Regulation of phospholipase C-γ1 by the actin-regulatory protein villin

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Received 31 January 2001; accepted in final form 3 May 2001

We previously demonstrated for the first time that villin, an actin-regulatory protein of epithelial cells, is tyrosine phosphorylated in vivo in response to phosphatidylinositol 4,5-bisphosphate; tyrosine phosphorylation of villin abolished villin’s ability to associate with phospholipase C-γ1 (PLC-γ1) in the brush border of intestinal epithelial cells. To study the mechanism of villin-associated PLC-γ1 activation, we reconstituted in vitro the tyrosine phosphorylation of villin and its association with PLC-γ1. Recombinant villin was phosphorylated in vitro by the non-receptor tyrosine kinase c-src or by expression in the TKX1 competent cells that carry an inducible tyrosine kinase gene. Using in vitro binding assays, we demonstrated that tyrosine-phosphorylated villin associates with the COOH-terminal Src homology 2 (SH2) domain of PLC-γ1 (21) and forms a complex with the brush-border membrane-bound phospholipase C-γ1 (PLC-γ1) (22). These observations suggest a role for villin, not only in actin organization but also in the PLC-γ1-mediated signal transduction. Because tyrosine-phosphorylated villin associates with PLC-γ1 (22), our hypothesis is that tyrosine phosphorylation of villin is intimately linked to regulation of PLC-γ1 catalytic activity. Villin can bundle, sever, cap, or nucleate actin filaments in a Ca2+- and phospholipid-dependent manner (18). For over a decade, it has been known that villin binds phosphatidylinositol 4,5-bisphosphate (PIP2) and that the association of villin with PIP2 inhibits villin’s actin-severing property (19). This observation lends further support to the idea that villin may regulate both the cortical cytoskeleton and phosphoinositide-mediated signal transduction pathways.

In this study we attempt to understand the molecular steps involved in the tyrosine phosphorylation of villin, its association with both the substrate and the enzyme, namely, PIP2 and PLC-γ1, respectively, and thus its regulation of phosphoinositide-mediated signal transduction pathways. Using recombinant villin and purified PLC-γ1, we reconstituted in vitro our previous in vivo observation (22). The advantage of reconstitution is that it allows selected and controlled conditions to be defined to explore the molecular basis of the phenomenon, thus making it easier to interpret the observations. The complexity of the various actin-remodeling abilities of villin, in addition to its association with several signaling molecules, makes such an approach most useful to dissect the in vivo function of villin.

Our previous work in the intestinal epithelial cells and the recent observation in the opossum kidney cell
line demonstrate that tyrosine phosphorylation of villin and its association with PLC-γ1 regulate changes in the microfilament structure that are crucial to the vectorial function of ion transport in epithelial cells (22, 28). Both PLC-γ1 and the microfilament network have been demonstrated to regulate ion transport functions in epithelial cells of the intestine and kidney (Refs. 22 and 28; for review see Ref. 21); however, our understanding of the mechanisms involved remains rudimentary. This study may help us understand the molecular and cellular mechanisms by which PLC-γ1 and the microfilament network regulate cell morphology and function in epithelial cells.

METHODS

Materials. PLC-γ1 was purified from HeLa cells that had been transfected with recombinant vaccinia virus containing a full-length sequence of PLC-γ1 (2). Native villin, purified from chicken brush-border membranes (stored in a buffer containing 0.5 mM β-mercaptoethanol), was a kind gift from Dr. S. W. Craig (Johns Hopkins University, Baltimore, MD). Monoclonal antibodies to villin, nck, and grb-2 were from Transduction Laboratories; monoclonal antibodies to phosphotyrosine, clone PY20, were from ICN; monoclonal antibodies to β-actin were from Pierce; BL21 and TKX1 competent cells were from Stratagene. Recombinant c-src kinase expressing the kinase domain was purchased from UBI. Glutathione Sepharose 4B fast flow was from Amersham-Pharmacia; GelCode blue was from Pierce; BL21 and TKX1 competent cells were from Stratagene. The nonmuscle actin polymerization kit was purchased from Cytoskeleton (Denver, CO).

Preparation of GST fusion proteins. Recombinant proteins of the SH2 and SH3 domains of PLC-γ1 were made as described earlier (2). Both domains were amplified by PCR using primers containing restriction sites at the ends. Amplicons were digested with their respective enzymes and cloned into pGEX-2T. Amino acid sequences of the NH2- and COOH-terminal SH2 and SH3 domains correspond to residues 550–657 (N-SH2), 668–745 (C-SH2), 550–745 [(N + C)-SH2], and 758–851 (SH3). Escherichia coli BL21 cells were transformed with the constructs and cultured at 37°C. Expression of the fusion proteins was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and the cells were harvested by centrifugation at 2,000 g for 15 min, sonicated in phosphate-buffered saline (PBS), and centrifuged at 5,000 g for 15 min. The resulting supernatant was mixed with 2 ml of a 50% (vol/vol) slurry of glutathione Sepharose 4B and incubated at room temperature for 30 min. After centrifugation the supernatant was removed, and the resin was washed with 50 bed volumes of PBS. This procedure was repeated twice. Glutathione S-transferase (GST)-fused recombinant proteins were eluted from the beads using 5 mM reduced glutathione. The eluted proteins were dialyzed, and the purity of the proteins was determined by separating the proteins by SDS-PAGE and staining the gel with Coomassie blue or GelCode blue. Full-length villin (human) cDNA cloned in pGEX-2T was also purified as described above.

Polymerization kinetics of actin using recombinant and native villin. The effects of villin on the polymerization of actin are well documented (9). In the presence of low concentrations of neutral salts, G-actin polymerizes to form long, double-stranded F-actin filaments. The kinetics of filament formation can be followed by measuring the change in viscosity of the protein. Actin polymerization by recombinant and native villin (purified from chicken brush-border membranes) was measured using the Ostwald capillary viscometer (type 100 Cannon Instrument, PA). Negative controls included GST protein and the buffer cocktail. Specific viscosity is defined as flow time of sample solution divided by flow time of the corresponding buffer, minus 1 (9).

The kinetics of actin polymerization were also determined using a nonmuscle actin polymerization kit according to the instructions of the manufacturer. The basis of this assay is that the fluorescence intensity of pyrene actin is much greater for polymeric than monomeric actin. (29). 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. G-actin (6 μM) in buffer containing 5 mM Tris-HCl, 0.2 mM ATP, and 0.2 mM CaCl2 was preincubated with native or recombinant villin (60 nM) for 10 min on ice. Polymerization was induced by the addition of 150 mM KCl and 1 mM MgCl2. The formation of fluorescence that occurs when pyrene G-actin forms pyrene F-actin was measured 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 rate of polymerization determined from the rate of fluorescence increase is proportional to the number of pointed ends formed and, therefore, the relative nucleation activity of villin (29). 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 (29). Fluorescence measurements were performed at 25°C using the Fluorolog 3 fluorometer. The excitation wavelength was set at 365 nm, and the emission wavelength was set at 388 nm.

In vitro kinase assay. Recombinant villin (concentrations indicated in RESULTS) was phosphorylated in vitro by c-src (2.5 U) in an assay mixture containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM EGTA, 20 mM MgCl2, 20 μM ATP, and 2–5 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 and transferred to nitrocellulose membrane, and a Western analysis was done using phosphotyrosine monoclonal antibodies.

Tyrosine phosphorylation of villin in TKX1 cells. Full-length villin cDNA (human) cloned in pGEX-2T was expressed in the Epicurian coli TKX1 cells. TKX1 cells carry a plasmid with the elk tyrosine kinase (tk) gene controlled by the trp promoter. The elk tyrosine kinase has broad specificity and has been shown to tyrosine phosphorylate a number of proteins in Epicurian coli (31). A two-step protocol involving first the induction of expression of villin protein gene (by addition of IPTG) followed by induction of the tk gene (by addition of 3-β-indoleacrylic acid (IAA), allowed for the accumulation of GST-tagged tyrosine-phosphorylated villin (VILT/WT). Phosphorylated villin was affinity purified from bacterial lysates using a glutathione Sepharose 4B column. The proteins were eluted with 5 mM glutathione in 1-ml fractions. Purity of the fractions was assessed by SDS-PAGE and staining with GelCode Blue. Tyrosine phosphorylation of villin was determined by Western analysis of the samples using phosphotyrosine monoclonal antibodies. TKX1 cells
cultured in the absence of IAA were used to obtain nonphosphorylated villin (VIL/WT).

Preparation of lipids. PIP2 and PE obtained as chloroform solutions were dried in a stream of nitrogen, and micelles were prepared by dissolving the lipid in 10 mM Tris-HCl, pH 8.0, at a concentration of 3.4 mg/ml. The lipids were sonicated in a Branson 1210 sonicator for 5 min at maximum power. The lipids were stored in aliquots at −80°C, and before use the lipids were sonicated for an additional 5 min.

Association of villin with PIP2. The association of villin with PIP2 was determined by a procedure described by Fukami et al. (15). Briefly, nonphosphorylated villin (VIL/WT, 1 μM) and phosphorylated villin (VILT/WT, 10 μM) were incubated with PIP2 (100 μM) for 15 min at 37°C in a buffer containing 140 mM NaCl, 1 mM EGTA, 0.5 mM dithiothreitol (DTT), 0.5 mM NaN3, 0.05% Tween 20, 20 mM MOPS-Tris, pH 7.4, and 0.2% BSA. The recombinant proteins were separated by SDS-PAGE and transferred to nitrocellulose, and a Western analysis was done with monoclonal antibodies to PIP2 (1).

The lipid binding assay described by Tsuchara et al. (36) was used to determine the association of PIP2 with villin. Briefly, fusion proteins (VIL/WT, VILT/WT, and GST, 2 μg) were incubated with PIP2 vesicles (final concn 1.7 mg/ml), and the reaction mixture was diluted to a final volume of 40 μl with PBS. After incubation at room temperature for 10 min and on ice for 5 min, the tubes were centrifuged at 100,000 rpm for 15 min at 4°C. The supernatant (S) was saved, and the pellet (P) was rinsed once with PBS. The pellet was resuspended in 60 μl of laemmli sample buffer, and 20 μl of this was loaded on a gel. The total (T, 2 μg protein) and 10 μl of the supernatant were mixed with 10 μl of sample buffer and loaded on the same gel. The gels were stained with GelCode blue, and the amounts of protein in T, S, and P were compared using the Eagle Eye II imager system (Stratagene).

Alternatively, the procedure described by Davletov and Sudhof (10), with minor modifications, was used to determine the association of PIP2 with villin. Twenty micrograms of fusion proteins bound to glutathione Sepharose beads were resuspended in 100 μl of buffer containing 50 mM HEPES, pH 7.2, 0.1 M NaCl, 0.5 mM EGTA (incubation buffer), and lipid vesicles containing 0.8 mg/ml of PIP2 and 3H-labeled PIP2 (20,000 cpm). The mixture was incubated at room temperature for 15 min with shaking. The fusion proteins bound to GST Sepharose were collected by centrifugation and washed three times with 1 ml of the incubation buffer. Lipid binding was quantified by liquid scintillation counting of the beads.

Preparation of ileal brush-border membranes. Distal ileum from New Zealand male rabbits was used to prepare brush-border membrane fractions by a method of differential centrifugation and Mg2+ precipitation, as described earlier (22). Rabbit distal ileum was exposed to carbachol (1 μM, 30 s), conditions previously shown to lead to tyrosine phosphorylation of villin (21). Brush-border membranes from control tissue were made in parallel (22).

In vitro binding studies. GST fusion proteins of the SH2 and SH3 domains of PLC-γ1 immobilized on glutathione Sepharose 4B were incubated for 1 h at 4°C with brush-border membrane extracts containing tyrosine-phosphorylated villin (prepared from carbachol-treated ileum, 1 μM, 30 s) or nonphosphorylated villin (prepared from control ileum). The recombinant proteins were collected by centrifugation and washed several times using a buffer containing 1% Triton X-100 and 150 mM NaCl. The samples were separated by SDS-PAGE and transferred to nitrocellulose, and a Western analysis was done using monoclonal antibodies to villin, phosphotyrosine, or GST.

PLC activity assay. The PLC-γ1 activity was measured using small unilamellar vesicles of 3H-labeled PIP2 as described earlier (2). The assay mixture contained ~10 μg of PIP2, 0.8 mM sodium deoxycholate, ~20,000 cpm of [3H]PIP2, 3 mM MgCl2, 100 mM NaCl, 2 mM EGTA, 2 mM CaCl2 (final free Ca2+ concn of 45 μM), and 0.1 mM DTT in 50 mM HEPES buffer, pH 7.0. For preparation of the substrate solution, PIP2 (0.75 mg) and [3H]PIP2 (1.5 μCi) were mixed and dried under a stream of nitrogen. Five milliliters of 65 mM HEPES (pH 7.0)-100 mM NaCl were added to the dried lipids, and the samples were sonicated for six 3-min intervals interspersed with periods of cooling on ice under a stream of nitrogen. Purified PLC-γ1 (40 ng) was used as the source of the enzyme. Recombinant villin was incubated with the substrate before the addition of PLC-γ1 for 5 min at 37°C. After the reaction mixture was incubated with PLC-γ1 for an additional 10 min at 37°C, the reaction was stopped by adding 500 μl of chloroform:methanol (1:1), and the lipids were extracted in the presence of 1 N HCl containing 5 mM EGTA. The aqueous and organic phases were separated by centrifugation, and a 350-μl portion of the upper aqueous phase was removed for liquid scintillation counting. The PLC activity was measured in the presence and absence of recombinant villin, either phosphorylated or not. The PLC activity was also measured using native villin and a recombinant protein expressing GST alone.

RESULTS

Recombinant villin demonstrates biological activity like native villin. Villin cDNA cloned in pGEX-2T was expressed as a GST fusion protein (Fig. 1A). The effects of villin on the polymerization of actin and the kinetics of filament formation were followed by measuring the change in viscosity of the protein as described in METHODS. Change in viscosity of actin was measured using three different molar concentrations of recombinant villin (recombinant villin:G-actin molar ratios of 1:65, 1:139, and 1:270). These concentrations were chosen on the basis of prior studies of the effects of villin on actin polymerization and are physiologically meaningful based on the ratio of villin to actin in purified brush border (9). Viscosity measurements using native villin (native villin:G-actin molar ratios of 1:139 and 1:270) were done in parallel. GST (GST:G-actin molar ratio, 1:65) and the buffer cocktail were used as negative controls. As shown in Fig. 1B, recombinant villin polymerizes actin nearly as well as the native protein.

Actin-polymerizing activity was also measured by analyzing the effect of recombinant and native villin on the polymerization of pyrene G-actin to pyrene F-actin. As shown in Fig. 1C, compared with the polymerization kinetics of actin alone (control), the addition of villin in the presence of 20 μM Ca2+ abolished the lag phase and increased the initial rate of actin polymerization. The recombinant villin polymerized actin as well as the native protein. We also analyzed the severing activity of recombinant and native villin by measuring the decrease in fluorescence of pyrenyl F-actin over time. As shown in Fig. 1D, recombinant villin depolymerizes actin as well compared with SDS-PAGE.
as the native protein. These studies demonstrated that full-length recombinant villin behaves like the native protein, thus allowing us to reconstitute in vitro our previous in vivo observations.

Villin can be tyrosine phosphorylated in vitro. Native villin (760 ng) and recombinant villin (3 μg) were tyrosine phosphorylated in vitro using c-src kinase. Unlike the native villin, recombinant villin was not...
tyrosine phosphorylated in vitro by c-src (Fig. 2A). Previous studies on phosphorylation of gelsolin suggested that addition of PIP2 to the kinase assay significantly increased the tyrosine phosphorylation of gelsolin in vitro (11). Thus it was possible that PIP2 might alter the ability of c-src to phosphorylate recombinant villin in vitro. However, addition of PIP2 to the kinase assay mixture did not phosphorylate recombinant villin (data not shown). In contrast, in vitro phosphorylation of recombinant villin required the presence of the reducing agent, β-mercaptoethanol (at concentrations ≥0.5 mM; Fig. 2B). Lower concentrations of β-mercaptoethanol (<2 mM) did not induce significant phosphorylation, whereas concentrations >5 mM did not lead to any further increase in the tyrosine phosphorylation of recombinant villin (data not shown). The native villin was stored in 0.5 mM β-mercaptoethanol. However, such a small amount (1 μl) of the purified protein was used (which was further diluted in the kinase reaction mixture) for phosphorylation by c-src that the final concentration of β-mercaptoethanol in the kinase mixture was insignificant. That the native protein is reduced in vivo by some physiological equivalent of β-mercaptoethanol cannot be ruled out. The addition of exogenous β-mercaptoethanol did not significantly increase the tyrosine phosphorylation of native villin (data not shown). These results suggest that the recombinant villin may form disulfide bonds and assume a conformation that masks the phosphorylation site, and reducing agents such as β-mercaptoethanol may be required to make this accessible for in vitro phosphorylation of recombinant villin; or alternatively, the recombinant villin may be folded differently from the native villin. However, it is possible that the cleavage of disulfide linkages within the villin protein may actually be important for the tyrosine phosphorylation of villin. A physiological role for disulfide bonds has been shown in gelsolin and severin (25, 39).

Tyrosine phosphorylation of villin in vitro is not affected by PIP2. Previous studies with gelsolin suggested that PIP2 augmented the tyrosine phosphorylation of the protein by c-src (11). To further examine a role for PIP2 in mediating the tyrosine phosphorylation of villin, we tested the effect of PIP2 on phosphorylation of villin in the presence of nonsaturating concentrations of β-mercaptoethanol. Recombinant villin (1 μM) was tyrosine phosphorylated in vitro in the presence of β-mercaptoethanol (1 mM) and varying concentrations of PIP2 (0–50 μM). As shown in Fig. 3A, the addition of exogenous PIP2 to the in vitro phosphorylation assay had no significant effect on the tyrosine phosphorylation of villin. Similar data were obtained using native villin purified from chicken brush border (Fig. 3B). In fact, the addition of PIP2 appears to inhibit the phosphorylation of villin, with significant (78%, P < 0.05, n = 5) inhibition at 50 μM PIP2 (Fig. 3A). PE also did not increase the phosphorylation of villin and, like PIP2, appears to inhibit (at 200 μM, 49%, P < 0.05, n = 3) the tyrosine phosphorylation of villin (Fig. 3C). The concentrations of PIP2 and PE used in this study were based on similar studies done with gelsolin (11). The data indicate that villin differs from gelsolin in its requirement for exogenous PIP2 for tyrosine phosphorylation in vitro.

Stoichiometry of villin phosphorylation. The amount of phosphate incorporated in villin was determined in an in vitro kinase assay (as described in METHODS) using 2.6 nmol of recombinant villin. Villin was phosphorylated in vitro by c-src in a reaction mixture containing 30 μCi of [γ-32P]ATP (20 μM ATP, sp act = 3,000 Ci/mmol) and 5 mM β-mercaptoethanol in a final volume of 50 μl. The phosphorylated recombinant villin was affinity purified using glutathione Sepharose, and the radioactivity associated with the phosphorylated villin was measured in a liquid scintillation counter. Phosphorylation of GST was negligible and was subtracted from the phosphorylated villin samples. Using this method, we obtained 0.61 mol phosphate/mol of villin. These amounts are comparable to those reported for gelsolin (0.86 mol phosphate/mol gelsolin) (11) and

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suggest nearly quantitative phosphorylation, provided only one major site is phosphorylated in villin. Similar numbers were obtained with native villin (0.70 mol phosphate/mol of villin).

Tyrosine phosphorylation of villin in TKX1 cells. The phosphorylation of villin by c-src is shown in these studies to characterize the phosphorylation of villin by PIP₂, to determine the stoichiometry of villin phosphorylation, and to compare in vitro phosphorylation of villin with other proteins of its family, including gelso-lin. However, since differences in folding between recombinant and native villin cannot be ruled out, an alternative approach to obtain tyrosine-phosphorylation recombinant villin was used. This approach obliterates the need to use either exogenous kinase or β-mercaptoethanol to phosphorylate the recombinant villin. Full-length villin cDNA was expressed in the TKX1 cells, which carry an inducible tyrosine kinase gene (see METHODS). Recombinant villin was purified from the TKX1 cells as a GST-tagged tyrosine-phosphorylated protein (Fig. 4). Batch purification was not used to collect tyrosine-phosphorylated villin from GST Sepharose because the eluted protein contains phosphorylated bands in addition to villin (see fraction 4, Fig. 4B). Phosphorylated villin (VILT/WT) collected from fractions 7–13 was pooled and used in these studies. VILT/WT polymerized actin like the native

Fig. 3. In vitro phosphorylation of villin is not regulated by phosphatidylinositol 4,5-bisphosphate (PIP₂). A: recombinant villin was phosphorylated in vitro by c-src in absence or presence of varying concentrations of PIP₂ (1–50 μM). This is an immunoblot with anti-phosphotyrosine antibodies. The Western blot is representative of 5 experiments with similar results. B: native villin purified from chicken brush border was phosphorylated in vitro by c-src in absence or presence of PIP₂ (25 μM). This is an immunoblot with anti-phosphotyrosine antibodies. The Western blot is representative of 3 experiments with similar results. C: recombinant villin was phosphorylated in vitro by c-src in absence or presence of varying concentrations of phosphatidylethanolamine (PE; 40–200 μM). The phosphorylated proteins were separated by SDS-PAGE and immunoblotted with anti-phosphotyrosine antibodies. This immunoblot is representative of 3 experiments with similar results.

Fig. 4. Tyrosine phosphorylation of villin in TKX1 cells. A: full-length villin (human), cloned in pGEX-2T, was expressed in TKX1 cells as described in METHODS. This is a GelCode blue-stained gel of proteins eluted from the glutathione Sepharose 4B column. Molecular mass markers are shown at right. B: an immunoblot of samples shown in A using phosphotyrosine monoclonal antibodies. The GST fusion proteins were eluted from the column in 1-mI fractions as described in METHODS. Fraction numbers (F15, F13, F10, F7, F4, F1) are shown at top of Western blot. This blot is representative of 5 experiments with similar results.
protein (data not shown). Villin phosphorylated in TKX1 cells was used in all the subsequent experiments.

Tyrosine-phosphorylated villin does not bind PIP_2. Although direct interaction of villin with PIP_2 has not been demonstrated, two PIP_2-binding domains have been identified in villin (17). This and our previous observation that tyrosine-phosphorylated villin associates with PLC-γ led us to speculate that tyrosine phosphorylation may regulate the ability of villin to bind both the enzyme and the substrate involved in phosphoinositide signal transduction, namely PLC-γ_1 and PIP_2. We sought to determine if the ligand-binding properties of villin could be regulated by its tyrosine phosphorylation. To determine the association of villin with PIP_2, recombinant villin phosphorylated (VIL/WT) or not (VIL/WT) was incubated in vitro with PIP_2. The recombinant proteins incubated with (Fig. 5, A and B) or without (Fig. 5C) PIP_2 were analyzed by Western analysis using PIP_2 monoclonal antibodies.

The PIP_2 monoclonal antibodies used in these studies (also called KT10 and kt3g in various citations)
were first described over a decade ago by Uno et al. (38) and have been used successfully to establish several PIP2-protein interactions (14, 16, 38). As suggested by earlier studies and as shown in Fig. 5A, nonphosphorylated villin associates with PIP2. The association of PIP2 with villin even under the denaturing conditions required for SDS-PAGE has been observed with all other PIP2-associated actin- regulatory proteins studied using this approach, including α-actinin, vinculin, and gelsolin (1, 13, 15). PIP2 does not dissociate from villin during SDS-PAGE and does not affect its migration, similar to what has been reported for other PIP2-binding proteins (1, 13, 15). In contrast, even in the presence of a 10-fold higher concentration of VILT/WT (Fig. 5B), tyrosine phosphorylation abolished the ability of VIL/WT to associate with PIP2 (Fig. 5A). The PIP2 monoclonal antibody did not cross-react with the villin protein (Fig. 5C). These data show that tyrosine phosphorylation of villin may modulate its ability to sequester PIP2, thus regulating phosphoinositol-mediated signal transduction.

To test phospholipid binding to villin, the binding of VIL/WT and VILT/WT to small unilamellar vesicles containing ³H-labeled PIP2 was determined. Binding was monitored by a count of ³H associated with the fusion proteins using a liquid scintillation counter. The experiments revealed that VIL/WT showed significant (P < 0.01 compared with GST, n = 3) association with PIP2, while VILT/WT showed no significant binding to the lipid vesicles. The nonspecific binding of lipid vesicles was determined by its association with GST (Fig. 5D). Alternatively, to assess lipid binding, a centrifugal liposome-based binding assay was also used. Liposomes pellet in the ultracentrifuge under these conditions and carry with them those proteins that bind to the liposomes (8, 36). As a control for protein precipitation or nonspecific lipid binding, protein solutions were also centrifuged without the added liposomes. Figure 5E summarizes the binding observed for VIL/WT, VILT/WT, and GST. Liposomes containing PIP2 bound and pelleted VIL/WT but not VILT/WT or GST alone. Thus our data show that 1) nonphosphorylated villin associates with PIP2, and 2) tyrosine phosphorylation of villin abolishes its ability to associate with PIP2.

Tyrosine-phosphorylated villin binds to the COOH-terminal SH2 domain of PLC-γ1. We have previously reported that tyrosine-phosphorylated villin associates with PLC-γ1 in vivo (22). PLC-γ1 contains two SH2 domains and one SH3 domain. SH2 domains from several different proteins, including PLC-γ1, have been shown to bind specifically to tyrosine-phosphorylated cellular proteins (32). We hypothesized that tyrosine-phosphorylated villin may associate with the SH2 domain of PLC-γ1. Plasmids containing the SH2 domains of PLC-γ1 were expressed as GST fusion proteins (Fig. 6A). To determine the association of tyrosine-phosphorylated villin with SH2 domains of PLC-γ1, we exposed rabbit distal ileum to carbachol (1 μM, 30 s), conditions previously shown to lead to tyrosine phosphorylation of villin. Brush-border membranes from control tissue were made in parallel as described in METHODS. The recombinant PLC-γ1 proteins immobilized on glutathione Sepharose beads were incubated with brush-border membrane extracts from carbachol-treated ileum, the proteins bound to the beads were separated by SDS-PAGE, and Western blot analysis was done using villin monoclonal antibodies. As shown in Fig. 6B, villin associates with the COOH-terminal SH2 domain of PLC-γ1 and with the recombinant protein containing both the COOH- and the NH2-terminal domains of PLC-γ1. The tyrosine phosphorylation of the villin associated with the SH2 domain of PLC-γ1 is shown in Fig. 6C. Tyrosine-phosphorylated villin does not associate with the NH2-terminal SH2 domain of PLC-γ1 (Fig. 6, B and C). Brush border extracts from control ileum incubated with PLC-γ1 recombinant proteins showed no association of villin with PLC-γ1 (Fig. 6D).

Figure 6E shows Western analysis of the same samples as shown in Fig. 6, B, C, and D, using GST monoclonal antibodies. The villin-PLC-γ1 complex was also probed for other known ligands of PLC-γ1, including nck, actin, and grb-2, all of which were negative (data not shown). A GST fusion protein expressing the SH3 domain of PLC-γ1 showed no association with either nonphosphorylated or phosphorylated villin (data not shown).

Nonphosphorylated villin inhibits PLC-γ1 catalytic activity in vitro. Having shown that villin can associate with both PIP2 and PLC-γ1, we next sought to determine if villin could regulate the activity of PLC-γ1. Purified PLC-γ1 (40 ng) used in these studies was determined to be 98% pure by HPLC as described earlier (2). PLC-γ1 catalytic activity was measured in the presence or absence of different concentrations of recombinant villin (VIL/WT, 0–30 μM). As shown in Fig. 7A, villin inhibits PLC-γ1 activity in a dose-dependent manner (n = 11). Fifty percent of the PLC-γ1 activity was inhibited by villin at a concentration of 12.4 mM. This concentration dependency is comparable to that observed for the inhibitory effect of gelsolin on PLC-γ activity (34). The PLC-γ1 catalytic activity was also measured in the presence of native villin, and similar results were obtained. There was no change in the PLC-γ1 activity in the presence of GST. PLC-γ1 activity was measured in the presence of 10 μM villin and increasing concentrations of PIP2 (Fig. 7B). Increasing concentrations of PIP2 reversed the inhibitory effect of villin on the PLC-γ1 catalytic activity. The simplest interpretation of these data is that villin inhibits PLC-γ1 by substrate competition.

Tyrosine-phosphorylated villin does not inhibit PLC-γ1 activity in vitro. To determine the effect of tyrosine-phosphorylated villin on PLC-γ1 catalytic activity, we used recombinant villin phosphorylated in TKX1 cells (VIL/WT). Nonphosphorylated villin (VIL/WT) was obtained from TKX1 cells grown in the absence of IAA. The PLC-γ1 activity was measured in vitro in the presence of villin (5 and 10 μM) that was phosphorylated or not. As seen in Fig. 7C, recombinant villin inhibited PLC-γ1 activity at both concentrations. In contrast, phosphorylated villin did not inhibit...
PLC-γ1 at either concentration used. These data show that tyrosine phosphorylation abolishes the ability of villin to inhibit PLC-γ1 catalytic activity in vitro. On the basis of these data, we propose that tyrosine phosphorylation of villin is fundamental to the regulation of PLC-γ1 catalytic activity by villin, because it determines villin's ability to associate with both PIP2 and PLC-γ1.

AJP-Cell Physiol • VOL 281 • SEPTEMBER 2001 • www.ajpcell.org
DISCUSSION

Villin, an actin-regulatory protein described in the epithelial cells of the intestine and kidney, is now being reported in other epithelial cells such as the merkel cells (37) and the alveolar cells (20), suggesting that villin may be a protein ubiquitous to all epithelial cells. We have previously shown that tyrosine phosphorylation of villin and its association with PLC-γ1 in the intestinal epithelial cells is involved in the cytoskeletal rearrangement associated with receptor activation (21, 22). Since our first report, tyrosine phosphorylation of villin and its association with PLC-γ1 has been shown to be involved in rearrangement of the actin cytoskeleton in the opossum kidney cell line (28). Villin is abundantly expressed in renal proximal tubule and intestinal microvilli, and its association with PLC-γ1 has been shown to regulate ion transport in both the renal and intestinal epithelial cells. More recently, a role for villin in renal ischemia has been reported (40). The actin cytoskeleton of proximal tubule cells contributes significantly to development of acute, ischemic kidney tubule cell damage (30). Alterations in the actin cytoskeleton associated with the actin-severing properties of villin thus appear to be important both in physiology and pathophysiology of epithelial cells of the intestine and kidney.

Villin belongs to a family of proteins (that includes gelsolin and severin) that share F-actin-severing activity. Several actin-binding proteins, including gelsolin, CapG, fragmin, and profilin, have been shown to be tyrosine phosphorylated in vitro (11). Although these proteins have not been demonstrated to be tyrosine phosphorylated in vivo, tyrosine kinases of the Src family have been shown to regulate the physiological functions of these proteins (6, 26). These observations suggest that tyrosine phosphorylation of this family of proteins may be a general mechanism that may lend new properties to these proteins and add yet another level of regulation that may be recognized by future studies involving the identification of the phosphorylated tyrosine residues and other functional assays.

We now report for the first time the in vitro tyrosine phosphorylation of villin. Recombinant villin can be tyrosine phosphorylated in vitro by tyrosine kinases such as elk (Fig. 4), c-src (Fig. 2), and c-yes (Khurana, unpublished observation). The brush border of intestinal epithelial cells expresses both c-src and c-yes (23). Because it is suggested that phosphorylation of gelsolin is regulated by c-src, it is reasonable to speculate that villin phosphorylation in vivo may also be regulated by the Src family tyrosine kinases, c-src and/or c-yes. Although the recombinant villin protein exhibits actin-regulatory properties similar to those of the native protein (Fig. 1), it required ≥2 mM of β-mercaptoethanol for phosphorylation (Fig. 2B). Tyrosine phosphorylation of gelsolin and CapG in vitro by c-src is significantly enhanced in the presence of PIP2 (11). The mechanism of PIP2 stimulation is hypothesized to involve exposure of key tyrosine residues due to a phospholipid-dependent conformational change in the pro-
teins. This effect was specific for PIP$_2$ and could not be duplicated by other anionic and neutral phospholipids, including phosphatidylserine or PE. In our studies, we could not substitute β-mercaptoethanol with PIP$_2$ or any other phospholipid, including PE, to tyrosine phosphorylate recombinant villin in vitro. Also, unlike gelsolin, PIP$_2$ did not enhance the phosphorylation levels of villin in vitro. This observation could mean one of two things: that villin may actually differ from these proteins in its requirement for PIP$_2$ for phosphorylation or, alternatively, that the β-mercaptoethanol present in the kinase buffer may function like the phospholipid in unmasking the tyrosine residues. These differences in the phosphorylation of villin and gelsolin by PIP$_2$ may even be physiologically relevant and perhaps important to our understanding of the unique function performed by each of these proteins in vivo. This is important in light of the fact that both villin and gelsolin are expressed in intestinal epithelial cells.

Two phosphoinositide binding sites have been identified in villin (amino acids 111–118 and 137–145), which are also the site of F-actin binding to villin before severing (18). Like villin, gelsolin and CapG, members of an actin filament-severing and capping protein family, are also activated by Ca$^{2+}$ and inhibited by phosphoinositides, particularly PIP$_2$ (18). There is emerging evidence that actin-regulatory proteins binding to PIP$_2$ may have functions beyond a direct effect on the cytoskeleton. For instance, in vitro gelsolin alters the activity of phosphoinositide-specific PLC (3, 7), phospholipase D (4, 33), and phosphoinositide 3-OH kinase (6, 7). The association of villin with the phosphoinositide, PIP$_2$, and the phosphoinositide-hydrolyzing enzyme, PLC-γ$_1$, suggests that villin may be involved in regulating the phosphoinositide-mediated signal transduction pathways. These studies suggest that proteins of this family, including villin, may be components of a signaling complex that transduces external stimuli to the cortical cytoskeleton.

We have previously shown that tyrosine-phosphorylated villin associates with PLC-γ$_1$ in vivo, suggesting that tyrosine phosphorylation plays a key role in the interaction between villin and PLC-γ$_1$. Using recombinant proteins to the SH2 domain of PLC-γ$_1$, we now show that phosphorylated villin binds specifically to the COOH-SH2 domain of PLC-γ$_1$. Gelsolin, which shares villin’s actin-severing but not actin-bundling properties, has also been shown to associate in vivo with PLC-γ (5). However, the site of interaction between gelsolin and PLC-γ remains to be determined. Future studies with other members of this family may demonstrate that PLC enzymes may also be common ligands for this family of proteins.

The association of villin with both the substrate and the enzyme, namely PIP$_2$ and PLC-γ$_1$, suggests that villin’s interaction with PLC-γ$_1$ may have a functional consequence. To determine the effect of villin association on PLC-γ$_1$ activity, we measured the catalytic activity of purified PLC-γ$_1$ in the presence or absence of villin. Villin inhibits PLC-γ$_1$ activity in a dose-dependent manner. This is consistent with the idea that proteins of the villin family may regulate PLC activity, since gelsolin and CapG have been demonstrated to inhibit PLC-γ activity both in vitro and in vivo (5, 34, 35). The simplest hypothesis to explain this observation is that since villin binds PIP$_2$, sequestration of the substrate by villin may be the mechanism for the observed inhibition of PLC activity by villin. Such an observation has been made with gelsolin (34). Reversal of villin’s inhibitory effect on PLC-γ$_1$ activity in the presence of increasing concentrations of PIP$_2$ (Fig. 7B) lends support to this hypothesis. The inability of tyrosine-phosphorylated villin to inhibit PLC-γ$_1$ activity (Fig. 7C) would then suggest that tyrosine phosphorylation of villin may modulate its ability to bind PIP$_2$.

![Fig. 8. Model for molecular mechanism of actin remodeling and catalytic activation of PLC-γ$_1$ by tyrosine phosphorylation of villin. Tyrosine phosphorylation of villin recruits PLC-γ$_1$ to the brush-border membrane of intestinal epithelial cells and alters the ability of villin to sequester PIP$_2$. Removal of PIP$_2$ from villin vacates this site for F-actin binding before severing. Activation of PLC-γ$_1$ in proximity of villin generates 1,4,5-trisphosphate (IP$_3$), which releases Ca$^{2+}$ from intracellular stores, leading to a localized increase in intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]). Removal of PIP$_2$ from villin as well as binding of Ca$^{2+}$ to villin would lead to an increase in the actin-severing properties of villin. Diacylglycerol (DAG) and IP$_3$ are products of PLC-γ$_1$ activation. Y, tyrosine residue; PO$_4$–, phosphorylated tyrosine residue.](http://ajpcell.physiology.org/10.1152/ajpcell.00079.2001)
Indeed, using in vitro binding studies (Fig. 5, A–C), a centrifugal liposome-based binding assay (Fig. 5E), as well as association of fusion proteins to $^3$H-labeled PIP2 vesicles (Fig. 5D), we demonstrate that, while nonphosphorylated villin binds PIP2, tyrosine phosphorylation of villin abolishes its ability to associate with the phospholipid. Our studies demonstrate that phosphorylation of villin determines its association with PLC-$\gamma_1$ and PIP2. Thus tyrosine phosphorylation of villin regulates its interaction with both the substrate and the enzyme associated with phosphoinositide signal transduction. The present work presents biochemical proof of the functional relevance of tyrosine phosphorylation of a cellular protein.

The data presented in this study indicate that there is significant cross-talk between components of the transmembrane signal machinery and the actin cytoskeleton at multiple levels, including the generation of important second messengers. Our working model (Fig. 8) is that binding of villin to PLC-$\gamma_1$ brings the enzyme in proximity of the substrate (PIP2), thus activating PLC-$\gamma_1$. However, the enzyme may be activated only when villin no longer sequesters PIP2, i.e., when villin is tyrosine phosphorylated. The identified PIP2 binding site in villin is also the site for actin binding to villin before severing (17); thus removal of PIP2 from this site would make it accessible for actin to bind to villin and for villin to sever F-actin filaments. Decades of in vitro work with villin have suggested that very high $Ca^{2+}$ concentrations (>5 $\mu$M) are required to activate villin's actin-severing function (27). Because this is not a physiologically relevant intracellular $Ca^{2+}$ concentration ([Ca$^{2+}$]), it has been assumed for years that villin's actin-bundling and not actin-severing properties are physiologically significant. Studies done with the villin-knockout mice report normal bundling of F-actin filaments in the intestinal microvilli, suggesting that in the absence of villin, the actin-bundling properties associated with villin can be substituted by other proteins in the microvilli (28). A second study with the villin-knockout mice reports that villin is required for F-actin severing rather than bundling in the microvilli of intestinal cells (12). On the basis of these observations, it seems it may be time to revisit villin's actin-severing rather than actin-bundling functions.

According to our model, the regulation of villin's actin-severing function may be intimately linked to its tyrosine phosphorylation. Tyrosine phosphorylation of villin would lead to the recruitment of PLC-$\gamma_1$ and alter the ability of villin to sequester PIP2. Removal of PIP2 from villin would vacate this site for F-actin to bind to this severing site (17). Activation of PLC-$\gamma_1$ in proximity of villin would generate $d$-myo-inositol 1,4,5-trisphosphate (IP$_3$), which would release Ca$^{2+}$ from intracellular stores, leading to a localized increase in [Ca$^{2+}$]. Intracellular Ca$^{2+}$ stores are now identified in the apical membrane of epithelial cells of the intestine (24). Tyrosine phosphorylation of villin could also modulate its ability to regulate the cortical cytoskeleton in submicromolar concentrations of Ca$^{2+}$. Concurrent with this model, we have recently reported a decrease in the F-actin content as well as a redistribution of villin in response to carbachol treatment in the intestinal cell line, Caco-2 (21).

We thank Dr. Susan W. Craig (Johns Hopkins University, Baltimore, MD) for assistance with the actin polymerization experiments and the kind gift of villin purified from chicken brush-border membranes, and Dr. Tony Guererro (Johns Hopkins University) for assistance with the fluorometric measurements of actin polymerization.

These studies were supported by a grant from the American Digestive Health Foundation (Industry Research Scholar Award) and by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-54755 to S. Khurana.

REFERENCES


REGULATION OF PLC-γ1 BY VILLIN


AJP-Cell Physiol • VOL 281 • SEPTEMBER 2001 • www.ajpcell.org