Nedd4-2 Functionally Interacts with CIC-5

IN Volvement in Constitutive Albumin Endocytosis in Proximal Tubule Cells*

Deanne H. Hryciw‡, Jenny Ekberg‡, Aven Lee‡, Ingrid L. Lensink§, Sharad Kumar§, William B. Guggino¶, David I. Cooki, Carol A. Pollock** and Philip Poronnik‡‡

From the ‡School of Biomedical Sciences, The University of Queensland, Brisbane, Queensland 4072, Australia, §Hanson Institute, Institute of Medical and Veterinary Sciences, Adelaide, South Australia 5000, Australia, the ¶Department of Physiology, School of Medicine, The Johns Hopkins University, Baltimore, Maryland 21205, the ‡‡Department of Physiology, University of Sydney, New South Wales 2006, Australia, and the Kolling Institute, Royal North Shore Hospital, University of Sydney, Sydney, New South Wales 2065, Australia

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 \(\mu\)g/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, pathophysiological concentrations of albumin (100 and 1000 \(\mu\)g/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 intravesicular 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 isoform 3 (NHE3),1 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 Ga-interacting protein (12), and CIC-5 interacting with cofilin (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/func-

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†† To whom correspondence should be addressed: School of Biomedical Sciences, The University of Queensland, St. Lucia, Queensland 4072, Australia. Fax: 61-7-3365-1766; E-mail: p.poronnik@uq.edu.au.

1 The abbreviations used are: NHE3, Na\(^{+}\)-H\(^{+}\) exchanger isoform 3; OK, opossum kidney; CHQ, chloroquine; BSA, bovine serum albumin; T-TR-albumin, albumin conjugated to Texas Red; MOPS, 4-morpholinepropanesulfonic acid; LLVY-AMC, N\(\alpha\)-succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin; MG-132, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; siRNA, small interfering RNA.
For that °C. The beads were then washed by centrifugation and incubated with glutathione-Sepharose 4B beads (Amersham Biosciences) as previously described. For the GST Pull-down Assay—

Immunoprecipitation with Anti-HECT Antibody—

EXPERIMENTAL PROCEDURES

Cell Cultures—The opossum kidney (OK) cell line was obtained from Dr. D. Markovich (University of Queensland, Australia). Cells were maintained in Dubnoff’s modified Eagle’s medium (Gibco, Grand Island, NY) 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 μg/ml 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 Nedd4 and Nedd4-2 (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 preclared lysate was then incubated with anti-HECT or control antibodies at 4 °C overnight. Protein A-agarose (50 μl) was again added to the sample, which was incubated for 3 h at 4 °C. The pellet was 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 Laemmli gel sample buffer, separated on a 5% SDS-PAGE gel, and then transferred to nitrocellulose membranes.

GST Pull-down Assay—The carboxyl terminus of CIC-5 (residue Arg1085 to the stop codon at position 1087) 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-f 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 Laemmli gel sample buffer, separated on a 10% SDS-PAGE gel, and transferred to nitrocellulose membranes. The Western blots were probed with anti-Nedd4 and anti-Nedd4-2 primary antibodies and secondary horseradish peroxidase-conjugated antibodies and detected with SuperSignal West Pico substrate (Pierce).

Xenopus Oocyte Expression of CIC-5 and Nedd4-2—Capped RNA transcripts encoding full-length human CIC-5, CIC-5 containing a Y672A mutation (CIC-5 PYmut), Nedd4-2, or the ligase-defective cysteine to serine mutants of Nedd4 and Nedd4-2 (Nedd4-2(Nedd4-2 Cys mut)) were synthesized using a mMESSAGE mMACHINE mMachine in vitro transcription kit (Ambion). Xenopus laevis stage V-VI oocytes were removed and treated with collagenase (Sigma Type I) for defolliculation. The oocytes were then injected with the cRNA of the CIC-5 channel (25 ng/oocyte) with or without the Nedd4 or Nedd4-2 constructs (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 technique. Microelectrodes were filled with 3 M KC1 and typically had resistances of 0.3–1.5 megohms. All recordings were made at room temperature (20–23 °C) using a bath solution containing the following components: 100 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 0.3 mM CaCl₂, 20 mM Heps, pH 7.5, with NaOH. During recording, oocytes were perfused continuously at a rate of ~1.5 μl/min. Using a GeneClamp 500B amplifier and pCLAMP 8 software (Axon Instruments Inc, Union City, CA), current responses were low pass filtered at 1 kHz, digitized at 10 kHz, and leak subtracted on-line using a −P/6 protocol and analyzed off-line. 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 CIC-5 was determined by the method of Zerangue et al. (32). Oocytes were injected with the following RNA at 10 ng/oocyte: HA-tagged CIC-5, a kind gift of Prof. Thomas Jentsch (Hamburg, Germany), with or without Nedd4-2/Nedd4-2 Cys 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-coupled secondary antibodies (goat anti-mouse 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 Eleno Femto maximum sensitivity substrate solution (Pierce) and incubated at room temperature for 5 min. Chemiluminescence was measured using a Fluostar Optima microplate reader (BMG Technologies). In Vivo Ubiquitination of CIC-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 carbobenzyoxy-L-leucyl-L-leucyl-L-leucinal (MG-132) (6 μM) for 6 h at 37 °C. Triton X-100-soluble fractions were obtained as described above. For cells expressing the HA-Ub, immunoprecipitation to detected 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, 1.5 mM MgCl₂, and 10% glyceral and three times in lysis solution. In each case, bound proteins were eluted into Laemmli 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-LC-Biotin (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 Strepavidin (Pierce) for 15 min on ice. The beads were pelleted, and the supernatant that contained the cytosolic (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 Laemmli sample buffer.
buffer. Equal protein amounts of the cytosolic and of the biotinylated fraction were resolved on a 5% 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 Nedd4-2 (AAGTGGTTGACTCCAACGACT) (pSuperNedd4-2) and Nedd4 and Nedd4-2 Cys mut. In addition, cells were transiently transfected with pSuper (40) containing a sequence for siRNA for Nedd4-2 (AAATGTTTACCTCAAGCAGT) (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 Effectene (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 in cells exposed to albumin for 1 min. Receptor-mediated albumin uptake is effectively abolished by disruption of the actin cytoskeleton (5, 37), with total TR-albumin uptake being reduced to less than 80% of control values in the presence of latrunculin A or cytochalasin B. The residual (>20%) component represents nonspecific albumin uptake mechanisms present in all cell types (e.g. fluid phase uptake). In the current study, we defined receptor-mediated TR-albumin uptake as that component of albumin uptake that is sensitive to inhibition of actin polymerization by latrunculin A. At the end of the uptake period, cells were washed in HEPES buffer, pH 6, at 4 °C and then solubilized in MOPS lysis buffer (20 mM MOPS, 0.1% Triton X-100, pH 7.4). The TR-albumin fluorescence was determined using 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 Devaeyst, 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 Ned4 or Ned4-2. In the presence of GST-CIC-5, both Ned4 (~120 kDa) and Ned4-2 (~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 Ned4 or Ned4-2 were observed. These data confirmed that OK cells did contain both Ned4 and Ned4-2 and that the C terminus of CIC-5 interacted with both of these proteins.

**Nedd4/Nedd4-2 and ClC-5 Channel Activity**—The Xenopus expression system was used to investigate whether Ned4/Nedd4-2 had any effect on the whole-cell currents mediated by CIC-5. Xenopus oocytes were co-injected with the cRNA for ClC-5 and wild type or Ned4/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 un.injected 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 Ned4 or Ned4-2 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 Ned4-2, 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 Ned4-2 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 Ned4-2. In contrast, overexpression of the Ned4-2 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 Ned4-2 and ClC-5 and pointed to a specific role for Ned4-2 in regulating the magnitude of ClC-5 currents.

Ned4/Nedd4-2 typically bind a PY motif in the C terminus of plasma membrane proteins, and mutations in this motif result in a loss of Ned4/Nedd4-2 binding (34, 39). We therefore used ClC-5 PY mut and repeated the above oocyte experiments. In oocytes injected with ClC-5 PY mut, the Cl− currents increased to the same levels (142.5 ± 7.2%; n = 15; p < 0.0001) as those in oocytes co-expressing wild type ClC-5 with Ned4-2.

**Fig. 2.** Effects of Ned4/Nedd4-2 on ClC-5 currents in Xenopus oocytes. A, representative current traces from oocytes expressing ClC-5 alone, ClC-5 plus Ned4-2, ClC-5 plus Ned4-2 Cys mut, or ClC-5 PY mut. B, current-voltage relationships for oocytes expressing ClC-5 with the various Ned4/Nedd4-2 constructs or ClC-5 PY mut expressed alone. Individual points represent the mean ± S.E. of 15–20 oocytes from three or four separate batches. C, bar graphs showing the percentage changes in the Cl− current amplitude at +50 mV relative to the control (I/Io) (oocytes expressing only ClC-5). ClC-5 was co-expressed in oocytes with the various Ned4/Nedd4-2 constructs, or ClC-5 PY mut was expressed alone. Data are expressed as the mean ± S.E. of 15–20 oocytes from three or four separate batches.
Fig. 3. Cell surface expression of ClC-5 is regulated by Nedd4-2 in Xenopus oocytes. Surface expression of HA-tagged ClC-5 in the absence and presence of Nedd4-42 or Nedd4-2 Cys mut. Surface levels of HA-CIC-5 were measured using an antibody-based chemiluminescence assay. Oocytes injected with ClC-5 without a HA tag were used as a negative control. Data are expressed in relative light units (RLU) per oocyte, normalized to HA-tagged ClC-5 expressed alone. Data are expressed as mean ± S.E. of 40 oocytes from 12 batches (\( * \), \( p < 0.001 \) relative to control).

Cys mut. Furthermore, when the CIC-5 PY mut was co-expressed with either wild type Nedd4/Nedd4-2 or Nedd4/Nedd4-2 Cys mut, the currents remained elevated above the controls. This clearly indicated that abolition of the PY domain prevented any effect of Nedd4-2 on CIC-5. The increases in CIC-5 currents under these conditions suggested constitutive endogenous Nedd4/Nedd4-2 activity in oocytes that influenced the surface levels of CIC-5. Overexpression of Nedd4-2 Cys mut overrode this effect, resulting in CIC-5 currents that were identical in magnitude to those observed in oocytes expressing the CIC-5 PY mut.

The above data were consistent with the decrease in CIC-5 currents in response to Nedd4-2 due to a reduction in the number of CIC-5 channels at the cell surface. We confirmed this hypothesis using a luminescence-based cell surface assay and a CIC-5 channel expressing an extracellular HA-epitope (HA-CIC-5) (19). Oocytes were coinjected with the mRNA for the HA-CIC-5 channel expressing an extracellular HA-epitope (HA-ClC-5) (19). Oocytes were coinjected with the mRNA for the HA-ClC-5 with or without Nedd4-2 or Nedd4-2 Cys mut, and the surface HA-ClC-5 was detected in a luminometer using a luciferase secondary antibody system. Results were calculated as relative light units per oocyte standardized to HA-ClC-5 (Fig. 3). Control oocytes expressing HA-ClC-5 had a strong signal (\( n = 42 \); standardized to 100 ± 4.3%). Coexpression with Nedd4-2 reduced surface levels of HA-ClC-5 to 72.4 ± 4.8% of control levels (\( n = 40; \ p < 0.001 \)). In contrast, coexpression with Nedd4-2 Cys mut resulted in an increase in the cell surface levels of HA-CIC-5 to 193.8 ± 12.2% compared with control (\( n = 35; \ p < 0.0001 \)). In oocytes expressing a nontagged CIC-5, there was almost no background signal detected (2.2 ± 0.7% of control (\( n = 40; \ p < 0.0001 \)). The data were obtained from oocytes from three separate batches with at least 12 oocytes per group analyzed in each experiment. These experiments clearly demonstrated that the Nedd4-2-mediated changes in CIC-5 currents were due to changes in cell surface levels of the channel.

Effects of Albumin on Total CIC-5 Levels—Given that CIC-5 has an obligate role in albumin uptake, it is possible that the cells may autoregulate the levels of CIC-5 to maintain constant levels during albumin uptake. This may involve up-regulation of the total levels of CIC-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 CIC-5 following treatment with the proteasomal inhibitor MG-132 (Fig. 4A). However, in parallel experiments in cells exposed to albumin (10 \( \mu \)g/ml) for 3 h, there was a significant increase in the levels of CIC-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 CIC-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 CIC-5. Thus, the presence of normal tubular levels of albumin results in a rapid elevation in the levels of total CIC-5 protein, presumably reflecting an increased requirement for CIC-5 upon initiation of albumin uptake.

Cell Surface Biotinylation—The above data were consistent with physiological levels of albumin leading to increased CIC-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 CIC-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 CIC-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 CIC-5, most likely due to the known effect of proteasome inhibition on endocytosis and recycling (45, 46). Importantly, exposure to albumin (10 \( \mu \)g/ml) for 3 h caused a significant increase in the amount of CIC-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 CIC-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 CIC-5 above those observed in the presence of albumin. In contrast, no significant changes were observed in the levels of cytosolic CIC-5, indicating that the increase in total CIC-5 in response to albumin is primarily due to increased CIC-5 in the membrane fraction.

Ubiquitination of CIC-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 CIC-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 Ni\(^{2+}\)-nitrioltriacetic acid beads. In both cases, the bound proteins were eluted and run on Western blots and probed with an antibody against CIC-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 CIC-5 and only very faint bands in the presence of MG-132. In cells incubated with albumin, there was a faint band for CIC-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 CIC-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 CIC-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 proteasomal pathways are required for efficient albumin processing by OK cells.

*Nedd4* and *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 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 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).

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. 9, A and B). This effect was exclusive for Nedd4-2, since no change in the protein levels of Nedd4 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. 9C). 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, the activity was only 131 ± 18% (n = 3; p < 0.01), 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. 9C). 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,

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 < 0.0001) of control levels (Fig. 7C). These data clearly show that Nedd4-2 is a specific physiological regulator of constitutive albumin uptake in OK cells.
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 Na₅₁₂ and Na₅₁₋₇ 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, Na₅, 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 pg 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 in 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 therefore 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

² A. B. Fotia and S. Kumar, unpublished data.
contribute in part to the tubular hypertrophy (58) typically observed in diabetic renal disease, where reduced proteasomal activity has also been reported (59).

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, Na1.8 (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 present at the cell surface that is not ubiquitinated. (ii) 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, Na1.8 (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.
Nedd4-2 and Proximal Tubule Albumin Uptake

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