Nucleated Polymerization of Actin from the Membrane-associated Ends of Microvillar Filaments in the Intestinal Brush Border

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ABSTRACT We examined the nucleated polymerization of actin from the two ends of filaments that comprise the microvillus (MV) core in intestinal epithelial cells by electron microscopy. Three different in vitro preparations were used to nucleate the polymerization of muscle G-actin: (*a*) MV core fragments containing "barbed" and "pointed" filament ends exposed by shear during isolation, (*b*) isolated, membrane-intact brush borders, and (*c*) brush borders demembranated with Triton-X 100. It has been demonstrated that MV core fragments nucleate filament growth from both ends with a strong bias for one end. Here we identify the barbed end of the core fragment as the fast growing end by decoration with myosin subfragment one. Both cytochalasin B (CB) and *Acanthamoeba* capping protein block filament growth from the barbed but not the pointed end of MV core fragments. To examine actin assembly from the naturally occurring, membrane-associated ends of MV core filaments, isolated membrane-intact brush borders were used to nucleate the polymerization of G-actin. Addition of salt (75 mM KCl, 1 mM $MgSO_4$) to brush borders preincubated briefly at low ionic strength with G-actin induced the formation of 0.2–0.4 μm "growth zones" at the tips of microvilli. The dense plaque at the tip of the MV core remains associated with the membrane and the presumed growing ends of the filaments. We also observed filament growth from the pointed ends of core filaments in the terminal web. We did not observe filament growth at the membrane-associated ends of core filaments when the latter were in the presence of 2 μM CB or if the low ionic strength incubation step was omitted. Addition of G-actin to demembranated brush borders, which retain the dense plaque on their MV tips, resulted in filament growth from both ends of the MV core. Again, 2 μM CB blocked filament growth from only the barbed (tip) end of the core. The dense plaque remained associated with the tip-end of the core in the presence of CB but usually was dislodged in control preparations where nucleated polymerization from the tip-end of the core occurred. Our results support the notion that microvillar assembly and changes in microvillar length could occur by actin monomer addition/loss at the barbed, membrane-associated ends of MV core filaments.

The arrangement of actin filaments within the microvilli of intestinal epithelial cells is precisely determined with respect to filament length and polarity. Each microvillus contains a bundle or core of approximately 20 filaments of the same length. The "barbed" ends of the core filaments are embedded in a dense plaque which effects the attachment of the core to the plasma membrane at the tip of the microvillus. The "pointed" ends of the filaments are at the rootlet-end of the core in the terminal web (the barbed/pointed terminology is based on the arrowhead morphology of actin filaments decorated with heavy meromyosin [HMM] or myosin subfragment 1 [S_1]; see reference 22 for a review on brush-border cytoskeletal structure and function). Actin polymerization may play an important role in the formation of this precise array of actin filaments. This notion is based on the observations of Tilney and Cardell (29) on the reformation of microvilli in salamander intestinal epi-

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thelium exposed to high hydrostatic pressure. These investigators observed that pressure treatment induced a complete breakdown of the brush-border surface which then reformed within 30 min after returning the tissue to atmospheric pressure. Ultrastructural analysis of the reformation process indicated that microvilli first appeared as short protrusions on the apical surface which presumably elongated to form microvilli. Each protrusion contained a membrane-associated dense plaque with short core filaments protruding from it. Presumably, the dense plaque is the same structure found at the tip of the reassembled microvillus. From these observations, Tilney and Cardell (29; see also references 14, 30) suggested that microvilli form from the nucleated polymerization of core filaments from the membrane-associated dense plaques.

Once the microvillus is formed, actin assembly/disassembly may also be responsible for the changes in microvillar length which occur in vivo. For example, microvilli gradually elongate as epithelial cells migrate from the crypt to the tip of the intestinal villus. Reversible shortening of microvilli occurs in fasted animals (1, 19) and in intestinal tissue treated in organ culture with cycloheximide (16).

Assembly of microvillar core filaments and changes in filament length may be regulated by controlling the actin monomer concentration in the cell. In addition, the rates of monomer association and/or dissociation at the two ends of core filaments may be regulated by the presence of actin binding proteins which can affect the addition or loss of actin monomer at either the barbed or pointed end of the core filament (for further discussion, see references 8, 14). This is a potentially powerful mode of control because an actin filament grows, at least in vitro (10, 15, 31), with a strong bias for monomer addition onto its barbed end. In the microvillus, the presumed fast-growing ends of core filaments are "attached" to the plasma membrane. Recently, several laboratories have characterized actin-binding proteins from a variety of cell types that can either block monomer addition onto the barbed or pointed end of the actin filament (reviewed in reference 8). In fact, such a "capping" function has been postulated for the dense plaque at the tip of the microvillus core (24, 30).

In this and a companion study (26) we have addressed three questions regarding the assembly properties of microvillar core filaments. First, we have used fragments of microvillar cores to nucleate the polymerization of actin. These studies were done to determine if core filaments, like S_1-decorated filaments (10, 15, 31), exhibit a bias for monomer addition onto their barbed ends. Secondly, we have used isolated, membrane-intact and demembranated brush borders, which, unlike core fragments, contain the naturally occuring ends of core filaments to nucleate the polymerization of actin. These experiments allowed us to examine for the presence of capping proteins associated with either the barbed ends of core filaments at the tip of the microvillus, or at the pointed ends of core filaments in the terminal web. Finally, we have examined the effects of the drug, cytochalasin B (CB), an inhibitor of actin assembly (4–7, 9, 11, 17, 18) and *Acanthamoeba* capping protein (13) on the nucleated assembly of actin from the two ends of microvillar core filaments.

From our results reported here, we suggest the possibility that microvillar core filament assembly and changes in core filament length may occur by actin monomer addition/loss at the membrane-associated ends of core filaments as well as at their "free," slow-assembly-ends in the terminal web. Preliminary accounts of some of the results described here have appeared elsewhere (20, 25).

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. (23). Demembranation of brush borders with Triton X-100 was accomplished by the methods described in Mooseker and Tilney (24), except that 0.1 mM phenylmethylsulfonyl fluoride was included in the detergent solution. Microvillar core fragments were isolated by extensive homogenization of brush borders followed by differential centrifugation as described in Howe et al. (12). This homogenization procedure breaks the underlying filament core at the base of the microvillus, leaving behind the rootlet portion of the core still embedded in the terminal web. Thus the pointed ends of the core filaments are "fresh" ends generated by shear. The microvillar membrane was removed from these filament bundles, which will be referred to in this report as core fragments, by several washes with Triton X-100 as described in Howe et al. (12). Unlike the situation with intact brush borders (24), complete demembranation of microvillar core fragments required extensive shearing in a 9-in Pasteur pipette. This procedure generates core fragments that are free of membrane and usually have little, if any, detectable dense plaque material associated with either end, as determined by electron microscopy of negatively stained preparations.

Nucleated Assembly of Actin Filaments from Microvillus Core Fragments, Membrane-intact Brush Borders and Demembranated Brush Borders

Nucleation of actin polymerization from the two ends of microvillus core fragments and subsequent decoration with S_1 was conducted by analysis of negatively stained specimens exactly as prepared by methods described in Pollard and Mooseker (26). Nucleation of actin from the two naturally occurring ends of the microvillus core filaments was analyzed by addition of actin monomer to isolated membrane-intact and demembranated brush borders. Small pellets (25–50 μl) of intact or demembranated brush borders were suspended in 1.0 ml of brush-border buffer (BB buffer) consisting of 75 mM KCl, 1 mM $MgSO_4$, 1 mM EGTA, 0.2 mM dithiothreitol (DTT), 10 mM imidazole-Cl, pH 7.2. Monomeric actin prepared by the method of Spudich and Watt (27) was added from a concentrated stock (1–10 mg/ml) in buffer G consisting of 2.0 mM Tris-HCl, pH 8.0, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM DTT. After 3–5 min of incubation at room temperature, 1.0 ml of cold 4.0% glutaraldehyde in double strength BB buffer was added with very gentle mixing. The brush borders or demembranated brush borders were collected by centrifugation for 5 min at 1,000 *g*. The supernates were removed and fresh fixative consisting of 2.0% glutaraldehyde, 0.2% tannic acid, 0.1 M sodium phosphate buffer pH 7.0, was added for an additional 45 min on ice. The remainder of the fixation protocol is exactly as described in Begg et al. (2). The samples were embedded in Epon and thin sections were examined with a Philips 201 electron microscope.

To allow access of actin monomer to the "tip" end of the microvillus core in membrane-intact brush borders, small pellets of isolated brush borders were suspended in 1.0 ml of a low ionic strength buffer consisting of 1 mM EGTA, 0.2 mM DTT, 10 mM imidazole-Cl, pH 7.2. Actin monomer was added (or an equivalent volume of buffer G in control samples) to a concentration of 48 μM. The brush borders were "soaked" in actin monomer, on ice, for 30–60 min, and the polymerization of actin was then induced by addition of 1.0 ml of double strength BB buffer. The brush borders were incubated for 5 min at room temperature and then processed as above for electron microscopy.

To test the effects of CB on nucleated assembly of actin filaments from the two ends of the microvillus core, the above experiments were conducted exactly as described with the inclusion of 2 μM CB (Sigma Chemical Co., St. Louis, MO) in the initial suspension buffer. To insure that the newly formed filaments protruding from the ends of the microvillus cores were the result of nucleated assembly and not annealing of preformed actin filaments to the ends of the core filaments, brush borders and demembranated brush borders were incubated for 5 min, at room temperature, in the presence of 24 μM F-actin. The samples were then processed for thin-section electron microscopy exactly as described above. A partially purified preparation of *Acanthamoeba* "capping" protein was made by the methods of Isenberg et al. (13). This fraction contains two polypeptides of 31,000 and 28,000 daltons.

RESULTS

Nucleated Assembly of Actin from the Two Ends of Microvillus Core Fragments

As we reported previously (26), addition of G-actin (1.2–7.2 μM) to microvillar core fragments in the presence of buffers

containing either 20 or 75 mM KCl and 5 mM $MgSO_4$ results in the growth of filaments from both ends of the core fragments (see Fig. 2, reference 26). However, filaments grow much faster from one of the two ends of the core fragment. Unfortunately, it is usually impossible to determine by direct examination which end of the core fragment is the fast growing end (i.e., barbed or pointed end). Occasionally we have observed, however, what appears to be remnants of the dense plaque material associated with the fast growing end of such core fragments (Fig. 1), suggesting that the tip or barbed end of the core is the preferred assembly end. This is confirmed by decoration of nucleated filaments with myosin, S_1 (Fig. 2). The decoration with S_1 also demonstrates that the polarity of the newly formed filaments is identical to the core filaments which nucleated their assembly.

In the presence of 2 μM CB (Fig. 3), nucleated assembly from the barbed end of core fragments is blocked but growth from the pointed end is still observed. Thes results confirm those of Maclean-Fletcher and Pollard (18), who have noted a similar inhibitory effect of CB on the nucleated polymerization of actin from S_1-decorated actin filament "seeds."

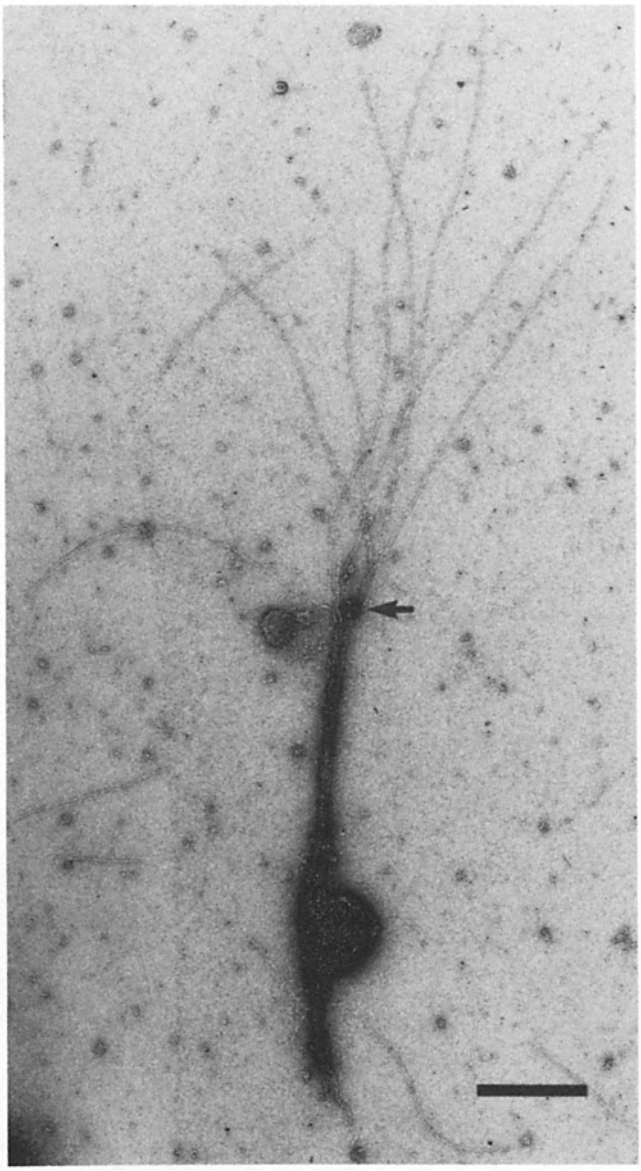


FIGURE 1 A negatively stained microvillus core fragment used to nucleate the polymerization of 2.8 μM actin for 20 s in the presence of 75 mM KCl, 5.0 mM $MgSO_4$. There is a strong bias for filament growth from the upper end of the core fragment. Remnants of dense plaque material (arrow) remain associated with the fast growing end. Bar, 0.5 μm. × 31,000.

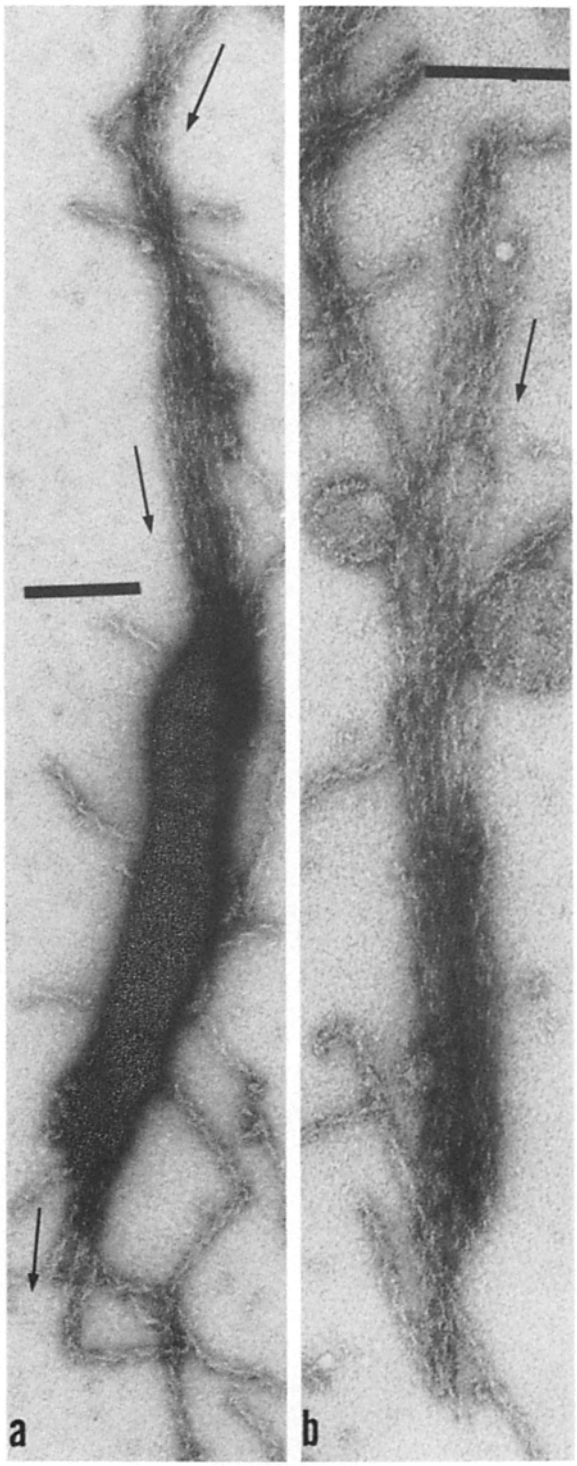


FIGURE 2 (*a* and *b*) S_1-decoration of core fragments which have nucleated the polymerization of 7.2 μM actin in the presence of 20 mM KCl for 2 min. "Arrowheads" on the "fast" assembly end of the core fragments contain the barbed ends of the filaments. The "arrowheads" on the short filaments nucleated by the lower ends of the core fragment point away from the core identifying the pointed end of the microvillus core as the slow assembly end. Arrows indicate filament polarity. Bar, 0.2 μm. (*a*) × 78,000. (*b*) × 98,000.

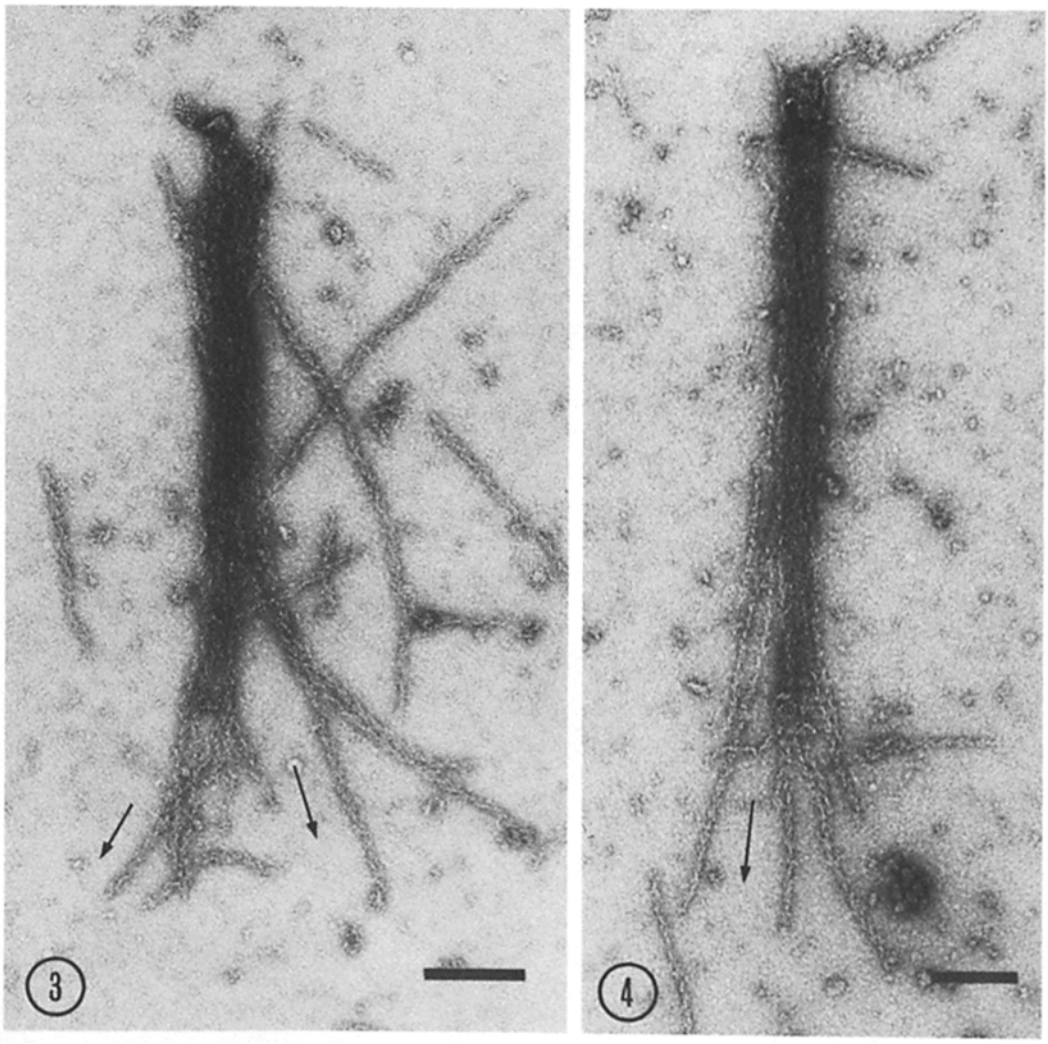


FIGURE 3 and 4 Fig. 3: Nucleated polymerization in the presence of cytochalasin B. S_1-decorated core fragment which has nucleated polymerization of 2.8 μM actin for 40 s in the presence of 75 mM KCl, 5 mM $MgSO_4$, and 2 μM CB. No filament growth from the upper, barbed end of the core fragment has occurred but nucleated assembly from the pointed ends of core filaments has not been blocked. Bar, 0.2 μm. × 74,000. Fig. 4: Nucleated polymerization in the presence of *Acanthamoeba* capping protein. S_1-decoration of core fragment which has nucleated polymerization of actin (conditions as in Fig. 3) in the presence of 2 μg/ml *Acanthamoeba* capping protein. Like CB, capping protein blocks monomer addition onto the barbed, but not pointed end of the core fragment. Bar, 0.2 μm. × 64,000.

To test the utility of core fragments for assaying the effects of actin-binding proteins on actin assembly, we examined the effect of *Acanthamoeba* "capping" protein on the nucleation of filaments from core fragments. Like CB, this actin-binding protein blocks monomer growth from the barbed end of S_1-decorated actin seeds (13). As expected, the capping protein blocks monomer addition onto the barbed, but not pointed ends of microvillar core filaments (Fig. 4).

Nucleated Assembly of Actin from the Two Ends of Microvillus Core Filaments in Membrane-free Brush Borders

These studies with isolated microvillus core fragments demonstrated the potential of the filaments in the core as nucleating sites for actin polymerization, but the two ends of such fragments are exposed artificially by shear during isolation. To assay for the presence of assembly capping proteins it is necessary to examine the "nucleating potential" of the naturally occurring filament ends on intact microvillus cores. To accomplish this, we used isolated brush borders demembranated with Triton-X 100 to nucleate the polymerization of actin. Detergent treatment removes the microvillar membrane without disrupting the structural integrity of the core or the dense plaque bound to its tip-end (24; see Fig. 6 *a*). The assumption here is that the functional as well as structural properties of the protein(s) which comprise the dense plaque are not affected by the detergent treatment.

Incubation of membrane-free brush borders with 4.8–24 μM G-actin for 5 min results in the growth of actin filaments from both ends of their microvillar filament bundles (Fig. 5). Examination of the terminal web region at high magnification (Fig. 5 *b*) indicates that most filament ends on the rootlet portion of the microvillus cores have nucleated assembly of actin filaments. Although the number of actin filaments nucleated by the basal ends of the cores appears to be greater than that from the tip-ends, this is an artifact of thin sectioning. Like a bouquet of flowers, the microvillar bundles of the isolated brush border are more tightly packed at their bases in the terminal web region. Consequently, within a thin section the number of filament ends attributable to the rootlet portion of the microvillar cores is considerably greater than that from the tip-ends. It is also important to note that lengths of filaments grown from the two ends of microvillus cores cannot be determined in thin-sectioned material.

The nature of nucleated assembly from the tip-ends of microvillus cores is more difficult to interpret because several distinct morphologies have been observed, examples of which are found in Fig. 5 *a* and *c*. As is the case for basal growth, the site of nucleation is readily determined because the filaments of the core remain tightly packed, and the newly formed filaments fan out from the presumed site of nucleation on the

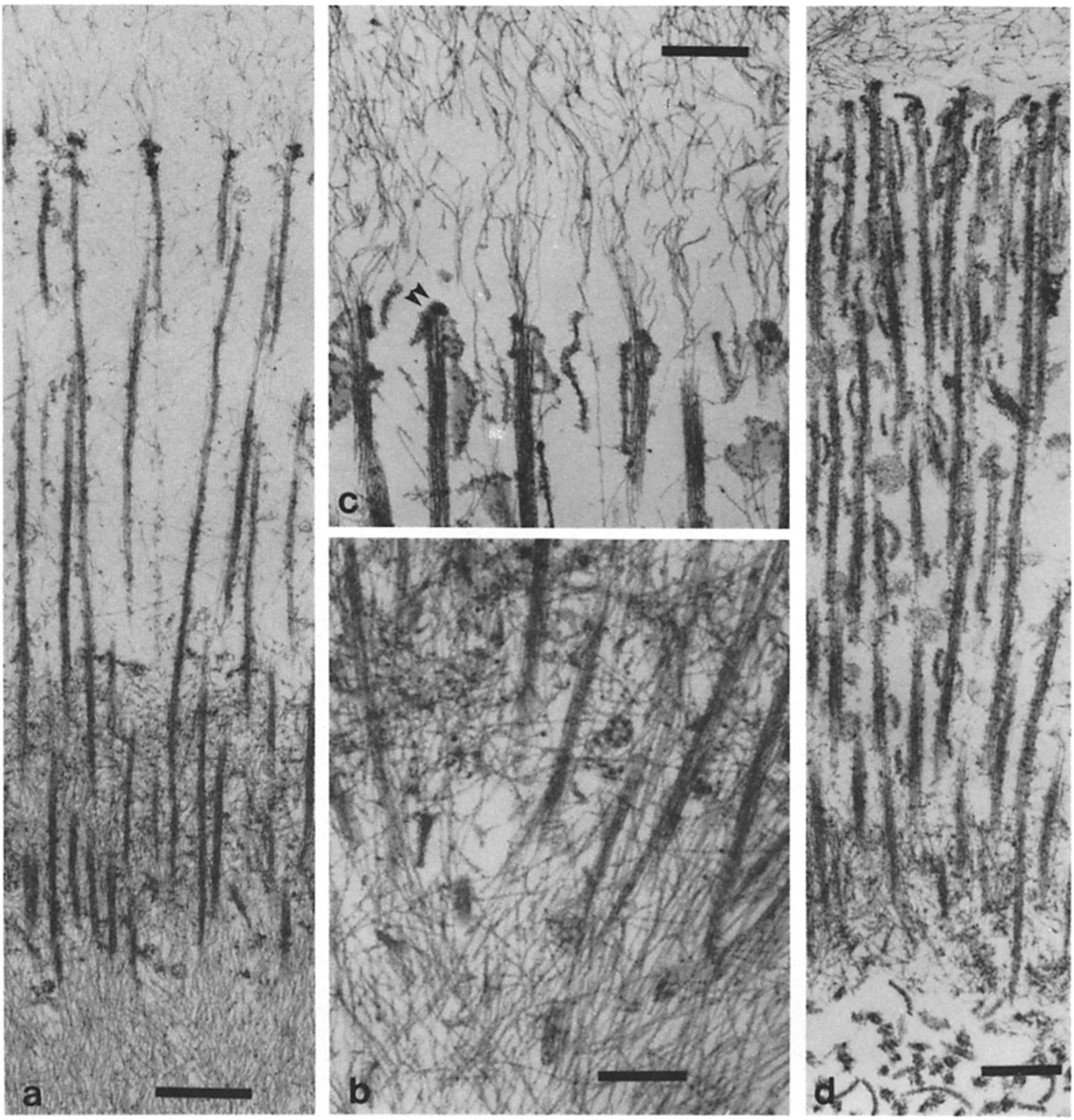


FIGURE 5 Nucleated polymerization of actin from the two ends of microvillar core filaments in membrane-free brush borders. (*a*) Demembranated brush border incubated for 5 min in the presence of 24 μM actin monomer. Both ends of the microvillar core filaments have nucleated assembly of actin. Bar, 0.4 μm. × 36,000. (*b*) Terminal web region of a demembranated brush border which has nucleated actin polymerization. Newly assembled actin filaments fan out from the basal ends of microvillus cores. Bar, 0.2 μm. × 71,000. (*c*) Nucleated polymerization of actin from the tip-end of microvillus cores. Most microvilli have nucleated filament growth from their tip-ends. Only those microvilli which retain a sleeve of membrane surrounding the tip of the core fail to nucleate actin polymerization (double arrows). The dense plaques, are, in most instances, partially "dislodged" from the former tip-end of the cores. Bar, 0.2 μm. × 67,000. (*d*) Demembranated brush border incubated for 5 min. with 24 μM preassembled F-actin. Neither the tip or rootlet ends of microvillus cores have exogenously added F-actin annealed to their filament ends. Bar: 0.4 μm, × 48,000.

distal ends of the core filaments. In most instances, there are remnants of the dense plaque associated with the core at the site of nucleation, but usually it appears to have been partially dissociated from the filament ends of the core either before nucleation had occurred, or has been "pushed aside" as a result of the new filament growth. Occasionally, we observed tips of microvilli from which no detectable assembly of filaments has occurred. Invariably, these microvillus cores have an intact dense cap and remnants of the microvillar membrane surrounding the tip end of the core (Fig. 5*c*; double arrows). The remaining membrane may act as a diffusion barrier which either prevents or restricts monomer access to the filament

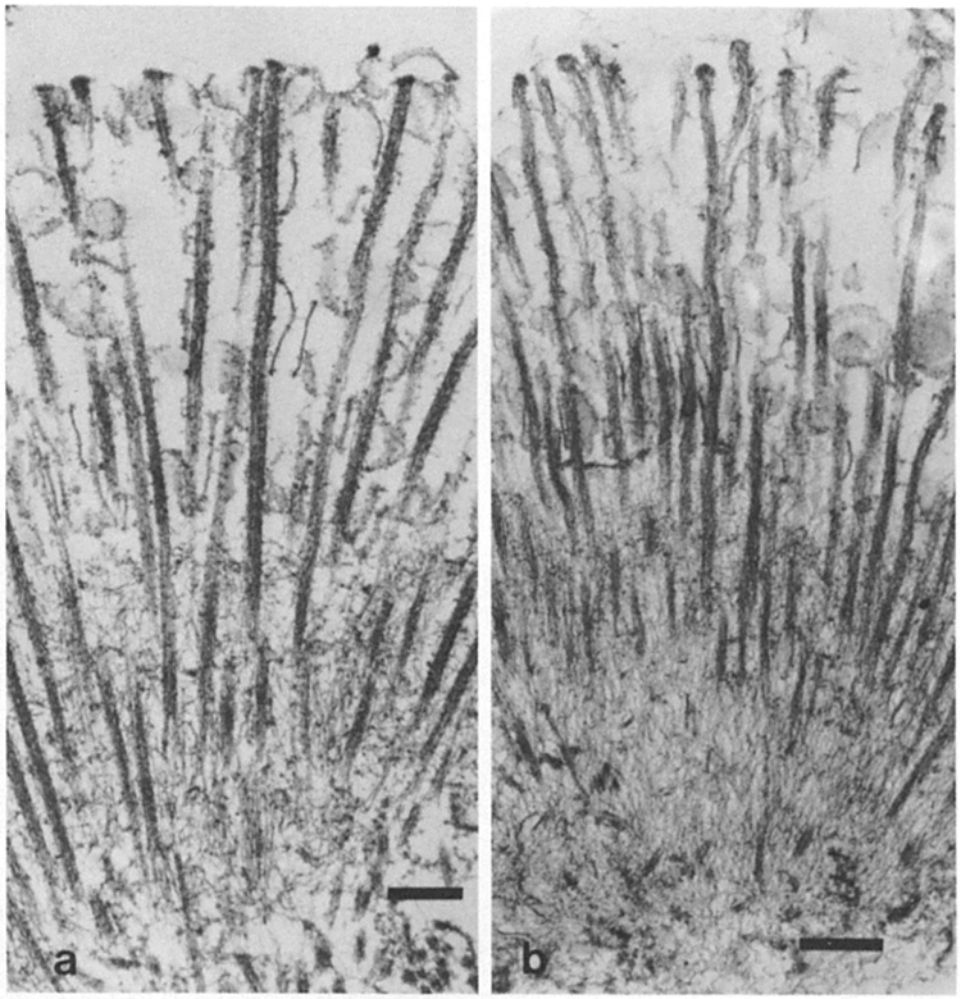


FIGURE 6 Effect of CB on the nucleated assembly of actin from the two ends of microvillar core filaments in demembranated brush borders. (*a*) Demembranated brush border treated with 2 μM CB with no actin monomer added. The morphology of the demembranated brush borders is unaffected by treatment with CB (for a control micrograph see Fig. 4, reference 24). Note that the microvillar core retain dense plaques associated with their tip-ends. Bar, 0.2 μm. × 46,000. (*b*) Membrane-free brush border which has nucleated polymerization of 24 μM actin monomer in the presence of 2 μM CB. The basal, pointed ends of core filaments have nucleated filament assembly, but no growth from the tip-ends of core filaments is observed. The microvillar cores retain dense plaques on their tip-ends. Bar, 0.3 μm. × 34,000.

ends, or alternatively, the lack of filament growth from these cores could represent a functionally intact plaque-membrane complex which in an undamaged state can prevent the assembly of actin. Another type of tip-nucleation observed in these preparations is from the ends of microvillus cores that had probably been broken before addition of actin monomer. Such cores are always present in preparations of demembranated brush borders and can be identified because the dense plaque is absent from the tip-end of the core, and the core is shorter than adjacent filament bundles. The broken microvillar bundles, as expected, nucleate the assembly of filaments, but in thin-sectioned material, it is difficult to determine if there are any qualitative or quantitative differences in the effectiveness of nucleated assembly from broken cores vs. cores with intact dense plaques.

The results of the nucleating studies using membrane-free brush borders indicate that the dense plaque associated with the microvillus tip does not prevent the nucleated assembly of actin from the barbed ends of core filaments under these in vitro conditions. However, the structural integrity of the dense cap is disrupted considerably in brush-border preparations which have been used as nucleating seeds when compared to that observed in control preparations (see Fig. 6*a*). The dislodgement of dense plaque material may in fact be causally related to the assembly reaction. Support for this notion comes from nucleation experiments conducted exactly as above but in the presence of 2.0 μM cytochalasin B. When nucleated assembly of actin from the tip-end of cores is prevented by addition of CB, most cores retain a structurally intact dense plaque at their tips (Fig. 6*b*). As expected, CB does not block the nucleated assembly of actin from the basal ends of the microvillus cores. These results demonstrate that the dense plaque does not interfere with the inhibitory effect of CB on the assembly of actin from the barbed ends of the core filaments, and conversely, CB does not appear to have an effect on the interaction of the dense plaque protein(s) with those filament ends (Fig. 6*a*).

We have conducted a series of control experiments in which F-actin, rather than G-actin, was added to demembranated (Fig. 5*d*) or membrane-intact brush borders (results not shown). This was done to insure that the observed nucleation of filament assembly onto the two ends of the microvillus core was the result of monomer addition rather than annealing of preassembled filaments. No annealing of F-actin onto either end of the microvillus core was observed (Fig. 5*d*).

Nucleated Assembly of Actin from Naturally Occurring, Filament Ends in Membrane-intact Brush Borders

Since it is possible that the detergent treatment used to remove the brush border membrane may also remove or destroy the function of capping proteins associated with the ends of microvillus core filaments, we also used membrane-intact brush borders to nucleate actin filament polymerization. Direct addition of actin monomer (2.4–24 μM) to isolated brush

borders in assembly buffer containing 75 mM KCl and 1 mM Mg^{++} results in nucleated polymerization from the basal (pointed) ends of core filaments in the terminal web (Fig. 7). This demonstrated the absence of an endogenous inhibitor of actin assembly associated with the basal, pointed ends of core filaments, at least after brush border isolation. No detectable nucleation of actin from the tip-end of the core was observed—nor was it expected since there was probably not sufficient time for actin monomer to diffuse underneath the microvillar membrane to the core tip. (This interpretation is based on observations from S_1-decoration experiments on which it was noted that at least 30–60-min incubation of brush borders with S_1 was required to obtain adequate decoration of core filaments within the sleeve of the microvillar membrane. The rootlet portion of the core becomes decorated with S_1 much more readily [Mooseker, unpublished observations] presumably because the brush borders are open at the terminal web side.)

To obviate the problem of monomer access to the tip-end of the microvillus core, we conducted a series of experiments in which brush borders with intact membranes were "pre-soaked" in actin monomer, at low ionic strength so that diffusion of actin monomer up to the microvillus tip could occur. After 30–60 min of incubation, the salt concentration was raised to induce actin assembly. As in the previous experiment (Fig.7), actin filaments grew from the basal ends of the microvillus cores (Fig. 8*a*). There is also strongly suggestive evidence that growth from the tip ends, at the site of membrane-filament attachment, occurs in these preparations. This conclusion is based on the unusual morphology of the tip-region of microvilli in these preparations (Fig. 8) as compared to that in control preparations (Fig. 9) or in preparations in which actin monomer was added in the presence of CB (Fig. 10). In both control and CB-treated preparations (CB should prevent monomer addition onto the tip-ends of core filaments), the tip regions of microvilli have normal morphology; that is, the filaments within the microvillus are closely packed along the full length of the microvillus to the point of termination in the dense plaque. In most instances one can also observe numerous radial links between the core and the surrounding membrane along the entire length of the microvillus (Figs. 9 and 10). In contrast, brush borders treated with actin monomer in these pre-soak experiments have at many of their microvillus tips a 0.2–0.4 μm presumptive "growth zone" (Fig. 8). This zone is characterized by a sharp discontinuity in the microvillus core above which the filaments are loosely packed and lacking radial links to the membrane. However, the distal ends of the filaments terminate in a dense plaque at the membrane which is of normal morphology. It is also important to note that microvilli in control preparations (Fig. 9) are quite uniform in length. In brush borders which have nucleated actin polymerization, the microvilli have irregular lengths and the longest microvilli also have the longest growth zones (Fig. 8), suggesting that elongation of microvilli has occurred. Since this type of "tip morphology" is never seen in control or CB-treated preparations, where monomer addition onto the tip-ends of core filaments should not occur, the most likely explanation is that monomer has added onto the barbed ends of the core filaments at the junction between those filament ends and the dense plaque on the membrane. Most importantly, this polymerization, and resultant microvillus elongation occurs without disrupting the structural integrity of this apparent membrane-filament attachment site.

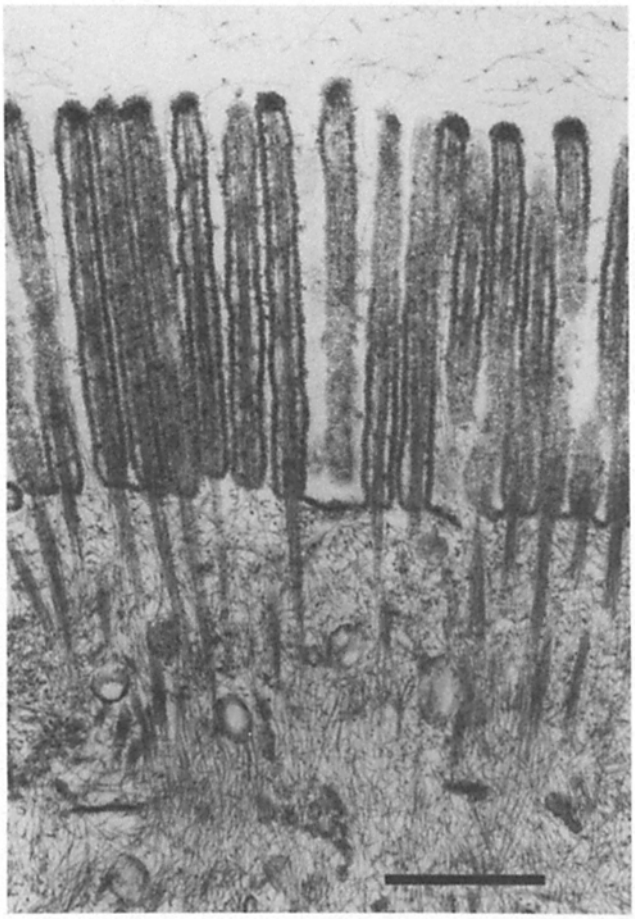
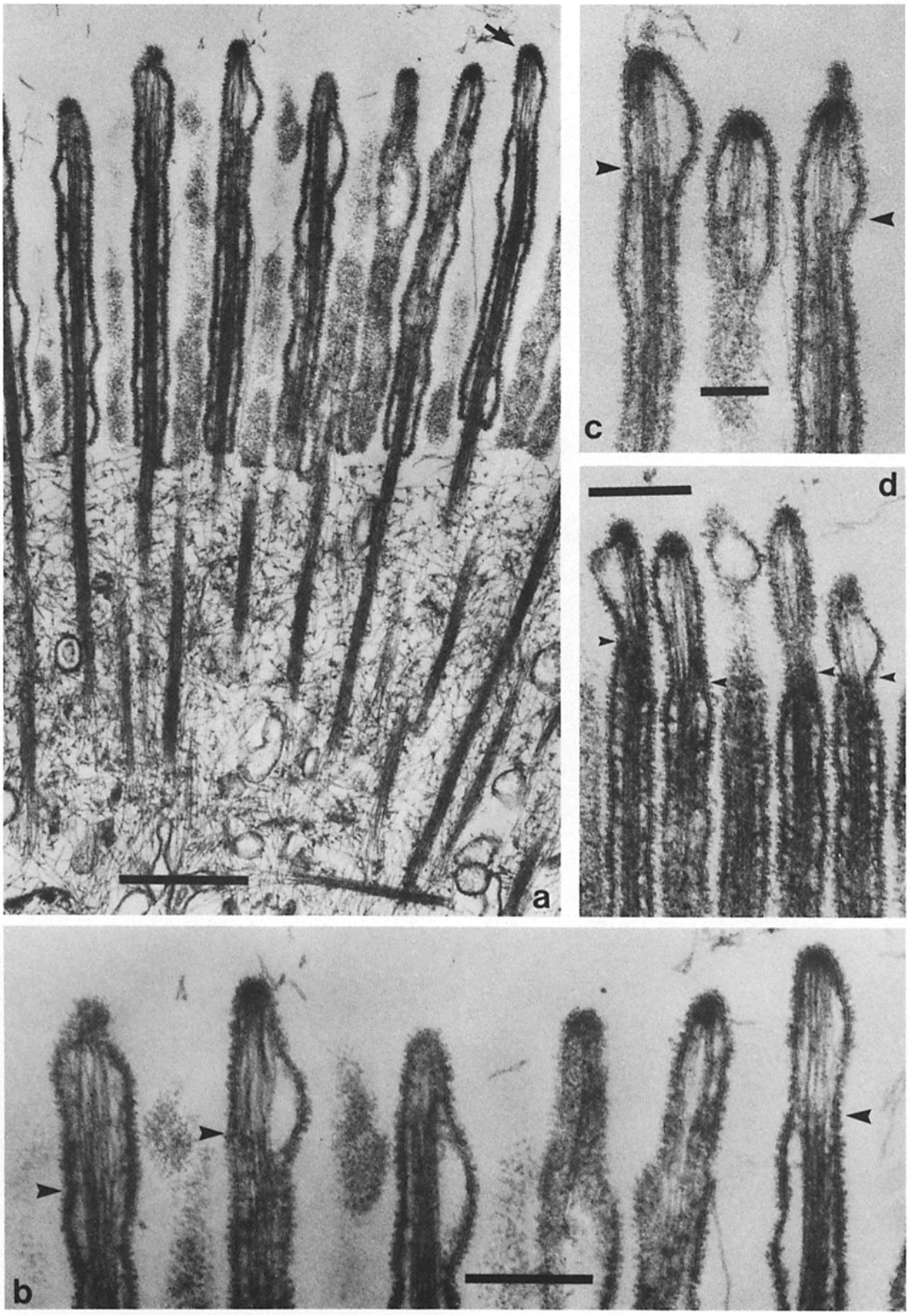


FIGURE 7 Nucleated polymerization of actin from the rootlet-ends of microvillar core filaments in an isolated brush border. G-actin (24 μM) was added to brush borders suspended in polymerization buffer (75 mM KCl, 1 mM $MgSO_4$). Numerous actin filaments fan out from the basal ends of microvillar bundles as a result of nucleated polymerization of actin. No detectable assembly of actin onto the tip-ends of core filaments is observed. Bar, 0.5 μm. × 48,000.

DISCUSSION

Microvillar Core Filaments Exhibit Biased Growth in Vitro

In the first paper of this series (26) we demonstrated the utility of microvillar core fragments as nucleating "seeds" for quantitative analysis of actin assembly. Over the range of actin monomer concentrations (1.2–7.2 μM) we measured rates of assembly from the fast and slow growing ends of core fragments that increased linearly as a function of actin monomer concentration. This enabled us to calculate, by the method of Bergen and Borisy (3), the polymerization rate constants for the two ends of an actin filament under two conditions—one barely permissive for polymerization (20 mM KCl) and the other (75 mM KCl, 5 mM Mg^{++}) optimal for assembly. Here we have completed our previous analysis (26) by identifying the fast and slow growing ends of the microvillus core filaments as the barbed and pointed ends, respectively. We also have demonstrated the usefulness of core fragments for qualitative analysis of the effects of actin-binding proteins (e.g., *Acanthamoeba* capping proteins) and drugs (e.g., cytochalasin B) on actin assembly. Most importantly, given the context of this current study which focuses on the assembly properties of the micro-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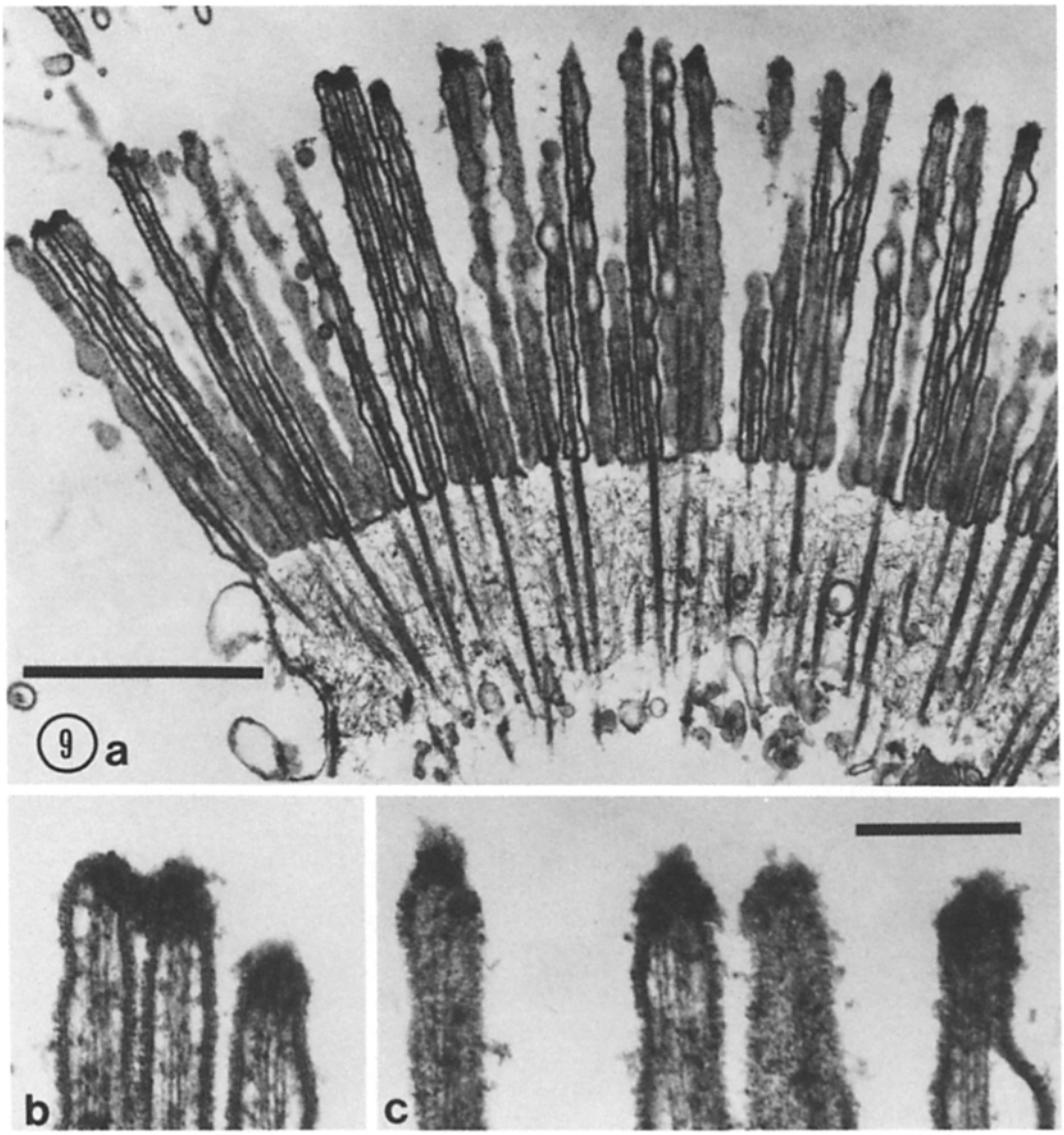

FIGURE 9 (*a*) Isolated brush border treated as in Fig. 8, except that actin monomer was omitted from the low ionic strength "pre-soak." Microvilli have uniform lengths and have normal tip morphology. The close packing of the core filaments is continuous along the entire length of the core to the point of insertion in the dense plaque at the tip of the microvillar membrane. Bar, 0.4 μm. × 43,000. (*b* and *c*) Higher magnification of some of the microvillar tips on the brush border in *a*. The normal morphology of the filament-membrane attachment site at the tips of microvilli is structurally unaffected by the low-ionic strength presoak. Bar, 0.2 μm. × 108,000.

villar core filaments, we demonstrate that "native" microvillar filaments exhibit a bias for monomer addition onto their barbed ends. This is an important point, because although the core fragments used do not contain the original filament ends of the core, and thus are free of any capping proteins which might be present, these filaments do contain various binding proteins along their length (e.g., microvillar 95 and 110 kdalton, fimbrin and calmodulin; reviewed in reference 22) which could affect monomer addition. In fact, these accessory proteins along the length of the core filaments may inhibit monomer addition, particularly at the pointed end. At the relatively low actin monomer concentrations used in these nucleation studies (low concentrations are required to detect biased growth rates by EM), we invariably observed fewer filaments nucleated from the pointed end of these core fragments (Fig. 1; see also Fig. 2 in reference 26). The incomplete participation of microvillar filament ends in nucleating polymerization could be due to the presence of core accessory proteins, but it is also plausible that

FIGURE 8 Elongation of microvilli in isolated brush borders induced by polymerization of actin from the membrane-associated ends of core filaments. (*a*) Isolated brush border "pre-soaked," at low ionic strength, with 24 μM G-actin before addition of salt to induce actin assembly. As in Fig. 7, the rootlet ends of core filaments have nucleated filament assembly. Several microvilli have unusual tip morphology, possibly resulting from nucleated polymerization of actin onto the membrane-associated ends of core filaments (see text). The dense plaques at the tips of microvilli remain associated with the presumed growing ends of the newly formed filaments. Note that, unlike brush borders in control preparations (Fig. 9) microvilli have nonuniform lengths, and the longest microvilli have the longest "growth zones" at their tips. The arrow indicates a microvillus in which elongation may have caused the membrane to slide up the core. Bar, 0.4 μm. × 56,000. (*b*) Higher magnification of the tip region of the brush border in *a*. The junction between the presumed ends of the core filaments and the newly assembled filaments nucleated from those ends is indicated by arrows. Bar, 0.2 μm. × 108,000. (*c* and *d*) Other examples of "growth zones" at the tips of microvilli in brush borders treated as in *a*. Note that the microvillar membrane appears to be pulled "taut" over the newly assembled filaments. (*c*) Bar, 0.1 μm. × 113,000. (*d*) Bar, 0.2 μm × 88,000.

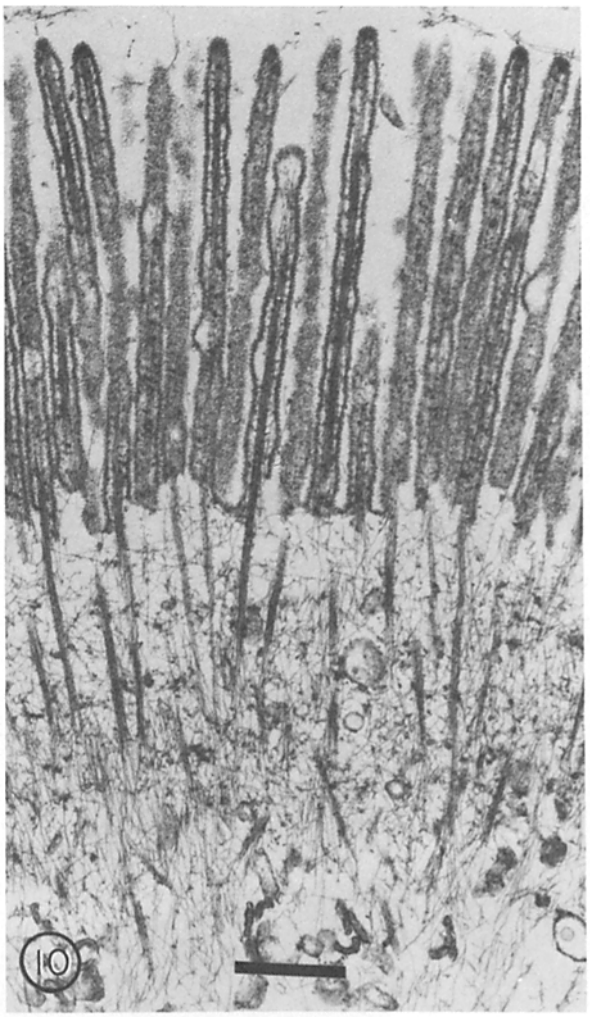

FIGURE 10 Isolated brush border treated as in Fig. 8 with the addition of 2 μM CB. The rootlet ends of core filaments have nucleated filament assembly, but "growth zones" at the tips of microvillar are not present, presumably due to the inhibition of filament growth on the membrane-associated ends of core filaments by CB. Bar, 0.4 μm. × 35,000.

the high shear forces used to isolate core fragments damaged some of the filament ends. This explanation is supported by observations on nucleated assembly of actin from intact microvillus cores in membrane-free brush borders (Fig. 5). As well as can be determined from thin-sectioned material, most, if not all core filaments nucleate polymerization from both ends even at low (2.4 μM) actin monomer concentrations (results not shown for 2.4 μM).

The "Rootlet" Ends of Microvillar Core Filaments Are Not Capped In Vitro

We have shown that the rootlet (pointed) ends of core filaments nucleate polymerization in both membrane-intact and demembranated brush borders (Figs. 5, 7, and 8). However, we do not feel that this constitutes convincing evidence for the absence of capping proteins on the pointed ends of core filaments in vivo. Unlike the membrane-associated end of the core, whose structural, if not chemical integrity can be judged morphologically (see discussion below), we have no similar assay for intactness of the rootlet ends of core filaments in isolated brush borders. In addition, this end of the core is constantly exposed to the imbibing media used for isolation. It is plausible that dissociation of capping protein(s) present on the rootlet ends of core filaments could occur.

The Membrane-associated Ends of Microvillar Core Filaments Are Not Capped In Vitro

The main focus of this study was to determine if actin monomer can add onto the membrane-associated ends of microvillar core filaments. Our task would have been much simpler if the barbed ends of microvillar core filaments in membrane-free brush borders had failed to nucleate actin polymerization. The postulated "capping" function for the dense plaque at the tip of the microvillus core would have been demonstrated. Instead, the dense plaque does not inhibit actin monomer addition under these in vitro conditions (Fig. 5 *a* and *c*). Does this mean that core filaments are not capped at their membrane-associated ends, or have we simply removed or destroyed the endogenous capping proteins once present in vivo by the procedures used for isolation and demembranation of the brush borders?

Obviously the question raised above remains an open one. Nevertheless, the results of nucleation studies using membrane-intact brush borders provide at least suggestive evidence that core filaments can elongate (or shorten) by monomer exchange at their membrane-associated ends. We have shown that elongation of the tip-end of microvilli can be induced by nucleated polymerization of actin from the barbed ends of core filaments without disrupting the structural organization of filament-membrane attachment site at the microvillus tip (Fig. 8). This attachment site consists of 20–30 filament ends embedded in a dense plaque which in turn is attached to the plasma membrane. Given this structural complexity, which is unaltered in microvilli with elongated tips, it seems unlikely that monomer addition onto the barbed ends of core filaments occurred because the "native" configuration of this attachment site was disrupted during brush-border isolation.

We have interpreted the results of nucleation studies using membrane-intact brush borders (Fig. 8) as a demonstration that actin monomer can add onto the membrane-associated ends of core filaments. If this interpretation is correct, then we are left with an interesting puzzle regarding how the microvillar membrane accommodates the artificial elongation of the microvillus. Perhaps the microvillus membrane stretches, or there is sufficient "slack" in the membrane to allow some extension. In support of this notion is the observation that many microvilli with "growth zones" (e.g., Fig. 8 *c* and *d*) have thinner diameters at their tips, suggesting that the membrane has been pulled "taut" by the elongation of the filaments underneath it. The fact that the length of the growth zones is quite uniform and rarely exceeds 0.4 μm also supports the idea that the extent of elongation may be restricted by the degree of slack and/or stretch in the membrane. Another possibility is that the membrane can slide up the core during elongation because it is (or becomes) detached from the rest of the brush-border membrane at the base of the microvillus. If so, one would expect to observe a region of "bare" filament bundle exposed at the base of the microvillus roughly equal to the length of the growth zone at the tip. In fact, microvilli with this morphology are frequently observed in these preparations (e.g., Fig. 8 *a*).

We suggested from the observations on the polymerization-induced elongation of microvilli that association or attachment of a core filament at its barbed end to the plasma membrane does not prevent growth at that end—the end identified as the

preferred assembly-end using core fragments as nuclei for polymerization (Figs. 1 and 2). On the other hand, we cannot rule out the possibility that capping of filament ends and attachment of those ends to the microvillar membrane are carried out by two different sets of proteins. The endogenous capping proteins could have been lost during brush-border isolation without detaching the filaments from the microvillar membrane. This raises the important problem of how one defines the attachment or association of core filaments with the plasma membrane. We say that core filaments are attached to the membrane at the tip of the microvillus based on ultrastructural evidence. Although electron microscopy is perhaps the best assay for identifying specific interactions of filaments with membranes, there is no evidence, as yet, which demonstrates a physical connection between core filaments and the membrane. We can only say that the barbed ends of core filaments are embedded in a densely staining region of cytoplasm which in turn is very close to the plasma membrane. Given this limited definition, our results indicate that "attachment" of an actin filament to the membrane by its barbed end may not prevent growth at that end, and conversely, that monomer addition may not interfere with attachment. Additional evidence for growth of actin filaments at their membrane-associated ends has been provided by the recent studies of Tilney et al. (28) on the assembly of acrosomal filaments during spermiogenesis in *Limulus*.

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