Akt recruits Dab2 to albumin endocytosis in the proximal tubule

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Akt recruits Dab2 to albumin endocytosis in the proximal tubule. Am J Physiol Renal Physiol 307: F1380–F1389, 2014. First published September 24, 2014; doi:10.1152/ajprenal.00454.2014.—Proximal tubule epithelial cells possess a highly sophisticated endocytic machinery to retrieve the albumin in the glomerular filtrate. The megalin-cubulin complex and the endocytic adaptor disabled-2 (Dab2) play a pivotal role in albumin endocytosis. We previously demonstrated that protein kinase B (Akt) regulates albumin endocytosis in the proximal tubule through an interaction with Dab2. Here, we examined the nature of Akt-Dab2 interaction. The pleckstrin homology (PH) and catalytic domains (CD) of Akt interacted with the proline-rich domain (PRD) of Dab2 based on yeast-two hybrid (Y2H) experiments. Pull-down experiments utilizing the truncated constructs of Dab2 demonstrated that the initial 11 amino acids of Dab2-PRD were sufficient to mediate the interaction between Akt and Dab2. Endocytosis experiments utilizing Akt1- and Akt2-silencing RNA revealed that both Akt1 and Akt2 mediate albumin endocytosis in proximal tubule epithelial cells; therefore, Akt1 and Akt2 may play a compensatory role in albumin endocytosis. Furthermore, both Akt isoforms phosphorylated Dab2 at Ser residues 448 and 449. Ser-to-Ala mutations of these Dab2 residues inhibited albumin endocytosis and resulted in a shift in localization of Dab2 from the peripheral to the perinuclear area, suggesting the physiological relevance of these phosphorylation sites in albumin endocytosis. We conclude that both Akt1 and Akt2 are involved in albumin endocytosis, and phosphorylation of Dab2 by Akt induces albumin endocytosis in proximal tubule epithelial cells. Further delineation of how Akt affects expression/phosphorylation of endocytic adaptors and receptors will enhance our understanding of the molecular network triggered by albumin overload in the proximal tubule.

Proximal tubule epithelial cells possess a highly sophisticated endocytic machinery programmed to retrieve 3-5 g of albumin/day via receptor-mediated endocytosis (46). Megalin, a 640-kDa LDL superfamily-type 1 transmembrane receptor and its adaptor protein disabled-2 (Dab2) mediate albumin endocytosis through a complex network of endocytic mediators that include clathrin, adaptor protein (AP)-2, and membrane phosphoinositides. Dab2 is also involved in multiple signaling events governing cell cycle progression, differentiation, and epithelial cell positioning. Dab2 binds to the NPXY motif in the cytoplasmic tail of megalin. Dab2 conditional knockout (KO) mice display decreased endocytosis, proteinuria, and impaired megalin/cubulin trafficking in the visceral endoderm characterized by redistribution of megalin from endosomes to microvilli (41, 43). In vitro and in vivo albumin overload surpassing the endocytic capacity of the proximal tubule results in a profibrogenic, inflammatory response and apoptosis (1, 15). Importantly, an association between endocytic proteins and progression of kidney diseases was shown in human genome-wide studies. A missense variant (I2984V) in the cubulin gene was discovered to be associated with a 41% increased risk for development of persistent microalbuminuria (MA) in type 1 diabetes mellitus in a prospective study. Genome-wide studies have identified Dab2 as one of the candidate genes that determine kidney function, susceptibility to chronic kidney disease, and albuminuria, which emphasizes the potential yet undetermined role of Dab2 in the progression of kidney diseases (5, 10, 36). Taken together, these data strongly suggest that alterations in expression and interactions between endocytic receptors and adaptor proteins play an important role in the development and progression of kidney diseases.

Protein kinase B (Akt) plays a pivotal role in cellular signaling events mediating proliferation, cell survival, and insulin signaling. We have demonstrated that Akt regulates albumin endocytosis through its interaction with the proline-rich domain (PRD) of Dab2, suggesting that Akt is a key component of the endocytic machinery in the proximal tubule (35). Akt was shown to interact with megalin most likely through Dab2 (9). The Akt family is composed of three different isoforms with >80% homology. Despite the great degree of homology between Akt isoforms, KO mouse models reveal their diverse functions (23, 26). Knockout of Akt1 in mice causes defects in fetal and postnatal growth and increased mortality. Akt2 KO mice display a diabetes-like phenotype, whereas Akt 3 KO has a negative impact on brain development (48). Akt possesses three different domains: pleckstrin homology (PH), catalytic (CD), and regulatory (RD) (6). Akt-PH is responsible for lipid-protein interactions, regulating translocation of Akt to the plasma membrane by binding membrane phosphoinositides (13, 45). Akt is a serine and threonine kinase with a diverse group of substrates. We proposed that the interaction between Dab2 and Akt bolster a phosphorylation event between Akt and Dab2.

In this study, our goal was to delineate the nature of Akt-Dab2 interaction. We first examined the isoform and domain of Akt that partners with Dab2. We examined phosphorylation of Dab2 by Akt as a potential pathway for induction of albumin endocytosis by Akt. Furthermore, we examined the relevance of this phosphorylation event to albumin endocytosis by functional studies. We believe that delineating the role of Akt in albumin endocytosis will shed light on our understanding of the link between albumin endocytosis and
cell signaling in the proximal tubule and help decipher the mechanism of tubular epithelial cell injury observed in albumin overload/proteinuric states.

METHODS

Cell lines. Human kidney proximal tubule clone-8 (HKC-8) cells (courtesy of Dr. L. Racussen, Johns Hopkins University) were grown in DMEM/F12 (Life Technologies) supplemented with 5% certified fetal bovine serum. HKC-8 cells express megalin and cubilin. Cells were incubated in serum-free media (SFM) for 16 h before albumin endocytosis experiments. Cell lysates were prepared and immunoprecipitation was performed as previously published (35). Akt1, Akt2, and Akt3 expression in HKC-8 cells was evaluated by Western blotting utilizing corresponding antibodies (Cell Signaling) (35).

Plasmids and small interfering RNA. HKC-8 cells were transfected with either Akt1, Akt2, or Akt1 + Akt2 small interfering RNA (siRNA) or control siRNA (Dharmacon SMART pool, Thermo Scientific). Briefly, cells were plated in a 24-well plate at 4 × 10^4/well density and transfected with siRNA at 60–70% confluence. To prepare the transfection complex, Dharmafect 1 agent (1 μl/well) was incubated with Akt siRNA (50 nM final concentration) or control siRNA at room temperature for 25 min in SFM as per the manufacturer’s instructions. Transfected cells were utilized in experiments 72 h after transfection. Albumin uptake was determined by fluorometric method (35).

Fig. 1. Akt has three isoforms with >80% homology. They all possess a pleckstrin homology (PH) domain, a catalytic domain (CD), and a regulatory domain (RD). The threonine and serine phosphorylation sites are adjacent to each other and located at the CD and RD (A). Akt isoforms display tissue specificity. Both Akt1 and Akt2 are displayed in immortalized human proximal tubule epithelial cells (HKC-8; B). No significant expression of Akt1 and Akt2 was detected in Akt1 knockout (KO) and Akt2 KO kidneys, respectively. Akt1 KO kidneys displayed some decrease in Akt2 expression (C). HKC-8 cells expressed both megalin and cubilin (D).
Potential phosphorylation sites of Dab2 by Akt were predicted by Scansite software (http://scansite.mit.edu) as Ser-448 and -449 based on the consensus site R-X-R-X-X-S/T. Dab2 Ser-448 and Ser-449 were mutated to Ala individually and in combination (448-449 AA) by site-directed mutagenesis (Stratagene). In phosphorylation experiments, wild-type Dab2 and Dab2 448-449 AA were cloned into the C terminus of the hemagglutinin (HA)-pCMV vector (Clontech) with restriction sites EcoRl and BamHl. All cloned constructs were confirmed by DNA sequencing.

Glutathione S-transferase fusion pull-down experiments. The recombinant proteins containing Dab2 1–206 (PTB), Dab2 1–368, Dab2 335–610 (M15), Dab2 600–730 (PRD) were fused to glutathione S-transferase (GST). The Dab2 constructs were cloned into pGEX-4T-1 by PCR using Dab2 cDNA as a template. Truncated Dab2-PRD (600–730) constructs were generated by site-directed mutagenesis. All constructs were verified by automated sequencing. GST and GST fusion proteins containing Dab2 constructs were expressed in Escherichia coli B21 cells under the control of the isopropyl-β-D-thiogalactoside (IPTG)-inducible tac promoter following the same protocol published previously (35).

Binding assays. Dialyzed GST fusion proteins were bound to glutathione-Sepharose and then mixed with clarified HKC-8 cell lysate to give a final concentration of ~7.5 mg/ml in 300 μl of total volume. After incubation at 4°C for 60 min, the beads were separated by centrifugation, and aliquots corresponding to one-sixtieth of each supernatant (S) and one-fifth of each washed pellet (P) were resolved by SDS-PAGE and transferred to nitrocellulose membranes. The blot was probed with pan-Akt antibody (no. 9272, Cell Signaling).

In vitro phosphorylation assays. Experiments were carried out at room temperature in a final volume of 25 μl with 10 mm HEPES, pH 7.4, 10 mm MgCl₂, and 2 mm EDTA. In this buffer, purified fusion proteins (2 g), either GST alone, GST-Dab2 PTB (1–206), GST-Dab2 (1–368), GST-Dab2-M15 (335–610), or GST-PRD (600–730), were incubated with purified recombinant human Akt2 (Active motif). Reactions were initiated via the addition of γ-[³²P]ATP (1 μCi; 2.2 × 10⁹ cpm) followed by 25 μl of reaction mix at room temperature, and reactions were terminated by spotting 1 μl on a P81 phosphocellulose membrane (Whatman, Inc.). Membranes were washed three times in 1% Triton X-100 in 50 mm sodium phosphate buffer and then twice in 10% ethanol. Membranes were exposed to x-ray film and quantified by densitometry.

Fig. 2. The role of Akt1 and Akt2 in albumin endocytosis was investigated. Significant knockdown of Akt1 and Akt2 was accomplished by small interfering (si) RNA treatment of the HKC-8 cells. Individual inhibition of Akt1 and Akt2 did not cause any cross-inhibition between isoforms (A). Akt1 and Akt2 siRNA caused a 30–40% decrease in albumin endocytosis (B; n = 5). *P < 0.05.

Fig. 3. Yeast two-hybrid experiment to delineate the Akt domain responsible for interaction with adaptor disabled-2 (Dab2)-proline-rich domain (PRD) by p53 and laminin transformed with PGADT7-T (Gal4AD fused with SV40 large T-antigen) were utilized as positive and negative control, respectively. PGADT7, PGADT7-Akt-PH, PGADT7-Akt-CD, PGADT7-Akt-RD, and PGBK7-Dab2PRD were transformed in competent Y2HGold cells. Transformants were grown on DDO-X (SD/-Leu/-Trp supplemented with X-Gal), TDO/X/A (SD/-His/-Leu/-Trp supplemented with X-Gal and aureobasidin), and QDA/X/A (SD/-Ade/-His/-Leu/-Trp supplemented with X-Gal and aureobasidin) medium. PGBK7-Dab2-PRD/PGADT7-Akt-PH and PGBK7-Dab2-PRD/PGADT7-Akt-CD grew blue colonies on the most stringent QDA/X/A medium, suggesting the presence of a robust interaction.
Amersham Pharmacia Biotech) and were allowed to proceed for 30 min at room temperature before being stopped with SDS-PAGE sample buffer. Phosphorylated samples were run on a 4–20% SDS-PAGE gradient gel, transferred to a nitrocellulose membrane, and then stained with Ponceau S before exposure of the membrane to a phosphoscreen. The extent of phosphorylation was quantified via phosphoimager analysis followed by densitometric analysis of the stained bands on the same membrane.

Additional in vitro phosphorylation assays utilizing immunoprecipitated proteins. HEK-293 cells were transfected with either wild-type or mutant pCMV-HA-tagged Dab2 constructs (Clontech). Dab2 was immunoprecipitated with an HA antibody (Roche) and incubated with purified Akt1 or Akt2 in the presence of γ-[32P]ATP to allow in vitro phosphorylation to occur, followed by washing, SDS-PAGE, transfer, immunoblotting, and phosphoscreen imaging of the same nitrocellulose membrane as published previously (24).

Confocal microscopy. HKC-8 cells were grown on collagen-coated cover slides and transfected with HA-tagged wild-type Dab2 and Dab2 448-449 AA at 80% confluence. Slides were washed with cover slides and transfected with HA-tagged wild-type Dab2 and nitrocellulose membrane as published previously (24).

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Confocal microscopy. HKC-8 cells were grown on collagen-coated cover slides and transfected with HA-tagged wild-type Dab2 and Dab2 448-449 AA at 80% confluence. Slides were washed with PBS** and fixed in 4% paraformaldehyde for 10 min and blocked with 10% goat serum/0.2% Triton X-100 in PBS. Cells were stained by clathrin heavy chain (Abcam) and HA antibody (Covance) at 4°C overnight and incubated with fluorescence-labeled secondary antibodies. After mounting, slides were visualized by Olympus confocal microscopy.

Yeast two-hybrid experiments. The Matchmaker Gold yeast two-hybrid system (Clontech) was utilized. Truncated Akt constructs, PH (amino acids 1–106), CD (148 – 418), and RD (419 – 480) were generated by PCR using full-length Akt as a template (Fig. 1A) and verified by automatic sequencing. Akt constructs were cloned into PGADT7 between restriction sites EcoRI and BamHI. Dab2-PRD was cloned into PGBK7T between EcoRI and NdeI restriction sites. HIS3 expression Bait (Dab2PRD) was cloned into PGBK7T, whereas prey, Akt-PH, -CD, and -RD were cloned into the PGADT7 vector. After the plasmids mapping interaction sites were subcloned into the EcoRI and BamHI sites in the LexA DNA binding domain of the bait vector, the Saccharomyces cerevisiae strain was transformed with the appropriate plasmid combinations and selected first on SD minimal medium plates lacking Leu and Trp. Individual clones were selected and then streaked/ spotted onto plates of SD medium lacking Leu and Trp (double dropout; DDO), on plates without His, Leu, and Trp (triple dropout; TDO), or onto plates lacking Ade, His, Leu, and Trp (quadruple dropout; QDO) with X α-gal (X) and antibiotic aureobasidin A (A) to eliminate any false positive background interactions. Clones representative of the growth pattern for the interaction being tested were then resuspended, normalized for by optical density, and spotted identically onto dropout plates, and yeast were grown at 30°C for 4 days.

Animal experiments. Kidneys of homozygous Akt1, Akt2 KO mice, and wild-type littermates were obtained from the University of Pennsylvania (Birnbaum MJ). All mice-breeding experiments were reviewed and approved by the University of Pennsylvania Institutional Animal Care and Use Committee in accordance with the guidelines of the US National Institutes of Health.

Kidneys of male mice at 6–10 wk of age were frozen in OTC and cut into 5-μm sections using a cryostat for immunofluorescence staining. Kidney sections were stained for Dab2 (rabbit, 1: 200 courtesy of Linton Traub) after fixation with 4% PFA, and slides were blocked with 5% goat serum in PBS+0.1% Triton X-100 at RT for 1

![Fig. 4. Representation of Dab2 constructs. Dab2-PTB (1–206), Dab2 (1–368), Dab2 PRD (600–730; A).](image-url)

Truncated Dab2PRD constructs was generated and fused into glutathione-S-transferase (GST). Aliquots corresponding to 1/60 of each supernatant (S) and 1/5 of each washed pellet (P) were resolved by SDS-PAGE and probed with an Akt antibody. GST pull-down experiments revealed that the initial 12 amino acids (600–611) were sufficient for interaction with Akt (B). GST-fused Dab2 PRD revealed a strong interaction with myosin VI. Endocytic protein clathrin heavy chain (HC) that interacts with the PTB domain of Dab2 was utilized as a negative control and did not reveal any interaction with Dab2 PRD.
Kidney lysates were homogenized in lysis buffer with protease inhibitors (Roche) on ice with a Dounce homogenizer. The homogenate was centrifuged at 1,000 g for 5 min, and then the supernatant was centrifuged at 15,000 g for 30 min at 4°C. Equal amounts of protein were loaded onto an SDS-PAGE gel, and Western blotting was performed using the same antibodies listed above.

Statistical analysis. Student’s t-test was utilized for two independent sample sets. Densitometric measurement comparisons of Western blots were performed by Student’s sample t-test by the SPSS statistical program. The program performed a two-tailed Student’s t-test given a null hypothesis that the two means were equal. A P value of <0.05 was considered significant.

RESULTS

Akt1 and Akt2 are the dominant isoforms of Akt in the proximal tubule. Three different isoforms of Akt, Akt1 (Pkbα), Akt2 (Pkbβ), and Akt3 (Pkbγ) have been described (Fig. 1A). Akt1 is ubiquitously expressed and implicated in cell growth and survival, whereas Akt2 is involved in insulin-mediated glucose regulation and is expressed highly in insulin-sensitive tissues. Expression of Akt3 is restricted to the testes and brain (20, 26). We demonstrated that Akt1 and Akt2 are both expressed abundantly, whereas Akt3 has only minimal expression in the proximal tubule (Fig. 1B). Kidney lysates of Akt1 and Akt2 KO mice demonstrated complete KO of Akt1 and 2, respectively. Negligible Akt3 expression was found in both KO and wild-type mice kidneys (Fig. 1C). Human kidney proximal tubule epithelial cells (HKC-8) cells expressed both megalin and cubulin, demonstrating the suitability of this cell line to study proximal tubule cell function.

Functional overlap between Akt1 and Akt2 in regulation of proximal tubule albumin endocytosis. We previously demonstrated that Akt modulates albumin endocytosis through its interaction with Dab2 (35). Despite the diverse phenotypes of Akt1 and Akt2 KO mice, functional redundancy between Akt1 and Akt2 has been reported (32). We next examined the Akt isoform that is involved in albumin endocytosis in the proximal tubule. Inhibition of either Akt1 or Akt2 resulted in ~40 and 30% inhibition in albumin endocytosis, respectively. There was no potentiation of this effect when both Akt1 and Akt2 were inhibited simultaneously (Fig. 2, A and B). We propose that this is due to competition between Akt1 and Akt2 binding, indicating that both isoforms may regulate the same target process (i.e., they are functionally redundant). In addition, one may also speculate that the effect of Akt1, Akt2, and Akt1 + Akt2 inhibition may have a saturable maximum effect on albumin endocytosis, which was accomplished by individual inhibition of Akt1 and Akt2.

PH and CD domains of Akt interact with Dab2-PRD. Akt isoforms consist of an N-terminal PH domain, a CD harboring the Thr-308 residue that is phosphorylated by phosphoinositide-dependent kinase 1 (PDK1), and a C-terminal hydrophobic RD encompassing the Ser-473 phosphorylation site. A yeast two-hybrid interaction screen was performed after transformation of the pGBK7-Dab2-PRD fused to the Gal4 DNA binding domain with Akt- PH domain (1–110), CD (111–412), and RD (412–481) ligated to the pGADT7 vectors. The Akt-PH and -CD domains both had a robust interaction with Dab2-PRD (Fig. 3). Binding of the Akt-PH domain to phosphatidylinositol [PtdIns (3,4) P2] and PtdIns (3,4,5) P3 regulates its recruitment to membranes and phosphorylation and hence activation of Akt. We previously demonstrated that Akt modulates albumin endocytosis through its interaction with Dab2 (35). Despite the diverse phenotypes of Akt1 and Akt2 KO mice demonstrated complete KO of Akt1 and 2, respectively. Negligible Akt3 expression was found in both KO and wild-type mice kidneys (Fig. 1C). Human kidney proximal tubule epithelial cells (HKC-8) cells expressed both megalin and cubulin, demonstrating the suitability of this cell line to study proximal tubule cell function.

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Fig. 5. Akt2 phosphorylates GST-Dab2 domain protein constructs in vitro. GST-tagged Dab2 protein fragments were expressed in Escherichia coli, purified and subjected to in vitro phosphorylation labeling in the presence of γ-[32P]ATP and purified Akt2 before SDS-PAGE and transfer to nitrocellulose membranes as described in METHODS. A: representative Ponceau S membrane staining to determine protein expression levels of the various constructs. B: representative phosphoscreen image of the same membrane indicating the positions of the phosphorylated bands corresponding to the various protein constructs (arrowheads) in the presence (+) or absence (−) of purified Akt2. A faint band at ~60 kDa in each lane represents autophosphorylated Akt2. C: summary of densitometric determinations of relative phosphorylation normalized by protein expression for each of the constructs in the presence (+) or absence (−) of purified Akt2. #P = 0.08 relative to 1–206 construct in presence of Akt; n = 3.
to the plasma membrane (3). We propose that phosphorylation of Akt at the Thr-308 residue in the CD and translocation of the Akt-PH domain bring Akt in close proximity to Dab2. Interactions between these two domains and Dab2 may then facilitate Dab2 phosphorylation by Akt.

The initial 12 amino acids (600–611) in Dab2-PRD are minimally sufficient for interaction with Akt. Dab2 interacts with multiple partners in the endocytic pathway, including membrane phosphoinositides, clathrin, and myosin VI. The majority of these protein-protein interactions occur via the N-terminal PTB domain (1–206) and Dab2 residues 1–368, with the exception of myosin VI (41, 29). To delineate the specific region of Dab2-PRD that interacts with Akt, we generated various GST fusion proteins representing PRD truncation mutants: full-length Dab2 PRD (600–730), Dab2 (600–711), Dab2 (600–683), Dab2 (600–639), and Dab2 (600–611). The minimally sufficient functional unit for interaction with Akt was Dab2 (600–611) (Fig. 4, A and B). GST pull-down experiments involving GST-Dab2 PRD (600–730) and myosin VI (positive control) and clathrin heavy chain (negative control) were utilized to validate the interaction between Dab2 PRD and Akt. It is also noteworthy that the interaction between Dab2-PRD and myosin VI appears to be stronger than that of Dab2 PRD and Akt. We believe this may be characteristic of an interaction that is required for phosphorylation which occurs in the presence of a stimulus/ligand.

Akt phosphorylates Dab2. Akt is a Ser/Thr kinase with multiple substrates involved in cell proliferation, differentiation, and survival (37). In vitro phosphorylation experiments revealed that Akt phosphorylated full-length Dab2. As an initial screening experiment to predict the residue(s) phosphorylated by Akt, in vitro phosphorylation experiments were performed utilizing GST-bound, truncated Dab2 mutants. This experiment demonstrated that the Dab2-M15 (335–610) domain was most strongly phosphorylated by Akt (Fig. 5, A–C). Ser-448 and Ser-449 were predicted as likely phosphorylation sites within Dab2-M15 by Scansite software based on the consensus Akt phosphorylation target motif X-R-X-X-S/T (2). Phosphorylation experiments were repeated after generation of individual and combined Ser-to-Ala mutations of Dab2 Ser-448 and Ser-449 by site-directed mutagenesis. The quantitative evaluation of phosphorylation revealed that both Akt1 and Akt2 phosphorylate full-length Dab2 at S448/S449 in vitro. Specifically, there was a significant decrease in phosphorylation of the Dab2 S448A/S449A mutant, suggesting that both
Phosphorylation of Dab2 by Akt regulates albumin endocytosis. To test the functional relevance of Dab2 phosphorylation, we examined albumin endocytosis in HKC-8 cells. Expression of the Akt phosphorylation-deficient Dab2 S448A/S449A mutant reduced albumin endocytosis by ~25% relative to cells expressing WT-Dab2, suggesting that Dab2 phosphorylation at these sites plays a significant role in the regulation of albumin endocytosis (Fig. 6D). The reduction in albumin endocytosis accomplished by the Dab2 S448A/S449A mutant was less than that with Akt 1 and Akt2 knockdown (Fig. 2B). We thus propose that Akt may also potentially target other Dab2 residues or endocytic proteins in the proximal tubule.

Apical expression of Dab2 is downregulated in Akt1 and Akt2 KO mice kidney proximal tubule epithelial cells. Confocal imaging of WT, Akt1 KO, and Akt2 KO mouse kidneys revealed that the absence of Akt1 or Akt2 reduced apical Dab2 expression (Fig. 7) without any decrease in tissue expression (data not shown), suggesting mistrafficking of Dab2.

Phosphorylation of Dab2 by Akt regulates albumin endocytosis. We previously demonstrated that Akt regulates albumin endocytosis through an interaction with the PRD domain of Dab2. To understand the spatial mechanism of this novel interaction, we investigated the Akt residues that interact with Dab2-PRD. We discovered that the Akt-CD and Akt-CD domain-domain interactions between Akt-CD and Akt-CD P3/PtdIns (3,4) P2 induce a conformational change, resulting in translocation of Akt to the plasma membrane. This facilitates phosphorylation at the Thr-308 site located in the Akt-CD by PDK1 (21, 31). Previous studies have shown that the PRD domain of Akt may serve as a membrane-targeting module, but it is not required for Akt activation or phosphorylation (3). Dab2 binds to clathrin, phosphoinositides, and AP-2 through its C-terminus PTB domain and myosin VI through the PRD domain (29, 39, 41). We postulate that the interaction between the Akt-CD and -PH domains with Dab2-PRD at the plasma membrane level serves as a hub, which ignites activation of the network of endocytic proteins encompassing membrane phosphoinositides, clathrin, AP-2, and myosin VI.

Considering the role of Akt as a Ser/Thr kinase, we next investigated whether Dab2 could undergo phosphorylation by Akt. We demonstrated that Akt phosphorylates Dab2 at residues Ser-448 and -449. Both Akt1 and Akt2 isoforms phosphorylate Dab2, demonstrating parallel functional redundancy in both phosphorylation and their role in albumin endocytosis in the proximal tubule. Furthermore, Dab2 phosphorylation appears to be a physiologically relevant event, as albumin endocytosis was also inhibited by mutating the Ser-448 and -449 residues to Ala. Of note, there may be additional Akt phosphorylation site(s) in Dab2 as residual phosphorylation did not occur outside of the M15 domain (Fig. 5), and mutation of these particular Dab2 residues did not fully abrogate phosphorylation in vitro (Fig. 6). We propose that phosphorylation of...
Dab2 by Akt tethers it to the plasma membrane and brings Dab2 in close physical proximity to the other endocytic proteins clathrin, membrane phosphoinositides, and AP-2. The role of membrane phosphoinositides in facilitating additional interactions between Dab2-PRD and other potential Akt substrates in clathrin-mediated endocytosis remains to be investigated.

We report that the initial 12 amino acids of Dab2-PRD (600–611) are sufficient for interaction with Akt. Although Akt and Dab2 interact at the PRD domain, the discovered phosphorylation sites are located within the M15 region (at Dab2 Ser-448 and -449). This distinction between the location for kinase binding vs. phosphorylation of a target protein has precedents in the literature. For example, the metabolic sensor AMP-activated protein kinase (AMPK) binds to the C-terminal cytoplasmic tail of the cystic fibrosis transmembrane conductance regulator (CFTR), but AMPK phosphorylates CFTR in the CFTR R-domain (24, 34). We postulate that binding may more efficiently target Akt to Dab2 and other associated proteins, or that Dab2 may undergo allosteric regulation through Akt binding that facilitates conformational changes required for phosphorylation.

Dab2 is a putative tumor suppressor, and downregulation of Dab2 has been demonstrated in several cancers (4, 40, 47). Mutation of Dab2 phosphorylation sites caused a shift in intracellular location of Dab2 from the periphery to a perinuclear staining.

Fig. 8. Human kidney proximal tubule cells were grown on collagen-coated cover slides. At ~90% confluence, cells were transfected with HA-tagged vector only, HA-WT Dab2, and HA-Dab2 SS448–9AA plasmid. Immunofluorescence staining with HA antibody and clathrin HC antibody was performed to localize clathrin and Dab2. Dab2 colocalizes peripherally in the endocytic vesicles colocalizing with clathrin in HKC-8 cells transfected with WT-Dab2. Cells transfected with HA-Dab2 SS448–9AA displayed more prominent perinuclear Dab2 staining.
clear region. It is thus conceivable that phosphorylation of Dab2 by Akt at baseline directs Dab2 to endocytic machinery, and mutation of the phosphorylation sites shifts Dab2 to regulate cell cycle progression. These findings are consistent with previously published reports that endocytic proteins may be channeled to mitosis during cell cycle progression, with clathrin-mediated endocytosis being shut down (19). Negative regulation of endocytic function of Dab2 was observed during mitosis, resulting in its disintegration from peripheral clathrin heavy chain and relocation from the plasma membrane to a perinuclear distribution (11).

We previously reported that Akt expression is downregulated in an in vitro albumin-overload model, along with Dab2 and megalin (35). We propose that agents that are targeted to bolster Akt expression in the proximal tubule may promote cell survival while inducing albumin endocytosis in proteinuric states where megalin and Akt expression may be downregulated. We predict that Akt most likely has other targets in addition to Dab2 in the endocytic pathway. Identifying other potential targets and elucidating additional roles of Akt in regulation of albumin endocytosis in the proximal tubule could lead to the discovery of novel pharmaceutical agents to diminish proteinuria-induced renal injury in glomerulopathies.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: K.K., H.L., K.R.H., and E.E. analyzed data; M.J.B. and E.E. edited and revised the manuscript; E.E. approved final version of manuscript.

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