Microinjection of Villin into Cultured Cells Induces Rapid and Long-lasting Changes in Cell Morphology but Does Not Inhibit Cytokinesis, Cell Motility, or Membrane Ruffling

Zsofia Franck, Matthew Footer, and Anthony Bretscher

Section of Biochemistry, Molecular and Cell Biology, Biotechnology Building, Cornell University, Ithaca, New York 14853

Abstract. Villin, a Ca^{2+}-regulated F-actin bundling, severing, capping, and nucleating protein, is a major component of the core of microvilli of the intestinal brush border. Its actin binding properties, tissue specificity, and expression during cell differentiation suggest that it might be involved in the organization of the microfilaments in intestinal epithelial cells to form a brush border. Recently, Friederich et al., (Friederich, E., C. Huet, M. Arpin, and D. Louvard. 1989. *Cell.* 59:461–475) showed that villin expression in transiently transfected fibroblasts resulted in the loss of stress fibers and the appearance of large cell surface microvilli on some cells. Here, we describe the effect of villin microinjection into cells that normally lack this protein, which has allowed us to examine the immediate and long-term effects of introducing different concentrations of villin on microfilament organization and function. Microinjected cells rapidly lost their stress fibers and the actin was reorganized into abundant villin containing cortical structures, including microspikes and, in about half the cells, large surface microvilli. This change in actin organization persisted in cells for at least 24 h, during which time

they had gone through two or three cell divisions. Microinjection of villin core, that lacks the bundling activity of villin but retains all the Ca^{2+}-dependent properties, disrupted the stress fiber system and had no effect on cell surface morphology. Thus, the Ca^{2+}-dependent activities of villin are responsible for stress fiber disruption, and the generation of cell surface structures is a consequence of its bundling activity.

Microinjection of villin led to the reorganization of myosin, tropomyosin, and α-actinin, proteins normally associated with stress fibers, whereas both fimbrin and ezrin, which are also components of microvillar core filaments, were readily recruited into the induced surface structures. Vinculin was also redistributed from its normal location in focal adhesions. Despite these changes in the actin cytoskeleton, cells were able to divide and undergo cytokinesis, move, spread on a substratum, and ruffle. Thus, we show that a single microfilament-associated protein can reorganize the entire microfilament structure of a cell, without interfering with general microfilament-based functions like cytokinesis, cell locomotion, and membrane ruffling.

E ACH eukaryotic cell employs a specific set of proteins that associate with actin to build and dictate the functions of the microfilaments in the cell (reviewed in Pollard and Cooper, 1986; Stossel et al., 1985). Two basic mechanisms are used to achieve specificity of these actin-based microfilaments. One is compartmentalization: different associated proteins are used in the various microfilament arrangements within a single cell. For example, in cultured fibroblasts myosin is associated with the stress fibers, but is typically much reduced in the microfilament-rich ruffling membrane (Weber and Groeschel-Stewart, 1974). In a complementary fashion, fimbrin is largely absent from the stress fiber system, but is enriched in the ruffling membrane (Bretscher and Weber, 1980a). The other is cell type specificity: the expression of certain microfilament-associated proteins is under cell type control. For example, differentiated intestinal epithelial cells express the tissue specific actin-binding

proteins villin and the 110K-calmodulin complex (Bretscher et al., 1981; Drenkhahn and Dermietzel, 1988; Robine et al., 1985; Glenney et al., 1981b). One way to probe the distinct contributions of each of the various microfilament-associated proteins to cellular structure is to either eliminate them from a cell that normally contains them, or to introduce them into cells that normally lack them.

The intestinal microvillus cytoskeleton isolated in the absence of free Ca^{2+} is a simple structure that contains four major proteins: actin (43 kD), villin (95 kD), fimbrin (68 kD), and the 110K-calmodulin complex (Bretscher and Weber, 1978, 1980a,b; Matsudaira and Burgess, 1979; Howe and Mooseker, 1983). Villin and fimbrin cross-link the actin filaments into a uniformly polarized bundle in which all the actin filaments are in lateral register (reviewed in Mooseker, 1985; Bretscher, 1986a; Louvard, 1989). This bundle is attached to the plasma membrane by a regular array of heli-

cally disposed lateral arms that are composed, at least in part, of the 110K-calmodulin complex (Matsudaira and Burgess, 1979; Howe and Mooseker, 1983; Coluccio and Bretscher, 1989). In support of this model, we recently demonstrated that structures very similar to isolated microvillus cytoskeletons could be assembled in vitro from purified actin, villin, fimbrin, and 110K-calmodulin (Coluccio and Bretscher, 1989). Since these four proteins are able to determine the structure of the microvillar cytoskeleton in vitro, we wished to explore whether they are able to reconstitute the structure in vivo in cells that lack a brush border. Since two of the major proteins, villin and the 110K-calmodulin complex, are tissue specific, their role in the assembly of microvilli can be assessed by introducing each protein individually into cultured cells that lack a brush border.

Recently, Friederich et al. (1989) described the consequences of long-term (48-72 h) high-level expression of villin, its core and headpiece domains, in transiently transfected CV-1 fibroblasts. In cells expressing high levels of villin, numerous long microvilli were found on the dorsal surface and the cells showed a disruption of their stress fibers. Immunocytochemistry revealed that villin was present in the long surface microvilli. They also noted that not all villin-expressing cells displayed the morphological changes. The Ca^{2+}-dependent severing, capping, and nucleating activities of villin are located in a 90-kD domain, known as villin core, whereas a carboxy-terminal F-actin binding 8.5-kD domain, known as villin headpiece, is needed for filament bundling (Glenney et al., 1981a; Glenney and Weber, 1981; Janmey and Matsudaira, 1988). Friederich et al. (1989) also showed that cells transfected to express villin core did not show any modifications of the cytoskeleton or cell surface and the protein exhibited a diffuse intracellular distribution. In contrast, when the headpiece domain was expressed it was localized to short microvilli on the cell surface, but did not modify the dorsal cell surface.

In a preliminary report, Bretscher et al. (1981) using single label immunofluorescence microscopy found that microinjected villin associated with membrane ruffles in PtK_2 cells. Here we reinvestigate the consequences of microinjecting villin and villin core into cultured cells. Microinjection offers certain advantages over transfection. It has allowed us to explore the concentration dependence of the observed phenomena, the rapidity and long-term consequences of villin microinjection, and to quantitate the effects in a homogeneous cell population. Our findings confirm and extend many of the results of Friederich et al. (1989). We find that injection of villin results in the very rapid disruption of stress fibers and the apparent redistribution of actin into cortical structures, including microspikes and large surface microvilli. The morphology of the injected cells is significantly altered due to the rearrangement of the actin cytoskeleton by villin, and remains this way for at least 24 h. Despite these significant changes, the cells can still undergo cell division, are motile, and ruffle, cellular processes that require the participation of microfilaments. However, introduction of villin core results in disruption of the stress fiber system without any effect on surface structures. Thus, the disruption of stress fibers by villin can be attributed to the core domain, whereas the generation of surface structures is a consequence of its bundling activity. Our results indicate that introduction of a tissue-specific microfilament-associated pro-

tein can have a global effect on microfilament distribution without interfering with some vital microfilament-based processes.

Materials and Methods

Cells

Cells used in this study were mouse NIH 3T3 fibroblasts; human A431 and KB epidermoid carcinoma cells; and Madin-Darby bovine kidney (MDBK) cells. The 3T3 and A431 cells were grown in DME supplemented with 10% FCS, the MDBK cells in MEM supplemented with 10% FCS, and the KB cells in MEM with 10% calf serum. For microinjection, cells were plated on glass cover slips that had been scored with a diamond pencil on the surface to facilitate identification of injected cells. Cells were used 24 to 48 h after plating.

Induction of Membrane Ruffles

Membrane ruffling was induced in KB cells essentially as described by Kadowaki et al. (1986). 24 h after plating cells, the culture medium was replaced with buffer consisting of 0.3% BSA, 130 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 10 mM Hepes, pH 7.4. Ruffling was induced by the addition of EGF to a final concentration of 10 nM. Cells were fixed and processed for immunofluorescence microscopy at the peak of their ruffling activity, which is 5 min after growth factor addition.

Proteins

Villin was purified as described (Bretscher and Weber, 1980b; Bretscher, 1986b) and concentrated by anion-exchange chromatography or ammonium sulphate precipitation. For injection, villin was dialyzed into injection buffer (120 mM KCl, 10 mM Tris-HCl, pH 7.4), clarified in a microfuge for 15 min, and the protein concentration determined using the method of Bradford (1976) using BSA as standard. Villin core was prepared as described by Glenney and Weber (1981).

Microinjection

Needle microinjection was performed essentially as described by Graessmann and Graessmann (1976). Glass micropipettes were pulled from self-filling borosilicate glass capillaries on a P-77B Brown-Flaming Micropipette Puller (Sutter Instruments Co., Novato, CA). Microinjection was performed on an Olympus IMT-2 inverted microscope with the aid of a micromanipulator (E. Leitz, Inc., Rockleigh, NJ). Constant pressure was applied to the needle with an attached syringe, so that the solution was continuously flowing from the needle during the injection procedure. Previous studies have determined that the mean value of the percent volume injected is ~10% (Cooper et al., 1987). Cells injected with >15% of their volume die soon after injection.

Antibodies and Indirect Immunofluorescence Microscopy

Affinity-purified rabbit antibodies to chicken intestinal brush border villin (Bretscher et al., 1981), to chicken intestinal brush border myosin and human ezrin (Bretscher, 1989), to bovine brain tropomyosin, and to chicken gizzard α-actinin (Bretscher and Weber, 1980b) have been described. Antibodies to chicken gizzard vinculin, purified as described (Feramisco and Burridge, 1980), were elicited in rabbits and affinity purified on immobilized vinculin. For indirect immunofluorescence microscopy, primary antibodies were used at the following concentrations: antivillin, 25 or 50 µg/ml; antimyosin, 50 µg/ml; antitropomyosin, 25 µg/ml; anti-α-actinin, 50 µg/ml; antiezrin, 5 µg/ml; and antivinculin, 35 µg/ml. Cells were fixed in 3.7% formaldehyde for 10 min, permeabilized in −20° ethanol for 2 min, rehydrated in PBS, and incubated with primary antibody for 30 min. Alternatively, cells were extracted and fixed in two steps as described by Cao and Wang (1990) before incubation with the primary antibody. This method removed excess villin and facilitated the identification of villin-containing surface structures. After extensive washings, the samples were incubated with fluorescein-conjugated goat anti-rabbit IgG (ICN K & K Laboratories, Inc., Plainview, NY) mixed with a 1:20 dilution of rhodamine-conjugated phalloidin to visualize F-actin (Molecular Probes Inc., Junction City, OR). Before use, the secondary antibody was extensively preabsorbed on fixed
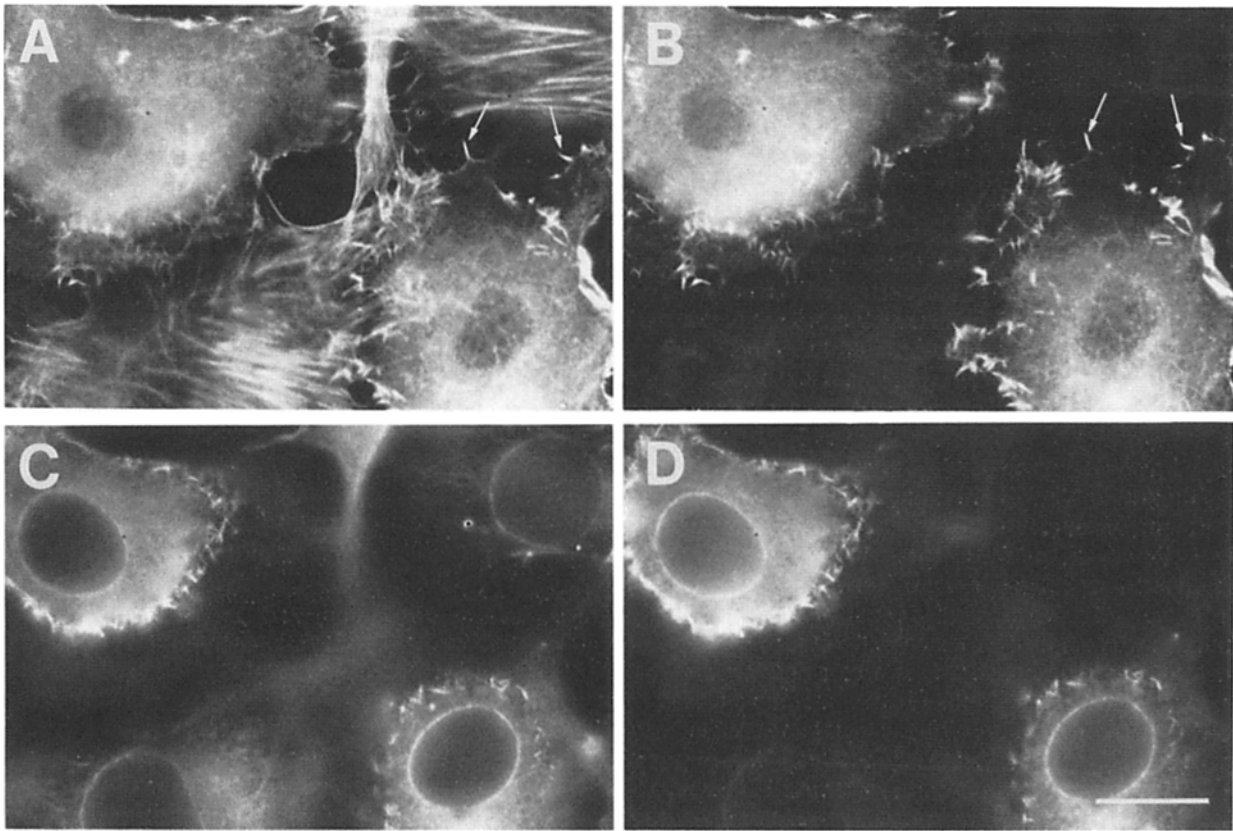
Figure 1. Microinjection of villin results in the disruption of stress fibers and induces the formation of surface microvilli and microspikes. 3T3 cells were injected with 6 mg/ml villin and the actin (*A* and *C*) and villin (*B* and *D*) distributions are shown 1 h after injection in two different focal planes. Note the loss of stress fibers and the formation of microspikes at the cell borders (*A* and *B, arrows*) and long microvilli on the upper surface (*C* and *D*) in the injected cells. Notice also the perfect colocalization of actin and villin in these structures. Bar, 20 μm.

3T3 cells to reduce nonspecific staining. After a further set of extensive washings, the preparations were mounted in 10 mM *p*-phenylenediamine, 90% glycerol, and observed using a Zeiss Universal Microscope fitted with epifluorescence optics. Images were photographed using Kodak T-MAX P3200 film shot at an EI of 3200.

Results

Microinjection of Villin Results in Rapid and Long-lasting Changes in Cell Morphology and in the Actin Cytoskeleton

Villin was injected into cultured mouse 3T3 fibroblasts, which normally lack the protein, and the effect this had on cell morphology and on the actin cytoskeleton was examined 1 h after injection (Fig. 1). The microinjected cells were identified by immunofluorescence microscopy using villin-specific antibody (Fig. 1, *B* and *D*) and the actin cytoskeleton was visualized by rhodamine-labeled phalloidin staining (Fig. 1, *A* and *C*). The injected cells showed extensive formation of villin containing surface structures, including numerous microspikes (Fig. 1 *A* and *B, arrows*) and large surface microvilli on the upper cell surface (Fig. 1, *C* and *D*). Actin staining revealed a perfect colocalization with villin in the newly formed surface structures. The formation of new surface structures was accompanied by the disruption of

stress fibers in the injected cells which also showed a diffuse cytoplasmic actin staining (Fig. 1 *A*).

The disruption of stress fibers and the expression of microspikes around the perimeter of cells always occurred after villin injection; however, the formation of long microvilli on the dorsal surface was not found in all cells. An example of a cell in which surface microvilli were not noticeably induced is shown in Fig. 2, *A* and *B*. Because 3T3 cells can normally express surface microvilli, to demonstrate that villin induces their formation and does not only colocalize with them, we quantitated the effect that villin injection had on their expression. We found that uninjected cells had short surface microvilli whose appearance was favored by high cell density, reaching ~10–15% of the population in near confluent cultures. Villin injection increased this number to ~50% in the same culture, and the microvilli were strikingly longer. Fig. 2, *C–F* show a field of injected and control cells at relatively high density. Note the extensive formation of microspikes around the cell borders (Fig. 2 *D*) and the disruption of stress fibers in all the injected cells (Fig. 2 *C*). Here all the villin-containing cells are covered with long processes (Fig. 2 *F*); an uninjected cell is indicated with microvilli that are noticeably shorter than those on the villin-containing cells (Fig. 2 *E, arrow*). At higher magnification this difference is even more striking: (Fig. 2 *G*) shows three cells, two of which had been injected with villin (Fig. 2 *H*)
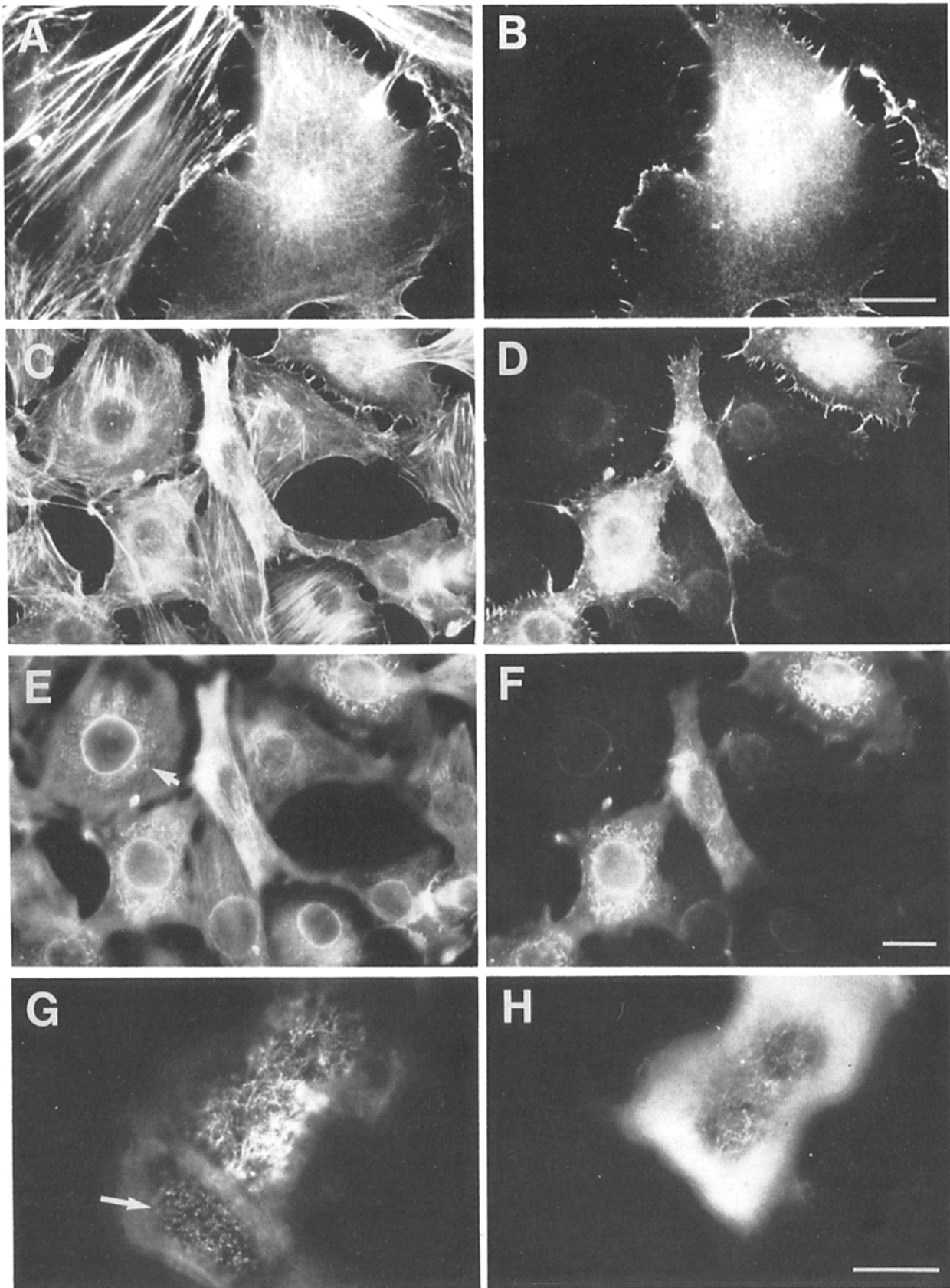
Figure 2. Villin induces the formation of surface microvilli in about half the injected cells that are larger than normal 3T3 cell surface microvilli. (*A* and *B*) This is an example of a villin-injected cell that did not develop surface microvilli; notice the loss of stress fibers (*A*) and the generation of a "spiky" cell border that has actin- (*A*) and villin- (*B*) containing microspikes. (*C–F*) Low power light micrographs of the distribution of actin (*C* and *E*) and villin (*D* and *F*) in the same field of cells photographed at two different focal planes.
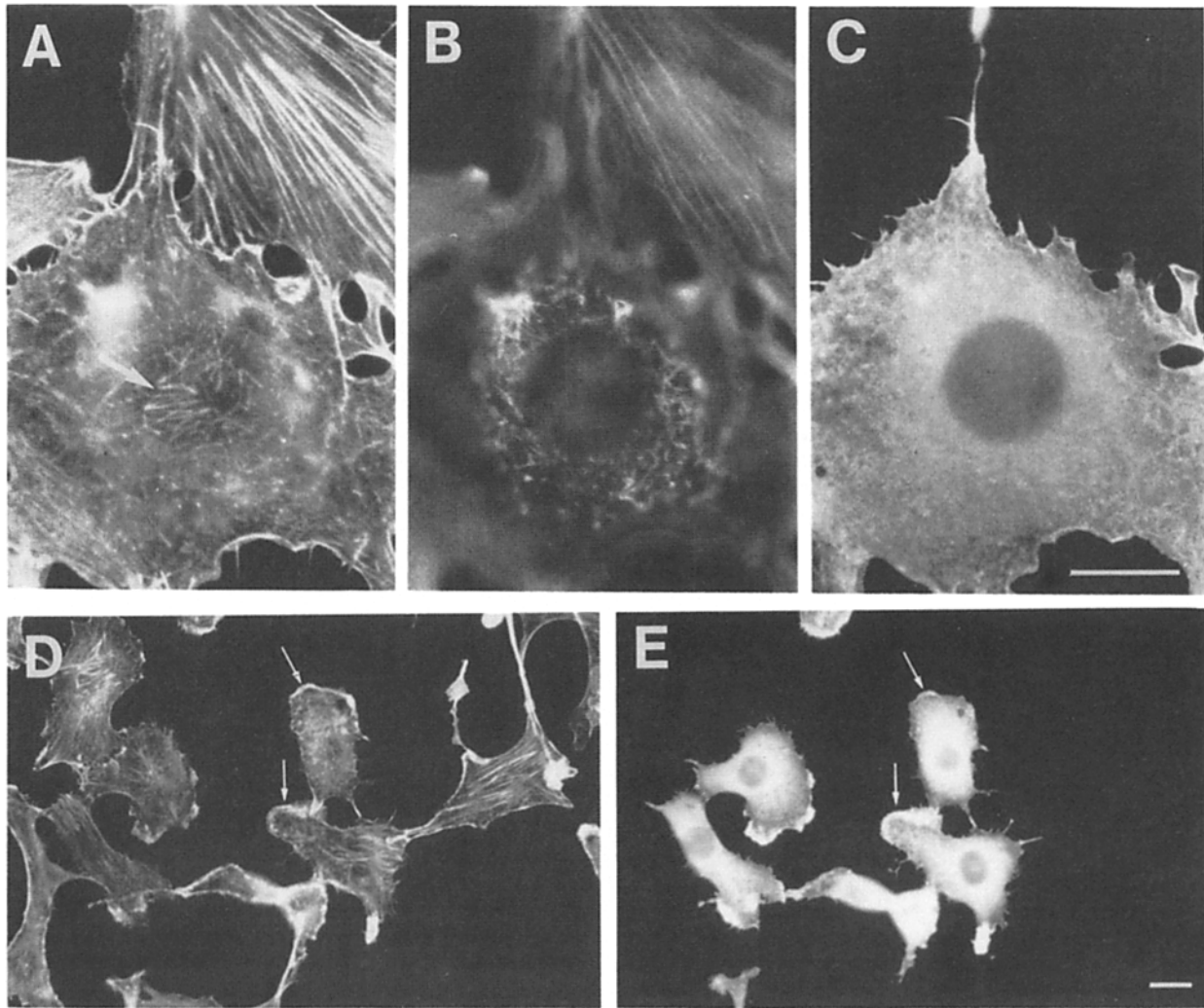
Figure 3. Effect of villin injection on the morphology of 3T3 cells as a function of time. 3T3 cells were injected with 6 mg/ml villin and fixed 24 h (*A–C*) or 48 h (*D* and *E*) after injection. For the upper panels, *A* and *B* show the actin localization at two different focal planes, *C* the villin localization. Note the long microvilli on the surface of the cell (*B*) and the short radial stress fibers that have already reappeared in the perinuclear area (*A, arrow*). The lower panels show the siblings of a single cell that were injected with villin, identified by the localization of villin (*E*). Many of the cells are still devoid of stress fibers (*D*) and have moved away from each other after cell division. Note the colocalization of villin and actin in the ruffling membranes of these cells (*D* and *E, arrows*). Bars, 20 μm.

and display long surface processes, whereas the third control cell is covered with short dot-like processes (Fig. 2 *G*).

Microinjection of different concentrations of villin (1.1–7.5 mg/ml) induced morphological changes similar to those described above. The injection volume using the micropipette technique is generally ∼10% of the cell volume (Cooper et al., 1987), so the final concentration of villin in cells at the highest concentration injected was ∼0.7 mg/ml. If we assume that the actin concentration in a cell is ∼5 mg/ml, this represents a molar ratio of about one villin molecule for ev-

ery 14 actin monomers. This is very similar to the ratio of the two proteins in the isolated intestinal brush border (Bretscher, 1983), the structure that is the richest source of villin.

The morphological changes induced by villin injection were rapid and long lasting. Most cells fixed immediately after injection (5 min) had already developed microspikes and microvilli but many of them showed only partial loss of stress fibers, with the ones in the perinuclear area preferentially remaining. By 15 min after injection the changes described

Four cells were injected with 6 mg/ml villin and fixed 1 h after injection, all of these have lost their stress fibers and are very "spiky" around their perimeters (*C* and *D*). All the injected cells developed long microvilli on their upper surface (*E* and *F*); these are much larger than those found on an uninjected cell (*E, arrow*). (*G* and *H*) Higher power micrographs of the upper surface of three cells, two of which were injected with villin. Note that the villin-induced microvilli as revealed by actin (*G*) and villin (*H*) staining are much larger than those of the uninjected cell (*G, arrow*). Bar, 20 μm.
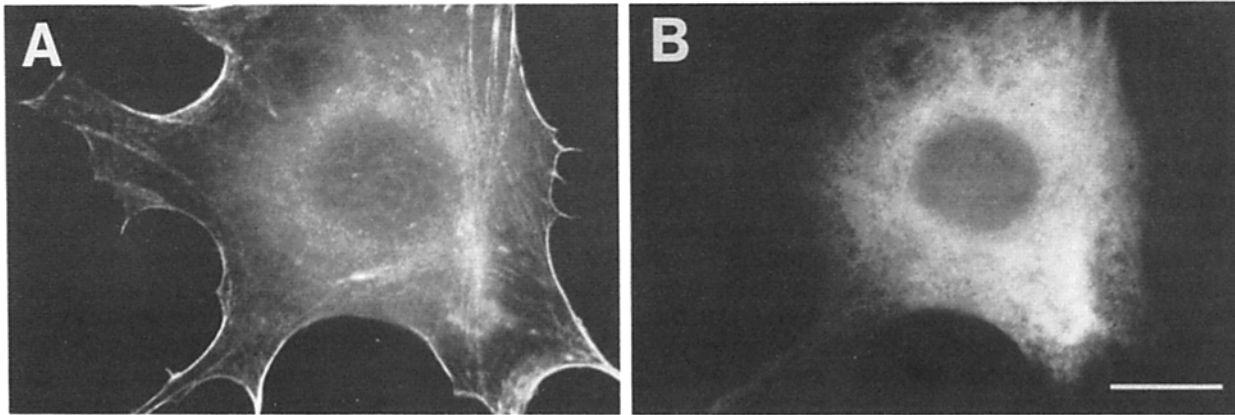
Figure 4. Injection of villin core leads to the disruption of stress fibers but does not induce surface structure formation. Actin (*A*) and villin core (*B*) distribution in a cell 30 min after injection with 5.5 mg/ml villin core. Note the relatively uniform difuse staining for the core in *B* and its lack of localization with the microfilaments at the cell border. Bar, 20 μm.

above were fully developed and remained evident in cells fixed even 24 h after injection (Fig. 3, *A–C*). However, by this time radial stress fibers have started to reappear in the perinuclear area (Fig. 3 *A, arrow*) indicating that after such a long incubation and several cell divisions (see below) daughter cells have begun to overcome the effect of villin injection.

The villin-induced morphological changes were not restricted to cells of fibroblastic origin. Villin injection caused the same morphological changes in a normal epithelial cell line (MDBK cells, not shown). Human carcinoma A431 and KB cells normally express very few stress fibers, and injection of villin into these cells resulted in less obvious morphological changes. However, they often expressed long and numerous microvilli all over the surface of the cells regardless of whether the injected cell was in an epithelial sheet or on the periphery (Fig. 5, *G* and *H*).

Microinjection of Villin Core Results in the Reversible Disruption of Stress Fibers but Does Not Induce Surface Structure Formation

Microinjection of villin core into 3T3 cells had a markedly different effect on the cells than the microinjection of villin. The surface of the injected cells remained unchanged and the injected protein did not localize to preexisting microspikes or surface microvilli but showed uniform diffuse cytoplasmic staining (Fig. 4 *B*). Examination of the actin distribution in the injected cells showed a dramatic time dependence. At 15 and 30 min after 5.5 mg/ml villin core was injected into cells, >85% of the cells lost their stress fibers. By 45 min after injection, only 40–60% appeared to have lost their stress fibers, and by 60 min essentially all the cells had a normal stress fiber distribution. Microinjection of buffer or control proteins (IgG and BSA) never showed similar effect on the stress fiber system.

Microinjection of Villin Leads to the Redistribution of Microfilament-associated Proteins

As documented above, the microinjection of villin results in rapid and long-lasting morphological changes in cells, with the loss of stress fibers and the redistribution of actin into peripheral structures, like microspikes and microvilli. We,

therefore, investigated the distribution of actin-binding proteins known to be associated with stress fibers (myosin, tropomyosin, and α-actinin) and of proteins that are present in microvilli (fimbrin and ezrin). We also examined the effect villin microinjection had on focal contacts as determined by vinculin localization.

We were not able to localize both villin and other proteins by immunofluorescence microscopy as all our primary antibodies were made in rabbits. Therefore, we recorded the position of injected cells by reference to a scratch on the coverslip. Injected cells are indicated by arrows in Fig. 5. In these myosin (Fig. 5, *A* and *B*), tropomyosin (Fig. 5, *C* and *D*) or α-actinin (not shown) revealed diffuse cytoplasmic staining instead of the typical periodic distribution down the stress fibers as seen in the uninjected controls. Vinculin disappeared from adhesion sites and disintegrated into small patches with only a few sites remaining intact at the margins of the cell (Fig. 5, *E* and *F*).

The distribution of the actin-associated proteins fimbrin and ezrin, which specifically associate with microfilaments in surface structures, remained largely unaltered and these proteins were recruited, along with villin, into the newly formed microspikes and microvilli (ezrin, Fig. 5, *G* and *H*; and fimbrin [not shown]). In other experiments, the distribution of microtubules as revealed using tubulin antibody was not significantly changed.

Effect of Villin Injection on Microfilament-based Cell Functions

Although 3T3 cells microinjected with villin undergo a change in cell morphology, they are able to proceed through the cell cycle at about the normal rate and undergo cell division. When a single cell on a coverslip was injected with villin, and the cells incubated for 24 or 48 h, 4 and 18 cells were found, respectively, that contained villin as determined by immunofluorescence microscopy. The cells in Fig. 3 (*A–C*) show the villin and actin distribution in a cell 24 h after injection, during which time the injected cell had gone through three cell divisions. The daughter cells have reduced stress fibers (Fig. 3 *A*), but express long microvilli (Fig. 3 *C*). After a 48-h incubation, a single injected cell gave rise to 18 cells that contained detectable villin; the daughter cells were
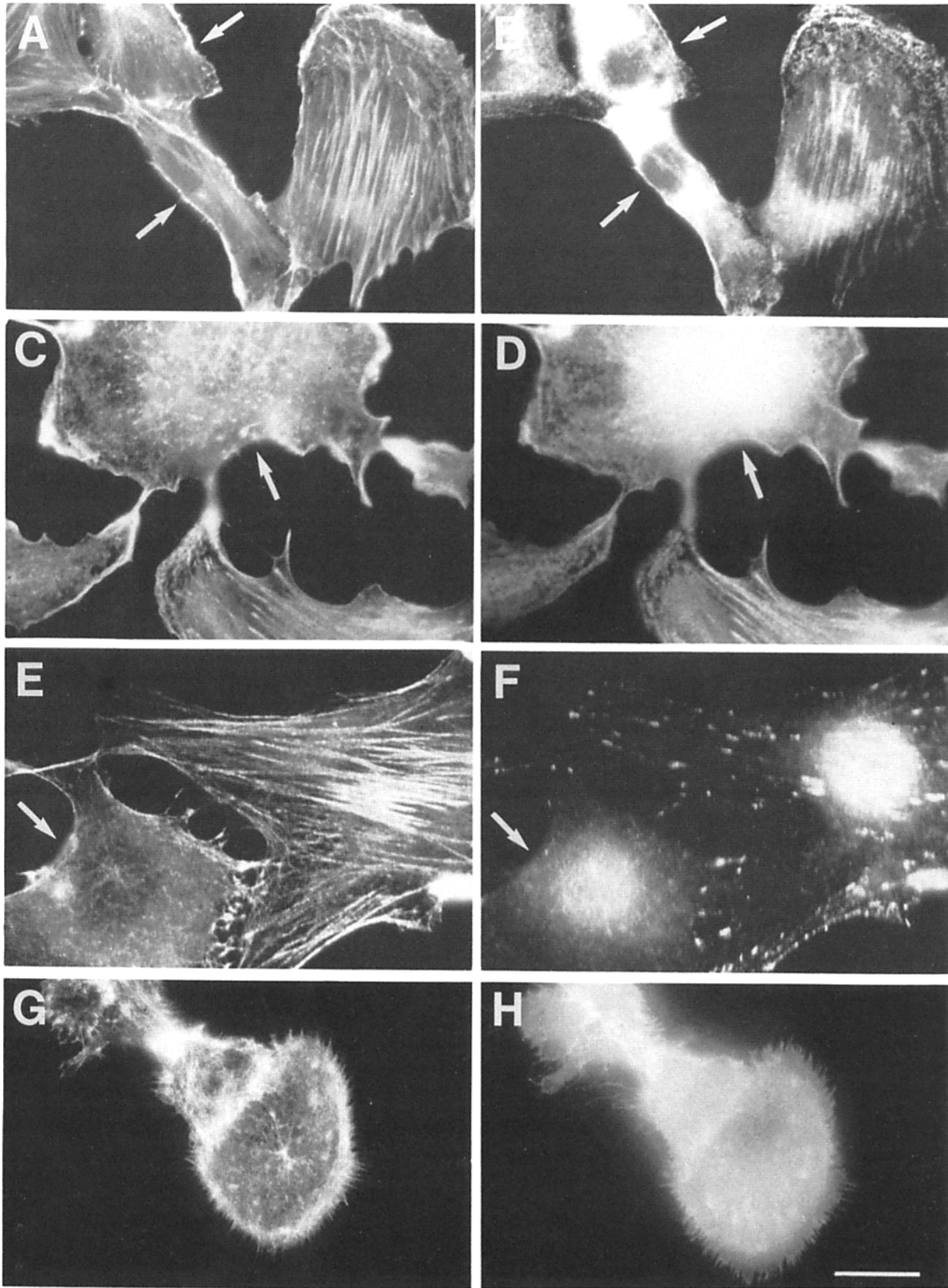
Figure 5. Localization of microfilament-associated proteins in cells injected with villin. Cells were injected with 6 mg/ml villin and processed for fluorescence microscopy 1 h later. The corresponding distributions of actin (*A, C, E,* and *G*) and of myosin (*B*), tropomyosin (*D*), vinculin (*F*), and ezrin (*H*) in 3T3 cells (*A–F*) and A431 cells (*G* and *H*) is shown. The injected cells are indicated with an arrow. Bar, 20 μm.
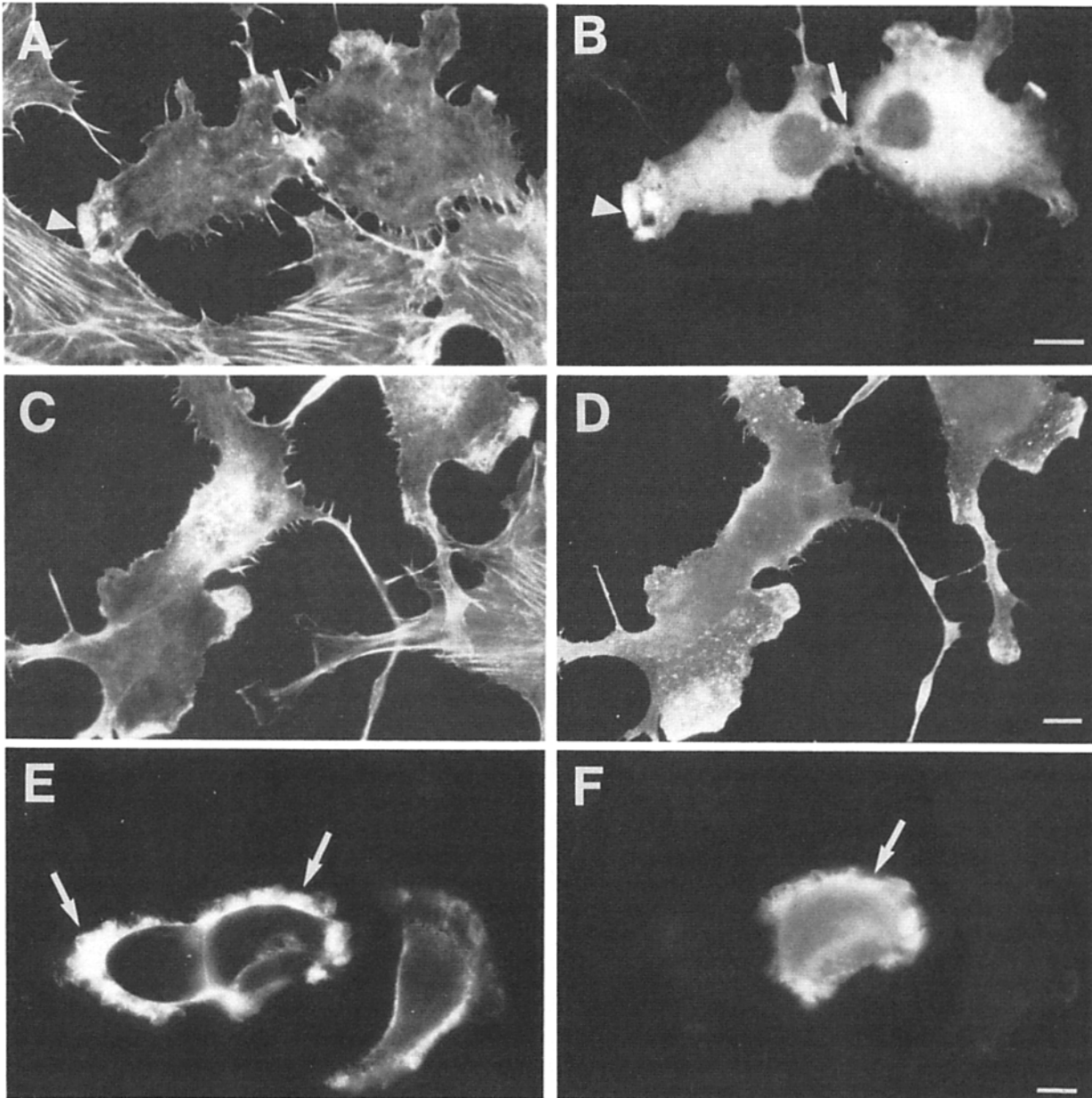
Figure 6. Villin microinjection does not interfere with cytokinesis, cell spreading, and membrane ruffling. (*A* and *B*) Localization of actin (*A*) and villin (*B*) in an injected cell undergoing cytokinesis. The cell was injected with 6 mg/ml villin 24 h before fixation. Notice the enrichment of actin in the contractile ring (*arrow*) and in membrane ruffles (*arrowhead*), and the enrichment of villin in the ruffles but not in the contractile ring. (*C* and *D*) Trypsinized 3T3 cells were allowed to reattach for 50 min, injected with 6 mg/ml villin, and then allowed to spread for 24 h. They were then processed and stained for actin (*C*) and villin (*D*). Notice the aberrant shapes of the villin-containing cells as compared to the villin-free cells (*C*) and the colocalization of actin and villin in microspikes on some of the cells (*C* and *D*). (*E* and *F*) Villin does not interfere with growth factor–induced membrane ruffling in human carcinoma KB cells. One cell was injected with 6 mg/ml villin, starved of growth factors for 1 h, and induced to ruffle by the addition of EGF. The cells were fixed 5 min later. Notice that the actin localization (*E*) reveals abundant ruffles (*arrows*) on both uninjected and injected cells; villin (*F*) is present in the induced ruffles. Bars, 20 μm.

not found in a clump, but had moved away from each other and had noticeable ruffling membranes enriched in actin and villin (Fig. 3, *D* and *E*). This demonstrates that the injected cells are motile. Comparison of villin-injected cells with cells injected with nonimmune IgG showed no differences in their growth rates. We also found direct evidence that in villin-injected cells the contractile ring is formed normally. In Fig. 6 (*A* and *B*) a cell is in the process of cytokinesis,

where the contractile ring is highly enriched in actin (Fig. 6 *A*), but not in villin (Fig. 6 *B*). Note that in this same dividing cell, actin and villin colocalize and both are enriched in ruffling membranes.

We examined two other microfilament-based functions in cells microinjected with villin: cell spreading and the ability to ruffle. To look at the effect of villin on the ability of cells to spread, freshly plated 3T3 cells were injected with 6 mg/

ml villin at a stage where they had just started to reattach but had not yet spread out. They were then incubated for a further 24 h. No significant loss of injected cells was found, despite the documented effect of villin on the integrity of the focal contacts. After this period, both the uninjected and injected cells were fully spread, but the injected cells could be readily distinguished by their very aberrant morphology (Fig. 6, *C* and *D*). All the injected cells were devoid of stress fibers, had many long processes and an enhanced expression of microspikes around their periphery (Fig. 6 *C*).

Motile cells have a microfilament-rich structure at the leading edge known as the ruffling membrane. Microinjection of villin into 3T3 cells resulted in the accumulation of villin into membrane ruffles when they are present on the cells. Membrane ruffles can also be induced in certain receptive cells following addition of appropriate stimuli. One of the best systems is the human carcinoma KB cell line which ruffles intensively after the addition of EGF (Kadowaki et al., 1986), with actin being preferentially recruited into the membrane ruffles (Fig. 6 *E*). If KB cells were first injected with villin, then subjected to EGF treatment, they showed ruffling in the same way as their uninjected counterparts. The injected villin is redistributed into the newly formed surface structures (Fig. 6 *F*).

Discussion

In an attempt to understand the physiological role of villin in the assembly and maintenance of the brush border cytoskeleton we explored the effects of introducing villin into cells that normally lack this protein. Microinjection of villin had a rapid and long-lasting effect on both the actin distribution and morphology of the cells. Injected cells rapidly lost their stress fibers, formed microspikes, and some developed numerous long microvilli on their upper surface. This change in the actin cytoskeleton was accompanied by a distinct alteration in cell shape, but did not interfere with cell growth or a number of microfilament-based processes, including membrane ruffling, cytokinesis, and cell locomotion. Moreover, the effects of villin injection were reversible. Although the overall changes in the actin cytoskeleton and cell morphology were still evident 24 h after injection, during which period the cells had undergone two to three cell divisions, the cells began to regain their stress fiber system and their normal morphology. Thus, villin has the capacity to rapidly, but reversibly, reorganize the actin cytoskeleton giving rise to new cortical actin-containing structures.

These results confirm and complement the report of Friedrich et al. (1989) exploring the consequences of transient expression of villin in fibroblasts. Both studies find that introduction of villin results in a redistribution of actin from the stress fiber system into cortical structures, in our case, abundant microspikes and often large surface microvilli. Because of the nature of the microinjection technique, we are able to demonstrate that villin induces these changes very rapidly, and the effects are long-lasting without the further expression or introduction of villin. This indicates that the observed morphological changes are direct consequences of the presence of villin in the cells, thus ruling out the possibility that they are due to another protein(s) whose synthesis is induced by villin. Our injection experiments with villin core explain why Friederich et al. (1989) did not detect mor-

phological changes on expression of this domain of villin. We show here that villin core, in which resides the Ca^{2+}-dependent F-actin severing, nucleating, and capping activities of villin, is able to transiently disrupt stress fibers in cultured cells. It is likely that this short-lived effect is a reflection of villin core's instability; this would explain why the expression studies, where a protein has to be synthesized and accumulate over a period of hours, showed no effect on the actin cytoskeleton.

It is interesting to compare the results of villin core injection with that of gelsolin, a close relative of the core domain that shows ∼50% sequence identity with villin (Arpin et al., 1988; Bazari et al., 1988). Sanger et al. (1987) reported that PtK₂ cells microinjected with gelsolin lost their stress fibers and rounded up 30 min after injection, but then regained their normal shape and stress fiber system in 3 h after injection. In contrast, Cooper et al. (1987) found no effect on actin distribution, cell shape, deformability, or ruffling activity in fibroblasts or macrophages injected with gelsolin. The results of the injection of villin core therefore seem more analogous to the observations of Sanger et al. (1987) and may suggest that villin core and gelsolin can disrupt stress fibers in a similar way. Thus, this part of the molecule is responsible for the disruption of stress fibers seen with intact villin. The whole villin molecule, which bundles F-actin in the absence of Ca^{2+} in vitro, must therefore be involved in the generation of the cell surface structures. This provides the first example of a mechanism by which a single protein with dual activities on actin structure (severing/capping and bundling) brings about the reorganization of the microfilament cytoskeleton.

Disruption of the stress fibers was accompanied by a redistribution of proteins normally associated with the microfilament system. Myosin, tropomyosin, and α-actinin, which are normally found in a striated pattern along stress fibers (Weber and Groeschel-Stewart, 1974; Lazarides, 1975; Lazarides and Burridge, 1975), were diffusely distributed in the injected cells. Vinculin, normally found in focal adhesions at the termini of stress fibers (Geiger, 1979), was also redistributed in injected cells, although this was not accompanied by cell detachment from the plate. In contrast to the proteins associated with stress fibers, fimbrin and ezrin, which are normally found in cell surface structures and are also components of the microvillar core (Bretscher and Weber, 1980a; Bretscher, 1983), were recruited into the newly formed microspikes and microvilli in the injected cells.

We have found that villin-injected cells can participate in general microfilament-based functions that require elevated levels of Ca^{2+}. The ability of cells to ruffle in response to the addition of a growth factor indicates that the disruption of microfilaments by villin does not interfere with the signaling pathway and the additional reorganization of the actin pool that is required for this process to occur. The observation that injected villin readily associates with the newly formed ruffles suggests that the large Ca^{2+} transient noted immediately after growth factor addition (Gonzalez et al., 1988) may not extend to the membrane ruffles. In contrast to the localization to membrane ruffles, villin seems to be excluded from the contractile ring of dividing cells. Contraction of this structure almost certainly involves elevated levels of Ca^{2+}, as phosphorylation of myosin by myosin light chain kinase requires activation by calcium-calmodulin. The con-

tractile ring has many similarities to both stress fibers of cultured cells and the microfilament bundle associated with belt desmosomes at the level of the zonula adherens in epithelial cells. All these structures contain in common actin, myosin, tropomyosin, caldesmon, filamin, and α-actinin (summarized in Bretscher and Lynch, 1985). As we have shown, villin does not associate with stress fibers but disrupts them, yet the contractile ring appears to be unaffected in villin-injected cells. Similarly, intestinal epithelial cells contain a high level of villin, yet rather little is associated with the microfilament bundles of belt desmosomes. Taken together, this suggests that the contractile ring is more similar to the microfilament bundle associated with belt desmosomes, and both are different from the stress fibers of cultured cells.

What is the role of villin in the assembly of the brush border? The protein has three properties that suggest it might play a critical role in remodeling the intestinal epithelial cells as they differentiate and develop a brush border. First, villin's calcium-regulated properties are those anticipated for a regulatory protein. Second, villin is tissue specific; it is only expressed in cells that have a brush border or in cells with the same embryonic origin (Bretscher et al., 1981; Robine et al., 1985). Third, before the assembly of the brush border, villin synthesis increases and its distribution changes so that it becomes localized under the apical plasma membrane (Robine et al., 1985; Dodouet et al., 1987; Shibayama et al., 1987; Maunoury et al., 1988; Ezzell et al., 1989; Heintzelman and Mooseker, 1990). The results in this paper show that villin has the ability to disrupt cytoplasmic microfilaments and to induce the formation of surface microspikes and microvilli; a similar mechanism may operate during differentiation. In this process, villin might disrupt microfilament-containing structures as it concentrates in the apical cytoplasm to provide a pool of actin (perhaps in the form of short filaments) for microvillar core assembly. Expression of its filament-bundling activity under appropriate conditions would stabilize the core filaments and thereby facilitate their formation from specific sites on the plasma membrane.

In this report we have shown that villin has profound effects on the overall microfilament organization of a cell, yet apparently does not interfere with many known microfilament-based cell functions. This shows that the overall organization of the actin cytoskeleton, and therefore of the whole cell, can be determined by the expression of a single protein, rather than requiring a subtle blend of a large number of actin-associated proteins. Most of the microfilament-based activities are needed for the normal functioning of cells, so most of the microfilament-associated proteins are expected to be constitutively expressed. Specialized cells may also have unique microfilament structures that are involved in carrying out their specific functions. They can employ one or several cell type-specific proteins to reorganize the microfilament arrangements and assemble the necessary structures during differentiation. Such a scenario seems to be mimicked by the microinjection of villin into fibroblasts.

References

Arpin, M., E. Pringault, J. Finidori, A. Garcia, J. -M. Jeltsch, J. Vandekerckhove, and D. Louvard. 1988. Sequence of human villin: a large duplicated domain homologous with other actin-severing proteins and a unique small carboxy-terminal domain related to villin specificity. *J. Cell Biol.* 107:1759–1766.

Bazari, W. L., P. T. Matsudaira, M. Wallek, J. Smeal, R. Jakes, and Y. Ahmed. 1988. Villin sequence and peptide map identify six homologous domains. *Proc. Natl. Acad. Sci. USA.* 85:4986–4990.

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.

Bretscher, A. 1983. Purification of an 80,000-dalton protein that is a component of the isolated microvillus cytoskeleton, and its localization in non-muscle cells. *J. Cell Biol.* 97:425–432.

Bretscher, A. 1986a. The molecular structure of the microvillar cytoskeleton. *In* Ion gradient-coupled transport. *Inserm (Inst. Natl. Sante. Rech. Med.) Symp.* 26:13–21.

Bretscher, A. 1986b. Purification of the intestinal microvillus cytoskeletal proteins villin, fimbrin and ezrin. *Methods Enzymol.* 134:24–37.

Bretscher, A. 1989. Rapid phosphorylation and reorganization of ezrin and spectrin accompany morphological changes induced in A-431 cells by epidermal growth factor. *J. Cell Biol.* 108:921–930.

Bretscher, A., and K. Weber. 1978. Localization of actin and microfilament-associated proteins in the microvilli and terminal web of the intestinal brush border by immunofluorescence microscopy. *J. Cell Biol.* 79:839–845.

Bretscher, A., and K. Weber. 1980a. Fimbrin: a new microfilament-associated protein present in microvilli and other cell surface structures. *J. Cell Biol.* 86:335–340.

Bretscher, A., and K. Weber. 1980b. Villin is a major protein of the microvillus cytoskeleton which binds both G- and F-actin in a calcium dependent manner. *Cell.* 20:839–847.

Bretscher, A., and W. Lynch. 1985. Identification and localization of immunoreactive forms of caldesmon in smooth and nonmuscle cells: a comparison with the distributions of tropomyosin and α-actinin. *J. Cell Biol.* 100:1656–1663.

Bretscher, A., M. Osborn, J. Wehland, and K. Weber. 1981. Villin associates with specific microfilamentous structures as seen by immunofluorescence microscopy on tissue sections and cells microinjected with villin. *Exp. Cell Res.* 135:213–219.

Cao, L., and Y. Wang. 1990. Mechanism of the formation of contractile ring in dividing cultured animal cells. I. Recruitment of preexisting actin filaments into a cleavage furrow. *J. Cell Biol.* 110:1089–1095.

Coluccio, L. M., and A. Bretscher. 1989. Reassociation of microvillar core proteins: making a microvillar core in vitro. *J. Cell Biol.* 108:495–502.

Cooper, J. A., J. Bryan, B. Schwab III, C. Frieden, D. J. Loftus, and E. L. Elson. 1987. Microinjection of gelsolin into living cells. *J. Cell Biol.* 104:491–501.

Dudouet, B., S Robine, C. Huet, C. Sahuquillo-Merino, L. Blair, E. Coudrier, and D. Louvard. 1987. Changes in villin synthesis and subcellular distribution during intestinal differentiation of HT29-18 clones. *J. Cell Biol.* 105:359–369.

Drenkhahn, D., and R. Dermietzel. 1988. Organization of the actin filament cytoskeleton in the intestinal brush border: a quantitative and qualitative immunoelectron microscope study. *J. Cell Biol.* 107:1037–1048.

Ezzell, R. M., M. M. Chafel, and P. T. Matsudaira. 1989. Differential localization of villin and fimbrin during development of the mouse visceral endoderm and intestinal differentiation. *Development (Camb.).* 106:407–419.

Feramisco, J. R., and K. Burridge. 1980. A rapid purification of α-actinin, filamin and a 130,000-dalton protein from smooth muscle. *J. Biol. Chem.* 255:1194–1199.

Friederich, E., C. Huet, M. Arpin, and D. Louvard. 1989. Villin induces microvilli growth and actin redistribution in transfected fibroblasts. *Cell.* 59:461–475.

Geiger, B. 1979. A 130K protein from chicken gizzards: its localization at the termini of microfilament bundles in cultured chicken cells. *Cell.* 18:193–205.

Glenney, J. R., and K. Weber. 1981. Calcium control of microfilaments: uncoupling of the F-actin severing and -bundling activity of villin by limited proteolysis in vitro. *Proc. Natl. Acad. Sci. USA.* 78:2810–2814.

Glenney, J. R., P. Kaulfus, and K. Weber. 1981a. F-actin assembly modulated by villin: Ca^{++}-dependent nucleation and capping of the barbed end. *Cell.* 24:471–480.

Glenney, J. R., M. Osborn, and K. Weber. 1981b. The intracellular localization of the microvillus 110K protein, a component considered to be involved in side-on membrane attachment of F-actin. *Exp. Cell Res.* 138:743–751.

Gonzalez, F. A., D. J. Gross, L. A. Heppel, and W. W. Webb. 1988. Studies on the increase in cytosolic free calcium induced by epidermal growth factor, serum, and nucleotides in individual A431 cells. *J. Cell. Physiol.* 135:269–276.

Graessmann, M., and A. Graessmann. 1976. 'Early' simian-virus-40-specific RNA contains information for tumor antigen formation and chromatin replication. *Proc. Natl. Acad. Sci. USA.* 73:366–370.

Heintzelman, M. B., and M. S. Mooseker. 1990. Assembly of the brush border cytoskeleton: changes in the distribution of microvillar core proteins during enterocyte differentiation in adult chicken intestine. *Cell Motil. Cytoskeleton* 15:12–22.

Howe, C. L., and M. S. Mooseker. 1983. Characterization of the 110 kdalton actin-, calmodulin-, and membrane-binding protein from microvilli of intestinal epithelial cells. *J. Cell Biol.* 97:974–985.

Janmey, P. A., and P. T. Matsudaira. 1988. Functional comparison of villin and gelsolin. Effects of Ca^{2+}, KCl, and polyphosphoinositides. *J. Biol. Chem.* 263:16738–16743.

Kadowaki, T., S. Koyasu, E. Nishida, H. Sakai, F. Takaku, I. Yahara, and M. Kasuga. 1986. Insulin-like growth factors, insulin, and epidermal growth factor cause rapid cytoskeletal reorganization in KB cells: clarification of the roles of type I insulin-like growth factor receptors and insulin receptors. *J. Biol. Chem.* 261:16141–16147.

Lazarides, E. 1975. Tropomyosin antibody: the specific visualization of tropomyosin in non-muscle cells. *J. Cell Biol.* 65:549–561.

Lazarides, E., and K. Burridge. 1975. α-Actinin: Immunofluorescent localization of a muscle structural protein in non-muscle cells. *Cell.* 6:289–298.

Louvard, D. 1989. The function of the major cytoskeletal components of the brush border. *Curr. Opin. Cell Biol.* 1:51–57.

Matsudaira, P. T., and D. R. Burgess. 1979. Identification and organization of the components of the isolated microvillus cytoskeleton. *J. Cell Biol.* 83:667–673.

Matsudaira, P. T., and P. A. Janmey. 1988. Pieces in the actin-severing protein puzzle. *Cell.* 54:139–140.

Maunoury, R., S. Robine, E. Pringault, C. Huet, J. L. Guenet, J. A. Gaillard, and D. Louvard. 1988. Villin expression in the visceral endoderm and in the gut anlage during early mouse embryogenesis. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:3321–3329.

Mooseker, M. S. 1985. Organization, chemistry, and assembly of the cytoskeletal apparatus of the intestinal brush border. *Annu. Rev. Cell Biol.* 1:209–241.

Pollard, T. D., and J. A. Cooper. 1986. Actin and actin-binding proteins. A critical evaluation of mechanisms and functions. *Annu. Rev. Biochem.* 55:987–1035.

Robine, S., C. Huet, R. Moll, C. Sahuquillo-Merino, E. Coudrier, A. Zweibaum, and D. Louvard. 1985. Can villin be used to identify malignant and undifferentiated normal digestive epithelial cells? *Proc. Natl. Acad. Sci. USA.* 82:8488–8492.

Sanger, J. M., B. Mittal, A. Wegner, B. M. Jockusch, and J. W. Sanger. 1987. Differential response of stress fibers and myofibrils to gelsolin. *Eur. J. Cell Biol.* 43:421–428.

Shibayama, T., J. M. Carboni, and M. S. Mooseker. 1987. Assembly of the intestinal brush border: appearance and redistribution of microvillar core proteins in developing chick enterocytes. *J. Cell Biol.* 105:335–344.

Stossel, T. P., C. Chaponnier, R. M. Ezzell, J. H. Hartwig, P. A. Janmey, D. J. Kwiatkowski, S. E. Lind, F. S. Southwick, H. L. Yin, and K. S. Zaner. 1985. Non-muscle actin binding proteins. *Annu. Rev. Cell Biol.* 1:353–402.

Weber, K., and U. Groeschel-Stewart. 1974. Myosin antibody: the specific visualization of myosin containing filaments in non-muscle cells. *Proc. Natl. Acad. Sci. USA.* 71:4561–4564.