Nedd4-2 Functionally Interacts with CIC-5

INVOLVEMENT IN CONSTITUTIVE ALBUMIN ENDOCYTOSIS IN PROXIMAL TUBULE CELLS*

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Constitutive albumin uptake by the proximal tubule is achieved by a receptor-mediated process in which the Cl⁻ channel, CIC-5, plays an obligate role. Here we investigated the functional interaction between CIC-5 and ubiquitin ligases Nedd4 and Nedd4-2 and their role in albumin uptake in opossum kidney proximal tubule (OK) cells. In vivo immunoprecipitation using an anti-HECT antibody demonstrated that CIC-5 bound to ubiquitin ligases, whereas glutathione S-transferase pull-downs confirmed that the C terminus of CIC-5 bound both Nedd4 and Nedd4-2. Nedd4-2 alone was able to alter CIC-5 currents in Xenopus oocytes by decreasing cell surface expression of CIC-5. In OK cells, a physiological concentration of albumin (10 µg/ml) rapidly increased cell surface expression of CIC-5, which was also accompanied by the ubiquitination of CIC-5. Albumin uptake was reduced by inhibiting either the lysosome or proteasome. Total levels of Nedd4-2 and proteasome activity also increased rapidly in response to albumin. Overexpression of ligase defective Nedd4-2 or knockdown of endogenous Nedd4-2 with small interfering RNA resulted in significant decreases in albumin uptake. In contrast, pathophysiologically concentrations of albumin (100 and 1000 µg/ml) reduced the levels of CIC-5 and Nedd4-2 and the activity of the proteasome to the levels seen in the absence of albumin. These data demonstrate that normal constitutive uptake of albumin by the proximal tubule requires Nedd4-2, which may act via ubiquitination to shunt CIC-5 into the endocytic pathway.

One of the major roles of the renal proximal tubule is to constitutively reabsorb proteins such as albumin that are filtered across the glomerulus (1). In humans, the kidneys filter ~180 liters of blood per day. The concentration of albumin in the glomerular filtrate in humans has recently been estimated to be 3.5 mg/liter, within the range measured in rodents and dogs (<1–50 mg/liter) (2). This translates into at least 600 mg of albumin crossing the glomerular barrier in humans each day, yet less than 30 mg is normally excreted in the urine per day (3), with the rest reabsorbed by the proximal tubule (1). The linear sequence of events by which this is accomplished is characteristic of receptor-mediated endocytosis: (i) albumin acts as a ligand for the megalin-cubulin scavenger receptor; (ii) after binding, the albumin-receptor complex is internalized into clathrin-coated pits; (iii) as the early endosome progresses to a late endosome, the intraendosomal fluid is acidified, and the albumin dissociates from the receptor complex; and (iv) the albumin is degraded in the lysosome to its constituent amino acids (1, 4). The exact molecular mechanisms, however, and the protein-protein interactions that mediate this highly active endocytic apparatus remain largely unresolved. It is now apparent that a macromolecular complex is required for efficient uptake of albumin (5). This complex includes the albumin receptor, megalin-cubulin, as well as several plasma membrane ion transporters/channels; v-type H⁺-ATPase, Na⁺-H⁺ exchanger isofrom 3 (NHE3), and the Cl⁻ channel CIC-5 (6). Each of these proteins has specific ion transporting functions with key roles in regulating the ionic composition of the vesicle during endosomal formation and acidification (6–8). More recently, it has been recognized that these proteins may have roles additional to their ion transporting activity. These involve macromolecular complex assembly and the recruitment of various signaling molecules, mediated by interactions of the intracellular carboxyl termini with diverse cytosolic proteins. Examples of this include PDZ-mediated interactions between NHE3 and the cytoskeleton and other transporters/receptors (9–11), megalin interacting with Gα-interacting protein (12), and CIC-5 interacting with coflin (5).

One of the most pronounced examples of defective albumin uptake due to a genetic disorder is observed in Dent’s disease. In this disease, patients present with persistent low molecular weight proteinuria and microalbuminuria (13). Dent’s disease is due to mutations in CIC-5 that disrupt its trafficking/function (8) with similar increases in urinary protein excretion observed in CIC-5 knock-out mice models (14, 15). In both cases, the increased protein in the urine is due to defective endocytosis in the proximal tubule. These findings point to an obligate role for CIC-5 in albumin uptake. Initially, the reduction in albumin uptake was thought to be due to CIC-5 failure to act as an anion shunt to neutralize v-type H⁺-ATPase-mediated H⁺ movement during endosomal acidification (16).

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† The abbreviations used are: NHE3, Na⁺-H⁺ exchanger isoform 3; OK, opossum kidney; CHQ, chloroquine; BSA, bovine serum albumin; Ub, ubiquitin; TR-albumin, albumin conjugated to Texas Red; MOPS, 4-morpholinepropanesulfonic acid; LLVY-AMC, 7-amino-4-methylcoumarin; MG-132, carbobenzoxy-L-leucyl-L-leucinal; siRNA, small interfering RNA.
Moreover, however, it has been shown that in Dent’s disease, defects in ClC-5 also result in mis trafficking of both megalin and v-type H+ -ATPase (17, 18). Furthermore, we have demonstrated that ClC-5 interacts with coflin to modulate the actin cytoskeleton in the local vicinity of the endocytic complex (5). These findings highlight the importance of understanding the mechanisms that underlie the trafficking of ClC-5, because the availability of ClC-5 at the plasma membrane could be predicted to be a rate-limiting factor in albumin uptake by the proximal tubule.

One mechanism for regulating cell surface levels of membrane proteins is ubiquitination by WW-HECT ubiquitin-protein ligases, such as Ned4d, Ned4d-2, and WWP2, which typically results in ubiquitination of the target protein leading to its removal from the membrane and degradation in proteasomes (19). These ligases have been shown to regulate surface expression and activity of epithelial Na+ channels in native cells (20–23), and a much wider range of channels and transporters has been observed to be regulated in this way in the Xenopus oocyte expression system, including the voltage-gated K+ channel Kv1.3 (24), the voltage-gated Na+ channel SCN5A (25), other neuronal voltage-gated Na+ (Na+) channels (26), the Na+ phosphate transporter NaPiIIb (27), the glutamate transporter EAAT1 (28), and the glutamine transporter SN1 (29) as well as the Cl− channel CIC-2 (30). The plasma membrane levels of CIC-5 have also been shown to be regulated by WWP2 in Xenopus oocytes (28). This then raises the question as to whether WWP2, or perhaps Ned4d-2/4-2, are physiological regulators of ClC-5 in vivo and whether they regulate only the surface levels of the channel itself or also CIC-5-dependent albumin endocytosis by the proximal tubule. The aim of the current study was therefore to determine whether Ned4d-4-2 has a role in constitutive albumin uptake in a cell culture model of the proximal tubule.

EXPERIMENTAL PROCEDURES

Cell Cultures—The opossum kidney (OK) cell line was obtained from Dr. D. Markovich (University of Queensland, Australia). Cells were maintained at 37 °C in D-MEM (Invitrogen) supplemented with 10% fetal bovine serum, 1% penicillin, and streptomycin and incubated at 37 °C in 5% CO2. For all experiments, OK cells were seeded to confluence and grown for 5 days to allow the formation of a polarized monolayer. The cells were then incubated for 2 days in 5 mM glucose Dulbecco’s modified Eagle’s medium/F-12 medium under serum-free conditions.

Immunoprecipitation with Anti-HECT Antibody—An antibody that recognizes the HECT domains of Ned4d and Ned4d-2 (31) was used to isolate proteins from OK cell lysate. Briefly, OK cells were lysed in lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100 (pH 7.5), and Complete Protease inhibitors (Roche Applied Science). Protein A-agarose (5 μl; Roche Applied Science) was incubated with the lysate for 3 h at 4 °C. The precleared lysate was then incubated with anti-HECT or control antibodies at 4 °C overnight. Protein A-agarose (50 μl) was then added to the sample, which was incubated for 3 h at 4 °C. The pellets were washed three times in 500 μl of wash buffer (50 mM Tris-HCl, 500 mM NaCl, 0.1% Triton X-100, pH 7.5), and the samples were eluted into Laemml gel sample buffer, separated on a 5% SDS-PAGE gel, and then transferred to nitrocellulose membranes. The blot was probed with anti-CIC-5 antibody as described previously (5).

GST Pull-down Assay—The carboxyl terminus of ClC-5 (residue Arg868 to the stop codon at position 747) was cloned into the EcoRI and XhoI sites on the vector pGEX-6P-1 (Amersham Biosciences). The GST fusion protein, GST-CIC-5-ct, was produced using the GST purification module (Amersham Biosciences) as previously described (5). For the pull-down assay, GST or GST-CIC-5-ct fusion protein (50 μg) was incubated with glutathione-Sepharose 4B beads (Amersham Biosciences) for 3 h at 4 °C. The beads were then washed by centrifugation and incubated with the Triton X-100-soluble fraction (1 mg) from OK cells at 4 °C for 18 h. The beads were then washed, and the samples were eluted into Laemml gel sample buffer, separated on a 10% SDS-PAGE gel, and transferred to nitrocellulose membranes. The Western blots were probed with anti-Ned4d and anti-Ned4d-2 primary antibodies and secondary horseradish peroxidase-conjugated antibodies and detected with SuperSignal West Pico substrate (Pierce).

Xenopus Oocyte Expression of CIC-5 and Ned4d/Nedd4-2—Capped RNA transcripts encoding full-length human ClC-5, CIC-5 containing a Y672A mutation (CIC-5 PY mut), Ned4d, Ned4d-2, or the ligase-defective cysteine to serine mutants of Ned4d and Ned4d-2 (Nedd4/Nedd4-2 Cyts mut) were synthesized using a mMESSAGE mMACHINE in vitro transcription kit (Ambion). Xenopus laevis stage V-VI oocytes were oocytes were then injected with the cRNA of the CIC-5 channel (25 ng/μl) with or without the Ned4d or Nedd4/Nedd4-2 cRNAs (10 ng/oocyte). The oocytes were incubated at 18 °C in ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 5 mM HEPES, 5 mM pyruvic acid, and 50 μg/ml gentamicin, pH 7.5) prior to recording. Three days after cRNA injection, whole cell Cl− channel currents were recorded from oocytes using the two-electrode (virtual ground circuit) voltage clamp amplifier and pCLAMP 8 software (Axon Instruments Inc, Union City, CA). The cell dialysate was low-pass filtered at 1 kHz and leak-subtracted online using a −P/6 protocol and analyzed offline. Inward Cl− currents were generated by holding the cells at −70 mV and applying step depolarizations to membrane potentials from −30 mV to +80 mV.

Surface expression of ClC-5 was determined by the method of Zerangue et al. (32). Oocytes were injected with the following RNA at 10 ng/μl: HA-tagged ClC-5 C-terminal (HA-ClC-5 C-ter), with or without Ned4d-2/Nedd4-2 Cyts mut and, as a negative control, CIC-5 without the HA tag. After 2–3 days at 18 °C, oocytes were placed in ND96 with 1% BSA to block unspecific binding and then incubated for 60 min with a rat monoclonal anti-HA antibody (1 μg/ml; 3F10; Roche Molecular Biochemicals) in 1% BSA/ND96, washed for 60 min with 1% BSA/ND96, and incubated with horseradish peroxidase-conjugated secondary antibody (goat antirabbit IgG conjugated to horseradish peroxidase (Pierce)) in 1% BSA/ND96 for 60 min. Oocytes were washed as previously described and transferred to frog Ringer solution (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl2, 5 mM HEPES, pH 7.5) without BSA. All incubations and washes were performed at 4 °C. Individual oocytes were placed in 50 μl of Supersignal Elenos Femto maximum sensitivity substrate solution (Pierce) and incubated at room temperature for 5 min. Chemiluminescence was detected using a FluorStar Optima microplate reader (BMG Technologies).

In Vivo Ubiquitination of ClC-5—Either HA-tagged ubiquitin (HA-Ub) (33) or His-tagged ubiquitin (His-Ub) (34) was transiently transfected into OK cells. Confluent monolayers were incubated in albumin (10 μg/ml) and/or carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG-132) (6 μM) for 3 h at 37 °C. Triton X-100-soluble fractions were obtained as described above. Cells expressing the HA-Ub, immunoprecipitation to detect ubiquitinated proteins was performed using the HA antibody (Roche) as described above, except that the wash solution lacked detergent. For cells expressing His-Ub, the ubiquitinated proteins were isolated using the method of Staub et al. (35). Briefly, cells were lysed in 100 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, and 6 μg MG-132, and the cell lysate was incubated with Ni2+-nitrilotriacetic acid-agarose beads (Qiagen) at 4 °C for 4 h. The beads were washed two times with 20 mM HEPES, pH 7.5, 300 mM NaCl, 0.1% Triton X-100, 10% glycerol and three times in lysis solution. In each case, bound proteins were eluted into Laemml sample buffer and separated on a 5% SDS-PAGE gel, transferred to nitrocellulose, and immunoblotted with anti-CIC-5 antibody as described previously (5).

Cell Surface Expression of CIC-5—Cell surface proteins were biotinylated using the method of Lisanti et al. (36). Briefly, confluent OK cell monolayers were exposed to albumin (10 μg/ml) and/or MG-32 (6 μM) for 3 h at 37 °C. Monolayers were washed three times in cold phosphate-buffered saline and then biotinylated with 1.22 mg/ml EZ-Link NHS-activated Strep-Tactin agarose (Pierce) at 4 °C with gentle agitation. Monolayers were washed three times in cold phosphate-buffered saline, and the cells were lysed as described previously. The biotinylated proteins were isolated by binding to ImmunoPure immobilized Streptavidin (Pierce) for 15 min on ice. The beads were pelleted, and the supernatant that contained the cysteolic (unbiotinylated) fraction was recovered by centrifugation at 4500 × g for 6 min at 4 °C. The membrane (biotinylated) fraction was washed, and the pellet was suspended in Laemml sample buffer.
buffer. Equal protein amounts of the cytosolic and of the biotinylated fraction were resolved on a 4% SDS-polyacrylamide gel and transferred to nitrocellulose, and Western blot was performed with the anti-ClC-5 antibody as previously described (5).

**Albumin Uptake**—Albumin uptake was measured using a modification of standard method as previously described (5, 37, 38). Confluent monolayers were grown in 48-well plates and treated with the different experimental conditions. Cells pretreated with MG-132 (6 μM) and/or chloroquine (CHQ) (100 μM) were incubated for 1 h at 37 °C. OK cells were transiently transfected with Nedd4 (31) and Nedd4-2 (39) and Nedd4 and Nedd4-2 Cys mut. In addition, cells were transiently transfected with pSuper (40) containing a sequence for siRNA for the Nedd4-2 (AAGTGGTTGACTCCAACGACT) (pSuperNedd4-2). In transfection experiments, the cells were transfected with the cDNA plasmids or mock-transfected with empty vector (control) and then grown for 7 days before experimentation. Transfection of the plasmid was performed following the manufacturer’s protocol with Effectorne (Qiagen), for the Nedd4/Nedd4-2 constructs and Fugene (Roche Applied Science) for the Nedd4-2 siRNA. To measure albumin uptake, the treated cells were exposed to 50 μg/ml of albumin conjugated to Texas Red (TR-albumin) (Molecular Probes) for 120 min. Nonspecific binding was determined using the method of Kirkpatrick et al. (41).

**Proteasome Activity Assay**—Proteasomal activity in OK cells was determined using the method of Kirkpatrick et al. (41). Cells were incubated with albumin (10, 100, or 1000 μg/ml) for 2 h. Cells were lysed in 0.1% Triton X-100/phosphate-buffered saline, and 250-μg aliquots of the lysates were added to 75 μM N-succinyl-Leu-Leu-Leu-Tyr-7-amino-4-methylcoumarin (LLVY-AMC) to a final volume of 100 μl. The reaction mixture was incubated at 30 °C for 1 h, and the fluorescence intensities were measured in a Fluostar Optima plate reader with wavelengths of 360-nm excitation and 460-nm emission. Background fluorescence, consisting of the reaction mixture incubated at 30 °C for 1 min, was subtracted from the fluorescence values. Data are presented as percentage of control (100%), cells not treated with albumin.

**Materials**—The generation of the polyclonal antibodies has been described previously: anti-ClC-5 (5) (kindly provided by Prof. Olivier Devayast, Division of Nephrology, Université Catholique de Louvain Medical School, Belgium) and anti-HECT (31) and anti-Nedd4-2 (42). Anti-Nedd4 antibody was kindly provided by Dr. Daniela Rotin (The Hospital for Sick Children, University of Toronto, Canada). The HA-tagged ClC-5 in the Xenopus expression vector was kindly provided by Prof. Thomas Jentsch (Centre for Molecular Neurobiology, Hamburg University, Germany). The His-tagged ubiquitin was provided by Prof. Ron Kopito (Department of Biological Sciences, Stanford University, Stanford, CA). CHQ was obtained from Sigma, MG-132 was from Calbiochem, and LLVY-AMC was from Biomol. Secondary antibodies conjugated to horseradish peroxidase were from Pierce.

**Quantification of Results and Statistical Analysis**—Densitometric analysis of the Western blot data was performed using Fujifilm ScienceLab 99 Image Gauge (version 3.3). Statistical analyses of the data were performed using analysis of variance and Dunnett’s Multiple Comparison Test or Student’s t test (oocyte data). A p value of less than 0.05 was considered significant.

**RESULTS**

**In Vitro Interaction between ClC-5 and Nedd4/Nedd4-2**—Both Nedd4 and Nedd4-2 are ubiquitin ligases that contain HECT domains; therefore, in order to determine whether ClC-5 interacted with these HECT domains, we first performed a co-immunoprecipitation in OK cell lysate using an antibody that cross-reacts with both Nedd4 and Nedd4-2 HECT domains. The subsequent Western blots clearly demonstrated that there was a physical interaction between ClC-5 and a HECT domain (Fig. 1A). We then performed a series of pull-down experiments with GST-ClC-5-ct to determine whether Nedd4 or Nedd4-2 interacted with ClC-5 in an in vitro system. GST-ClC-5-ct bound to glutathione-Sepharose beads was incubated with OK cell lysate, and the proteins bound to the beads
were eluted and then Western blotted and probed with antibodies against either Nedd4 or Nedd4-2. In the presence of GST-ClC-5-ct, both Nedd4 (~120 kDa) and Nedd4-2 (~115 kDa) were detected (Fig. 1B). The doublets observed were probably due to splice variants (43, 44). In contrast, in control samples incubated with GST alone, no bands for Nedd4 or Nedd4-2 were observed. These data confirmed that OK cells did contain both Nedd4 and Nedd4-2 and that the C terminus of ClC-5 interacted with both of these proteins.

**Nedd4/Nedd4-2 and ClC-5 Channel Activity**—The Xenopus expression system was used to investigate whether Nedd4/Nedd4-2 had any effect on the whole-cell currents mediated by ClC-5. *Xenopus* oocytes were co-injected with the cRNA for ClC-5 and wild type or Nedd4/Nedd4-2 Cys mut and standard two-electrode voltage clamp used to determine the whole-cell currents. In oocytes injected with ClC-5 cRNA alone, we observed strongly rectifying Cl⁻ currents that started between +10 to +20 mV, characteristic of ClC-5 (Fig. 2, A and B). These currents were not observed in control uninjected oocytes and furthermore, these currents were reduced in the presence of iodide, an anion that has a lower conductance through ClC-5 (data not shown). When either Neddd4 or Nedd4 Cys mut was co-expressed with ClC-5, there were no significant effects on the magnitude of the ClC-5 currents. However, in oocytes overexpressing Nedd4-2, there was a significant reduction in whole-cell currents measured at +50 mV to 72.4 ± 3.13% (n = 19; p < 0.0001) of control values (Fig. 2). Increasing the amount of Nedd4-2 cRNA injected per oocyte to 50 ng resulted in a further reduction to 54.9 ± 5.8% (n = 19; p < 0.0001) of controls. This value was significantly less (p < 0.01) than the value obtained at 10 ng of Nedd4-2. In contrast, overexpression of the Nedd4-2 Cys mut (10 ng/oocyte) resulted in a pronounced increase in whole-cell currents to 145.8 ± 7.3% (n = 20; p < 0.0001) of control values. The magnitude of this effect was not altered in oocytes injected with 50 ng of cRNA (139.4 ± 8.1%; n = 11; p < 0.0001). These data clearly demonstrated a specific and functional interaction between Nedd4-2 and ClC-5 and pointed to a specific role for Nedd4-2 in regulating the magnitude of ClC-5 currents.

**Nedd4/Nedd4-2 and Proximal Tubule Albumin Uptake**
Effects of Albumin on Total ClC-5 Levels—Given that ClC-5 has an obligate role in albumin uptake, it is possible that the cells may autoregulate the levels of ClC-5 to maintain constant levels during albumin uptake. This may involve up-regulation of the total levels of ClC-5 to cope with the increased levels of endocytosis in the presence of albumin. In control cells not exposed to albumin, there was no significant effect on the levels of ClC-5 following treatment with the proteasomal inhibitor MG-132 (Fig. 4A). However, in parallel experiments in cells exposed to albumin (10 μg/ml) for 3 h, there was a significant increase in the levels of ClC-5 to 150 ± 6% (n = 4; p < 0.001) of the levels in the absence of albumin. Proteasome inhibition in the presence of albumin further increased in ClC-5 levels to 218 ± 22% of control levels (n = 4; p < 0.001), a level significantly greater with albumin alone (p < 0.01; Fig. 4B), indicating that the proteasome also plays a role in regulating levels of ClC-5. Thus, the presence of normal tubular levels of albumin results in a rapid elevation in the levels of total ClC-5 protein, presumably reflecting an increased requirement for ClC-5 upon initiation of albumin uptake.

Cell Surface Biotinylation—The above data were consistent with physiological levels of albumin leading to increased ClC-5 at the plasma membrane in order to initiate albumin endocytosis. We therefore used cell surface biotinylation to determine any changes in the surface levels of ClC-5 in response to albumin. Each experimental protocol was repeated three times on separate batches of cells. Representative blots and results are shown in Fig. 4. In control cells (not exposed to albumin), there was a detectable level of ClC-5 in the plasma membrane (Fig. 4, C and D). Under these conditions, inhibition of the proteasome with MG132 resulted in a significant increase in the surface levels of ClC-5, most likely due to the known effect of proteasome inhibition on endocytosis and recycling (45, 46). Importantly, exposure to albumin (10 μg/ml) for 3 h caused a significant increase in the amount of ClC-5 at the cell surface to 554 ± 95% (n = 3; p < 0.01) of control levels (Fig. 4, C and D), consistent with the increase in total ClC-5 observed in response to albumin (Fig. 4A). Inhibition of the proteasome in the presence of albumin caused a further small but significant (p < 0.05) increase in the cell surface levels of ClC-5 above those observed in the presence of albumin. In contrast, no significant changes were observed in the levels of cytosolic ClC-5, indicating that the increase in total ClC-5 in response to albumin is primarily due to increased ClC-5 in the membrane fraction.

Ubiquitination of ClC-5—Removal of a plasma membrane protein by Nedd4-2 is generally associated with polyubiquitination of that protein. We used different methods to determine the ubiquitination status of ClC-5 in the presence of albumin. OK cells transfected with either HA-Ub or His-Ub were exposed to albumin and MG-132. The HA-tagged samples were immunoprecipitated using anti-HA antibody, whereas the His-tagged samples were harvested using Ni²⁺-nitrilotriacetic acid beads. In both cases, the bound proteins were eluted and run on Western blots and probed with an antibody against ClC-5. Each experimental protocol was repeated three times on separate batches of cells. Representative results are shown in Fig. 5, A and B. Under control conditions (no albumin), there were no bands visible for ClC-5 and only very faint bands in the presence of MG-132. In cells incubated with albumin, there was a faint band for ClC-5 in the cells expressing the HA-Ub. In contrast, in cells exposed to albumin and MG-132, there were large single bands for both HA- and His-Ub. These data clearly show that ClC-5 is ubiquitinated in the presence of albumin, but this species is short lived, since it can only be detected under conditions in which the normal albumin uptake pathway is disrupted with MG-132.
Degradation Pathways and Albumin Uptake—The monoubiquitination of ClC-5 in the presence of albumin suggested that the channel was being shunted into the endocytic/lysosome pathway as a result of albumin uptake. Blocking the lysosomes with CHQ reduced TR-albumin uptake to 60 ± 7% (n = 3; p < 0.05) of control levels (Fig. 6). Similarly, inhibiting the proteasome with MG-132 reduced TR-albumin uptake to 59 ± 6% (n = 3; p < 0.05) of control levels (Fig. 6). Treatment
of cells with both MG-132 and CHQ further reduced TR-albumin uptake to 29 ± 6% (n = 3; p < 0.05) of the control levels, a level significantly lower than that observed for either treatment alone, suggestive of an additive effect (Fig. 6). These experiments demonstrated that both the lysosomal and proteosomal pathways are required for efficient albumin processing by OK cells.

**Nedd4/Nedd4-2 and Albumin Uptake**—The data in the oocytes showed that the cell surface levels of CIC-5 could be regulated by Nedd4-2 and could hence represent a rate-limiting step in albumin uptake. We therefore investigated the effects of Nedd4 and Nedd4-2 overexpression or suppression on albumin uptake. OK cells were transfected with wild type Nedd4 or Nedd4-2 or the Cys mutants. Overexpression of wild type Nedd4 or Nedd4-2 or Nedd4 Cys mut had no significant effects on TR-albumin uptake (Fig. 7A). In contrast, overexpression of the Nedd4-2 Cys mut reduced TR-albumin uptake to 67 ± 7% (n = 3; p < 0.05) of control levels. It is important to note, as we have previously published (5), that the transfection efficiency of the OK cells under these conditions is of the order of 50–60%; therefore, the ~33% reduction we observe in TR-albumin uptake in cells overexpressing Nedd4-2 Cys mut.

Since overexpression strategies may alter the expression of other proteins, we then used a silencing RNA approach to confirm the specificity of the role of Nedd4-2 in regulating albumin uptake. Cells were transiently transfected with pSuper control plasmid or pSuperNedd4-2. We first investigated whether this siRNA specifically suppressed Nedd4-2 in OK cells. Cells were transiently transfected and Western blots for Nedd4 and Nedd4-2 performed on Triton X-100-soluble fractions. The presence of the siRNA caused a pronounced reduction in the levels of Nedd4-2 to 30 ± 9% (n = 3; p < 0.01) of control, cells transfected with pSuper alone, whereas the levels of Nedd4 remained unchanged (Fig. 7B). The large effect of the siRNA in these Western blot experiments reflects a higher expression efficiency of the siRNA driven by a promiscuous promoter, such that even cells that only contain only a minimal amount of the plasmid DNA still produce enough silencing RNA to knock down the levels of Nedd4-2. We then measured albumin uptake in cells expressing pSuperNedd4-2 and found that TR-albumin uptake was significantly reduced to 72 ± 2% (n = 4; p < 0.001) of control levels (Fig. 7C). These data clearly show that Nedd4-2 is a specific physiological regulator of constitutive albumin uptake in OK cells.

**Proteasome Activity**—The previous experiments suggested a role for the proteasome in regulating albumin uptake. We therefore investigated the effects of albumin on the activity of the proteasome itself. We used a fluorescent proteasome substrate (LLVY-AMC) to directly measure proteasomal activity in OK cells. Exposure to albumin (10 μg/ml) for 2 h significantly increased proteasomal activity with LLVY-AMC fluorescence increasing to 171 ± 25% (n = 4; p < 0.01) of control levels (Fig. 8). We repeated the experiments in the presence of higher concentrations of albumin. Interestingly, as the concentration of albumin increased, proteasome activity progressively decreased, such that at 1000 μg/ml albumin, the activity was only 131 ± 18% (n = 4; p < 0.01) of control, a value significantly less than that observed with 10 μg/ml albumin (Fig. 8).

**Protein Levels of Nedd4 and Nedd4-2**—Elevated levels of ubiquitin ligases have been reported in chronic disease states such as muscle wasting (47). We used Western blotting to investigate whether albumin uptake was associated with any acute changes in the protein levels of CIC-5 and Nedd4/Nedd4-2 upon activation of the albumin uptake pathway. Exposure to albumin (10 μg/ml) for 2 h resulted in a rapid and pronounced increase in the levels of Nedd4-2 protein to 187 ± 8% (n = 3; p < 0.001) of control levels (Fig. 9). This effect was exclusive for Nedd4-2, since no change in the level of Nedd4 was observed under these conditions. Although the levels of Nedd4-2 remained elevated at higher concentrations (100 μg/ml) of albumin (163 ± 13%; n = 3; p < 0.05), there was a reversal toward control levels, with the level at 1000 μg/ml albumin being significantly lower than that observed at 10 μg/ml albumin (131 ± 5%; n = 3; p < 0.01,

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**Fig. 5. CIC-5 is ubiquitinated in the presence of albumin.** A. OK cells were transiently transfected with His-UB and the ubiquitinated proteins isolated using Ni²⁺-nitrilotriacetic acid-agarose beads following exposure to albumin (10 μg/ml) and/or MG-132 (6 μM) for 3 h. B. OK cells were transiently transfected with HA-UB, and the ubiquitinated proteins were isolated by immunoprecipitation using anti-HA antibody following exposure to albumin (10 μg/ml) and or MG-132 (6 μM) for 3 h. These data suggest that CIC-5 was rapidly ubiquitinated in the presence of albumin.
Similarly, exposure of cells to 10 μg/ml albumin caused a pronounced increase in the levels of ClC-5 (206 ± 20%; n = 3, Fig. 9, C and D). This increase persisted in cells exposed to 100 μg/ml albumin (158 ± 29%, Fig. 9, C and D). However, when the cells were exposed to 1000 μg/ml albumin, the levels of ClC-5 protein returned to control levels (Fig. 9, C and D). This reduction in ClC-5 protein at 1000 μg/ml albumin was significantly different from that in cells exposed to 10 μg/ml albumin (n = 3, p < 0.01).

**DISCUSSION**

ClC-5 appears to play an obligate role in facilitating albumin uptake by the proximal tubule at least at two levels. First, it is involved in formation of the endocytic complex (5, 15), and...
second, it plays an important role as an anion shunt during the acidification of the endosomes (16). Therefore, the cell surface availability of CIC-5 could be predicted to be a rate-limiting step in albumin uptake, and CIC-5 must be routed into the albumin degradative pathway. Physiological levels of albumin appear to trigger an endocytic pathway that involves a significant increase in the turnover of plasma membrane components and presumably up-regulation of the proteins involved. This is supported by studies from our group and others that show that albumin causes an increase in the protein levels and activity of NHE3 (48, 49).

The molecular mechanisms that govern the assembly of the albumin endocytic complex and how surface levels of CIC-5 are maintained remain largely unclear but are likely to involve C-terminal interactions between the various proteins within the complex and other cytosolic regulators. The current study further characterizes the molecular changes that take place in OK cells to enable constitutive albumin uptake in response to physiological levels of albumin.

FIG. 8. **Proteasomal activity in response to albumin.** OK cells were exposed to increasing concentrations of albumin for 2 h, and then the relative fluorescence intensity of the proteasomal substrate LLVY-AMC was determined. Data are expressed as mean ± S.E. of three separate experiments (*, p < 0.01 relative to control; +, p < 0.05; 1000 μg/ml albumin relative to 10 μg/ml albumin).

FIG. 9. **Acute effects of albumin on cellular levels of Nedd4/Nedd4-2 and CIC-5.** A, representative Western blots of lysates from OK cells exposed to albumin (10 μg/ml) for 2 h. Blots were probed with antibodies directed against either Nedd4 or Nedd4-2. B, densitometric analysis of Nedd4/Nedd4-2 levels in lysates from OK cells exposed to albumin (10 μg/ml). Data are expressed as mean ± S.E. of three separate experiments (*, p < 0.001). C, representative Western blot of Nedd4-2 and CIC-5 levels in lysates from OK cells exposed to increasing concentrations of albumin. D, densitometric analysis of Nedd4-2 (black) and CIC-5 (gray) levels in lysates from OK cells exposed to increasing concentrations of albumin. Data are expressed as mean ± S.E. of three separate experiments (*, p < 0.01 relative to control; +, p < 0.05 relative to 10 μg/ml albumin).
In this paper, we demonstrate protein-protein interactions between the C terminus of CIC-5 and the ubiquitin ligases Nedd4/Nedd4-2 and that Nedd4-2 is a physiological regulator of constitutive albumin uptake by cells of proximal tubule origin. We also show that albumin increases both the total amount of CIC-5 and the levels of CIC-5 at the plasma membrane and that CIC-5 is ubiquitinated, providing a mechanism to route CIC-5 into the endocytic pathway.

Using in vitro techniques, we showed that CIC-5 interacted with both Nedd4 and Nedd4-2. In Xenopus oocytes, however, we found that overexpression of Nedd4-2 maximally reduced CIC-5-mediated currents and cell surface expression of CIC-5 by ~50%, whereas Nedd4 had no effect. This contrasts with the regulation of ENaC and Na$_{v}$1.2 and Na$_{v}$1.7 in Xenopus oocytes, where maximal levels of Nedd4-2 almost completely abolish ENaC currents, whereas Nedd4 only partially inhibits the currents (21, 26, 33, 50, 51). The reasons for the differential efficacy of Nedd4-2 in Xenopus oocytes on ENaC, Na$_{v}$, and CIC-5 currents remain unresolved. It is possible that this reflects the need for an adaptor protein that is not present in oocytes in concentrations sufficient to optimize Nedd4-2 actions on CIC-5 or, alternatively, another E3 ubiquitin ligase being the endogenous effector in oocytes.

The observation that Nedd4-2 acts on CIC-5 via the C-terminal PY motif is consistent with the previously observed action of the WW domains of WWP2 (19). Similarly, the effect of the Nedd4-2 Cys mut in increasing CIC-5 currents is comparable with that reported for the ligase-defective WWP2 (19). In contrast, the Nedd4 Cys mut had no effect on CIC-5 currents, confirming that Nedd4 has no action on CIC-5 in oocytes. Interestingly, overexpression of wild type WWP2 was reported to have no effect on CIC-5 currents in Xenopus oocytes (19). In the current study, however, we observed a pronounced inhibition of CIC-5 currents in the presence of wild type Nedd4-2 that increased from ~25 to ~50% inhibition as the concentration of Nedd4-2 cRNA was increased from 10 to 50 ng per oocyte. Thus, the lack of effect of WWP2 on CIC-5 currents may be simply due to the fact that a lower concentration of cRNA (5 ng/oocyte) was used in these experiments (19) or, alternatively, that Nedd4-2 has a higher affinity than WWP2 for CIC-5 in this system. Importantly, the effect of Nedd4-2 on the whole-cell currents was due to an actual reduction in the number of CIC-5 channels at the plasma membrane, highlighting the ability of Nedd4-2 to regulate the cell surface availability of CIC-5.

Nedd4-2 induces the internalization of its target protein by ubiquitination. ENaC is polyubiquitinated, leading to its removal from the membrane and degradation in the lysosome (34) and proteasome (52). An important finding of the current study is that in response to albumin, CIC-5 is ubiquitinated. A previous study investigating the interactions of WWP2 with CIC-5 reported no success in demonstrating the ubiquitination of CIC-5, an effect that was attributed to the possibility that the ubiquitinated species may be short lived in a cellular system (19). In support of this, we found that we could only detect the significant levels of the ubiquitinated species induced by the presence of albumin when the endocytic pathway is inhibited by blocking the proteasome. Furthermore, preliminary in vitro studies also suggest that CIC-5 is ubiquitinated.$^{2}$ Our data suggest the presence of a monoubiquitinated species of CIC-5; however, due to the limitations of the methods, we cannot rule out that CIC-5 is polyubiquitinated. Monoubiquitination is regarded as a signal for endocytosis (45, 53), resulting in the trafficking of the target protein to multivesicular bodies and subsequently the lysosome (46), or alternatively, the protein may be deubiquitinated and returned to the recycling endosomes (54). We therefore demonstrate that albumin activates a specific pathway that is not active in its absence. Inhibition of the proteasome or control cells also increased surface levels of CIC-5, although CIC-5 is not ubiquitinated under these conditions. This can be explained by the known actions of proteasomal inhibition in generally disrupting the endocytosis of membrane proteins (54, 55). For example, inhibition of proteasome was found to promote epidermal growth factor receptor recycling and to block its degradation (46) and also to block the normal trafficking of low density lipoprotein receptor-related protein into the internal multivesicular bodies (45), with low density lipoprotein receptor-related protein rapidly recycling and accumulating at the cell surface. It is likely that such a phenomenon also underlies the effects of MG132 on surface levels of CIC-5 in OK cells not exposed to albumin.

The role of the proteasome in regulating levels of CIC-5 contrasts markedly with the findings for the sodium phosphate transporter type II, where inhibition of the proteasome in OK cells had no effect on the rates of parathyroid hormone-induced degradation of this membrane transporter (56). In comparison, both lysosomal and proteasomal inhibition have been shown to regulate surface levels of ENaC (34, 52). The observation that inhibition of both the proteasome and the lysosome has an additive effect in reducing albumin uptake suggests that under normal conditions, these pathways act in concert to degrade various protein components of the endocytic complex. Albumin uptake increases with the extracellular concentration of albumin within the physiological range (37). There must be a corresponding increase in the degradation of the proteins involved. The fact that we observe a dramatic reversal in the levels of total cellular CIC-5 and Nedd4-2 at a high (pathophysiological) concentration of albumin (1 mg/ml) presumably reflects a shutdown of this pathway due to depletion of key components. The ubiquitin proteasome pathway also tightly controls the levels of key regulatory molecules involved in a myriad of cellular pathways, from cyclins to transcription factors through to ion channels, receptors, and endocytosis (57). Thus, the reduced proteasomal activity and Nedd4-2 levels we observe at higher pathophysiological levels of albumin may

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$^{2}$ A. B. Fotia and S. Kumar, unpublished data.
Proteins containing PY motifs may interact with multiple members of the Nedd4 family of ubiquitin ligases, yet each of these ligases are themselves subject to specific regulation by other proteins (24, 27, 60). Thus, it is critical to determine which ubiquitin ligase is the true physiological regulator in a given cell type. We therefore employed both dominant negative mutant and silencing RNA strategies to determine that Nedd4-2 had a role in constitutive albumin uptake in OK cells. We found that only Nedd4-2 Cys mut was able to significantly reduce albumin uptake, whereas Nedd4 Cys mut was without effect, a result consistent with the effects of these mutants on CIC-5 activity in oocytes. The physiological role of Nedd4-2 was confirmed by the use of the siRNA plasmid directed against Nedd4-2, which also inhibited albumin uptake. The specificity of this effect was confirmed by the fact that in cells transfected with the siRNA against Nedd4-2, endogenous Nedd4 levels remained unchanged. The fact, however, that we do not observe strong suppression of albumin uptake despite a ~70% reduction in Nedd4-2 protein suggests either the involvement of an intermediate protein or possibly that another ubiquitin ligase (e.g. WWP2) can partially substitute for Nedd4-2. The data from the current study are in agreement with the recent findings of Snyder and co-workers, who used small silencing RNA oligonucleotides to demonstrate that Nedd4-2 but not Nedd4 was the physiological regulator of ENaC in two epithelial cell lines (Fischer rat thyroid and H441) (23). More recently, we have also shown that Nedd4-2 is the specific ubiquitin ligase that acts on the voltage-gated Na\(^+\) channel, Na\(_1\).8 (26). The specific role of Nedd4-2 in albumin uptake is further strengthened by our data showing a rapid increase in protein levels of Nedd4-2 but not Nedd4 in response to albumin, which parallels the increase in CIC-5. Acute increases in total Nedd4/Nedd4-2 protein levels in response to activation of a cellular pathway have not previously been reported. Given that the OK cells used in this study express both Nedd4 and Nedd4-2, it is unclear why only Nedd4-2 is specifically activated by albumin.

In conclusion, the current study identifies Nedd4-2 as a physiological regulator of albumin uptake and further defines the obligate role of CIC-5 in albumin uptake by the proximal tubule. We present a new model for the regulation of albumin uptake (Fig. 10). (i) In the absence of albumin, CIC-5 is located primarily in recycling endosomes (14), similar to NHE3 (10), with some presence at the cell surface that is not ubiquitinated. (ii) The presence of albumin triggers the formation of an endocytic complex that includes CIC-5. (iii) Nedd4-2 is recruited to this complex and ubiquinates CIC-5. This ubiquitination by Nedd4-2 shunts CIC-5 into the albumin uptake/degradative pathway. Some CIC-5 may recycle back to the plasma membrane pool. (iv) In response to the increased requirement of these proteins, the cell produces more CIC-5 and Nedd4-2. (v) The increase in membrane turnover/endocytosis and resultant degradation of albumin is accompanied by a significant increase in pro teaseal activity. In this case, the proteasome presumably plays a key role in maintaining the integrity of the endocytic apparatus by regulating the levels of specific components of this pathway. Our data show that, in addition to its role in regulating Na\(^+\) reabsorption in the distal tubules, Nedd4-2 plays a key role in mediating another constitutive function of the kidney, namely albumin uptake by the proximal tubule. It will be of interest to determine the extent to which ubiquitination also regulates the levels of other membrane proteins associated with the albumin uptake complex, such as NHE3 or megalin.
Nedd4-2 Functionally Interacts with CIC-5: INVOLVEMENT IN CONSTITUTIVE ALBUMIN ENDOCYTOSIS IN PROXIMAL TUBULE CELLS
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