Villin-induced growth of microvilli is reversibly inhibited by cytochalasin D

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SUMMARY

Villin is an actin-binding protein that is associated with the cytoskeleton of brush border microvilli. In vitro, villin nucleates, caps or severs actin filaments in a Ca\(^{2+}\)-dependent manner. In the absence of Ca\(^{2+}\), villin organizes microfilaments into bundles. Transfection of a villin-specific cDNA into cultured cells that do not produce this protein results in the growth of long surface microvilli and the reorganization of the underlying actin cytoskeleton. Here we studied the effects of low concentrations of cytochalasin D on the induction of these plasma membrane-actin cytoskeleton specializations. Transfected cells were treated with concentrations of cytochalasin D that prevent the association of actin monomers with the fast-growing end of microfilaments in vitro. In villin-positive cells, cytochalasin D inhibited the growth of microvilli and promoted the formation of rodlet-like actin structures, which were randomly distributed throughout the cytoplasm. The formation of these structures was dependent on large amounts of villin and on the integrity of an actin-binding site located at the carboxy terminus of villin, which is required for microfilament bundling in vitro and for the growth of microvilli in vivo. The effect of cytochalasin D was reversible. The observation of living cells by video-imaging revealed that when cytochalasin D was removed, rapid disassembly of actin rodlets occurred after a lag phase. The present data stress the important role of the plasma membrane in the organization of the actin cytoskeleton and suggest that the extension of the microvillar plasma membrane is dependent on the elongation of microfilaments at their fast-growing end. Inhibition of microfilament elongation near the plasma membrane by cytochalasin D may result in the ‘random’ nucleation of actin filaments throughout the cytoplasm.

On the basis of the present data, we propose that villin is involved in the assembly of the microvillar actin bundle by a mechanism that does not prevent monomer association with the prefered end of microfilaments. For instance, villin may stabilize actin filaments by lateral interactions. The functional importance of the carboxy-terminal F-actin binding site in such a mechanism is stressed by the fact that it is required for the formation of F-actin rodlets in cytochalasin D-treated cells. Finally, our data further emphasize the observations that the effects of cytochalasin D in living cells can be modulated by actin-binding proteins.

Key words: villin, actin-binding, brush border, membrane protrusion, transfection

INTRODUCTION

Brush border microvilli are plasma membrane extensions present at the apical surface of some absorptive epithelial cells (for review see Arpin and Friederich, 1992; Louvard et al., 1992). These finger-like membrane interdigitations, which are supported by an axial array of microfilaments, are a suitable model with which to investigate how plasma membrane-actin cytoskeleton interactions contribute to the modulation of cell shape. We have previously provided evidence that villin, one of the major actin-binding proteins associated with the axial microfilament bundle of intestinal microvilli (Bretscher and Weber, 1979; Matsudaïra and Burgess, 1979), actively participates in the assembly of the microvillar structure (Friederich et al., 1989). Transfection of a cDNA coding for human villin into fibroblast-like cells (the CV-1 cell line), which normally do not produce this protein, causes the growth of surface microvilli and the concomitant reorganization of the underlying microfilament network (Friederich et al., 1989, 1992). In vitro, villin modulates the state of actin polymerization and organization by its Ca\(^{2+}\)-dependent microfilament bundling, capping, nucleating or severing activities (Craig and Powell, 1980; Bretscher and Weber, 1980; Mooseker et al., 1980; Glenney et al., 1981a; for review see Mooseker, 1985; Friederich et al., 1990). In the present study we have investigated the assembly of the microvillar actin cytoskeleton near the plasma membrane and how villin is involved in this process. Towards this goal CV-1 transfected with villin cDNA were exposed to the actin-binding drug cytochalasin D (CD) during or after microvilli...
formation. In order to avoid the numerous effects of this drug on actin polymerization by its interaction with actin monomers (Goddette and Frieden, 1986a,b; for review see Cooper, 1987). We used low CD concentrations (2×10⁻⁹ to 2×10⁻⁷ M). Under these conditions CD inhibits the association of actin monomers to the fast-growing end of microfilaments by 90% and thereby mimicks the activity of capping proteins (Brown and Spudich, 1979; Brenner and Korn, 1979; Flanagan and Lin, 1980; Bonder and Mooseker, 1986; Sampath and Pollard, 1991). Previous studies carried out by others used this behaviour of CD to investigate the turnover of microfilaments in living cells. The results obtained during those studies suggested that actin monomer addition at the uncapped barbed end of actin oligomers is necessary for the extension of lamellar membrane protrusions in motile cells (Yahara et al., 1982; Forscher and Smith, 1988).

Here we show that in cells producing large amounts of villin CD impairs the formation of the spike-like F-actin bundles that constitute the cytoskeleton of surface microvilli and provokes the appearance of thick cytoplasmic actin bundles that do not interact with the plasma membrane. The formation of these F-actin structures requires the carboxy-terminal actin-binding site of villin (Friederich et al., 1992), which is also necessary for microfilament bundling in vitro (Glenney and Weber, 1981). Our results are in favor of the existence of uncapped actin oligomers in close contact with the dorsal plasma membrane, which may serve as nuclei for the assembly of microfilaments supporting the long, rigid microvilli seen in cells producing large amounts of villin.

MATERIALS AND METHODS

Plasmid DNA

Standard procedures as described by Sambrook and collaborators (1989) were used for large-scale preparation of the constructs pSV51-villin and pSV51-villin Δ7 (Friederich et al., 1989, 1992) as previously described.

Cell culture

Unless otherwise indicated, CV-1 cells were grown in Dulbecco’s minimum essential medium (DMEM) supplemented with 10% fetal calf serum (complete medium), at 37°C, under a 10% CO₂ atmosphere.

Transient cDNA expression in CV-1 cells

CV-1 cells were transfected using the DEAE-dextran-mediated DNA transfer method as previously described (Friederich et al., 1989) with the following modifications. In order to limit cell damage, the DNA-DEAE-dextran incubation step was reduced to 30 min. After removal of the DNA-DEAE-dextran-containing medium, cells were treated for 2-3 h with culture medium (see above) containing 50 µM chloroquine, which increased transfection efficiency. Cells were analyzed after addition of DNA at the time points indicated in the figure legends.

Cytochalasin D treatment

The cytochalasin D (Sigma Chem. Co., St. Louis, MO) stock solution (2 mM in ethanol) was kept at −20°C and diluted in complete culture medium just before use. After addition of the DNA the cells were incubated in the absence of CD for various time periods (see figure legends). The prewarmed CD-containing medium was then added and the cells were further incubated for the time periods indicated in the figure legends. In most cases cells were briefly washed (less than 30 s) with phosphate-buffered saline (PBS) containing 10 µM CaCl₂ and 10 µM MgCl₂, and processed for immunofluorescence labeling as described above. In recovery experiments, cells were washed three times with prewarmed complete culture medium after CD treatment and incubated in the absence of CD for 1-4 h.

Villin antibodies

The production and properties of the monoclonal (ID2C3) and the polyclonal antibodies against villin, as well as affinity purification of the polyclonal antibodies have been described (Dudouet et al., 1987; Coudrier et al., 1981).

Fluorescence labeling of the cells

In most of the experiments, transfected cells were fixed with 3% of paraformaldehyde, detergent permeabilized with Triton X-100 (0.4%) and labeled as described (Reggio et al., 1983). For immunofluorescence staining of villin, cells were incubated with purified monoclonal anti-villin antibodies (10 µg/ml) and then with mouse IgG-specific antibodies conjugated to fluorescein (Amersham). In order to visualize F-actin, rhodamine-conjugated phalloidin (0.3 µg/ml; Sigma Chem. Co.) was added to the anti-villin antibodies. In order to visualize the cell surface, living cells on coverslips were washed with PBS at 20°C and then labeled for 5 min at 0°C with wheat germ agglutinin conjugated to rhodamine (10 µg/ml, Sigma Chem. Co.). After three washes with cold PBS, 3% of paraformaldehyde was added and cells were allowed to warm up to 20°C. After fixation, cells were processed for immunofluorescence labeling as described above.

Fig. 1. CD inhibits the villin-induced reorganization of the actin cytoskeleton in CV-1 in a dose-dependent manner. Cells were transfected with a DNA construct encoding human villin. CD was added after 24 h. Cells were incubated for further 24 h in the presence of CD and then processed for double-fluorescence labeling. Untransfected cells were treated in parallel. Parafformaldehyde-fixed and detergent-permeabilized cells were double-labeled for villin with a villin-specific monoclonal antibody, followed by incubation with a fluorescein-coupled secondary antibody, and for F-actin using rhodamine-conjugated phalloidin. Untransfected cells were stained for F-actin with rhodamine-conjugated phalloidin. Left and middle panels are micrographs of the double-stained transfected cells. Left panels: villin immunofluorescence stainings. Middle panels: micrographs of the corresponding phalloidin labelings. Right panels: phalloidin labelings of the untransfected cells. (A, B, C) controls (no CD); (A, B) note the presence of many long actin-containing spikes present on the dorsal face of the villin-positive cell; (C) lamellar F-actin containing membrane protrusions (arrowheads) and stress fibers are present at the ventral face of untransfected cells. (D, E, F) 5 nM CD; (D, E) the number of villin-induced F-actin structures is strongly reduced. Moreover, this cell is devoid of stress fibers; (F) untransfected cells present an almost unmodified actin cytoskeleton (lamellipodia are indicated by arrowheads). (G, H, I) 50 nM CD; (G, H) note the presence of a few thick actin rodlets and the lack of the fine, regular-shaped actin spikes; (I) these prominent F-actin structures are not detectable in villin-negative cells; note the absence of lamellipodia at the cell periphery (arrowheads). (J, K, L) 50 nM CD followed by wash-out of CD for 3 h; (J, K) spike-like F-actin structures are present on the villin-positive cells; (L) untransfected cells have lamellipodia (arrowhead) and stress fibres. Bars, 10 µm.
Cytochalasin D inhibits microvilli growth

Fig. 1
Proteins

Rabbit skeletal muscle actin was prepared according to the method of Spudich and Watt (1971). Actin was labeled with rhodamine following to the procedure described by Kreis and colleagues (1982). Frozen samples were stored at −70°C.

Sequential microinjection of pSV51-villin and rhodamine-actin into CV-1 cells

CV-1 cells were plated on 22 mm glass coverslips. Microinjection was performed 24 h after plating using an automated microinjection system (Pepperkok et al., 1988) and microinjection capillaries (GC-100 F/10). DNA (0.2 mg/ml) mixed with 1% FITC-coupled dextran (150000; Sigma Chem. Co.) was injected in sterilized water. Before microinjection rhodamine-actin was diluted in G-buffer (2 mM Tris-HCl, 0.2 mM CaCl₂ and 0.2 mM ATP, pH 7.6) lacking β-mercaptoethanol to a final concentration of 0.5 mg/ml and cleared by centrifugation at 75,000 g for 30 min. The supernatant was kept on ice and immediately used for microinjection. Cells were microinjected in HEPES-buffered DMEM medium lacking NaHCO₃. First, the pSV51-villin DNA was microinjected into the nucleus of the cells. After microinjection of DNA, cells were incubated for 6 h at 37°C. Cells were then exposed to 50 nM CD for a further 12 h, by which time villin-induced reorganization of the actin cytoskeleton had occurred. The microscope used for microinjection was equipped with fluorescence illumination to detect the fluorescein signals of the cells microinjected with the DNA-FITC-dextran solution. These cells were then microinjected with rhodamine-actin and incubated for 30 min at 37°C after the second injection. Microinjection of one coverslip (~30 cells) was achieved within 15 min.

RESULTS

We assessed the effect of low concentrations of cytochalasin D on the villin-induced morphogenesis of microvilli. CV-1 cells were transiently transfected with a DNA construct encoding human villin and CD was added at various times after transfection. The effects of low doses of CD on the formation of rodlet-like F-actin structures in the presence of CD depends on large amounts of villin. Cells were transfected with a DNA construct encoding human villin. CD was added 8 h later and was present until 24 h after transfection. Cells were processed for fluorescence double-labeling of villin and F-actin as described in Fig. 1. Left panels are micrographs of the villin immunofluorescence stainings. Right panels are micrographs of the corresponding phallolidin labelings. (A,B) no CD, note the absence of F-actin spikes. Villin staining accumulates at the cell periphery and colocalizes with cortical microfilaments. (C, D) 100 nM CD. The villin-label colocalizes with the CD-resistant cortical actin filaments. No rodlet-like actin structures are detectable. Bar, 10 µm.

Fig. 2. The formation of rodlet-like F-actin structures in the presence of CD depends on large amounts of villin. Cells were transfected with a DNA construct encoding human villin. CD was added 8 h later and was present until 24 h after transfection. Cells were processed for fluorescence double-labeling of villin and F-actin as described in Fig. 1. Left panels are micrographs of the villin immunofluorescence stainings. Right panels are micrographs of the corresponding phallolidin labelings. (A,B) no CD, note the absence of F-actin spikes. Villin staining accumulates at the cell periphery and colocalizes with cortical microfilaments. (C, D) 100 nM CD. The villin-label colocalizes with the CD-resistant cortical actin filaments. No rodlet-like actin structures are detectable. Bar, 10 µm.
the reorganization of the microfilament network into spike-like F-actin structures and on the formation of microvilli on the dorsal cell face were analyzed by double-labeling fluorescence microscopy.

**Exposure of CV-1 cells to CD impairs the reorganization of the actin cytoskeleton induced by large amounts of villin**

Analysis of transfected cultures of cells showed that the reorganization of the actin cytoskeleton in villin-producing CV-1 cells occurred within 24 to 48 h after addition of DNA (Figs 1A,B, 2A,B). Thus, to investigate the effect of CD on this process, cells were exposed to the drug during this period. The effect of a range of various concentrations of CD (2-100 nM) were determined. After 48 h cells were double-stained for F-actin using rhodamine-coupled phalloidin and for villin by indirect immunostaining. As evaluated from the intensity of the immunofluorescence signal, cells examined under these conditions contained large amounts of villin. In the absence of CD, cells exhibited a reorganized actin cytoskeleton. Fluorescence-phalloidin labeling of microfilaments of a villin-positive cell showed the redistribution of F-actin into long spike-like structures located at the dorsal face of the cell (Fig. 1A,B). Villin-label was localized in these structures. Villin-negative cells had normal, well-developed stress fibers and numerous membrane protrusions under these conditions (Fig. 1C). CD concentrations equal to or higher than 5 nM affected F-actin spike formation (Fig. 1). Villin-positive cells treated with 5 nM CD exhibited a reduced number of spike-like actin structures and lacked stress fibres (Fig. 1D,E). In contrast, villin-negative cells had almost unaffected stress fibres at their ventral faces (Fig. 1F). Exposure to CD concentrations higher than 20 nM completely impaired the appearance of fine needle-like F-actin structures in villin-positive cells. Cells incubated with 50 nM CD are shown in Fig. 1G,H,I. Surprisingly, villin-containing cells exhibited actin rodlets to which the villin-label was associated (Fig. 1G,H). These F-actin structures were never observed in CD-treated villin-negative cells (Fig. 1I). However, under these conditions not only villin-producing cells but also those lacking villin had a reduced number of the stress fibres (Fig. 1I). Moreover, CD impaired the formation of lamellar F-actin-containing membrane extensions (Fig. 1I, arrowheads).

We also investigated the reversibility of the CD effect. After exposure to 50 nM CD the drug was washed out for 3 h (Fig. 1J,K). Villin-producing cells exhibited spike-like F-actin structures, very similar to those observed in the untreated villin-positive cell (Fig. 1A,B). However, while in villin-negative cells (Fig. 1L) F-actin-containing membrane protrusions (Fig. 1L, arrowheads) had reappeared already 30 min after CD removal, the disassembly of the thick actin bundles and the subsequent formation of fine F-actin spikes required longer incubation periods (2 h).

Cells were also exposed to CD between 6 and 24 h after transfection of villin cDNA, a time period when levels of synthesized villin are low, as evaluated from the weak immunofluorescence signal (Fig. 2). Under these conditions, villin did not reorganize the actin cytoskeleton in untreated cells (Fig. 2A,B; and Friederich et al., 1989). In parallel, villin-positive cells exposed to CD (100 nM) did not exhibit thick cytoplasmic F-actin rodlets (Fig. 2C,D). This finding indicates that the formation of these F-actin structures was dependent on large amounts of villin. Interestingly, villin codistributed with CD-resistant microfilaments that were frequently found at the periphery of non-confluent cells (Fig. 2C,D).

**Parallel to the inhibition of the formation of F-actin spikes, low CD concentrations inhibit the growth of long microvilli**

The organization of the dorsal cell surface of transfected cells was investigated by fluorescence double-labeling for villin and for the outer plasma membrane using fluorescent wheat germ agglutinin as a cell surface probe (Fig. 3). Cells that were not treated with CD displayed numerous, elongated membrane projections on their dorsal face (Fig. 3A,B), previously identified by transmission and scanning electron microscopy as long microvilli (Friederich et al., 1989). Exposure of cells to increasing concentrations of CD resulted in a dose-dependent reduction of the density of these long microvilli (Fig. 3C,D,E,F). Villin-positive cells treated with CD concentrations equal to or higher than 20 nM contained F-actin rodlets, as indirectly visualized here by the associated villin label (Fig. 3E,F). The fact that these cells were free of long surface projections strongly suggests that the F-actin rodlets are not extending the dorsal plasma membrane. However, it is important to stress that even in the presence of 50 to 100 nM CD rudimentary microvilli were still found on most cells (Fig. 3G,H). The morphology of these microvilli was not affected by the presence or absence of villin. Thus, only the formation of villin-induced long microvilli appeared to be inhibited by CD.

The effect of CD on the formation of long microvilli was also reversible. Villin-positive cells that, after long-term exposure to 50 nM CD, were incubated for 3 h in the absence of the drug displayed long membrane protrusions on their dorsal face (Fig. 3I,J). These microvilli could be clearly distinguished by their length and shape from those found on villin-negative cells.

**Removal of CD results, after a lag phase, in the rapid disassembly of the cytoplasmic F-actin rodlets**

In order to gain an insight into the contribution of CD to the stabilization of the F-actin rodlets we investigated their disassembly dynamics after removal of CD. Living villin-positive CV1 cells that had been treated with 50 nM CD were microinjected with rhodamine-actin as a probe for the dynamic distribution of actin, and then observed by time-lapse video-intensified fluorescence microscopy (Fig. 4). Most of the microinjected rhodamine-actin was incorporated into the thick F-actin rodlets, as visualized in Fig. 4A, immediately before wash-out of CD (Fig. 4). In parallel, microinjected cells that had been fixed and fluorescently immunostained for villin displayed a clear colocalization of villin and rhodamine-actin label (data not shown). The micrograph in Fig. 4B represents an enlarged area of the same cell, recorded 50 min after CD removal.
Fig. 3
Cytochalasin D inhibits microvilli growth and shows that F-actin rodlets were still present in the cytoplasm. After this lag phase, the actin rodlet rapidly disassembled; the most prominent rodlet shortened within 9 min from 13 to 4 µm (Fig. 4C). It is interesting to note that the actin rodlet appeared to disassemble exclusively at one extremity (Fig. 4B,C; see arrowheads). Assuming that the rodlet is formed exclusively by long protofilaments of 13 µm, the disassembly rate of such a filament, which theoretically has 4,745 subunits (Egelman et al., 1982), would be 6 subunits s⁻¹.

Exposure to CD causes rapid shortening of spike-like F-actin structures and slow subsequent formation of cytoplasmic F-actin bundles

The data reported above indicate that villin-induced growth of microvilli is extremely sensitive to CD. We have also explored the sensitivity of ready-formed F-actin spikes to CD. It is important to stress that assembled F-actin spikes were only affected when cells were exposed to ≥20 nM CD. An untreated villin-positive cell and a cell exposed for 15 min to 100 nM CD are shown in Fig. 5. While the untreated cell exhibited long needle-like structures (Fig. 5A,B), the cell incubated in the presence of CD contained numerous short F-actin structures (Fig. 5C,D). F-actin rodlets were exclusively observed in cells exposed for 2 h or longer to CD (data not shown).

The carboxy-terminal actin-binding site of villin is required for the formation of cytoplasmic actin bundles

We put forward the hypothesis that villin may participate in the formation of the prominent F-actin rodlets by its bundling activity. To test this hypothesis we used a villin variant lacking seven amino acid residues from the carboxy terminus of the headpiece domain. In vitro as well as in vivo studies allowed us to demonstrate that this truncation inactivates an actin-binding site (Friederich et al., 1992), which has been shown by others to be required for microfilament bundling (Glenney et al., 1981b). Untreated cells that produced this truncate (Fig. 6A,B) contained an...
unmodified stress fiber system at their ventral face and lacked spike-like F-actin structures. The villin variant accumulated at the cell periphery, near the plasma membrane (Fig. 6A). When exposed to 100 nM CD, cells producing this villin truncate exhibited a highly reduced number of stress fibres as did the surrounding untransfected cells (Fig. 6C,D) but never contained actin rodlets as observed with wild-type villin.

**DISCUSSION**

**CD inhibits the villin-induced morphogenesis of long surface microvilli**

In the present study we further characterized the mechanism of assembly of surface microvilli. We took advantage of the fact that villin induces the reorganization of the actin cytoskeleton in transfected CV-1 cells, accompanied by the growth of microvilli. Although we cannot exclude the possibility that CD may have another target besides actin, recent work on the effects of cytochalasin on cells containing a cytochalasin-resistant β-actin variant has provided direct evidence that cytochalasin interacts exclusively with actin in living cells (Ohmori et al., 1992). Long-term exposure to low concentrations of CD did not irreversibly damage the cells, since the effects of CD on the cytoskeleton were always fully reversible in our study.

Our results demonstrate that the villin-induced microvilli and the formation of spike-like F-actin structures are affected by low concentrations of CD at which untransfected control cells exhibited an almost unmodified actin cytoskeleton. Since stress fibers of villin-positive cells were disrupted, even when cells were almost free of spike-like F-actin structures, it is likely that the disassembly of stress fibers is not a direct consequence of the recruitment of actin...
Cytochalasin D inhibits microvilli growth

Cytochalasin D inhibits microvilli growth monomers into F-actin-containing surface structures. CD was used at concentrations that have been shown to inhibit the association of actin monomers to the plus-end of microfilaments in vitro. Therefore, we consider that the most likely mechanism for the inhibition of microvilli growth by CD is the inhibition of the elongation of microfilaments at their fast-growing end. It is unlikely, however, that CD might act by competing with villin, since in vitro experiments demonstrated that villin and the actin-binding drug cytochalasin B, which is closely related to CD, bind to two different sites at the barbed end of microfilaments (Cribbs et al., 1982).

We also considered the possibility that CD may prevent attachment of microfilaments to the plasma membrane by competing with proteins involved in this process. Although we cannot exclude such an interaction, the presence of short actin-containing surface protrusions in CD-treated cells suggests that actin filaments still persist near the microvillar plasma membrane.

The formation of actin rodlets is a cooperative effect of villin and CD

At CD concentrations that completely impaired the growth of long surface microvilli, villin induced the formation of randomly distributed cytoplasmic actin rodlets. The formation of these F-actin structures was dependent on large amounts of villin and was never observed in untransfected cells. This surprising finding suggests that blocking nucleation sites near the plasma membrane results in the ‘random’ nucleation of microfilaments throughout the cyto-

Fig. 6. The formation of rodlet-like actin structures in the presence of CD is dependent on an actin-binding site located at the carboxy terminus of the villin headpiece domain. Cultures of cells were transfected with a DNA construct encoding the Δ7 villin truncate (Friederich et al., 1992). CD was added after 24 h. Cells were incubated in the presence of CD for further 24 h and then analyzed. Cells were processed for double-labeling of villin and F-actin as described for Fig. 1, with the difference that polyclonal villin-specific antibodies were used as first antibodies and fluorescein-coupled rabbit IgG-specific antibodies were used as second antibodies. Left panels are micrographs of the immunofluorescence stainings. Right panels are micrographs of the corresponding phalloidin labelings. (A,B) no CD; note the absence of F-actin spikes. (C,D) 100 nM CD; note the absence of F-actin rodlets. Villin label colocalizes with CD-resistant cortical F-actin. Bar, 10 µm.
plasm and stresses the important role of the plasma membrane in the organization of the actin cytoskeleton. After wash-out of CD, the actin rodlets disassembled with an estimated rate of 6 monomers s$^{-1}$, a value that is very close to that of the off-rate of ADP-actin from the barbed end of actin filaments in vitro (7 monomers s$^{-1}$) (Pollard, 1986). This suggests that the fast-growing ends of these microfilaments were blocked by CD and that the rodlets may have formed slowly by monomer addition at the pointed and/or the CD-capped barbed ends. The fast disassembly at one extremity of the rodlet suggests a polarized orientation of the microfilaments. It remains to be investigated whether villin is responsible for this organization by bundling microfilaments in an oriented manner.

The finding that the villin truncate is not able to induce the formation of the thick actin rodlets in the presence of CD: (1) strongly suggests that the carboxy-terminal actin-binding site is required for the formation of these structures; (2) provides indirect evidence that the bundling activity of villin is implicated in this process; (3) is further proof that the formation of actin rodlets is dependent on the presence of villin. Moreover, our results qualitatively demonstrate that in living cells CD not only causes the disassembly of existing actin structures but may also contribute to the formation of other new ones. The fact that the formation of these structures is dependent on villin demonstrates that the action of CD can be modulated by actin-binding proteins. Moreover, this finding may explain why controversial results have been obtained in quantitative studies measuring the G/F-actin content in CD-treated living cells (Morris and Tannenbaum, 1980; Casella et al., 1981; Fox and Phillips, 1981; Rao et al., 1992).

Implications of CD-impaired villin-induced microvilli formation on the mechanism of the assembly of microvilli

Our data suggest that CD impairs the elongation rather than the formation of short microvilli containing membrane-associated actin oligomers. Thus it is very likely that the fast-growing ends of the microvillar microfilaments are not capped, or are only unstably capped, at least during the elongation phase, which is critical for the extension of the microvillar plasma membrane. This is in line with the findings of Mooseker and colleagues (1982), who observed actin monomer addition at the barbed end of isolated intestinal brush border microvilli. Although the present approach does not allow us to determine the polarity of the microvillar microfilaments, we predict from our findings that the barbed ends of the microvillar microfilaments are oriented towards the tip of the villin-induced microvilli. Such an orientation of membrane-associated microfilaments has been found in other membrane protrusions such as microspikes (Small et al., 1978). It will be important in the future to verify this prediction by myosin S1 subfragment decoration.

A possible role for villin in the formation of long microvilli may be the nucleation or stabilization of actin microfilaments near the plasma membrane. In vitro experiments have shown that nucleation of actin polymerization by villin is followed by capping of the barbed end (Glenny et al., 1981a). However, our results suggest that during the elongation phase the barbed ends of microvillar actin filaments are unblocked. Thus, if villin nucleates actin filaments this should occur by a mechanism that results in uncapped or in unstably capped barbed filament ends. Villin may, for instance, stabilize actin filaments by lateral interactions. This hypothesis is strengthened by the observation that the actin-binding site of villin, which we demonstrated here to be essential for the formation of the cytoplasmic actin rodlets, is not only involved in microfilament bundling but also induces G-actin to adopt a conformation favorable for microfilament formation (Friederich et al., 1992). This property may also play a role in the stabilization of F-actin oligomers near the plasma membrane as well as of the actin rodlets formed in the presence of CD.

The effect of cytochalasin B has been previously tested on developing microvilli of intestinal epithelial cells, using cultured explants of chick embryo intestines (Burgess and Grey, 1974). Since cytochalasin B was added at embryonic stages (day 12 to 18) where intestinal epithelial cells already presented microvilli with an organized actin core made of actin-binding proteins such as fimbrin and villin (Shibayama et al., 1987; Ezzell et al., 1989), it is difficult to compare our findings on CV-1 cells with those obtained during these studies. However, it is interesting to note that microvilli of developing intestinal epithelial cells were sensitive to cytochalasin, which altered their shape, length and spatial distribution. As we observed in transfected CV-1 cells, cytochalasin caused the formation of new F-actin structures. Treatment of explants with cytochalasin B within a range of 0.1-2.5 µg/ml resulted in the formation of thick actin bundles plunging deeply into the cytoplasm (Burgess and Grey, 1974). In contrast to the actin rodlets that we observed in the cell body of transfected cells, these long bundles supported, on their upper part, the plasma membrane, forming normal-sized microvilli. Very high concentrations of cytochalasin B induced branching or elongation of part of the microvilli as well as the redistribution of their spatial arrangement, some areas of the apical cell surface being devoid of microvilli (Mak et al., 1974). In contrast to the microvilli of embryonic cells, those of adult intestinal cells are resistant to cytochalasin B (Mak et al., 1974), indicating that the dynamics of the microvillar actin cytoskeleton change during development. The observation that villin-induced microvilli in CV-1 cells were still sensitive to CD may reflect the fact that these structures are lacking factor(s) that protect the actin cytoskeleton of intestinal microvilli from the effect of this drug.

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