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Calcium control of microfilaments: Uncoupling of the F-actin-severing and -bundling activity of villin by limited proteolysis *in vitro*

(epithelia/microvilli/cytoskeleton)

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ABSTRACT **Villin is a major F-actin-bundling protein present in the microfilament bundle underlying the plasma membrane of the microvilli present on intestinal epithelial cells. Mild *in vitro* proteolysis converts villin (M_r, 95,000) into a large fragment, the villin core (apparent M_r, 90,000). Villin core has lost the F-actin-bundling activity expressed by villin in the absence of calcium but retains the micromolar K_d calcium-binding site and the calcium-dependent restriction of actin filament length (F-actin severing) of intact villin. This finding suggests a common structural and functional relatedness between the known calcium-dependent F-actin-severing proteins from different cell types, even though not all of them reveal F-actin-bundling activity.**

Ca^{2+} regulation of the submembranous microfilament organization, turnover, and transport has often been considered to be of vital importance for the expression of various cellular activities. With progress in the biochemical analysis of this complex organization, interest has focused on two targets. The first involves the activation of myosin light-chain kinase by calmodulin (1), the general eukaryotic Ca^{2+} regulatory protein (2). The specifically phosphorylated myosin shows increased F-actin-activated myosin ATPase activity, assumed to be the basis of microfilament contractility (1) because myosin is often associated with this structure (3). The second target has been recognized as being myosin-independent and resides in the three dimensional organization of F-actin and its numerous associated proteins (4, 5). Identification of this target as F-actin itself revealed an unexpected Ca^{2+}-dependent mechanism, which has been described as "F-actin severing" (6–8).

Within the last year, three Ca^{2+}-dependent F-actin-severing proteins have been described from cell types as diverse as rabbit alveolar macrophages, chicken intestinal epithelial cells, and *Physarum polycephalum* (6–8). All three factors are nearly globular monomeric proteins and are major constituents in their respective cells. They reveal Ca^{2+} binding with μM dissociation constants, sever F-actin into short filaments at μM free Ca^{2+}, and, at sufficiently high concentrations, form stoichiometric complexes with monomeric actin, even when F-actin buffers are used (6–9). In spite of these similarities, the three proteins differ in molecular weight and reveal different F-actin interactions in the absence of Ca^{2+}. Thus, villin from intestinal cells (M_r, 95,000) acts as an F-actin-bundling protein, in agreement with its presence in the microvillus core filaments (7, 9, 10), once Ca^{2+} is depleted or replaced by ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetate (EGTA). A similar property has not been found for fragmin from *Physarum* (M_r, 43,000) (8), and gelsolin (M_r, 91,000) (6) from macrophages.

In an attempt to elucidate the different actin-binding properties of villin, we aimed at derivatives with less complex actin interactions. Here we show that mild *in vitro* proteolysis results in a large villin core fragment (apparent M_r, 90,000). This core retains Ca^{2+}-dependent F-actin-severing activity but has lost F-actin-bundling activity.

MATERIALS AND METHODS

Villin was isolated from chicken intestinal epithelial cells (7). A proteolytic fragment of villin ("*villin core*") was generated by incubating villin (1.5 mg/ml) in 75 mM KCl/10 mM imidazole/0.1 mM $MgCl_2$, 1 mM EGTA/5 mM $CaCl_2$, pH 7.3, with 10 μg of *Staphylococcus aureus* V-8 protease per ml (Miles-Yeda, Israel). After 15 min at room temperature, freshly prepared 0.25 M phenylmethylsulfonyl fluoride in ethanol was added (final concentration, 1 mM). Bovine serum albumin (Miles-Yeda, Israel) was added to 10 mg/ml, and the solution was quickly applied at 4°C to a DNase-I-agarose column previously saturated with G-actin (7). After extensive washing with buffer to remove the enzyme and the albumin, villin core was eluted by the addition of EGTA to a final concentration of 10 mM.

Villin or villin core was subjected at room temperature to the sedimentation assay in the presence of bovine skeletal muscle actin as described (7), except that the buffer contained 0.1 M KCl, 1 mM $MgCl_2$, and 10 mM 1,4-piperazinediethanesulfonic acid (Pipes) (pH 7.0) with either 2 mM EGTA or 2 mM EGTA and 2 mM $CaCl_2$. The latter solution is assumed to contain approximately 10 μM free Ca^{2+} (11). Electron microscopy with negative stain analysis (2% uranyl acetate) was carried out by using a Philips 301 microscope. Samples were diluted 5-fold with the polymerization buffer immediately before application to the grid.

Sucrose gradient analysis of actin/villin or actin/villin core complexes was performed as described (9). $NaDodSO_4$ (10%)/polyacrylamide gel electrophoresis was used. Two-dimensional gels were run as described by O'Farrell (12). Chicken skeletal muscle α-actinin, bovine skeletal muscle G-actin, and bovine brain calmodulin were purified as described (7, 9). Catalase was from Boehringer Mannheim and DNase was from Sigma.

RESULTS

Isolation of a M_r 90,000 Villin Core. Limited proteolysis of villin by V-8 protease produced a stable high M_r core (Fig. 1). The core was effectively separated from the protease by actin-affinity chromatography on immobilized DNase I as described

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Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetate; Pipes, 1,4-piperazinediethanesulfonic acid.

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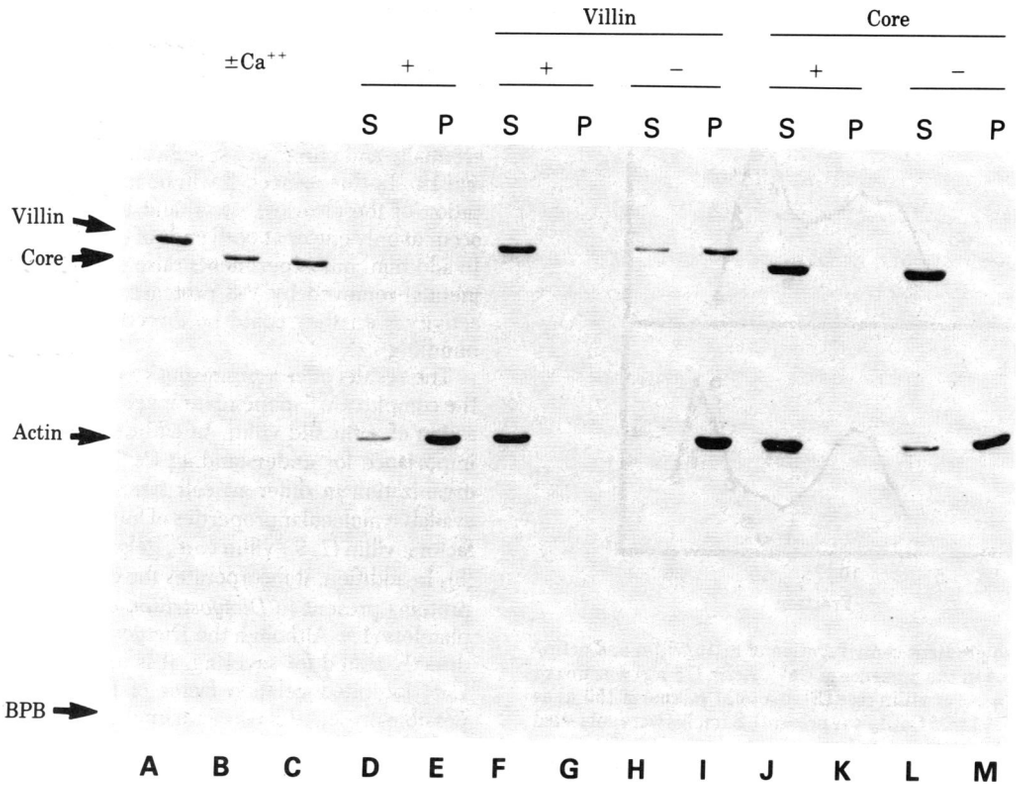

FIG. 1. Limited proteolysis of villin, isolation of villin core, and Ca^{2+}-dependent interaction with actin. Villin (lane A) was incubated with V-8 protease, and the digest (lane B) was subjected to actin affinity chromatography. Villin core was eluted with EGTA buffer (lane C). Actin alone (lanes D and E), villin and actin (lanes F–I), or villin core and actin (lanes J–M) were adjusted to 0.1 M KCl/1 mM $MgCl_2$/10 mM Pipes, pH 7.0, containing either 2 mM EGTA (−) or 2 mM EGTA and 2 mM $CaCl_2$ (approximately 10 μM in free Ca^{2+}) (+). After 30 min at room temperature (total volume 100 μl; 0.5 mg of actin and 0.25 mg of villin or core per ml), the solution was centrifuged for 20 min at 100,000 × g. Supernatants (S) and pellets (P) adjusted to equivalent final volumes were analyzed by gel electrophoresis. BPB, Tracking dye bromophenol blue. Note the non-sedimentable actin in the presence of Ca^{2+} and villin or villin core (lanes G and K). Also note the incorporation of villin but not villin core in the actin pellets in the presence of EGTA (lanes I and M).

for villin (7). Villin core bound to immobilized monomeric actin in the presence of Ca^{2+} and was released from the affinity column free of actin, once Ca^{2+} was replaced by EGTA. The limited structural modification by the proteolytic treatment is reflected in the two-dimensional gel pattern (Fig. 2), which shows the two isoforms of villin (α and β) (7), shifted slightly on the M_r axis towards the acid side when compared with intact villin. Longer incubation with V-8 protease produced increasing amounts of fragments with M_r 40,000–50,000. We also explored the proteases papain, thermolysin, and chymotrypsin. They resulted in the production of a similar M_r 90,000 core, although in these cases the core was contaminated by intact villin and by lower M_r polypeptides. Thus, we concentrated on the core produced by V-8 protease.

Ca^{2+}-Dependent F-Actin Severing by Villin Core. Three lines of evidence, in addition to its isolation procedure, show

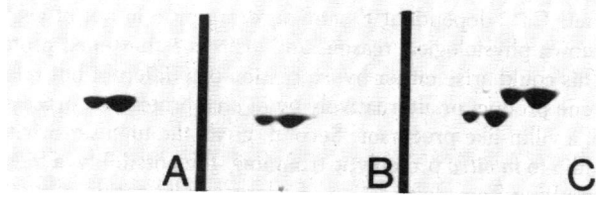

FIG. 2. Two-dimensional electrophoretic analysis of villin core. Only the relevant portion of the electropherograms are shown (basic end to the right) for intact villin (*A*), isolated villin core (*B*), and a mixture of the two (*C*).

that villin core retains the Ca^{2+}-dependent F-actin-severing capacity typical of intact villin. First, F-actin polymerization experiments showed that in the presence of villin core and 10 μM free Ca^{2+}, actin is not sedimentable at 100,000 × g for 20 min (Fig. 1) under buffer conditions in which actin alone yields easily sedimentable F-actin polymers [actin/villin ratio of 2:1 (wt/wt)]. Second, sucrose gradient centrifugation of the mixture of actin and villin core in the presence of Ca^{2+} demonstrated a discrete complex (Fig. 3) similar to the actin/villin complex present in Ca^{2+} extracts of microvilli cytoskeletons (9) sedimenting at 9.4 S. These results show that the complexes can contain only a few monomeric actin molecules (perhaps 2–4) because villin alone sediments at 5.1 S and behaves as a nearly globular protein (7). Third, further direct evidence for Ca^{2+}-dependent severing by villin core was obtained by electron microscopy with negative-stain analysis (Fig. 4). F-actin polymerized alone in the presence of Ca^{2+} gives rise to the well-known long filaments. These were conspicuously absent in actin/villin core mixtures (molar ratio, 10:1) which revealed instead an abundance of short filaments. Indistinguishable results were obtained on similarly processed actin/villin mixtures (see also ref. 7). Thus, villin and villin core restrict F-actin filament length in the presence of Ca^{2+}.

Villin Core Has Lost the Ability to Bundle F-Actin in the Presence of EGTA. When actin is polymerized with villin in the presence of EGTA, thick bundles of tightly packed F-actin filaments are observed by electron microscopy with negative-stain analysis (7) (Fig. 4*C*). In contrast, actin polymerized in the

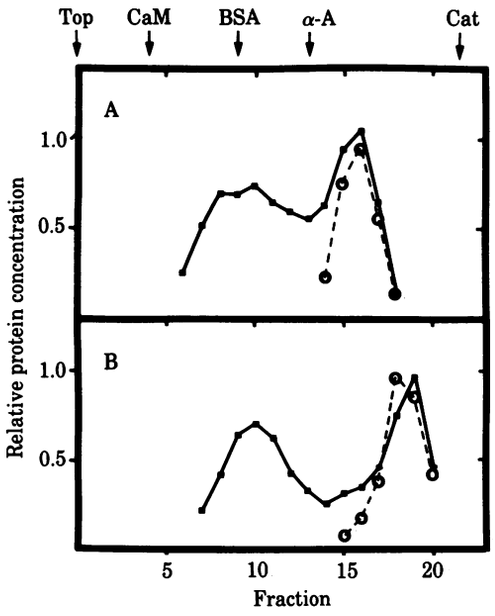

FIG. 3. Sucrose gradient centrifugation of actin/villin and actin/villin core mixtures in the presence of Ca^{2+}. Actin (75 μg) was mixed with 37 μg of villin (*A*) or villin core (*B*) in a total volume of 150 μl as in Fig. 1 (except that 1 mM $CaCl_2$ was present). Samples were subjected to sucrose gradient centrifugation in the same solvent. Fractions were analyzed by gel electrophoresis, stained, and scanned (9). ■——■, Actin; ○·····○, villin or villin core distribution. Standards in a comparison gradient were calmodulin (CaM, 1.85 S), bovine serum albumin (BSA, 4.2 S), α-actinin (α-A, 6.2 S), and catalase (Cat, 11.3 S).

presence of villin core at a similar molar ratio and under the same conditions resulted in the formation of long individual filaments indistinguishable from actin polymerized alone. Examination of a large number of negatively stained specimens gave no indication of the tightly packed F-actin bundles observed in the presence of intact villin. The modifications in the buffer conditions used in the present study appear to be more favorable for villin-induced actin bundling than those as described (7). This may be either due to the lower pH (i.e., pH 7.0 instead of pH 7.6), the use of a Pipes buffer instead of a Tris·HCl buffer, or the presence of a higher concentration of Mg^{2+}. Not only did villin core not bundle F-actin in the presence of EGTA, but also the core was not recovered in the resulting F-actin pellet (Fig. 1). Thus, although the core binds monomeric actin in the presence of Ca^{++}, its binding to F-actin in the presence of EGTA differs markedly from that previously found for villin.

DISCUSSION

Villin has two morphologically and structurally distinct effects on F-actin organization (i.e., F-actin bundling in the absence of Ca^{2+} and F-actin severing in the presence of Ca^{2+}) (7, 9, 10). The transition between two such strikingly different physiological properties seems to be governed by the presence of a Ca^{2+}-binding site with a dissociation constant of approximately 2 μM, even in the presence of mM free Mg^{2+} (9). In agreement with this model, increasing concentrations of villin finally result in low M_r complexes between villin and actin, provided Ca^{2+} is present (9).

The bundling and severing activities of villin can be uncoupled. Mild *in vitro* proteolysis yields a large M_r core fragment. The cleavage by V-8 protease seems very specific as judged by one- and two-dimensional gel electrophoretic analysis. Villin core retains both the Ca^{2+} binding site and the Ca^{2+}-dependent restriction of F-actin filament length but has lost the bundling expressed in the absence of Ca^{2+}.

The restricted action of proteolytic enzymes on native proteins can be envisioned as a process in which certain hinge regions connecting tightly packed functional domains are preferentially and often very specifically cleaved (e.g., see refs. 13 and 14). In this respect, it will be interesting to identify the location of the cleavage site(s) and to determine whether they occur at only one or at both ends of the villin polypeptide chain. In addition, our experiments raise the possibility that the fragment(s) removed by V-8 protease may have some functional activity (i.e., they could be directly or indirectly involved in bundling).

The results offer a promising step towards the unraveling of the complex Ca^{2+}-dependent mechanisms governing the interaction of actin and villin. In addition, they may be of general importance for understanding Ca^{2+}-dependent microfilament organization in different cell types. Table 1 summarizes the available molecular properties of four Ca^{2+}-dependent severing factors: villin (7, 9), villin core, gelsolin (6, 15, 16), and fragmin (8). In addition, it incorporates the data so far described for two proteins present in *Dictyostelium discoideum* (11) and human platelets (17). Although the *Dictyostelium* protein has not been directly tested for severing; it is included because it acts as a Ca^{2+}-inhibited gelation factor of F-actin (11), indicating the possible presence of two actin binding sites. The platelet protein and macrophage gelsolin have not been assayed for bundling; however, the tightly packed microfilament bundles present in activated platelets (18, 19) could indicate a functional role of a putative bundling protein.

The proteins summarized in Table 1 behave as monomeric proteins, are nearly globular in shape, and have very similar molecular weights. They display very strong Ca^{2+} binding, characterized by a micromolar dissociation constant, which seems responsible for their actin-severing activity occurring at the same concentration. In addition, they are all major cytoplasmic proteins in the cell type from which they are isolated. Thus, they seem both functionally and structurally related. Do they all display bundling activity in the absence of Ca^{++}? This is difficult to predict because gelsolin and the platelet protein have not been assayed for this property. As to the other three proteins, villin (7) and the *Dictyostelium* protein (11) gelate or bundle F-actin, whereas fragmin, characterized by a lower M_r, clearly does not (8). However, proteolytic trimming of villin is accompanied by a loss of bundling activity, and the resulting villin core resembles fragmin in many biochemical properties (Table 1).

Because we have found upon prolonged *in vitro* proteolysis further villin derivatives capable of Ca^{2+}-dependent binding to monomeric actin and displaying M_r similar to that of fragmin (M_r, 40,000–50,000), two possibilities arise, if one assumes a general mechanism of Ca^{2+} control over F-actin organization. First, although villin and fragmin are functionally related, certain cell types, such as *Physarum*, avoid bundling activity of their Ca^{2+}-dependent F-actin-severing protein out of yet unknown physiological reasons and express a shortened protein. This could arise either by expression of a different but related gene product or alternatively by *in vivo* proteolytic processing of a villin-like precursor. Second, given the high sensitivity of villin to *in vitro* proteolytic trimming, the possibility of fragmin resulting from degradation of a larger polypeptide cannot yet be ruled out. In this context, we note a further similarity between fragmin and villin core. Although both form discrete complexes with monomeric actin in the presence of Ca^{2+} and show severing, their lack of bundling activity in the presence

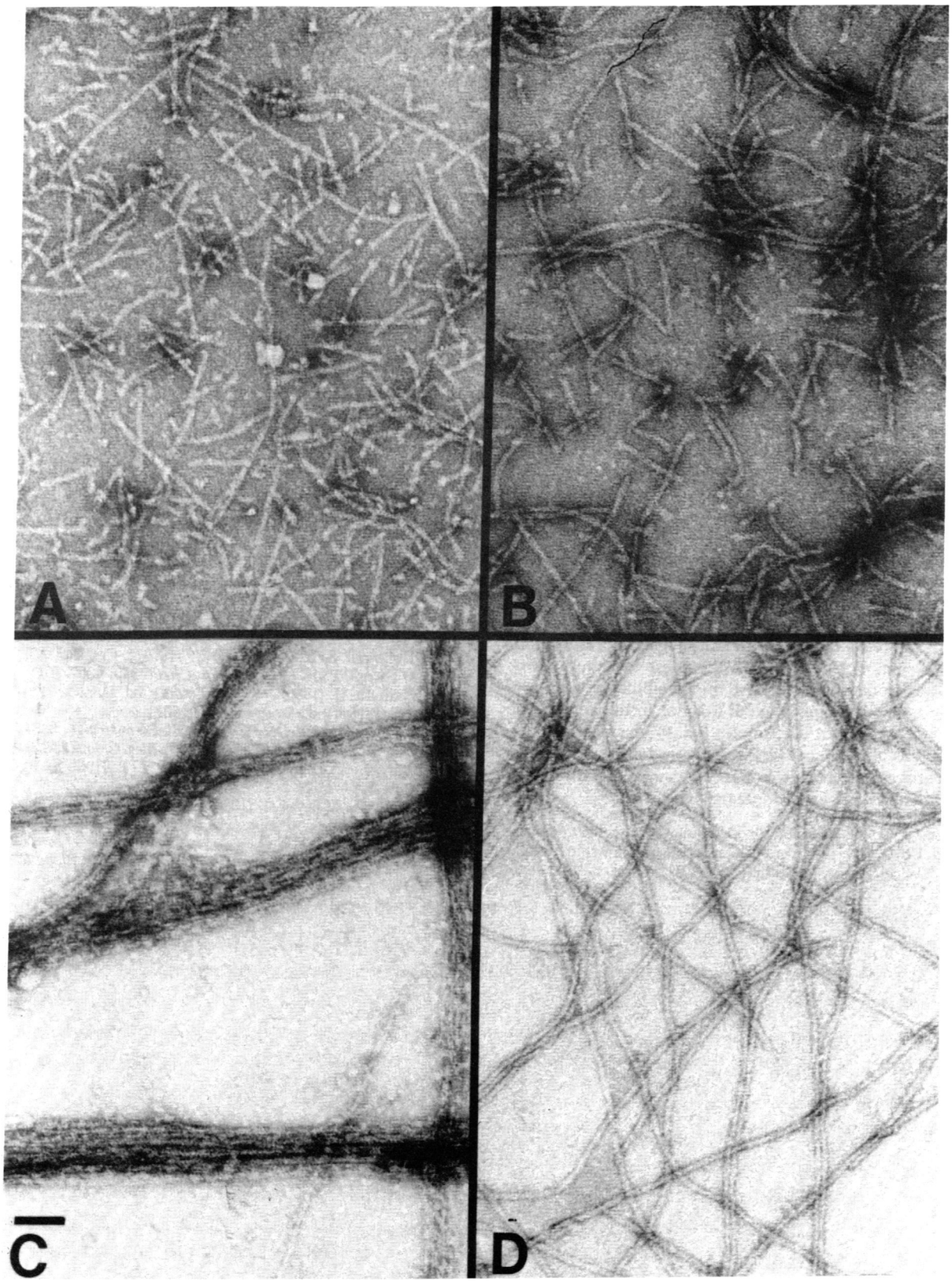

FIG. 4. Electron microscopic analysis of the structures obtained from mixtures of villin, villin core, and actin in the presence or absence of Ca^{2+}. Actin/villin (*A*, *C*) or actin/villin core (*B*, *D*) mixtures were incubated with 10 μM free Ca^{2+} (*A*, *B*) or 2 mM EGTA (*C*, *D*) as described in Fig. 1, except that villin (or core) /actin molar ratios were 1:10 (*A*, *B*) and 1:4 (*C*, *D*). (×140,000; bar = 70 nm.) Note the thick filament bundles in the presence of villin and EGTA (*C*), which are not observed when villin core substitutes for villin (*D*). Note the numerous short filaments in the presence of Ca^{2+} when either villin or its core is present.

Table 1. Comparison of the molecular properties of different Ca^{2+}-dependent F-actin-severing proteins

Property	Villin	Villin core	Gelsolin	95 P	95 D	Fragmin
Source	Intestine	Proteolysis	Macrophage	Platelet	*Dictyostelium*	*Physarum*
Major cellular protein	+		+	+	+	+
Part of cytoskeleton	+		?	?	?	?
$M_r \times 10^{-3}$	95	90	91	95	95	43
Number of subunits	1	1	1	?	?	1
Ca^{2+} binding	μM	μM	μM	+	+	+
Ca^{2+}-dependent F-actin severing	+	+	+	+	?	+
Ca^{2+}-dependent monomeric actin binding	+	+	?	+	?	+
EGTA-dependent F-actin bundling or gelation	+	−	?	?	+	−
Coupling of severing and bundling	+	−	?	?	?	−
Refs.	(7, 9)	This paper	(6, 15)	(17)	(11)	(8)

?, Not yet established; + or −, presence or absence either stated in the appropriate references or predicted from our interpretation of the corresponding data; D, *Dictyostelium*; P, platelet.

of EGTA is accompanied by either no binding or very limited binding to F-actin.

Many molecular details of the processes of F-actin severing and bundling have so far remained unexplored because current studies have concentrated on the striking results observed by electron microscopy or by sedimentation and other similar assays (6–9, 11, 16). Thus, both phenomena currently are described in phenotypical terms based mainly on morphological criteria.

Although a very recent study on gelsolin (16) favors a direct action on preformed F-actin, the authors stated that they could not exclude the possibility that the results can be explained as the consequence of nucleation of new filaments and parallel shortening of preexisting filaments. Although the present studies of villin cannot distinguish between these two alternatives, future experiments should explore the possibility of villin promoting or enhancing nucleation of F-actin. In this respect the villin core may provide the simpler tool to elucidate the molecular details of F-actin severing, because intact villin acts in a necessarily more complex manner by coupling severing and bundling activity in a Ca^{2+}-dependent manner.

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