Chloride channel CIC-5 binds to aspartyl aminopeptidase to regulate renal albumin endocytosis

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1Q Centre for Clinical Research, The University of Queensland, Herston, Brisbane, Queensland, Australia; 2School of Biomolecular and Biomedical Sciences, University College Dublin, Belfield, Dublin, Republic. of Ireland; 3Department of Nephrology, Monash University Department of Medicine, Monash Medical Centre, Clayton, Victoria, Australia; 4Department of Physiology, The University of Melbourne, Parkville, Victoria, Australia; 5Department of Pharmacology, Mount Sinai School of Medicine, New York, New York; 6Department of Medicine, Saint Vincent’s Hospital, Fitzroy, Victoria, Australia; 7Children’s Medical Research Institute, The University of Sydney, Westmead New South Wales, Australia; and 8School of Medical Sciences and the Bosch Institute, The University of Sydney, New South Wales, Australia

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Lee A, Slattery C, Nikolic-Paterson DJ, Hryciw DH, Wilk S, Wilk E, Zhang Y, Valova VA, Robinson PJ, Kelly DJ, Poronnik P. Chloride channel CIC-5 binds to aspartyl aminopeptidase to regulate renal albumin endocytosis. Am J Physiol Renal Physiol 308: F784–F792, 2015. First published January 13, 2015; doi:10.1152/ajprenal.00322.2014.—CIC-5 is a chloride/proton exchanger that plays an obligate role in albumin uptake by the renal proximal tubule. CIC-5 forms an endocytic complex with the albumin receptor megalin/cubilin. We have identified a novel CIC-5 binding partner, cytosolic aspartyl aminopeptidase (DNPEP; EC 3.4.11.21), that catalyzes the release of N-terminal aspartate/glutamate residues. The physiological role of DNPEP remains largely unresolved. Mass spectrometric analysis of proteins binding to the glutathione-S-transferase (GST)-CIC-5 C terminus identified DNPEP as an interacting partner. Coimmunoprecipitation confirmed that DNPEP and CIC-5 also associated in cells. Further experiments using purified GST-CIC-5 and His-DNPEP proteins demonstrated that the two proteins bound directly to each other. In opossum kidney (OK) cells, confocal immunofluorescence studies revealed that DNPEP colocalized with albumin-containing endocytic vesicles. Overexpression of wild-type DNPEP increased cell-surface levels of CIC-5 and albumin uptake. Analysis of DNPEP-immunoprecipitated products from rat kidney lysate identified β-actin and tubulin, suggesting a role for DNPEP in cytoskeletal maintenance. A DNase I inhibition assay showed a significant decrease in the amount of G actin when DNPEP was overexpressed in OK cells, suggesting a role for DNPEP in stabilizing the cytoskeleton. DNPEP was not present in the urine of healthy rats; however, it was readily detected in the urine in rat models of mild and heavy proteinuria (diabetic nephropathy and anti-glomerular basement membrane disease, respectively). Urinary levels of DNPEP were found to correlate with the severity of proteinuria. Therefore, we have identified another key molecular component of the albumin endocytic machinery in the renal proximal tubule and describe a new role for DNPEP in stabilizing the actin cytoskeleton.

CIC-5; albumin endocytosis; albuminuria; aspartyl aminopeptidase

THE RENAL PROXIMAL TUBULE reabsorbs significant amounts of water, ions (Na+, Cl−, HCO3−, PO43−), glucose, and amino acids from the glomerular filtrate (31). In addition to ions and small molecules, anionic proteins such as albumin cross the glomerular barrier (14). The concentration of albumin in normal filtrate in humans has been estimated to be ~3.5 mg/l (35). As the kidneys filter ~180 liters of blood/day (20), this translates to ~720 mg of albumin entering the kidneys on a daily basis; however, <30 mg of albumin is excreted in the urine. Therefore, ~95% of the filtered load of albumin is constitutively reabsorbed in the renal proximal tubules by clathrin-mediated endocytosis (14). This process involves I) albumin binding to the megalin/cubilin scavenger receptor (9); 2) internalization of the albumin complex; 3) dissociation of albumin from the receptor in endosomes (8, 14); and 4) degradation of albumin into its constitutive amino acids in lysosomes that are then returned into the blood, while megalin/cubilin is recycled back to the cell membrane (11).

Albumin endocytosis requires the formation of a multiprotein scaffold that includes the scavenger receptor megalin/cubilin-aminonion complex, Na+/H+ exchanger isoform 3 (NHE3), V-type H+-ATPase, and CIC-5, which appears to play a central role (14, 23, 32, 41). The importance of both megalin and cubilin in albumin uptake was demonstrated in megalin-knockout mice (48) and cubilin-deficient dogs (2), both of which had pronounced low-molecular-weight proteinuria and albuminuria. Cubilin is an extracellular protein, and megalin is a larger protein with a transmembrane domain (8). Studies in cubilin-deficient mice revealed it is essential for the reabsorption of albumin while megalin mediates the internalization of the cubilin-albumin complex (1). NHE3 is known to bind the C terminus of megalin and has been proposed to play a role in the initial acidification of the nascent endosome by acting to dissipate the high intravesicular Na+ concentration in exchange for cytosolic H+. Albumin uptake is effectively abolished in NHE3-deficient opossum kidney (OK) cells (18), and NHE3 knockout mice have proteinuria (19). Taken together, these observations demonstrate a key role for NHE3 in albumin uptake. Acidification of the late endosome and lysosome is driven by the V-type H+-ATPase (15).

The evidence for the role of CIC-5 itself as a central orchestrator of albumin uptake continues to grow. CIC-5 was originally thought to act as an anion shunt to maintain electroneutrality during endosomal acidification. Changes in the cell surface availability of CIC-5 have profound implications for the membrane proteins involved in albumin uptake. For exam-
ple, patients with Dent’s disease, who have mutations in ClC-5, present with significant proteinuria. Similarly, ClC-5 knockout mice also have proteinuria due to impairment of endocytosis (44). This is accompanied by the selective loss of plasma membrane proteins at the brush border, including megalin and cubulin (10). In patients with Dent’s disease, mutations that delete the C terminus of ClC-5 result in V-H^+ATPase mis- trafficking to the basolateral membrane, contrasting with its apical location in the normal kidney (34). In addition, NHE3 endocytosis is reduced in ClC-5 knockout mice (38).

Recent studies from our group have shed light on the cytoskeletal interactions mediated by the C terminus of ClC-5 that interacts directly with the PDZ scaffold NHERF2, an interaction that is likely to be important for tethering the complex to the actin cytoskeleton (22). The endocytic complex scaffolded by the C terminus of ClC-5 also recruits in accessory proteins involved in the remodeling of actin filaments that are required during the formation of the endosome (25). Together, these findings suggest that ClC-5 plays a central role in nucleating the formation of the albumin endocytic complex. This involves the C terminus of ClC-5 acting to scaffold interactions with key proteins directly involved with albumin endocytosis (V-H^+ATPase) (34) and megalin/cubulin (8) and accessory proteins (cofilin and NHERF2) (22, 25). The present study was undertaken to identify new ClC-5 binding proteins to further understand the molecular basis of albumin uptake by the proximal tubule. We used a proteomic approach based on a glutathione-S-transferase (GST) pulldown followed by mass spectrometry. We identified aspartyl aminopeptidase (DNPEP) as a new binding partner of ClC-5 that plays a significant role in albumin uptake in cultured renal proximal tubular cells and which serves as a potential marker of renal disease.

MATERIALS AND METHODS

Plasmid construction. Generation of heme agglutinin (HA)-tagged, enhanced green fluorescent protein (EGFP)-tagged, or GST fusion constructs was carried out by high-fidelity PCR with primers containing in-frame restriction sites. The PCR products were digested and subcloned into either a pHM6 vector (Clontech), a pEGFP vector (Clontech), or a pGEX6P-1 vector (Amersham Pharmacia). The PCR fragment containing the entire mouse DNPEP coding sequence was fused at the EcoR I/Hind III sites in the pHM6 vector (to produce pHM6-DNPEP) and at the Bgl II/EcoR I sites in the pEGFP vector (to produce pEGFP-DNPEP). The PCR fragment containing the entire ClC-5 coding sequence was fused at the Kpn I/EcoR I sites in the pHM6 vector to produce pHM6-CIC-5. The PCR fragment containing the coding sequence of the CIC-5 C-terminal region was fused at the EcoR I/Xho I sites of pGEX6P-1 to produce pGEX6P-1-CIC-5ct. The DNPEP mutant construct [pHM6-(H94F) DNPEP] was generated using a Quick Change Site-Directed Mutagenesis kit (Stratagene) from the wild-type pHM6-DNPEP construct. Fidelity of the plasmid constructs was confirmed by DNA sequencing.

Antibodies. An antibody against ClC-5 (12) was kindly provided by Prof. Olivier Devuyst (Division of Nephrology, CELL Unit, Christian de Duve Institute of Cellular Pathology, Université Catholique de Louvain Medical School, Brussels, Belgium) and the antibody against DNPEP (46) have been described previously. An anti-HA antibody was purchased from Roche Applied Science (Indianapolis, IN). An anti-His antibody was purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). An anti-NHERF1 antibody was kindly provided by Associate Prof. Chris Yun (Department of Medicine, Emory University, Atlanta, GA).

Expression and purification of His-DNPEP. His-DNPEP fusion proteins were expressed in the Escherichia coli strain BL21 codon plus (Stratagene). Optimal expression of fusion proteins was achieved by adding a final concentration of 1 mM isopropyl-β-D-thiogalacto- pyranoside to the bacterial culture, with overnight incubation at 20°C. The bacteria were harvested by centrifugation at 3,000 g for 30 min and resuspended in lysis buffer containing 50 mM NaPi (pH 7.8), 300 mM NaCl. The suspension was then sonicated and centrifuged for 20 min at 27,000 g. The supernatant was incubated with Talon beads (Clontech) with end-to-end rotation at 4°C for 3 h, and the suspension then decanted into a 2-cm-diameter column. The column was washed with buffer containing 50 mM NaPi (pH 7.8), 300 mM NaCl, 10% glycerol, and 20 mM imidazole. His-DNPEP fusion protein was finally eluted with elution buffer containing 50 mM NaPi (pH 7.8), 300 mM NaCl, and 200 mM imidazole.

Cell culture and transfections. The OK cell line was maintained in DMEM/Ham’s F-12 (DMEM/F-12) media supplemented with 10% fetal bovine serum, penicillin/streptomycin, and incubated at 37°C in 5% CO2. Cells were transfected with different combinations of DNA constructs using Lipofectamine LTX (Invitrogen) precisely following the manufacturer’s instructions.

GST pull-down experiments. Lysates were prepared from dicig whole rat kidneys and homogenizing equal tissue weights in HNT buffer containing 20 mM HEPES-Tris (pH 7.5), 120 mM NaCl, 0.6% Triton X-100, 5 mM EDTA, and a protease inhibitor cocktail (Roche Diagnostics). After gentle rotation for 2 h at 4°C, homogenates were centrifuged at ~30,000 g for 50 min at 4°C, and the supernatant was collected. The kidney lysate was then incubated with GST or GST-CIC-5ct fusion protein beads in HNT buffer at 4°C overnight with end-to-end rotation. Bound proteins were washed five times with HNT buffer and resuspended in Laemmlı buffer. Samples were heated at 95°C for 5 min, resolved on SDS-PAGE, and stained with Coomassie brilliant blue.

In-gel digestion and matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Protein bands were excised from Coomassie blue-stained gels, rinsed with water, and destained with several washes of 50 mM ammonium bicarbonate in 50% acetonitrile. The gel pieces were dehydrated with 100% acetonitrile and dried in a rotary vacuum concentrator. Sequencing grade modified trypsin (12.5 ng/μl in 25 mM ammonium bicarbonate, pH 8.0, Promega) was added for 30 min at 4°C and then incubated overnight at 37°C. The resulting peptide mixture was collected and concentrated to a minimal volume in a rotary vacuum concentrator. An aliquot of the peptide mixture (0.5 μl) was spotted onto the matrix-assisted laser desorption ionization (MALDI) target plate and overlaid with 0.5 μl of α-cyano-4-hydroxycinnamic acid solution (Agilent G2037A).

A Voyager-DE PRO MALDI-time-of-flight (TOF) mass spectrometer (Applied Biosystems) equipped with delayed extraction was employed, and data were acquired in positive reflector mode using external calibration. Peptide mass fingerprint (PMF) analysis and protein identification were done using an in-house Mascot search engine (www.matrixscience.com) against a nonredundant protein database (NCBI). Similarity searches against the nonredundant protein database were performed using BLAST from NCBI. The full-length DNPEP cDNA was then cloned from mouse kidney cDNA by high-fidelity PCR with primers corresponding to the sequence from GenBank accession number NP_058574.2.
150 mM NaCl, pH 7.6), and the protein complexes were eluted three times with 0.1 M glycine (pH 2.8). Eluates were pooled and concentrated using a rotary vacuum concentrator. Proteins were separated on SDS-polyacrylamide gel and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) by electroblotting. Blots were incubated with appropriate antibodies overnight at 4°C in 5% nonfat milk, 100 mM Tris (pH 7.5), 306 mM NaCl, and 0.1% Tween 20. Blots were washed three times with Tris-NaCl-Tween buffer, incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (Pierce, Rockford, IL), and washed again. Immunoreactive proteins were detected by enhanced chemiluminescence using a SuperSignal kit (Pierce).

**Cell surface biotinylation.** OK cells were transiently transfected with pHM6-DNPEP, pHM6-(H94F) DNPEP, or pHM6 empty vector (control) using Lipofectamine 2000 (Invitrogen). Forty-eight hours posttransfection, confluent OK cell monolayers were washed three times in ice-cold PBS, and then biotinylated with EZ-Link NHS-SS-Biotin (Pierce) at 4°C with gentle agitation. Monolayers were washed three times in cold PBS, and the cells were lysed in lysis buffer containing 150 mM NaCl, 20 mM HEPES, 1 mM EDTA, 1% NP-40, and complete protease inhibitor cocktail (Roche). The biotinylated proteins were isolated by binding to ImmunoPure Immobilized Streptavidin (Pierce) for 20 min at 4°C. The beads were pelleted, and the supernatant that contained the cytoplasmic (unbiotinylated) fraction was recovered by centrifugation at 4,000 g for 5 min at 4°C. The membrane (biotinylated) fraction was washed three times, and the pellet was resuspended in Laemmli sample buffer. An equal protein amount of the biotinylated fraction was resolved on a 7% SDS-PAGE gel, transferred to a nitrocellulose membrane (Bio-Rad), and immunoblotted with antibodies against CIC-5 or NHERF1.

**Albumin uptake.** OK cells are a widely accepted model for the study of renal albumin endocytosis. Standard fluorescent methods were used to measure albumin uptake in OK cells (13, 25, 28). Control OK cells (mock transfected with empty vector) or OK cells transfected with the various constructs were grown to confluence in 48-well plates. To determine albumin uptake, cells were exposed to 50 μg/ml albumin conjugated to Texas red (TR; Molecular Probes) for 120 min. Nonspecific binding was determined in cells exposed to 0.1 mM MOPS with 0.1% Triton X-100. The TR-albumin fluorescence was standardized to total albumin fluorescence. TR-albumin uptake was standardized to total albumin fluorescence.

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independently confirm the mass spectrometry data, GST pull-catalyzes the release of N-terminal aspartate/glutamate residues of the DNPEP could be identified; sequenced amino acid residues are indicated in bold grey.

Identification of DNPEP as a novel CIC-5-interacting protein. A GST-pull-down experiment coupled with mass spectrometry was used to identify proteins in the rat kidney lysate that interact with the C terminus of human CIC-5. Two main proteins (~45 and ~55 kDa, respectively) were pulled down using GST-CIC-5ct but not with GST alone (Fig. 1A). The ~45 kDa protein was identified as actin, while the ~55-kDa protein was identified as the cytosolic protein DNPEP (EC 3.4.11.21) with 30% amino acid sequence coverage (Fig. 1B), which catalyzes the release of N-terminal aspartate/glutamate residues from target peptides (46, 47).

DNPEP interacts with CIC-5 in vitro and in cells. To independently confirm the mass spectrometry data, GST pull-down products from rat kidney lysate were subjected to immunoblot analysis with anti-DNPEP antibody. GST-CIC-5ct but not GST alone binds to DNPEP (Fig. 2A). To confirm that the CIC-5-DNPEP complex exists endogenously in kidney, total lysates from rat kidney were incubated with the anti-DNPEP antibody, and immunoprecipitated proteins were analyzed by immunoblotting with anti-CIC-5 antibody. CIC-5 was detected as a band migrating at ~83 kDa, but no signal was detected when lysates were incubated with the IgG control (Fig. 2B). To determine whether the interaction between the C terminus of CIC-5 and DNPEP was direct and not through an intermediate protein, we performed an in vitro binding assay with purified His-DNPEP and GST-CIC-5ct. His-DNPEP was incubated with either GST or GST-CIC-5ct that had been immobilized to glutathione-Sepharose beads. Proteins that remained bound to the beads after extensive washing were then separated by SDS-PAGE and visualized by anti-DNPEP immunoblotting. GST alone did not bind, but an interaction occurred between CIC-5ct and DNPEP (Fig. 2C).

Immunolocalization of DNPEP in the rat kidney. The specificity of the DNPEP antibody for its target was initially tested on immunoblots of electrophoretically separated SDS-solubilized kidney lysate, which revealed a single labeled band at ~55 kDa (Fig. 3A), which accords with the molecular weight of the purified enzyme (47). Immunohistochemical analysis of rat kidney sections revealed that DNPEP protein was located in epithelial cells of the proximal tubule (Fig. 3B) and collecting duct (Fig. 3C). The glomeruli, endothelia, and podocytes were all unstained. No staining was detected in the negative control, where the primary antibody was not used (data not shown). Peptide preabsorption also eliminated staining with this antibody (Fig. 3D), demonstrating specificity of the DNPEP immunohistochemistry.

Effect of DNPEP on albumin uptake and CIC-5 expression. CIC-5 is essential for albumin uptake, and we demonstrated that CIC-5 interacts with DNPEP. Therefore, we investigated whether DNPEP expression could alter albumin endocytosis in cultured proximal tubule cells. In OK cells overexpressing wild-type DNPEP but not catalytically inactive DNPEP, TR-albumin uptake was significantly increased by ~20% (n = 3; P < 0.05) compared with that of mock-transfected levels (Fig. 4A). As reported previously, the transfection efficiencies of OK cells in these experiments are typically on the order of 30–40% (25); therefore, the ~20% increase we observe in TR-
albumin uptake by the total population of cells is an underestimate of the true increase in TR-albumin uptake by those cells that express DNPEP. The availability of CIC-5 to the endocytic complex is a limiting factor for albumin uptake; therefore, the observed increase in albumin uptake when DNPEP is overexpressed should be paralleled by increased cell surface levels of CIC-5. Figure 4B shows that overexpression of wild-type DNPEP but not catalytically inactive DNPEP resulted in a significant (−22%) increase in CIC-5 at the cell surface, compared with the control ($n = 3$, $P < 0.05$).

Colocalization of DNPEP with albumin-containing vesicles.

The subcellular localization of DNPEP in OK cells was investigated by confocal microscopy using antibodies against DNPEP and TR-BSA as an endocytic tracer that follows the clathrin-mediated endocytosis pathway. DQ-BSA, a highly quenched fluorescent derivative of BSA, was also included in these experiments due to its dramatic increase in fluorescence intensity following proteolytic degradation. The fluorescence of DQ-BSA is largely quenched by several adjacent Bodipy dyes until DQ-BSA is hydrolyzed by proteases, producing fluorescent products (42). Hence, the DQ-BSA probe is a useful tool for the labeling of endocytic vesicles associated with albumin degradation. In the absence of albumin, endogenous expression of DNPEP in OK cells was within many cytoplasmic puncta, and upon albumin exposure (20 min) the punctate staining for DNPEP concentrated near the subapical membrane compatible with endosomal reactivity was observed (Fig. 5A). Colocalization studies were performed after 20-min

![Image](F788 ClC-5 BINDS ASPARTYL AMINOPEPTIDASE)

Fig. 4. Overexpression of DNPEP increased albumin uptake and CIC-5 cell surface levels. A: control opossum kidney (OK) cells (pHM6 empty vector transfected) and OK cells expressing either wild-type DNPEP (wt DNPEP) or catalytically inactive DNPEP (H94F DNPEP) were exposed to TR-albumin (50 μg/ml) for 2 h. Albumin uptake increased −20% in OK cells expressing wt DNPEP (but not H94F DNPEP) compared with control. Values are means ± SD of 3 separate experiments. *$P < 0.05$ relative to pHM6-transfected control. B: representative Western blots showing effects of wt DNPEP or H94F DNPEP expression on CIC-5 cell surface levels in OK cells; detection of NHERF1 served as a loading control. Bar graph shows densitometric analysis of CIC-5 expression from 3 separate experiments. CIC-5 levels were normalized to NHERF1 (loading control) and show surface expression of CIC-5 increased −22% in OK cells expressing wt DNPEP (but not H94F DNPEP) compared with control. Values are means ± SD. *$P < 0.05$ relative to control.

![Image](AJP-Renal Physiol • doi:10.1152/ajprenal.00322.2014 • www.ajprenal.org)

Fig. 3. Immunolabeling of DNPEP in rat kidney. A: Western blot analysis of homogenized rat kidney lysate (10 μg) using anti-DNPEP antibody. Immunohistochemistry using rat kidney sections showed strong immunolabeling for DNPEP was present on epithelial cells lining the proximal tubules (B) and collecting duct (C). The glomeruli, endothelia, or podocytes were unstained. No staining was detected in the negative control (D; primary antibody preabsorbed with the peptide). Original magnification ×400.
incubation with TR-BSA or DQ-BSA. When the signals were combined with immunofluorescence for DNPEP, it was evident that a significant fraction of DNPEP was colocalized with endocytic vesicles containing TR-BSA (Fig. 5B). In contrast, DNPEP was not colocalized with endocytic vesicles containing DQ-BSA (Fig. 5C). These results suggest that a substantial fraction of DNPEP in the proximal tubular model of OK cells is colocalized in the endosomal compartment responsible for the receptor-mediated uptake of albumin.

Effect of DNPEP on polymerization of actin microfilaments. To gain insights into the possible physiological role of DNPEP in the kidney, we isolated DNPEP-coimmunoprecipitating proteins from rat kidney lysate and performed mass spectrometry to identify additional DNPEP-interacting proteins. This led to
the identification of β-actin and tubulin (data not shown), suggesting a role for DNPEP in cytoskeletal maintenance. In particular, β-actin was detected with high sequence coverage of ~64% and was also identified prominently in GST-CIC5 pull-down samples. DNPEP-β-actin interaction was independently confirmed by coimmunoprecipitation experiments (Fig. 6). However, DNPEP and β-actin did not bind each other directly in binding studies using purified recombinant proteins (data not shown). Therefore, the interaction between DNPEP and β-actin is indirect. We then performed a DNase I inhibition assay to investigate the effect of overexpressed DNPEP on actin cytoskeleton dynamics in the OK cell line. This method represents a widely accepted and reliable technique for quantitative determinations of the ratio of monomeric (G) to filamentous (F) actin, i.e., the G/F-actin ratio (3, 43). Extracts obtained from OK cells overexpressing DNPEP had significantly increased F-actin, while the total actin in both DNPEP-overexpressed cells and control cells remained constant (Table 1).

Detection of DNPEP protein in urine of STZ-induced diabetic and anti-GBM rats. STZ-induced diabetes in rats is a commonly used model of diabetic nephropathy. While DNPEP was detected in normal rat serum, it was not detectable in urine of normal (control) rats or 4 wk after the induction of diabetes was detected in normal rat serum, it was not detectable in urine of normal (control) rats or 4 wk after the induction of diabetes. While DNPEP remained constant (Table 1).

Effect of overexpressed DNPEP on the polymerization of actin in OK cells.

Table 1. Effect of overexpressed DNPEP on the polymerization of actin in OK cells

<table>
<thead>
<tr>
<th>G/F-Actin Ratio</th>
<th>F-Actin/Protein, µg/mg</th>
<th>Total Actin/Protein, µg/mg</th>
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<tbody>
<tr>
<td>DNPEP</td>
<td>0.69 ± 0.07*</td>
<td>38.13 ± 1.102*</td>
</tr>
<tr>
<td>Control</td>
<td>1.02 ± 0.09</td>
<td>31.78 ± 2.135</td>
</tr>
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Values are means ± SD. DNPEP, aspartyl aminopeptidase. Opossum kidney (OK) cells were transfected with pHM6 empty vector (control) or a pHM6-DNPEP construct (DNPEP) and harvested 48 h later. Measurements of monomeric (G) and total cellular actin was performed by DNa I inhibition assay to determine the G/F-actin ratios, the ratio of F-actin content to total cellular protein, and the ratio of total actin content to total cellular protein. *P < 0.05; n = 3.

DISCUSSION

This study reports a new CIC-5-interacting partner, DNPEP, that plays a role in the endocytosis of albumin in kidney cells. This represents a new physiological role for this aminopeptidase. DNPEP is a widely distributed and abundant cytosolic metalloproteinase enzyme that removes N-terminal aspartic or glutamic acid residues from target peptides. The DNPEP monomer is a ~55-kDa metalloprotease that belongs to the M18 family of the MH clan of metalloproteinases and is the only known mammalian member of this family. Recent crystal structures have revealed that it assembles into a dodecameric tetrahedral complex that in part dictates the access of peptide substrates to the active site (7). However, its physiological role as a peptidase remains unclear. DNPEP can convert angiotensin II to angiotensin III in vitro, and substrate-specificity studies confirm that it is an acidic amino acid-prefering aminopeptidase (47). The fact that DNPEP interacted with CIC-5 in the kidney suggested a role for DNPEP in albumin endocytosis. This possibility was further supported by our findings that DNPEP colocalizes with endocytosed albumin in OK cells. We were also able to demonstrate that the peptidase activity of DNPEP was required for the modulation of albumin uptake and that this also influenced the cell surface levels of CIC-5.
An interesting finding in this study was the potential mode of action. The pull-down approach revealed that DNPEP associates with actin. This is in accord with another recent study on malaria (26) that also showed DNPEP binding to actin. Our data extend this finding by revealing a potential role for this interaction; that DNPEP may serve to stabilize the cytoskeletal F-actin filaments. It is well established that the actin cytoskeleton plays a key role in cell polarity, cell shape, and vesicle trafficking (4). In epithelial cells, actin plays a further role in the assembly of the brush border (16). Actin dynamics are mediated by various cross-linking and bundling proteins which are involved in the ultrastructure of the microvilli and in the recruitment and anchorage of the key membrane proteins responsible for normal epithelial function (40). We have demonstrated previously that albumin endocytosis not only depends on an intact cytoskeleton (e.g., disruption of the actin cytoskeleton with cytochalasin significantly reduces uptake by ~80%) but also involves significant remodeling of the cytoskeleton (24). We also showed that the actin-depolymerizing protein coflin associates with CIC-5 and plays a facilitatory role in albumin uptake by promoting remodeling of the cytoskeleton during endosomal formation (25). In the current study, we have identified a novel role for DNPEP in albumin uptake that involves interaction with actin during its association with CIC-5. The trafficking of NHE3, which is essential for albumin uptake, is relatively well understood. The interaction of NHE3 with the cytoskeleton via actin binding proteins such as ezrin and the NHERF PDZ scaffolds regulates its availability and mobility at the cell surface (6). Depolymerization of the actin cytoskeleton is also essential for the normal trafficking of NHE3 within the proximal tubule cell (5). It is likely that similar mechanisms also regulate the trafficking of CIC-5 (22, 23, 25, 45). In particular, a recent study has shown a central role for CIC-5 in the exocytosis of NHE3, a finding that highlights the importance of CIC-5 in regulated exocytosis and endocytosis of apical membrane proteins (29). These findings in turn demonstrate the importance of characterizing the accessory and scaffold proteins that associate with CIC-5 and govern its trafficking.

In addition to the new role for DNPEP in albumin uptake that we describe, there is some interest in urinary aminopeptidases as potential biomarkers of tubular damage (33). Increased urinary DNPEP has been reported in response to increased salt intake in hyperthyroid rats (37). In another study, urinary DNPEP activity increases soon after cisplatin injection in rats and correlates with renal dysfunction (39). We show that DNPEP is also present in the urine of rodent models of diabetic nephropathy and anti-GBM disease. All these disease states result in proteinuria and tubular albumin overload. It is well known that increased tubular albumin concentrations cause significant impairment of the albumin endocytic apparatus (13, 17, 42). Under conditions where there is a failure of normal albumin endosomal processing, components of the pathway may well be shed into the urine (10, 21, 44).

Conversely, we show that DNPEP is present in serum but not in urine samples from normal rats. Therefore, the DNPEP that we observe in the urine in disease states may primarily reflect glomerular damage and leakage of proteins in the ~55-kDa range such as DNPEP. Further studies will be required to determine the origins of urinary DNPEP in various renal disorders. Our data, taken together with other reports, suggests that DNPEP should be investigated for its potential value as a biomarker for tubular dysfunction in renal disease.

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**DISCLOSURES**

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

**AUTHOR CONTRIBUTIONS**


**REFERENCES**

CIC-5 Binds Aspartyl Aminopeptidase


