In Vivo Analysis of Functional Domains from Villin and Gelsolin

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Abstract. Transfected CV1 cells were used to compare the in vivo effects of various domains of villin and gelsolin. These two homologous actin modulating proteins both contain a duplicated severin-like sequence. Villin has in addition a carboxy-terminal domain, the headpiece, which accounts for its bundling activity.

The effects of the villin-deleted mutants were compared with those of native villin. Our results show that essential domains of villin required to induce the growth of microvilli and F-actin redistribution are present in the first half of the core and in the headpiece. We also show that the second half of the villin core cannot be exchanged by its homolog in gelsolin.

When expressed at high levels of CV1 cells, full length gelsolin completely disrupted stress fibers without change of the cell shape. Addition of the villin headpiece to gelsolin had no effect on the phenotype induced by gelsolin alone. Expression of the first half of gelsolin induced similar modifications as capping proteins and rapid cell mortality; this deleterious effect on the cell structure was also observed when the headpiece was linked to the first half of gelsolin. In cells expressing the second half of gelsolin, a dotted F-actin staining was often seen. Moreover elongated dorsal F-actin structures were observed when the headpiece was linked to the second gelsolin domain.

These studies illustrate the patent in vivo severing activity of gelsolin as well as the distinct functional properties of villin core in contrast to gelsolin.

VILLIN and gelsolin are two highly homologous calcium-regulated proteins modulating actin polymerization. Villin, first isolated by Bretscher and Weber (1979), is a specific marker for epithelial cells involved in absorptive processes. Gelsolin (Yin and Strossel, 1979), widely distributed in mammalian cells (Yin et al., 1981, 1984; Kwiatkowski et al., 1988), exists in two isoforms, a cytoplasmic one and a secreted one, which derive from a single gene by alternative splicing (Kwiatkowski et al., 1986, 1988). Cytoplasmic gelsolin has been proposed to be implied in a number of physiological functions requiring rapid actin cytoskeleton reorganization, such as endocytosis (Yin et al., 1981), signal transduction (Chaponnier et al., 1987), and cell locomotion (Cunningham et al., 1991).

Villin and gelsolin (respective molecular masses of 92 kD and 81 kD), can sever, cap, and nucleate actin filaments in a calcium-regulated manner (Yin and Stossel, 1979; Glenney et al., 1981*a*; Matsudaira et al., 1985). Some of these activities are also modulated by polyphosphoinositides (Janmey and Matsudaira, 1988). However, gelsolin severs F-actin in the presence of micromolar calcium (Yin and Stossel, 1979; Coue and Korn, 1985), while half-maximal villin severing activity requires 200 μM calcium (Northrop et al., 1986). In addition, villin, but not gelsolin, bundles actin filaments (Bretscher and Weber, 1980) at low calcium concentration (<1 μM). A large number of experiments carried out with proteolytic fragments from villin and gelsolin have allowed the definition of functional domains in each protein. In summary (see Fig. 1 for references), villin's capping, nucleating, and severing functions as well as its calcium and polyphosphoinositide regulation have been localized to an 87-kD aminoterminal domain of the protein, named core. Villin's bundling activity requires both the core and the carboxy-terminal 8.8 kD of the molecule named headpiece. In gelsolin, severing, capping, and polyphosphoinositides regulation have been assigned to the amino-terminal half of the protein, while nucleation and at least part of the calcium regulation have been localized to the carboxy-terminal half.

Analysis of the cDNA sequences of villin (Arpin et al., 1988; Bazari et al., 1988) and gelsolin (Kwiatkowski et al., 1986) showed 45% homology between the two proteins. Both contain a duplicated protein segment (that we denote V1 and V2 in villin, G1 and G2 in gelsolin), homologous with the 40-kD severing proteins from lower eucaryotes: severin (André et al., 1988) and fragmin (Ampe et al., 1987). In addition, villin has a carboxy-terminal domain coding for the headpiece, unique among all actin binding

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proteins so far described (see Fig. 2). The structural symmetry observed in villin core and in gelsolin does not parallel the functional asymmetry noted above for each protein.

We have previously reported (Friederich et al., 1989) that expression of the villin cDNA in CV1 cells induced the growth of long microvilli at the cell surface accompanied by redistribution of F-actin; these effects required the presence of the headpiece. Similar observations were later made by Franck et al. (1990) using microinjection to introduce villin into 3T3 cells. In contrast, microinjection of gelsolin into fibroblasts has given variable results (Cooper et al., 1987; Huckriede et al., 1990).

In this paper, we compared in the same in vivo system (transfected CV1 cells), the expression of several mutants of villin and gelsolin. We studied two parameters: (*a*) the subcellular localization of the proteins and their association with the F actin filaments, and (*b*) their ability to induce modifications of the actin cytoskeleton.

Materials and Methods

Cell Culture

Unless otherwise indicated, CV1 cells were grown in Dulbecco's minimum medium supplemented with 10% FCS at 37°C, under a 10% CO_2 atmosphere.

Transient cDNA Expression in CV1 Cells

CV1 cells were transfected using DEAE dextran as described previously (Friederich et al., 1989) with the following modifications. To limit cell damage the DNA-DEAE dextran incubation step was reduced to 30 min. After removal of the DNA-DEAE dextran mixture, the cells were treated for 2–3 h with culture medium containing chloroquine (50 μg/ml; Sigma Chemical Co., St. Louis, MO) under a 5% CO_2 atmosphere. Cells were analyzed 40–72 h after addition of DNA.

Recombinant DNA Constructions

For DNA engineering, standard procedures as described by Sambrook et al. (1989) were used.

All the constructs were engineered in pSP, pGEM (Promega Biotec, Madison, WI), Bluescript or Bluescribe (Stratagene, La Jolla, CA) vectors, and then inserted into expression vector pSV51 (Huylebroeck et al., 1988). Oligonucleotide linkers were purchased from Pharmacia Fine Chemicals (Pharmacia, France). The position of the restriction sites used in the constructions are indicated in Fig. 2 (*Villin Deletion Mutants*).

For construction of the V1+ headpiece mutant, the full-length villin cDNA (Arpin et al., 1988) was digested with BamHI. Ends were filled in with the Klenow fragment of DNA polymerase I. After ligation of an octomeric KpnI linker, the plasmid was recircularized. Digestion with KpnI removed a 906-bp fragment encoding the second half of the core. The religated plasmid (V1+ headpiece), coded for a mutant in which villin residue 411 was directly linked to residue 714. For the construction of the ΔNT-villin mutant, villin cDNA was digested with SmaI and an octomeric AatII linker was ligated to the plasmid as described above. Digestion with AatII removed a 351-bp fragment. The religated plasmid coded for a mutant in which villin residue 16 was directly connected to residue 134.

Gelsolin and Gelsolin Deletion Mutants. Gelsolin cDNA (Kwiatkowski et al., 1986) was excised from Bluescript and directly inserted into pSV51. The construction encoding the first domain of gelsolin was obtained by deletion of the cDNA segment comprised between two NcoI sites (bp positions 1310 and 2342 in cytoplasmic gelsolin cDNA). The religated plasmid coded for a mutant in which cytoplasmic gelsolin residue 380 was directly linked to residue 725. To construct the mutant encoding the second half of gelsolin, the cDNA fragment located downstream from the BglII site (position 1345) was purified. To reconstitute the amino-terminal part of the second gelsolin domain, an adaptor was created by annealing two 40 mers oligonucleotides. This adaptor included an EcoRI site, an initiator ATG as well as the nucleotides encoding gelsolin residues 388–396. The resulting construct then coded for a gelsolin mutant comprising residues 387–730.

Gelsolin-Villin Chimeras. Chimera 1 (Gelsolin + headpiece) was sequentially assembled in a Bluescribe vector before subcloning in pSV51. A NcoI/BamHI adaptor (C ATG GAT CCC TTC AAG) was used to link cytoplasmic gelsolin residue 725 to villin residue 713 directly. Chimera 2 (G1 + headpiece) and chimera 3 (G2 + headpiece) were derived from the chimera 1 construct in a similar way as described above for construction of gelsolin deletion mutants. Chimera 2 connected the gelsolin residues 1–380 with the villin fragment 713–825. Chimera 3 connected gelsolin residues 387–725 with the villin fragment 713–825. Chimera 4 (V1 + G2 + headpiece) was assembled as follows. A villin cDNA fragment encoding the V1 domain (see villin constructs) was subcloned in pSP72 vector. This fragment and a 28 oligonucleotide portion of the vector polylinker was inserted upstream the BglII site in chimera 3 (G2 + headpiece). Chimera 4 then comprised: villin residues 1–410, a linker portion encoding 7 extra amino acids (S-/W-/N-/S-/S-/M-/M), gelsolin residues 397–725 and the villin residues 713–825.

Villin and Gelsolin Antibodies

Monoclonal anti-human villin antibodies have been previously described (Dudouet et al., 1987). Polyclonal anti-gelsolin antibodies were raised in rabbits using purified human plasma gelsolin (generous gift from J. Bryan, Baylor University, Houston, TX) and affinity purified as previously described (Coudrier et al., 1981).

Immunoblotting

Transfected cells plated on 3.5-cm culture dishes were washed twice with ice-cold PBS and lysed by addition of 200 μl of Laemmli sample buffer (Laemmli, 1970). Cell lysates were immediately transferred to an Eppendorf tube and boiled for 5 min. 10 μl of each cell lysate was used for immunoblotting analysis. Proteins were separated by SDS-PAGE under reducing conditions. Transfer to nitrocellulose and antibody incubations were performed according to the method described by Burnette (1981) and modified as previously described (Coudrier et al., 1983).

Indirect Immunofluorescence

Transfected cells were fixed with 3% paraformaldehyde, permeabilized with 0.2% Triton X-100 and analyzed by indirect immunofluorescence as described (Reggio et al., 1983). Staining was performed by incubation with either the monoclonal villin antibody or the affinity-purified polyclonal anti-gelsolin antibodies (0.5 μg/ml for each), followed by incubation with fluorescein-conjugated anti-IgG antibodies (Biosys, Compiègne, France) which were rabbit IgG specific in the case of polyclonal anti-gelsolin antibodies, or mouse IgG specific in the case of the monoclonal anti-villin antibody. For simultaneous visualization of F-actin distribution, rhodamine-phalloidin (2 U per dish; Sigma Chemical Co.) was added to the anti-villin or anti-gelsolin antibodies.

Results

Recombinant DNA Constructions

DNA constructs are summarized in Fig. 2. Based on the previous in vitro studies with villin proteolytic fragments (Fig. 1), two villin deletion constructs were generated (Fig. 2, *left column*). In the first one, denoted V1 + headpiece, the first half of villin core was directly joined to the headpiece. Referring to villin proteolytic fragments this construct connected the 44T domain to the headpiece. Since all the villin functions so far identified have been located to these two fragments, we hypothesized that this mutant should have similar functions as native villin. In the second deletion mutant, denoted ΔNT-villin, we removed a region corresponding to the 14-kD proteolytical fragment described by Janmey and Matsudaira (1988).

Two constructs representing the amino-terminal and carboxy-terminal halves of gelsolin were engineered from cytoplasmic gelsolin cDNA (Fig. 2, *middle column*).

Four gelsolin-villin chimeras were constructed (Fig. 2,

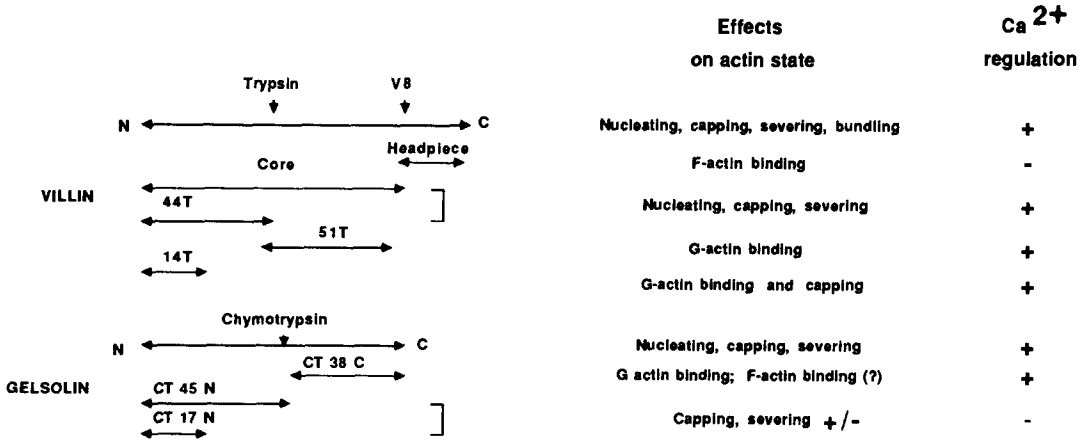


Figure 1. Summary of the functional properties of proteolytic fragments from villin and gelsolin assayed in vitro. The protease V8 cuts villin into a large amino-terminal 87-kD fragment, the core, and a small carboxy-terminal 8.8-kD fragment, the headpiece (Glenney et al., 1981*b*). The core retains the calcium-regulated nucleating, capping, and severing activities exhibited by native villin. The headpiece binds F-actin in a calcium-independent manner. The bundling function is lost by the cleavage of the molecule in these two domains indicating that one of the F-actin binding sites is located in the core while the other is located in the headpiece. Trypsin cleaves villin in two fragments: (Matsudaira et al., 1985; Janmey and Matsudaira, 1988) the amino-terminal one, *44T*, retains the same functions as the core except that nucleation ability is reduced. The carboxy-terminal fragment, *51T*, binds G-actin in the presence of calcium. Further digestion of 44T led to a 14-kD fragment (*14T*) which binds G-actin and has a partial capping activity (Janmey and Matsudaira, 1988). Chymotrypsin divides gelsolin in two fragments (Kwiatkowski et al., 1985; Yin et al., 1988; Bryan, 1988). The amino-terminal one, *CT 45*, caps and severs actin, but has no calcium regulation and no nucleating activity. The carboxy-terminal domain. *CT 38*, binds G-actin in a calcium-regulated manner; it could also have some F-actin binding properties as suggested by cross-linking studies (Sutoh and Yin, 1989). The amino-terminal segment *CT17* binds G-actin but has a poor capping activity (Chaponnier et al., 1986).

right column). In chimera 1 (Gelsolin + headpiece), we replaced the complete villin core domain by the homologous gelsolin sequence. In this construction, the last five amino acids from gelsolin are missing, however, earlier in vitro studies have indicated that deletion of the last 17 gelsolin residues had no effect on its function (Kwiatkowski et al., 1989). Chimera 2 (G1 + headpiece) and chimera 3 (G2 + headpiece) both derived from chimera 1 by deletion of either the amino-terminal or the carboxy-terminal half of gelsolin. Such chimeras were analyzed to test if addition of the villin headpiece could interfere with gelsolin domain functions. In chimera 4 (V1 + G2 + headpiece), the amino-terminal half

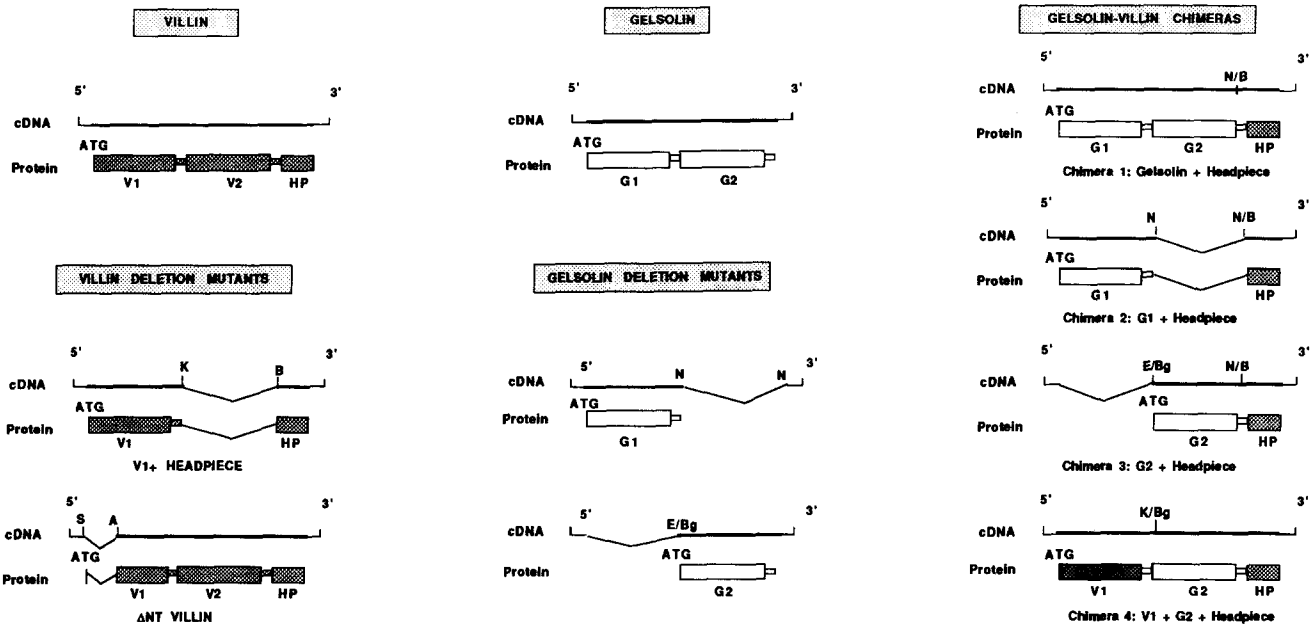


Figure 2. Schematic representation of the structure of villin and gelsolin. Design of villin and gelsolin deletion mutants and of the gelsolin–villin chimeras. Wild-type villin and gelsolin structures are represented. Each protein contains a duplicated severin-like protein segment that we denote *V1* and *V2* in villin, *G1* and *G2* in gelsolin. *HP* indicates the villin headpiece. The restriction enzyme sites used in the various constructions are indicated with the first letters of the enzyme (*K* for KpnI; *B* for BamHI; *S* for SmaI; *A* for AatII; *N* for NcoI; *E* for EcoRI; and *Bg* for BglII).

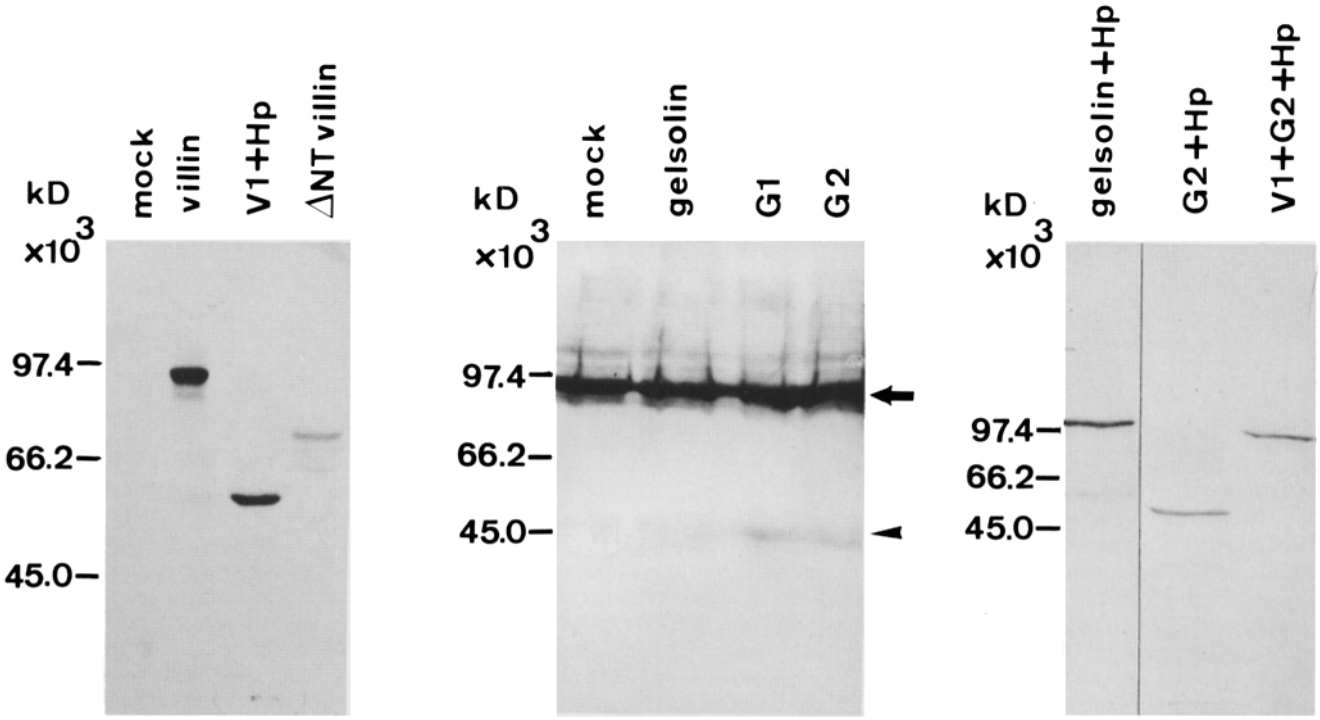


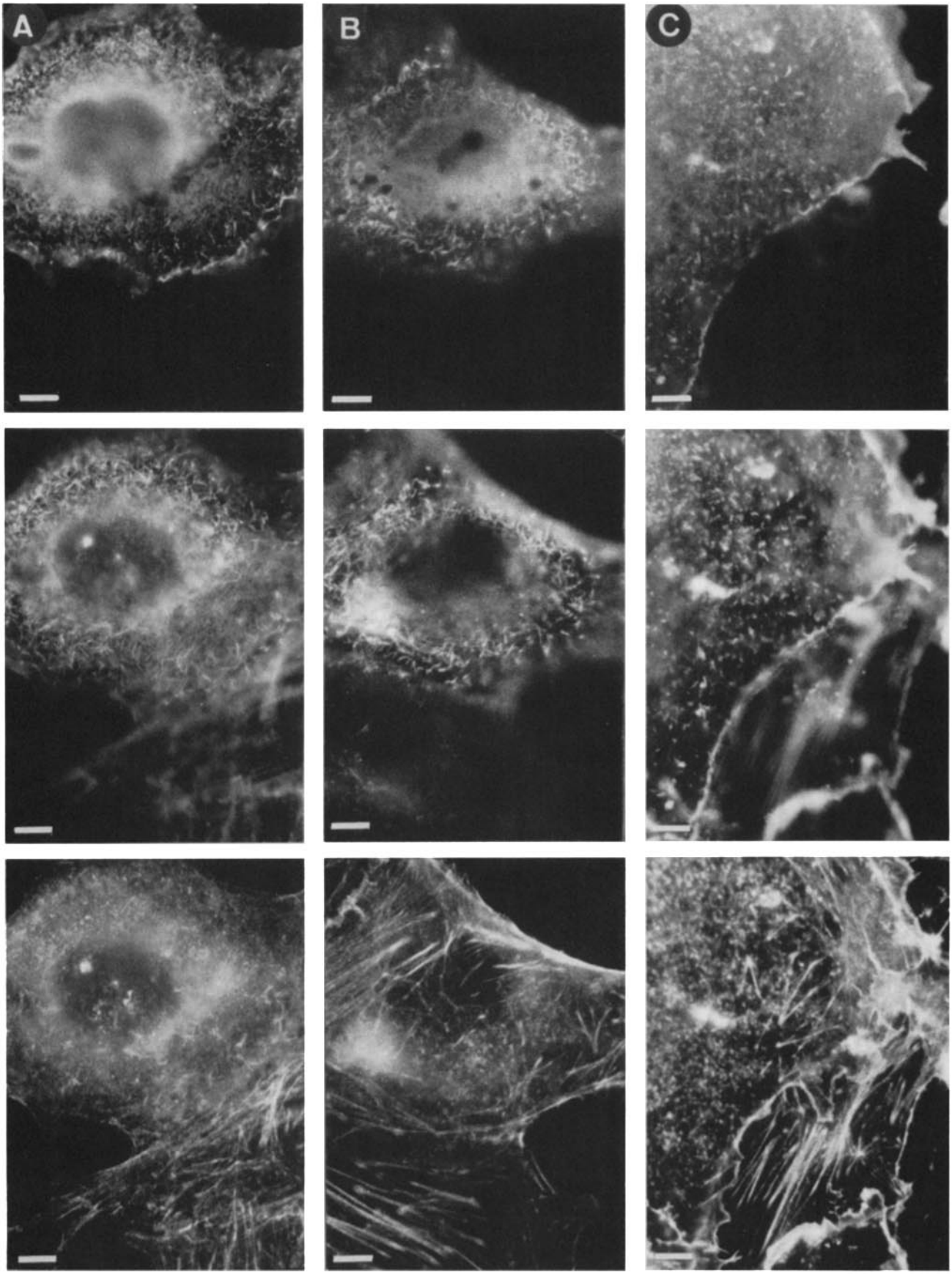
Figure 3. Immunoblotting analysis of villin, gelsolin, and mutants produced in transfected CV1 cells. Lysates from cells transfected with villin or villin deletion mutants were analyzed with the specific headpiece antibody (*left panel*). Mock transfected cells and cells transfected with wild-type villin cDNA were used as negative and positive controls, respectively. The apparent molecular masses of the villin deletion mutants were 60 and 84 kD. Lysates from cells transfected with gelsolin or gelsolin domains were incubated with the polyclonal antibody raised against human plasma gelsolin (*middle panel*). Mock transfected cells were used as positive control. Transfected gelsolin comigrates with endogenous gelsolin. The estimated molecular masses of the gelsolin deletion mutants (*G1* and *G2*) were both 46 kD. Lysates of cells transfected with gelsolin–villin chimeras were analyzed with the headpiece specific antibody (*right panel*). The apparent molecular masses of the gelsolin–villin chimeras were 97, 52.5, and 92 kD, respectively.

of the villin core was linked via a seven residue spacer to chimera 3. In this mutant, the second domain of the villin core (V2), is then replaced by the equivalent domain of gelsolin (G2).

Monitoring the Synthesis of the Mutant Proteins

Immunoblotting was only used as an assay to monitor that all the constructs gave rise to the right size polypeptides (Fig. 3). Villin deletion mutants (*left panel*) were detected using a villin specific mAb. Since this antibody recognizes an epitope at the carboxy-terminal end of the headpiece (E. Friederich, unpublished observations), the use of this reagent also confirmed the conservation of the reading frame in the headpiece containing constructs. Cells transfected with wild-type villin were used as positive controls. Immunoblots of lysates from CV1 cells producing villin deletion mutants revealed protein bands with an apparent molecular mass of 84 and 60 kD, respectively, corresponding to that calculated from the amino acid sequences (Arpin et al., 1988). No band was visualized in the lane corresponding to mock transfected CV-1 cells. Mutants containing gelsolin or gelsolin fragments were detected using a polyclonal gelsolin antibody (*middle panel*). In untransfected cells, this antiserum revealed a strong band with an apparent molecular mass of 87 kD, corresponding to endogenous gelsolin of CV1 cells. In lysates from cells transfected with the full length gelsolin cDNA, no additional band could be detected, suggesting comigration of the endogenous gelsolin and of the recombinant gelsolin coexpressed in positive cells. Moreover, immunofluorescence observations (see below) confirmed the overexpression of gelsolin in transfected cells. Faint bands of the expected size, 46 kD, were detected in lysates from cells transfected with the first or second domain of gelsolin. The gelsolin–villin chimeras were analyzed using the monoclonal villin antibody allowing their specific identification (*right panel*). The apparent molecular masses were in agreement with those predicted for each chimera (97, 52.5, and 92 kD).

Figure 4. Effects of wild-type villin and villin deletion mutants on F-actin organization in CV1 cells. Expression of wild-type villin (*column A*), V1+Headpiece mutant (*column B*), or ΔNT villin mutant (*column C*) was analyzed 72 h after transfection. Double fluorescence stainings are shown. The transfected proteins were labeled with the villin headpiece specific mAb and a fluorescein-conjugated secondary antibody. F-actin was stained with rhodamine-phalloidin. The upper panels are micrographs of villin and villin mutant-transfected cell: they illustrate the protein labeling at the top (dorsal face) of the cells. The same cells are shown in the middle and lower panels; those visualize the F-actin distribution on the dorsal faces (*middle panels*) or on the ventral faces (*lower panels*) of the cells. Bars, 10 μm.


A
B
C

Effects of Mutants and Chimeras on F-Actin Organization in CV-1 Cells

Transfected cells were examined by double-fluorescence staining to visualize both mutant or chimeric protein distribution and the F-actin architecture (rhodamine–phalloidin). Since the effects of wild-type villin on actin cytoskeleton are dependent on high synthesis levels (Friederich et al., 1989), we report here our observations on cells producing large amounts of the transfected proteins, as evaluated from the intensity of the immunofluorescence signal. This procedure is indeed not quantitative but has the advantage of examining a cell population on the basis of a single cell.

Villin Mutants. The effects of villin deletion mutants on F-actin organization were compared to those observed with wild-type villin (Fig. 4). We looked specifically for two easily detectable morphological modifications seen in villin transfected cells: the formation of spike-like F-actin structures on the dorsal face and the loss of stress fibers at the ventral face of CV1 cells (Friederich et al., 1989). Upper panels represent micrographs focused on the top of the cells stained with a villin antibody. Middle and lower panels show images of phalloidin stainings focused on the top and on the bottom of the cells, respectively.

Column *A* in Fig. 4 shows a representative cell expressing high levels of villin and illustrates the effects of the wild-type villin on F-actin network organization. A large number of elongated spike-like F-actin structures, which ultrastructural observations have shown to be long microvilli (Friederich et al., 1989), are seen on the dorsal face of CV1 cells (*middle panel*). The colocalization of villin label (*upper panel*) with these actin structures is only clearly visible here in some parts of the cells, probably because of the high level of villin expression causing the blurred staining. Focusing on the bottom of the same cell (*lower panel*) demonstrated an absence of stress fibers, which were unperturbed in the adjacent villin-negative cell.

High level expression of the mutant V1 + headpiece (Fig. 4, *column B*) caused modifications of the F-actin cytoskeleton comparable to that observed with the wild-type villin. As seen in the middle panel, long spike-like F-actin structures also occurred, which were absent from the neighboring negative cell. The protein label (*upper panel*) co-distributed with that of F-actin structures (*middle panel*). A partial disruption of stress fibers and a diffuse phalloidin label were observed at the ventral face of the cells (*lower panel*). In this area of the cell, the villin mutant staining was diffuse (data not shown). However, in comparison with cells producing wild-type villin, those synthesizing the V1 + headpiece mutant, displayed less frequently and to a smaller degree these morphological changes.

Analysis of cells producing the ΔNT-villin mutant (Fig. 4, *column C*) showed that this mutant did not induce the formation of spike-like F-actin structures (*middle panel*). These structures were never observed, even in cells producing very large amounts of the mutant protein. However, the protein label co-distributed with dotted F-actin stainings (*upper* and *middle panels*). We have previously shown that this pattern corresponds to F-actin structures forming the cytoskeleton of the rudimentary microvilli present on the dorsal faces of normal CV1 cells (Friederich et al., 1989). At the ventral face of the same ΔNT-villin transfected cell (*lower panel*), actin staining revealed a significant reduction of stress fibers, and in addition, a dotted F-actin staining pattern.

Effects of Gelsolin and Gelsolin Deletion Mutants. Labeling of gelsolin and gelsolin mutants are shown in Fig. 5, *A*, *C*, and *E*, while *B*, *D*, and *F* show the corresponding actin staining.

In cells expressing high levels of gelsolin (*A*), the distribution of the protein was diffuse and a patchy appearance of the labeling was often seen. Notice here that endogenous staining in adjacent untransfected cells was very faint compared with the intensity of the fluorescence in highly positive cells, explaining why it was not possible to visualize endogenous gelsolin on the same micrograph. But the most striking effect, in cells expressing large amounts of gelsolin, was the nearly complete disruption of stress fibers (*B*) without change in the gross shape of the cells. In cells expressing lower levels of gelsolin, colocalization of the protein staining with the short F-actin microfilaments in the rudimentary microvilli on the dorsal face of the cells was observed and stress fibers did not appear significantly affected (data not shown). However, we did not observe an association of gelsolin with stress fibers, possibly because of the strong diffuse labeling caused by an increased amount of cytoplasmic gelsolin.

Expression of the first half of gelsolin had marked deleterious effects on the cells (*C* and *D*, the cell on the left side of each micrograph). Transfected cells exhibited an abnormal shape and a complete disruption of the actin cytoskeleton, which appeared retracted and concentrated into foci into which the mutant protein colocalized. We could also visualize on the same micrograph, endogenous gelsolin labeling in untransfected cells, which was essentially associated with stress fibers (*C* and *D*, cells on the right side of each micrograph). It is possible that, in spite of the fact that detergent permeabilization was done after fixation of the cells, some of the cytoplasmic gelsolin was lost (Cooper et al., 1988).

In cells producing the second half of gelsolin (*E* and *F*), a dotted distribution of the protein labeling was often observed at the dorsal face of the cells (*E*). This staining colocalized with the normal F-actin structures on the dorsal face. In cells expressing large amounts of this mutant, a partial disorganization of stress fibers occurred and a dotted F-actin label was seen near the ventral face of the cells (*F*). We did not notice any cell mortality in transient transfection experiments with G2.

Villin and Gelsolin Chimeras. Fig. 6 shows double-immunofluorescence of cells transfected with three of the chimeras. The chimeric protein distributions (visualized using the headpiece specific antibody) are shown in *A*, *C*, and *E*, while the corresponding F-actin network organizations are shown in *B*, *D*, and *E*. Analysis of cells producing chimera 1 (Gelsolin + headpiece) revealed a protein labeling pattern similar to that observed with wild-type gelsolin, but with brighter staining of the cell periphery in comparison with gelsolin. Part of the chimeric protein colocalized with stress fibers (*A*). However, rhodamine–phalloidin staining in the same area (*B*) revealed that only a few stress fibers were present in the positive cell, compared to the strong phalloidin labeling in the surrounding negative cells. This effect was essentially similar to that observed in cells expressing high amounts of gelsolin. Importantly, whatever the amount of the chimeric protein synthesized, as we could judge from

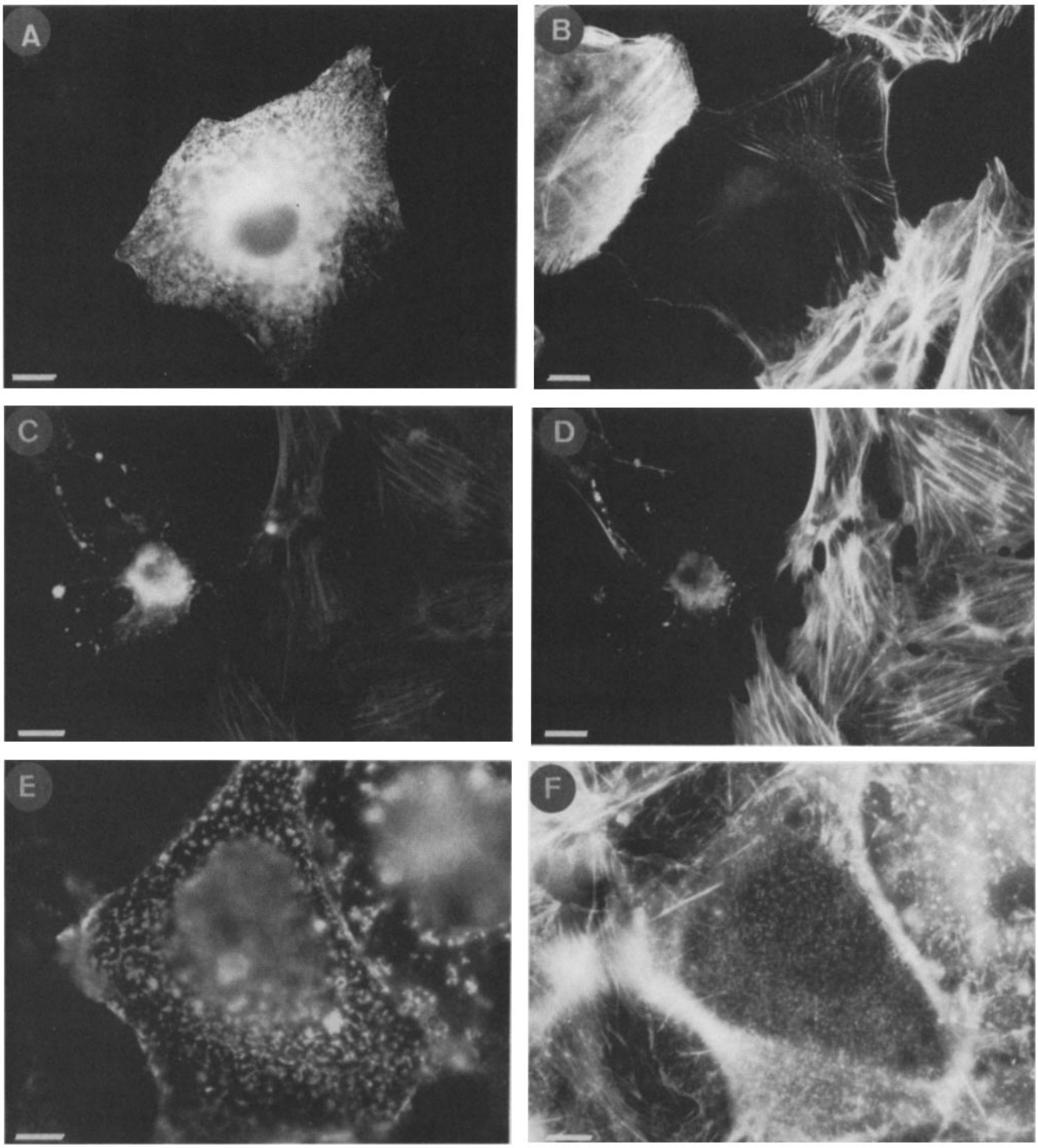


Figure 5. Expression of human cytoplasmic gelsolin and gelsolin mutants in CV1 cells. Expression of wild-type gelsolin (*A* and *B*), the first half of gelsolin (*C* and *D*), and the second half of gelsolin (*E* and *F*). Cell cultures were fluorescently labeled for the transfected proteins using an affinity purified gelsolin antibody and a fluorescein-conjugated secondary antibody (*A*, *C*, and *E*). F-actin stainings (rhodamine-phalloidin) are shown in *B*, *D*, and *E*. A representative cell expressing the first half of gelsolin (G1) is visualized in the left part of *C* and *D*. The lower expression level of this mutant required longer exposition times, explaining why we could also detect on the same micrograph, endogenous gelsolin in untransfected cells (right part of *C* and *D*). *E* shows the dorsal face of a CV1 cell transfected with the second domain of gelsolin (G2) and *F* illustrates the F-actin distribution at the bottom of the same cell. Bars, 10 μm.

the fluorescence intensity, we never observed any spike formation on the dorsal faces of the cells. In cells expressing chimera 2 (G1 + headpiece), morphological alterations were very similar to those described in cells transfected with the first domain of gelsolin alone (data not shown). Cells expressing chimera 3 (G2 + headpiece) showed different modifications than those transfected with the second domain of gelsolin alone. Fig. 6 *D* visualizes the F-actin structures

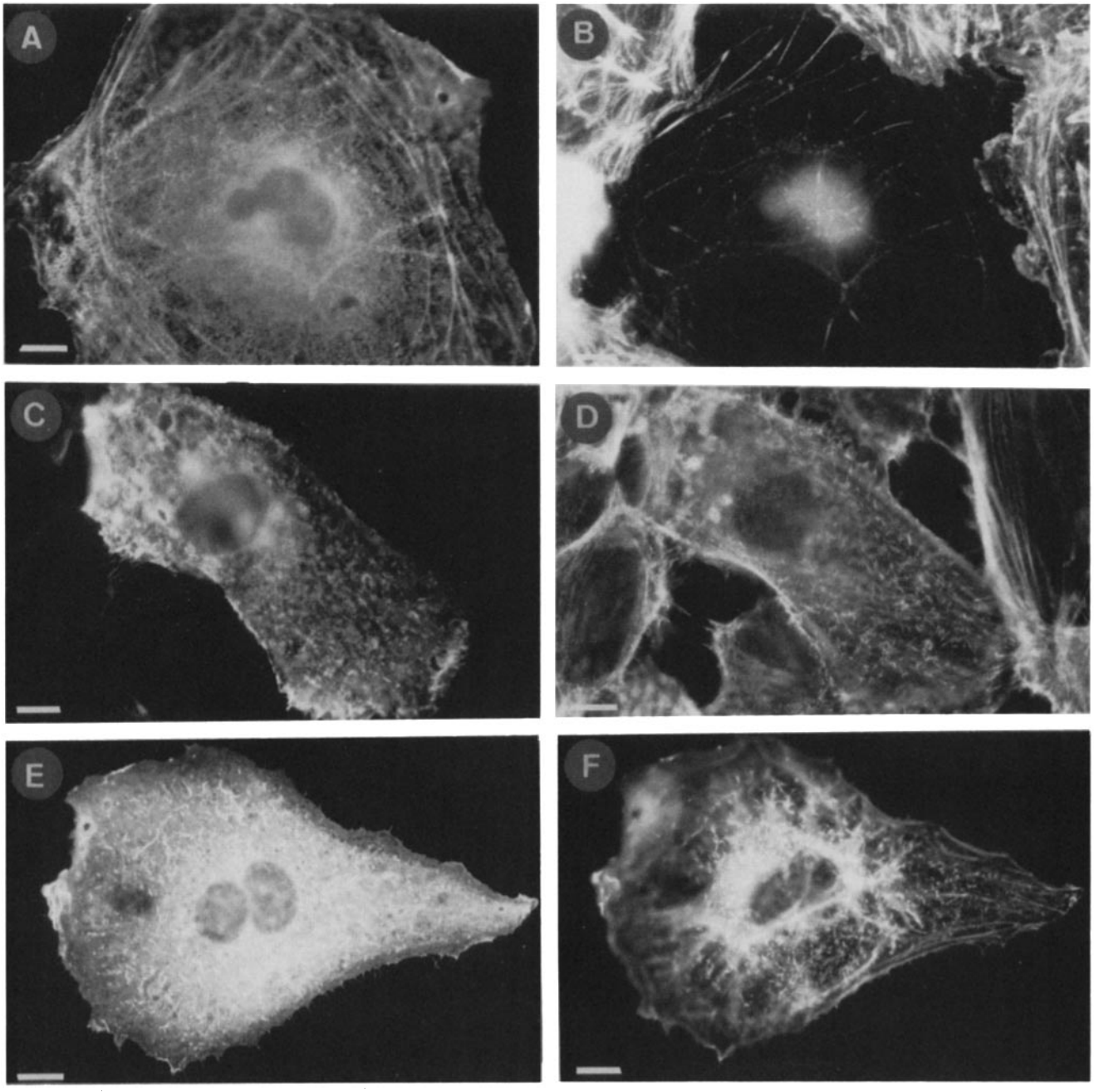


Figure 6. Effects of gelsolin–villin chimeras on the formation of F-actin spike-like structures and actin cytoskeleton reorganization. Expression of gelsolin–villin chimeras (described in Fig. 2). Cell cultures were fluorescently double labeled for the transfected protein and for F-actin as described in Fig. 4. Protein stainings are shown in *A*, *C*, and *E* and F-actin stainings in *B*, *D*, and *F*. *A* and *B*: chimera 1 (Gelsolin + headpiece); *C* and *D* show the top of a cell transfected with chimera 3 (G2 + headpiece). *E* and *F* illustrate a cell transfected with chimera 4 (V1 + G2 + headpiece); notice here that *E* and *F* photos are different focal planes of the same cell: *E* (top of the cell); *F* (bottom of the cell). Bars, 10 μm.

at the dorsal face of a positive cell, which are slightly more numerous, longer, and straighter than the rudimentary microvilli seen in negative cells. This effect was less intense than that seen with the mutant V1 + headpiece (Fig. 4, *column B*), but was consistently observed in numerous transfection experiments. The expressed construct colocalized to these modified F-actin structures at the dorsal face of the cells (Fig. 6 *C*). No modification of the stress fiber organization was seen in cells transfected with the chimera 3 (data not shown). Chimera 4 (V1 + G2 + headpiece) had no reproducible effect on the F-actin organization at the dorsal face of the cells and did not induce any modification of the ventral stress fibers system (*E* and *F*). Colocalization of part of this chimeric protein with F-actin was seen in the stress fibers and in the rudimentary microvilli on the dorsal face of the cells.

Discussion

Most of the data on the activities of villin and gelsolin do-

mains have been obtained from in vitro studies using either proteolytically derived fragments (see Introduction and Fig. 1 for references) or gelsolin deletion mutants expressed in *Escherichia coli* (Way et al., 1989, 1990; Pope et al., 1991) or in Cos cells (Kwiatkowski et al., 1989). In vivo studies are another approach to better understand the physiological role of these proteins. Here, we have compared the effects of villin and gelsolin domains in CV1 cells transfected with cDNAs encoding a variety of mutants and chimeras. The chimeras are of particular interest, as they permit direct comparison between homologous domains of villin and gelsolin. The effects of the various mutants on spike formation and actin cytoskeleton disorganization are summarized in Table I.

All mutant proteins colocalized with microfilament-containing structures, suggesting that they associated with F-actin and were at least partially functional. This observation favors the hypothesis that in all cases conformationally correct proteins resulted from the cDNA constructions. We have previously shown that the effects of villin on actin cytoskeleton are dependent on high synthesis levels. In this study, we only describe the effects that we observed for each mutant in highly expressing cells, as judged from the intensity of the immunofluorescence signal. However, as it is not possible to quantify, in transient transfection experiments, the amount of protein produced per cell, it is difficult to directly correlate the effects observed with expression levels of the various constructs. Consequently, it is possible that some activities of the constructs were not detected in our transfection experiments because of an expression level insufficient to produce a detectable morphological effect. Nevertheless transient transfection experiments provide a useful approach since they permit visualization of phenotypic effects that may not constitute a selective advantage in permanent transformants as seems to be the case for cells overexpressing gelsolin (Cunningham et al., 1991).

Table I. Summary of the Effects of the Various Mutants on the Formation of Spike-like F-Actin Structures and on the Disruption of Stress Fibers

Constructs	F-actin spike-like induction	Actin cytoskeleton modifications
Villin and villin deletion mutants		
Villin	++	+
V1 + headpiece	+	±
ΔNT villin	–	+
Gelsolin and gelsolin deletion mutants		
Gelsolin	–	+++
G1	–	+++
G2	–	+
Gelsolin–villin chimeras		
Gelsolin + headpiece	–	+++
G1 + headpiece	–	+++
G2 + headpiece	±	–
V1 + G2 + headpiece	–	–

Most of the Information for Villin Function Is Contained in the First Half of the Core and in the Headpiece Domain

Based on previous reports indicating that, in vitro, functionally relevant villin sequences are located in these two domains (Fig. 1), we examined the in vivo effects of a villin mutant missing the second half of villin core. Our observations suggest that this mutant conserves the minimal structural requirements for villin function. However, an alteration in the spacing/three dimensional presentation of domains in the core and the headpiece may explain that the mutant was less efficient than native villin in reorganizing the actin cytoskeleton. Alternatively, the second half of villin core may directly contribute to villin function. We examined also whether or not, the second domain of gelsolin could functionally replace the second half of villin core. Although chimera 4 (V1 + G2 + headpiece) contained all sequence present in the truncated villin construct (V1 + headpiece), it never induced F-actin spikes formation. These observations indicate that the second half of villin core and its homolog in gelsolin are not functionally equivalent.

To investigate the functional importance of the amino terminal end of villin, we constructed a mutant (ΔNT- villin), missing a sequence corresponding to the 14-kD proteolytic fragment (Fig. 1). This fragment binds to G-actin and partially caps actin filaments in vitro (Janmey and Matsudaira, 1988). Moreover, it contains a sequence (amino acid residues 72–93) which is highly homologous in villin, severin, and gelsolin. In severin, this sequence contains an actin-binding site and is crucial for capping (Eichinger et al., 1991). As the ΔNT-villin mutant never induced the formation of F-actin spikes, we suggest that the capping activity of villin may be necessary to induce spike formation. Capping and nucleating activities are closely related in villin (Glenney et al., 1981*a*; Janmey and Matsudaira, 1988), so nucleation may also be necessary for spike induction. It is therefore important to determine in vitro whether or not this mutant retains nucleating activity.

Disorganization of the actin cytoskeleton was observed in cells transfected with villin or villin mutants. The recruitment of actin monomers in the newly formed F-actin structures could account, at least partially, for the loss of stress fibers. However, the fact that in cells transfected with the ΔNT-villin mutant, the formation of F-actin spikes and stress fiber disruption are uncoupled, favors the idea that other mechanisms may be involved. Such mechanisms could also occur in cells transfected with the second half of gelsolin and, as previously reported, in those transfected with the villin headpiece (Friederich et al., 1989). We speculate that these various mutant proteins disrupt stress fibers by the displacement of stress fiber stabilizing proteins. This hypothesis is strengthened by the observation that both villin and gelsolin, compete in vitro for F-actin binding with tropomyosin, a protein known to stabilize stress fibers (Burgess et al., 1987).

Overexpression of Gelsolin and Gelsolin Halves Disrupts Stress Fiber Organization

Overexpression of gelsolin had very drastic effects on the CV1 actin cytoskeleton. This observation is similar to those reported by Cunningham et al. (1991) and with some, but not all experiments carried out by microinjection of gelsolin (Franck et al., 1990; Cooper et al., 1987). Significant differences in the amount of proteins accumulated in the cytoplasm may account for these results. In our transfection experiments, double immunofluorescence staining showed that the extent of stress fiber disruption paralleled the intensity

of gelsolin labeling. Whether or not actin filament severing, the most characteristic in vitro activity of gelsolin, is involved in the disparition of most stress fibers remains to be elucidated. Our observations indicate that fully regulated gelsolin expressed at high levels can have important effects on the equilibrium between intracellular pools of G- and F-actin without immediately affecting cell viability or morphology.

In contrast, the first domain of gelsolin (G1) induces an accumulation of G1/F-actin aggregates and cell retraction. Similar effects were observed after microinjection of the first domain of gelsolin (Cooper et al., 1987) or after microinjection of capping proteins (Fuchtbauer et al., 1983). Since the capping and severing activities of the first gelsolin domain are no longer regulated by Ca^{2+} in vitro, irreversible interactions of the mutant with F-actin microfilaments may be responsible for its deleterious effect on actin organization, and finally cell mortality. Alternatively, the first domain of gelsolin may not only disrupt stress fibers but also affect the organization of focal contacts.

The Addition of the Villin Headpiece to Gelsolin Did Not Reconstitute a Villin-like Protein

Villin core and gelsolin share the ability to bind F-actin exclusively in the presence of Ca^{2+} (Fig. 1, Glenney et al., 1981*b*; Glenney and Weber, 1981). Villin headpiece gives villin core the ability to bind F-actin in the absence of Ca^{2+}. We therefore hypothesized that addition of villin headpiece to gelsolin might confer villin activity on the chimera. However, expression of this chimera did not induce spike-like F-actin structures as observed with villin, but drastically disrupted stress fibers, as observed with wild-type gelsolin. This result clearly shows that addition of the headpiece to gelsolin is not sufficient to create a molecule with villin-like properties. Moreover, villin headpiece appeared to have very little influence on the in vivo activities of gelsolin.

We also examined the effects of each gelsolin domain linked to the villin headpiece. In cells expressing chimera 2 (G1 + headpiece), we observed the same phenotype as that observed in cells expressing the first half of gelsolin alone (see above). In contrast to the preceding two chimeras, addition of the headpiece to the second gelsolin domain modified the effects of this domain in transfection experiments. In cells transfected with such chimera, F-actin structures on the dorsal face of cells appeared elongated. According to the results obtained with proteolytically derived fragments, the second half of gelsolin has weak Ca^{2+} regulated actin binding activity (Kwiatkowski et al., 1985). However cross-linking studies have suggested that it may contain an F-actin binding site (Sutoh and Yin, 1989). The potential F-actin–binding site of the gelsolin fragment and the villin headpiece may act cooperatively to produce this effect. The possible actin-bundling or actin cross-linking properties of this chimera would be usefully examined in vitro. Nevertheless, it was surprising to observe that in cells transfected with chimera 4 (V1 + G2 + headpiece) such modification of the dorsal microvilli did not occur. Thus, it is possible that the additional actin-binding sites in this chimera are not properly arranged.

Our observations with gelsolin villin chimera support the view that in evolution villin does not result from the simple addition of the headpiece domain to a pre-existent gelsolin structure. Divergent evolution in the nonhomologous domains of the two proteins probably explains the large increase in calcium required for villin severing activity in comparison with gelsolin. Villin severing could be an ancestral function that does not occur under physiological conditions. These data are in agreement with the recent comparison of the genomic organization of human villin gene to that of human gelsolin: structural divergences between the two genes exclude a simple evolutionary link (Pringault et al., 1991).

Conclusions

Transfection in CV1 cells of villin and gelsolin mutants or chimeras allowed us to study the contribution of individual domains for the actin regulatory activities of the two proteins. In some respects, our data are consistent with the in vitro differences in calcium sensitivities and activities of the proteolytically isolated domains. Other results can be explained by several hypotheses relative to the direct actin regulatory properties of the mutants or their possible competition with other actin binding proteins in the cell. However, as we created mutants that cannot be obtained by proteolytic digestions, new experiments should investigate their in vitro properties. We anticipate that this work will pave the way for further studies, namely crystallographic work, that ultimately will help to understand in details the structural differences and similarities of villin and gelsolin accounting for their functional particularities.

We thank J. C. Benichou for his help with the photographic work.

This work was supported by grants from the Association pour la Recherche sur le Cancer (N° 6379), the Fondation pour la Recherche Médicale, the Institut National de la Santé et de la Recherche Médicale (N° 86-7008), and the Ligue Nationale Française contre le Cancer.

Received for publication 19 September 1991 and in revised form 14 November 1991.

References

Ampe, C., and J. Vandekerckhove. 1987. The F-actin capping proteins of *Physarum polycephalum* cap 42a is very similar, if not identical, to fragmin and is structurally and functionally very homologous to gelsolin; cap 42b is *Physarum* actin. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:4149–4157.

André, E., F. Lottspeich, M. Schleicher, and A. Noegel. 1988. Severin, gelsolin, and villin share a homologous sequence in regions presumed to contain F-actin severing domains. *J. Biol. Chem.* 263:722–727.

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

Bretscher, A., and K. Weber. 1979. Villin: the major microfilament-associated protein of the intestinal microvillus. *Proc. Natl. Acad. Sci. USA.* 76:2321–2325.

Bretscher, A., and K. Weber. 1980. 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.

Burgess, D. R., K. O. Broschat, and J. M. Hayden. 1987. Tropomyosin distinguishes between the two actin-binding sites of villin and affects actin-binding properties of other brush border proteins. *J. Cell Biol.* 104:29–40.

Burnette, W. N. 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* 112:195–203.

Bryan, J. 1988. Gelsolin has three actin binding sites. *J. Cell Biol.* 106:1553–1562.

Chaponnier, C., P. A. Janmey, and H. L. Yin. 1986. The actin filament-severing domain of plasma gelsolin. *J. Cell Biol.* 103:1473–1481.

Chaponnier, C., H. L. Yin, and T. P. Stossel. 1987. Reversibility of gelsolin/

actin interactions in macrophages: evidence for a Ca^{2+}-dependent and Ca^{2+}-independent pathways. *J. Exp. Med.* 165:97–106.

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

Cooper J. A., D. J. Loftus, C. Frieden, J. Bryan, and E. E. Elson. 1988. Localization and mobility of gelsolin in cells. *J. Cell Biol.* 106:1229–1240.

Coue, M., and E. D. Korn. 1985. Interaction of plasma gelsolin with G-actin and F-actin in the presence and absence of calcium ions. *J. Biol. Chem.* 260: 15033–15041.

Coudrier, E., H. Reggio, and D. Louvard. 1981. Immunolocalization of the 110,000 molecular weight cytoskeletal protein of intestinal microvilli. *J. Mol. Biol.* 152:49–66.

Coudrier, E., H. Reggio, and D. Louvard. 1983. Characterization of an integral membrane glycoprotein associated with microfilaments of pig intestinal microvilli. *EMBO (Eur. Mol. Biol. Organ.) J.* 2:469–475.

Cunningham, C. C., T. P. Stossel, and D. D. Kwiatkowski. 1991. Enhanced motility in NIH 3T3 fibroblasts that overexpressed gelsolin. *Science (Wash. DC).* 251:1233–1236.

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 HT 29-18 clones. *J. Cell Biol.* 105: 365–369.

Eichinger L., A. Noegel, and M. Shleicher. 1991. Domain structure in actin binding proteins: expression and functional characterization of truncated severin. *J. Cell Biol.* 112:665–676.

Franck, Z., M. Footer, and A. Bretscher. 1990. 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. *J. Cell Biol.* 111:2475–2485.

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

Fuchtbauer, A., B. M. Jockusch, H. Maruta, M. W. Kilimann, and G. Isenberg. 1983. Disruption of microfilament organization after injection of F-actin capping proteins into living tissue culture cells. *Nature (Lond.).* 304:361–364.

Glenney, J. R., Jr., 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., Jr., P. Kaulfus, and K. Weber. 1981*a*. F-actin assembly modulated by villin: Ca^{2+}-dependent nucleation and capping of the barbed end. *Cell.* 24:471–480.

Glenney, J. R., Jr., N. Geisler, P. Kaulfus, and K. Weber. 1981*b*. Demonstration of at least two different actin-binding sites in villin, a calcium-regulated modulator of F-actin organization. *J. Biol. Chem.* 256:8156–8161.

Huckriede, A., H. Hinssen, A. Fuchtbauer, C. Chaponnier, A. Weeds, and B. M. Jockusch. 1990. Differential effects of gelsolins on tissue culture cells. *Cell Motil. and Cytoskeleton.* 16:229–238.

Huylebroeck, D., G. Maertens, M. Verhoeyen, C. Lopez, A. Raeymakers, W. Min You, and W. Fiers. 1988. High level transient expression of influenza virus proteins from a series of SV 40 late and early replacement vectors. *Gene (Amst.).* 66:163–181.

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

Kwiatkowski, D. J., P. A. Janmey, J. E. Mole, and H. L. Yin. 1985. Isolation and properties of two actin-binding domains in gelsolin. *J. Biol. Chem.* 260: 15232–15238.

Kwiatkowski, D. J., T. P. Stossel, S. H. Orkin, J. E. Mole, H. R. Colten, and H. L. Yin. 1986. Plasma and cytoplasmic gelsolins are encoded by a single gene and contain a duplicated actin-binding domain. *Nature (Lond.).* 323: 455–458.

Kwiatkowski, D. J., R. Mehl, and H. L. Yin. 1988. Genomic organization and biosynthesis of secreted and cytoplasmic forms of gelsolin. *J. Cell Biol.* 106: 375–384.

Kwiatkowski, D. J., P. A. Janmey, and H. L. Yin. 1989. Identification of critical functional and regulatory domains in gelsolin. *J. Cell Biol.* 108:1717–1726.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature (Lond.).* 227:680–685.

Matsudaira, P., R. Jakes, and J. E. Walker. 1985. A gelsolin-like Ca^{2+}-dependent actin-binding domain in villin. *Nature (Lond.).* 315:248–250.

Northrop, J., A. Weber, M. S. Mooseker, C. Franzini-Armstrong, M. F. Bishop, G. R. Dubyak, M. Tucker, and T. P. Walsh. 1986. Different calcium dependence of the capping and cutting activities of villin. *J. Biol. Chem.* 261:9274–9281.

Pope, B., M. Way, and A. G. Weeds. 1991. Two of the three actin binding domains of gelsolin bind to the same subdomain of actin. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 280:70–74.

Pringault, E., S. Robine, and D. Louvard. 1991. Structure of the human villin gene. *Proc. Natl. Acad. Sci. USA.* 88:10811–10815.

Reggio, H., P. Webster, and D. Louvard. 1983. Use of immunocytochemical techniques in studying the biogenesis of cell surface in polarized epithelia. *Methods Enzymol.* 98:379–396.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 545 pp.

Sutoh, K., and H. L. Yin. 1989. End-label fingerprintings show that the NH_2 and COOH termini of actin are in the contact site with gelsolin. *Biochemistry.* 28:5269–5275.

Way, M., J. Gooch, B. Pope, and A. G. Weeds. 1989. Expression of human plasma gelsolin in *Escherichia coli* and dissection of actin binding sites by segmental deletion mutagenesis. *J. Cell Biol.* 109:593–605.

Way, M., B. Pope, J. Gooch, M. Hawkins, and A. G. Weeds. 1990. Identification of a region in segment 1 of gelsolin critical for actin binding. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:4103–4109.

Yin, H. L., and T. P. Stossel. 1979. Control of cytoplasmic actin gel-sol transformation by gelsolin, a calcium-dependent regulatory protein. *Nature (Lond.).* 281:583–586.

Yin, H. L., J. H. Albrecht, and A. Fattoum. 1981. Identification of gelsolin, a Ca^{2+}-dependent regulatory protein of actin gel-sol transformation, and its intracellular distribution in a variety of cells and tissues. *J. Cell Biol.* 91: 901–906.

Yin, H. L., D. J. Kwiatkowski, J. E. Mole, and F. S. Cole. 1984. Structure and biosynthesis of cytoplasmic and secreted variants of gelsolin. *J. Biol. Chem.* 259:5271–5276.

Yin, H. L., K. Ida, and P. A. Janmey. 1988. Identification of a polyphosphoinositide-modulated domain in gelsolin which binds to the sides of actin filaments. *J. Cell Biol.* 106:805–812.