of the calcium sensitivity of the filament bundle. We suggest that villin may act as a calcium-sensitive factor regulating microfilament assembly and disassembly and that calmodulin serves as a buffer modulating the free calcium concentration. This hypothesis may explain some aspects of the physiological process of calcium uptake in the intestine and of the effects of calcium fluxes on the submembranous organization of microfilaments in other cells and tissues.**

Local changes in the cytoplasmic free calcium concentration, which is assumed to be in the micromolar or submicromolar range, trigger many important cellular functions. Currently, the best understood such process is that involving muscle contraction and relaxation, which can be explained in molecular terms (1). Ca^{2+} changes also regulate many important events of intermediate metabolism through the modulation of key enzymatic activities by calmodulin, the widespread calcium-binding protein of eukaryotic cells (2). In addition, Ca^{2+} regulates the morphology and locomotion of various nonmuscle cells. Because the latter processes are intimately connected with the organization and turnover of actin-containing microfilaments, much recent interest has focused on calcium control of these ubiquitous cellular organizations. At least two Ca^{2+}-dependent events have been discerned in this context. The first is the action of a specific calmodulin-dependent myosin kinase, which phosphorylates a myosin light chain and thereby regulates the activity of the F-actin-activated myosin ATPase (2, 3). The second is the Ca^{2+} ion inhibition of the formation of three-dimensional gels of F-actin and actin-binding proteins in both crude cellular extracts and subfractions derived from them (4). Several proteins have been implicated in the calcium sensitivity of F-actin gelation such that changes in the free calcium concentration at micromolar levels induce phase transitions (4–8).

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The core filament organization of the microvilli present on intestinal epithelial cells provides an ideal system for the analysis of microfilament organization and its regulation by calcium. Microvilli can be isolated intact and free of other contaminating filaments. The microvillus core filaments are stable even after removal of the plasma membrane, retaining the cross-bridges that normally connect the microvillus core with the plasma membrane (9–11). Because the intestinal epithelium is the place of calcium transport into the body (12), calcium fluxes would be expected in the microvillus, as well as a mechanism for the control of the internal free calcium level. The microvillus core has a relatively simple protein composition—in addition to actin, there are four major associated proteins (7, 11, 13, 14). Here we explore the function of two of them, calmodulin and villin, as Ca^{2+}-binding proteins in an isolated microfilament structure.

MATERIALS AND METHODS

Chicken intestinal brush borders were prepared as described (10), except that solution I [75 mM KCl/0.1 mM $MgCl_2$/1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA)/10 mM imidazole, pH 7.3; ref. 11] was used throughout. Microvilli were prepared (10) and demembranated in 1% Triton X-100 for 15 min at 22°C with gentle stirring. All solutions were 0.25 mM in phenylmethylsulfonyl fluoride. Harvested microvillus cores were suspended and added to solution I containing 10-[3-(4-methyl-1-piperazinyl)propyl]-2-trifluoromethylphenothiazine (trifluoperazine) dihydrochloride, free Ca^{2+}, or both, as given by the molar ratio of Ca/EGTA. After a 10-min incubation at 4°C, centrifugation at 12,000 × *g* for 10 min (Eppendorf centrifuge) gave a supernatant and a pellet fraction that were estimated by subjecting equivalent percentages of each fraction to $NaDodSO_4$/polyacrylamide gel electrophoresis using 6–15% gradient slab gels. Calcium concentrations were monitored as total calcium by atomic absorption and as free calcium by using the Arsenazo method (15). Free calcium in balanced Ca/EGTA buffers was estimated as described (8). Villin purified to homogeneity and free of calmodulin (7) was extensively dialyzed and then subjected to equilibrium dialysis with ^{45}Ca in 10 mM imidazole/75 mM KCl/1 mM $MgCl_2$, pH 7.3 at 4°C for 20 hr. Protein contents were monitored by the Lowry procedure. Known amounts of homogeneous bovine brain calmodulin purified as described (16) and monitored by quantitative amino acid analysis were run in parallel on $NaDodSO_4$/polyacylamide gels to assess the calmodulin content of the various fractions by

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; trifluoperazine, 10-[3-(4-methyl-1-piperazinyl)propyl]-2-trifluoromethylphenothiazine.

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densitometry. Electron microscopy of embedded material was as in ref. 7.

RESULTS

Calmodulin. A typical gel pattern of isolated microvilli from intestinal epithelial cells before and after demembranation with Triton X-100 is shown in Fig. 1. The purified core filaments contained, in addition to actin, four major structural components: the 105,000 M_r polypeptide, villin (M_r = 95,000), fimbrin (M_r = 68,000), and a polypeptide of apparent M_r of 17,000 (see also refs. 7, 11, 13). The latter protein is calmodulin, as shown by Howe and Mooseker by coelectrophoresis experiments, activation of phosphodiesterase, and heat resistance (14). We confirm this assignment. Calmodulin isolated from a boiled extract of microvilli has the typical amino acid composition of brain calmodulin, including the presence of a single residue of trimethyllysine (2), and binds specifically to Sepharose-immobilized phenothiazine (17). Among the minor components present were the four very-low-molecular-weight proteins (12,000–15,000) previously reported (11). We found the amount of these proteins to be very variable and have avoided preparations in which they become prominent. The relative content of calmodulin in several preparations was monitored by gel scanning analysis and, when expressed as a molar ratio to actin, increased from 1:7.5 in brush borders to 1:3 in core filaments, confirming that calmodulin is a major protein of this structure (14). Its concentration is higher than that of the other three associated proteins, which seem to be present in an approximate ratio of 1:10 compared with actin (11, 18). As long as the isolation buffer contains EGTA, we have obtained reproducible calmodulin values that are not reduced even if 10 mM EGTA is included. Atomic absorption showed that isolated core filaments contain 0.9 (±0.3 SD) nmol Ca/mg protein or 0.48 (±0.13 SD) mol Ca/per mol calmodulin present. Clearly, the three or four tight Ca^{2+} binding sites of calmodulin (2, 19) are not all occupied in the isolated structure. The data suggest instead that, at most only half the calmodulin molecules of the core filaments contain a single Ca^{2+} under the conditions of microvillus core isolation; thus, at least part of the calmodulin is present on the microfilament core without bound Ca^{2+}. Although it is generally assumed that calmodulin binds to its receptors only in the presence of calcium (2), there are at least two other examples where dissociation does not occur in the presence of EGTA (20, 21).

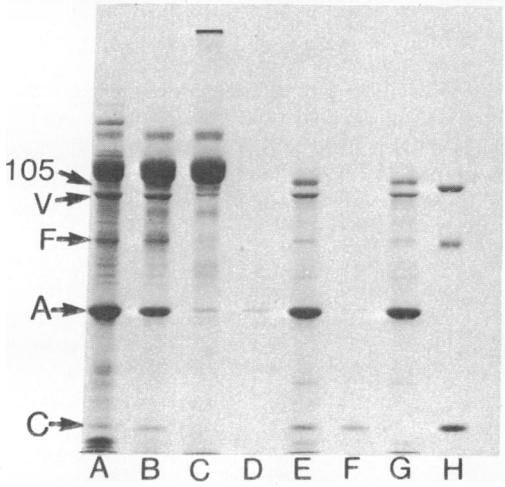

FIG. 1. NaDodSO$_4$/polyacrylamide gel electrophoresis of brush borders (A), microvilli (B), 1% Triton-soluble proteins (C), and core filaments (D–G). Core filaments were added to solution I (D and E) or to solution I containing trifluoperazine (0.25 mM) and having a Ca/EGTA ratio of 0.6 (F and G). D and F were supernatants, and E and G were pellet fractions after 10-min incubation at 0°C. Equivalent amounts of fractions are shown. Markers were α-actinin (M_r = 100,000), bovine serum albumin (M_r = 68,000), and calmodulin (M_r = 17,000) (H). Microvillus proteins marked are calmodulin (C), actin (A), fimbrin (F), villin (V), and the 105,000 M_r protein (105).

Reversible Release of Calmodulin. To assess a possible ultrastructural importance of calmodulin in the core filaments, we tried to remove it specifically. Increase of calcium levels in buffer I to a Ca/EGTA ratio of 0.6 in the presence of 0.25 mM trifluoperazine, which is known to bind tightly to calmodulin (2, 17, 22), gave core filaments devoid of calmodulin but still containing actin and the other three associated proteins—fimbrin, villin, and the 105,000 M_r protein (Fig. 1). Thus, although trifluoperazine alone resulted in the release of ≈50% of the calmodulin, complete calmodulin release was observed only when the Ca/EGTA ratio was 0.6, a level that has little effect on the core structure (see below). Electron microscopy of calmodulin-depleted core filaments showed a normal ultrastructure, including the presence of cross-bridges (9–11), which, it has been suggested, are composed of the 105,000 M_r protein (11). Calmodulin dissociation from core filaments is a reversible process. Core filaments freed of trifluoperazine and returned to solution I incorporated purified bovine brain calmodulin to a final level corresponding to 85% of that of isolated core filaments (using the actin content as internal standard; see Fig. 2).

Villin Is a Calcium-Binding Protein. Villin is currently the only other F-actin-associated protein of the microvillus core purified to homogeneity. It shows calcium-dependent interaction with both G- and F-actin in reconstituted systems (7). Therefore villin, free of calmodulin, was subjected to equilibrium dialysis. A single tight calcium-binding site (K_d = 2.5 μM) was found (Fig. 3).

Calcium-Dependent Disassembly of Core Filaments. The finding of two Ca^{2+}-binding proteins—calmodulin and villin—in isolated core filaments implies a functional importance of calcium ions in microvillus organization and stability. Therefore, isolated core filaments were subjected to a series of graded calcium solutions, and their stability in the standard assay was determined (Figs. 4 and 5). At a Ca/EGTA ratio of 0.9 (approximately 5×10^{-6} M free Ca^{2+}) drastic disassembly of the core filaments occurred. Fimbrin and villin became nonsedimentable to about 90%, whereas incomplete release of

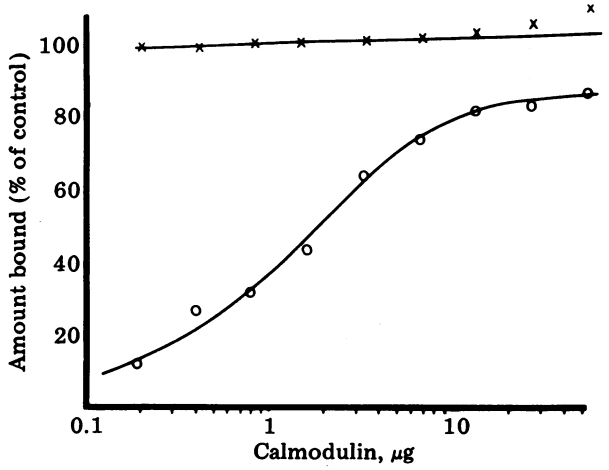

FIG. 2. Uptake of exogenous calmodulin by depleted cores. Cores were prepared as in Fig. 1 and, after drug removal, challenged in solution I with various concentrations of calmodulin for 15 min at 0°C. Collected cores were analyzed by gel electrophoresis, and the bound calmodulin was determined by densitometry. O, Calmodulin-depleted cores; X, control cores.

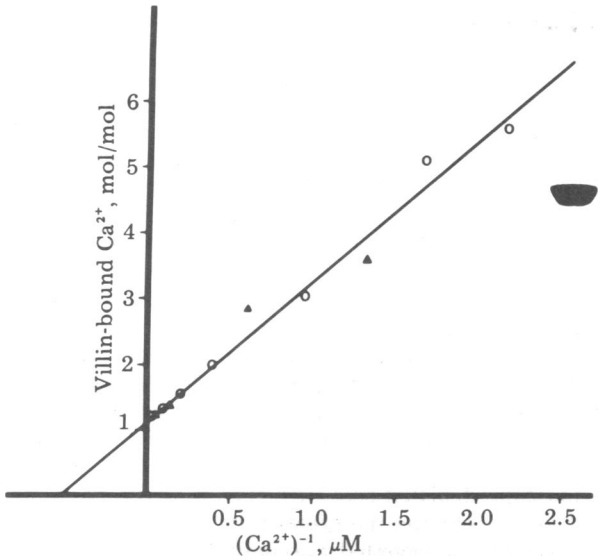

FIG. 3. Calcium binding of villin in equilibrium dialysis using ^{45}Ca in 10 mM imidazole/75 mM KCl/1 mM $MgCl_2$, pH 7.3 at 4°C. o, Normal villin preparation; x, villin after chromatography on DE52 (7). Protein used had been previously dialyzed into the buffer used. Insert shows stained gel of villin preparation.

actin (<60%) and calmodulin (<40%) was evident and the 105,000 M_r protein remained in the pellet fraction (<15% released). At Ca/EGTA ratios of <0.6 (≈7×10^{-7} M free calcium), there was very little release of actin, calmodulin, and the 105,000 M_r protein (<10%), although some villin and fimbrin were released (<15%). Thus, core filaments are stable at concentrations of free Ca^{2+} of less than 7×10^{-7} M and disassemble at 10^{-6} M free Ca^{2+}. Even at the highest calcium concentrations tested, calmodulin release was reproducibly incomplete, and release of the 105,000 M_r protein was barely detectable. The isoelectric focusing and two-dimensional gel pattern of proteins released by Ca^{2+} treatment of microvillus cores is shown in Fig. 6. The ratio of the β- and γ-non-muscle actin species is similar to that in the unfractionated microvilli cores (10). In addition, the two variants (7) of villin, fimbrin and calmodulin, are observed.

The supernatant fraction from core filaments disassembled at a Ca/EGTA ratio of 5 was subjected to sucrose gradient centrifugation (see Fig. 7). All the villin was contained in a peak at 9.4 S, which also contained some of the actin, whereas actin-free villin sediments at 5.1 S (7). By assuming equivalent staining of the two proteins by Coomassie brilliant blue, we calculated that the molar ratio of the complex is approximately

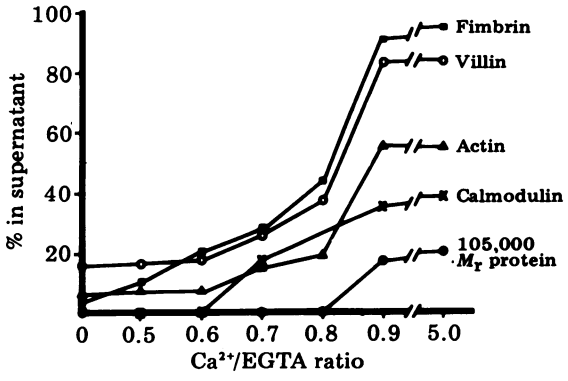

FIG. 4. Stability of cores at different Ca/EGTA ratios in solution I at 4°C. Treatment was for 10 min. Equivalent fractions of pellet and supernatant were analyzed by gel electrophoresis followed by densitometry.

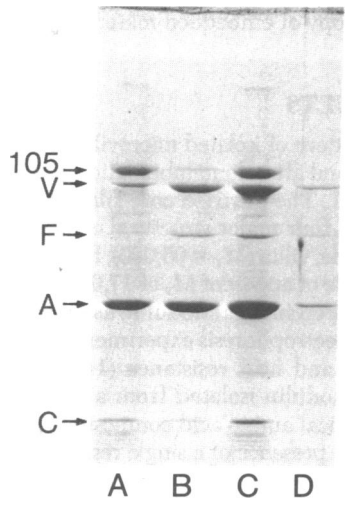

FIG. 5. $NaDodSO_4$/polyacrylamide gel electrophoresis of microvillus core preparations exposed to Ca/EGTA ratios of 0.9 (A and B) and 0.6 (C and D). Equivalent amounts of supernatants (B and D) and pellets (A and C) were analyzed. Proteins marked as in Fig. 1.

three actins per villin. When DNase I, a known G-actin-binding protein (24), was added to the calcium extract of microvilli, gradient centrifugation showed a defined complex of DNase–actin–villin sedimenting at approximately 9.7 S. The molar ratio of the three proteins in the complex was computed to be 2.1 actin to 1.8 DNase I to 1.0 villin. These results confirm the suggestion (7) that villin binds to monomeric actin in the presence of calcium and suggests two actin binding sites per villin molecule. Calmodulin and fimbrin are clearly resolved from the actin–villin complexes, indicating that, at least under these experimental conditions, no stable association of these proteins occurs.

Because of the sensitivity of core filaments to increased free calcium concentrations, it was important to distinguish calcium sensitivity due to calmodulin binding from calcium sensitivity due to villin. To do this, core filaments free of calmodulin were prepared as described and subjected to the same graded series of free calcium ions. Again, by using the standard assay, extensive disassembly was observed at 5×10^{-6} M. Thus, the calcium-induced disassembly process occurs independent of the presence of calmodulin.

DISCUSSION

The ultrastructurally intact microfilament organization of isolated intestinal microvilli provides a suitable system to study some aspects of the structural, functional, and regulatory properties of native microfilaments *in vitro*. The isolated

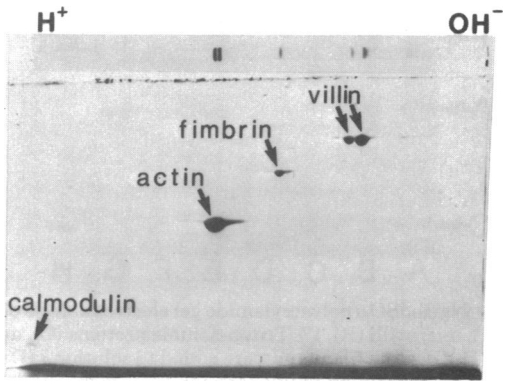

FIG. 6. Two-dimensional gel analysis of microvillus core filament proteins, released by treatment with 5 mM $CaCl_2$. Soluble proteins were clarified by high speed centrifugation and subjected to one- and two-dimensional electrophoresis by the method of O'Farrell (23). Horizontal dimension, isoelectric focusing; vertical dimension, $NaDodSO_4$/polyacrylamide gel electrophoresis.

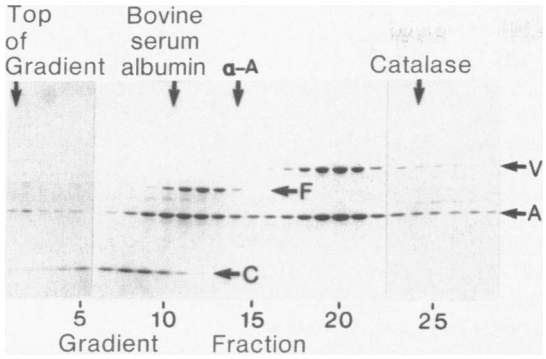

FIG. 7. Soluble proteins obtained after exposure of microvilli cores to 5 mM $CaCl_2$ in solution I were clarified by high speed centrifugation and then analyzed on 5–20% sucrose gradients in the same solvent (15 hr at 4°C, 40,000 rpm Beckman SW60 rotor). Fractions were concentrated and analyzed by polyacrylamide gel electrophoresis. Proteins marked as in Fig. 1. Parallel gradients had the following markers: α-actinin (6.2 S), bovine serum albumin (4.2 S), and catalase (10.3 S).

core-filament bundle has a relatively simple composition that is characterized by a parallel arrangement of F-actin fibers and four major associated proteins, two of which—calmodulin and villin—bind calcium tightly ($K_d \approx 10^{-6}$ M), thus covering the physiological range. We have tried to explore the possible functions of both proteins for microvillus structure and function.

Calmodulin remains tightly bound to the core filaments despite the use of EGTA buffers during their isolation. Next to actin, it seems the most abundant structural protein. By assuming a ratio of one calmodulin for every three actins, 370 actin monomers per μm length of actin filament (25), a minimum of 20 F-actin filaments per microvillus core, and a microvillus core diameter of 0.1 μm (9), we calculated that calmodulin is present at a level of about 0.6 mM in the microvillus. As there are three to four high-affinity Ca^{2+} binding sites per calmodulin molecule (2, 19), the maximal calcium-binding equivalent is approximately 2 mM, which suggests an effective Ca^{2+}-buffering capacity.

Calmodulin can be selectively and specifically released from the microvillus core by the calmodulin-specific phenothiazine drug, trifluoperazine. The resulting core-filament preparation, free of calmodulin but containing the other major proteins, have a normal ultrastructural appearance. The process is reversible; exogenously supplied calmodulin can nearly saturate the calmodulin-depleted structure. The calmodulin-binding proteins in the structure have so far not been identified, but the observation that no additional calmodulin will bind to the control cores (Fig. 2) suggests saturation under these conditions. There are several indirect arguments that this general calcium-regulating protein may be bound at more than one site. These include not only the relative abundance of calmodulin but also its complex release pattern (which requires both an increase in free calcium concentration and trifluoperazine). In addition, despite the extensive disassembly of core filaments by 5 mM calcium, the larger amount of calmodulin remains with the insoluble material, which contains an appreciable amount of actin and nearly the full complement of the 105,000 M_r protein but hardly any villin or fimbrin. Thus, the direct identification of the binding proteins of calmodulin must await further studies, including ultrastructural analysis, because F-actin by itself seems not to provide the direct binding partner. Because the isolation of brush borders occurs in the presence of EGTA, calmodulin requiring Ca^{2+} for binding, for instance to the terminal web, would be lost.

The interaction between isolated villin and actin is complex and calcium dependent. In the presence of Ca^{2+} ($>10^{-7}$ M), villin forms a complex with G-actin and restricts F-actin polymer formation whereas, in the absence of calcium, villin acts as a bundling protein or gelation factor. The resulting structures have packing densities and arrangements reminiscent of native core filaments (7). The Ca^{2+} sensitivity of these reactions is now confirmed by the finding that villin has a tight calcium binding site (K_d 2.5×10^{-6} M in the presence of 1 mM Mg^{2+}). Thus actin-binding proteins, which act as crosslinking and gelation factors, can be subdivided into calcium-insensitive factors such as filamin (4, 5) and calcium-sensitive factors such as villin. As discussed (7), villin may be the prototype of calcium-sensitive factors, which may include gelsolin from rabbit macrophages (5), actinogelin from *Ascites* cells (6), and the 95,000 M_r protein from *Dictyostelium discoideum* (8). These proteins, as shown for villin, are incorporated as structural crosslinking proteins into microfilaments in the absence of calcium and are probably major structural proteins of the cell. A local flux of Ca^{2+} ions may trigger their transition from a crosslinking function to a phenotypically F-actin severing function, leading to a collapse of the three-dimensional F-actin gel or the ordered F-actin bundle, probably accompanied by the release of short F-actin filaments that could be translocated in the cell (7). In the case of the microvillus core filaments, the calcium-dependent disassembly *in vitro* seems not dependent on the abundant amount of calmodulin present, because it also occurs in calmodulin-depleted core filaments. The Ca^{2+}-dependent action of villin on F-actin (7), the reported ATP-dependent release of the 105,000 M_r protein (11), and the location of myosin in the terminal web (26, 27) point to some yet-unknown dynamics of microvillar function, perhaps more complex than that implied by the myosin-mediated contraction seen in demembranated brush borders (28).

Some calmodulin in the microvillus cytoskeleton may function as the usual calcium-dependent regulator of the various enzymatic activities known to be modulated by this multifunctional protein (2). Future experiments should explore this possibility for the microvillus and clarify the postulated role of calmodulin in intestinal secretion (29). In addition, the protein may play a general structural role. It has been implicated in the microfilament bundles of cultured cells (30) and in the mitotic apparatus (16, 30). Thus calmodulin may be a component of certain microfilaments, although its direct binding protein(s) remain to be identified. In the case of the microvillus core filaments, the abundance of calmodulin is startling. One possible explanation concerns the physiological function of intestinal epithelial cells. Their enormous microvillus-dependent luminar surface extension is an optimal adaptation for the uptake for nutrients, among which is the full complement of calcium. Indeed vitamin D-dependent expression of calcium-binding proteins has long been noticed in the intestinal epithelium (12). Although increased expression of calmodulin has so far not been shown to be a vitamin D-regulated process, this possibility has now to be considered. Given the influx of Ca^{2+} into the intestinal epithelium, together with the action of villin as an F-actin bundling protein necessary for microvillar integrity and its destructive action as an F-actin severing protein at Ca^{2+} concentrations greater than micromolar, one role of calmodulin may be to act as a buffer modulating the free calcium concentration and thereby protecting, when necessary, the microvillus structure from concentrations of Ca^{2+} high enough to saturate the villin binding site. Such a model should also be explored in other cell types, where Ca^{2+} influxes occur at specialized membrane arrays. The microfilament organization underneath all plasma membranes may also contain

calmodulin, not only as the Ca^{2+}-dependent regulator for adenylcyclase, phosphodiesterase, and other crucial enzymatic activities, but also as a modulator of the level of free calcium.

Note Added in Proof. The results of Howe and Mooseker (14) have now been documented in detail (31). There is extensive agreement between their results and ours on the Ca^{2+} sensitivity of the isolated core filament structure. Our interpretation differs significantly from theirs, because they were not aware of Ca^{2+}-mediated F-actin severing activity of villin.

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