

Direct Electron Microscopic Visualization of Barbed End Capping and Filament Cutting by Intestinal Microvillar 95-kdalton Protein (Villin): A New Actin Assembly Assay Using the *Limulus* Acrosomal Process

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ABSTRACT We have re-examined the Ca^{++} -dependent interaction of an intestinal microvillar 95-kdalton protein (MV-95K) and actin using the isolated acrosomal process bundles from *Limulus* sperm. Making use of the processes as nuclei for assembling actin filaments, we quantitatively and qualitatively examined MV-95K's effect on filament assembly and on F-actin, both in the presence and in the absence of Ca^{++} . The acrosomal processes are particularly advantageous for this approach because they nucleate large numbers of filaments, they are extremely stable, and their morphology can be used to determine the polarity of any nucleated filaments. When filament nucleation was initiated in the presence of MV-95K and the absence of Ca^{++} , there was biased filament assembly from the bundle ends. The calculated elongation rates from both the barbed and pointed filament ends were virtually indistinguishable from control preparations. In the presence of Ca^{++} , MV-95K completely inhibited filament assembly from the barbed filament end without affecting the initial rate of assembly from the pointed filament end. The inhibition of assembly results from MV-95K binding to and capping the barbed filament end, thereby preventing monomer addition. This indicates that, while MV-95K is a potent nucleator of actin assembly, it is also a potent inhibitor of actin filament elongation. To examine the effects of MV-95K on F-actin in the presence of Ca^{++} , we developed an assay where MV-95K is added to filaments previously assembled from acrosomal processes without causing filament breakage during mixing. These results clearly demonstrated that rapid filament shortening by MV-95K results through a mechanism of disrupting intrafilament monomer-monomer interactions. Finally, we show that tropomyosin-containing actin filaments are insensitive to cutting, but not to capping, by MV-95K in the presence of Ca^{++} .

Actin exhibits amazing flexibility in participating in both cytoplasmic structure and motility (for review on actin function, see references 8, 17). Very few, if any, of actin's cytoplasmic roles can be attributed solely to its in vitro assembly characteristics. The presence of numerous actin binding proteins within the cell confers remarkable versatility to actin. Among the most fascinating of these is a family of Ca^{++} -sensitive actin binding proteins identified in a wide variety of cell types (e.g., gelsolin [46], fragmin [14], actinogelin [25], platelet 90,000 [38], severin [6]; see also reference 8) which regulate filament structure, filament-filament interaction, and filament assembly. The most

"versatile" member of this family to be characterized, thus far, is a 95,000-dalton protein of the brush border microvillar core, variously referred to as villin (3) or MV-95K (26, 27). Although the in vivo function of this protein is unknown, the in vitro interaction of MV-95K with actin has been intensely studied in several laboratories including our own (see reference 8). These studies include analysis of the Ca^{++} -dependent effects of MV-95K on actin assembly as well as its effects on preformed filaments. In the absence of Ca^{++} , this protein has little detectable effect on filament assembly (12, 26). At Ca^{++} concentrations greater than $\sim 10^{-6}$ M, MV-95K increases the rate

of polymerization, presumably by acting as a potent nucleator of assembly (6, 12, 27).

The interaction of MV-95K with F-actin is also Ca^{++} -dependent. In $<10^{-6}\text{M}$ Ca^{++} , MV-95K cross-links actin filaments into bundles (3, 24, 26). Addition of MV-95K to solutions of F-actin, in the presence of Ca^{++} , produces a rapid decrease in viscosity (6, 12, 24, 27) and reduction in sedimentability without a measured decrease in polymer. These viscosity and sedimentation changes are due to the reduction in the average filament length (3, 24, 26, 29).

We further investigated the Ca^{++} -dependent effects of MV-95K on actin by a simple new assay employing the isolated acrosomal process from *Limulus* sperm. In situ the *Limulus* acrosomal process is a 50 μm long tapered bundle of tightly packed and uniformly polarized actin filaments (35, 36). The bundle contains 85–100 filaments at one end and gradually tapers to only 13 filaments at the other end. This actin bundle is readily isolated as shorter segments (referred to as acrosomal processes) ranging from ~ 5 to 20 μm in length which are extremely stable under a wide range of salt conditions (35). Incubation of the isolated acrosomal processes in G-actin and salt results in rapid nucleation of filaments having uniform length from both bundle ends (2). The polarity of the nucleated filaments can be determined by comparing the diameters of the acrosomal bundles at their two ends. The thin end of the acrosomal process corresponds to the "barbed" end of an S1-decorated filament while the thick end corresponds to the "pointed" filament end (36). Taking advantage of the acrosomal processes as nuclei for filament polymerization, we examined the Ca^{++} -dependent effects of MV-95K both on filament assembly and on pre-assembled filaments.

MATERIALS AND METHODS

Preparation of *Limulus* Acrosomal Processes, MV-95K, Actin, and Tropomyosin: *Limulus* sperm acrosomal processes were prepared according to the procedure of Tilney (35). Chicken intestinal MV-95K protein was prepared following the methods of Mooseker et al. (26). After isolation, the protein was dialyzed into 75 mM KCl, 10 mM imidazole (pH 7.2), 0.2 mM DTT, and 0.02% sodium azide. Muscle actin was extracted from an acetone powder of chicken breast muscle according to Spudich and Watt (34). Purified G-actin was obtained by gel filtration on Sephadex G-150 (Pharmacia Fine Chemicals, Uppsala, Sweden) as outlined by MacLean-Fletcher and Pollard (21). Tropomyosin (TM) was extracted from chicken breast according to Bailey (1) and further purified by hydroxyapatite chromatography following the procedure of Eisenberg and Kielley (10). After purification the TM was dialyzed into 75 mM KCl, 10 mM imidazole, pH 7.2, 0.2 mM DTT, and 0.02% sodium azide.

Growth Rate Determination: Actin filament growth rates were determined by using the acrosomal processes to nucleate filament assembly. This was carried out by a slight modification of the procedure of Pollard and Mooseker (31) for nucleation of actin filaments from isolated microvillar core fragments. Processes were suspended in nucleation buffer (75 mM KCl, 1 mM MgSO_4 , 10 mM imidazole [pH 7.2] with either 1 mM EGTA or 0.2 mM CaCl_2) both in the presence and in the absence of MV-95K. To this mixture, freshly gel-filtered G-actin was added. The final concentrations of MV-95K and actin were 0.1 and 2 μM , respectively. After incubation for 15–60 s, the assembly mixture was gently diluted with four volumes of ice-cold nucleation buffer and then negatively stained. This dilution limits any further filament elongation by both decreasing the temperature and lowering the actin monomer concentration (31). The lengths of nucleated barbed and pointed end filaments were obtained by direct measurements from electron micrographs. Typically, between 25 and 100 filaments were measured per time point. Growth rates were obtained by plotting the average filament length as a function of time and calculating the slope by linear regression.

Capping Assay: Acrosomal processes were incubated for 20 min on ice in nucleation buffer containing 0.2 mM CaCl_2 and 0.3 μM MV-95K. The acrosomal processes were then washed several times by centrifugation and resuspended in nucleation buffer plus Ca^{++} . After the final resuspension in nucleation buffer plus Ca^{++} , an aliquot was removed and added to gel filtered G-actin to a final concentration of 2 μM actin. After 45 s, the sample was diluted and negatively stained. Controls were performed by using 95,000-dalton dialysis

buffer during the initial 20-min incubation.

Cutting Assay: Gel-filtered G-actin was added to acrosomal processes in nucleation buffer containing 0.2 mM CaCl_2 . After 45 s, MV-95K (0.1–0.6 μM) was gently added to the nucleation mixture. This length of incubation gave readily measurable filament growth from both the barbed and pointed filament ends using 2 μM actin. Incubation was continued for an additional 15 s, at which time the mixture was diluted and negatively stained. In control preparations, an equal volume of MV-95K dialysis buffer was substituted for MV-95K.

Inhibition of Cutting by Tropomyosin: The above cutting assay was repeated on TM-containing actin filaments. In these experiments, actin filament nucleation was carried out in the presence of saturating amounts of TM to actin (0.5 and 2 μM , respectively).

Electron Microscopy: Negative staining was carried out on copper grids coated with parlodion and stabilized by a thin carbon film. Immediately before use, the grids were glow discharged to improve wetting. All samples were stained using unbuffered 1% aqueous uranyl acetate. Grids were examined on either a Philips 201 or 300 at an accelerating voltage of 80 kV.

Other Methods: All samples were electrophoresed on 10% gels according to Laemmli (18) or Matsudaira and Burgess (23). Gels were stained with Coomassie Brilliant Blue. Protein concentrations were determined following the methods of Lowry et al. (20). Actin and MV-95K concentrations were also determined spectrophotometrically using extinction coefficients of $A_{280}^{1\%} = 6.5$ (21) and $A_{280}^{1\%} = 13$ (12), respectively.

RESULTS

Effects of MV-95K on Actin Assembly in the Presence and Absence of Calcium

Addition of pure G-actin to isolated acrosomal processes in the presence of nucleation buffer results in filament growth from both ends of the bundle. The starting point of assembly is easily identified because the nucleated filaments tend to splay out from the end of the acrosomal processes which consists of tightly packed actin filaments (Fig. 1). In addition, the filaments within the bundle are slightly thicker in diameter than pure actin filaments (35). These features facilitate accurate length measurement of filaments nucleated from the ends of the acrosomal process. Under the chosen experimental conditions, the measured growth rates from both ends of the bundle were linear over the time course of the experiment. Increasing the time of incubation resulted in greater filament breakage. The polarity of the newly grown filaments was identified by comparing the differences in diameter at the ends of the bundle resulting from the gradual change in the number of filaments along the length of the acrosomal process. Using 2 μM actin, the growth rate from the barbed filament end was approximately ten times faster than from the pointed end (see Fig. 4*a*). Pollard and Mooseker (31) observed a comparable bias in growth rates (eightfold) using similar, but not identical, assembly conditions.

When MV-95K was added to the assembly mixture, filaments were nucleated from both ends of the acrosomal process (Fig. 2). The bias in assembly was the same as the EGTA controls, with the barbed filament end, or thin bundle end, being the preferred end for assembly. The calculated growth rates from the barbed and pointed ends of the processes are virtually identical to control growth rates. This observation is in agreement with other reports (reviewed in reference 8) finding little detectable effect on actin polymerization by MV-95K in the absence of calcium.

Previously, it had been shown that in the absence of Ca^{++} MV-95K cross-linked actin filaments into bundles in a concentration- and pH-dependent manner (3, 24, 26). In the experiments reported here the nucleated filaments, while not bundled, possess a striking periodic studding (Fig. 2, *inset*). This pronounced studding was never seen in control preparations and could very well be attributed to the binding of MV-95K along

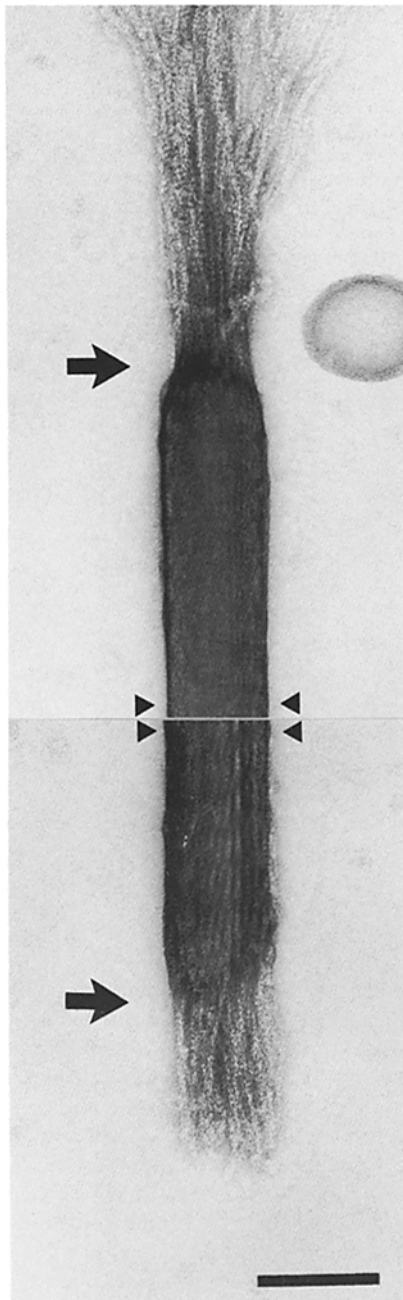


FIGURE 1 Nucleated polymerization of actin filaments, in the presence of Ca^{++} , from the two ends of the acrosomal process after 30 s of incubation using 2 μm actin (for details, see Materials and Methods). The figure is a composite of the two ends from a single bundle. The difference in diameter at the acrosomal process ends is readily seen by comparing the upper and lower halves of the figure where the two bundle ends meet. In this particular example, the bundle diameter in the upper part of the figure is 80 nm while the bundle diameter in the lower half measures 90 nm (measurement made at points designated by arrowheads). The bundle exhibits biased assembly, with the thin bundle end growing filaments faster than the thick bundle end. These ends correspond to the barbed and pointed filament ends, respectively (see reference 36). This figure also illustrates the difference in organization between the filaments within the acrosomal process and those that are newly grown, thus enabling precise determination of the starting point of filament nucleation (see arrows).

Note the large number of filaments nucleating with uniform length from the thick bundle end. Bar, 0.1 μm , $\times 162,000$.

the actin filament. In these experiments the nucleated filaments were not bundled because the conditions were not optimal for bundling (24). When these experiments were repeated at both optimal pH and MV-95K concentrations the newly grown filaments were organized into bundles (E. M. Bonder, unpublished observations).

The same types of nucleation experiments were repeated in the presence of 0.2 mM CaCl_2 . In the absence of MV-95K, filaments readily polymerized from both ends of the acrosomal processes and the bias for barbed end growth remained the same as in the EGTA controls. The measured growth rates from both ends of the process were somewhat slower in the presence of Ca^{++} than in its absence.

Addition of MV-95K to the calcium assembly mixture gave

a much different picture. Long filaments were no longer seen growing from the thin bundle end (barbed filament end). Instead, a dense mat of very short filaments was spread across the grid background. These short filaments were assembled within the first 15 s of the assay, illustrating the potent nucleating action of MV-95K on actin polymerization (6, 12, 27). Instead of the acrosomal process nucleating filaments off both ends, there was a complete absence of growth from one end

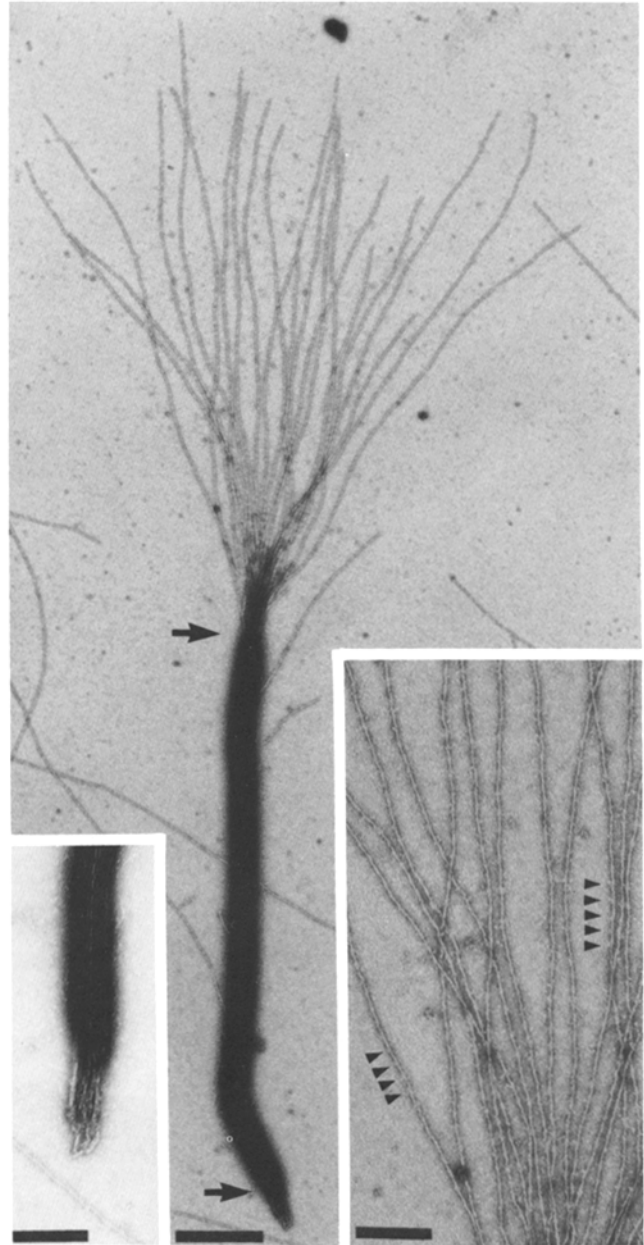


FIGURE 2 Nucleated polymerization of actin filaments in the presence of MV-95K and the absence of Ca^{++} . An acrosomal process after 45 s of incubation, showing biased growth with the preferred assembly end at the barbed filament end. Arrows indicate the starting point of filament nucleation. Notice the uniform length of the nucleated filaments from both bundle ends. (Left inset) Pointed filament growth is shown at higher magnification. (Right inset) A higher magnification of the filaments grown from the barbed end. Notice the highly periodic studding (indicated by arrowheads) of the actin filaments when nucleated in the presence of MV-95K and the absence of Ca^{++} . Bar, 0.5 μm ; $\times 25,200$. Insets: Bar, 0.2 μm ; $\times 50,100$.

and a ragged collection of short filaments at the other (Fig. 3 *b* and *c*). A comparison of the bundle diameters shows that the thin bundle end did not nucleate any detectable filaments while a small filament tuft was grown from the thick bundle end (Fig. 3 *b* and *c*). The assembly off the pointed filament end occurred in the initial 15 s of the assay, with very little increase in filament length over the duration of the experiment (compare Fig. 3 *b* with *c*, also see Fig. 4 *b*). The average length of the filaments nucleated during the initial 15 s in the presence of MV-95K was virtually identical to controls measured in the absence of MV-95K. In addition, the filaments nucleated from the pointed end were not so uniform in length as the pointed end filaments nucleated in control preparations. This non-uniform or ragged filament growth is never seen in either the

EGTA or calcium controls and must be due to the presence of MV-95K. Over the 60-s period, little detectable growth was observed from the barbed filament end.

Barbed End Capping by MV-95K

The absence of filament growth from the thin end of the processes suggested that MV-95K blocked monomer addition to the barbed end of a filament. This is consistent with the experiments of Glenney et al. (12) and the proposal of Craig and Powell (7) that MV-95K interacts with filament ends.

To directly assay for barbed end capping by MV-95K, we incubated acrosomal processes in a buffer containing Ca^{++} and MV-95K prior to their use as assembly nuclei. After this

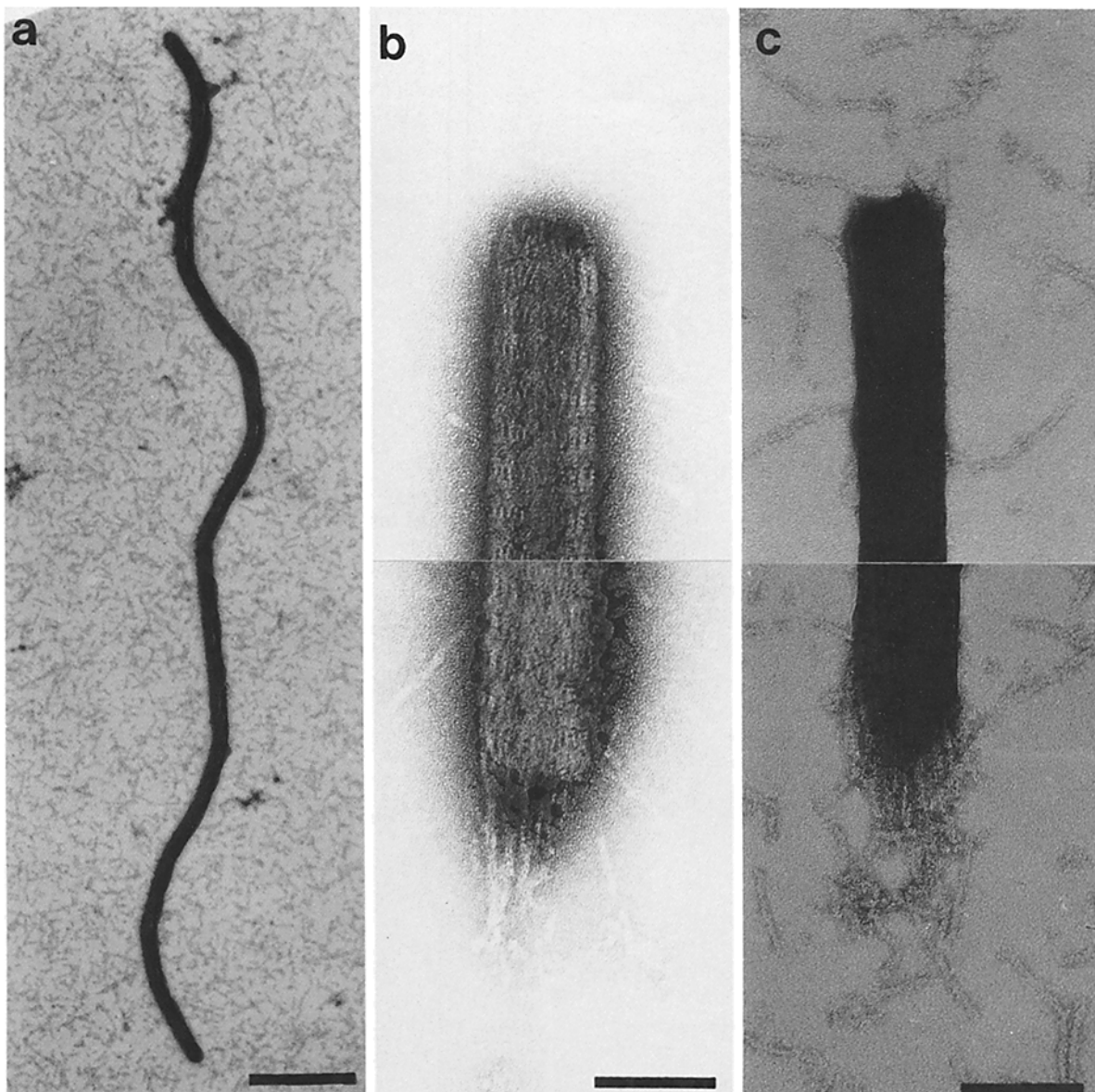


FIGURE 3 Filament nucleation off acrosomal processes in the presence of Ca^{++} and MV-95K. (a) The presence of MV-95K reduces filament elongation from the acrosomal process bundles while inducing the rapid assembly of short filaments seen in the background. This particular process was negatively stained 30 s after addition of actin. (b) A composite of the bundle ends of the acrosomal process in a at higher magnification. Filaments are no longer nucleated from the thin bundle end, while the opposite end has nucleated some filaments that are not uniform in length. (c) Another composite of an acrosomal process 60 s after actin addition. Again, filaments have not nucleated from the barbed filament end. The pointed filament end still has nucleated filaments, but their length has shown no increase. (a) Bar, 1 μm ; $\times 14,900$. (b and c) Bar, 0.1 μm ; $\times 176,300$.

incubation step, the processes were washed by repeated centrifugation to remove any unbound MV-95K. SDS PAGE of these steps (data not presented) showed an excess of MV-95K remaining in the supernate, indicating that all MV-95K binding sites on the processes were probably saturated. Actin was then added to the processes, and the bundles were examined for inhibition of monomer addition to either bundle end.

Unlike the situation in control preparations, addition of G-actin to the pre-incubated processes resulted in little filament assembly off the bundle ends (compare Fig. 5 *a* and *b*). The assembly pattern was similar to that in the previous growth studies, where in the presence of MV-95K there was a limited amount of growth from one bundle end (Fig. 5 *c*). This type of nucleation was consistent across the grid, and, of 50 processes randomly assayed, only one had growth from both bundle ends. Comparing the diameters of the two bundle ends showed that only the thick bundle end nucleated filaments and those filaments were also uniform in length (Fig. 5 *C*). The grids also

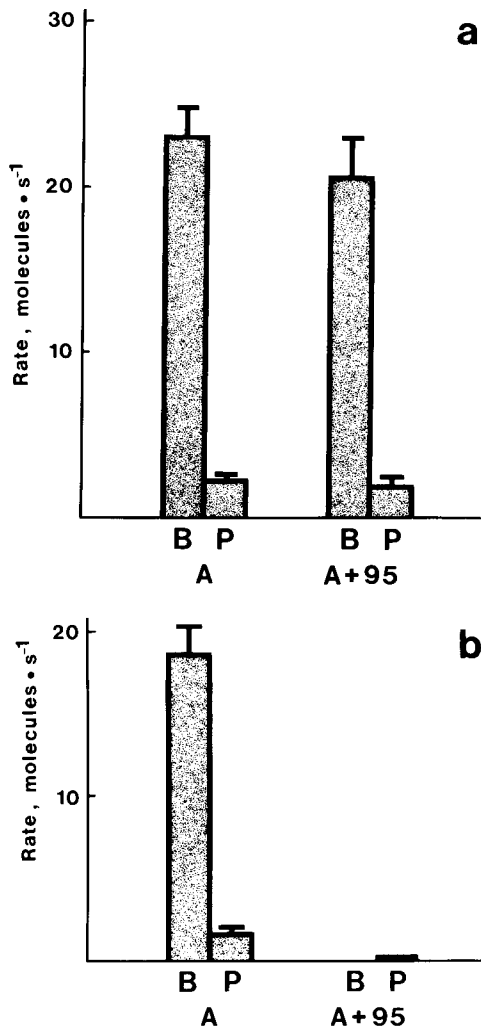


FIGURE 4. Calculated filament elongation rates from acrosomal processes, both in the presence and in the absence of MV-95K and Ca⁺⁺. (a) Growth rates from the barbed (B) and pointed (P) filament ends in the absence of Ca⁺⁺ for actin alone (A) and actin plus MV-95K (A + 95). (b) Same as in a, except in the presence of Ca⁺⁺. Note the absence of any growth from the barbed filament end in the presence of MV-95K. Filament elongation from the pointed end occurred in the initial 15 s of the assay, and then the average length did not change for the rest of the experiment. Error bars indicate one standard deviation.

lacked any spontaneously nucleated filaments, verifying that all unbound MV-95K was removed during the centrifugation step (compare Figs. 3 *a* to 5 *b*). These experiments provide a direct and unequivocal demonstration that, in the presence of Ca⁺⁺, MV-95K can bind to the barbed filament end and block actin monomer addition. This capping activity is specific for the barbed filament end since pointed end assembly was not substantially different from that of the controls.

Mechanism of MV-95K Induced Shortening of Pre-assembled Actin Filaments

Addition of MV-95K to F-actin in the presence of calcium produces a rapid reduction in the average filament length. Two mechanisms have been proposed: (a) by binding to filament ends, MV-95K prevents re-annealing after filament breakage (7) or (b) by disrupting monomer-monomer interaction, MV-95K cuts filaments along their length (12, 26). Distinguishing between these mechanisms is experimentally difficult because actin filaments are believed to be highly susceptible to shear, leading to filament breakage (8). In addition, the filaments may undergo spontaneous breakage in the absence of shear, as recently reported by Wegner (43). Therefore, any assay employing mixing or shearing makes differentiating between the two possibilities quite difficult. We developed an assay, using the acrosomal process to circumvent the problem of filament breakage. In these experiments we examined the effect of MV-95K on filaments pre-assembled from the two ends of the acrosomal process. Since we can nucleate filaments of pre-determined and uniform length off the acrosomal processes, any filament breakage during addition of MV-95K buffer in control preparations would result in the loss of that uniformity. In such control preparations, filaments nucleated from both ends of the acrosomal processes remained uniform in length, with also very few free filaments scattered across the grids (Fig. 6). These controls were virtually identical to unmixed preparations. Therefore, we believe that this experimental approach imposes minimal shear on the filaments, hence minimal filament breakage.

When MV-95K was added to acrosomal processes having pre-assembled filaments, there was a rapid and extensive loss of filaments from both bundle ends (Fig. 7 *a*). Examination of the bundles at higher magnification shows that small filament tufts sometimes remained on some of the bundle ends (Fig. 7 *b* and *c*) and that there was no specificity as to which bundle end retained these tufts (compare Fig. 7 *b* and *c*). Also, the filaments remaining within the tufts were not uniform in length. When the experiment was repeated with a higher concentration of MV-95K, neither filaments nor filament tufts were retained on any of the acrosomal processes (Fig. 7 *d*). With the absence of appreciable growth from the acrosomal processes, there was an enormous increase in short filaments spread across the grid (Fig. 7 *a*). The high density of short filaments cannot be attributed solely to the loss of filaments previously assembled off the bundle ends. Rapid nucleation of G-actin into short filaments by MV-95K must also have been occurring, given the potent nucleating action of this protein. The background filaments in this experiment also appear to be much more varied in length than those in the previous growth study where all of the short filaments were nucleated by MV-95K (compare Figs. 7 *a* and 3 *a*). As discussed below, these results indicate that the action of MV-95K on actin filaments involves a disruption of monomer-monomer interaction, resulting in the cutting of a filament at random sites along its length.

Resistance of Tropomyosin-containing Actin Filaments to Cutting by MV-95K

Previously, it was reported that suspension of brush borders in a Ca^{++} -containing buffer resulted in solation of the microvilli but not the terminal web rootlets of those microvilli (27). Since the brush border contains TM, which has been localized to the terminal web rootlet portion of the microvillus core (9), it may play a role in the rootlet's resistance to solation by MV-95K. Using Ostwald viscometry, Mooseker et al. (27) showed that TM inhibited the cutting action of MV-95K on F-actin while having little inhibitory effect on the potent nucleating action of MV-95K during filament assembly. It was also shown that

both in the presence and in the absence of Ca^{++} , TM did not nucleate filament assembly or enhance its steady state plateau viscosity.

The resistance of filaments assembled in saturating quantities of TM to cutting by MV-95K in the presence of Ca^{++} was re-examined using the above acrosomal process assay. TM-containing actin filaments were assembled onto both ends of the acrosomal process, as in the previous section, and then MV-95K was added to the mixture. Unlike the earlier results, the newly assembled filaments remained attached to the bundles with little indication of filament cutting (Fig. 8). In these preparations, the thin bundle end had filaments that were approximately five times longer than the filaments nucleated

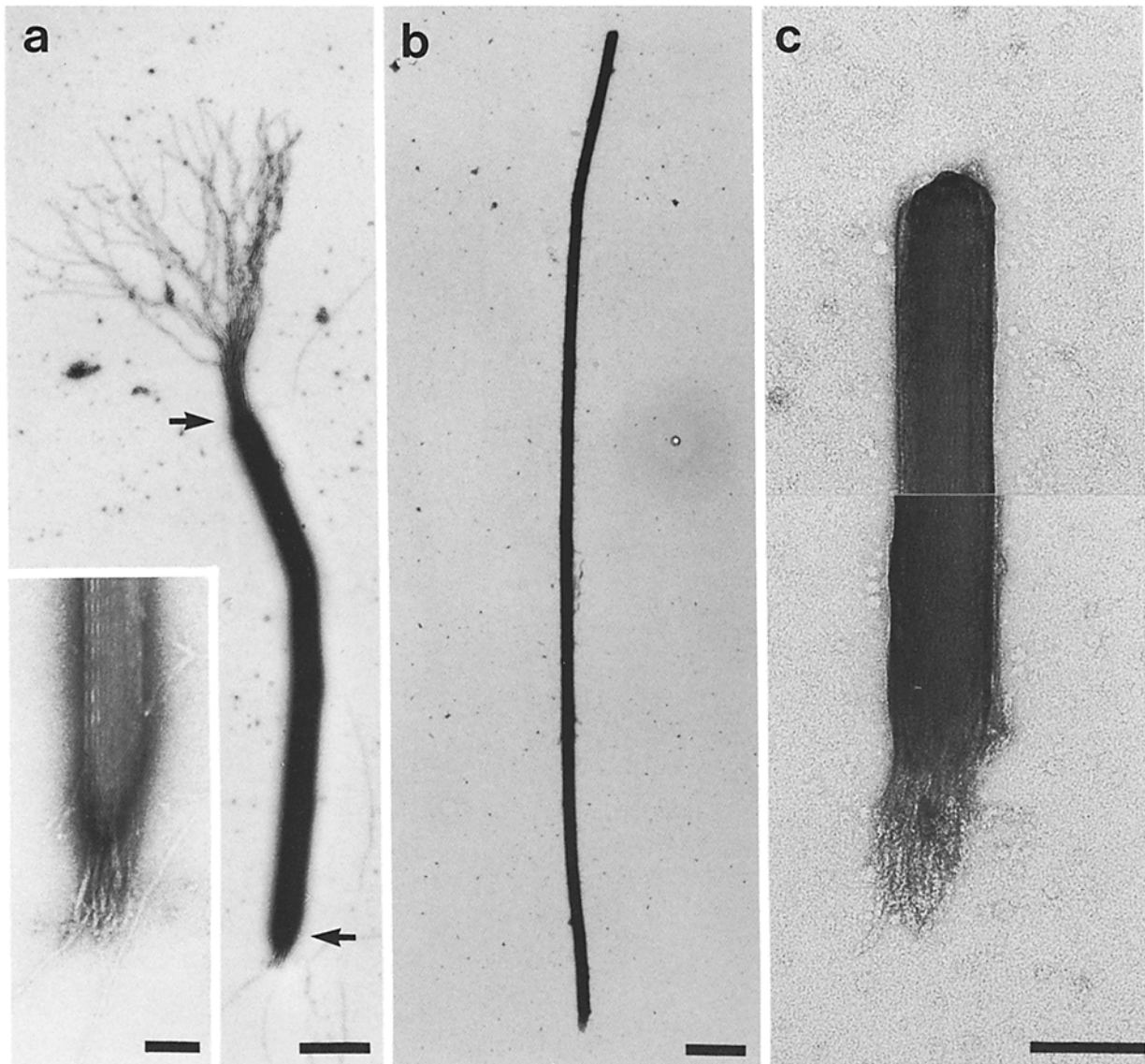


FIGURE 5 Capping of the barbed filament end by MV-95K. Prior to their use in a nucleation assay, the acrosomal processes were incubated in nucleation buffer containing both MV-95K and Ca^{++} and then washed free of any unbound MV-95K (see Materials and Methods for details). (a) A control preparation where dialysis buffer was substituted for MV-95K in the initial incubation. The acrosomal process nucleated biased filament growth, with the barbed end being the preferred assembly end. Arrows indicate the bundle ends. (Inset) The thick bundle end (pointed filament end) at higher magnification showing uniform filament growth. (b) An acrosomal process after the incubation step in MV-95K, showing greatly reduced filament nucleation. Note the absence of any extraneous background filaments. (c) A composite showing the two ends of the acrosomal process in *b* at higher magnification. There is a complete absence of growth from the thin bundle end, that is, the barbed filament end, resulting from the binding of MV-95K. Pointed end filament growth was indistinguishable from that of control preparations. Bar, $0.5 \mu\text{m}$; $\times 21,700$. (Inset) Bar, $0.1 \mu\text{m}$; $\times 76,600$. (b) Bar, $0.5 \mu\text{m}$; $\times 17,500$. (c) Bar, $0.1 \mu\text{m}$; $\times 167,500$.

off the thick end as compared to approximately the eightfold bias in control preparations (data not shown). This shift in bias probably resulted from MV-95K capping the barbed filament end preventing monomer addition while elongation continued from the pointed filament end for an additional 15 s.

MV-95K's action as a potent nucleator even in the presence of TM was evidenced by the many short filaments spread across the grid background (Fig. 8) which were never seen in controls. The absence of free background filaments in the TM-containing controls showed that TM does not nucleate filament assembly, further substantiating the earlier viscometric data (27).

DISCUSSION

Advantages of the Acrosomal Process Nucleation Assay for Actin Assembly Studies

The results presented here demonstrate the usefulness of this assay for analysing the effects of actin-binding proteins on both actin assembly and actin filament structure. Actin polymerization is a complex process consisting of up to three distinct

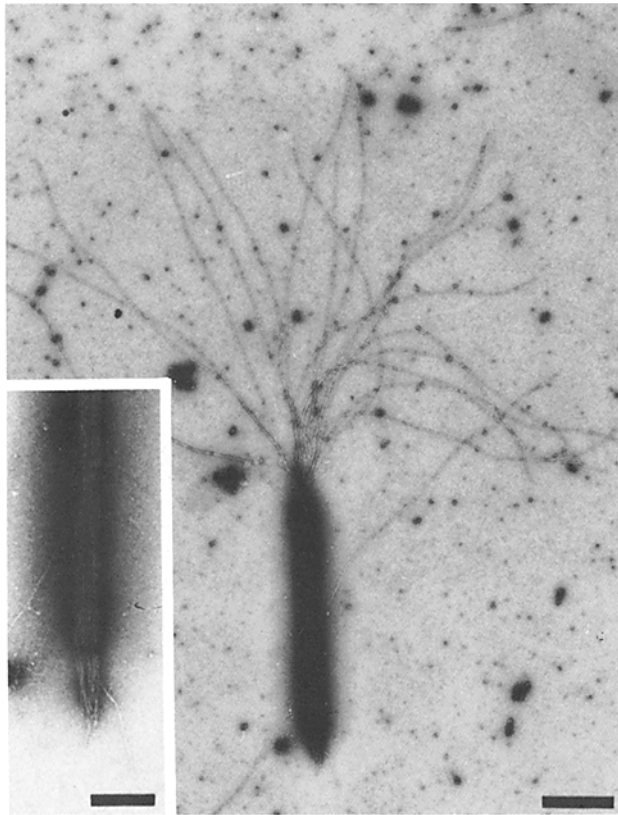


FIGURE 6 A negative stain from a control preparation examining the effect of mixing on filaments previously assembled from the acrosomal processes. Filaments were assembled from the acrosomal processes in the presence of Ca^{++} for 45 s, at which time an aliquot of buffer was added and mixed. Negative staining followed after 15 s. Notice the long unbroken filaments growing from the barbed filament end. The degree of filament uniformity and the average length of the filaments from both the barbed and pointed filament ends are virtually identical to controls where buffer was not added, indicating the absence of filament breakage during this assay. *Inset*: The thick bundle end, at high magnification, showing that these filaments were also unaffected by buffer addition and mixing. Bar, 0.2 μm ; $\times 18,600$. (*Inset*) 0.2 μm ; $\times 42,300$.

assembly steps: nucleation, elongation, and breakage-annealing, which all presumably occur simultaneously (see reference 32). Other techniques such as sedimentation, viscometry, flow birefringence, or spectrophotometry measure the net result of all three assembly steps. Another level of complexity is added by an actin filament having two ends, each with its own assembly rate constants (31, 42). Consequently, actin exhibits biased assembly, with the barbed filament end growing faster than the pointed end (15, 16, 45). At steady state, the filaments can exhibit net polymerization at one end and net depolymerization at the other end, giving rise to a phenomenon called treadmilling (42). Treadmilling is in turn probably dependent upon the salt conditions of the experiment (31). Addition of an actin binding protein to actin can only complicate matters by affecting one or any combination of the above parameters.

By using acrosomal processes to nucleate filament assembly we can limit our observations to filament elongation from a constant number of assembly nuclei. This approach eliminates the effects that uncontrolled nucleation and breakage-annealing have on obtaining both quantitative and qualitative data from an experiment. It also allows the independent examination of assembly from both the barbed and pointed filament ends. The advantages of such an approach were first demonstrated for actin in studies using microvillus core fragments as nuclei (28, 31). For example, using the core fragments (28, 31), cytochalasin B was found to completely inhibit assembly from the barbed filament end, resulting in an 80% reduction in elongation rate. Under similar experimental conditions using viscometry, cytochalasin B was shown to reduce the assembly rate by only 20% (22).

However, because of the intrinsic properties of the acrosomal process bundles (see introduction) they are far superior to either microvillus core fragments or other nuclei as seeds for actin-assembly studies. For example, microvillus cores are unstable at both high and low ionic strength, cannot be used in the presence of Ca^{++} (because of MV-95K's presence), and tend to become unbundled, making identification of the origin of new filament growth difficult. Finally, filament polarity cannot be determined unless the newly grown filaments are decorated with S1. Other nuclei that have been used include glutaraldehyde-fixed, S1-decorated filament fragments (22, 37) and polystyrene beads (4, 5, 12). The S1-decorated fragments are fixed before use, and therefore possibly altering actin filament structure, as recently reported by Lehrer (19). Assembly from the pointed end is also very slow, suggesting that this end may not be a good filament nucleator (37). Although polystyrene beads coated with either polylysine (5) or MV-95K (12) have been used for qualitative analysis of nucleated filament assembly, no attempts at quantitative analysis have been reported.

MV-95K Caps the Barbed End of Actin Filaments in the Presence of Ca^{++}

Previous studies summarized above and reviewed elsewhere (8, 27) have demonstrated that, in the presence of Ca^{++} , MV-95K (a) is a potent nucleator of filament assembly, (b) restricts the length that filaments achieve at steady state, and (c) may interact with the barbed end of the filament. Until now, direct evidence for barbed end interaction has not been obtained.

The results presented here on the nucleated polymerization of actin from acrosomal processes in the presence of Ca^{++} and MV-95K provide a graphic illustration of MV-95K's nucleating potential. As long as freshly gel-filtered G-actin is used, fila-

ment growth is observed only from the acrosomal processes in control preparations. In the presence of MV-95K, short filaments cover the entire grid, indicating that it stimulates nucleation. Most importantly, these experiments (Figs. 3–4) provide direct and quantitative evidence that MV-95K blocks monomer addition at the barbed filament end. In addition, even though the initial rate of filament elongation at the slow or pointed filament end is unaffected by MV-95K (Fig. 4*b*) the complete absence of filament growth from the barbed filament end provides direct evidence that this protein is actually a potent inhibitor of the elongation phase of actin assembly. This was completely missed by the other techniques used to study MV-95K-actin interactions.

These experiments also provide evidence for MV-95K blocking barbed end growth by binding to that end of the filament rather than by altering the polymerization properties of the

monomer pool. First, MV-95K blocked barbed end growth at substoichiometric ratios to actin monomer. Second, the latter mechanism which relies on lowering the free monomer pool available for assembly would be expected to inhibit the initial rate of pointed end growth, which was not observed (Fig. 4*b*).

The binding of MV-95K to the barbed end is directly demonstrated by the results of the experiment in which acrosomal processes pre-soaked in MV-95K were used to nucleate assembly. Even though free MV-95K was not present, barbed end growth was completely inhibited. Moreover, the fact that MV-95K remains bound to the processes after several washes, infinitely diluting any free MV-95K, indicates that it binds to the end of an actin filament with very high affinity.

Although our results provide unequivocal evidence that MV-95K binds to and prevents monomer addition to barbed filament end, these experiments do not reveal whether this protein

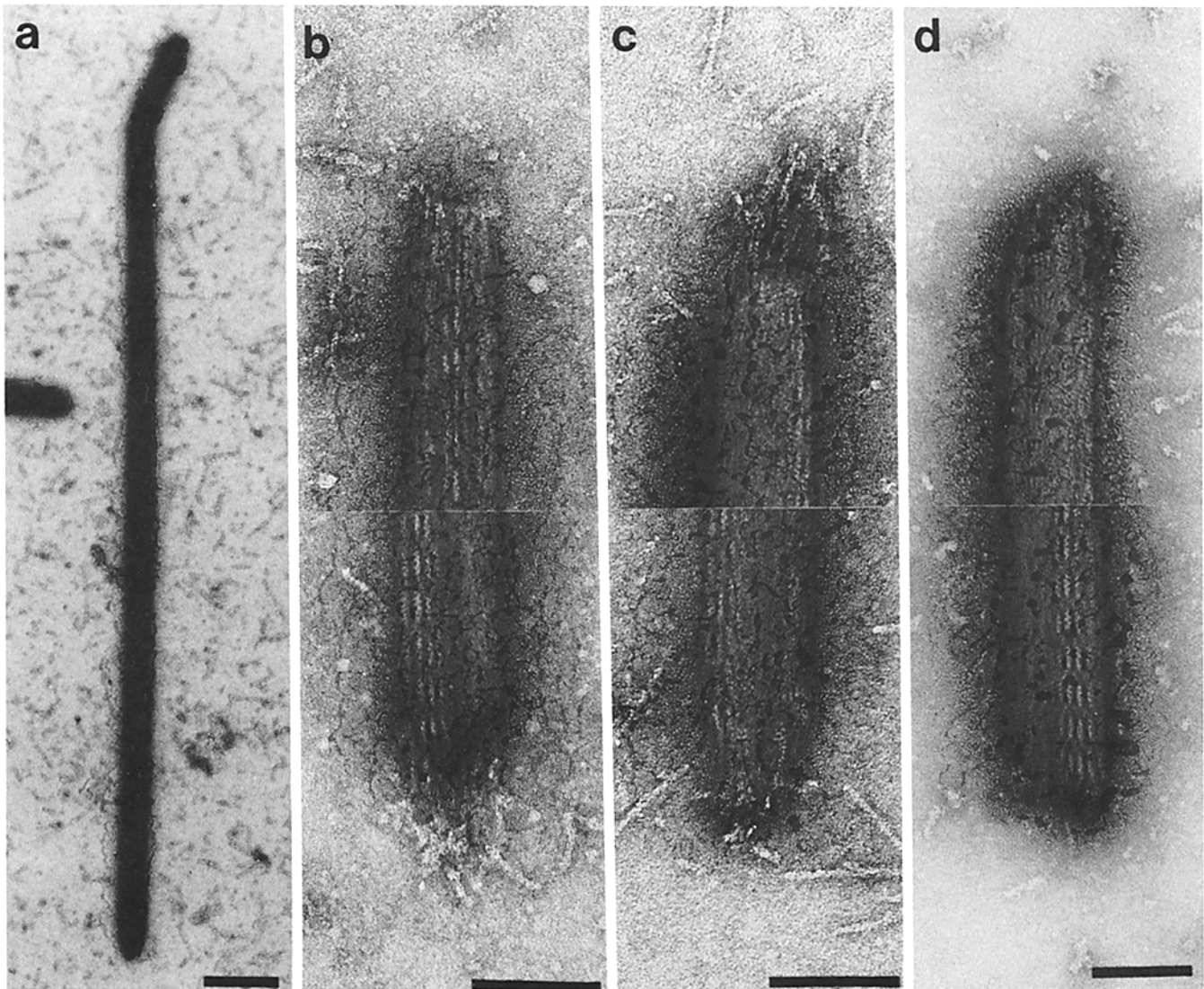


FIGURE 7 Effect of addition of MV-95K to filaments nucleated from acrosomal processes in the presence of Ca^{++} . Conditions are identical to those in Fig. 6, except that MV-95K was added. (a) An electron micrograph showing the loss of previously nucleated filaments from an acrosomal process, along with a substantial increase of short filaments in the background after addition of $0.1\text{-}\mu\text{m}$ MV-95K. (b) A composite of the two bundle ends from a. Note the absence of filaments on the barbed filament end and a small tuft of filaments having variable length remaining at the pointed end. (c) Same conditions as in b, but, this time, resulting in a small tuft at the barbed end and a few short filaments at the pointed end. (d) The ends of an acrosomal process after addition of $0.6\text{-}\mu\text{m}$ MV-95K. There is now a complete loss of filaments from both bundle ends. Note the small clumps in the background, which are probably MV-95K-actin oligomers. (a) Bar, $0.5\text{ }\mu\text{m}$; $\times 22,600$. (b and c) Bar, $0.1\text{ }\mu\text{m}$; $\times 193,500$. (d) Bar, $0.1\text{ }\mu\text{m}$; $\times 153,800$.



FIGURE 8 Resistance of TM-containing actin filaments to Ca^{++} -mediated cutting by MV-95K. Acrosomal processes nucleated actin filaments in the presence of saturating amounts of TM and 0.2 mM Ca^{++} followed by addition of MV-95K to the newly assembled filaments (see Materials and Methods). (a) An acrosomal process with TM-actin filaments nucleating from both ends, even after addition of MV-95K. The bias in length at the barbed end is only five times that of the pointed end, whereas in controls there was an eightfold bias in length. A second, shorter acrosomal process is also present, but its preferred end is somewhat obscured. Arrows indicate the ends of the acrosomal processes. Notice how the two bundles have nucleated pointed end filaments that are almost identical in length. (b) The same acrosomal process as in a at higher magnification, comparing the two bundle ends. The nucleated barbed and pointed end filaments are still attached to the bundles and show little sign of breakage, indicating that they are resistant to cutting by MV-95K. Note the presence of short filaments in the background of a and b, which result from the nucleating action of MV-95K. Bar: (a) 0.5 μm ; $\times 25,600$. (b) 0.2 μm ; $\times 73,900$.

also inhibits the rate of monomer loss. Recent experiments done in collaboration with Dr. A. Weber provide convincing data that MV-95K also inhibits the rate of monomer dissociation from the barbed end. Using 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD)-actin, a fluorescent derivative of actin (41), it was shown that, in the presence but not absence of Ca^{++} , MV-95K inhibits the rate of filament depolymerization when filaments were diluted below the critical concentration. We conclude from these experiments that MV-95K is a true capper of the barbed end, inhibiting both monomer association and dissociation.

Since MV-95K is a barbed end capper, one would predict that the critical concentration for actin assembly should change to that of the pointed end. A higher critical concentration has been estimated (2, 31) for the pointed filament end, under physiological conditions. Recently, we examined (Bonder, E. M., D. Fishkind, and M. S. Mooseker, unpublished observations) the minimal actin concentrations (critical concentration) necessary for filament assembly off the ends of the acrosomal process. Using salt conditions virtually identical to those used here (20 μM Ca^{++} instead of 200 μM Ca^{++}), we measured different critical conditions for the two ends of a filament. In addition, we found the critical concentrations to be strongly dependent on the salt conditions used during assembly. This last observation may help explain the widely varying monomer flux rates being measured by various laboratories (30, 33, 39, 42, 44). These data predict that under the conditions tested MV-95K should increase the critical condition in the presence of Ca^{++} . Experiments done in collaboration with Dr. A. Weber, using NBD-actin (41), and Drs. Wang and Taylor, using fluorescence resonance energy transfer (40), have confirmed this prediction. In the presence of Ca^{++} , MV-95K raises the critical concentration for assembly and causes a net depolymerization after addition to filaments.

Finally, the experiments analysing nucleated polymerization of actin in the presence of MV-95K and absence of Ca^{++} indicate that this protein has no significant effect on elongation rates under these conditions, (Fig. 4a) even though it binds to F-actin (3, 24, 26).

MV-95K Shortens Filaments by Disrupting Monomer-Monomer Interactions within the Filament

The experiments discussed above prove that MV-95K binds tightly to the barbed end of an actin filament. However, the results of the experiments in which MV-95K was added to filaments pre-grown off acrosomal processes demonstrate that this protein can also rapidly bind along the length of an actin filament and cut it by disrupting intrafilament monomer interactions.

These results clearly rule out the mechanism for filament shortening based solely on inhibition of re-annealing after filament breakage. Such a mechanism would not have resulted in any loss of filaments previously nucleated from the acrosomal process ends because the assay used produces a minimal amount of filament breakage (also see Results).

Although our results rule out the breakage-annealing mechanism for filament shortening, we still must consider a third possibility based on the fact that MV-95K is a potent nucleator of assembly. In the assay we have devised to examine cutting of filaments, the only filaments present in the mixture, before

addition of MV-95K, are those that have grown off the acrosomal processes. It should be remembered that in these preparations the bulk of the actin is still monomer. Addition of MV-95K causes the rapid nucleation of the available G-actin as well as causing the rapid loss of the pre-assembled filaments. Perhaps the rapid nucleation by MV-95K could cause a redistribution of monomers from the previously assembled filaments onto the newly formed nuclei, giving rise to large numbers of short filaments. A mechanism such as this can be ruled out by two lines of evidence. First, this mechanism requires that the filaments grown off the acrosomal processes must depolymerize faster than they polymerize. The possibility is highly unlikely, given the calculated monomer dissociation rate constants measured at the two filament ends (2, 31). Second, Glenney et al. (12) showed that addition of a 3:1 actin-villin core (a 90,000-dalton fragment of MV-95K) complex to solutions of F-actin did not rapidly reduce its viscosity, as would the addition of free MV-95K.

A mechanism we have not discussed had been proposed by one of our paper's reviewers, whereby MV-95K simply prevents filament re-annealing after transient breaking of intra-strand bonds between adjacent monomers, so-called breathing. Such a mechanism would require that the breathing rate be sufficiently rapid so as to reduce 2 μ m long filaments down to one-tenth that size in 15 s. If breathing were occurring, then one would predict that the transient breaks would also be a site for monomer insertion. Using radioactively labeled actin, Simpson and Spudich (33) have shown that monomer addition to an actin filament is limited to the ends of filaments and does not occur along the filament length. Other experiments using fluorescence resonance energy transfer further substantiate the notion that if breathing is occurring it occurs at a very slow, as yet, unmeasured rate (30). Given the significant lack of evidence for filament breathing, we do not believe that the extremely rapid cutting of actin filaments by MV-95K proceeds through the reviewer's mechanism.

Relationship between Ca^{++} -dependent Capping and Cutting by MV-95K

The results presented both above and elsewhere (11, 13, 26) are highly suggestive that MV-95K may have at least two different Ca^{++} -dependent interactions with an actin filament. Earlier studies found that at micromolar Ca^{++} concentrations addition of MV-95K to G-actin resulted in the restriction of filament length while addition to F-actin did not produce rapid filament shortening (26). The TM experiments reported here illustrate an example where MV-95K is able to cap the barbed filament end even though those filaments are not susceptible to cutting (see also reference 27). Recently, the Ca^{++} threshold for cutting and capping was re-examined and the findings suggest that, at 1–5 μ M Ca^{++} , MV-95K caps only filament ends while in 10–20 μ M Ca^{++} it can both cap and cut actin filaments (41; M. S. Mooseker and D. Fishkind, unpublished observations). The above observations open up the possibility that there may also be Ca^{++} -dependent capping proteins that cannot rapidly shorten actin filaments in addition to the capping/cutting proteins already identified. This suggestion is not so untenable since a barbed end capper that does not cut filaments and is Ca^{++} independent has already been isolated, namely *Acanthamoeba* capping protein (8).

These findings on the differential regulation of MV-95K's interaction with actin by both Ca^{++} and other actin-binding

proteins might shed some light as to its function in the brush border of intestinal epithelial cells. MV-95K may play an essential role in microvillus core assembly and structure by regulating microvillar elongation during cell migration from the crypts to the tip of the villus or microvillar length changes associated with fasting (see references 27, 28 for additional discussion).

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