Regulation of Actin Dynamics by Tyrosine Phosphorylation: Identification of Tyrosine Phosphorylation Sites within the Actin-Severing Domain of Villin†

Liwei Zhai,‡ Narendra Kumar,‡ Alfredo Panebra,‡ Peilin Zhao,‡ Abby L. Parrill,§ and Seema Khurana*,‡

Department of Physiology, University of Tennessee Health Science Center, Memphis, Tennessee 38163, and Department of Chemistry, University of Memphis, Memphis, Tennessee 38152

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ABSTRACT: We have previously shown that villin, an epithelial cell actin-binding protein, is tyrosine phosphorylated both in vitro and in vivo and that villin's actin-modifying functions are regulated by phosphorylation. Here as a first step toward understanding the role of villin tyrosine phosphorylation, we sought to identify the major phosphorylation site(s) in human villin and study its role in actin filament assembly. We generated a series of carboxyl-terminal truncation mutants of villin and cloned them in the prokaryotic expression vector pGEX-2T. Full-length villin and the truncation mutants were expressed in TKX1 cells, which carry an inducible tyrosine kinase gene. Using this approach, we identified a region in the amino-terminal actin-severing domain of villin as the site of phosphorylation (amino acids 1−261). Five phosphorylation sites were identified by direct mutation of candidate tyrosines (Y) to phenylalanine (F), namely, Y46, -60, -64, -81, and -256. Changing all of these sites to phenylalanine resulted in a villin mutant that neither was phosphorylated in TKX1 cells nor was a substrate for *c-src* kinase in an in vitro kinase assay. Using a pyrene actin-based fluorescence assay, we mapped the various phosphorylated tyrosine residues with the actin-nucleating and -depolymerizing functions of villin. Phosphorylation of any one of the identified sites inhibited the actin-nucleating function of villin, whereas phosphorylation at Y46 and/or Y60 increased the actin-severing activity of villin. Since there is significant homology between the amino-terminal end of villin and other actin-severing proteins, the results provide a structural basis for the actin-severing mechanism and help understand the relationship of phosphorylation with this function.

Villin, an epithelial cell-specific protein, belongs to a family of actin-binding proteins that contain segments which display internal homology with each other (*1*). Members of the villin family include, among others, gelsolin, fragmin, and severin. Like many actin-binding proteins, the actin-modifying functions of villin have only been described in vitro. The in vitro interaction of villin with actin has been extensively studied. At low calcium concentrations (<1 μM), villin will bundle F-actin (*2*). In micromolar calcium, villin acts as a barbed-end capper of F-actin, and at calcium concentrations above 100 μM, villin will sever actin filaments (*2*, *3*). Villin binds Ca^{2+} and phosphatidylinositol 4,5-bisphosphate (PIP_2),[1] both of which modulate its actin regulatory functions (*4*, *5*). Regulation of villin's actin-modifying functions in response to changes in the concentrations of Ca^{2+} and PIP_2, which are often elevated during agonist activation, suggests that villin may lie in the pathway between cell surface receptor activation and cytoskeletal remodeling. Villin is also tyrosine phosphorylated both in intestinal epithelial cells (*6*) and in vitro (*7*), adding yet another level of regulation. Thus, regulation of filament assembly by villin may be a function of not only the concentrations of various intracellular messengers such as Ca^{2+} and PIP_2 but also the phosphorylation state of villin.

Rearrangement of the apical membrane cytoskeleton plays an essential role in a wide variety of physiological and pathophysiological processes in epithelial cells. Our previous work in intestinal epithelial cells and the recent observation in an opossum kidney cell line demonstrate that tyrosine phosphorylation of villin regulates changes in the microfilament structure that are crucial to the function of ion transport in epithelial cells (*6*, *8*). In addition, rearrangement of the microvillar cytoskeleton and concomitant redistribution of villin have been reported in studies with several enteroinvasive bacteria (*9*, *10*), in intestinal restitution (*11*, *12*), in renal ischemia and ischemia of the small intestine (*13*−*16*), and in colonic adenocarcinomas (*17*). Despite intense investigation, the mechanisms regulating spatial and temporal control of microvilli retraction and formation and the actin cytoskeletal organization associated with these processes are not yet well established. Understanding the regulation of villin's actin-modifying properties might help clarify the role of villin in these processes. Precise actin cytoskeletal remodeling requires tight spatial and temporal regulation of actin filament assembly and organization. Since the actin

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* To whom correspondence should be addressed: University of Tennessee Health Science Center, 894 Union Ave., Nash 402, Memphis, TN 38163. Phone: (901) 448-3410. Fax: (901) 448-3505. E-mail: skhurana@utmem.edu.

‡ University of Tennessee Health Science Center.

§ University of Memphis.

[1] Abbreviations: F, phenylalanine; Gst, glutathione *S*-transferase; IAA, 3-β-indoleacrylic acid; IPTG, isopropyl β-D-thiogalactopyranoside; PIP_2, phosphatidylinositol 4,5-bisphosphate; TCA, trichloroacetic acid; VIL/WT, full-length recombinant human villin; VILT/WT, full-length recombinant human tyrosine-phosphorylated villin; Y, tyrosine.

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filaments are themselves quite stable kinetically, the actin filament dynamics in the cell depend on actin-binding proteins that can sever, cap, nucleate, and cross-link the filaments. The actin-modifying properties of these proteins are regulated in vitro by several different factors, including calcium (*4*), phospholipids (*18*), pH (*19*), serine/threonine phosphorylation (*20*, *21*), and tyrosine phosphorylation (*7*, *22*−*25*). Less well characterized are the tyrosine phosphorylation of these proteins and the effect of phosphorylation on the actin-modifying properties of these proteins.

Our previous studies identify tyrosine phosphorylation as a negative regulator of villin's actin-polymerizing and a positive regulator of its actin-depolymerizing functions (*22*). Therefore, determining the site(s) of phosphorylation in villin is of substantial interest. 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 (*26*, *27*). Thus, tyrosine phosphorylation may 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.

In the study presented here, we use a series of carboxyl-terminal truncation mutants to localize the phosphorylation sites responsible for modulating the actin regulatory functions of villin, and we report that several different sites contribute to this behavior. We identify an area in the amino terminus of villin as the site of phosphorylation. The amino terminus of villin is essential for actin depolymerization and its association with phospholipids, which in turn also regulates its actin-severing activity (*4*, *28*). Since there is significant homology between the amino-terminal end of villin and other actin-severing proteins, the structural and functional relationships between tyrosine phosphorylation of villin and actin polymerization kinetics are likely to extend to these actin-binding proteins, including gelsolin, fragmin, and severin.

EXPERIMENTAL PROCEDURES

Materials. Epicurian coli TKX1 and BL21 competent cells and the QuikChange site-directed mutagenesis kit were from Stratagene. Glutathione Sepharose 4B Fastflow and [^{32}P]ATP were from Amersham-Pharmacia. Glass fiber filters were from Millipore. GelCode Blue was from Pierce. Monoclonal antibodies to phosphotyrosine (clone PY20) were from ICN. Monoclonal antibodies to villin were from Transduction Laboratories. Monoclonal antibodies to *c-src* and recombinant *c-src* kinase expressing the kinase domain were purchased from UBI. The nonmuscle and muscle actin polymerization kits were purchased from Cytoskeleton (Denver, CO); all other chemicals were from Sigma or Invitrogen.

Tyrosine Phosphorylation of Villin in TKX1 Cells. Full-length or mutant villin cDNA cloned in pGEX-2T were expressed in *E. coli* TKX1 cells as described previously (*7*, *22*). TKX1 cells carry a plasmid with the elk tyrosine kinase (tk) gene controlled by the trp promoter. The TKX1 cells were transformed with pGEX-2T containing full-length or mutant villin (human) cDNA. A two-step protocol involving first the induction of expression of the villin protein gene [by addition of isopropyl β-D-thiogalactopyranoside (IPTG)] followed by induction of the tk gene [by addition of indoleacrylic acid (IAA)] allowed for the accumulation of glutathione *S*-transferase (Gst)-tagged tyrosine-phosphorylated villin. Recombinant villin was affinity purified from bacterial lysates using a Glutathione Sepharose 4B column. The protein was eluted using 5 mM reduced glutathione in 1 mL fractions. The purity of the fractions was assessed by SDS−PAGE and by staining the gels with GelCode Blue. Tyrosine phosphorylation of villin was assessed by Western analysis using a phosphotyrosine monoclonal antibody. Densitometric analysis was carried out using Scion Image software. TKX1 cells were cultured in the absence of IAA to obtain unphosphorylated villin controls. Alternatively, *E. coli* BL21 cells were transformed with the full-length or mutant villin cDNA to obtain unphosphorylated villin controls.

Carboxyl-Terminal Truncation Mutants of Villin. To map the villin phosphorylation site(s), we created truncation mutants. Briefly, we designed complementary primers to introduce stop codons at positions 475, 338, 261, and 67 of human villin cDNA using the QuikChange site-directed mutagenesis kit, as recommended by the manufacturer. The introduction of stop codons was verified by sequencing. TKX1 cells were transformed with full-length or mutant villin cDNA. Recombinant truncation proteins (VT1−VT4) were purified from TKX1 cells as described above.

Substitution of Tyrosine with Phenylalanine in Villin Truncation Mutants. The putative phosphorylatable tyrosine (Y) residues in the truncation mutants VT3 and VT4 were changed to phenylalanine (F) by designing complementary primers in which a Y codon was replaced with an F codon. Tyrosines at positions 46, 60, 64, 81, 110, 134, and 256 were replaced with phenylalanine using the QuikChange site-directed mutagenesis kit and Pfu Turbo DNA polymerase to make single-base changes from TAT and TAC to TTT and TTC, respectively. The mutation primers were as follows: Y46F, 5′-GATGGTGACTGCTTCATCATCCTG-GC-3′ (forward) and 5′-GCCAGGATGATGAAGCAGT-CACCATC-3′ (reverse); Y60F, 5′-AGCAGCCTGTCC-TTTGACATCCACTAC-3′ (forward) and 5′-GTAGTG-GATGTCAAAGGACAGGCTGCT-3′ (reverse); Y64F, 5′-TATGACATCCACTTCTGGATTGGCCAG-3′ (forward) and 5′-CTGGCCAATCCAGAAGTGGATGTCATA-3′ (reverse); Y81F, 5′-GCAGCTGCCATCTTCACCACACAGATG-3′ (forward) and 5′-CATCTGTGTGGTGAAGATGGCAGCT-GC-3′ (reverse); Y110F, 5′-CTTCCGAGGCTTCTTCAAG-CAAGGCC-3′ (forward) and 5′-GGCCTTGCTTGAA-GAAGCCTCGGAAG-3′ (reverse); Y134F, 5′-GAGACC-AACTCCTTTGACGTCCAGAGG-3′ (forward) and 5′-CCTCTGGACGTCAAAGGAGTTGGTCTC-3′ (reverse); and Y256F, 5′-TGCACTCAAACTGTTCCATGTGTCT-GAC-3′ (forward) and 5′-GTCAGACACATGGAACAG-TTTGAGTGCA-3′ (reverse). We confirmed the introduction of the desired codon by sequencing and transformed TKX1 competent cells with the recombinant plasmids. Recombinant villin mutants were purified from TKX1 cells as described above.

Urea Denaturation Assay. To determine the effects of specific mutations on the overall stability of the villin molecules, fluorescence-monitored urea denaturation was performed on each recombinant protein as described previously (*29*). Wild-type and mutant villin proteins were used at a final concentration of 1 μM in buffer containing 10 mM

Tris (pH 7.5), 50 mM NaCl, and different concentrations of urea (0−8 M). The proteins were incubated in the appropriate buffered urea at room temperature for 1 h. Fluorescence measurements were taken at an excitation wavelength of 280 nm and at an emission wavelength of 355 nm. The midpoint of the unfolding transition was determined for each protein.

In Vitro Kinase Assay. To assess the in vitro phosphorylation of recombinant villin by *c-src* kinase, recombinant villin proteins were expressed in BL21 competent cells as unphosphorylated proteins. Recombinant villin was phosphorylated in vitro by *c-src* as described previously (*7*). Briefly, recombinant villin was phosphorylated with *c-src* (2.5 units) in an assay mixture containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5 mM EGTA, 20 mM $MgCl_2$, 20 μM ATP, and 5 mM β-mercaptoethanol. Villin was phosphorylated for 60 min at 37 °C, and the reaction was stopped by the addition of Laemmli sample buffer. Tyrosine-phosphorylated proteins were separated by SDS−PAGE (10%), transferred to a nitrocellulose membrane, and probed with phosphotyrosine monoclonal antibodies.

Stoichiometry of Villin Phosphorylation. The amount of phosphate incorporated in villin was determined in an in vitro kinase assay (as described above) using 2.6 nmol of recombinant villin. VT3BL and VT3BL(Y46,60,64,81,256F) were phosphorylated in vitro by *c-src* in a reaction mixture containing 30 μCi of [γ-^{32}P]ATP (20 μM ATP, SA = 3000 Ci/mmol), in a final volume of 50 μL. The reaction was stopped by adding an equal volume of 10% ice-cold trichloroacetic acid (TCA) and the mixture incubated on ice for 20 min. The reaction mixture was loaded onto glass fiber filters (GF/C, Millipore) in a manifold device under vacuum. Filters were washed with 20 mL of 5% cold TCA, 10 mL of 95% ethanol, and 30 mL of diethyl ether. Filters were dried, and the amount of radioactivity associated with the proteins was measured in a liquid scintillation counter. Each experiment was performed in triplicate. Phosphorylation of Gst was negligible and was subtracted from the phosphorylated villin samples. Similarly, autophosphorylation of *c-src* kinase was subtracted from the phosphorylated villin sample.

Measurement of Actin Polymerization Kinetics by Phosphorylated and Unphosphorylated Villin Using Fluorescence Spectroscopy. The kinetics of actin polymerization were determined using a nonmuscle or muscle actin polymerization kit according to the instructions of the manufacturer and as described previously (*7*, *22*). The basis of this assay is that the fluorescence intensity of pyrene-labeled actin is much greater for polymeric than monomeric actin (*30*). The ability of villin to nucleate actin assembly or to sever actin filaments was determined by its effect on the rate and extent of increase or decrease, respectively, of fluorescence of pyrene-labeled actin. Fluorescence measurements were performed at 25 °C using the FluoroMax 3 spectrofluorometer. The excitation wavelength was set at 365 nm, and the emission wavelength was set at 388 nm.

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 $CaCl_2$ was preincubated with villin or villin mutants (120 nM) for 10 min on ice. Polymerization was induced by the addition of 150 mM KCl and 1 mM $MgCl_2$. The increase in fluorescence that occurs when pyrene G-actin forms pyrene F-actin was measured over time. To test the nucleating activity of full-length unphosphorylated villin

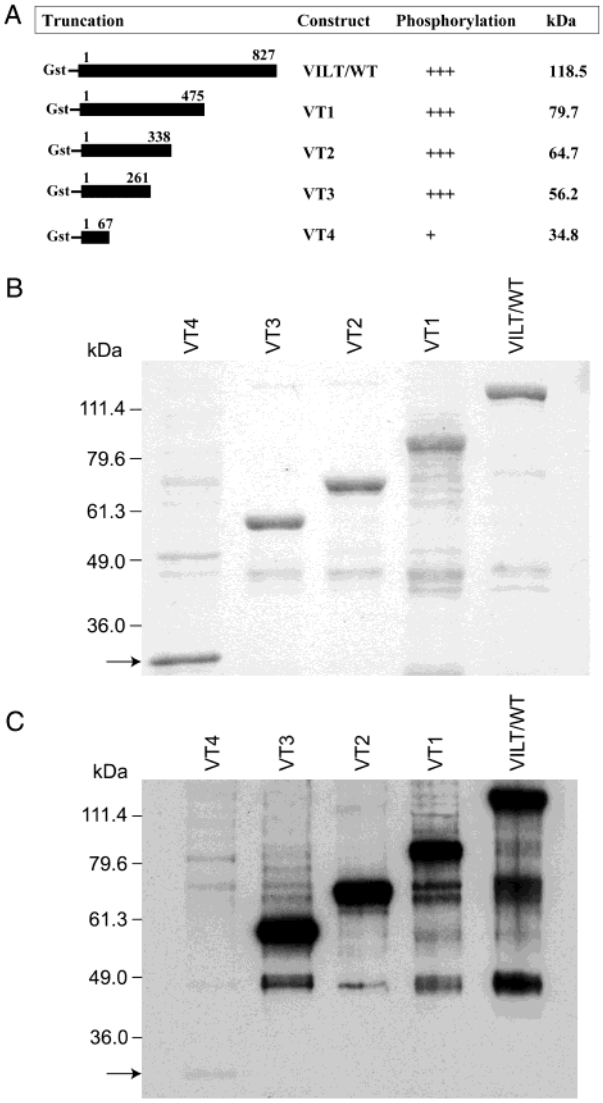

FIGURE 1: Tyrosine phosphorylation of wild-type villin and truncation mutants of villin. (A) Schematic representation of wild-type villin and carboxyl-terminal truncation mutants of villin that were expressed as Gst-tagged phosphorable proteins in TKX1 cells. (B) SDS−PAGE analysis of recombinant, phosphorylated wild-type villin (VILT/WT) and truncation mutants of villin. The gels were stained with GelCode Blue. (C) Western blot of VILT/WT and truncation mutants of villin with phosphotyrosine monoclonal antibodies. The arrow indicates the small amount of tyrosine phosphorylation seen in truncation mutant VT4. Data are representative of six experiments with similar results.

(VIL/WT), full-length phosphorylated villin (VILT/WT), and the various mutants of villin, we measured their effect on the initial phase of actin polymerization by recording the increase in fluorescence every 15 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 in solutions containing wild-type villin or villin mutants (60 nM). To compare the severing activity of VIL/WT, VILT/WT, and the villin mutants, we measured the decrease in fluorescence per minute in the linear range of the curve as described previously (*20*). The decrease in fluorescence was recorded every 15 s.

Cosedimentation of Wild-Type and Mutant Villin. F-Actin (3 μM) was incubated with VIL/WT or VILT/WT(Y46,60,-

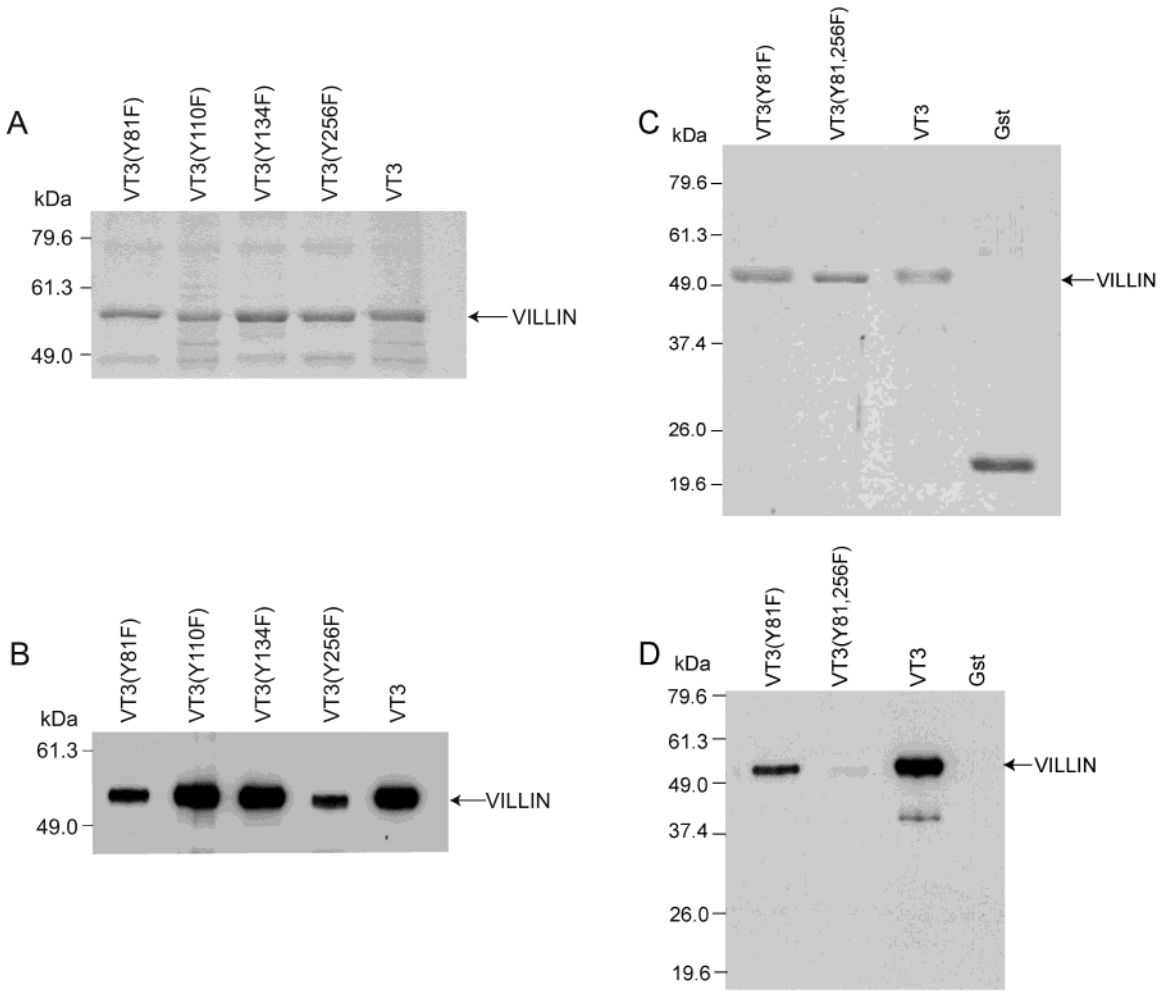

FIGURE 2: Substitution of a tyrosine residue at position 81 or 256 with phenylalanine in villin truncation mutant VT3 decreases the phosphotyrosine content of recombinant mutant villin proteins. Mutant villin proteins were generated by substitution of a tyrosine residue at position 81, 110, 134, or 256 with phenylalanine in villin truncation mutant VT3 [VT3(Y81F), VT3(Y110F), VT3(Y134F), or VT3-(Y256F) respectively]. The villin mutants were expressed as Gst-fused phosphorable proteins in TKX1 cells (see Experimental Procedures). The samples were normalized for protein content and subjected to SDS−PAGE and Western analysis. (A) SDS−PAGE of villin mutants, stained with GelCode Blue. (B) Western blot of villin mutants probed with phosphotyrosine antibodies. (C) A villin mutant was generated by substituting both Y81 and Y256 with F [VT3(Y81,256F)]. SDS−PAGE of the VT3(Y81,256F) villin construct purified from TKX1 cells and stained with GelCode Blue. (D) Western blot of VT3(Y81,256F) probed with phosphotyrosine antibodies. Data are representative of eight experiments with similar results.

81,256F) in the presence of 2 mM EGTA [as described previously (*22*)]. F-Actin was incubated with villin for 10 min at room temperature and sedimented for 15 min at 200000*g* in a Horiba ultracentrifuge. The supernatant was acetone-precipitated with 2 volumes of acetone. The pellet and the acetone-precipitated proteins were resuspended in Laemmli sample buffer and analyzed by SDS−PAGE. The acrylamide gels were stained with GelCode Blue, and the proteins were quantified using the Bio-Rad system. Densitometric analysis was carried out using Scion Image software.

RESULTS

The Major Tyrosine Phosphorylation Site Is Located in the Amino Terminus of Villin. We have previously reported that full-length recombinant villin behaves like the native protein (*7*), thus allowing us to reconstitute in vitro our previous in vivo observations (*7*, *22*). To determine the site of tyrosine phosphorylation in villin, truncation mutants of villin were constructed as summarized in Figure 1A. The constructs consisted of carboxyl-terminal deletions of human villin cDNA engineered into the prokaryotic expression vector pGEX-2T. Four truncation mutants were generated as Gst fusion proteins in TKX1 cells. The expression of full-length villin and truncation mutants of villin was assessed by SDS−PAGE and staining with GelCode Blue (Figure 1B). The apparent molecular masses were in agreement with those predicted for each deletion mutant. To assess tyrosine phosphorylation of these mutants, equal amounts of the proteins were analyzed by Western analysis using phosphotyrosine antibodies. The densitometric analysis showed that full-length villin (VILT/WT) and its three truncation mutants (VT1−VT3) exhibited similar levels of tyrosine phosphorylation (Figure 1C). In contrast, there was a dramatic decrease in the level of tyrosine phosphorylation of the shortest fragment, VT4. In the villin mutant VT4, phosphorylation was essentially undetectable; only very long exposure of the film revealed a faint band (Figure 1C). These data suggested to us that the major phosphorylation site(s) in villin was located between amino acids (aa) 67 and 261 in the amino terminus of human villin.

Identification of Five Phosphorylation Sites in the Amino Terminus of Villin. Human villin contains five tyrosine

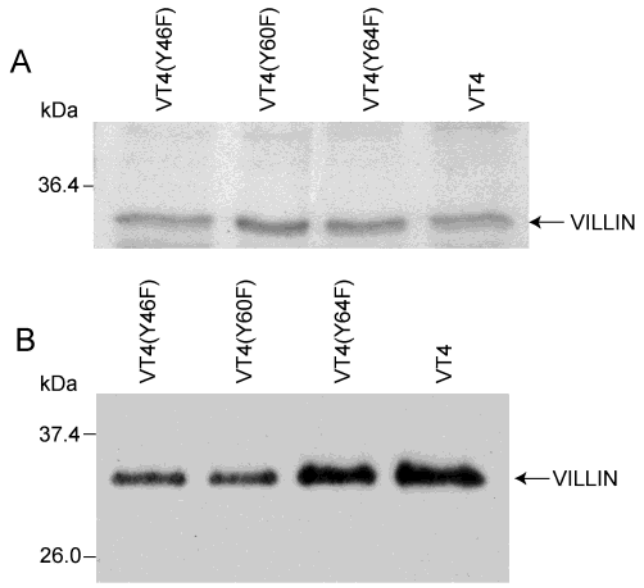

FIGURE 3: Substitution of a tyrosine residue at position 46, 60, or 64 with phenylalanine in truncation mutant VT4 decreases the phosphotyrosine content of recombinant mutant villin proteins. Mutant villin proteins were generated by substitution of a tyrosine residue at position 46, 60, or 64 with phenylalanine in villin truncation mutant VT4 [VT4(Y46F), VT4(Y60F), or VT4(Y64F) respectively]. The villin mutants were expressed as Gst-fused phosphorylatable proteins in TKX1 cells as described in Experimental Procedures. The samples were normalized for protein content and subjected to SDS−PAGE and Western analysis. (A) SDS−PAGE of villin mutants, stained with GelCode Blue. (B) Western blot of villin mutants probed with phosphotyrosine antibodies. Data are representative of six experiments with similar results.

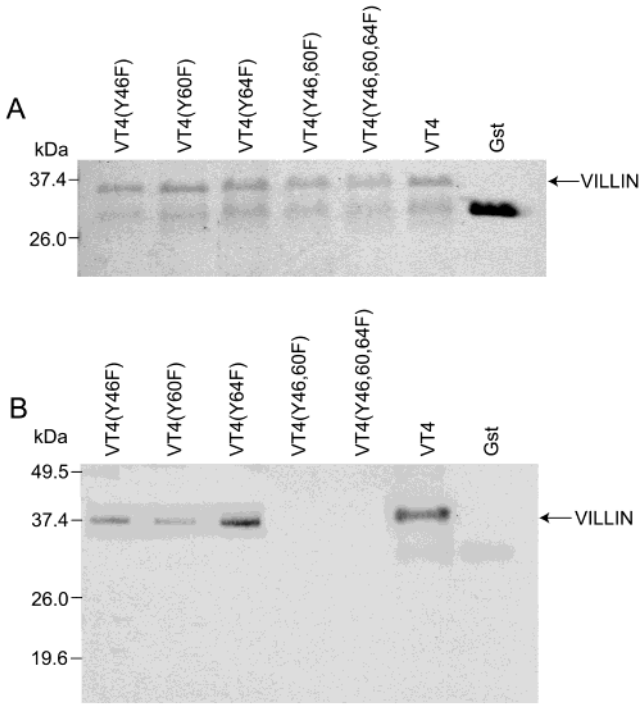

FIGURE 4: Substitution of all three tyrosine residues at positions 46, 60, and 64 with phenylalanine eliminates tyrosine phosphorylation in villin mutant VT4. Double [VT4(Y46,60F)] and triple [VT4(Y46,60,64F)] mutants of villin were expressed in TKX1 cells. The samples were normalized for protein content and subjected to SDS−PAGE and Western analysis. (A) SDS−PAGE of villin mutants stained with GelCode Blue. (B) Western blot of villin mutants probed with monoclonal antibodies to phosphotyrosine. Data are representative of eight experiments with similar results.

residues (Y81, -110, -134, -207, and -256) between aa 67 and 261. Alignment of villin sequences from human, mouse, chicken, and *Dictyostelium* demonstrated that four of these tyrosine residues were conserved (Y207 is not). We used site-directed mutagenesis to substitute each of the conserved tyrosine residues with phenylalanine in truncation mutant VT3. The mutants were expressed in TKX1 cells (Figure 2A), and their tyrosine phosphorylation status was determined by Western analysis using phosphotyrosine antibodies (Figure 2B). Mutation of Y81 or Y256 to F significantly decreased the level of tyrosine phosphorylation in the villin construct VT3. However, mutation of both of these sites [VT3-(Y81,256F), Figure 2C] did not completely abolish the phosphorylation of VT3 (Figure 2D). There are three additional tyrosine residues between aa 1 and 67 in villin mutant VT3, and we speculated that perhaps one or more of these might also be a minor phosphorylation site, particularly since we see some phosphorylation in villin mutant VT4, which is comparable to that of VT3(Y81,256F) (compare Figure 2D with Figure 1C).

To identify additional phosphorylation sites in villin, we used villin truncation mutant VT4 which contains these three tyrosine residues, namely, Y46, -60, and -64. Each of these tyrosines was individually mutated to phenylalanine. As shown in Figure 3A, all three mutants [VT4(Y46F), VT4-(Y60F), and VT4(Y64F)] corresponded to the expected size. Equal amounts of each of these constructs were analyzed for tyrosine phosphorylation (Figure 3B). Densitometric analysis demonstrated that mutation of Y46 or Y60 to F significantly decreased the level of phosphorylation in villin construct VT4. In contrast, mutation of Y64 decreased the level of phosphorylation in villin construct VT4 by less than 15%. This suggested that Y46 and Y60 were the two major sites of phosphorylation in this fragment of villin. Thus, we identified two major (Y81 and -256) and three minor (Y46, -60, and -64) phosphorylation sites in the amino terminus of human villin.

Tyrosines 46, 60, 64, 81, and 256 Are the Only Sites of Phosphorylation in the Amino Terminus of Villin. To confirm that the individual tyrosine sites that were identified were the only sites of phosphorylation in the amino-terminal domain of villin, we substituted all of these tyrosines with phenylalanine in various combinations. We generated double [Y46 and Y60 mutated to F; VT4(Y46,60F)] and triple [Y46, Y60, and Y64 mutated to F; VT4(Y46,60,64F)] mutations in villin truncation VT4 (Figure 4A). As shown in Figure 4B, substitution of all three tyrosine residues with phenylalanine completely abolished the phosphorylation of VT4. Similarly, we generated several mutations in villin truncation mutant VT3 (Figure 5A) and demonstrated that substituting all five identified tyrosine residues with phenylalanine [VT3-(Y46,60,64,81,256F)] led to the complete loss of phosphorylation in villin mutant VT3 (Figure 5B). We also examined other possible combinations of mutations in these five residues (data not shown) and collectively concluded that the two major tyrosine phosphorylation sites in villin are residues Y81 and Y256 and the minor sites are Y46, -60, and -64.

Villin Mutants Lacking the Identified Phosphorylation Sites Are Not Substrates for c-src Kinase. We have previously demonstrated that recombinant villin is a substrate for *c-src*

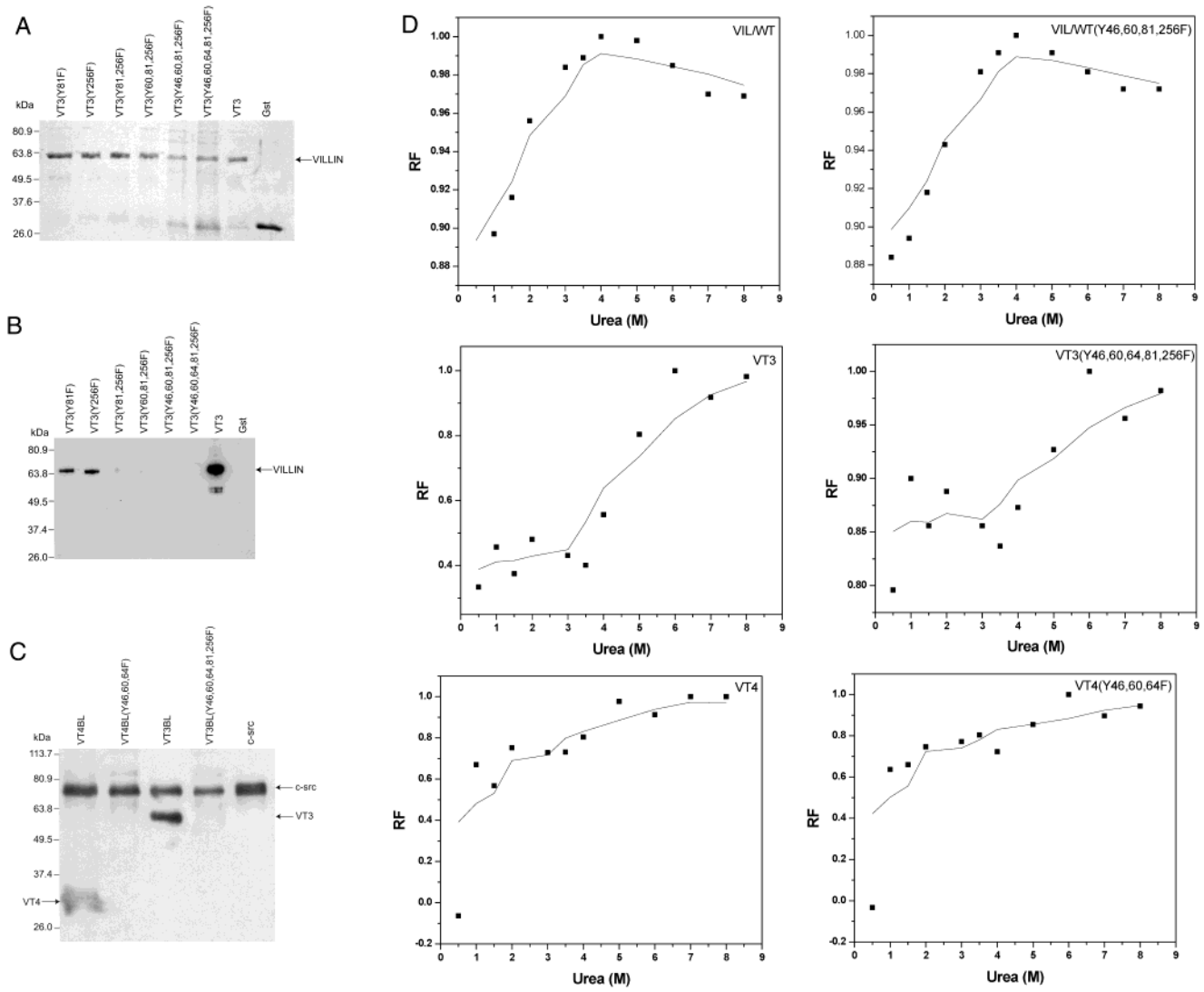

FIGURE 5: Substitution of all five tyrosine residues at positions 46, 60, 64, 81, and 256 with phenylalanine eliminates tyrosine phosphorylation in villin mutant VT3. Villin mutant proteins were expressed with multiple tyrosine residues substituted with phenylalanine [VT3(Y81F), VT3(Y81,256F), VT3(Y46,81,256F), VT3(Y46,60,81,256F), and VT3(Y46,60,64,81,256F)] as described in the text. The mutant proteins were analyzed by SDS−PAGE and Western analysis. (A) SDS−PAGE of villin mutants stained with GelCode Blue. (B) Western blot of villin mutants probed with monoclonal antibodies to phosphotyrosine. (C) Villin truncation mutants VT3 and VT4 as well as the two mutants described above, VT3(Y46,60,64,81,256F) and VT4(Y46,60,64F), were expressed as unphosphorylated proteins in BL21 cells [VT3BL, VT4BL, VT3BL(Y46,60,64,81,256F), and VT4BL(Y46,60,64F) respectively]. The recombinant villin mutants were tyrosine phosphorylated in vitro by *c-src* kinase as described in Experimental Procedures. Samples were separated by SDS−PAGE (10%), transferred to nitrocellulose, and immunoblotted using phosphotyrosine antibodies. The *c-src* protein is seen in these blots due to autophosphorylation and activation of the kinase. Arrows show the phosphorylated proteins VT3 and VT4. Data are representative of five experiments with similar results. (D) The stability of the recombinant villin proteins was measured by fluorescence-monitored urea denaturation as described in Experimental Procedures. The left panels show protein unfolding of unphosphorylated villin proteins VIL/WT, VT3BL, and VT4BL, and the panels on the right show protein unfolding of villin proteins with mutations, VIL/WT(Y46,60,81,256F), VT3BL(Y46,60,64,81,256F), and VT4BL(Y46,60,64F). The relative fluorescence units are shown on the *y*-axis, and the urea concentrations (molar) are shown on the *x*-axis. Note that villin proteins with mutations unfold at the same urea concentrations as villin proteins with mutations.

kinase (*7*). To further explore the correlation between the phosphorylation of villin and *c-src*, we assessed in vitro the phosphorylation of villin mutant VT3(Y46,60,64,81,256F), which lacks all five identified tyrosine phosphorylation sites, and of VT4(Y46,60,64F), which lacks three of these sites. For these studies, villin truncation mutants VT3 and VT4 were expressed in BL21 competent cells to obtain unphosphorylated proteins (VT3BL and VT4BL, respectively). Substitution of all three identified tyrosine residues in VT4BL with phenylalanine resulted in a villin mutant [VT4BL(Y46,60,64F)] that is not phosphorylated in vitro by *c-src* kinase (Figure 5C). Similarly, substitution of all the five identified tyrosine residues with phenylalanine in VT3BL resulted in a villin mutant [VT3BL(Y46,60,64,81,256F)] that was not phosphorylated in vitro by *c-src* kinase. Please note that the minor phosphorylation of villin construct VT4BL by *c-src* (Figure 5C) is comparable to the phosphorylation of VT4 by elk kinase (see Figure 1C). While VT4BL has no other phosphorylatable tyrosine residues (all three are mutated to phenylalanine), VT3BL has other tyrosine residues (including Y110, -134, and -207); however, these are not phosphorylated by *c-src* kinase. These data are consistent with the interpretation that villin may be a substrate for *c-src* kinase. These studies were also carried out in the presence of radiolabeled ATP (as described in Experimental Procedures). Using this method, we obtained 2.3 mol of

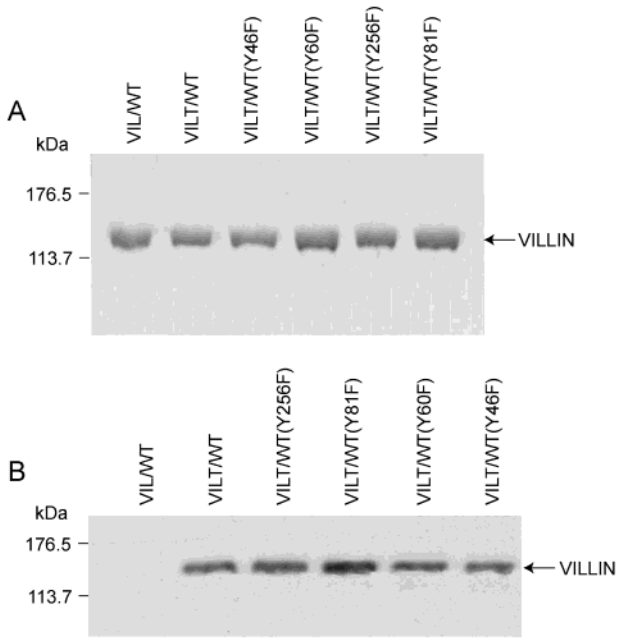

FIGURE 6: Expression of full-length villin proteins with point mutations at position 46, 60, 81, or 256. Tyrosine residues at positions 46, 60, 81, and 256 were substituted with phenylalanine in full-length villin, and they were expressed as recombinant proteins [VILT/WT(Y46F), VILT/WT(Y60F), VILT/WT(Y81F), and VILT/WT(Y256F) respectively] in TKX1 cells. The mutant proteins were characterized by SDS−PAGE and staining with GelCode Blue (A) or Western analysis using phosphotyrosine antibodies (B). Data are representative of four experiments with similar results. VIL/WT is the recombinant full-length, unphosphorylated villin, and VILT/WT is the recombinant full-length tyrosine-phosphorylated villin. This is not a quantitative Western blot.

phosphate/mol of villin. These studies suggest nearly quantitative phosphorylation, provided only two major sites are phosphorylated in villin, and further confirm the sites identified by mutational analysis.

To determine the effect of mutation on the conformation of the villin constructs, urea denaturation of the proteins was monitored by changes in tryptophan fluorescence. The magnitude of intensity of fluorescence is often used as a probe of perturbations in the folded state of a protein. The fluorescence of aromatic residues such as tryptophan varies with the folding of the protein, and changes in intrinsic fluorescence can be used to monitor structural changes in a protein. As shown in Figure 5D, wild-type, VT3, and VT4 villin proteins with or without mutation (Y to F) exhibit similar unfolding transitions. Full-length villin and full-length villin with four mutations show a midpoint for the transition at ~4 M urea. VT3, VT3(Y46,60,64,81,256F), VT4, and VT4(Y46,60,64F) exhibit a midpoint for the transition around 6 M urea. These data indicate that mutations of Y to F do not significantly perturb the folding of villin proteins.

Multiple Sites of Tyrosine Phosphorylation Regulate Villin's Nucleating and Depolymerizing Functions. To study the role of the specific tyrosine phosphorylation sites in the actin regulatory functions of villin, we constructed a series of mutant cDNA in which each of the four identified phosphorylation sites was substituted with phenylalanine individually. The point mutations were made in full-length villin cDNA, and expressed in TKX1 cells (Figure 6A). These villin mutants were tyrosine phosphorylated (Figure 6B) but lacked one of the identified tyrosine residues [VILT/WT(Y46F), VILT/WT(Y60F), VILT/WT(Y81F), and VILT/WT(Y256F)]. Figure 6B is not a quantitative Western blot but shows that full-length and mutant villin proteins are tyrosine phosphorylated. Full-length unphosphorylated villin (VIL/WT), phosphorylated villin (VILT/WT), and the different tyrosine mutants of phosphorylated villin were used in a pyrene-based actin polymerization and depolymerization assay as described in Experimental Procedures. Unphosphorylated villin mutants (namely, Y to F mutants but expressed in BL21 cells vs TKX1 cells) behave like unphosphorylated wild-type villin (VIL/WT; data not shown).

As shown in Figure 7A, the addition of VIL/WT (120 nM) abolished the lag phase and increased the initial rate of actin polymerization compared to the polymerization kinetics of actin alone (control). In contrast, VILT/WT resulted in a lag phase and decreased the rate of actin polymerization, consistent with our previous report (*22*). Mutation of any one of the identified Y to F did not restore the actin-nucleating properties of the mutants, analogous to that of VIL/WT. These data demonstrate that phosphorylation of any one of the identified tyrosine residues inhibits actin nucleation by villin, and further suggest that phosphorylation at any of these sites could regulate the actin-nucleating function of villin in vivo.

Next, we examined the effect of tyrosine phosphorylation of villin on F-actin depolymerization. VIL/WT significantly increased the level of depolymerization of F-actin compared with control (Figure 7B). Furthermore, phosphorylation of villin improved its actin-depolymerizing property. These results are consistent with our previous report (*22*). Mutation of Y46 or Y60 to F resulted in actin severing by villin comparable to that of VIL/WT, whereas the villin mutants VILT/WT(Y81F) and VILT/WT(Y256F) behaved like VILT/WT. Mutation of both Y46 and Y60 to F [VILT/WT-(Y46,60F)] resulted in actin-severing activity comparable to that of VIL/WT. These data suggest that from the total pool of tyrosine-phosphorylated villin, a minor pool consisting of tyrosine phosphorylation of Y46 and Y60 regulates the actin-severing function of villin.

Since it is known that fragments derived from the amino-terminal half of villin (villin core and 44T) retain the Ca^{2+}-dependent actin-severing but not -nucleating properties of intact villin (*4*, *31*), we measured the actin-severing ability of villin using the phosphorylated truncation mutant VT3 (which encompasses 44T) and VT3 lacking the four major phosphorylation sites, namely, VT3(Y46,60,81,256F). The truncated phosphorylated mutant of villin, namely, VT3, depolymerized actin like the full-length phosphorylated villin, VILT/WT (Figure 7C). In contrast, mutating Y46, Y60, or all the four major phosphorylation sites to F [VT3(Y46F), VT3(Y60F), and VT3(Y46,60,81,256F)] inhibited the actin-severing property of phosphorylated villin mutant VT3. These data demonstrate that like the nucleating function of villin, actin severing by villin can be regulated by phosphorylation.

To authenticate that higher concentrations of villin did not change the kinetics of actin polymerization, the actin polymerization experiments were carried out in the presence of 0.5 μM villin and its mutants (compared to 120 nM). As shown in Figure 7D, higher concentrations of villin and its

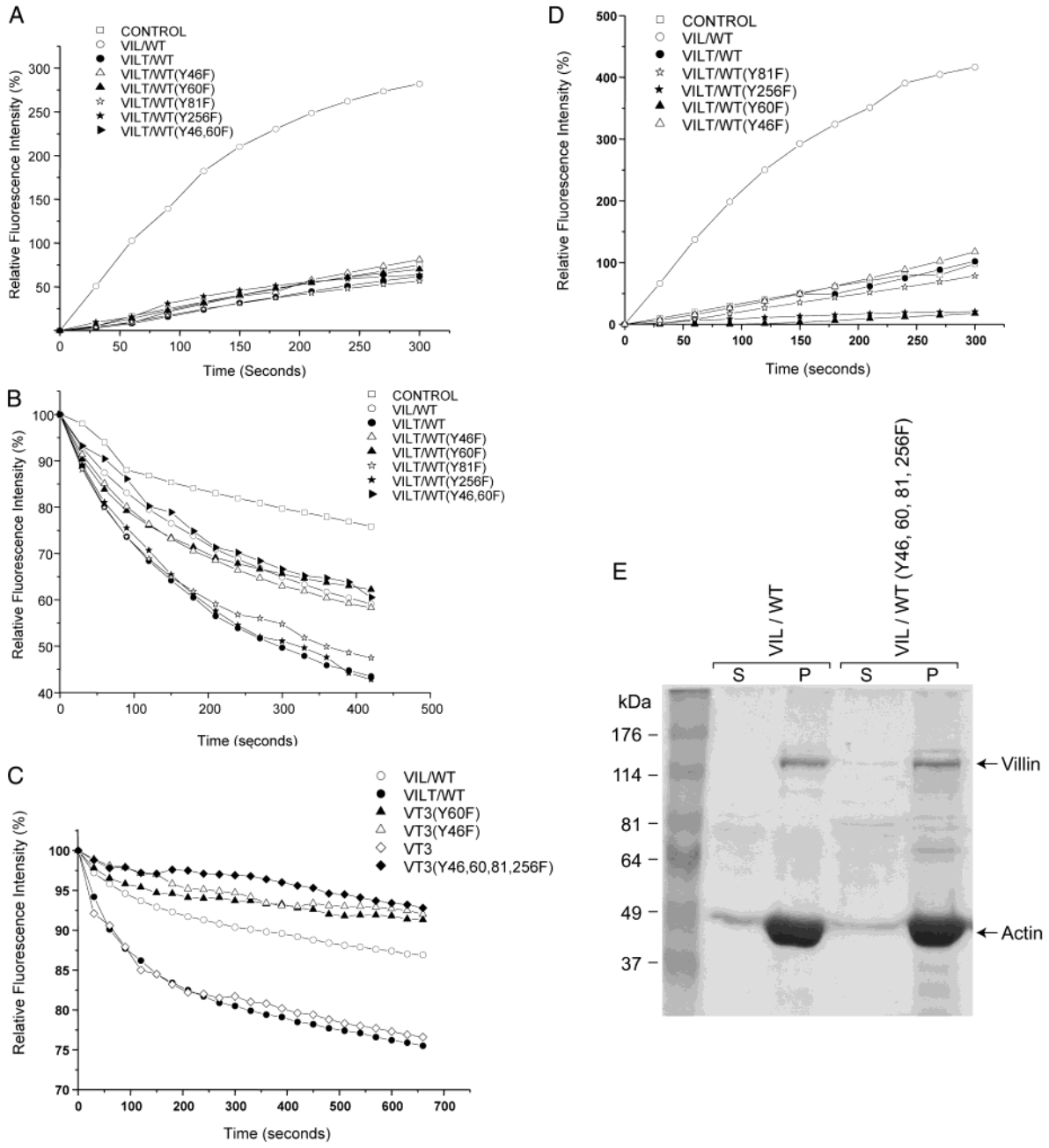

FIGURE 7: Effects of phosphorylated villin and villin mutants on actin dynamics. (A) Effect of wild-type and mutant villin proteins on the kinetics of actin polymerization. Pyrene-labeled G-actin (6 μM) was incubated with unphosphorylated (VIL/WT), phosphorylated (VILT/WT), or mutant villin protein (120 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 15 s as described in Experimental Procedures. (B) Effect of wild-type and mutant villin protein on actin depolymerization. Pyrene-labeled F-actin (1 μM) in the presence of VIL/WT, VILT/WT, or mutant villin (60 nM) was diluted to 0.1 μM in actin-depolymerizing 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 15 s as described in Experimental Procedures. (C) Effect of VT3 and the villin mutant VT3(Y46,60,81,256F) on actin depolymerization. Actin depolymerization in the presence of the phosphorylated villin truncation mutant VT3 and the villin mutants VT3(Y46F), VT3(Y60F), and VT3(Y46,60,81,256F) was assessed as described above. Values represent the means of four independent experiments. (D) Wild-type and mutant villin were polymerized as described for panel A, except higher concentrations of the protein samples were used (0.5 μM). (E) Wild-type (VIL/WT) and mutant villin [VIL/WT(Y46,60,81,256F)] were added to prepolymerized actin (3 μM), 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 (S) and pellet (P) fractions was determined by GelCode Blue staining. Molecular mass standards are shown on the left. Arrows show villin and actin. Note there is no difference in the amount of protein in the pellet associated with actin with both wild-type and mutant villin proteins. Data are representative of four experiments with similar results.

mutants (0.5 μM) did not change the actin polymerization kinetics. The data shown in Figure 7D are comparable to those shown in Figure 7A, suggesting that this is a quantitative assembly assay. To confirm that the regulation of the actin regulatory functions of villin by tyrosine phosphorylation was not due to conformational changes resulting in a loss of actin binding when Y residues are mutated to F, we determined the actin binding activity of unphosphorylated wild-type villin (VIL/WT) and mutant villin VIL/WT-(Y46,60,81,256F). As shown in Figure 7E, mutation of Y

to F does not result in any significant change in the binding of villin and its mutants with F-actin. This and the data shown in Figure 5D confirm that the regulation of actin-severing and -nucleating activities of villin by tyrosine phosphorylation mutants is not due to structural changes resulting in a loss of villin binding to actin. Taken together, these data demonstrate that the ability of villin to regulate actin assembly or disassembly is highly sensitive to changes in the structure of the amino-terminal domain of the protein.

DISCUSSION

We previously described the tyrosine phosphorylation of villin both in vivo (*6*) and in vitro (*7*) and demonstrated that the biological properties of villin strongly rely upon phosphorylation (*22*). Several lines of evidence indicate that tyrosine phosphorylation of villin plays an important role in the organization of the actin network in epithelial cells. Previous in vivo work from our laboratory shows that tyrosine phosphorylation of villin is accompanied by the redistribution of phosphorylated villin and a concomitant decrease in the F-actin content of intestinal epithelial cells (*6*, *32*). These studies suggest a causal relationship between tyrosine phosphorylation of villin and changes in the distribution and/or kinetics of actin polymerization in epithelial cells. Reconstitution in vitro demonstrates that tyrosine phosphorylation of villin regulates its actin regulatory properties, including its binding affinity for actin, bundling of actin, nucleation of actin, and actin severing (*22*). These observations suggest that in response to specific physiological stimuli, tyrosine phosphorylation of villin could regulate the organization of the microvillar cytoskeleton.

To initiate our understanding of the significance of villin phosphorylation in vivo, we sought to identify the in vitro sites of phosphorylation in villin and to correlate phosphorylation at these sites with villin's actin-modifying functions. The complexity of the various actin-remodeling abilities (nucleating, capping, severing, and bundling) of villin makes such an approach most useful in dissecting the in vivo effect of villin phosphorylation on the actin network. Identification of phosphorylation sites in vitro has provided a powerful tool in understanding the biological significance of phosphoproteins. Phosphorylation sites identified in vitro have been supported by subsequent in vivo studies as the sites of physiological significance (*33*−*35*). In other instances such as phosphorylation of the human progesterone receptor, in vitro phosphorylation studies revealed additional sites, which were later confirmed to be authentic in vivo phosphorylation sites, thus revealing phosphopeptides that remained unidentified in previous in vivo studies (*36*).

In the study presented here, we provide the first evidence that villin is phosphorylated at tyrosines 46, 60, 64, 81, and 256 and that the actin-nucleating and actin-severing activities of villin strongly rely upon phosphorylation of one or more of these tyrosine residues. The integrity of these tyrosine residues is required for actin nucleation and depolymerization by villin, since phosphorylation of any one site impairs villin's capacity to nucleate actin, and likewise mutation of Y46, Y60, or Y46 and Y60 together to phenylalanine severely impairs phosphorylated villin's ability to sever actin filaments. In other words, villin's ability to regulate the actin dynamics is dependent on its phosphorylation. In recent years, several actin-binding proteins have been demonstrated to be tyrosine phosphorylated either in vitro or in vivo (*26*, *27*, *37*). Phosphorylation (serine/threonine) of the few actin-binding proteins studied so far demonstrates a decrease in the actin binding affinities, or actin bundling properties, and/or a decrease in the degree of nucleation of actin (*20*, *21*, *23*, *38*−*40*). Thus, phosphorylation, whether of tyrosine or serine/threonine residues, appears to be a negative regulator of actin assembly. We primarily characterized the amino terminus-associated functions of villin in this paper, and showed that both nucleating and severing by villin are affected by villin phosphorylation.

The identity of the kinase(s) that phosphorylates villin is not known. Recombinant villin can be tyrosine phosphorylated in vitro by tyrosine kinases such as *elk*, *c-src*, and *c-yes* (*7*). The brush borders of intestinal and renal epithelial cells express both *c-src* and *c-yes* (*41*). Because it is suggested that phosphorylation of other proteins of the villin superfamily, including gelsolin, is regulated by *c-src* (*42*, *43*), it is reasonable to speculate that villin phosphorylation in vivo may also be regulated by the Src family tyrosine kinases. Five tyrosine phosphorylation sites were identified in villin, and changing these sites to phenylalanine resulted in a villin mutant that could not be phosphorylated in vitro by *c-src*, even though other tyrosine residues are present in this villin mutant (Figure 5C). These data suggest that the sites phosphorylated by elk kinase are also the sites phosphorylated by *c-src*, thus confirming that two kinases, which may have different phosphorylation profiles, phosphorylate the same site. These data suggest that in vivo, villin may be a substrate for *c-src* kinase. It is interesting to note that the most prominent *c-src* substrates are proteins that affect the organization of the actin cytoskeleton (*23*, *44*). Now it is also well recognized that *c-src* modulates the actin cytoskeleton in epithelial cells (*45*).

Villin belongs to a family of calcium-regulated actin-binding proteins that also include adseverin, scinderin, CapG, gelsolin, fragmin, and severin, all of which nucleate, cap, or sever actin filaments. The tyrosine residues identified in this study are conserved motifs also found in these homologous proteins (Figure 8). While Y60 and Y256 are relatively well conserved, Y46 is the most conserved site among these proteins. Y64 on the other hand is restricted to villin and gelsolin, the two actin-severing proteins found in higher eukaryotes (*46*), and Y81 is restricted to fragmin and severin, the two actin-severing proteins found in lower eukaryotes, and to villin (*47*, *48*). It is known that despite widely divergent evolutionary origins, actin-severing proteins are remarkably similar in their primary structure and function; thus, it is reasonable to speculate then that their actin-modifying functions could be regulated by tyrosine phosphorylation much like villin. Identification of phosphorylation sites in these proteins will be instructive in defining more precisely the functional similarities and differences between these proteins.

Previous in vitro studies have demonstrated that the amino-terminal region of villin retains the calcium-dependent actin-severing and -capping activities and contains several of villin's ligand binding sites, including two putative PIP_2-binding sites (*4*), Ca^{2+} binding sites (*5*), and the site of F-actin binding prior to severing (*49*). It is suggested that PIP_2 regulates villin through competition with actin for the same

SPECIES	Y46	Y60	Y64	Y81	Y256
VILLIN					
MOUSE	DGDC**Y**VVL	TLS**Y**DIH	**Y**WIGQ	EQGAAAI**Y**TTQM	ALKL**Y**HVSD
HUMAN	DGDC**Y**IIL	SLS**Y**DIH	**Y**WIGQ	EQGAAAI**Y**TTQM	ALKL**Y**HVSD
CHICKEN	EGDC**Y**VLL	GFS**Y**NIH	**Y**WLGK	EQGAAAI**Y**TTQM	ALKL**Y**HVSD
DICTYOSTELIUM	TNKS**Y**LLL	IKT**Y**NIH	**Y**WLGK	ELERIIK**Y**NTQM	QIKL**Y**QVEN
GELSOLIN	TGDA**Y**VIL	NLQ**Y**DLH	**Y**WLGN		LAKL**Y**KVSN
ADSEVERIN	VGEP**Y**LIL	GFS**Y**RLH			MAKL**Y**MVSD
CAPG	SGDS**Y**LVL				AAAL**Y**KVSD
SCINDERIN	VGDA**Y**LVL	GFT**Y**HLH			MAKL**Y**MVSD
FRAGMIN	TGDS**Y**IVL	KLA**Y**DVH		DEAGTAA**Y**KTVE	
SEVERIN	TGDS**Y**IVI			DEAGTAA**Y**KTVE	EKVL**Y**KLSD

FIGURE 8: Sequence alignment of villin and related proteins of the villin superfamily. Comparison of sequences for the amino-terminal region of villin and related proteins. The top panel shows alignments of villin sequences from mouse, human, chicken, and *Dictyostelium*. The bottom panel shows alignment of homologous sequences in amino-terminal domain of gelsolin, adseverin, severin, CapG, scinderin, and fragmin.

or overlapping sites in the amino-terminal core of this protein (*49*). This site consists of a cluster of basic amino acids that have been shown to promote villin binding to actin (*50*), as well as PIP_2 (*49*). Since phosphorylated villin exhibits a lower binding affinity for both PIP_2 (*7*) and F-actin (*22*), this could explain both why introduction of negative charges by phosphorylation weakens villin−actin interactions and why phosphorylation promotes the actin-severing functions of villin. Capping and nucleating activities are closely related in villin (*4*, *50*, *51*), which may also contribute to the effects on actin nucleation by phosphovillin. The identification of tyrosine phosphorylation sites near the actin and lipid binding domain of villin suggests that this site may be more sensitive to changes in the relative charge. For instance, tyrosine phosphorylation may induce conformational changes in this highly conserved region that may play an essential role in the regulation of actin assembly and disassembly in epithelial cells. Such a conformational change has been reported in villin in the presence of Ca^{2+} (*52*). Specific phosphorylation states may regulate the interaction of villin with the actin cytoskeleton or the plasma membrane, thereby providing a regulated mechanism for villin function within distinct microvillar domains.

Studies with mutant proteins have improved our understanding of the contribution of phenolic hydroxyl groups of tyrosine to the stability as well as catalytic activity of several proteins (*53*). The structure of villin (chicken) amino-terminal domain 1 has been determined in solution using NMR and NOE spectroscopy (*54*). On the basis of this structure, Y46, Y60, and Y64 are located in the two antiparallel β-sheets (β4 and β5), while Y81 is located in an α-helical conformation (α2). In addition, Y46 and Y64 are located across from each other in the two opposite β-sheets. Visualization of these residues and the surrounding amino acids indicates several possible new ion pairs that could form with the addition of a negative charge upon phosphorylation. The first of these is indicated by the proximity of the phenolic oxygen atom of Y60 to the ammonium group of K14 (4.7 Å). The second new ion pair that might form involves Y64 and R97, with a 4.9 Å separation between the tyrosine phenolic oxygen atom and the guanidine moiety of the arginine. Even more interesting, however, is the possible consequence of phosphorylating Y46. Y46 is located 5.0 Å from R23 and 3.8 Å from E25. R23 and E25 are ion-paired in the unphosphorylated villin structure, with a separation between their ionic

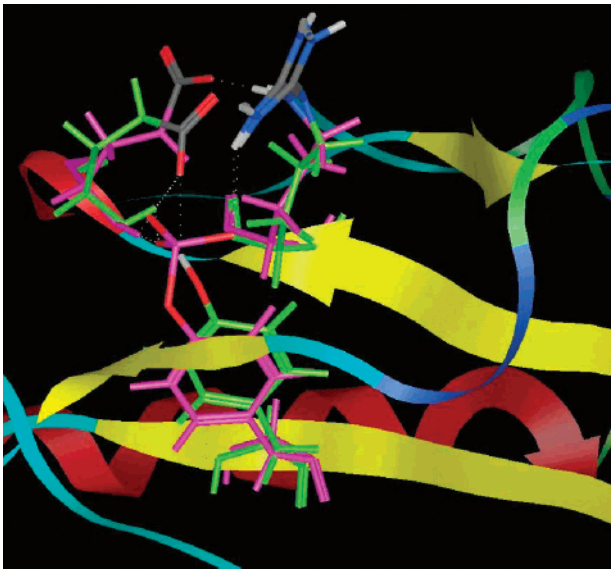

FIGURE 9: Comparison of energy-minimized villin 14T (entry 2vik from the Protein Data Bank) and the energy-minimized villin 14T modified to include phosphorylated tyrosine at position 46. Energy minimization was performed using the 1994 Merck Molecular Forcefield (MMFF94) as implemented in the MOE program. Residues Y46, R23, and E25 are shown in stick form. The end of each residue is colored by atom type, with red indicating oxygen, gray carbon, blue nitrogen, white hydrogen, and magenta phosphorus. The remainder of each residue is colored to indicate whether it is unmodified villin (green) or the phosphorylated villin (magenta).

groups of 3.0 Å. Thus, phosphorylation of Y46 might not only form a new ion pair between Y46 and R23 but also disrupt the existing ion pair between R23 and E25 in the process. To test this possibility, simple energy minimizations of both the unphosphorylated villin structure and the Y46-phosphorylated villin structure were performed. The villin structure that was used was entry 2vik in the Protein Data Bank (*54*). The Y46-phosphorylated structure was generated by adding a phosphate group to Protein Data Bank entry 2vik in the MOE program (version 2001, Chemical Computing Group). Both structures were energy minimized to a root-mean-square gradient of 0.001 kcal mol^{-1} $Å^{-1}$ using the Merck molecular force field (*55*). The introduction of a phosphate group on Y46 does indeed repel the negatively charged side chain of E25, causing it to reorient toward the exterior of the protein (Figure 9). This minor change can

therefore significantly change the surface charge distribution, which might strongly influence the interaction between villin and its ligands (F-actin, PIP_2, or Ca^{2+}). This potential structural and electrostatic consequence of Y46 phosphorylation is particularly interesting considering that Y46 is the most conserved tyrosine residue in villin as well as in other proteins of the villin superfamily (Figure 8).

Our identification of the phosphorylation sites of villin and their potential role in filament assembly and rearrangement offers an impetus and a model system for further characterization of the role of these sites on filament rearrangement or interaction with other cellular elements, including actin, phospholipids, and Ca^{2+}. Our working hypothesis is that phosphorylation at multiple sites may determine several potential rearrangements of the microfilament structure and therefore diverse functional consequences. The conformation of these phosphorylation sites in vivo and subsequent use of selected tyrosine to phenylalanine mutants as dominant negatives may allow us to selectively block, in vivo, events hypothesized to depend on villin tyrosine phosphorylation.

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