Villin Function in the Organization of the Actin Cytoskeleton

CORRELATION OF IN VIVO EFFECTS TO ITS BIOCHEMICAL ACTIVITIES IN VITRO

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Villin is an actin-binding protein of the intestinal brush border that bundles, nucleates, caps, and severs actin in a Ca2⁺-dependent manner in vitro. Villin induces the growth of microvilli in transfected cells, an activity that requires a carboxyl-terminally located KKEK motif. By combining cell transfection and biochemical assays, we show that the capacity of villin to induce growth of microvilli in cells correlates with its ability to bundle F-actin in vitro but not with its nucleating activity. In agreement with its importance for microfilament bundling in cells, the KKEK motif of the carboxyl-terminal F-actin-binding site is crucial for bundling in vitro. In addition, substitutions of basic residues in a second site, located in the amino-terminal portion of villin, impaired its activity in cells and reduced its binding to F-actin in the absence of Ca2⁺ as well as its bundling and severing activities in vitro. Altogether, these findings suggest that villin participates in the organization and stabilization of the brush border core bundle but does not initiate its assembly by nucleation of actin filaments.

Villin is a 92.5-kDa, tissue-specific actin-binding protein associated with the microvillar actin core bundle of intestinal and renal brush border, a highly organized structure implicated in absorption (1–5). In vitro, villin caps, nucleates, severs, and bundles actin filaments in a Ca2⁺ and phosphoinositide-regulated manner (see Fig. 1A and Refs. 6–9). Villin is composed of six repeats, each containing approximately 150 residues that together constitute the core domain followed by the carboxyl-terminal headpiece domain of 87 residues (10–12). The core domain contains Ca2⁺-dependent capping, nucleating, and severing activity, whereas the carboxyl-terminal headpiece domain is required for actin filament bundling and binds to F-actin, independently of Ca2⁺ (10, 13).

Villin is structurally and functionally related to two classes of proteins. The first group comprises Quail, a bundling protein in Drosophila required for the formation of cytoplasmic F-actin bundles in ovary nurse cells (14) as well as protovillin of Dictyostelium that has been proposed to be an ancestral form of villin (15). The second group includes the severing proteins gelsolin, the crystal structure of which has been solved recently (16), and scinderin found in higher eukaryotes (17) as well as fragmin and severin of lower eukaryotes (18, 19). Whereas proteins of the first group are organized into seven domains like villin, the members of the second group have an organization similar to the villin core domain. So far, villin is unique among these proteins because it is the only one that bundles and severs actin filaments in a Ca2⁺-regulated manner.

A role for villin in the assembly of the microvillus core bundle was determined by reconstitution experiments in vitro (20). Addition of villin and fimbrin, another actin-bundling protein associated with the microvillar core, to homogenous F-actin is sufficient for the formation of bundles with a basic structure similar to that of the brush border microvilli in vivo. Villin overproduction or villin mRNA ablation in cultured cells also suggested that villin supports assembly of the actin core bundle of microvilli (21–23). Fibroblast-like cells transfected with a cDNA encoding villin (21) or microinjected with villin protein (22) are covered by long microvilli containing bundled actin filaments to which villin is associated. Mutational analysis of villin showed that these morphological modifications require the first half of the core, also named villin 44T, and the headpiece domain (see Fig. 1B and Refs. 21 and 24). The headpiece domain of human villin contains at its carboxyl terminus a cluster of predominantly basic residues (KKEK), which is essential for the morphogenic activity of villin in transfected cells (25). Peptides derived from this region or recombinant headpiece bind to F-actin (25, 26). The second repeat of the core domain (V2) comprises a stretch of basic residues (27) that is important for the severing activity of the 44T villin subdomain (28). It is likely that this sequence also participates in bundling of actin filaments in vivo and in vitro. However, such a mechanism of action has, to our knowledge, never been tested experimentally.

To address the action mechanism of villin, we determined how mutations affecting villin-induced growth of microvilli in transfected cells can also affect activity of villin in vitro. Because information obtained from previous studies (21, 25) suggests that microfilament bundling is an important activity for villin function, we focused on the two F-actin-binding sites located in the headpiece and core domain, respectively. We have also investigated for the first time how residue substitutions influencing F-actin binding properties of villin-derived peptides or domains affect the activities of the whole villin molecule in vitro.

We show that F-actin bundling but not nucleation activity of villin is important for induction of F-actin spikes in transfected cells, suggesting that villin may stabilize and organize the F-actin bundle of microvilli but might not initiate the formation of these structures.

EXPERIMENTAL PROCEDURES

Reagents
Rhodamine-conjugated phalloidin was purchased from Sigma. N-Pyrethryliodoacetamide was obtained from Molecular Probes (Eugene, OR).
Antibodies
Monoclonal antibodies (1D2C2) recognizing an epitope in the carboxyl terminus of human villin, as well as their purification, was described previously (29). Fluorescein-coupled anti-mouse IgG antibodies were purchased from Cappel (Organan Teknika, Turnhout, Belgium). Phosphatase coupled anti-mouse IgG antibodies were obtained from Amer sham Pharmacia Biotech.

Construction of Recombinant DNAs
CDNA constructions were prepared by standard procedures as described by Sambrook and colleagues (30).

DNA Constructions for Production of Wild Type Villin or Villin Variants in Eukaryotic Cells—For expression in eukaryotic cells, a cDNA encoding wild type villin (4) was inserted into the pc86 expression vector comprising a cytoskeletal virus promoter sequence.

To introduce the double amino acid substitution R137A,K144A, a sense-primer specific of bp^2403–436 of the human villin coding sequence and an antisense primer located ~500 bp downstream of this sequence were used in a polymerase chain reaction. Both primers contained add-on sequences for subcloning, corresponding to a XbaI and an EcoRI site, respectively. The sense-primer was a 41-mer oligonucleotide: 5′-CCCTCTAGAGCTCCAGCGCTGCTGCACTGTCAGGCGCGCC-CA-3′ (mutations are underlined). The sequence of the antisense primer was complementary to bp 888–903 of the villin coding sequence, located upstream of the unique BglII restriction site of the villin sequence. The resulting polymerase chain reaction products were ligated through XbaI and EcoRI into the pSp64 vector. An ~180-bp fragment corresponding to the mutated villin 5′-sequence was removed from this construct by BglII and HpaII digestion and ligated to the AatII/HpaII-cut pSp64-villin yielding pSp64-villin RK/AA. A DNA fragment of 2.7 kilobase pairs encoding the mutagenized complete villin sequence was ligated through the XbaI/EcoRI sites into the pc86 expression vector, yielding pc86-villin RK/AA that encodes villin variant RK/AA.

A third amino acid substitution was introduced into the villin protein sequence by a similar cloning strategy using a 46-mer oligonucleotide 5′-CCCTCTAGAGCTCCAGCGCTGCTGCACTGTCAGGCGCGCC-CA-3′ as mutagenic primer (the mutations are underlined). The resulting construct was named ppc86-villin RK/AE and encodes the villin variant R137A,K144A,R145E.

DNA Constructions for Production of Wild Type Villin or Villin Variants in Escherichia coli—for expression in E. coli, all cDNAs were introduced into the prokaryotic pGEX-2T expression vector (31). For in-frame ligation to the 3′-end of the glutathione S-transferase coding sequence, the 5′-end of the villin cDNA coding region was modified by the polymerase chain reaction. For expression of the cDNA encoding the triple substitution variant RRKR/AE in E. coli, the pCc68-villin RK/AAE DNA was Smal/EcoRI-digested. An ~2.4-kilobase pair DNA fragment corresponding to bp 51–2481 of the villin coding sequence was removed from this construct by BglII and BglII digestion and ligated to the AatII/ BglII-cut pSp64-villin yielding pSp64-villin RK/AAE. A DNA fragment of 2.7 kilobase pairs encoding the mutagenized complete villin sequence was ligated through the XbaI/EcoRI sites into the pc86 expression vector, yielding pc86-villin RRKR/AAE that encodes villin variant RRKR/AAE.

To study severing, incubations were performed according to the method described by Burnette (34). Transfer onto nitrocellulose membrane and antibody incubations were performed according to the method described by Burnette (35). Purified monoclonal antibodies were used as first antibodies (5 µg/ml). Proteins were detected using phosphatase-coupled anti-mouse IgG antibodies following the manufacturer’s instructions (Amersham Pharmacia Biotech).

Fluorescent Labeling of the Cells
Transfected CV-1 cells plated on coverslips were fixed with 3% paraformaldehyde, detergent permeabilized with 0.4% Triton X-100, and labeled as described (36). For indirect immunofluorescence staining of villin, cells were incubated with purified monoclonal anti-villin antibodies (5 µg/ml) and then with mouse IgG-specific antibodies conjugated to fluorescein. To visualize F-actin, rhodamine-conjugated phal-loidin (0.3 µg/ml) was added at the same time as the secondary antibodies. In some experiments, cells were treated with 0.5% Triton X-100 for 20 s prior to fixation and immunostained as described (25).

Production and Purification of Proteins
Actin was prepared from rabbit skeletal muscle and labeled with N- pyrene-1,10-phenanthroline as described (25, 37, 38) and kept in G buffer (2 mM Tris-HCl, pH 7.2, 0.2 mM ATP, 1 mM diethiothreitol, 0.2 mM CaCl2).

Villin-glutathione S-transferase fusion proteins were purified from the soluble fraction of sonicated E. coli BL21 bacteria by glutathione-Sepharose chromatography following standard procedures. The bead-bound fusion protein was digested with throramin (purified from human serum). Thrombin was removed by incubating eluates in the presence of p-aminobenzamidine agarose beads (Sigma) for 30 min at 4 °C. Proteins were dialyzed against storage buffer (39). For longer storage or concentration of proteins, eluates were dialyzed against storage buffer containing 50% glycerol and kept at ~20 °C. We used this storage procedure because we noticed that storing villin in liquid nitrogen considerably lowered the bundling activity when compared with freshly prepared protein. Protein concentration was determined by the Bradford procedure (40) or by densitometry of Coomassie Blue stained protein bands separated on SDS-polyacrylamide gels, using bovine serum albumin as a standard.

Electron Microscopic Analysis of Bundling and Severing Activities
To test bundling activity, 15 µM G-actin in G buffer was polymerized by addition of 150 mM KCl and 1 mM MgCl2 for 3 h at 20 °C. F-actin and villin proteins were dialyzed against buffer A (5 mM K2HPO4, pH 7, 150 mM KCl, 1 mM MgCl2, 2 mM EGTA, 1 mM diethiothreitol). F-actin (3 µM) was incubated overnight at 8 °C with wild type villin or villin variants at concentrations indicated under “Results.” To study severing, incubation of 2 µM F-actin and 0.7 µM protein was performed for 1 min in the presence of 15 µM CaCl2. Actin filaments were then competitively stained by addition of 1% uranyl acetate in acetate buffer, pH 5.5, and observed with an electron microscope on carbon-coated grids.

Sedimentation of Protein with F-actin
F-actin (3 µM) in buffer A, diluted from a 15 µM stock solution was incubated with 0.7 µM wild type villin or villin variants in the same buffer for 10 min at 20 °C. F-actin was sedimented for 15 min at 30 psi in a Beckman Airfuge or for 15 min at 200,000 × g in a Beckman TL100 ultracentrifuge. The pellet and the acetone precipitated proteins of the supernatant were resuspended in 15 µl of Laemmli sample buffer (34) containing 1 mM diethiothreitol and analyzed by SDS-polyacrylamide gel electrophoresis. Coomassie Blue-stained proteins were quantified by densitometry using VilberLourmat software. The value found for protein cosedimenting with F-actin was corrected for the amount of protein that sedimented in the absence of F-actin. Trapping of soluble proteins in the pellet, as estimated by sedimenting F-actin in the presence of bovine serum albumin (5 µg/µl) was negligible under the experimental conditions used.

Actin Polymerization or Depolymerization Assays by Fluorescence Spectroscopy
Fluorescence measurements were performed at room temperature using an SLM25 Kontron Instrument or a Spex Fluoromax fluorimeter. The excitation wavelength was set at 365 nm, and the emission wavelength was set at 388 nm with a sample volume of 700 µl.

Sedimenting of Actin Filaments—Severing activity of proteins was ana-lyzed by the approach described by Janney and Matsudaira (33). Actin (2 µM, 100% pyrene-labeled) was polymerized in F buffer (5 mM Tris-
HCl, pH 7.2, 1 mM MgCl_2, 150 mM KCl, 0.2 mM ATP, 1 mM dithiothreitol), overnight at 4 °C. F-actin was diluted below the critical concentration of the pointed end (300 nM) into F buffer containing 200 μM CaCl_2 or 2 mM EGTA and incubated with villin or villin variants at concentrations indicated under “Results.” The depolymerization rate has been shown to be proportional to the number of pointed ends and thus to the number of cuts introduced by villin. To compare the severing activity of wild type villin to that of villin variants, we measured the decrease in fluorescence/min in the linear range of the curve as described previously (41). The severing activity of villin and villin variants was measured as described by Eichinger and colleagues (42). Hence, the severing activity is the number of cuts introduced by villin. To compare the severing activity of wild type villin to that of villin variants, we measured the decrease in fluorescence/min in the linear range of the curve as described previously (41).

Nucleation of Actin Polymerization—To test the nucleating activity of villin and villin variants, we measured their effect on the initial phase of actin polymerization. G-actin (2 μM, 50% pyrenyl-labeled) in G buffer containing 20 μM CaCl_2 or 2 mM EGTA, depending on the experiment, was preincubated in the presence of villin proteins for 10 min on ice. Polymerization was induced by addition of 150 mM KCl and 1 mM MgCl_2. The increase of fluorescence was measured over time.

Capping of Actin Filaments—The capping activity of villin and villin variants was measured as described by Eichinger and colleagues (42). Preformed unlabeled actin filaments (800 nM) diluted into F buffer from a stock of 3 μM F-actin, were used as “nuclei” for the polymerization of G-actin (6 μM, 33% pyrenyl-labeled). Villin proteins were preincubated with F-actin nuclei in F buffer containing 20 μM CaCl_2 or 2 mM EGTA for 10 min on ice. The F-actin-villin mixture was rapidly added to monomeric actin in G buffer containing 20 μM CaCl_2 or 2 mM EGTA, and polymerization was induced by addition of 100 mM KCl and 1 mM MgCl_2. The increase of fluorescence was measured over time.

RESULTS

Production of Wild Type Villin and Villin Variants in Transfected Cells—We wanted to determine whether the F-actin-binding site located in the subdomain 2 (V2) of the core is important for formation of F-actin bundles in cells (Ref. 27 and Fig. 1A). To inactivate this site, we replaced both Arg_137 and Lys_144 by the neutral amino acid alanine (Fig. 1B, villin RK/AA). These substitutions when introduced individually have been shown to reduce severing activity of villin 44T by 83 and 55%, respectively (28). We also constructed a triple mutant in which, in addition to the first two substitutions, Arg_145 was replaced by glutamic acid (Fig. 1B, villin RKR/AAE). The corresponding three residues of the gelsolin sequence, Arg_161, Arg_168, and Arg_169 are contained within a solvent-exposed
structural entity that is essential for interactions of gelsolin with F-actin (16).

Production of wild type villin and the two variants in HeLa cells, transfected with the corresponding cDNAs inserted into a eukaryotic cytomegalovirus-derived expression vector, was analyzed by immunoblotting using villin-specific antibodies (Fig. 1C). Specific protein bands of an apparent molecular mass of 95 kDa, corresponding to that of human villin, could be detected in lysates of cells transfected with wild type villin, villin RK/AA, or villin RKR/AAE. No protein band was detected in the lysate of mock-transfected HeLa cells (control). The amounts of wild type villin or of villin variants produced in transfected cells were similar.

The Stretch of Basic Residues Located in V2 of Villin Is Important for the Reorganization of the Actin Cytoskeleton in Transfected Cells—Overproduction of wild type human villin in transfected fibroblast-like CV-1 cells results in the growth of microvilli and in a reorganization of the underlying actin cytoskeleton into spike-like structures. The appearance of these structures is frequently accompanied by the disruption of stress fibers (21). We used these phenotypic modifications as an assay for villin activity in cultured cells.

Transiently transfected cells were double-labeled to visualize F-actin and wild type villin or villin variants (Fig. 2, A–I). The dorsal face of cells producing the wild type villin were covered by spike-like F-actin structures (Fig. 2, A and B). A plane of focus at the ventral face of the same cell showed the absence of stress fibers (Fig. 2C). In contrast, prominent stress fibers were visible in neighboring untransfected cells. A similar organization of F-actin into surface spikes was observed in cells producing wild type villin or villin RK/AA. This variant causes similar phenotypic modifications as those observed with wild type villin. G–I, villin RKR/AAE. A cell producing this variant does not exhibit long F-actin spikes but punctuated F-actin structures corresponding to the cytoskeleton of small microvilli. Stress fibers are still present at the ventral face of the cell. Insets in G and H show the codistribution of the RKR/AAE variant with the small F-actin structures. J–L, cells were extracted with 0.5% Triton X-100 for 20 s before fixation and labeling for villin. J and K, villin label remains associated with long F-actin spikes after detergent extraction of cells producing wild type villin or villin RK/AA. L, the villin RKR/AAE variant is almost totally extracted. Bar, 15 μm.
in vivo

have also analyzed a villin variant composed of the first half of their properties in assays for actin bundling, severing, nucleation, and capping. We analyzed variants carrying truncations or substitutions of basic residues in sequences located in the villin headpiece (villin Δ7 and KKEK/KEEE) or core domain (RKR/AAE) that inactivate villin in transfected cells (25, Figs. 1B and 2) and are involved in binding to F-actin (25, 26, 28). We have also analyzed a villin variant composed of the first half of the core domain and the headpiece, named villin V1–3+HP, that retained in vivo activity (Ref. 24 and Fig. 1B).

Recombinant variants of villin, produced as glutathione S-transferase fusion proteins and subsequently cleaved by thrombin, were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 3). Wild type villin, villin Δ7, villin KKEK/KEEE, and villin RKR/AAE migrated as expected at 95 kDa, whereas villin V1–3+HP had an apparent molecular mass of 60 kDa. Proteins of lower molecular masses were identified by immunoblotting as villin degradation products (data not shown).

**Substitutions of Basic Residues in the F-actin-binding Sites of the Core and Headpiece Domains Affect Bundling Activity**—We determined whether residue exchanges in the headpiece and core domain impairing villin-induced rearrangement of F-actin into bundles in transfected cells (Fig. 1B) also affect bundling activity of villin in vitro. Experiments were carried out at physiological ionic strength (150 mM KCl) at pH 7, conditions under which villin efficiently bundles actin filaments (43). In addition, because subtle variations of villin:actin molar ratios have important consequences on bundle formation, we analyzed bundling activity at saturating quantities of villin relative to actin (villin:actin 1:2; Ref. 43), in the presence of EGTA (Fig. 4). In the sample containing F-actin alone, single or intermingled curved filaments were detected (Fig. 4A). When wild type villin was added to F-actin, straight bundles with closely aligned filaments were observed (Fig. 4, B and B'). The villin V1–3+HP variant comprising the two F-actin-binding sites (Fig. 1, A and B) was also able to induce the formation of actin bundles but to a lesser extent, as suggested from the presence of a large number of free filaments (Fig. 4D). Higher magnification images showed that actin filaments were more loosely packed in these bundles (Fig. 4D') than in those observed in the presence of wild type villin (Fig. 4B'). The Δ7 variant lacking the seven last residues, including the KKEK motif of the headpiece domain (Fig. 1B), was inactive for bundling, even at higher molar ratios of villin to actin (2:1; Fig. 4C). Substitution of lysines 821 and 823 in the headpiece domain (KKEK/KEEE variant: Ref. 25 and Fig. 1B) almost completely abolished bundling activity. In samples incubated in the presence of this variant only a few bundles with loosely associated filaments could be observed (Fig. 4E) exclusively at high villin to actin ratios (2:1). Surprisingly, triple replacements in the charged sequence of V2 of the core domain did not significantly affect the extent of bundle formation (Fig. 4F). However, bundles formed in the presence of the RKR/AAE variant were irregularly shaped, and higher magnification images revealed that they contained loosely packed filaments to which electron dense material was associated (Fig. 4F').

**Both F-actin-binding Sites Are Required for Efficient Binding to F-actin in the Absence of Ca2+**—Experiments with villin-derived peptides and domains suggested that truncated or substituted residues in the Δ7, KKEK/KEEE, or RKR/AAE variants participate in F-actin binding (25, 28). We therefore tested whether loss or reduction of bundling activity correlated with reduced binding affinity of villin variants for F-actin in cosedimentation experiments performed in the absence of Ca2+ (Fig. 5).

Most of wild type villin and the V1–3+HP variant (Fig. 5A), was found in the pellet fractions (71% and 73% of total protein, respectively). In contrast, the amount of villin Δ7 recovered in the pellet was not greater than that sedimenting in the absence of F-actin (Fig. 5A). Also at higher molar ratios of villin Δ7 to actin (3:3) this variant was not recovered in the pellet fraction (data not shown), suggesting that this variant has completely lost the ability to bind to F-actin. In agreement with in vivo observations predicting a reduced affinity for the KKEK/KEEE variant (25), cosedimentation of this polypeptide with F-actin was less efficient (41%) when compared with wild type villin (71%). The core domain of villin does not bind to F-actin in the absence of Ca2+ (9). Thus, it was interesting to note that the RKR/AAE variant also showed reduced ability to cosediment with F-actin (44%). Although affinity constants for these F-actin-binding sites cannot be calculated for the entire villin molecule, curves obtained by plotting the amount of bound protein as a function of total protein indicated that headpiece and V2 substitution variants have a reduced affinity for F-actin in the absence of Ca2+ (Fig. 5B).

**All Villin Variants Retain Severing Activity**—Because the RKR/AAE variant was still able to bundle actin filaments, we wanted to determine whether these substitutions would affect severing activity of the whole villin polypeptide. Severing activity was measured by analyzing the effect of villin and villin variants on the depolymerization of pyrenyl F-actin diluted to a concentration below the critical concentration of the pointed end. We studied severing at KCl and CaCl2 concentrations (150 and 200 μM, respectively) allowing the detection of differences in activity between wild type villin and villin domains produced by limited proteolysis (13). The RKR/AAE variant was still able to sever actin filaments in a concentration-dependent manner. However, depolymerization curves observed in the presence of equal concentrations of wild type villin or RKR/AAE variant showed that the severing activity of the mutant was reduced 2-fold (Fig. 6A). Severing activity of the RKR/AAE variant was also analyzed by electron microscopy. As observed with wild type villin, F-actin filaments were drastically shortened when incubated in the presence of the RKR/AAE mutant (data not shown).
We have also analyzed the severing activity of the V1–3+HP variant and the headpiece mutants. The depolymerization curve in the presence of V1–3+HP variant was not significantly different from that of wild type villin (Fig. 6B). Truncations or substitutions in the villin headpiece domain (villin D7 or KKEK/KEEE) did not affect severing activity (data not shown). The severing activities of all villin variants were Ca²⁺-dependent. When EGTA was added, depolymerization rates of F-actin in the presence of wild type villin, villin RKR/AAE, or V1–3+HP were almost identical to that of F-actin alone (Fig. 6C).

Domains 4–6 of Villin Are Required for Efficient Actin Nucleation but Not for Villin-induced Growth of Microvilli—Villin may induce growth of microvilli by initiating the assembly of new microvilli. In this case, nucleating activity of villin may be required for actin oligomer recruitment at the inner face of the plasma membrane. Villin 44T, a proteolitical fragment corresponding to domains 1–3, does not efficiently nucleate in vitro (13). Thus, it was important to determine whether villin variant V1–3+HP, which still induces the growth of microvilli, retains nucleating activity (Fig. 7).

In comparison with the polymerization kinetics of actin alone, the addition of villin (6 or 12 nM) in the presence of 20 μM Ca²⁺ abolished the lag phase and increased the initial rate of actin polymerization (Fig. 7A). In comparison with wild type villin, the V1–3+HP variant had very little nucleating activity, even when up to 30 nM of V1–3+HP was added (Fig. 7A). It is noteworthy, however, that the lag phase of polymerization kinetics was still reduced when V1–3+HP was added. Variants with deletions or substitutions in the F-actin-binding sites accelerated the rate of actin polymerization as well as wild type villin (data not shown). Villin or villin variants did not affect the polymerization rate of actin in the absence of Ca²⁺ (Fig. 7B). These results suggest that nucleating activity is not required for villin-caused reorganization of the actin cytoskeleton in transfected cells.

We have also analyzed the capping activity of wild type villin and villin variants by measuring their effect on the polymerization rate of pyrenyl-labeled G-actin in the presence of short filaments, used as “nuclei”. All villin variants capped actin filaments with an activity which was indistinguishable from that of wild type villin (data not shown).

**DISCUSSION**

Villin is a cell type-specific, multifunctional actin-binding protein that has been proposed to participate in the assembly of the actin core bundle of the intestinal brush border. We ana-
lyzed amino acid sequences important for villin-induced growth of microvilli in transfected cells and determined which of the villin in vitro activities are required for its morphogenic effect. We produced for the first time full-length, recombinant villin and villin variants comprising residue replacements. We found that the activity of recombinant human villin was similar to that previously reported for villin purified from tissue. The capacity of villin variants to organize actin filaments into tight bundles in vitro and to bind efficiently to F-actin is closely related to their ability to induce microvilli in cultured cells (Table I). In contrast, nucleation of actin polymerization in vitro is not correlated with induction of microvilli by villin (Table I). Hence, villin may participate in the organization and stabilization of the F-actin core bundle in vivo but most likely does not “nucleate” the microvillus cytoskeleton by stabilizing actin oligomers at the plasma membrane.

The V1–3+HP variant comprising the minimal structural information necessary for elongation of microvilli in transfected cells (24) does not nucleate actin polymerization in vitro, as compared with wild type villin (Table I). Based on this finding and the observation that villin 44T does not nucleate at low 44T to actin ratios, it is likely that one of the actin monomer-binding sites required for nucleation is located in domains 4–6. In this regard, it is interesting to note that a natural variant of mouse adseverin lacking the fifth repeat of its gelsolin-like core by an alternative splicing event lost specifically its nucleating capacity (44). Similarly, it has been shown for the closely related gelsolin polypeptide that not G1–3 but G2-G6 nucleates actin polymerization (45, 46).

Separated villin core or headpiece domains do not bundle actin filaments in vitro (10). We found that the V1+HP variant cosediments with F-actin to a similar extent as wild type villin and bundles actin filaments. This finding suggests that F-actin-binding sites comprised in the first half of the core domain and the headpiece are functional and participate both in bundling. However, because the bundles were less tightly packed than those observed with wild type villin, it is likely that the lack of the second half of the core domain affects the structural organization of the villin domains and may at least partially explain why the activity of this variant in cultured cells is reduced (Ref. 24 and Table I).

The seven carboxyl-terminal residues of the headpiece and particularly the included KKEK motif are essential for villin function in transfected cells (24). Our results show that the loss or reduction of the morphogenic activity of villin Δ7 and KKEK/KEEE, respectively, correlates with the loss or reduction of their F-actin binding and bundling activities in vitro (Table I). These findings emphasize the importance of the KKEK motif in the binding of villin to actin, as suggested from work with villin-derived peptides (25). Because villin core alone does not cosediment with F-actin in the absence of Ca2+ (10), it is likely that K821E,K823E substitutions do not totally inactivate the F-actin-binding site located in the headpiece but reduce its affinity for F-actin. Replacement of Lys822 by cysteine has little effect on the binding of the headpiece domain to actin in vitro (26). We have not analyzed the effect of K823E substitution in vitro, because this mutation does not affect villin activity in the transfection assay. Therefore, we cannot exclude the possibility that only Lys821 but not Lys823 is directly involved in actin interactions. However, NMR-resolved structure of the carboxyl-terminal subdomain of the headpiece revealed that these residues cluster on one face of an α-helix and are likely to be both in contact with actin (47, 48). Because each residue contributes a fraction of the total binding energy, single mutations in polypeptides may only partially inhibit binding to F-actin (28).

Domain 2 of villin contains an F-actin-binding site that is important for severing activity and for docking villin to F-actin in the presence of Ca2+ (28, 27). Replacements of three basic residues (Arg137, Lys144, and Lys145) in full-length villin reduced its binding to F-actin in vitro and its avidity for microfilaments in transfected cells, confirming that these residues are part of an F-actin site, as suggested from work with villin-derived peptides (Table I and Ref. 28). Villin core does not bind F-actin binding in the absence of Ca2+ (9). The observation that substitutions in the V2 domain reduce F-actin binding affinity of full-length villin in the absence of Ca2+ suggests that the sites located in the headpiece and core domain are both implicated in binding and that binding of the V2 site may occur in a cooperative manner.

Triple substitutions (RKR/AAE) in the V2 site impaired actin spike formation (Table I), suggesting that this site is required for actin bundle formation in cells. In addition, this villin variant had a reduced capacity to disrupt stress fibers. In parallel, we observed that these substitutions affected both bundling and severing activity in vitro. However, may be due to the presence of domains 4–7, their effect on the severing activity of full-length villin was less severe as expected from the results.
obtained with the 44T villin fragment (28). Similarly, whereas mutations in the F-actin-binding site of the headpiece domain rendered villin incapable of bundling, the triple RKR/AAE replacement variant still generated actin bundles that, however, were less well organized and exhibited electron dense material along their sides. Thus, because the F-actin-binding site located in the villin core is involved in severing and bundling, we cannot exclude the possibility that both activities are important for villin function in cells.

Although F-actin spike formation and disruption of stress fibers appeared to be closely coupled, it is noteworthy that loss of stress fibers can be observed independently of spike formation in cells producing wild type villin (21). In line with this observation, it has been shown that the microinjected core domain disrupts stress fibers in cells (Ref. 22 and Table I). Whether the loss of stress fibers is solely a consequence of F-actin severing is not yet clear. For instance, it is not excluded that villin may destabilize stress fibers by competing with other actin-binding proteins (21). Indeed, the villin variant ∆7 retains full severing activity yet does not efficiently disrupt stress fibers (Ref. 25 and Table I). The difference in the capacity of villin core and ∆7 to disrupt stress fibers (Refs. 22 and 21

**Fig. 6.** Ca$^{2+}$-dependent severing activity of villin or villin variants. Pyrenyl-labeled actin filaments were diluted under the critical concentration of the (−) end. Severing by villin or villin variants was measured as a decrease in fluorescence because of rapid depolymerization at the newly created pointed ends. Graphs represent the decrease of relative fluorescence intensity plotted as a function of time. The fluorescence scale was adjusted so that the fluorescence of F-actin was 100 and that of G actin was 0. A, F-actin was diluted in polymerization buffer containing villin (60 nM) or RKR/AAE (35 or 60 nM) in the presence of 200 μM Ca$^{2+}$. Control, depolymerization of F-actin in the absence of severing protein. B, F-actin was diluted in polymerization buffer containing 200 μM Ca$^{2+}$ and villin (60 nM) or V1–3+HP (60 nM). Control, depolymerization of F-actin in the absence of severing protein. C, F-actin was diluted in polymerization buffer containing 2 mM EGTA and villin (60 nM), V1–3+HP (60 nM), or RKR/AAE (60 nM). Control, depolymerization of F-actin in the absence of severing protein.

**Fig. 7.** Ca$^{2+}$-dependent nucleating activity of villin and villin variants. A, to evaluate the nucleating activity, the effect of villin (6 and 12 nM) or V1–3+HP (12 and 30 nM) on the initial rate of polymerization of 6 μM actin in the presence of 20 μM CaCl$_2$ was determined. Polymerization was started by addition of 150 mM KCl and 1 mM MgCl$_2$ to G buffer containing G-actin and nucleating protein. Control, polymerization of actin in the absence of nucleating protein. The increase of relative fluorescence intensity is plotted in function of time. B, the effect of villin (12 nM) or V1–3+HP (12 and 30 nM) on the initial rate of polymerization of 6 μM actin in the presence of 2 mM EGTA. Control, polymerization of actin in the absence of nucleating protein.
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and Table I) may be explained by distinct properties of these variants or by the fact they were introduced into cells by different approaches, microinjection and transfection, respectively.

Altogether, our data suggest that the F-actin-binding site of the headpiece domain has a unique function in the formation of actin filament bundles. Weak hydrophobic interactions of this sequence with actin might be stabilized by electrostatic interactions involving the KKEK motif. The neutralization of the negative charges exposed at the surface of the actin filament may favor their alignment into bundles, as proposed from F-actin bundling studies with polyacationic agents in vitro (49). Subsequently, the F-actin-binding site of the V2 subdomain would then cross-link the aligned microfilaments. We do not know yet how villin induces the elongation of actin filaments in transfected cells. It is interesting to note that T-plastin, which is exclusively a bundling protein, is able to induce growth of microvilli in transfected epithelial cells (50). A mechanism involving changes of the rates of association or dissociation of monomers from the filaments ends, as shown for the 30-kDa bundling protein of Dictyostelium (51), remains to be carefully evaluated.

Recently it has been shown that brush border assembly is not impaired in villin minus mice (52), indicating that villin function may be redundant with that of other bundling proteins of the brush border such as fimbrin or espin (50, 53). However, brush border of intestinal cells in villin-minus mice does not undergo Ca\(^{2+}\)-induced actin filament fragmentation as observed for that of wild type animals (54). This observation opens the possibility that by virtue of its severing activity, villin may play a role in remodeling the actin cytoskeleton during physiological situations such as repair of lesions of the intestinal epithelium.

** Bundles contain loosely arranged actin filaments and electron dense material.

| TABLE I | Summary of the activities of wild type villin and villin variants in transfected cells and in vitro |
|---|---|
| 1 | N | wild type villin | 826 C |
| 2 | villin core (1-749) | |-|
| 3 | V1+3 HP (Δ 412-713) | ++ |
| 4 | villin Δ 7 (Δ 820-826) | -|
| 5 | villin KKEK/KEE (KK/821/823/E) | ++ |
| 6 | villin RK/ AA (RK137/144/ AA) |++ |
| 7 | villin RK/AA (RK137/144/145/AA) |++ |

* Disruption of stress fibres was only observed with microinjected villin core (22), because villin core produced from transfected cDNA was unstable (21).

** Bundles contain loosely arranged actin filaments and electron dense material.
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