

Regulation of Microvillus Structure: Calcium-dependent Solation and Cross-linking of Actin Filaments in the Microvilli of Intestinal Epithelial Cells

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ABSTRACT The bundle of filaments within microvilli of intestinal epithelial cells contains five major proteins including actin, calmodulin, and subunits of 105-, 95-, and 70-kdaltons. It has been previously shown (Howe, C. L., M. S. Mooseker, and T. A. Graves. 1980. Brush-border calmodulin: a major component of the isolated microvillus core. *J. Cell Biol.* 85: 916-923) that the addition of Ca^{++} ($>10^{-6}$ M) to microvillus cores causes a rapid, drastic, but at least partially reversible disruption of this actin filament bundle. High-speed centrifugation of microvillus cores treated with Ca^{++} indicates that several core proteins are solubilized, including 30-50% of the actin and calmodulin, along with much of the 95- and 70-kdalton subunits. Gel filtration of such Ca^{++} extracts in the presence and absence of Ca^{++} indicates that microvillar actin "solated" by Ca^{++} is in an oligomeric state probably complexed with the 95-kdalton subunit. Removal of Ca^{++} results in the reassembly of F-actin, probably still complexed with 95-kdalton subunit, as determined by gel filtration, cosedimentation, viscometry, and electron microscopy. The 95-kdalton subunit (95K) was purified from Ca^{++} extracts by DEAE-Sephadex chromatography and its interaction with actin characterized by viscometry, cosedimentation, and EM in the presence and absence of Ca^{++} . In the presence, but not absence, of Ca^{++} , 95K inhibits actin assembly (50% inhibition at 1:50-60 95K to actin) and also reduces the viscosity of F-actin solutions. Similarly, sedimentation of actin is inhibited by 95K, but a small, presumably oligomeric actin-95K complex formed in the presence of Ca^{++} is pelletable after long-term centrifugation. In the absence of Ca^{++} , 95K cosediments with F-actin. EM of 95K-actin mixtures reveals that 95K "breaks" actin into small, filamentous fragments in the presence of Ca^{++} . Reassembly of filaments occurs once Ca^{++} is removed. In the absence of Ca^{++} , 95K has no effect on filament structure and, at relatively high ratios (1:2-6) of 95K to actin, this core protein will aggregate actin filaments into bundles.

Studies on the structure, chemistry, and contractility of the brush border of intestinal epithelial cells have been among the most convincing for establishing basic similarities in the organization and function of contractile proteins in muscle and nonmuscle cells (2, 3, 13, 18-20, 22, 23, 26, 27, 30-32). Nevertheless, recent work on the structure and chemistry of the brush-border microvillus has led us to hold serious doubts regarding a simple, sarcomere model (19, 20, 26, 30) for the functional organization of actin and myosin in the brush border. We have shown that the addition of Ca^{++} to isolated microvillus cores results in a drastic but at least partially

reversible disruption of core structure caused by a "breakdown" of microvillar actin filaments (12). After Ca^{++} addition, much of the core actin and several other core proteins are no longer sedimentable at speeds that should pellet F-actin. Morphological studies indicate that both bundles and actin filaments are absent from these preparations. Instead, microfilarmentous networks are observed. Removal of Ca^{++} results in at least partial reassembly of both filaments and filament bundles (12). In light of these observations, it seems quite plausible that dramatic changes in the structural organization of the microvillus core may play an important role in the transport function

of the brush border and perhaps in microvillar motility— if such movements actually occur *in vivo*.

In this report we have investigated the basis for the Ca^{++} -dependent transformation of microvillar actin. We have attempted to answer questions concerning the state of microvillar actin in the presence of Ca^{++} , the involvement of other core proteins, and the reversibility of this phenomenon. Our results demonstrate that the disruption of core structure is attributable to a reversible fragmentation of microvillar actin that is mediated by one of the major proteins of the microvillus core, the 95-k dalton subunit (95K). In the absence of Ca^{++} , this protein serves a second function as a filament-filament cross-linker. While this manuscript was in preparation and review, we learned that several other laboratories^{1,2} working independently have made similar observations regarding the actin-binding properties of the 95-k dalton subunit, including a recent report by Bretscher and Weber (6) that contains a more detailed study of the physical properties of this protein than is reported here.

MATERIALS AND METHODS

Isolation of Brush Borders and Microvilli

Brush borders were isolated from the small intestines of chickens by the method of Mooseker et al. (20). Microvilli and demembrated microvillus cores were prepared by methods described in Howe et al. (12). For the biochemical preparations described below, five to ten animals were used.

Characterization of Ca^{++} -solubilized Microvillus Core Proteins (Ca^{++} Extracts)

Pellets of demembrated microvilli (20 mg total protein) were suspended in Ca^{++} buffer: 0.2 mM CaCl_2 , 75 mM KCl, 5 mM MgSO_4 , 0.1 mM ATP, 0.1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM imidazole-Cl, pH 7.3. After a 30–45-min extraction on ice, the samples were spun at 100,000 g for 2 h. The supernate, which contains approximately half of the core actin and calmodulin together with most of the 95- and 70-kdalton components, was used for analysis of “reassembly” of microvillar actin and associated proteins in the presence and absence of Ca^{++} , by sedimentation, viscometry (see below), and turbidity measurements (see below). This supernate will be referred to as the “ Ca^{++} extract” of microvillus cores.

To determine the degree of association of microvillar actin and other core components in the Ca^{++} extract, samples were analyzed by gel filtration. Ca^{++} extracts (1.0–1.5 ml at 4 mg/ml) were applied to a 500 × 10-mm agarose column (A 1.5 M, exclusion limit, 1,500,000 daltons; Bio-Rad Laboratories, Richmond, Calif.) equilibrated with Ca^{++} buffer. To analyze reassembly of core components in the absence of Ca^{++} , 1.0 mM EGTA was added to Ca^{++} extracts (referred to as “reversed” Ca^{++} extract) to lower free Ca^{++} to $<10^{-9}$ M. After 2-h incubation on ice, the sample was applied to an identical column equilibrated with Ca^{++} buffer containing 1 mM EGTA rather than 0.1 mM CaCl_2 . These columns were calibrated with calf thymus DNA, blue dextran (void volume markers), bovine serum albumin, myoglobin, and 5 mM ATP (salt volume marker). All calibration markers were purchased from Sigma Chemical Company, St. Louis, Mo. Samples of purified F-actin from chicken skeletal muscle and brush-border calmodulin were also chromatographed for comparison of their elution profiles with those of microvillar actin and calmodulin in the Ca^{++} extracts. Elution profiles were analyzed by absorbance at 290 nm (protein), or 260 nm (DNA and ATP), and by SDS gel electrophoresis.

Purification of the 95-kdalton Core Protein

Pellets of demembrated microvilli (10–30 mg total protein) were extracted

¹ P. Matsudaira and D. Burgess (Department of Biology, Dartmouth College, Hanover, N. H.), personal communication.

² S. Craig and L. Powell (Department of Physiological Chemistry, Johns Hopkins Medical School, Baltimore, Md.). Regulation of actin polymerization by villin, a 95,000-dalton cytoskeletal component of intestinal brush borders. Manuscript submitted for publication; and personal communication.

with 3–4 vol of 0.3 M KCl, 0.2 mM CaCl_2 , 0.1 mM DTT, 0.1 mM ATP, 0.1 mM PMSF, 10 mM imidazole-Cl, pH 7.3. After 30-min incubation on ice, the preparation was centrifuged for 2 h at 100,000 g and the supernate was dialyzed for 3 h against DEAE buffer: 20 mM KCl, 0.1 mM ATP, 0.1 mM CaCl_2 , 0.1 mM DTT, 0.1 mM PMSF, 10 mM imidazole-Cl, pH 7.3. In some recent experiments, we have added solid KI to 0.5 M to the supernate immediately before dialysis. The KI treatment reduces the amount of the 95-kdalton subunit (95K) eluting as a bound complex with actin in the DEAE chromatography procedure outlined below. We have observed no differences in the properties of 95K isolated with and without KI. However, most of the observations reported here were made with 95K preparations isolated without KI. After dialysis, the sample was centrifuged at 10,000 g for 15 min to remove precipitated protein (mostly actin). The supernate, which contained 95K together with contaminating 70-kdalton subunit (70K), calmodulin, and actin, was applied to a 4-ml DEAE-Sephadex column (A 25–120; Sigma Chemical Co.) equilibrated with DEAE buffer. The sample was washed onto the column with 2 vol of DEAE buffer and elution of 95K and other core proteins was performed by addition of one to two column volume “steps” of DEAE buffer containing increasing KCl concentrations (50 mM, 100 mM, 150 mM, and 500 mM). Purified 95K elutes from the column with the 50 mM KCl step. About one-third to one-half of the total 95K applied to the column usually elutes in later fractions contaminated with actin and 70K. The 95K in these fractions can be recovered and concentrated by rechromatography (after dialysis against DEAE buffer) on a second DEAE Sephadex column. The pooled fractions were applied to a 0.5–1.0 ml DEAE Sephadex column equilibrated with DEAE buffer. The 95K was eluted in one to two column volumes by application of a 100 mM KCl step. The contaminating actin and 70K remain bound to the column. The same procedure is useful for concentration of dilute 95K fractions obtained from the first DEAE column. For the actin-binding studies reported here, fractions of purified 95K were used within 2 d of isolation because we have observed significant loss of binding activity, proteolytic degradation, and loss of Ca^{++} sensitivity in samples stored for longer periods of time. Total yield from 20 mg of microvillar core protein was ~0.5–1.0 mg of 95K.

Electron Microscopy

Pellets of microvillus cores, 95K-actin complexes, and sedimented microvillar core proteins from Ca^{++} extracts were fixed and embedded by the method of Begg et al. (1). Samples for negative staining were applied to Formvar-carbon-coated grids and stained with 2% uranyl acetate. 95K-actin samples were diluted with their buffer to an actin concentration of 0.05–0.1 mg/ml immediately before staining.

Turbidity Measurements

Turbidity measurements on microvillus cores and Ca^{++} extracts of microvilli were made at 350 nm using a Gilford recording spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio).

Viscometry

The reassembly of microvillar core proteins in Ca^{++} extracts, the interaction of 95K with F-actin, and the effect of 95K on actin polymerization were studied at 25°C by viscometry, using Ostwald capillary viscometers (type A 100; Cannon Instrument Co., University Park, Pa.), with a sample volume of 0.6 ml and a buffer flow time of ~60 s. The specific viscosity in centistokes (cs) was calculated as the (sample flow time/buffer flow time) – 1, assuming a sample density of ~1.

Other Methods

Actin was purified from chicken skeletal muscle by the method of Spudich and Watt (28). Actin concentration was determined by absorbance at 290 nm, using an extinction coefficient of 0.65 for a 1 mg/ml solution of G-actin (24). Other protein concentrations were determined by the method of Lowry et al. (15). Gel electrophoresis in the presence of SDS was performed according to the method of Laemmli (14).

RESULTS

Constituent Proteins of the Microvillus Core

The proteins of this actin filament bundle make up a subset of the cytoskeletal/contractile proteins of the brush border (Fig. 1a). In addition to actin, major proteins of the core include calmodulin, the 105-k dalton subunit (105K), 95K

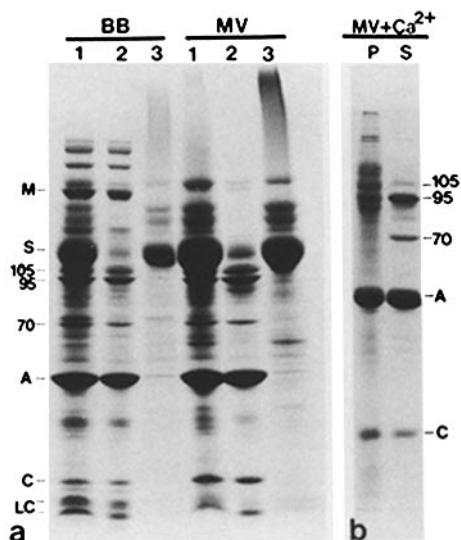


FIGURE 1 Proteins of the brush border and microvillus. (a) Comparison of the cytoskeletal and membrane components of the brush border (*BB*) and microvillus (*MV*). The proteins of the intact organelle (1), cytoskeletal fraction after detergent treatment (2), and solubilized membrane (3) are shown for both the brush border and microvillus by 4–16% gradient SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Abbreviations: *M*, myosin heavy chain; *LC*, myosin light chain; *S*, sucrose-isomaltase; *A*, actin; *C*, calmodulin; 105, 95, 70: mol wt $\times 10^3$ of three major proteins of the cytoskeletal fraction of the brush border and microvillus. See text for details. (b) Effect of Ca^{++} on microvillus cores: 10% SDS-PAGE of pellet (*P*) and supernate (*S*) fractions after 100,000 *g* spin of microvillus cores treated with 10^{-6} M Ca^{++} . Much of the actin has been “solated” together with about half of the core calmodulin and much of the 95,000-dalton and 70,000-dalton subunits (see also reference 12). Solution conditions: 75 mM KCl, 5 mM MgSO_4 , 0.1 mM DTT, 0.1 mM ATP, 0.1 mM PMSF, 2 mM EGTA, 1.6 mM CaCl_2 , ($\text{Ca}/\text{EGTA} = 0.8$, or $\text{Ca}^{++} = 10^{-6}$ M), 10 mM imidazole-Cl, pH 7.2.

(referred to as villin by Bretscher and Weber [4]),³ and 70K (2–5, 12, 16, 21). A number of other brush-border contractile proteins including myosin (20), tropomyosin (3, 19), and alpha-actinin (2, 7–9),⁴ are found only in the terminal web region of the brush border (Fig. 1*a*). Although it is not known whether the proteins of the microvillus core are also present in the terminal web region, several of these proteins, including calmodulin, 105K, and 95K, are enriched in the microvillus fraction, as compared to the whole brush border (12, 21).

We had speculated that 95-kdalton subunit was alpha-actinin (22), an assignment we and several other laboratories later proved incorrect (4, 7, 9, 21). Although comparison of 95K and 105K by peptide mapping indicated that these two proteins

³ The name villin has been given to the 95-kdalton subunit of the microvillus core by Bretscher and Weber (4), but we feel that this term is misleading because (a) it does not reflect the function of the protein, and (b) this protein is a component of the brush-border microvillus, not the intestinal villus. We tentatively propose an alternative name, flaccin (from flaccid), based on the possible role of this protein in the regulation of microvillus rigidity. Nevertheless, we will continue to refer to flaccin (or villin) as the 95-kdalton protein because it is quite possible that this protein may be related to other Ca^{++} -dependent gelation proteins that have been identified (and already named) in other cell types (see Discussion).

⁴ Although it has been convincingly shown that alpha-actinin is present in the terminal web region of intact cells (2, 8, 9), this protein is usually lost from the brush border during isolation (8, 21).

may be structurally related (21), the experiments of Matsudaira and Burgess (16) indicate that, functionally at least, these proteins are quite different. Using selective extraction procedures, these investigators provided evidence for identification of the 105-kdalton subunit as making up the cross filaments that laterally connect the core to the microvillus membrane (22, 23, 32), and 95K and/or the 70-kdalton subunit as filament-filament linker proteins. Recently, Bretscher and Weber have demonstrated, using antibody localization techniques, that the 70-kdalton subunit (referred to as fimbrin by these investigators) may also be present in the cortical cytoplasm of cultured cells (5).

Effect of Ca^{++} on the Structure of the Microvillus Core

We have shown that Ca^{++} ($>10^{-6}$ M) causes a drastic disruption of the isolated microvillus core that can be inhibited by the actin-stabilizing agent, phalloidin (12). After Ca^{++} treatment, ~30–50% of the core actin and calmodulin, along with most of the 95K and 70K, remain in the supernate fraction after sedimentation using conditions sufficient for pelleting of F-actin filaments (100,000 *g* for 2 h; see reference 12 and Fig. 1*b*). Electron microscopy of pelleted microvillus cores treated with Ca^{++} indicates that both filament bundles and actin filaments are absent. Instead, microfilamentous “meshworks” are observed (Fig. 2; see also Fig. 6 in reference 12). The reversibility of this effect is demonstrated by removal of Ca^{++} before sedimentation by addition of EGTA. In such preparations, straight actin filaments and corelike bundles of filaments are observed, indicating that at least partial reassembly of the core components has occurred (Fig. 2 and reference 12).

To assess the rate at which the microvillus core “solates” in the presence of Ca^{++} , we have monitored this reaction by measuring turbidity change. Addition of Ca^{++} causes a large decrease in turbidity so rapid that the maximum decrease is recorded as soon as mixing of the added Ca^{++} is completed (Fig. 3). This is followed by a gradual rise in turbidity for which we have no explanation. Addition of EGTA to lower the free Ca^{++} to $<10^{-9}$ M causes an equally rapid but smaller increase in turbidity that then continues to rise gradually during the course of the experiment. Presumably, the rise in turbidity after removal of Ca^{++} is the result of partial reassembly of the microvillus core proteins similar to that observed in the morphological studies described above (Fig. 2).

It should be noted that in the experiments described above, ATP (0.1 mM) as well as Ca^{++} were added to induce the drop in turbidity. Even though we have observed no requirement for ATP in the Ca^{++} disruption of the microvillus core by either biochemical or morphological criteria (results not shown), we have routinely included ATP in all our extraction procedures to minimize denaturation of any monomeric actin generated during Ca^{++} solation. Although the turbidity change induced by Ca^{++} in the absence of ATP (and the subsequent reversal in EGTA) is qualitatively identical to that in the presence of ATP, the magnitude of this turbidity decrease is ~60% of that observed in the presence of ATP (results not shown). This is probably the result of a separate, additive effect of ATP on core structure. The addition of ATP alone also causes a rapid decrease in turbidity, ~30% of that observed in the presence of ATP and Ca^{++} . The effects of ATP on core structure are currently under investigation and will be the subject of a later report.

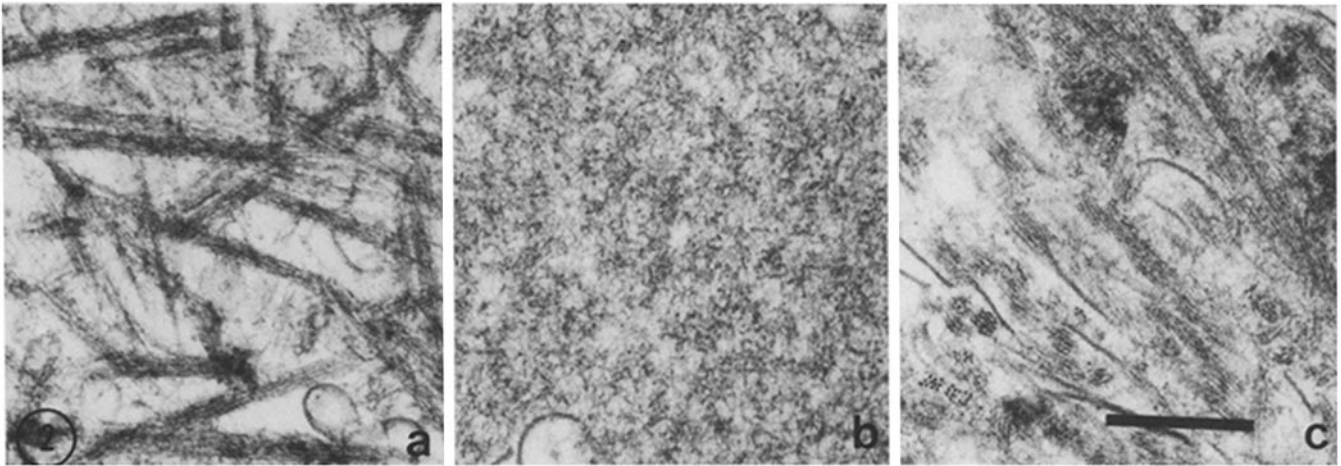


FIGURE 2 Reversible effects of Ca^{++} on microvillus core structure. Thin section electron micrographs of 100,000 *g* pellets of microvillus cores (a) in the absence of Ca^{++} , (b) 5×10^{-6} M in Ca^{++} , and (c) after removal of Ca^{++} by addition of EGTA before centrifugation. Ca^{++} treatment causes a complete disruption of bundle and filament structure that is apparently reversed after removal of Ca^{++} . Solution conditions as in Fig. 1 *b*. See also reference 12. Bar, $0.3 \mu\text{m} \times 65,000$.

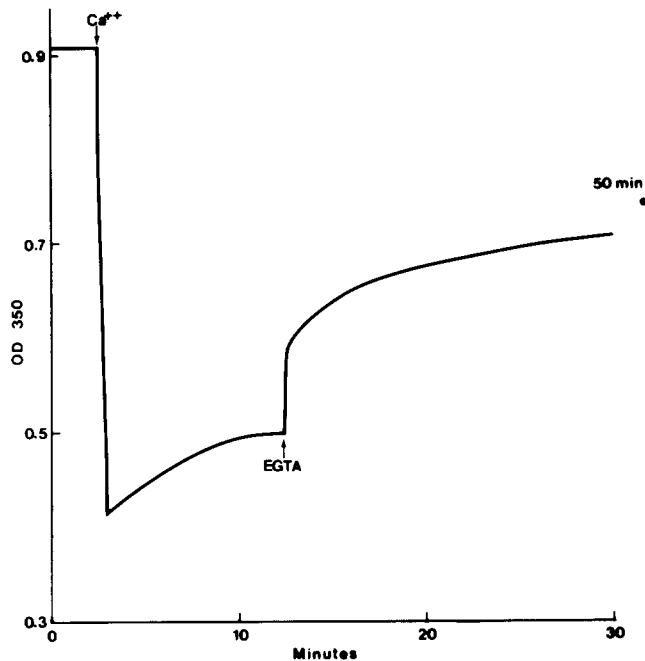


FIGURE 3 Ca^{++} -dependent turbidity changes in suspensions of microvillus cores. Ca^{++} (0.1 mM) and ATP (0.1 mM) were added to suspensions of microvillus cores (~ 1 mg/ml) at 3 min (Ca^{++} , arrow), causing a rapid decrease in turbidity (OD, 350 nm). At 13 min (EGTA, arrow) the Ca^{++} was reduced by addition of EGTA, causing a rapid, but smaller magnitude, increase in turbidity.

Analysis of Core Proteins Solubilized by Ca^{++}

One of the most important questions about the Ca^{++} -dependent disruption of microvillus structure concerns the basis for conversion of microvillar actin filaments into a nonpelletable form under conditions optimal for maintenance of F-actin filaments *in vitro*. To investigate this question, a series of experiments has been conducted to characterize the core proteins solubilized by Ca^{++} treatment. The term " Ca^{++} extract" will be used to refer to the 100,000 *g* supernate obtained after treatment of microvillus cores with Ca^{++} as described in Materials and Methods.

We have analyzed Ca^{++} extracts by gel filtration in the

presence and absence of Ca^{++} to (a) determine the aggregation state of actin, (b) identify what, if any, core proteins remain associated with microvillar actin in the solated state, and (c) determine what changes in the aggregation state of actin and associated core proteins occur after removal of Ca^{++} . Chromatography of Ca^{++} extracts was conducted using a filtration medium (agarose A 1.5 M; Bio-Rad Laboratories) of sufficiently large exclusion limit (1,500,000 daltons) to allow separation of relatively large oligomeric aggregates of actin (Fig. 4). In the presence of Ca^{++} , (Fig. 4*a*), only two of the core proteins in the Ca^{++} extract elute as discrete peaks—70K (partition coefficient, 0.4–0.5) and calmodulin (partition coefficient, 0.6–0.7). The elution profile for calmodulin is identical to that for purified brush-border calmodulin run on the same column. Several membrane proteins contaminating this preparation, including sucrase-isomaltase, also elute from the column as discrete peaks with the void volume. Actin and 95K both elute from the column in a broad front beginning with the void volume, suggesting the presence of 95K-actin complexes of varied sizes, most of which are considerably larger in molecular weight than monomeric actin. In the absence of Ca^{++} (Fig. 4*b*), the elution pattern observed is quite different. Only the elution of 70K is unchanged. Although there is a slight peak of calmodulin at the same position determined for "free" subunit, most of this protein elutes from the column in a broad front with partition coefficients ranging from 0 to 0.5. Although this result clearly suggests that calmodulin is in a bound state, presumably to one of the other proteins in the extract, there is no obvious candidate for such an association, except perhaps actin, and in other experiments we have shown that calmodulin does not bind to actin under these conditions (12). This "bound" state of calmodulin in the absence of Ca^{++} is homologous to the association of calmodulin with the intact microvillus core (12). As in the presence of Ca^{++} , the contaminating membrane proteins elute from the column at, and just after, the void volume, but in a somewhat broader peak. The most dramatic difference observed is in the elution of both actin and 95K. As in the presence of Ca^{++} , much of the actin elutes as a broad front beginning with the void volume. However, those fractions containing the largest aggregates of actin (the first three lanes on the gel in Fig. 4*b*) do not contain substantial amounts of 95K. Another important difference

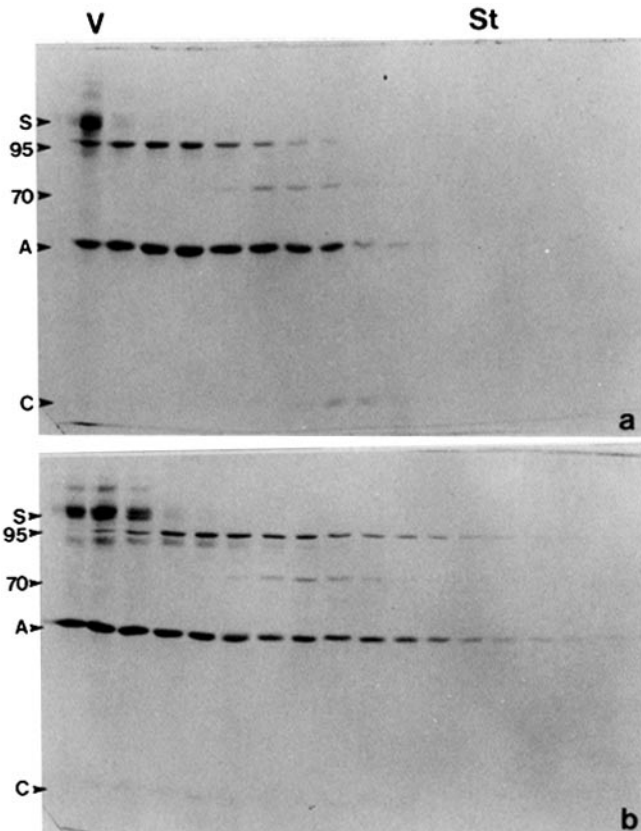


FIGURE 4 Gel filtration of Ca^{++} extracts of microvillus cores in the presence (a) and absence (b) of Ca^{++} . SDS gel electrophoresis of every other column fraction (~ 1 ml/fraction) starting with the void volume (V). Note the elution of actin (A) and 95K (95) in fractions after salt volume (St) in the absence of Ca^{++} . See text for details. Abbreviations as in Fig. 1.

observed is in the appearance of both actin and 95K in fractions that elute from the column long after the salt volume has been collected. This "leaching" of actin from the column is exactly the behavior we have observed in the gel filtration of purified F-actin under the same conditions. As suggested by one of our reviewers, the retarded elution of actin (and 95K) may result from the trapping or binding of actin filaments by the column matrix. Thus the elution profile of microvillar actin in the absence of Ca^{++} indicates that this actin is converted into a state that at least "behaves" chromatographically like actin filaments.

The gel filtration analysis of Ca^{++} extracts suggests that the "solated" form of actin in these extracts can be converted back into F-actin by removal of Ca^{++} with EGTA. The characterization of such "reversed" extracts by sedimentation, electron microscopy, turbidity, and viscometry also provide support for this conclusion. After removal of Ca^{++} , most of the actin and 95K and 30–50% of the calmodulin in these extracts is pelletable (Fig. 5). Addition of phalloidin to Ca^{++} extracts causes only a slight increase in the amount of actin and 95K pelletable in both the presence and absence of Ca^{++} as compared with control samples (Fig. 5). This is in contrast to the complete inhibition of Ca^{++} solation when this actin-stabilizing drug (33) is added to the intact microvillus core (12).

Electron microscopy of "reversed" and control extracts also demonstrates that partial reassembly of core actin filaments has occurred (Fig. 6). Negatively stained preparations of EGTA-treated extracts contain short filaments resembling F-

actin (Fig. 6d). Control preparations contain only globular material (Fig. 6c). In these negatively stained preparations, no evidence of filament-filament cross-linking has been observed, but some lateral association of filaments is evident in thin-sectioned material prepared from pellets of reversed Ca^{++} extracts. As in the negatively stained preparations, only short filaments, some of which are cross-linked into bundles, are observed (Fig. 6b). Sections prepared from the small pellets formed after recentrifugation of control extracts (i.e., in the presence of Ca^{++}) contain only amorphous granular material (Fig. 6a) somewhat similar in appearance to the pellets obtained from Ca^{++} -treated microvillus cores (Fig. 2). Biochemical analysis of this material indicates that it is composed mainly of actin and 95K (Fig. 5). This granular material may represent the fixed and embedded image of the oligomeric state of actin formed in the presence of Ca^{++} , or may simply be denatured protein.

The kinetics of this reassembly process have been analyzed by turbidity and viscosity measurements. After addition of EGTA to Ca^{++} extracts, there is a slow but substantial increase in both viscosity and turbidity (Fig. 7). Both changes occur with similar kinetics, suggesting that the same or at least closely related phenomena have been measured with these two techniques. The characteristics of both the turbidity and viscosity increase indicate that something other than a simple polymerization of actin has occurred. Given the concentration of actin in these extracts (~ 2 mg/ml), both the rate and extent of the viscosity change are much less than one would observe with pure actin at that concentration (a 2 mg/ml solution of actin achieves a maximum viscosity of 7 cs within the time required for the first measurement using the same viscometers and solution conditions employed in the Ca^{++} -extract studies). On the other hand, the turbidity increase is much greater than one would observe for the assembly of pure actin; reversed Ca^{++} extracts become quite cloudy after addition of EGTA. This dramatic increase in turbidity would suggest the formation of

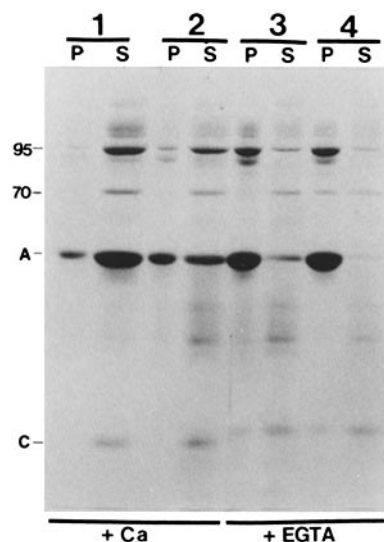


FIGURE 5 Sedimentation of Ca^{++} extracts in the presence and absence of Ca^{++} and phalloidin. SDS-PAGE of supernates (S) and pellets (P) from a 100,000 g spin of Ca^{++} extracts of microvilli; (1) Ca^{++} extract plus Ca^{++} (control); (2) Ca^{++} extract plus phalloidin (0.1 mg/ml) and Ca^{++} ; (3) "reversed" Ca^{++} extract (plus 2.0 mM EGTA); (4) reversed Ca^{++} extract plus phalloidin. Actin (A), 95K (95), and $\sim 30\%$ of the core calmodulin (C) are pelleted after reduction of free Ca^{++} by EGTA.

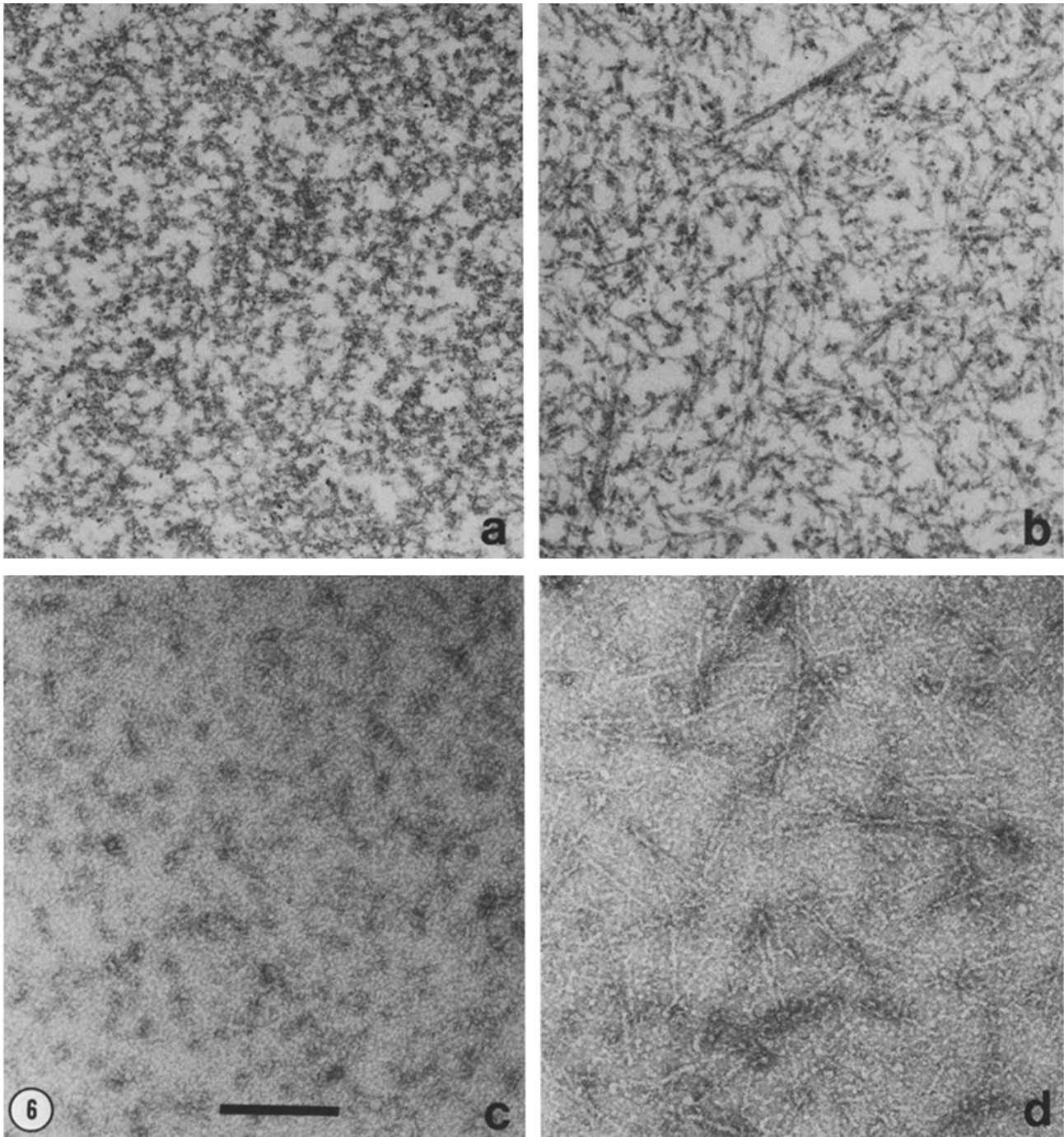


FIGURE 6 Reassembly of core filaments in Ca^{++} extracts after removal of Ca^{++} . Electron micrographs of thin sections (a and b), and negatively stained preparations (c and d) of Ca^{++} extracts before (a and c) and after (b and d) removal of Ca^{++} . Reduction of free Ca^{++} to $<10^{-9}$ M results in the appearance of short filaments, some of which are cross-linked into bundles (b). No filaments are present in the extracts with Ca^{++} present (a and c). Bar, $0.2 \mu\text{m} \times 96,000$.

large aggregates, perhaps between re-forming actin filaments. Except for the small bundles seen in thin-sectioned material (Fig. 6b), no evidence for such aggregates has been obtained by morphological studies. No increase in either turbidity or viscosity was seen in "nonreversed" Ca^{++} extracts (results not shown).

Purification of 95K

The results we have obtained from the analysis of Ca^{++} extracts of microvillus cores provide considerable indirect evi-

dence that 95K is somehow involved in the reversible, Ca^{++} -dependent disruption of the microvillar core filaments. To test this hypothesis directly, we have purified 95K and conducted an extensive series of experiments on the interaction of this protein with actin purified from chicken skeletal muscle.

Purification of 95K proved to be a relatively simple task based on the solubilization of this and other proteins of the core by Ca^{++} and the fractionation of 95K from actin and other subunits released from the core by Ca^{++} treatment, using ion exchange chromatography on DEAE Sephadex as detailed in Materials and Methods. Reasonably pure fractions of 95K

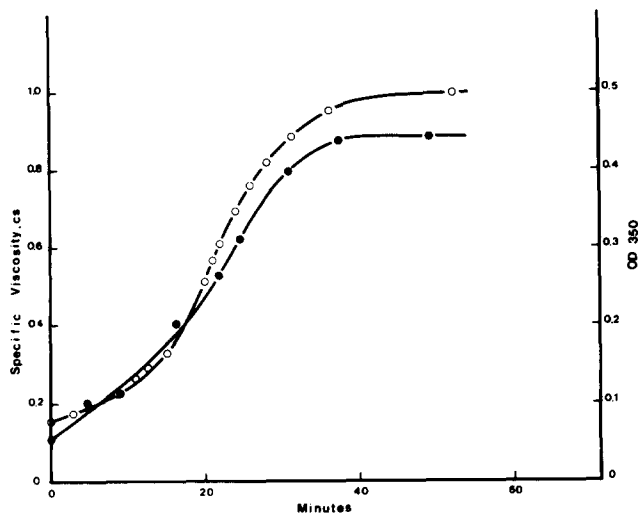


FIGURE 7 Viscometric and turbidometric analysis of Ca^{++} extracts of microvilli after removal of Ca^{++} . Addition of EGTA to Ca^{++} extracts (~ 4 mg total protein/ml) at zero time results in an increase in both viscosity (\bullet) and turbidity (\circ), measured at 350 nm.

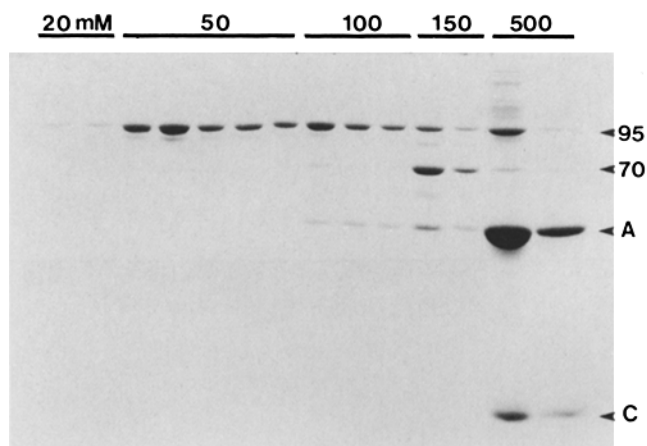


FIGURE 8 Purification of 95K. SDS-PAGE of DEAE-Sephadex fractions eluted with "steps" of increasing KCl concentration (20–500 mM). Purified 95K (95) elutes with the 50 mM step. See Materials and Methods for details of procedure.

of sufficient concentration for analysis (0.2–0.3 mg/ml) have been obtained by this procedure (Fig. 8).

Viscometric Analysis of 95K-Actin Interaction

We have begun a series of experiments to study the kinetics of the interaction of 95K with actin in the presence and absence of Ca^{++} , using the technique of Ostwald viscometry. The preliminary results outlined below convincingly demonstrate that 95K, in the presence of Ca^{++} , inhibits the assembly of actin and also reduces the viscosity of F-actin solutions. It is clear, however, that much more work is required to characterize the various parameters that may affect this interaction, including salt conditions, divalent cation and nucleotide requirements, pH dependence, and dependence on the concentration of Ca^{++} and 95K.

Even at relatively low subunit ratios of 95K to actin, 95K inhibits the assembly of actin in the presence of Ca^{++} (Fig. 9 a). In the presence of 95K at a ratio of 1:30 ($0.4 \mu\text{M}$ 95K: $12 \mu\text{M}$ actin), actin assembles to a final viscosity only 20–25% of that observed for actin alone. The addition of 95K to F-actin also

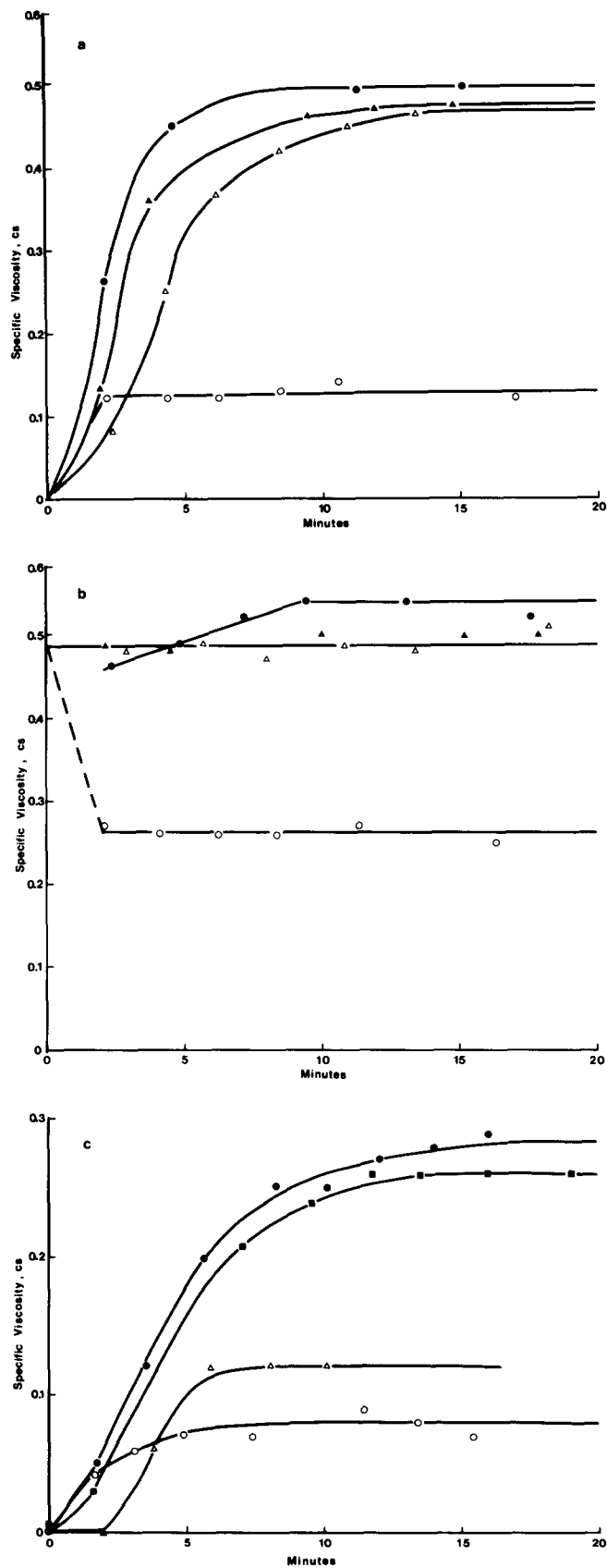


FIGURE 9 Viscometric analysis of 95K-actin interaction in the presence and absence of Ca^{++} . (a) The assembly of G-actin ($12 \mu\text{M}$) in the presence of 50 mM KCl, 1 mM MgSO_4 , 10 mM imidazole-Cl, pH 7.2, 0.1 mM ATP, and either 0.1 mM CaCl_2 (\circ , Δ) or 1 mM EGTA (\blacktriangle , \bullet). In the presence of 95K ($0.4 \mu\text{M}$, 1:30 molar ratio with actin)

causes a rapid decrease in viscosity in the presence of Ca^{++} (Fig. 9b). Under identical solution conditions, using the same protein samples as for the assembly experiments above, the addition of 95K to F-actin causes a drop in viscosity so rapid that the maximum decrease is measured by the first time-point. There is a 50% decrease in viscosity—or only half the inhibition of viscosity by 95K observed for actin assembly.

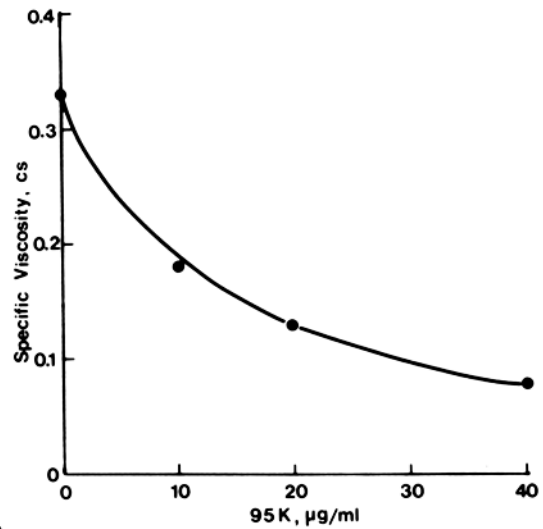
In the absence of Ca^{++} , 95K does not inhibit actin assembly but rather causes a slight but highly reproducible increase in both the rate and final extent of viscosity achieved as compared with that for actin alone.

In the experiment described in Fig. 9a, this difference in plateau values was 0.02 cs (0.49 vs. 0.47 cs). A similar effect is observed in mixtures of 95K and F-actin in the absence of Ca^{++} (Fig. 9b). After addition of 95K to F-actin, there is a slow rise in viscosity to a plateau value slightly greater than in the actin control (in Fig. 9b, these values are 0.54 vs. 0.49 cs). We have not determined the basis for either the acceleration of actin assembly by 95K in the absence of Ca^{++} or the increased viscosity of polymerized actin observed in the presence of this protein. The latter effect may be attributable to an increase in the amount of polymerized actin present, resulting from a 95K-dependent decrease in the critical concentration for assembly, or to an increase in filament-filament interaction.

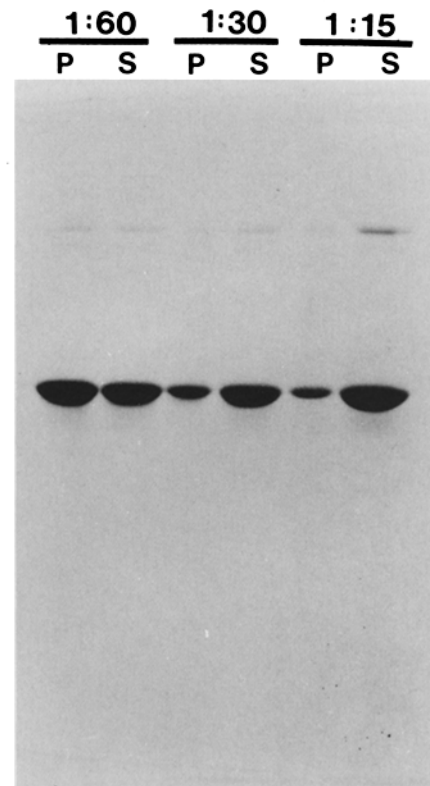
In the experiments described above, the concentration of Ca^{++} used was 10^{-4} M. Because the threshold for Ca^{++} effects on microvillus core structure is $\sim 10^{-6}$ M (12), we have examined the effect of 95K on actin assembly at this concentration of free Ca^{++} , using Ca/EGTA buffers (25). Although the inhibition of assembly by 95K is somewhat less than that observed in the presence of higher Ca^{++} , 95K is still a potent inhibitor of actin assembly at 10^{-6} M Ca^{++} (Fig. 9c). Curiously, 95K has much less of an effect on the reduction of F-actin viscosity at 10^{-6} M. For example, in one experiment with a 95K:actin ratio of 1:40 (0.2 μM 95K:8.0 μM actin), a 40% reduction in actin viscosity was observed in the presence of 10^{-4} M Ca^{++} , and only a 10% decrease at 10^{-6} M. The ineffectiveness of 95K in “breaking” F-actin at 10^{-6} M Ca^{++} may be a function of the low concentrations of 95K used in viscometry experiments. Several preliminary experiments using higher concentrations of 95K and assays by sedimentation rather than viscometry support this conclusion, but unfortunately we have observed Ca^{++} “sensitivity” at 10^{-6} M in only some of our preparations. The basis for this variability is currently under investigation.

We have also analyzed the dependence of reduction of F-actin viscosity (in the presence of 0.1 mM Ca^{++}) on the concentration of 95K. The results shown in Fig. 10a and b

and Ca^{++} (○), actin assembly is inhibited by 75% compared to actin alone (Δ). In the absence of Ca^{++} (●), 95K causes a slight increase in both the rate and extent of assembly compared to that for actin alone in the presence of EGTA (▲). (b) Effect of 95K on the viscosity of F-actin. Solution conditions and protein concentrations as in (a). 95K was added to F-actin solutions in the presence (○) and absence (●) of Ca^{++} at zero time. In the presence of Ca^{++} , 95K causes a rapid drop in viscosity to 50% that of actin alone (Δ). In the absence of Ca^{++} (●) 95K induces a slow increase in actin viscosity to a plateau value above that of actin alone (▲). (c) Effect of Ca^{++} concentration on 95K inhibition of actin assembly. Conditions as in (a) except that 8.0 μM actin and 0.26 μM 95K were used (1:30 ratio). Viscosity curves represent assembly of actin alone (■) at 10^{-4} M Ca^{++} , and actin in the presence of 95K at 10^{-4} M (○), 10^{-6} M (Δ) and $<10^{-9}$ M Ca^{++} (●).



a



b

FIGURE 10 Concentration dependence of 95K on the inhibition of F-actin viscosity and sedimentation in the presence of Ca^{++} (0.1 mM). (a) Viscosity of 8 μM actin solution (0.3 mg/ml) in the presence of increasing concentrations of 95K (0–0.4 μM ; 0–40 $\mu\text{g/ml}$). Salt conditions as in Fig. 9a. (b) Sedimentation (100,000 g) of F-actin/95K viscometry samples from a. The amount of actin remaining in the supernate (S) increases with increasing 95K concentration (expressed as molar ratio of 95K:actin).

demonstrate that 95K reduces the viscosity of F-actin in a concentration-dependent but nonlinear fashion. The incremental reduction of viscosity is much less at higher concentrations of 95K, one of the reasons a molar ratio of 1:30 was chosen in the previous experiments (Fig. 9). Sedimentation of the viscometry samples used to generate the data in Fig. 10a also reveals

a nonlinear increase in the amount of nonpelletable actin with increasing concentrations of 95K (Fig. 10*b*).

Analysis of Actin-95K Interaction by Cosedimentation

One of the problems with viscometry is that it is very expensive in terms of the amount of protein required for each data point. Because we have been forced to work with limited quantities of purified 95K, we have used techniques that require much less total protein, such as sedimentation and electron microscopy, to study the interaction of 95K with actin at molar ratios similar to that in the microvillus core (1:7–8; references 16 and 21).

In the absence of Ca^{++} , centrifugation (100,000 *g* for 2 h) of 95K/F-actin mixtures (1:6 molar ratio) results in the cosedimentation of these two proteins (Fig. 11). Because 95K alone does not sediment under these conditions, this indicates that 95K binds to actin filaments in the absence of Ca^{++} . In the presence of Ca^{++} (0.1 mM), most of the actin and 95K remain in the supernate, indicating that 95K has converted the F-actin into a nonpelletable form, essentially mimicking the effect of Ca^{++} on microvillar core filaments (12). Exactly the same results (not shown) are obtained in experiments in which the actin is first polymerized in the presence of 95K before sedimentation.

Although the above experiments demonstrate a binding interaction between 95K and F-actin in the absence of Ca^{++} , we cannot determine whether there is also an interaction between 95K and the “solated” form of actin in the presence of Ca^{++} . The gel filtration studies of Ca^{++} extracts of microvilli suggest that solated microvillar actin is in an oligomeric state coupled with 95K (Fig. 4*a*). To test this possibility, supernates from 95K-actin mixtures formed in the presence of Ca^{++} , such as those in Fig. 11, were subjected to long-term centrifugation (100,000 *g* for 12 h) in an attempt to pellet any oligomeric actin present. Under such conditions, neither G-actin nor 95K alone will sediment, but the actin and 95K in these supernates are pelletable, presumably as a bound complex (Fig. 12). Unfor-

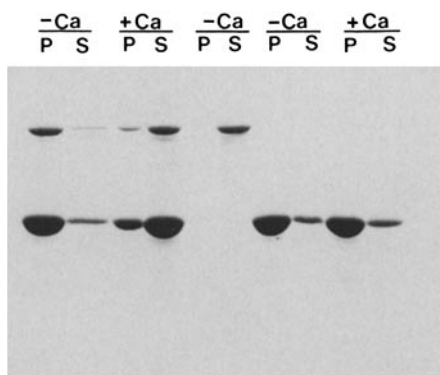


FIGURE 11 Cosedimentation of 95K and F-actin in the presence and absence of Ca^{++} . SDS-PAGE of 100,000 *g* supernates (S) and pellets (P) of 95K/F-actin mixtures (1 μM 95K:6 μM actin) in the presence and absence of 0.1 mM CaCl_2 . In the absence of Ca^{++} (first two lanes) 95K and actin cosediment; under these conditions, 95K alone (5th and 6th lanes) remains in the supernate. In the presence of Ca^{++} (3rd and 4th lanes), most of the actin and 95K remain in the supernate. No difference in the sedimentation of F-actin alone in the presence and absence of Ca^{++} is observed (last four lanes). Solution conditions as described in the legend to Fig. 1*b*, with 0.1 mM CaCl_2 or 1 mM EGTA.

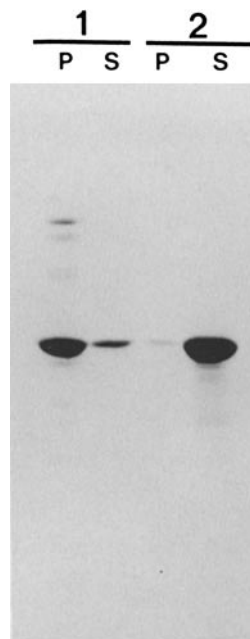


FIGURE 12 Sedimentation of the 95K-actin complex formed in the presence of Ca^{++} . The high-speed supernate from a 1:15 mixture of 95K and F-actin in the presence of Ca^{++} (same protein sample as in Fig. 10*b*) was subjected to long-term centrifugation (100,000 *g*, 12 h), and supernates (S) and pellets (P) were analyzed by SDS-PAGE (1). The 95K and most of the actin are pelletable under these conditions, but neither G-actin (2) nor 95K alone (not shown) will sediment. Note that extensive proteolysis of the 95K has occurred during long-term centrifugation.

tunately, we have thus far been unable to avoid some proteolytic degradation of the 95K during these long-term centrifugations; thus one has to consider the possibility that the observed proteolysis of 95K has resulted in re-formation of F-actin.

Ultrastructural Analysis of 95K-Actin Interaction

Morphological studies of 95K/F-actin mixtures corroborate the results of viscometric and cosedimentation analyses with respect to the solation effects of 95K on actin filaments in the presence of Ca^{++} . They also demonstrate that 95K in the absence of Ca^{++} can aggregate actin filaments into bundles, suggesting that this protein is a cross-linking component of the microvillus core (Figs. 13 and 14).

Samples of 95K-actin mixtures (molar ratio of 1:6; the samples used in Fig. 13 were the same as those depicted electrophoretically in Fig. 11) were analyzed by negative staining before sedimentation (Fig. 13*b*, *d*, and *f*), and by thin-section electron microscopy, using the pellets resulting from a 100,000 *g* spin (Fig. 13*a*, *c*, and *e*). In the absence of Ca^{++} , the morphology of actin filaments in the presence of 95K (Fig. 13*c* and *d*) is indistinguishable from pure actin filaments (Fig. 13*a* and *b*). However, most filaments in the 95K-actin pellet are aggregated into loosely packed bundles (Fig. 13*c*) that are not present in the control pellet (Fig. 13*a*). The lateral aggregation of actin filaments in the presence of 95K is much less evident in the negatively stained preparations of the same samples before centrifugation (Fig. 13*d*). Perhaps sedimentation forces or the increased protein concentration in the pellet favors the cross-linking of actin filaments by 95K. Alternatively, the shear forces involved in the preparation of the negatively stained

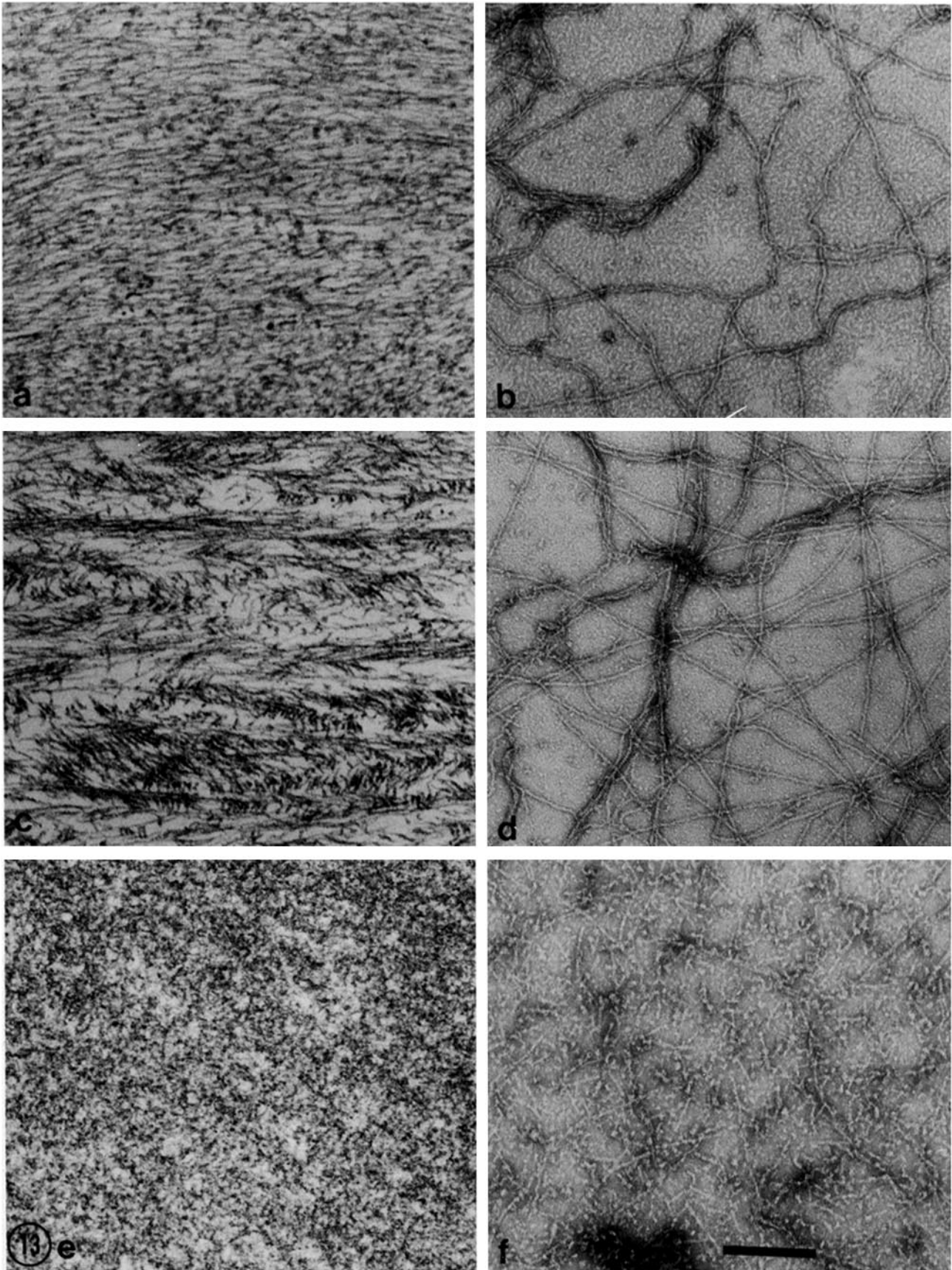


FIGURE 13 Morphology of 95K/F-actin interaction. Electron micrographs of thin-sectioned and negatively stained samples of F-actin (*a* and *b*) and 95K plus actin (1:6 molar ratio) in the absence (*c* and *d*) and presence (*e* and *f*) of Ca^{++} . These are the same protein samples as in Fig. 11. In the absence of Ca^{++} , filaments in the pellets of 95K-actin (*c*) are aggregated into loosely packed bundles not evident in the negatively stained sample (*d*) or in actin controls (*a* and *b*). In the presence of Ca^{++} (*e* and *f*), 95K causes a disruption of actin filaments visualized best in the negatively stained sample (*f*) as small, filamentous fragments. Bar, $0.2 \mu\text{m} \times 82,000$.

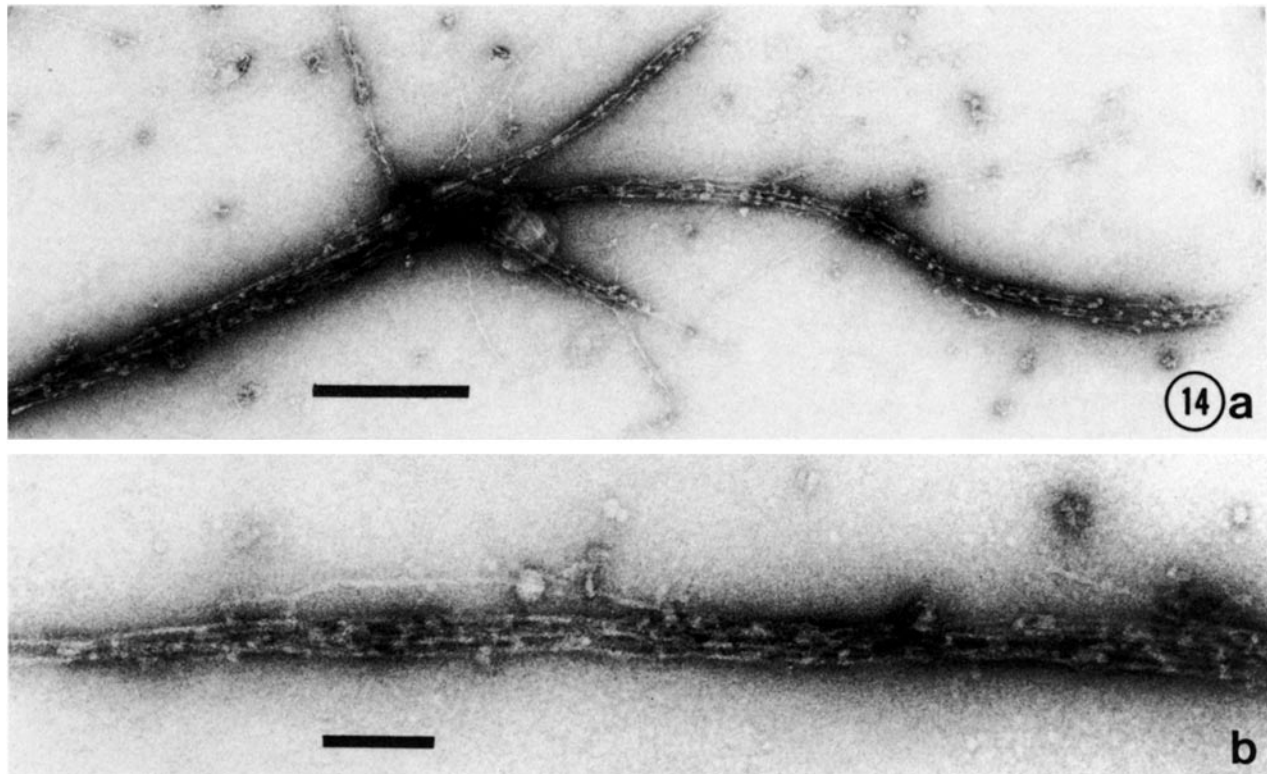


FIGURE 14 The bundling of F-actin by 95K in the absence of Ca^{++} . (a and b) Negatively stained preparations of F-actin ($2 \mu\text{M}$) in the presence of 95K ($1 \mu\text{M}$). At these high ratios of 95K:actin, tight aggregates or bundles of filaments variable both in length and diameter are formed. Note the mottled appearance of the bundles, presumably attributable to the presence of somewhat globular-shaped 95K cross-links. Solution conditions: 50 mM KCl, 5 mM MgSO_4 , 0.1 mM DTT, 1 mM EGTA, 0.1 mM ATP, and 10 mM imidazole, pH 7.0. (a) Bar, $0.3 \mu\text{m}$. $\times 68,000$. (b) Bar, $0.1 \mu\text{m}$. $\times 144,000$.

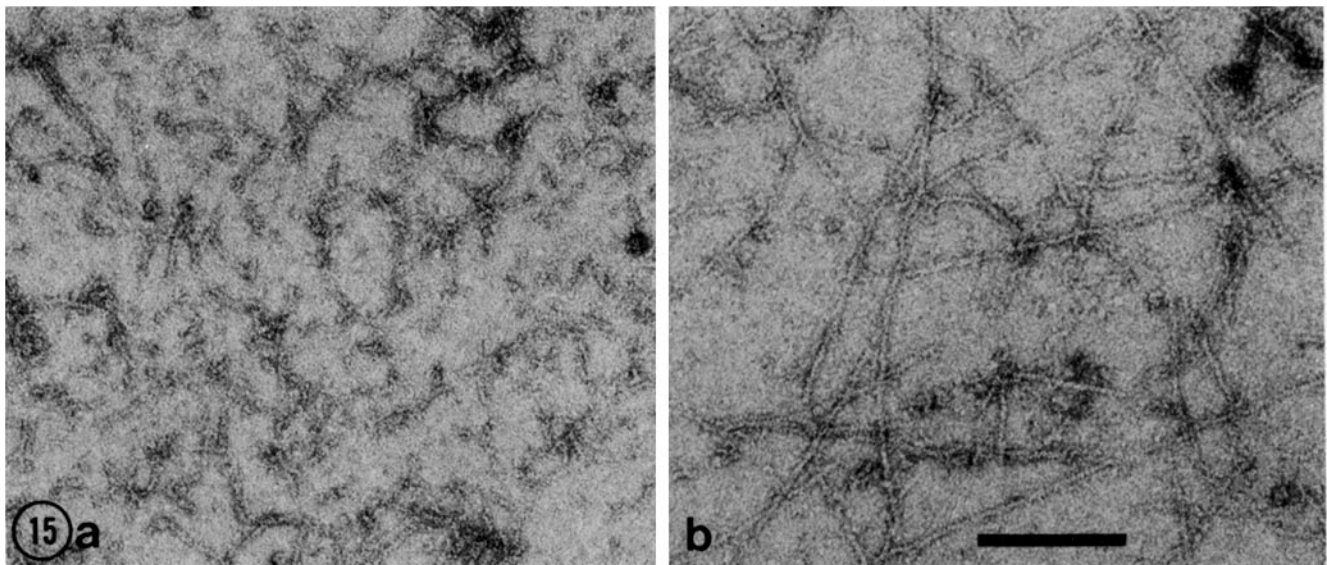


FIGURE 15 Reassembly of F-actin filaments from 95K-actin complexes formed in the presence of Ca^{++} . Negatively stained preparations of a 1:7 95K-actin mixture in the presence of Ca^{++} (a). After addition of EGTA to remove Ca^{++} , (b) reassembly of somewhat short F-actin filaments has occurred. Sample in b was negatively stained after 1 h of incubation (0°C) in the presence of EGTA. Bar, $0.2 \mu\text{m}$. $\times 120,000$.

samples may have disrupted filament-filament interactions.

At higher ratios of 95K to actin (1:2), the ability of 95K to cross-link actin filaments into bundles becomes much more obvious. Under these conditions, filament bundles of variable length ($5\text{--}20 \mu\text{m}$) and diameter are formed (Fig. 14). Unlike the bundles formed at a 1:6 ratio of 95K to actin (Fig. 13b)

these bundles contain obvious cross-linking structures. These cross-links are somewhat globular in morphology, giving the bundles a mottled appearance along their lengths. On the basis of these observations, one is tempted to assign a role for 95K as the “glue” that holds the filaments of the microvillus core together. However, the involvement of other core proteins,

currently under investigation in this and several other laboratories, cannot be ruled out at this time.

In the presence of Ca^{++} , the addition of 95K to actin filaments has a profound effect on their structure. In thin-sectioned preparations of the small pellets formed after centrifugation of such mixtures, no actin filaments are observed (Fig. 13*e*). Instead, these samples contain fibrillar material almost identical in appearance to the microvillar core material that sediments after Ca^{++} treatment (Fig. 2*b* and reference 12). The lack of filamentous material may be a fixation artifact, however. In negatively stained preparations of the same samples (Fig. 13*f*), small filamentous fragments of heterogeneous size are observed.

An obvious conclusion from these morphological studies is that the mechanism of actin filament solation by 95K is through the fragmentation of filaments into small pieces rather than through depolymerization by elevation of the "critical concentration" for assembly. The latter scheme would result in a lower concentration of filaments rather than a fragmentation of the whole population of filaments in the sample.

Reversibility of Actin Solation by 95K

We have shown that the disruption of the microvillus core in the presence of Ca^{++} is a reversible process (Fig. 2 and reference 12). In addition, we have also observed at least partial reassembly of actin filaments in Ca^{++} extracts of microvilli once Ca^{++} was removed by addition of EGTA. Although we have identified 95K as at least one of the causal agents in the solation of the microvillus core, the reassembly process may require other components, because in both of the above examples of reassembly, other core proteins, including calmodulin and 70K, were present. Examination of 95K-actin samples after removal of Ca^{++} with EGTA indicates that F-actin filaments are re-formed (Fig. 15). However, the filaments observed in these "reversed" samples are generally much shorter than those observed in preparations of pure actin (Fig. 13*b*) or mixtures of 95K and actin that have not been previously treated with Ca^{++} (Fig. 13*d*). Studies to determine the kinetics of this reassembly and the possible potentiation by other proteins of the microvillus core are now in progress.

DISCUSSION

We have pursued several lines of evidence in this and a previous report (12) investigating the effects of Ca^{++} on the organization of the microvillar filament bundle in the brush border of intestinal epithelial cells. These lines of evidence include the following: (a) The phenomenological characterization of Ca^{++} effects on microvillus core structure by ultrastructural, biochemical, and physical techniques (see Figs. 1-3 and reference 12). (b) The analysis of proteins solubilized from the core by Ca^{++} treatment under both the original extraction conditions (i.e., plus Ca^{++}) and after Ca^{++} removal, using techniques of gel filtration, sedimentation, viscometry, turbidity, and electron microscopy (Figs. 4-7). (c) The characterization of 95K, one of the core proteins implicated in the solation of the core from experiments in *a* and *b*, with respect to its Ca^{++} -dependent interaction with G- and F-actin. For these studies, techniques of viscometry, cosedimentation, and electron microscopy were used (see Figs. 8-15).

We conclude from studies on Ca^{++} extracts of microvilli that the disruption of core structure *in vitro* is attributable, at least in part, to the fragmentation of actin core filaments into an

oligomeric state rather than to their depolymerization into G-actin. Although the gel-filtration analysis of Ca^{++} extracts indicates that most of the solated microvillar actin is not in the form of monomeric actin, we have not yet defined the physical properties of this oligomeric state of actin. For example, solated actin could be in the form of very short F-actin filaments, or alternatively, in a complex with other core proteins that is nonfilamentous, or at least not F-actin in the sense that it is in a steady-state equilibrium with monomeric actin. The coelution of 95K with actin in the gel filtration of Ca^{++} extracts of microvillus cores strongly suggests that this protein is bound to both the solated form of actin in the presence of Ca^{++} and to the reassembled F-actin filaments that form after Ca^{++} is removed from the extract. The involvement of the 95K in the solation of core filaments is also supported by results of experiments reported here and by several other laboratories (see reference 6 and footnotes 1 and 2) on the interaction of purified 95K with muscle actin in the presence and absence of Ca^{++} (see Figs. 9-15). All four laboratories have observed similar effects of 95K on the Ca^{++} -dependent solation of F-actin and on the inhibition of actin assembly, using a wide variety of techniques.

Because the fragmentation of actin by 95K is a reversible process *in vitro*, we presume that the observed reassembly of core filaments after Ca^{++} removal (Fig. 2) is mediated at least in part by a change in the interaction of 95K with actin that permits and perhaps promotes reassembly. However, the "inefficiency" of filament reassembly from 95K-actin complexes formed in the presence of Ca^{++} (Fig. 15) leads us to suspect the involvement of other proteins of the core in this reassembly process. These suspicions are strengthened by a comparison of the slow kinetics of core filament reassembly in Ca^{++} extracts (Fig. 7) with the rapid increase in turbidity observed in suspensions of Ca^{++} -treated microvillus cores after removal of Ca^{++} . If this turbidity increase is a result of the reassembly of filaments and bundles, then an obvious conclusion from these results is that other components of the microvillus core absent from the Ca^{++} extract (this extract contains almost all the 95K present in the intact core [Fig. 1*b*]) may be necessary for the rapid reassembly of core filaments. Some of the core proteins absent from these Ca^{++} extracts include "nonsolated" actin, 105K, "bound" calmodulin (both the actin and calmodulin that remain in the pellet fraction after Ca^{++} treatment [Fig. 1*b*] are resistant to further extraction. [see reference 12]), and several as yet uncharacterized high molecular weight subunits (Fig. 1).

The observed cross-linking of muscle F-actin into bundles by purified 95K in the absence of Ca^{++} (Figs. 13 and 14, reference 6, and footnote 1) provides strong circumstantial evidence for the notion that this core protein is responsible for bundling of actin filaments in the microvillus. This possibility is also consistent with the results of Matsudaira and Burgess (16) in which they demonstrated that one could selectively extract both 95K and 70K from the core, leaving behind dissociated core filaments to which structures resembling the membrane cross-filaments were still attached. They concluded from these results that either 95K or 70K or both were involved in bundle formation. The molecular basis for the cross-linking of actin by 95K has not been established, but it may involve charge interactions as 95K is considerably more basic than actin, based on its elution from DEAE Sephadex and two-dimensional gel electrophoresis (reference 6 and Mooseker, unpublished observation). This, of course, raises the possibility

that the cross-linking of F-actin by 95K in vitro is the result of nonspecific charge interactions between these two proteins, as it has been shown that a variety of positively charged molecules can effect the lateral interaction of actin filaments (see reference 10). However, one can also turn this reasoning around to explain the nature of 95K-actin interaction in vivo, because we already know that some type of binding interaction between these proteins occurs in the cell.

Although we now have some important clues regarding the molecular basis for the Ca^{++} -dependent solation of the microvillus core, we are left with the very pressing question of the role of core solation in brush-border function, particularly with respect to microvillar motility. The literature is already top-heavy with speculation (mostly in our own papers!) about the molecular basis of microvillar movements in the almost complete absence of information regarding how and whether such movements occur in vivo. Consequently, we will refrain from any further model building. Although previous ideas (22, 26) about the sarcomerelike arrangement of actin and myosin in the brush border still seem to be correct (omitting, of course, alpha-actinin at the tips of microvilli), with respect to the static organization of these proteins, the dynamic aspects of such a model for generating microvillar movements must be reexamined, given the results presented in this report. At the very least, one must question any model that proposes that the microvillus core is a rigid rod moved around in some way by interactions with myosin at its basal end in the terminal web. It is plausible to suppose that a rise in intracellular Ca^{++} results in at least a partial "softening" of the microvillus, mediated by the interaction of 95K with core filaments—hence our proposed name, flaccin, for this core protein.³ Such a loss of core rigidity through fragmentation of core filaments and/or a loss of filament-filament interactions might promote contractility, much as has been proposed by Taylor, Condeelis, and Hellewell (see reference 11) in the solation-contraction coupling theory for amoeboid movement. On the other hand, fragmentation of the core filaments might, in fact, inhibit actomyosin-mediated movements of microvilli, because anchorage-dependent force generation would be prevented. In several simple but elegant experiments, Stendahl and Stossel (29) have obtained evidence for such inhibition of contraction in reconstituted actomyosin gels containing the macrophage protein, gelsolin (34), an actin-binding protein with properties similar to those of 95K of the microvillus core (see below). There is also the obvious possibility that the Ca^{++} -dependent solation of core filaments may result in a loss of microvillus rigidity sufficient to allow Brownian movements of these cellular protrusions. Such passive movements of microvilli might well be as effective in the facilitation of intestinal absorption as active microvillar motility (ordinarily, Brownian movements of microvilli are not detectable in the brush border of intact cells or in isolated brush borders in the absence of Ca^{++}).

It is tempting to interrelate the two Ca^{++} -dependent changes of cytoskeletal organization that have thus far been observed in vitro, namely core solation and core contraction by myosin interactions in the terminal web, into a new, improved model for microvillar motility. However, it is also possible that these two phenomena reflect separate rather than coordinated aspects of the cytoskeletal function in the brush border. This possibility is based on the observation that the basal end of the microvillus core within the terminal web region is not solated by Ca^{++} treatment (Mooseker, unpublished observations), possibly because of the absence of 95K, or alternatively because of the presence of other proteins (e.g., tropomyosin or myosin)

that may inhibit solation. Thus the contractile machinery in the terminal web may function independent of the "sol-gel" transformations occurring in the microvillus. In this regard, the terminal web contraction observed by Rodewald et al. (26), in which lateral constriction of the brush border rather than microvillar shortening occurred, may, in fact, more closely reflect the function of the contractile apparatus in the terminal web than the microvillar retractions we have observed in detergent-treated brush borders (19).

The above discussion also raises the important point that our earlier studies on microvillar contraction in isolated brush borders (19) may have been in error because of the misinterpretation of core solation as contraction. However, we have presented a number of observations (e.g., Figs. 4 and 7 in reference 19) that convincingly demonstrate the retraction of microvillus cores into the terminal web in detergent-treated brush borders after addition of Ca^{++} . Nevertheless, we are currently reexamining a variety of brush-border contractile models to sort out possible erroneous conclusions from our earlier work (19).

Another important question we raised in a previous report (12) concerns the role of calmodulin in the Ca^{++} -dependent solation of the microvillus core. Thus far we have obtained no evidence indicating a role for that regulatory protein in this process. There is no detectable calmodulin present in our preparations of purified 95K, and the addition of brush-border calmodulin to actin-95K complexes has no effect on these interactions either in the presence or absence of Ca^{++} . Furthermore, no detectable binding of calmodulin to actin-95K complexes has been detected by cosedimentation (results not shown). Thus the role of calmodulin in the microvillus and the nature of its binding to the core remain unknown.

Although the observations presented here shake the foundations of our previous ideas about the structure and function of the brush-border contractile apparatus, the properties of microvillus core solation are not unlike those of Ca^{++} -dependent gelation of cytoplasmic extracts from a variety of cell types. Furthermore, in several cell types, including *Dictyostelium* (11), macrophages (29, 34), and Ehrlich's tumor cells (17), Ca^{++} -dependent gelation proteins with properties similar to those of the 95K of the microvillus have been identified. For example, a protein of 95 k daltons in *Dictyostelium* will cause the gelation of F-actin solutions in the absence but not presence of Ca^{++} (11). Fragmentation of actin filaments by this protein in the presence of Ca^{++} was not observed (11). In macrophages, there is a 91-k dalton protein, gelsolin, that does cause the fragmentation of actin filaments in the presence of Ca^{++} and also inhibits the gelation of actin by macrophage actin-binding protein (29, 34). Gelation of actin by gelsolin alone, in the absence of Ca^{++} , was not reported. Finally, Mimura and Asano (17) have purified a 110-k dalton protein, actinogelin, from extracts of Ehrlich's tumor cells that will gel F-actin solutions in the absence but not presence of Ca^{++} . The effect of actinogelin on actin-filament structure was not reported. A direct comparison of these proteins will be required to determine whether any of these Ca^{++} -dependent gelation factors are chemically related. It is already clear, however, that there is a growing family of proteins that modulate, in a Ca^{++} -dependent fashion, both actin-actin interaction and actin-filament structure.

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