Tyrosine Phosphorylation of Villin Regulates the Organization of the Actin Cytoskeleton*

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We have previously shown that tyrosine phosphorylation of the actin-regulatory protein villin is accompanied by the redistribution of phosphorylated villin and a concomitant decrease in the F-actin content of intestinal epithelial cells. The temporal and spatial correlation of these two events suggested that tyrosine phosphorylation of villin may be involved in the rearrangement of the microvillar cytoskeleton. This hypothesis was investigated by analyzing the effects of tyrosine phosphorylation of villin on the kinetics of actin polymerization by reconstituting in vitro the tyrosine phosphorylation of villin and its association with actin. Full-length recombinant human villin was phosphorylated in vitro by expression in the TKX1-competent cells that carry an inducible tyrosine kinase gene. The actin-binding properties of villin were examined using a co-sedimentation assay. Phosphorylation of villin did not change the stoichiometry (1:2) but decreased the binding affinity (4.4 μM for unphosphorylated versus 0.6 μM for phosphorylated) of villin for actin. Using a pyrene-actin-based fluorescence assay, we demonstrated that tyrosine phosphorylation had a negative effect on actin nucleation by villin. In contrast, tyrosine phosphorylation enhanced actin severing by villin. Electron microscopic analysis showed complementary morphological changes. Phosphorylation inhibited the actin bundling and enhanced the actin severing functions of villin. Taken together, our data show that tyrosine phosphorylation of villin decreases the amount of villin bound to actin filaments, inhibits the actin-polymerizing properties of villin, and promotes the actin-depolymerizing functions instead. These observations suggest a role for tyrosine phosphorylation in modulating the microvillar cytoskeleton in vivo by villin in response to specific physiological stimuli.

Villin, an epithelial cell-specific protein, belongs to a family of actin-severing and -capping proteins, which includes gelsolin, severin, fragmin, and CapG among others. Villin is unique among this family of proteins in that it can also cross-link and bundle actin filaments. We have previously shown that villin is tyrosine-phosphorylated both in intestinal epithelial cells (1) and in vitro (2). Since our first demonstration of tyrosine phosphorylation of villin, other proteins of this family, including gelsolin, have been reported to be tyrosine-phosphorylated in vitro (3). Thus, tyrosine phosphorylation may also be a common feature of this family of proteins, and phosphorylation may play an important role in the organization of the actin network by these actin-binding proteins. Previous in vivo work from our laboratory shows that tyrosine phosphorylation of villin is accompanied by a decrease in the F-actin content of the cell (4). However, a causal relationship between tyrosine phosphorylation and changes in the distribution and/or kinetics of actin polymerization remains to be established.

In addition to actin, villin interacts with several signaling molecules including phosphatidylinositol 4,5-bisphosphate (5), Ca\(^{2+}\) (6), and phospholipase C-γ (1, 2). Tyrosine phosphorylation of villin and its ligand binding properties suggest that in addition to its role in regulating the actin cytoskeleton, villin may also be a regulatory target. Thus, villin may function as a structural scaffold for signaling proteins or participate in translating cell surface receptor-mediated biochemical reactions to the cell movement machinery. Severing of actin filaments and nucleation of actin polymerization are essential for the remodeling of the cortical actin network that accompanies nearly all types of cell activation. Precise actin cytoskeleton remodeling requires tight spatial and temporal regulation of actin filament assembly and organization. Cells accomplish this by stimulating or inhibiting the activity of several actin-associated proteins. The actin-modifying properties of these proteins are regulated by several different factors including calcium (6), phospholipids (5), pH (7), and serine/threonine phosphorylation (8, 9). In recent years several actin-regulatory proteins have been shown to be tyrosine-phosphorylated, adding yet another level of regulation. Less well characterized are the tyrosine phosphorylation of these proteins and the effect of phosphorylation on the actin-modifying properties of these proteins.

In the present study, we have used the approach of reconstitution in vitro to investigate the role of tyrosine phosphorylation of villin in the regulation of its actin-modifying functions. The complexity of the various actin-remodeling abilities of villin makes such an approach most useful in dissecting the in vivo effect of villin phosphorylation on the actin network. Using recombinant phosphorylated (VILT/WT) or unphosphorylated (VIL/WT) villin, we show that tyrosine phosphorylation promotes the actin-severing rather than actin-polymerizing functions of villin. The results of our studies allow us to propose a more general model for the actin-

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§ The abbreviations used are: VILT/WT, full-length recombinant human tyrosine-phosphorylated villin; VIL/WT, full-length recombinant human villin; GST, glutathione S-transferase; IAA, 3-β-indoleacrylic acid; PAGE, polyacrylamide gel electrophoresis.
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Nucleation of Actin Polymerization—G-actin (6 µM) in buffer containing 5 mM Tris-HCl, pH 7.0, 0.2 mM ATP, and 0.2 mM CaCl₂ was preincubated with VIL/WT or VILT/WT (60 nM) for 10 min on ice. Polymerization was induced by the addition of 150 mM KCl and 1 mM MgCl₂. The increase in fluorescence that occurs when pyrene G-actin associates with soluble F-actin was monitored over time. The rate at which actin polymerizes depends on the concentrations of free actin monomers and the filament ends. Because villin complexes with G-actin faster than spontaneous actin nuclei can form, the initial rate of polymerization determined from the rate of fluorescence increase is proportional to the number of pointed-end nuclei formed and, therefore, the relative nucleating activity of villin (12). To test the nucleating activity of VIL/WT and VILT/WT, we measured the effects on the initial rate of actin polymerization. The increase in fluorescence was recorded every 5 s.

Severing of Actin Filaments—For assays of filament-severing activity, a sample of pyrene-labeled F-actin was diluted below its critical monomer concentration into solutions containing villin (60 nM). Because actin filaments depolymerize only from their ends, the rate of fluorescence decrease, proportional to the depolymerization rate, depends on the number of ends and therefore on the number of cuts introduced by villin (12). To compare the severing activity of VIL/WT and VILT/WT, we measured the decrease in fluorescence/min in the linear range of the curve as described previously (8). The decrease in fluorescence was recorded every 2 s.

Electron Microscopic Analysis of Bundling and Severing Activities—To test the bundling activity, G-actin in buffer containing 5 mM Tris-HCl, pH 8.0, 0.2 mM ATP, and 0.2 mM CaCl₂ was polymerized by the addition of 150 mM KCl and 1 mM MgCl₂ for 1 h at 20 °C. F-actin (3 µM) was incubated overnight at 4 °C with VIL/WT or VILT/WT (1.5 µM) in the presence of EGTA (2 mM). To study severing, incubation of 2 mM F-actin and 0.7 mM VIL/WT or VILT/WT was performed for 1 min in the presence of 1 mM CaCl₂ at 20 °C. Samples were applied to carbon-coated Formvar grids. Excess sample was carefully withdrawn, and the grids were stained with 2% aqueous uranyl acetate after a brief fixation in 2% glutaraldehyde. For actin bundling studies, samples were diluted 1:1 in actin polymerization buffer immediately before applying the samples to the grids. Grids were examined at 60 kV in a JEOL 2000 EX-II electron microscope.

RESULTS AND DISCUSSION

Tyrosine Phosphorylation Decreases the Affinity of Villin for F-actin—The present work examines how villin and its phosphorylation may contribute to the regulation of actin dynamics. The finding that tyrosine phosphorylation of villin in intestinal epithelial cells coincides with the redistribution of villin from the actin cytoskeleton to the plasma membrane (1, 4) suggested that perhaps tyrosine phosphorylation regulates the actin binding properties of villin. To test this hypothesis, we first examined whether the actin binding activity of villin was regulated by phosphorylation. The binding affinity of villin for F-actin was determined by co-sedimentation experiments performed in the presence of villin (phosphorylated or not) and in the absence of Ca²⁺. Full-length human recombinant villin was purified from TKX1 cells either as a phosphorylated (VIL/WT) or unphosphorylated (VIL/WT) protein (Fig. 1A). We had previously demonstrated that full-length recombinant villin demonstrates all the actin modifying properties of the native protein (purified from chicken brush border) (2), thus allowing us to reconstitute in vitro the kinetics of actin polymerization by villin. Purified recombinant proteins (0–2 µM) were mixed with polymerized filamentous actin (3 µM) and then subjected to high-speed centrifugation. The partitioning of villin and actin between the supernatant and pellet fractions was analyzed by GelCode Blue staining and densitometry. The amount of villin bound to actin was plotted against the total villin concentration; the stoichiometry of binding and the k₅ values of the interaction between villin and F-actin were calculated by assuming a sigmoidal relationship between total villin concentration and villin bound to actin filaments (Hill equation). The stoichiometry of villin to actin was 1:2, consistent with two actin-binding sites/molecule of villin (13). Co-sedimentation experiments revealed that the stoichiometry of villin-actin bind-
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Fig. 1. Phosphorylation negatively regulates the binding of villin to actin. A, recombinant human villin phosphorylated (VILT/WT) or unphosphorylated (VIL/WT) was purified from TKX1 cells as described under “Experimental Procedures.” This is an immunoblot of VIL/WT and VILT/WT with monoclonal antibodies to phosphotyrosine (upper panel) or villin (lower panel). This blot is representative of three blots with similar results. B, increasing amounts of villin (VIL/WT or VILT/WT) were added to prepolymerized actin (5 μM). The samples were incubated for 10 min at 25 °C and then subjected to high-speed centrifugation. The supernatant and pellet fractions were separated by SDS-PAGE, and the partitioning of villin between the supernatant and pellet fractions was determined by GelCode Blue staining. The gels were analyzed by densitometry, and the percentage of villin in the pellet relative to the total amount of villin was calculated for each sample. Bound villin was plotted as a function of total villin. Data are the mean of three experiments and are fitted with the Hill equation.

Fig. 2. Effect of phosphorylated villin on actin dynamics. A, effect of villin phosphorylation on kinetics of actin polymerization. Pyrene G-actin (6 μM) was incubated with VIL/WT or VILT/WT (60 nM) in polymerization-inducing buffer, and fluorescence intensity was measured over time. Control represents the polymerization of actin in the absence of villin. Fluorescence was recorded every 5 s as described under “Experimental Procedures.” B, effect of villin phosphorylation on actin depolymerization. Pyrene-F-actin (1 μM) in the presence of VIL/WT or VILT/WT (60 nM) was diluted to 0.1 μM in actin-polymerizing buffer, and the decrease in fluorescence intensity was followed over time. Control represents the depolymerization of actin in the absence of villin. Fluorescence was recorded every 2 s as described under “Experimental Procedures.” Values represent the mean of three independent experiments.

Tyrosine Phosphorylation Decreases the Actin-nucleating Activity of Villin—Because the phosphorylated villin retained its ability to bind to F-actin, we wanted to determine whether phosphorylation would affect the nucleating activity of villin. We investigated the influence of villin phosphorylation on the kinetics of actin polymerization. First, the concentration of G-actin required for polymerization (critical concentration) in the presence of VIL/WT and VILT/WT was determined. Fluorescence measurements were made at steady state to determine polymer concentration as a function of total actin concentration. Tyrosine phosphorylation led to a very small change in the critical concentration of actin required for polymerization in the presence of villin (0.10 ± 0.008 μM for VIL/WT versus 0.15 ± 0.005 μM for VILT/WT). We next determined the actin nucleating activity of VIL/WT and VILT/WT. In comparison with the polymerization kinetics of actin alone (control), the addition of VIL/WT (60 nM) in the presence of 20 mM Ca²⁺ abolished the lag phase and increased the initial rate of actin polymerization (Fig. 2A). In contrast, VILT/WT resulted in a lag phase and decreased the rate of actin polymerization. It was noted that although actin polymerized in the presence of VILT/WT, phosphorylation significantly reduced the nucleating ability of villin (74.7 ± 2.5% with VIL/WT versus 97.2 ± 1.3% with VILT/WT). There was no change in actin nucleation in the presence of GST (data not shown). These data show that phosphorylation of villin inhibits its actin nucleating activity. Further, they suggest that tyrosine phosphorylation may decrease the ability of villin to initiate the formation of F-actin filaments in vivo, thus leading to the reorganization of the actin cytoskeleton.

Next, we examined the effect of tyrosine phosphorylation of villin on F-actin depolymerization. Pyrene F-actin (1 μM) in the presence of either VIL/WT or VILT/WT (60 nM) was diluted to a concentration of 0.1 μM, and the fluorescence intensity was monitored over time. VIL/WT significantly increased the depolymerization of F-actin compared with control (43.7 ± 3.2% versus 12.9 ± 0.9%, respectively; n = 9, p < 0.01). Furthermore,
phosphorylation of villin increased its actin-depolymerizing property. Fluorescence measurements show a 22% (n = 9, p < 0.01) increase in actin severing by VILT/WT compared with VIL/WT. These results are consistent with an increase in the actin depolymerizing function of phosphorylated villin. These data support our hypothesis that tyrosine phosphorylation of villin and a decrease in the intestinal cell F-actin content are not mutually exclusive.

Tyrosine Phosphorylation Alters the Actin Filament Organization—To further explore the correlation between phosphorylation and regulation of the actin-modulating properties of villin, we examined the effects of VIL/WT and VILT/WT on actin filament morphology. Actin polymerized in the absence of villin shows long, curved F-actin filaments that are not cross-linked (Fig. 3A). In the presence of saturating amounts of VIL/WT, the F-actin filaments are closely aligned, well organized, tight bundles (Fig. 3C). In contrast, phosphorylation of villin led to reorganization of the actin filaments, which now showed thicker bundles, which at higher magnification appeared to have loose, poorly organized filament structure. The bundles showed no distinct alignment of filament but contained loosely packed filaments with some associated electron dense material (Fig. 3C'). There was also an appreciable decrease in the average length of the filaments (Fig. 3C). Thus, although VILT/WT can bind actin filaments albeit with lower binding affinity for actin, it does not bundle filaments like VIL/WT. Negative charges exposed on the surface of the actin filaments favor their alignment into bundles, and polyamines can disintegrate actin bundles into single filaments (14). Basic amino acids in villin have been shown to promote villin binding to actin (15). This may explain why introduction of negative charges by phosphorylation weakens villin-actin interactions, and the phosphorylated villin shows poor bundling activity.

We next obtained electron micrographs of the actin-severing functions of villin. In the presence of Ca²⁺ and the absence of villin, the morphology of actin filaments is indistinguishable from pure actin filaments seen in the presence of EGTA (compare Figs. 4A and 3A). In the presence of Ca²⁺, the addition of VILT/WT to F-actin severs actin filaments (Fig. 4B). The filaments are much shorter than those observed in preparations of pure actin (Fig. 4B'). In contrast, the addition of VILT/WT has a profound effect on actin morphology. In samples briefly (1 min) treated with VILT/WT, no actin filaments were observed. The samples contained fibrillar material, which at higher magnification showed very small filamentous fragments of heterogeneous size (Fig. 4C'). Because VILT/WT does not significantly elevate the critical concentration for actin assembly, the obvious conclusion from these morphological studies is that phosphorylation promotes actin disassembly through fragmentation of filaments into small pieces rather than through depolymerization. Interestingly, in anoxic proximal tubule a coincident microvillar actin bundle disruption was shown to be associated with severing of actin bundles rather than depolymerization of F-actin (16). Tyrosine phosphorylation of villin could explain such severing of actin bundles during anoxia.

Analysis of the actin polymerization kinetics in the presence of tyrosine phosphorylated villin revealed that tyrosine phosphorylation promotes the actin disassembling properties rather than assembling properties of villin. In vivo, tyrosine-phosphorylated villin may dissociate actin bundles and promote severing, thus leading to a breakdown of the microvillar network. Phosphorylated villin could prevent actin assembly by distinct mechanisms including lower binding affinity for F-actin, inhibi-
iting nucleation of new filaments, and cutting pre-existing filaments. Phosphorylation of villin and a local decrease in the affinity of villin for actin could generate a dynamic state and an increase in the fluidity of the cytoskeleton. A decrease in the affinity of villin for actin could also affect the mechanical properties of the actin cytoskeleton. For instance it is known that a change in the affinity of cross-linkers for actin could change the cytoplasm from a solid to a fluid and thus from a rigid to a dynamic network. These temporal changes in the physical properties of the cytoskeleton could enhance cell motility, which might clarify the role of villin in intestinal restitution, for example (17, 18).

The structural and functional relationships between tyrosine phosphorylation of villin and actin polymerization kinetics are likely to extend to other actin-binding proteins. Identification of the tyrosine phosphorylation sites in villin will be instructive in understanding the structural basis of actin filament modifying activities and the relationship of phosphorylation with these functions, in both villin and other proteins that share sequence homology with villin. The kinetics of filament assembly may be a function of not only the concentrations of various intracellular messengers such as Ca\(^{2+}\) and phosphatidylinositol 4,5-bisphosphate but also the phosphorylation state of actin regulatory proteins. The actin-binding proteins may be novel substrates for tyrosine kinases, also suggesting that these proteins may target signaling molecules to effector sites as well as recruit actin complexes to modify the cytoskeleton at these sites. Interestingly, phosphorylation (serine/threonine phosphorylation) of the few actin-binding proteins studied so far demonstrates a decrease in the actin binding affinities, or actin bundling properties, and/or decrease in the nucleation of actin (19, 20). Thus, phosphorylation, whether on serine, threonine, or tyrosine residues in the actin-modifying proteins, appears to be a negative regulator of actin assembly. Phosphorylation of villin suggests that filament turnover in cells may be defined by the regulated action of actin-binding proteins interacting with signaling molecules. Thus, the cell may use villin phosphorylation as an important regulatory switch to modify the actin cytoskeleton in the epithelial microvillar core.

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