Shank2E binds NaP$_i$ cotransporter at the apical membrane of proximal tubule cells

Ryan R. McWilliams,1 Sophia Y. Breusegem,3 Kelley F. Brodsky,1 Eunjoon Kim,2 Moshe Levi1 and R. Brian Doctor1

1Department of Medicine, University of Colorado Health Sciences Center, Denver, Colorado; and 2Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daelon, Korea

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McWilliams, Ryan R., Sophia Y. Breusegem, Kelley F. Brodsky, Eunjoon Kim, Moshe Levi, and R. Brian Doctor. Shank2E binds NaP$_i$ cotransporter at the apical membrane of proximal tubule cells. Am J Physiol Cell Physiol 289:C1042–C1051, 2005. First published May 25, 2005; doi:10.1152/ajpcell.00568.2004.—Proteins expressing postsynaptic density (PSD)-95/Drosophila disk large (Dlg)/zonula occludens-1 (ZO-1) (PDZ) domains are commonly involved in moderating receptor, channel, and transporter activities at the plasma membrane in a variety of cell types. At the apical membrane of renal proximal tubules (PT), the type Ia NaP$_i$ cotransporter (NaP$_i$-IIa) binds specific PDZ domain proteins. Shank2E is a spliceoform of a family of PDZ proteins that is concentrated at the apical domain of liver and pancreatic epithelial cell types and is expressed in kidney. In the present study, immunoblotting of enriched plasma membrane fractions and immunohistology found Shank2E concentrated at the brush border membrane of rat PT cells. Confocal localization of Flag-Shank2E and enhanced green fluorescent protein-NaP$_i$-IIa in cotransfected OK cells showed these proteins colocalized in the apical microvilli of this PT cell model. Domain analysis showed that the PDZ domain of Shank2E specifically bound NaP$_i$-IIa and truncation of the COOH-terminalTRL motif from NaP$_i$-IIa abolished this binding, and Far Western blotting showed that the Shank2E-NaP$_i$-IIa interaction occurred directly between the two proteins. NaP$_i$-IIa activity is regulated by moderating its abundance in the apical membrane. High-Pi conditions induce NaP$_i$-IIa internalization and degradation. In both rat kidney PT cells and OK cells, shifting to high-Pi conditions induced an acute internal redistribution of Shank2E and, in OK cells, a significant degree of degradation. In sum, Shank2E is concentrated in the apical domain of renal PT cells, specifically binds NaP$_i$-IIa via PDZ interactions, and undergoes P$_i$-induced internalization.

PDZ domains; endocytosis; degradation; epithelia

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hormones such as parathyroid hormone and by dietary P$_i$ levels (16, 33). In opossum kidney (OK) cells, a proximal tubular cell model, low extracellular P$_i$ induces NaP$_i$-IIa insertion/retention in the apical membrane, while increased extracellular P$_i$ induces a rapid internalization and degradation of NaP$_i$-IIa (24). The COOH tail of NaP$_i$-IIa ends with a PDZ binding motif (-A-T-R-L), and NaP$_i$-IIa binds specific PDZ proteins, including PDZ kidney-1 (PDZK1; a/k/a NaP$_i$-Cap1), the ezrin, radixin, and moesin (ERM) family of actin-binding proteins binding phosphoprotein 50 (EBP50; a/k/a NHERF-1, or Na$^+/H^+$ exchanger regulatory factor-1) and NHE3 kinase-associated regulatory protein (E3KARP; a/k/a NHERF-2) (8, 9). The physiological significance of PDZ proteins in regulating NaP$_i$-IIa activity was revealed in a transgenic EBP50-knockout mouse model. These animals had decreased serum P$_i$, in the apical membrane, while increased extracellular P$_i$ inhib- 

Access to food was restricted to a 3-h period daily to condition rapid /H9262 were cotransfected with Flag-Shank2E (2 g) using Effectene reagent as directed by the manufacturer /H11002 vector (5) with /H11001 cyto cDNA with /H9262 digested with EcoRI, and ligated in-frame into the EcoRI-digested pHM6 vector (Roche, Indianapolis, IN). The GFP-NaP$_i$-IIa construct was characterized previ- 

MATERIALS AND METHODS

Animal tissues, cell culture models, and transfections. Kidneys from Sprague-Dawley rats (males —250 g) were harvested for whole tissue lysates, enriched membrane fractions and immunofluorescence studies. Animals were humanely euthanized (50 mg/kg pentobarbital sodium) as described in an Institutional Animal Care and Use Committee-approved protocol. Before tissue harvesting or fixation, the kidneys were cleared of blood by cannulation of the descending aorta and retrograde perfusion of phosphate-buffered saline (PBS).

The fate of proteins within renal PT cells during acute changes in serum P$_i$, was evaluated by moderating the P$_i$, levels in the diet. Rats were fed a low-P$_i$, diet (0.1% P$_i$, 0.6% Ca$^{2+}$) for 14 days before study. Access to food was restricted to a 3-h period daily to condition rapid feeding and allow for acute changes in serum P$_i$, levels (16). On the final day, one-half of the animals were maintained on low-P$_i$, chow while the other half were fed a high-P$_i$, diet (1.2% P$_i$, 0.1% Ca$^{2+}$). After 3 h of feeding, the animals were humanely killed and either fixed for immunohistochemistry or processed for Western blot analysis.

OK cells were used to evaluate the distribution and P$_i$,-induced degradation of proteins. OK cells were maintained in DMEM-Ham’s F-12 medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 100 U/ml penicillin, 100 U/ml streptomycin, and 5 mM t-glutamine at 37°C in a humidified atmosphere with 5% CO$_2$. OK cells were cotransfected with Flag-Shank2E (2 g) and hemagglutinin (HA)-NaP$_i$-IIa (2–6 g) or green fluorescent protein (GFP)-NaP$_i$-IIa (2 g) using Effectene reagent as directed by the manufacturer (Qiagen, Valencia, CA). OK cells were grown to confluence (36–48 h) before the study period. In a group of studies, the degradation rate of native OK cell proteins, including Shank2E, was evaluated. Initially, OK cells were grown for 8 days to allow for consistent differentiation. The cells were then synchronized with serum-free DMEM-Ham’s F-12 medium for 12–16 h and endocytosis/degrada- 

Human embryonic kidney (HEK)-293 cells were used to evaluate binding of specific Shank2E domains to NaP$_i$-IIa. These cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 U/ml streptomycin, and 5 mM t-glutamine at 37°C in a humidified atmosphere with 5% CO$_2$. HEK-293 cells were cotransfected at 50–75% confluence using Effectene reagent, 2 g of HA-NaP$_i$-IIa or HA-NaP$_i$-IIa (PDZ–) and 2 g of the individual Flag-Shank2E domain fusions proteins. Trans- 

Electrophoresis of tissue lysates, enriched membrane fractions and immunofluorescence studies was carried out as described previously (20). Briefly, blood was flushed from the kidneys with PBS (4°C), and all subsequent steps were performed at 4°C. Cortical slices were homogenized in isolation buffer (IB; 15 mM Tris-HCl, 5 mM EGTA, 300 mM mannitol, and 1 mM PMSF, pH 7.4) with a tight-fitting Dounce homogenizer, diluted homogenate was centrifuged (48,000 g, 30 min), and the pellet was resuspended using a loose Dounce. Crude BLM were precipitated at the addition of IB with 15 mM MgCl$_2$, followed by centrifugation (2,500 g, 15 min). Crude BLM was subjected to a second MgCl$_2$ precipitation, resuspended in water, homogenized with a loose-fitting Dounce, and centrifuged (750 g for 15 min) to remove the nuclei. The supernatant was centrifuged (48,000 g, 30 min), the pellet was resuspended in 50% sucrose, and the basolateral membranes were floated on a 41–37% sucrose step gradient by centrifugation (88,000 g, 3 h). The recovered BLM were washed with IB and pelleted (75,000 g, 10 min). For BBM, the supernatants from the first and second MgCl$_2$ precipitations were pooled, pelleted (48,000 g, 30 min), and subjected to a third MgCl$_2$ precipitation. Centrifugation (48,000 g, 30 min) of the remaining supernatant resulted in a pellet containing the enriched BBM.

To isolate material for immunoprecipitation studies, crude renal cortex membranes were prepared as previously described for liver membranes (28). Briefly, perfused kidneys were cut into slabs, and cortex was trimmed away from medulla and homogenized with a Potter-Elvex Dounce in IB. Nuclei and large debris were pelleted (10,000 g, 15 min), the supernatant was collected, and crude mem- 

For BBM, the supernatants from the first and second MgCl$_2$ precipitations were pellet (48,000 g, 30 min) and subjected to a third MgCl$_2$ precipitation. Centrifugation (48,000 g, 30 min) of the remaining supernatant resulted in a pellet containing the enriched BBM.

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Western blotting, Far Western blotting, and immunohistochemistry. Proteins from rat kidney, OK cells, and coimmunoprecipitation studies were assayed using Western blot analysis (4). Briefly, cells or tissues were solubilized in 5× PAGE buffer (5% sodium dodecyl sulfate, 25% sucrose, 5 mM EDTA, 50 mM Tris, pH 8.0, 5% β-mercaptoethanol, and Complete MiniTablet protease inhibitor cocktail; Roche), separated on a 4–14% gradient gel, and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA). These membranes were incubated and then washed in primary and then horse-radish peroxidase-conjugated secondary antibodies (1:60,000 dilution; Jackson ImmunoResearch, West Grove, PA). The antibody complexes were detected using enhanced chemiluminescence (Pierce, Rockford, IL) and captured using a photodocumentation system (UVP, Upland, CA).

For Far Western blotting, immunoprecipitated wild-type (WT) or truncated (~PDZ) HA-NaP-IIa proteins were run out in duplicate on an acrylamide gel and transferred onto nitrocellulose membrane. One blot was developed using Western blot analysis with an HA antibody to confirm equivalent expression levels of HA-NaP-IIa proteins. The second blot was incubated overnight in 5% nonfat milk in blot buffer, washed, overlaid with blot buffer containing Flag-2SHANK2E (~300 nM overnight at 4°C), and washed. The bound Flag-2SHANK2E was detected using Western blot analysis with an anti-Flag antibody as described above.

For immunohistochemistry, kidneys were perfused with 4% paraformaldehyde in PBS before processing for immunofluorescence staining (4). Briefly, kidney sections (5 μm) were blocked (10% goat serum, 3% nonfat dry milk, and 0.3% Triton X-100 in PBS) and incubated overnight at 4°C with primary antibody(s). After being washed, the sections were incubated (60 min, room temperature) with Alexa 488- or Alexa 568-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR), washed with PBS, and mounted in 90% glycerol, and 10% PBS containing 2.5% 1,4-diazabicyclo[2.2.2]octane (Sigma). OK cells were seeded on poly-L-lysine-coated coverslip (Lab-Tek, Naperville, IL), grown to confluence, fixed (20 min; 3% paraformaldehyde in PBS supplemented with Ca²⁺ and Mg²⁺), and quenched (10 min, 20 mM glycine) before being stained as described above. Stained kidney sections or OK cells were imaged using a laser scanning confocal microscope (model LSM510; Zeiss, Thornwood, NY) with a ×40 water-immersion lens objective. Confocal sections were acquired throughout the cell and summed for presentation. All image manipulations were performed using Zeiss LSM510 software.

Antibodies for Western blot and immunohistochemical analysis included Flag M2 antibody (Sigma), Shank2 (5, 14), NaP-IIa (32), EBP50 (7), actin (Chemicon, Temecula, CA), leucine amino peptidase (7), and Na⁺-K⁺-ATPase (Upstate Biotechnology, Lake Placid, NY).

RESULTS

Shank2E is expressed at the BBM of renal proximal tubule cells. An epithelial specific isoform of the Shank2 family, termed Shank2E, is expressed in a number of epithelial tissues, including the kidney (18). Other PDZ domain-containing proteins, including EBP50 and PDZK1, have previously been found to play pivotal roles in regulating the distribution and activity of specific transport proteins such as NaP-IIa in renal proximal tubule cells. The present study was designed to determine the intracellular distribution and binding partners of Shank2E in renal epithelial cells. The studies were run in both rat renal cortex tissue and OK cells, a well-characterized model of PT cells. Western blot analysis confirmed that Shank2E was expressed in both rat kidney cortex and OK cells (Fig. 1). As anticipated, NaP-IIa and EBP50 were also detected in both rat kidney cortex and OK cells.

The expression of Shank2E in OK cells suggests that Shank2E is expressed in PT cells. To confirm Shank2E expre-
tion in rat renal proximal tubules and determine the intracellular distribution of the protein, rat renal cortex sections were immunostained for Shank2E (Fig. 2A). PT segments were the most intensely stained segments in the renal cortex, and Shank2E staining was concentrated at the apical domain of these cells. This predominant apical distribution was confirmed through enrichment of the basolateral and apical membrane fractions from renal cortex (Fig. 2B). Significant enrichment of the apical and basolateral membrane fractions was demonstrated by the nearly complete separation of basolateral (Na\(^+\)-K\(^+\)-ATPase) and apical (leucine amino peptidase or LAP) membrane proteins. NaP\(_{\text{i}}\)-IIa was similarly concentrated in apical membrane fractions and Shank2E was significantly enriched in apical membrane fractions with little distribution in basolateral fractions. Apical colocalization of Shank2E and NaP\(_{\text{i}}\)-IIa was further demonstrated in OK cells (Fig. 2C). Immunolocalization of the native Shank2E in fixed OK cells was hampered by weak immunoreactivity.

Fig. 2. Shank2E is enriched at the brush-border membrane (BBM) of kidney epithelia. The subcellular localization of Shank2E was assessed by performing immunohistochemistry and membrane fractionation of rat kidney epithelia. A: immunofluorescence analysis of rat kidney found Shank2E was more abundant in the renal cortex compared with the medulla (top left). Closer examination of Shank2E in the renal cortex (bottom left) found Shank2E was largely absent from glomeruli (Glom), modestly expressed in distal segments (*), and prominently expressed in PT. Furthermore, Shank2E is concentrated predominantly at apical domain of proximal tubule segments, which is highlighted by F-actin staining (right; bar, 25 μm). B: biochemical enrichment of BBM or apical membranes and basolateral membranes (BLM) from rat renal cortical sections showed both NaP\(_{\text{i}}\)-IIa and Shank2E were highly enriched in the BBM fraction. Immunoblotting for leucine amino peptidase (LAP), an apical membrane protein, and Na\(^+\)-K\(^+\)-ATPase, a basolateral membrane protein, demonstrated the degree of BBM and BLM enrichment. C: similarly, in cotransfected OK cells, enhanced green fluorescent protein (EGFP)-NaP\(_{\text{i}}\)-IIa and Flag-Shank2E distributed to and colocalized (overlay) in apical microvilli. The staining pattern in the x-y plane is consistent with OK microvilli and the Z section shows the proteins are at the apical domain of the OK cell.
However, after cotransfection of Flag-Shank2E and EGFP-NaPi-IIa into OK cells, confocal imaging showed both proteins colocalized in OK cells. Z-axis images of the two proteins confirmed that their codistribution was in the apical domain of these cells.

Shank2E complexes with NaPi-IIa via PDZ domain interactions. The colocalization of Shank2E with NaPi-IIa within the apical domain of proximal tubules indicated the two proteins might reside within a common protein complex. This was evaluated by performing coprecipitation analysis of native proteins from rat renal cortex lysates and heterologously expressed proteins from cotransfected OK cells. Preclearing of rat renal cortical lysates with protein A/G beads failed to precipitate either NaPi-IIa or Shank2E. However, addition of anti-NaPi-IIa antibody resulted in both the immunoprecipitation of NaPi-IIa and the coprecipitation of Shank2E.

Similarly, in cotransfected OK cells, immunoprecipitation of Flag-Shank2E effectively coprecipitated HA-NaPi-IIa (Fig. 3B). HA-NaPi-IIa did not, however, coprecipitate when cotransfected with either the Flag vector (data not shown) or Flag-PRR domain, a domain that does not express a PDZ domain.

These studies indicate Shank2E and NaPi-IIa reside within a common protein complex in renal PT cells and suggest the interaction may occur directly. To establish that the interaction is direct and to characterize the domains responsible for the interaction, NaPi-IIa binding to three of the Shank2E protein domains was evaluated in cotransfected HEK-293 cells (Fig. 4A). Flag-tagged Shank2E domains included the ANK repeat, PDZ, and PRR domains. When cotransfected with full-length Flag-Shank2E or one of three individual Flag-tagged Shank2E domains along with either full-length HA-NaPi-IIa or truncated HA-NaPi-IIa, NaPi-IIa truncation removed the COOH-terminal T-R-L amino acids responsible for binding to PDZ domains. Analysis of lysates showed HA-NaPi-IIa was expressed in all cells and FlagIP showed that the Shank2E domains were also expressed. In OK cells with full-length NaPi-IIa wild type (WT), both the full-length Shank2E and the Shank2E PDZ domain coprecipitated NaPi-IIa (Co-IP). In contrast, the ankyrin repeat (ANK) and polyproline rich (PRR) domain fractions contained no detectable NaPi-IIa.

The specificity of the PDZ interaction was confirmed when truncation of the COOH-terminal PDZ binding motif (−PDZ) completely abolished the NaPi-IIa interaction with both the full-length and PDZ domain of Shank2E. B: to confirm that the Shank2E and NaPi-IIa binding was a direct protein-protein interaction, HA-NaPi-IIa and HA-NaPi-IIa (−PDZ) were immunoprecipitated, transferred onto nitrocellulose membrane, and overlaid with Flag-Shank2E. Flag-Shank2E showed preferential binding to the full-length NaPi-IIa.
COOH-terminal amino acids of NaPi-IIa participate directly in the binding interaction of NaPi-IIa to PDZ domains (8). As anticipated, truncation of the terminal four amino acids of NaPi-IIa (−PDZ) abolished the coprecipitation of any of the Shank2E domain proteins, including the full-length protein and the PDZ domain (Fig. 4A). To confirm that Shank2E binds directly with NaPi-IIa, immunoprecipitated Shank2E was overlaid onto a blot containing either HA-NaPi-IIa or HA-NaPi-IIa (−PDZ). Coomassie blue staining of Shank2E immunoprecipitates showed the predominance of a single protein (data not shown). Again, Shank2E bound preferentially to the full-length NaPi-IIa to demonstrate a direct protein-protein interaction between Shank2E and NaPi-IIa (Fig. 4B). In total, these studies demonstrate that NaPi-IIa, a known PDZ binding protein, associated directly with the PDZ domain of Shank2E.

Shank2E redistributes under high-Pi conditions. The demonstrated interaction of NaPi-IIa with Shank2E suggests Shank2E is involved in regulating NaPi-IIa activity. In renal PT cells, the predominant means of regulating NaPi-IIa activity is by moderating its abundance in the apical membrane. The abundance of NaPi-IIa in the apical membrane is a product of the relative rates of delivery, retention, and recovery of NaPi-IIa. The surface expression of NaPi-IIa is moderated by parathyroid hormone and by extracellular Pi concentrations. Under low-Pi conditions, NaPi-IIa accumulates in the membrane, while high-Pi conditions induce the retrieval and degradation of NaPi-IIa (16). NaPi-IIa degradation occurs within lysosomes and the degradation of NaPi-IIa in OK cells can be inhibited by incubating the cells with leupeptin. As an initial step in evaluating the physiological relationship between NaPi-IIa and Shank2E, the relative rates of degradation of the two proteins were assessed under low- and high-Pi conditions. When maintained under low extracellular Pi conditions, there was a variable but insignificant difference in NaPi-IIa levels in the presence of leupeptin (163 ± 48% of basal levels), indicating no significant NaPi-IIa degradation occurring during these 4 h (Fig. 5A). Similarly, EBP50 levels are unchanged in OK cells during 4 h of low-Pi incubation (105 ± 9% of basal levels). Interestingly, there is a significant increase in Shank2E

Fig. 5. Leupeptin protects against Shank2E degradation under basal conditions. To evaluate the relative rates of specific protein degradation in OK cells, cells were treated with leupeptin and protein levels were compared using immunoblotting. A: no discernible differences in NaPi-IIa or EBP50 levels were observed between control cells (−) and cells treated with leupeptin (+). In contrast, Shank2E levels were markedly increased in leupeptin-treated cells, indicating Shank2E has a significant rate of degradation under basal conditions. Actin served as a loading control. B: densitometric analysis confirmed this observation. While NaPi-IIa and EBP50 levels in leupeptin-treated cells were not significantly different from control cells, Shank2E levels in leupeptin-treated cells were 258 ± 51% of control levels (n = 5; P < 0.05).

Fig. 6. High P, induces parallel increases in Shank2E and NaPi-IIa degradation. After 20-h adaptation in low-Pi medium, OK cells were incubated in either low- or high-Pi medium for 4 h in either the absence or presence of leupeptin. A: in low-Pi conditions, leupeptin treatment resulted in Shank2E-specific accumulation. Incubation in high-Pi conditions had no discernible effect on EBP50 but resulted in a marked and parallel loss of both Shank2E and NaPi-IIa. This loss was inhibited by leupeptin. B: densitometric analysis of the loss of proteins in OK cells incubated in high-Pi conditions showed a significant and parallel loss of Shank2E and NaPi-IIa. Compared with levels in low-Pi conditions, Shank2E and NaPi-IIa levels were significantly decreased to 42 ± 9% and 35 ± 10%, respectively. EBP50 levels were not significantly different in high- vs. low-Pi conditions (n = 5; P < 0.05).
levels (258 ± 51% of basal levels) in leupeptin-treated cells during 4 h of low-Pi incubation (Fig. 5B). This suggests that despite being concentrated at the plasma membrane, Shank2E in OK cells undergoes a comparatively high rate of turnover.

Upon shifting from low- to high-Pi conditions, NaPi-IIa in OK cells is internalized and degraded (Fig. 6A). After 4 h in high-Pi medium, only 35 ± 10% of NaPi-IIa remained compared with NaPi-IIa levels measured in cells maintained in low-Pi medium (Fig. 6B). This degradation of NaPi-IIa is fully inhibited by coincubation with leupeptin. In contrast, the shift to high-Pi conditions has no effect on cellular levels of EBP50 (98 ± 8% of levels in low Pi), a known NaPi-IIa binding protein. Shank2E, however, showed a markedly similar level of degradation after a shift into high-Pi medium. After 4 h, Shank2E levels were only 42 ± 9% of levels measured in cells maintained in low-Pi medium, and this degradation was similarly inhibited by coincubation with leupeptin.

To determine whether this Pi-induced internal redistribution and degradation of Shank2E in OK cells also occurred in the whole kidney, the localization of Shank2E was compared in rats with low vs. high serum Pi levels. Serum Pi levels were moderated by using chronic feeding of a low-Pi diet, followed

![Fig. 7](http://apcell.physiology.org/)

**Fig. 7.** Acute high-Pi feeding induces parallel redistributions of Shank2E and NaPi-IIa. After chronic feeding of a low-Pi diet, rats were either maintained on the low-Pi diet or acutely switched to a high-Pi diet. After the 3-h feeding period, the intracellular distribution of NaPi-IIa and Shank2E were assessed in serial sections of rat kidney PT cortex. F-actin was stained to delineate the microvilli in PT cells and DIC images (right column) were included to denote the edges of the tubule segments. A: in rats maintained on a low-Pi diet, both NaPi-IIa and Shank2E were concentrated at the apical domain of PT cells. Overlays of F-actin and NaPi-IIa/Shank2E images (third column from left) showed both proteins codistributed within the microvillar region. B: in PT cells of animals acutely fed a high-Pi diet, NaPi-IIa and Shank2E were still found in the microvillar region but were also found within the cell interior, distinct from the microvillar domain. This indicates high-Pi induced internalization of both NaPi-IIa and Shank2E.

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by acute heavy feeding of high-\textit{P}$_1$ chow (16). In animals maintained on a low-\textit{P}$_1$ diet, serial sections of rat renal cortex showed that both NaPi-IIa and Shank2E were heavily concentrated within the microvillar region of PT cells (Fig. 7). After acute feeding of high-\textit{P}$_1$ chow, some NaPi-IIa remained in the microvillar region, but appreciable amounts were internalized into the cell interior. Strikingly, this intracellular redistribution of NaPi-IIa was paralleled by a similar redistribution of Shank2E into the cell interior (Fig. 7).

In paired studies, the comparative degradation of NaPi-IIa and Shank2E that occurs during the acute high-\textit{P}$_1$ feeding period was quantified with the use of Western blot and densitometric analyses (Fig. 8). As previously demonstrated, there was a significant decrease in NaPi-IIa levels (55 \pm 3\% of low-\textit{P}$_1$ controls; \textit{n} = 3) after 3 h. Despite the observed redistribution in PT cells, Shank2E levels in kidney cortex tissue of rats after high-\textit{P}$_1$ feeding were not significantly diminished (89 \pm 4\% of low-\textit{P}$_1$ controls; \textit{n} = 3). In addition, EBP50 levels were unchanged after high-\textit{P}$_1$ feeding (96 \pm 2\% of low-\textit{P}$_1$ controls; \textit{n} = 3). Thus, in contrast to OK cells, the Shank2E in rat renal PT cells does not appear to be targeted directly for degradation when internalized. While additional studies are needed to characterize the role of Shank2E in the high-\textit{P}$_1$-induced degradation of NaPi-IIa, the present study does demonstrate that the two interacting proteins are similarly induced by high-\textit{P}$_1$ conditions to be retrieved from the apical membrane, and, in OK cells, both enter the leupeptin-sensitive degradation pathway.

**DISCUSSION**

The distribution and activity of several apical membrane proteins in epithelial cells is moderated through interactions with PDZ domain proteins. NaPi-IIa, an essential protein in \textit{P}$_1$ recovery from renal tubular filtrate, is one such protein. The present study established that Shank2E, a PDZ protein recently identified in kidney, is concentrated at the apical domain of proximal tube cells (Fig. 2) and binds directly to NaPi-IIa via its PDZ domain (Figs. 3 and 4). The present study does not delineate the specific role of Shank2E in moderating NaPi-IIa activity but shows that Shank2E and NaPi-IIa are internalized in parallel in response to increased extracellular \textit{P}$_1$ (Fig. 7). In OK cells, shifting into high-\textit{P}$_1$ conditions induced a marked degradation of Shank2E that paralleled the degradation of NaPi-IIa under the same conditions (Fig. 6). This is in contrast to EBP50, another NaPi-IIa binding protein, whose levels were unaffected by alterations in extracellular \textit{P}$_1$.

**Shank2E binds specific proteins at the apical membrane of epithelial cells.** Shank protein family members were initially characterized within the PSD of neurons (22, 34, 3). There are three distinct Shank genes; each shares significant sequence homologies and domain organizations, including a single PDZ domain. Within the PSD, Shank proteins bind a number of PSD proteins and serves as a central organizing protein in the spatial and temporal distribution of proteins at the membrane-cytosol interface. At the apical membrane of pancreatic duct epithelial cells, Shank2 binds CFTR via its PDZ domain and inhibits the cAMP-regulated Cl$^-$ channel (14). Several ATP binding cassette (ABC) proteins, including CFTR and multidrug resistance protein 2 (mrp2), are bound by PDZ domain proteins. Among these ABC binding proteins, EBP50 and PDZK1 also bind NaPi-IIa via a PDZ interaction (8). Given that EBP50, PDZK1, and Shank2E each bound ABC proteins and EBP50 and that PDZK1 bound NaPi-IIa, it was hypothesized that Shank2E would also bind NaPi-IIa. The present study confirmed the hypothesis by showing native Shank2E and NaPi-IIa coprecipitated from rat renal cortex, recombinant Shank2E and NaPi-IIa coprecipitated when expressed in OK and HEK cells, and Shank2E bound directly to NaPi-IIa when NaPi-IIa had its PDZ binding motif (Figs. 3 and 4). Comparative binding studies using Shank2E domains and COOH-terminal truncations of NaPi-IIa further confirmed that the Shank2E-NaPi-IIa interaction occurred through the PDZ domain of Shank2E and the PDZ binding motif at the COOH terminus of NaPi-IIa (Fig. 4).

**PDZ domain proteins regulate NaPi-IIa activity.** Moderating NaPi-IIa activity in the renal proximal tubule is the primary physiological mechanism for regulating extracellular \textit{P}$_1$ concentrations. While the activity of many other membrane proteins is heavily influenced by phoshoregulation and recycling at the membrane, NaPi-IIa activity is regulated primarily by controlling the delivery, retention, and retrieval of the protein to the apical membrane with little or no apparent regulation of activity by phosphorylation or recycling (21). Instead, endocytosed NaPi-IIa is directly sorted and targeted for lysosomal degradation (12, 25). PDZ domain proteins have emerged as

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**Fig. 8. Fate of Shank2E in rat kidney cortex after high-\textit{P}$_1$ feeding.** The relative levels of specific proteins were also compared in renal cortical tissues in rats acutely fed a high-\textit{P}$_1$ diet vs. those maintained on low-\textit{P}$_1$ diets. A: Western blotting of cortical lysates showed a marked decrease in the levels of NaPi-IIa (55 \pm 3\% of low-\textit{P}$_1$ controls; \textit{n} = 3) after 3 h. B: densitometric analysis quantified a significant decrease in NaPi-IIa (55 \pm 3\% of low-\textit{P}$_1$ diet) with no significant decrease in Shank2E (89 \pm 4\% of low-\textit{P}$_1$ diet) or EBP50 (96 \pm 2\% of low-\textit{P}$_1$ diet). Values were normalized to actin (\textit{n} = 3).
pivotal entities in coordinating the activity of NaPi-IIa (9). After truncation of the COOH-terminal PDZ binding motif, NaPi-IIa still distributes to the apical membrane but also accumulates within intracellular compartments (11). In EBP50 knockout (EBP50−/−) mice, there is a marked decrease in NaPi-IIa at the apical membrane of proximal tubule cells, a significant increase in urinary Pi levels, and diminished NaPi-IIa activity in the apical membrane of PT cells in response to decreased serum Pi levels (31, 35). This indicates EBP50 is important for promoting NaPi-IIa activity through NaPi-IIa delivery or retention within the apical membrane of PT cells. In contrast, OKH cells, an OK cell clone deficient in EBP50 expression, has diminished parathyroid hormone-dependent inhibition of NaPi-IIa activity that is restored after EBP50 expression (17). A prominent difference between rat PT cells and OK cells was also observed in the present study. In OK cells, acute exposure to elevated Pi levels increased the internalization and degradation of NaPi-IIa (Fig. 6). In rat PT cells, elevated Pi levels also induced internalization (Fig. 7), but there was no significant loss of Shank2E (Fig. 8). This difference is not likely accounted for by the broader expression of Shank2E in the cells of the kidney cortex. While other cell types do appear to express Shank2 (Fig. 2), Shank2 expression is highest in the PT cells, and PT cells make up the majority of the cortical mass. Additional functional studies will elucidate the specific roles of PDZ domain proteins in moderating the distribution and activity of NaPi-IIa.

PDZ domain proteins have distinct roles in regulating NaPi-IIa activity. It is likely that the different NaPi-IIa-binding PDZ proteins do not have redundant functions but instead perform distinct roles in the regulated delivery, retention, and recovery of NaPi-IIa. First and foremost, the development of phosphousterity in EBP50−/− mice clearly indicates the inability of the other PDZ domain proteins to compensate for the loss of EBP50 function. Furthermore, analysis of three NaPi-IIa-binding PDZ domain proteins shows that they are markedly different with regard to their structural organization. EBP50 comprises two PDZ domains and an ERM binding motif, and PDZK1 comprises four PDZ domains with no other noted domains. In contrast, Shank2E has a single PDZ domain but also has six ANK repeats, an SH3 domain, a PRR domain, and a sterile α motif. Finally, the present study demonstrated a disparate response of Shank2E and EBP50 to a high-Pi environment. In OK cells, EBP50 levels were unaffected by the high-Pi environment, but Shank2E levels were significantly decreased (Fig. 6).

Shank2E parallels the endocytosis and degradation of NaPi-IIa. The present study suggests Shank2E may be linked to the internalization and degradation of NaPi-IIa (Figs. 6 and 7). Previous studies showed that Shank2 can bind dynamin II (23). Dynamin II, a broadly expressed isoform of dynamin, participates in the pinching off and internalization of endocytic vesicles. Thus Shank2E has established links to the endocytic machinery. In OK cells incubated under low-Pi conditions, Shank2E showed a comparatively high rate of degradation that was not observed with NaPi-IIa (Fig. 5). When OK cells were shifted into high-Pi conditions, Shank2E and NaPi-IIa underwent marked and parallel increases in degradation (Fig. 6). These biochemical measurements were mirrored by observations in the intact rat. Acute switching to a high-Pi diet induced Shank2E redistribution from the apical domain to the cell interior of PT cells (Fig. 7). This Shank2E redistribution was paralleled by the known redistribution of NaPi-IIa away from the apical membrane (Fig. 7) While additional studies are clearly required, the observations made in the present study support the hypothesis that Shank2E plays a pivotal role in segregating and trafficking NaPi-IIa into the lysosomal degradation pathway.

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