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PLC-γ1 Signaling Pathway and Villin Activation Are Involved In Actin Cytoskeleton Reorganization Induced by Na^+/P_i Cotransport Up-regulation

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Abstract

Background: The brief incubation of opossum kidney (OK) cells with low P_i results in Na^+/P_i cotransport up-regulation and in substantial, but transient, cytoskeletal reorganization. In this study, we examined signaling events involved in the depolymerization of microfilaments.

Results: Confocal laser scanning microscopy, immunoblot and immunoprecipitation experiments revealed villin co-localization with mainly actin short filaments and monomers, indicating that under the conditions used, villin acted as an actin-severing protein. Further analysis revealed that low concentrations of extracellular phosphate resulted in phospholipase Cγ1 (PLC-γ1) translocation to the actin cytoskeleton, without increases in its tyrosine phosphorylation. Additionally, tyrosine phosphorylation of a portion of insoluble villin was increased; whereas, only tyrosine phosphorylated villin associated with PLC-γ1. Although, tyrosine phosphorylation of PLC-γ1 was not observed during Na^+/P_i cotransport up-regulation, genistein treatment abolished the enzyme's translocation to the actin cytoskeleton, as well as its association with villin. In addition, villin was found to associate with the 85-KDa subunit (p85) of phosphatidylinositol (PI)-3 kinase, concomitant with PLC-γ1, in the cytoskeletal fraction of Na^+/P_i cotransport up-regulated cells.

Conclusions: Our observations suggest a signaling mechanism linking low ambient P_i levels to the acute up-regulation of its cotransport with sodium and the depolymerization of the subcortical actin cytoskeleton.

Introduction

The actin cytoskeleton participates in several cellular processes, including intracellular signaling, membrane trafficking, secretion, exo- and endocytosis (1–5). Interactions between the actin cytoskeleton and the plasma membrane are thought to be important in many cellular events, including transport protein activity (6–9). The actin cytoskeleton may affect transport activity directly, by connecting transport proteins subunits to cytoskeletal elements or indirectly, by interacting with signaling molecules (membrane receptors, G proteins, phospholipases and adenylate cyclase) that regulate transport protein activity (10).

The actin cytoskeleton includes, in addition to actin, several actin-binding proteins that modulate the polymerization and depolymerization of actin microfilaments. One example is villin, which is abundantly present in proximal tubule and intestinal microvilli (11,12). Villin belongs to a family of Ca^{2+} and polyphosphoinositide-regulated, actin-binding proteins (13,14) that nucleate, cap or sever actin filaments. At physiological levels of intracellular Ca^{2+}, villin bundles actin filaments; whereas, in the presence of micromolar Ca^{2+}, villin severs actin filaments (13,15). Differential activation of severing and nucleating activities of villin also occurs in response to altered phosphoinositide concentra-

*On January 30, 2000 an accidental death took Professor D.S. Emmanouel away from us. This article is dedicated in his memory.

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tions. In vitro studies have shown that villin associates especially with phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2]. PI(4,5)P2 binding inhibits the ability of villin to sever actin filaments (14). Various extracellular signals stimulate the hydrolysis of PI(4,5)P2 by activatin phospholipase C (PLC). The products of this reaction are two second messengers: inositol 1,4,5-trisphosphate [I(1,4,5)P3], which mediates the increase of intracellular free calcium levels, and diacylglycerol (16). It is also known that phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P3], the product of the reaction catalyzed by PI-3 kinase, activates PLC-γ isoenzymes by interacting with their Src homology-2 (SH2) domains in the absence of PLC-γ tyrosine phosphorylation (17), or by binding to the pleckstrin homology (PH) domain of PLC-γ1 leading to translocation of the latter to the plasma membrane (18). Thus, the actin-severing activity of villin could be augmented during activation of PLC-γ1, through both the hydrolysis-induced decrease in the concentration of PI(4,5)P2, as well as through the increase of cytosolic calcium brought about by the I(1,4,5)P3 production generated by the phospholipase's action.

Inorganic phosphate balance is essential for the maintenance of normal cellular functions and relies heavily on renal P_i reabsorption, which is mainly a proximal tubular function. Reabsorption of inorganic phosphate by renal tubular epithelia is performed by at least two Na^+/P_i cotransporter types, which are located in the apical membrane (19). The activity of the cotransport seems to be regulated mainly through changes of the system's $V_{max.}$ This can be due to alterations in the number of cotransporter units inserted into the luminal membrane (20) or to enhanced fluidity of the apical membrane. These mechanisms are consistent with the existence of recycling mechanisms capable of transferring them to and from a subapical cytoplasmic location to dock them apically (21). We previously showed that OK cells incubated with low extracellular P_i up-regulate Na^+/P_i cotransport, while undergoing a rapid, but transient, cytoskeletal reorganization (22). In the present study, we examined intracellular signaling events involved in the depolymerization of microfilaments. Using confocal laser scanning microscopy, quantitative immunoblot and immunoprecipitation analysis, we studied villin's redistribution in relation to microfilament depolymerization. Our findings provide evidence that Na^+/P_i cotransport up-regulation triggers PLC-γ_1 and PI-3 kinase activation and suggest that villin plays a key role in the signaling cascade causing microfilament depolymerization during the initiation of Na^+/P_i cotransport up-regulation by low ambient phosphate. To our knowledge, the association of villin with p85 ex vivo has not been previously described. It is also the first report providing a signaling cascade initiated by the low extracellular P_i incubation of OK cells.

Materials and Methods

Materials

Culture media DMEM-Ham's F12, trypsin and EDTA solutions were from Biochrom KG (Berlin, Germany); whereas, fetal calf serum was purchased from Flow Laboratories (Irving, Scotland). Bovine serum albumin and phalloidin were obtained from Sigma (St. Louis, MO). Rhodamine-phalloidin and fluorescein-labeled goat anti-mouse Immunoglobulin G (IgG) were from Molecular Probes, Inc. (Eugene, OR). Monoclonal anti-villin andibodies for immunofluorescence and immunobloting were purchased from Chemicon (Termecula, CA). Mixed monoclonal antibodies to PLC-γ1, monoclonal anti-phosphotyrosine and polyclonal anti-p85 antibodies, as well agarose beads were purchased from Upstate Biotechnology (Lake Placid, NY). The ECL Western blotting kit and monoclonal anti-actin andibody were purchased from Amersham Corp. (Arlington Heights, IL). Type IV collagen was from Gibco BRL (Life Technologies, Inc. Bethesda, MD). All other chemicals were obtained from *usual commercial sources* (Sigma, St. Louis, MO) at the highest grade available.

Cell Culture

OK cells were from the American Type Culture Collection (Rockville, MD) and were studied between passages 40 and 50. Cells were maintained in a humidified atmosphere of 5% CO_2, 95% air at 37°C and fed twice weekly with a 1:1 Dulbecco's modified Eagle's medium (DMEM)-Ham's F12 medium, supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine, 20 mM $NaHCO_3$, 22 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 50 IU/ml penicillin and 50 mg/ml streptomycin. Subcultivation was performed with Ca^{2+}- and Mg^{2+}- free phosphate-buffered saline (PBS), containing 0.25% trypsin and

5mM EDTA. Cells were cultured in 25 cm^2 for immunoblot analysis and in 75 cm^2 flasks for immunoprecipitation experiments, or in 35 mm plastic dishes for confocal laser scanning microscopy, for 48 hr with complete medium as described above. The medium was changed to serum-free medium, containing 0.1% bovine serum albumin (BSA), 15–20 hr prior to the actual experiments. Exposure of cells to low extracellular P_i concentration was performed as described previously (22). The tyrosine kinase inhibitor genistein (100 μM) or its vehicle (dimethylsulfoxide; DMSO) was added for the last 40 min of the 15–20 hr preincubation period. All experiments described below were performed while cells were still in their logarithmic growth phase.

Confocal Laser Scanning Microscopy

For morphological observations by confocal laser scanning microscopy, OK cells were cultured onto Type IV collagen covered glass slides (22 × 22 mm), so as to assure cellular attachment and orientation conditions analogous to those obtained for renal tubular epithelia in situ. In the appropriate experiments, cells were exposed for 5 min to low extracellular P_i concentration (0.1 mM). Cell fixation and direct staining for fluorescence of microfilaments by rhodamine-phalloidin or double-labeling with additional indirect staining for fluorescence of villin, by (FITC-IgG) were performed as described previously (23). The coverslips were analyzed using a confocal laser scanning module (Leica Lasertechnik, Heidelberg, Germany), attached to an inverted microscope (Zeiss IM35, Zeiss, Oberkochen, Germany), equipped with an argon-krypton ion laser, as described previously (24). Confocal images were acquired using a 63/1.25 oil immersion objective and dedicated Confocal Laser Scanning Microscope (CLSM) software (Leica Lasertechnik, Heidelberg, Germany).

Fluorescence Measurements of F-actin

The filamentous (F)-actin content was determined in OK cells, pre-treated as described above, by the rhodamine-phalloidin fluorescence assay (25), with minor modifications cells grown in 24-well tissue-culture plates at a seeding density of 10^4 cells/cm^2, were fixed by the addition of 3.7% formaldehyde in PBS for 15 min at room temperature. After permeabilization of cells with Triton X-100 (0.2% in PBS), rhodamine-phalloidin (1.5 μM in PBS) was added and left for 30 min. The cells were washed with PBS and dissolved in 0.5 ml of 0.1 N NaOH. Fluorescence of the samples was easured in a Perkin-Elmer (Oak Brook, IL) fluorometer using excitation and emission wavelengths of 550 nm and 580 nm, respectively.

Immunoblot Analysis of TritonX-100 Insoluble Cytoskeletal Pellets and Corresponding Supernatants

Detergent-insoluble cytoskeletal fractions were prepared by using a modification of a method described previously (26). Cells were incubated in 1 ml of cytoskeleton extraction buffer (consisting of 0.5% TritonX-100, 10 mM EGTA, 40 mM KCl, 5 μg/ml leupeptin, 1 μg/ml aprotinin, 1mM PMSF and 10 mM imidazole, pH 7.15, on ice) for 20 min at 4°C. The cell extracts were centrifuged for 4 min at 16,000 g and the resulting low speed pellet (LSP) was dissolved in a Tris/SDS buffer (consisting of 0.625 M Tris-HCl pH 7.4, 2% SDS and 10% glycerol). The remaining supernatant was centrifuged for 2.5 hr at 100,000 g to obtain a high speed pellet (HSP), which was also dissolved in the same volume of Tris/SDS buffer. Proteins in the remaing high-speed supernatant (HSS) were precipitated by mixing with 4 volumes of ice-cold acetone and subsequently dissolved in Tris/SDS buffer. The three fractions (LSP, HSP and HSS) of control and Na^+/P_i cotransport up-regulated cells were subjected to SDS/PAGE and Western blotting using monoclonal mouse anti-actin and anti-villin antibodies. Blots were developed using the ECL Western blots kit. Band intensities were quantitated by PC-based image analysis (Image Analysis Inc., Ontario, Canada).

Immunoprecipitation Analysis of TritonX-100 Soluble and Insoluble Fractions

For immunoprecipitation experiments, the TritonX-100-soluble fraction of control and Na^+/P_i cotransport up-regulated cells was prepared according to Golenhofen et al. (27), with minor modifications. Cells were incubated in 1 ml of Triton-extraction buffer (0.3% TritonX-100, 5 mM Tris, pH 7.4, 2 mM EGTA, 300 mM sucrose, 2 μM phalloidin, 1 mM PMSF, 10 μg/ml leupeptin, 20 μg/ml aprotinin, 1 mM sodium orthovanadate, and 50 mM NaF) for 5 min on ice. After removing the soluble proteins, the Triton-insoluble fraction remaining

on the plate was scraped directly into 500 μl RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% TritonX-100, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 1 mM EDTA, 1 mM dithiothreitol and 1 mM sodium orthovanadate) and any remaining insoluble material was removed by centrifugation. Equal amounts of protein (500 μg) from each fraction were immunoprecipitated with the appropriate antibodies overnight at 4°C with subsequent addition of 100 μl agarose-conjugated protein A for 2 hr. Immunoprecipitates were resuspended in 100 μl SDS sample buffer and subjected to SDS/PAGE and Western blotting.

Measurement of Intracellular Ca^{2+} Levels

Intracellular calcium levels of control and Na^+/P_i cotransport up-regulated cells were measured by using fura-2 fluorescence, according to Lang et al (28). Fluorescence measurements were made under an inverted phase-contrast microscope (IM-35, Zeiss, Germany) equipped for epifluorescence and photometry (Hamamatsu, Herrsching, Germany). Light from a xenon arc lamp (XBO75, Osram, Berlin, Germany) was directed through a gray filter, alternatively through a 340 nm or a 380 nm interference filter, and a diaphragm, and then was deflected by a dichroic mirror (FT425, Zeiss, Germany) into the objective (Plan-Neofluar 63 × oil immersion, Zeiss, Germany). The fluorescence values are expressed as the ratios obtained at the two different excitation wavelengths (340 nm/380 nm) (28).

Statistical Analysis

Results are expressed as mean ± SE (n = number of cell preparations). Statistical analysis was performed by unpaired Student's t-test, p-values < 0.05 were considered significant.

Results

Intracellular Redistribution of Actin and Villin During Na^+/P_i Cotransport Up-regulation

The rapidly mediated alterations by Na^+/P_i cotransport up-regulation of the actin microfilaments shown by previous biochemical approaches (22) were further explored by confocal laser scanning microscopy. In control cells, actin microfilaments were stained intensely, starting from their apical cytoplasmic regions and encompassing all sections scanned, including those of the basal attachment sites to the substratum (Fig. 1A–E). In OK cells exposed for 5 min to low extracellular phosphate at higher cytoplasmic scanning sections, intact microfilaments could be recognized only to a very limited extent; whereas,

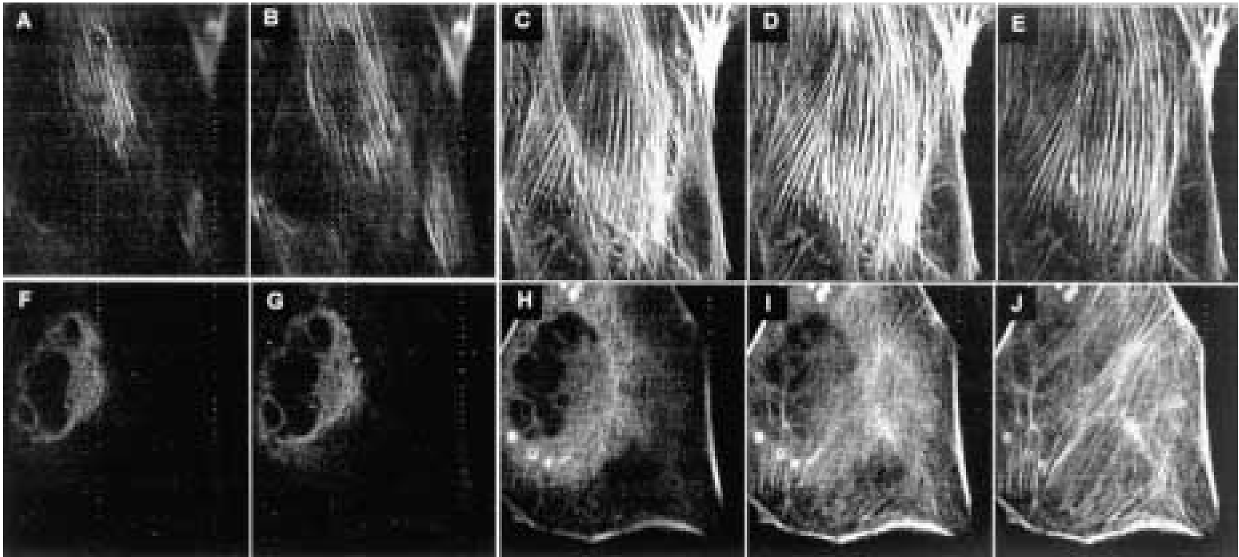

Fig. 1. Distribution of actin in opossum kidney cells. (A–E) Control cells. (F–J) Cells exposed to 0.1 mM P_i for 5 min. A, F to E, and J, respectively, represent scanning sections from the upper (apical) cytoplasmic region towards the (basal) attachment site of the cells. The step size of the optical sections was adjusted to 0.5 μm.

the lower scanning sections were characterized by intense submembranous and peripheral fluorescence (Fig. 1F–J). According to previously reported data by our group (22), the disorganization of the submembranous actin network at the higher cytoplasmic scanning sections would be expected to facilitate the trafficking of Na^+/P_i cotransporters towards the apical membrane, thus promoting luminal uptake of P_i. To the contrary, the intense peripheral fluorescence at the lower scanning sections may suggest a second functional role of polymerized actin, leading to activation/inactivation of ion transport proteins based on the basolateral membrane, although this remains to be demonstrated. The actin depolymerization induced by Na^+/P_i cotransport up-regulation was also demonstrated with the quantitative rhodamine-phalloidin fluorescence assay. Exposure of OK cells for 5 min to 0.1 mM P_i resulted in a significant decrease by 38.2% of arbitrary fluorescence units (from 93.13 ± 7.74 to 57.54 ± 8.71, $n = 4$, $p < 0.05$), consistent with a decrease in the amount of filamentous actin.

Double labeling for F-actin and villin in cells exposed to low P_i revealed that villin colocalized (yellow staining) mainly with blotchy rhodamine-fluorescence, which corresponded to short actin filaments and/or actin monomers; whereas, the characteristic microfilamentous network had almost completely disappeared (Fig. 2F–J). This observation indicated that villin may sever actin filaments, followed by capping of the barbed ends produced. Control experiments revealed that the intense green perinuclear staining may result from nonspecific binding of the FITC-IgG antibody (data not shown).

Redistribution of Villin to Nonsedimentable Actin Forms During Na^+/P_i Cotransport Up-regulation

The distribution of actin and villin shown by confocal microscopy was further examined using quantitative immunoblot analysis of detergent-insoluble cytoskeletal pellets and their corresponding supernatants (Fig. 3). In control preparations, 48 ± 4% of actin and 54 ± 4% of villin fractionated with the Low Speed Pellet (LSP; which corresponded to the microfilamentous network). In the remaining High Speed Pellet (HSP; which corresponded to short actin filaments) the proportion of total actin and villin were 15 ± 2% and 10± 2% respectively; whereas, 37 ± 3% of total actin and 36 ± 2% of total villin were in the High Speed Supernatant (HSS; which corresponded to monomeric actin). In Na^+/P_i cotransport up-regulated cells, the percentage of sedimentable actin and villin in the LSP decreased to 33 ±

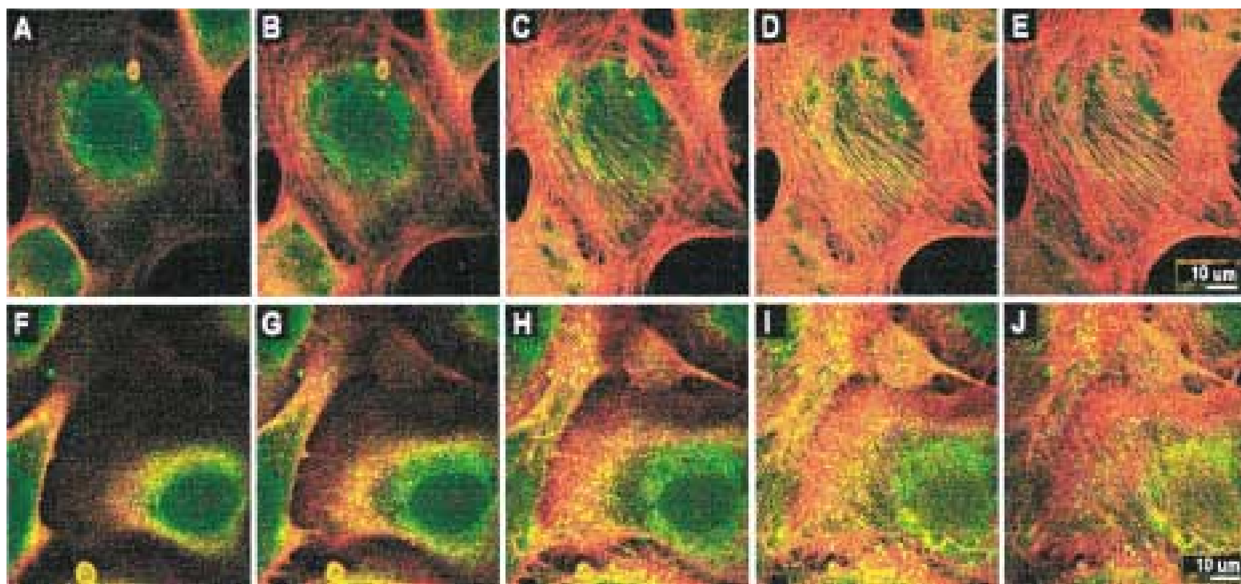

Fig. 2. Relative distribution of actin and villin in opossum kidney cells stained with rhodamine-phalloidin (red) and FITC-IgG (green), respectively. (A–E) Control cells. (F–J) Cells exposed in 0.1 mM P_i for 5 min. A, F to E, and J, respectively, represent scanning sections from the upper (apical) cytoplasmic region towards the (basal) attachment site of the cells. The step size of the optical sections was adjusted to 0.5 μm. Scale bar = 10 μm.

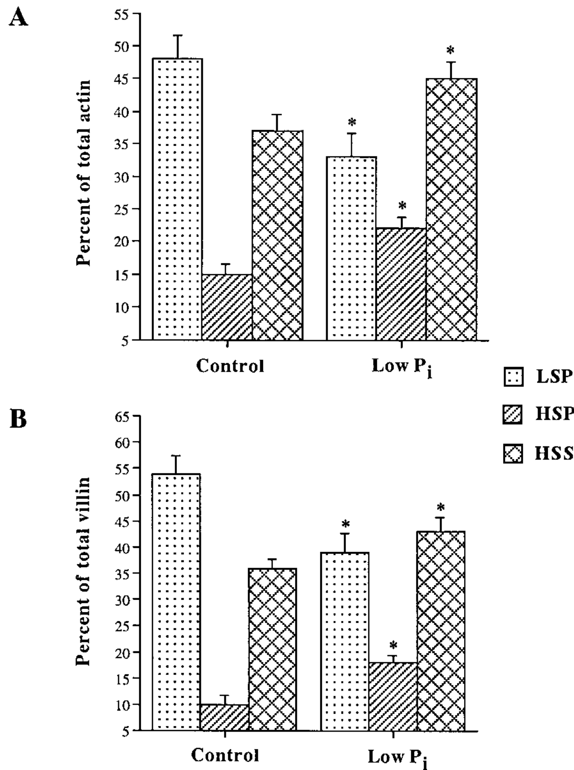

Fig. 3. Redistribution of villin to less sedimentable forms of actin during Na^+/P_i cotransport up-regulation. Cellular fractions (LSP, HSP, HSS) obtained by differential centrifugation of lysed control and Na^+/P_i cotransport up-regulated cells, analyzed for their contents of actin (A) and villin (B) by the Western blot method. LSP, low speed pellet; HSP, high speed pellet; HSS, high speed supernatant.

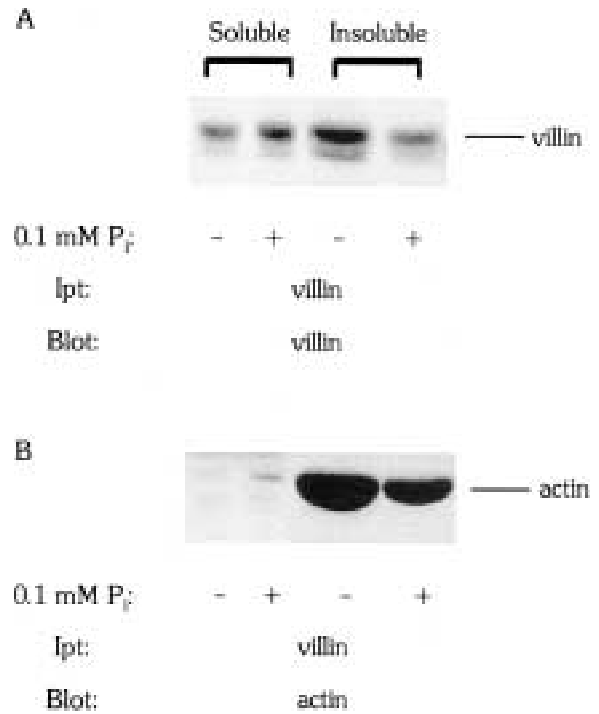

Fig. 4. Binding of villin to actin simultaneously with their redistribution from the TritonX-100-insoluble to soluble cytoskeletal fraction, during Na^+/P_i cotransport upregulation. Villin was immunoprecipitated from the TritonX-100-soluble and insoluble fractions of control (−) and Na^+/P_i cotransport up-regulated cells (+). Nitrocellulose membranes were probed with monoclonal antibodies against villin (A) and actin (B) (representative of a total of 4 experiments). Ipt, immunoprecipitation; Blot, Western Blot Analysis.

4% and 39 ± 4% ($n = 6$, $p < 0.05$), respectively. In contrast, actin and villin were increased to 22 ± 2% and 18 ± 2% ($n = 6$, $p < 0.05$) in the HSP, respectively, and to 45 ± 3% and 43 ± 3% ($n = 6$, $p < 0.05$) in the HSS, respectively. The increasing proportions of actin and villin in HSP and HSS may suggest that an actin-severing activity of villin resulted in the formation of shorter actin filaments, as well as actin oligomers and monomers, which associated with villin. All of these sediment poorly under the centrifugation conditions applied.

The association of villin with actin and their redistribution was confirmed by immunoprecipitation experiments (Fig. 4). Villin was immunoprecipitated from TritonX-100-soluble and insoluble extracts of control cells and cells exposed to low P_i medium. The immunoprecipitated proteins were separated by SDS/PAGE, transferred to nitrocellulose and probed with monoclonal antibodies to villin and actin (Fig. 4A, B). In Na^+/P_i cotransport up-regulated cells, the amount of villin was significantly decreased in the TritonX-100-insoluble and increased in the soluble fraction, compared with that in the respective cytoskeletal fractions of control cells (Fig. 4A). After stripping the membrane and reprobing with anti-actin antibody, a concomitant redistribution of actin was observed. The amount of actin co-immunoprecipitating with villin in the TritonX-100-insoluble fraction of Na^+/P_i cotransport up-regulated cells was substantially smaller than that of control cells (Fig. 4B). This suggested that villin, which in control cells mainly associated with polymerized actin-forming filament bundles, remained bound to monomers and short filaments of actin after its depolymerization. This indicated that villin, which functions as an actin-bundling protein in control cells, assumed an actin-severing role in Na^+/P_i cotransport up-regulated cells.

Finally, Na^+/P_i cotransport up-regulation induced a significant increase, by 21.8 ± 3.5% of the intracellular calcium levels, compared with control cells ($n = 16$, $p < 0.001$; significance of difference vs. 100%). This finding further supported the suggestion that villin-mediated events account for the actin depolymerization.

Translocation of PLC-γ1 to the Actin Cytoskeleton

Since villin is also regulated by phosphatidylinositol bisphosphate (PIP2)(14), which is in turn dependent on PLC-γ1 activity (16), we also investigated this signaling pathway. PLC-γ1 was immunoprecipitated from TritonX-100-soluble and insoluble extracts of control and Na^+/P_i cotransport up-regulated cells. The immunoprecipitated proteins were separated by SDS/PAGE, transferred to nitrocellulose and probed with monoclonal antibody to phosphotyrosine (Fig. 5A). Neither cells incubated with low extracellular phosphate nor control cells exhibited tyrosine phosphorylated PLC-γ1. Interestingly, when the Western blots analysis shown in Figure 5A was stripped and reprobed with anti-PLC-γ1 antibody, a clear translocation of PLC-γ1 to the actin cytoskeleton was observed. The amount of PLC-γ1 in the TritonX-100-insoluble fraction was increased significantly (Fig. 5B). The presence of PLC-γ1 in the cytoskeleton fraction of Na^+/P_i cotransport up-regulated cells was confirmed by the subsequent stripping of the nitrocellulose membrane and reprobing with monoclonal anti-actin antibody. Figure 5C shows that the TritonX-100-insoluble fraction of cells incubated with low extracellular P_i contained substantially greater amounts of actin, co-immunoprecipitating with PLC-γ1, than control cells.

To investigate further the association of PLC-γ1 with the actin cytoskeleton, tyrosine phosphorylated proteins were immunoprecipitated from the two cytoskeletal fractions, separated by SDS/PAGE, transferred to nitrocellulose and probed with monoclonal andibody to actin (Fig. 6A). In the TritonX-100-insoluble fraction of Na^+/P_i cotransport up-regulated cells, the immunoprecipitated actin amount was significantly larger than that of control cells. Although actin immunoprecipitated with anti-phosphotyrosine antibody, it was not itself phosphorylated, since it could not be detected when the stripped nitrocellulose membrane was reprobed with anti phosphotyrosine

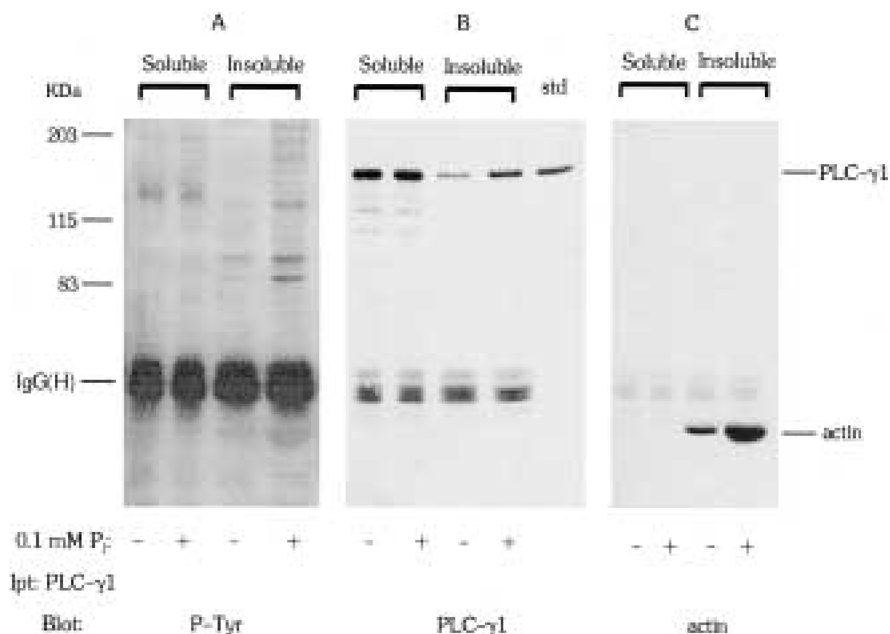

Fig. 5. PLC-γ1 translocation to the actin cytoskeleton. PLC-γ1 was immunoprecipitated from the TritonX-100-soluble and insoluble fractions of control (−) and Na^+/P_i cotransport up-regulated cells (+). Nitrocellulose membrane was probed with monoclonal antibodies against phosphotyrosine (A), PLC-γ1 (B) and actin (C). Std: positive control for PLC-γ1 (representative of a total of 8 experiments). PLC-γ1, phospholipase Cγ1; P-Tyr, phosphotyrosine; IgG(H), Immunoglobulin G (Heavy chain); Blot, Western Blot Analysis.

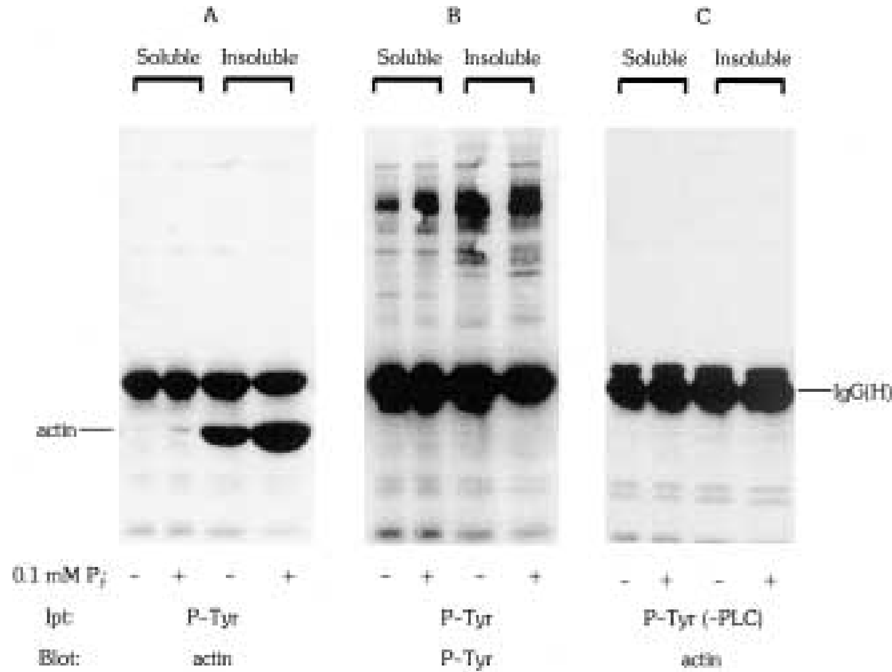

Fig. 6. A tyrosine phosphorylated protein mediates the association of PLC-γ1 with actin. Tyrosine phosphorylated proteins were immunoprecipitated from the TritonX-100-soluble and insoluble fractions of control (−) and Na^+/P_i cotransport up-regulated cells (+). Nitrocellulose membrane was probed with monoclonal antibodies against actin (A) and phosphotyrosine (B). In different cell extracts, PLC-γ1 was immunodepleted and the remaining soluble and insoluble fractions were reprecipitated with anti-phosphotyrosine antibody. Nitrocellulose membrane was probed with monoclonal antibody against actin (C) (representative of a total of 6 experiments). PLC-γ1, phospholipase Cγ1; P-Tyr, phosphotyrosine; IgG(H), Immunoglobulin G (H); Blot, Western Blot Analysis.

antibody (Fig. 6B). Actin, therefore, co-immunoprecipitated with a tightly associated tyrosine-phosphorylated protein(s). Interestingly, when PLC-γ1 was immunodepleted from TritonX-100-soluble and insoluble extracts of control and Na^+/P_i cotransport up-regulated cells, the remaining fractions were subsequently immunoprecipitated with anti-phosphotyrosine antibody and the membrane probed with anti-actin antibody, actin was not detected (Fig. 6C). The only difference between Figures 6A and 6C is that, in the latter, PLC-γ1 and the protein(s) associated with it are absent. This result indicates that the tyrosine-phosphorylated protein(s) with which actin co-immunoprecipitates associates with PLC-γ1, since neither PLC-γ1 nor actin are themselves phosphorylated under the experimental conditions used (Fig. 5A).

Association of Phosphorylated Villin with PLC-γ1

Since tyrosine phosphorylation of PLC-γ1 was not increased under the experimental conditions used and tyrosine-phosphorylated protein(s) seemed to be present in the complex of PLC-γ1 with actin, we probed other tyrosine-phosphorylated proteins that could mediate the PLC-γ1-actin association. As shown in Figure 5A, PLC-γ1 was immunoprecipitated from TritonX-100-soluble and insoluble extracts of control and Na^+/P_i cotransport up-regulated cells and the nitrocellulose membrane was probed first with anti-phosphotyrosine antibody (Fig. 7A). From the various tyrosine-phosphorylated proteins co-immunoprecipitating with PLC-γ1, one corresponds to villin (Fig. 7A), as was confirmed by the subsequent stripping and reprobing of the nitrocellulose membrane with monoclonal anti-villin antibody (Fig. 7B). The tyrosine phosphorylation, as well as the amount of villin that associated with PLC-γ1, was increased by low extracellular phosphate incubation of OK cells (Fig. 7A, B). Immunoprecipitation of villin from TritonX-100-soluble and insoluble extracts of control and Na^+/P_i cotransport up-regulated cells and probing of the

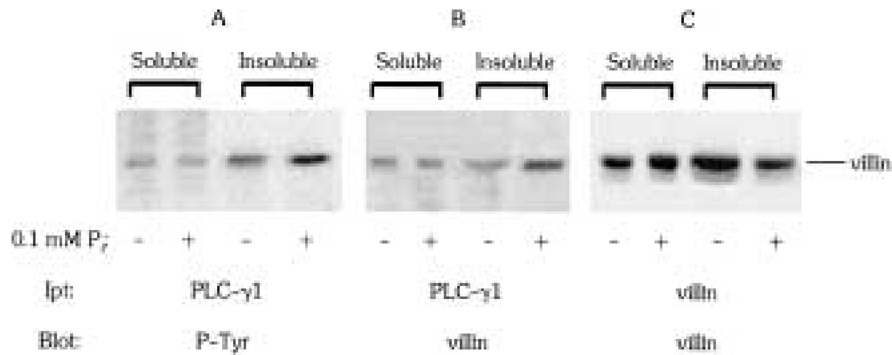

Fig. 7. Association of increased amount of tyrosine-phosphorylated villin with PLC-γ1 during Na^+/P_i cotransport up-regulation. PLC-γ1 was immunoprecipitated from the TritonX-100-soluble and insoluble fractions of control (−) and Na^+/P_i cotransport up-regulated cells (+). Nitrocellulose membrane was probed with monoclonal antibodies against phosphotyrosine (A) and villin (B). Villin was immunoprecipitated, under the same experimental conditions, and the nitocellulose membrane was probed with monoclonal antibody against villin (C) (representative of a total of 8 experiments). PLC-γ1, phospholipase Cγ1; P-Tyr, phosphotyrosine.

nitrocellulose membrane with anti-villin antibody, also indicated that the amount of villin associating with PLC-γ1 was smaller than the total amount of villin present in each fraction (Fig. 7C).

To examine whether a relationship existed between tyrosine phosphorylation of villin and its association with PLC-γ1, the latter was immunodepleted from TritonX-100-soluble and insoluble extracts of control and Na^+/P_i cotransport up-regulated cells and the remaining soluble and insoluble fractions were immunoprecipitated with anti-villin antibody. The immunoprecipitated proteins were separated by SDS/PAGE, transferred to nitrocellulose and probed with anti-phosphotyrosine antibody (Fig. 8A, B). As shown in Figure 8B, the insoluble fractions of cells in which PLC-γ1 was absent did not contain tyrosine-phosphorylated villin as well, suggesting that only the tyrosine-phosphorylated villin associated with PLC-γ1.

Translocation of PLC-γ1 to the Actin Cytoskeleton and Its Association with Villin

Control and Na^+/P_i cotransport up-regulated cells were pretreated with the tyrosine kinase inhibitor genistein. Equal volumes of TritonX-100-soluble and insoluble extracts were subse-

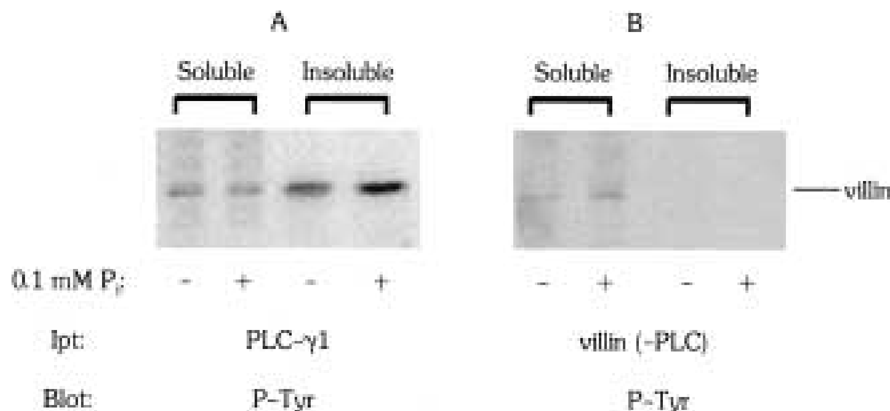

Fig. 8. Tyrosine phosphorylated villin preferentially associates with PLC-γ1. (A) PLC-γ1 was immunoprecipitated from the TritonX-100-soluble and insoluble fractions of control (−) and Na^+/P_i cotransport up-regulated cells (+). (B) The remaining soluble and insoluble fractions were reprecipitated with anti-villin antibody. Nitrocellulose membranes were probed with monoclonal antibody against phosphotyrosine (representative of a total of 3 experiments). PLC-γ1, phospholipase Cγ1; P-Tyr, phosphotyrosine.

quently immunoprecipitated with anti-PLC-γ1 antibody and the nitrocellulose membrane also was probed with anti-PLC-γ1 antibody (Fig. 9A, B). Figure 9B shows that treatment of OK cells with genistein blocked the PLC-γ1 translocation to the cytoskeleton. Quantitation of band intensities revealed that the ratio of soluble to insoluble PLC-γ1 in control cells was 8.82 and in untreated Na^+/P_i cotransport up-regulated cells, 6.05. In genistein-treated Na^+/P_i cotransport up-regulated cells, this ratio was 9.43, reaching the control levels. Additionally, when the above nitrocellulose membranes were stripped and probed with anti-villin antibody, it was observed that, in the TritonX-100-insoluble fraction of genistein-treated cells, villin was not co-immunoprecipitated with PLC-γ1 (Fig. 9D). These results suggested that translocation of PLC-γ1 to the cytoskeleton, as well as its association with villin, was dependent on tyrosine phosphorylation events. In addition to the observations described above, the overall results indicated that tyrosine phosphorylation of villin may be necessary for its association with PLC-γ1 and for the translocation of PLC-γ1 to the actin cytoskeleton during Na^+/P_i cotransport up-regulation.

Na^+/P_i Cotransport Up-regulation

Villin was immunoprecipitated from TritonX-100-soluble and insoluble extracts of control and Na^+/P_i cotransport up-regulated cells and the nitrocellulose membrane was probed with anti-phosphotyrosine antibody. As shown in

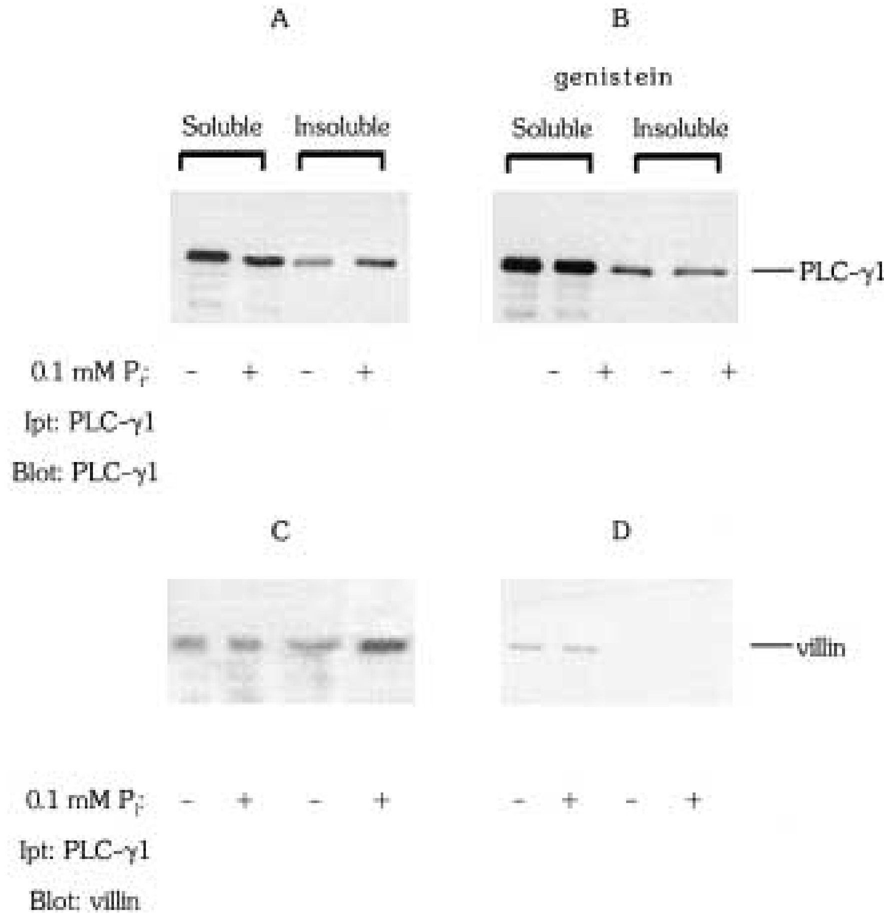

Fig. 9. The inhibition of tyrosine phosphorylation abolishes PLC-γ1 translocation to actin cytoskeleton, as well as its association with villin. Control (−) and Na^+/P_i cotransport up-regulated cells (+) were preincubated with genistein (100 μM, 40 min) or with its vehicle (dimethylsulfoxide; DMSO). PLC-γ1 was immunoprecipitated from equal volumes of TritonX-100-soluble and insoluble fractions. Nitrocellulose membranes of untreated and genistein treated cells were probed with monoclonal anti-PLC-γ1 (A and B) and anti-villin (C and D) antibody (representative of a total of 2 experiments). PLC-γ1, phospholipase Cγ1; P-Tyr, phosphotyrosine.

Figure 10A.1, a tyrosine phosphorylated protein, with molecular mass of 85Kda, co-immunoprecipitated with villin and its tyrosine phosphorylation increased strongly in the TritonX-100-insoluble fraction during Na^+/P_i cotransport up-regulation. The same tyrosine-phosphorylated protein co-immunoprecipitated with PLC-γ1 in the TritonX-100-insoluble fraction of Na^+/P_i cotransport up-regulated cells (Fig. 10A.2) and corresponded to the p85 subunit of PI-3 kinase. This was confirmed by its immunoprecipitation from the two cytoskeletal fractions and probing with anti-phosphotyrosine antibody (Fig. 10A.4), as well as by subsequent stripping and reprobing of the nitrocellulose membranes with monoclonal anti-p85 antibody (Fig. 10B.1,2,4). In addition to the increased tyrosine phosphorylation of p85 in the TritonX-100-insoluble fraction, p85 clearly translocated from the TritonX-100 soluble to the insoluble fraction during Na^+/P_i cotransport up-regulation (Fig. 10B.4). Since a pool of villin associates with PLC-γ1 in the TritonX-100-insoluble fraction, it was unclear whether the p85 subunit associated with the villin-PLC-γ1 complex itself or with each individual protein separately. To explore this question, PLC-γ1 was immunodepleted from TritonX-100-soluble and insoluble extracts of control and Na^+/P_i cotransport up-regulated cells and the remaining soluble and insoluble fractions were immunoprecipitated with anti-villin antibody. The immunoprecipitated proteins were separated by SDS/PAGE, transferred to nitrocellulose and probed with anti-phosphotyrosine and anti-p85 antibody (Fig. 10A.3, B.3). As shown in Figures 10A.3 and 10B.3, in the TritonX-100-insoluble fraction of Na^+/P_i cotransport up-regulated cells, villin not associated with PLC-γ1 did not bind with either phosphorylated or unphosphorylated p85. These results indicated that p85 associated with the villin-PLC-γ1 complex in the cytoskeletal fraction of cells that were exposed to low extracellular P_i.

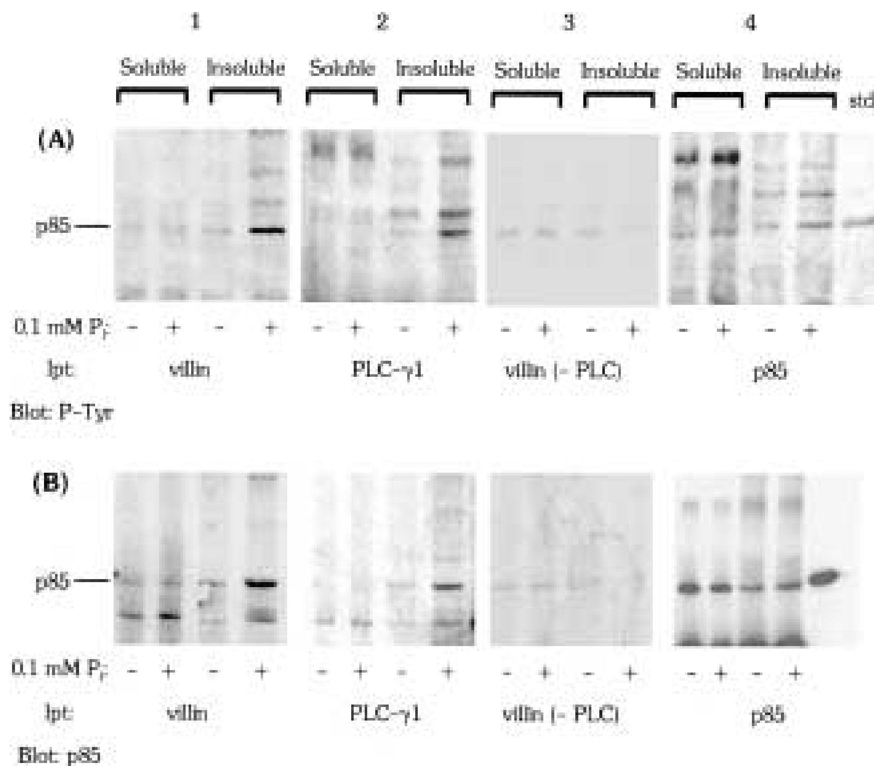

Fig. 10. Association of villin with the p85 subunit of PI-3 kinase. Villin (A), PLC-γ1 (B) and p85 (D) was immunoprecipitated from the TritonX-100-soluble and insoluble fractions of control (−) and Na^+/P_i cotransport up-regulated cells (+). In different cell extracts, PLC-γ1 was immunodepleted and the remaining soluble and insoluble fractions were reprecipitated with anti-villin antibody (C). Nitrocellulose membranes were probed with monoclonal antibodies against phosphotyrosine (A) and p85 (B). Std: positive control for p85 (representative of a total of 3 experiments). PI-3 kinase, phosphatidylinositol-3 kinase; p85, the 85 KDa subunit of PI-3 kinase; P-Tyr, phosphotyrosine.

Discussion

Previous work with OK cells has shown that brief (5 min) incubation of these cells with low (0.1 mM) P_i results in Na^+/P_i cotransport up-regulation and in a substantial and rapid, but transient, depolymerization of microfilaments and microtubules (22). Phosphate reabsorption is a tubular function performed by Na^+/P_i cotransporters located in the brush border membrane of proximal tubule cells (29). It has long been established that even short-term incubation of cultured cells in low- P_i media augments the rate of their phosphate uptake through Na^+-coupled phosphate cotransport (22,30,31). However, it is not known how the apical membrane of these cells "senses" the decrease in extracellular phosphate and what signaling mechanisms are subsequently initiated, leading to increases in P_i uptake. The results of this study provide a partial outline of the signal transduction events, causing the depolymerization of actin microfilaments, downstream of a hypothetical extracellular phosphate sensor.

We studied the mechanism of microfilament depolymerization during Na^+/P_i cotransport up-regulation by examining the distribution of the actin-bundling and -severing protein villin, which is abundant in the apical microvilli of proximal tubule (like OK cells) and intestinal cells (32). We were able to show that villin, which is present in three actin-associated pools in OK cells, redistributed to fractions containing short actin filaments and actin monomers during Na^+/P_i cotransport up-regulation. This suggests that, under the experimental conditions used, villin acted as an actin-severing protein, since it is known to remain bound to the fast-growing ends of short filaments, as well as actin oligomers produced after severing microfilaments (33–35). In this study, the measured increase of intracellular calcium levels known to augment the actin-severing activity of villin, further supports this interpretation.

The severing activity of villin also is influenced by polyphosphoinisitides, especially PIP2 (36), whose intracellular concentrations are regulated by the activity of PLC (37). In addition, several studies have demonstrated that proximal tubule cell microvilli are sensitive to Ca^{2+}-dependent disruption by a process involving polyphosphoinositide hydrolysis (38); whereas, in osteoblasts, stimulation of P_i uptake by platelet-derived growth factor (PDGF) occurs via mechanisms involving activation of PLC-γ1 and PI-3 kinase (39). A recent study (40) reported that only part of TritonX-100-soluble villin became tyrosine-phosphorylated and associated with PLC-γ1 that was recruited to the brush border membrane of the intestinal epithelial cells and that these events were linked to inhibition of NaCl absorption and of brush border Na^+/H^+ exchange. According to the above study, all the PLC-γ1 were present in the TritonX-100-soluble fraction of ileal villus brush borders. In contrast, our findings demonstrate that PLC-γ1 translocates to the TritonX-100-insoluble fraction of OK cells during Na^+/P_i cotransport up-regulation. However, the differences in the PLC-γ1 compartmentation observed between OK cells and intestinal epithelia (40) may very well reflect the different organ or species origins of the cells studied.

In the present study, we demonstrate that during Na^+/P_i cotransport up-regulation by low ambient phosphate (in addition to the depolymerization of microfilaments, the concomitant redistribution of villin and the increasing intracellular calcium levels, such as steps consistent with augmentation of the actin severing activity of villin) the following signaling events are concurrently taking place:

1. translocation of PLC-γ1 to the cytoskeleton, without alteration of its phosphorylation state;
2. involvement of tyrosine-phosphorylated protein(s) in the association of PLC-γ1 with actin;
3. increased tyrosine phosphorylation of a portion of TritonX-100-insoluble villin and association of only tyrosine phosphorylated villin with PLC-γ1;
4. necessity of tyrosine phosphorylation events for PLC-γ1 translocation to the actin cytoskeleton and for its association with villin; and
5. association of the villin-PLC-γ1 complex with increased amounts of the tyrosine-phosphorylated p85 subunit of PI-3 kinase in the TritonX-100-insoluble fraction.

It is of note, that during Na^+/P_i cotransport up-regulation, we could not demonstrate increases in tyrosine phosphorylation of PLC-γ1 that account for the activation of the enzyme (41,42). However, several studies have demonstrated that tyrosine phosphorylation of PLC-

γ1 is independent of, or even unnecessary for, activation of the enzyme (43–45). Other mechanisms may be involved in its activation, including the translocation of PLC-γ1 to the cytoskeleton or its targeting to the membrane (18,46,47). Indeed, when OK cells were incubated with low extracellular P_i, we observed a clear translocation of PLC-γ1 from the cytosol to the cytoskeleton where tyrosine-phosphorylated protein(s) seemed to be present in the complex of PLC-γ1 with actin. In TritonX-100-insoluble fraction, PLC-γ1 was observed to associate with a pool of villin that was tyrosine-phosphorylated. The tyrosine phosphorylation of villin, as well as the amount of villin that associates with PLC-γ1, was increased during Na^+/P_i cotransport up-regulation. The fact that only tyrosine-phosphorylated villin associated with PLC-γ1 suggests that this phosphorylation plays a key role in the interaction of villin with PLC-γ1. Predictably, genistein pretreatment of OK cells abolishes the association of villin with PLC-γ1, as well as its translocation to the actin cytoskeleton. Since Na^+/P_i cotransport up-regulation does not affect the phosphorylation state of PLC-γ1, the tyrosine phosphorylation of villin also can account for the translocation of PLC-γ1 to the cytoskeleton. Regarding the interaction between villin and PLC-γ1, it seems possible that this might be mediated through binding of villin to the SH2 domain of PLC-γ1. This is in analogy to other cytoskeletal proteins that have been demonstrated to associate with SH2 domains of signaling molecules, such as PLC-γ1 and the p85 subunit of PI-3 kinase (48). Thus, we hypothesize that villin may activate PLC-γ1 indirectly, by causing its translocation to the cytoskeleton and vice versa, that PLC-γ1 activation could augment the severing activity of villin via decreases of intracellular PI(4,5)P2 concentration and increased I(1,4,5)P3 production, with the latter causing the levels of intracellular calcium to rise.

The binding of villin to the 85-KDa subunit of PI-3 kinase suggests that villin may affect the lipid kinase activity of the enzyme by a mechanism reported to apply, in vitro, for two other actin-binding proteins (49), profilin and gelsolin, which along with villin, belong to the family of actin-binding proteins regulated by phosphoinositides (50). This is the first observation of villin associating with p85 ex vivo. Actually, villin and gelsolin, both composed of six homologous domains (51), belong to the same family of Ca^{2+}-regulated, actin-binding proteins (50). Activation of PI-3 lipid kinase results in phosphorylation of PI(4,5)P2 to produce PI(3,4,5)P3 (52,53), which activates PLC-γ1, by interacting with its SH2 domain, even without tyrosine phosphorylation of PLC-γ1 (17). Finally, the concomitant association of villin with PLC-γ1 and the 85-KDa subunit of PI-3 kinase in the TritonX-100-insoluble extracts suggests a coordinating function of villin, which could promote the binding of PLC-γ1 with the product of PI-3 kinase. Since it is assumed that TritonX-100-insoluble fraction is composed primarily of filamentous actin (27) that attaches to the plasma membrane at focal contact sites, an association of PLC-γ1 and PI-3 kinase with the actin cytoskeleton may promote the interaction of these enzymes with their lipid substrates. However, low extracellular P_i concentration induces tyrosine phosphorylation of a protein with molecular mass of 125 KDa (Fig. 6B), which, according to pilot immunoprecipitation experiments, corresponds to focal adhesion kinase (data not shown). This finding suggests involvement of focal adhesion molecules in the microfilament redistribution induced by Na^+/P_i cotransport up-regulation that is observed by confocal microscopy (Fig. 1) and we are in the process of examining them in depth in a separate project. The increased tyrosine phosphorylation of p85 in the TritonX-100-insoluble fraction of Na^+/P_i cotransport up-regulated cells suggests that the protein kinase activity of PI-3 kinase might also be increased. We interpret this as resulting from the activation of the hypothetical sensor that "recognizes" the decrease of extracellular phosphate.

In summary, we propose the following signaling mechanism causing microfilament depolymerization during the initiation of Na^+/P_i cotransport up-regulation by low ambient phosphate: following "recognition" by a hypothetical sensor of low extracellular phosphate, signaling downstream of this event results in the concomitant activation of PI-3 kinase and PLC-γ1. Activation of the hypothetical sensor causes the translocation of PI-3 kinase to the membrane cytoskeleton, where it could be activated as lipid kinase by its binding to villin; whereas, PLC-γ1 might be activated by PI(3,4,5)P3, the product of the reaction catalyzed by PI-3 lipid kinase. Subsequently, PLC-γ1 catalyzes the hydrolysis of PI(4,5)P2 to generate inositol 1,4,5-trisphosphate, which

increases intracellular free-calcium levels and diacylglycerol (16). The combination of the increased intracellular calcium levels and the decreased amount of PI(4,5)P2, as a consequence of PLC-γ1 activation, promote the actin-severing property of villin. The association of villin with PLC-γ1 (which may be mediated through the former's tyrosine phosphorylation) could account for the translocation of PLC-γ1 to the actin cytoskeleton, with villin acting as an anchoring protein. Lastly, the association of PLC-γ1 with the cytoskeleton may lead to its activation and also promote the interaction of the enzyme with its lipid substrates.

The overall result of the cascade outlined, would be the depolymerization of microfilaments during short-term Na^+/P_i cotransport up-regulation, since rapid dissolution of the submembranous actin network would be expected to facilitate the trafficking of Na^+/P_i cotransporters towards the apical membrane (22), thus promoting luminal uptake of P_i.

Footnote: The terms "interaction" or "association" refer to observations that result from co-immunoprecipitation of villin and PLC-γ1 or PI-3 kinase and, thus, it cannot be excluded that an unidentified protein that binds to villin has been co-depleted with PLC-γ1.

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