

## An Internalization Signal in ClC-5, an Endosomal Cl<sup>-</sup> Channel Mutated in Dent's Disease\*

Received for publication, November 26, 2000

Published, JBC Papers in Press, December 14, 2000, DOI 10.1074/jbc.M010642200

Michael Schwake‡, Thomas Friedrich§, and Thomas J. Jentsch¶

From the Zentrum für Molekulare Neurobiologie Hamburg, Hamburg University, Falkenried 94, D-20246 Hamburg, Germany

**The ClC-5 chloride channel resides mainly in vesicles of the endocytotic pathway and contributes to their acidification. Its disruption in mice entails a broad defect in renal endocytosis and causes secondary changes in calciotropic hormone levels. Inactivating mutations in Dent's disease lead to proteinuria and kidney stones. Possibly by recycling, a small fraction of ClC-5 also reaches the plasma membrane. Here we identify a carboxyl-terminal internalization motif in ClC-5. It resembles the PY motif, which is crucial for the endocytosis and degradation of epithelial Na<sup>+</sup> channels. Mutating this motif increases surface expression and currents about 2-fold. This is probably because of interactions with WW domains, because dominant negative mutants of the ubiquitin-protein ligase WWP2 increased surface expression and currents of ClC-5 only when its PY motif was intact. Stimulating endocytosis by expressing rab5 or its GTPase-deficient Q79L mutant decreased WT ClC-5 currents but did not affect channels with mutated motifs. Similarly, decreasing endocytosis by expressing the inactive S34N mutant of rab5 increased ClC-5 currents only if its PY-like motif was intact. Thus, the endocytosis of ClC-5, which itself is crucial for the endocytosis of other proteins, depends on the interaction of a carboxyl-terminal internalization signal with ubiquitin-protein ligases containing WW domains.**

CLC chloride channels form a gene family of chloride channels that has at least nine members in mammals (1). Based on homology, it can be divided into three branches. Channels of the first branch perform their physiological function in the plasma membrane. In some instances, this conclusion is also supported by inherited disease. Mutations in the ClC-1 chloride channel cause myotonia (2, 3) because its conductance is essential for the electrical stability of the muscle plasma membrane. Mutations in the human ClC-Kb kidney chloride channel are a cause of Bartter's syndrome (4), and disruption of the mouse ClC-K1 leads to renal diabetes insipidus (5). Both findings indicate that these channels are involved in the transport of salt and fluid across different nephron segments.

\* This work was supported by grants from the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie (to T. J. J.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Ph.D. student of the faculty of biology, chemistry, and pharmacy of the Freie Universität Berlin.

§ Present address: Max-Planck-Institut für Biophysik, Kennedyallee 70, D-60596 Frankfurt am Main, Germany.

¶ To whom correspondence should be addressed. Tel.: 49-40-4717-4741; Fax: 49-40-4717-4839; E-mail: Jentsch@plexus.uke.uni-hamburg.de.

In contrast, members of the two remaining branches of the CLC gene family probably function primarily in intracellular compartments (1). Again, an inherited disease has shed light on one of these channels. ClC-5, which belongs to the branch also comprising ClC-3 and ClC-4, is mutated in Dent's disease (6). This X-linked disorder is characterized by the urinary loss of low molecular weight proteins, calcium, and phosphate, and leads to the clinically important symptoms of kidney stones and nephrocalcinosis. The selective loss of low molecular weight proteins points to a defect of the renal proximal tubule of the kidney, where filtered proteins are normally reabsorbed by endocytosis. Indeed, ClC-5 is located in subapical endosomes of the proximal tubule where it colocalizes with the V-type H<sup>+</sup>-ATPase and with reabsorbed protein (7, 8). In transfected cells, besides some labeling of the plasma membrane, ClC-5 was mainly found in intracellular vesicles, where it colocalizes with endocytosed protein (7). Mice with a disrupted ClC-5 gene indeed had a broad defect in proximal tubular endocytosis (9). Fluid-phase endocytosis, receptor-mediated endocytosis, and the endocytotic removal of plasma membrane proteins were markedly inhibited but not totally abolished. Because several hormones or their precursors are endocytosed from the primary urine, this led to secondary changes in calciotropic hormones and changes in phosphate excretion (9). ClC-5 probably provides an electric shunt for the acidification of endosomes (7). Indeed, preliminary experiments revealed that renal endosomes isolated from ClC-5 knockout mice were acidified at slower rates (9).

When ClC-5 was expressed in *Xenopus* oocytes (10, 11) or in mammalian cells (10), it induced strongly rectifying chloride currents that were detectable only at voltages more positive than +20 mV. Point mutations that changed the voltage dependence, ion selectivity, and rectification demonstrated that ClC-5 directly mediates plasma membrane chloride currents (10). Thus, consistent with the immunocytochemistry of transfected cells (7), a small fraction of ClC-5 also resides in the plasma membrane.

The cytoplasmic carboxyl terminus of ClC-5 contains between its two conserved CBS<sup>1</sup> domains (12) a proline-rich stretch of amino acids (PPLPPY). A similar sequence is not found in the otherwise highly related ClC-3 and ClC-4 channels. This sequence bears resemblance to the PY motif that was recognized in the human epithelial Na<sup>+</sup> channel (ENaC) (13). Each of the three different subunits ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) of this heteromeric channel contains a carboxyl-terminal motif with the consensus sequence PPXY. The motifs on the  $\beta$ - and  $\gamma$ -subunits can interact with Nedd4, a ubiquitin-protein ligase, by binding

<sup>1</sup> The abbreviations used are: CBS, cystathionine- $\beta$  synthase; ENaC, amiloride-sensitive epithelial sodium channel; HECT, homologous to E6-AP carboxyl terminus; HA, hemagglutinin; WT, wild type; BSA, bovine serum albumin; BFA, brefeldin A; I-V, current-voltage.

to WW domains of the latter protein (14). This interaction leads to the ubiquitination of ENaC (15) and to its internalization by clathrin-mediated endocytosis (16) and to its degradation. This process is physiologically important, because the distal tubular Na<sup>+</sup> reabsorption mediated by ENaC needs to be tightly regulated. Mutations that truncate or disrupt the PY motif in the carboxyl termini of  $\beta$  or  $\gamma$  ENaC subunits lead to increased channel activity primarily due to a markedly prolonged surface retention time. This explains the gain of sodium channel activity observed in Liddle's syndrome, a rare and very severe form of human inherited hypertension (15–17). Mutational analysis revealed that the tyrosine residue of the PY motif is critical for the recognition and endocytotic retrieval of ENaC from the plasma membrane (16).

A peptide corresponding to the PY motif of CIC-5 was shown to bind to several WW domains within the proteins WWP1, WWP2, and WWP3 (18). WW domains are conserved protein motifs comprising ~35 amino acids. They are characterized by two highly conserved tryptophan residues, a central core of aromatic and hydrophobic residues, and a proline located three residues downstream from the second tryptophan (19). WWP2 (and probably the other, partially characterized proteins as well) belongs to the same protein class as Nedd4 (20) with which it shares several domains: a C2 (Ca<sup>2+</sup>/lipid binding) domain, multiple (three or four) WW domains, and a carboxyl-terminal ubiquitin-protein ligase (HECT) domain.

Given the pivotal role of CIC-5 in renal endocytosis (7, 9), it is of great interest to identify signals that are involved in CIC-5 trafficking. We therefore investigated whether the PY motif on CIC-5 plays a role in endocytosis of the channel protein. Indeed, disrupting this motif by site-directed mutagenesis led to increased surface retention and to higher currents. Coexpression with mutants of rab5 and WWP2 indicate that the PY motif is in fact important for endocytosis, a process probably depending on interactions with proteins of the Nedd4 family. Thus, we have identified a motif that is important for the endocytosis of CIC-5, which itself plays an important role in endocytotic processes (9).

## EXPERIMENTAL PROCEDURES

### Molecular Biology

**CIC-5 and WWP2 Constructs**—These cDNAs were cloned into pTLN (21), a vector containing *Xenopus* globin untranslated sequences and which is optimized for protein expression in *Xenopus* oocytes.

**rab5 Constructs**—This cDNA was cloned into the eukaryotic expression vector pFrog3 derived from pCDNA3 (Invitrogen) by flanking the multiple cloning site with the 5' and 3' untranslated region from the *Xenopus* globin gene (7). rab5 constructs were amino-terminally tagged with the Myc epitope (MEQKLISEEDLQS) (7). Point mutations were introduced by recombinant polymerase chain reaction. All polymerase chain reaction-derived fragments were entirely sequenced.

### Expression in *Xenopus laevis* Oocytes and Voltage Clamp Analysis

Capped cRNA was transcribed from constructs linearized by MluI using the mMessage mMachine kit (Ambion) and SP6 polymerase (for constructs in pTLN) or T7 polymerase (pFrog3 constructs). A total amount of 10 ng of cRNA was injected into defolliculated oocytes, *i.e.* 5 ng of cRNA each in coexpression studies. Oocytes were kept at 17 °C in modified Barth's solution (88 mM NaCl, 2.4 mM NaHCO<sub>3</sub>, 1 mM KCl, 0.41 mM CaCl<sub>2</sub>, 0.33 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 0.82 mM MgSO<sub>4</sub>, 10 mM HEPES, 50  $\mu$ g/ml tetracyclin, 20  $\mu$ g/ml gentamycin, pH 7.6). Standard two-electrode voltage clamp measurements were performed at room temperature 2–3 days after injection using TurboTEC 05 or 10C amplifiers (NPI Instruments, Tamm, Germany) and pClamp 5.5 software (Axon Instruments, Foster City, CA). Data are given as means  $\pm$  S.E. Currents were recorded in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5 mM HEPES, pH 7.4).

### Determination of Surface Expression

Surface expression of the CIC-5 protein was determined by the method of Zerangue *et al.* (22). An HA epitope was inserted by polymerase chain reaction mutagenesis into the extracellular loop between transmembrane domains D1 and D2 of wild type (WT) CIC-5 and mutants. After 3 days at 17 °C, oocytes were placed for 30 min in ND96 with 1% BSA at 4 °C to block unspecific binding, incubated for 60 min at 4 °C with 1  $\mu$ g/ml rat monoclonal anti-HA antibody (3F10, Roche Molecular Biochemicals, in 1% BSA/ND96), washed at 4 °C, and incubated with horseradish peroxidase-coupled secondary antibody (goat anti-rat Fab fragments, Jackson ImmunoResearch, in 1% BSA for 30–60 min at 4 °C). Oocytes were washed thoroughly (1% BSA, 4 °C, 60 min) and transferred to frog Ringer solution (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl<sub>2</sub>, 5 mM HEPES, pH 7.5) without BSA. Individual oocytes were placed in 50  $\mu$ l of Power Signal Elisa solution (Pierce) and incubated at room temperature for 1 min. Chemiluminescence was quantified in a Turner TD-20/20 luminometer (Turner Design, Sunnyvale, CA).

### Western Blot Analysis

The oocytes used to measure the surface expression of channel subunits were subsequently pooled and stored at –20 °C. After homogenization of the pooled oocytes in an ice-cold solution containing 250 mM sucrose, 0.5 mM EDTA, 5 mM Tris-HCl, pH 7.4, and a protease inhibitor mix (Complete®, Roche Molecular Biochemicals), yolk platelets were removed by three low speed centrifugation steps (500  $\times$  g, 2 min). The resulting supernatant was mixed with Laemmli sample buffer, and the protein equivalent to one oocyte was analyzed by SDS-polyacrylamide gel electrophoresis (10% polyacrylamide). After transfer to nitrocellulose, blots were blocked in Tris-buffered saline (150 mM NaCl, 25 mM Tris, pH 7.4) containing 5% milk powder and 0.1% Nonidet P-40. Primary (rat anti-HA monoclonal 3F10; 200 ng/ml; Roche Molecular Biochemicals) and secondary (horseradish peroxidase-conjugated goat anti-rat IgG; 1:10,000; Jackson ImmunoResearch) antibodies were diluted in Tris-buffered saline blocking solution. Washes were in Tris-buffered saline with 0.1% Nonidet P-40. Reacting proteins were detected by using the Renaissance reagent (PerkinElmer Life Sciences) and photographic films (Kodak).

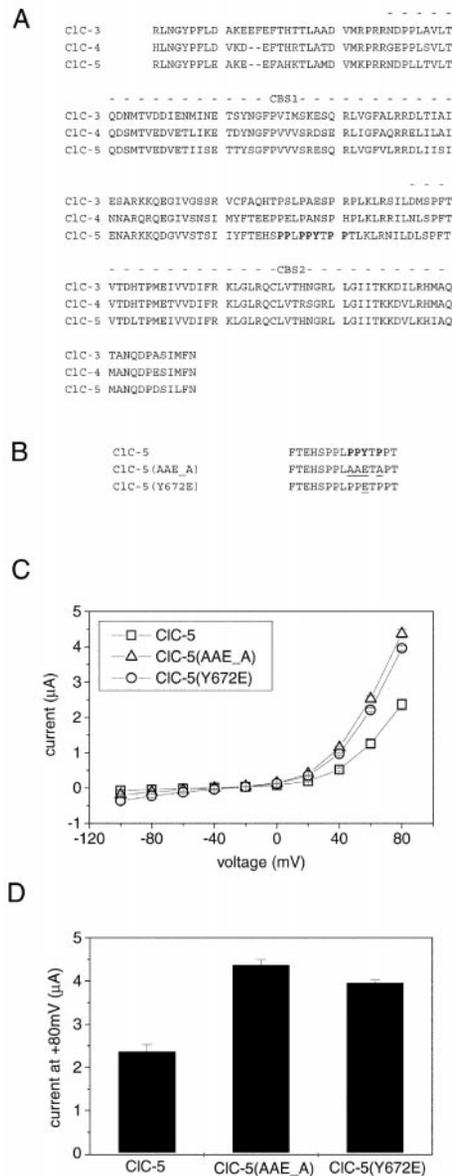
### Northern Blot Analysis

A human multiple tissue Northern blot of polyadenylated RNA (2  $\mu$ g/tissue; CLONTECH) was hybridized under high stringency conditions with a <sup>32</sup>P-labeled cDNA probe encompassing the entire open reading frame of human WWP2 using standard conditions. Autoradiography used a phosphorimager (Fuji Bas-1500) or photographic films.

## RESULTS

The carboxyl terminus of CIC-5 carries a proline-rich stretch of amino acids that shows similarity to a PY motif and that is not present in the highly related CIC-3 and CIC-4 proteins (Fig. 1A). We mutated several prolines and the tyrosine residue within the motif (Fig. 1B) and measured the effect of these mutations on currents in the *Xenopus* oocyte expression system. There was no change in their macroscopic biophysical properties, like rectification (Fig. 1C) or ion selectivity (data not shown). However, these mutations consistently yielded approximately 2-fold higher currents (Fig. 1D).

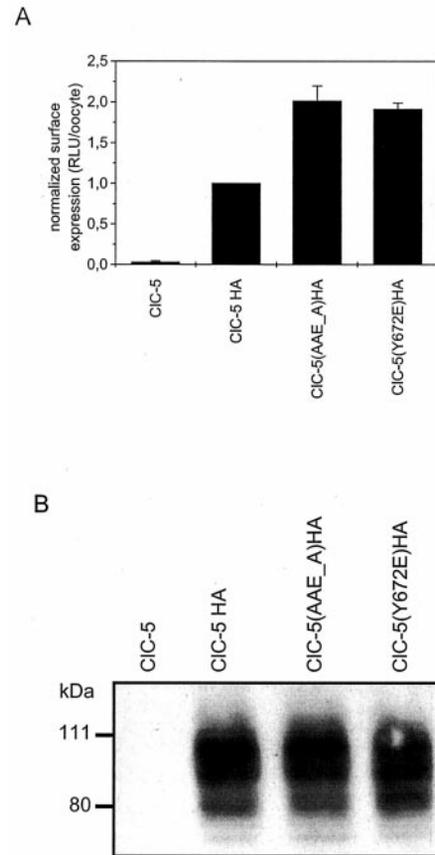
To investigate whether mutations in the PY motif enhance currents by increasing the surface expression of the channel protein, we inserted an HA epitope into the extracellular loop between D1–D2 of WT and the two CIC-5 mutants. The tagged constructs yielded currents that were indistinguishable from wild type (data not shown). The amount of HA epitopes at the cell surface was quantified by incubating nonpermeabilized oocytes with a monoclonal anti-HA antibody, followed by an enzymatic amplification procedure that uses chemiluminescence as the final step (22). As shown in Fig. 2A, the surface expression correlated well with the current amplitudes measured in *Xenopus* oocytes. To rule out effects on translation efficiency or overall protein stability, the total amount of CIC-5 was determined by Western blotting extracts of the same oocytes used for the surface expression assay. The CIC-5(AAE\_A) mutant is expressed to the same overall amount in oocytes as



**FIG. 1. Mutational analysis of the PY motif of CIC-5.** *A*, comparison of the carboxyl-terminal regions of CIC-3, CIC-4, and CIC-5. The two conserved CBS domains are indicated, and the proline-rich PY motif present exclusively in CIC-5 is shown in *bold*. *B*, mutations of the CIC-5 PY motif used in this study. *C*, I-V relationships of CIC-5, CIC-5(AAE\_A), and CIC-5(Y672E) currents ( $n = 10$ ). Steady-state currents measured by a two-electrode voltage clamp of *Xenopus* oocytes are shown. *D*, bar diagram of current levels obtained from WT and mutant CIC-5 at +80 mV ( $n = 10$ ; error bars indicate  $\pm$  S.E.).

WT CIC-5 (Fig. 2*B*). The effect of the CIC-5(AAE\_A) mutants could be due to the replacement of tyrosine by the negatively charged glutamate. This might mimic a tyrosine phosphorylation. We therefore compared currents of the CIC-5(Y672E) mutant with those of CIC-5(Y672A) but could not detect any differences (data not shown), making this explanation unlikely.

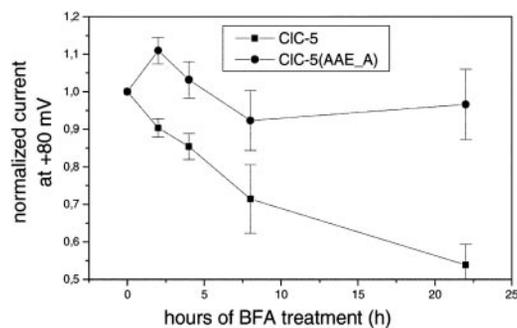
We next investigated the influence of the PY motif on the surface retention time of CIC-5 under conditions in which the insertion of newly synthesized channels into the plasma membrane is inhibited by brefeldin A (BFA). BFA blocks the anterograde vesicular transport from the endoplasmic reticulum and causes a redistribution of Golgi cisternae to the endoplasmic reticulum (23). Although BFA has multiple targets in vesicular transport, it does not affect clathrin-mediated endocytosis in the plasma membrane (24). It is known that BFA is also efficient in *Xenopus* oocytes (16, 25). Thus, in the presence of BFA



**FIG. 2. Surface expression of CIC-5, CIC-5(AAE\_A), and CIC-5(Y672E).** *A*, surface expression of epitope-tagged wild type CIC-5 and the two PY motif mutants of CIC-5. Oocytes injected with CIC-5 not containing the epitope served as a negative control (*first bar*). The assay used chemiluminescence for detection, and results are expressed in relative light units (RLU) per oocyte. The values for surface expression were normalized to CIC-5 HA. The surface expression of CIC-5 HA, CIC-5(AAE\_A)HA, and CIC-5(Y672E)HA correlates with the current level at +80 mV shown in Fig. 1*D*. (Three independent oocyte batches and cRNA preparations were used. At least six oocytes were analyzed for each experiment and construct; error bars indicate  $\pm$  S.E.) *B*, Western blot analysis of oocytes expressing epitope-tagged CIC-5, CIC-5(AAE\_A), and CIC-5(Y672E), respectively. WT CIC-5 served as a control. The oocytes that were used for each experiment shown in *A* were pooled and assayed for the overall expression of HA-tagged chloride channels using an antibody directed against the HA epitope.

plasma membrane currents reflect the half-lives of plasma membrane resident channels, without complications arising from newly synthesized proteins. However, BFA treatment does not exclude the possibility that endocytosed channels are recycled back to the surface. Oocytes were injected with cRNA encoding WT and AAE\_A mutant CIC-5. When currents had reached steady-state levels after 3 days, BFA was added to oocytes, and currents were determined over a period of 22 h. Wild type currents decreased with a half-time of about 23 h. In contrast, the decay of currents from the AAE\_A mutant was dramatically slowed (Fig. 3). Thus, mutating the PY motif in CIC-5 markedly increases its stability in the plasma membrane.

CIC-5 was colocalized with markers of early endosomes (7, 8) and was present in the large vesicles created by overexpressing the GTPase-deficient Q79L mutant of rab5 (7). rab5 is an important regulator of endocytosis. Overexpression in *Xenopus* oocytes of rab5 or its Q79L mutant increases fluid phase endocytosis, whereas the opposite is true for the dominant negative S34N mutant (26). To test whether global changes in endocytosis affect CIC-5 currents, we coexpressed WT and mutant



**FIG. 3. Effect of BFA on channel activity of CIC-5 and the CIC-5(AAE\_A) mutant.** CIC-5 and CIC-5(AAE\_A) cRNA were injected into *Xenopus* oocytes. Three days later, oocytes were transferred to modified Barth's solution containing 10  $\mu$ g/ml BFA at time zero. Data points represent the mean  $\pm$  S.E. (current at +80 mV) from three separate experiments, each performed with six oocytes for each time point and construct. Currents were normalized to the respective currents at  $t = 0$ .

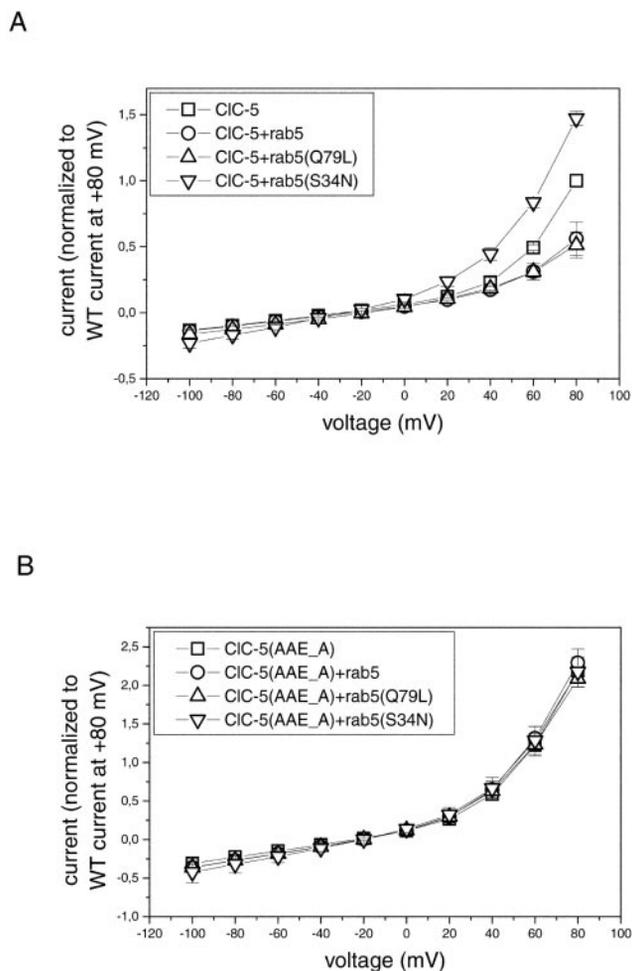
CIC-5 with several rab5 constructs in *Xenopus* oocytes. Currents were markedly decreased when WT CIC-5 was coexpressed with either WT or rab5(Q79L) (Fig. 4A). Conversely, currents were increased upon expression of rab5(S34N), indicating that constitutively occurring endocytosis decreases the steady-state level of CIC-5 in the plasma membrane. Both effects depended on an intact PY motif, because there was no appreciable effect of any of these rab5 constructs on the CIC-5(AAE\_A) mutant (Fig. 4B).

We next investigated whether the surface expression and currents of CIC-5 can be modulated by coexpressing a Nedd4-related protein. We chose WWP2 because its fourth WW domain interacted strongly with a synthetic peptide encompassing the PY motif of CIC-5 (18). Like Nedd4, WWP2 comprises an amino-terminal C2 domain, four WW domains, and a carboxyl-terminal HECT domain with a predicted ubiquitin-protein ligase activity. Northern analysis revealed that WWP2 is expressed in all tissues examined (Fig. 5A). This includes kidney, suggesting that interaction with CIC-5 might occur under physiological conditions. *Xenopus* oocytes have endogenous Nedd4-like activities, sometimes obscuring effects of overexpressing Nedd4 (27, 28). Therefore several nonfunctional WWP2 constructs were generated that might antagonize the effect of oocyte homologs (Fig. 5B). In the first construct, the HECT domain was deleted by truncation (termed WW $\Delta$ HECT). The next construct consisted only of WW domains 3 and 4 (termed WW3-4), and the third mutant carried a Cys to Ala point mutation in the HECT domain at a site that is necessary for ubiquitin binding (termed WWP2CA) (28, 29).

When WT WWP2 was coexpressed with CIC-5, no effects on the current were observed. However, coexpression with any of the three nonfunctional WWP2 constructs led to an approximately 2-fold increase of currents (Fig. 5C). No such effect was found for the CIC-5(AAE\_A) mutant (Fig. 5D). Thus, WWP2 mutants probably antagonize effects of an endogenous Nedd4-like protein that normally down-regulates CIC-5 currents. This interaction depends on an intact PY motif in CIC-5. Furthermore, these experiments strongly suggest that an intact HECT domain on the WW-containing protein is necessary for the down-regulation of CIC-5 plasma membrane expression. Additional experiments (data not shown) demonstrated that the increase in currents elicited by the WWP2 mutants correlated again with an increased surface expression of CIC-5.

#### DISCUSSION

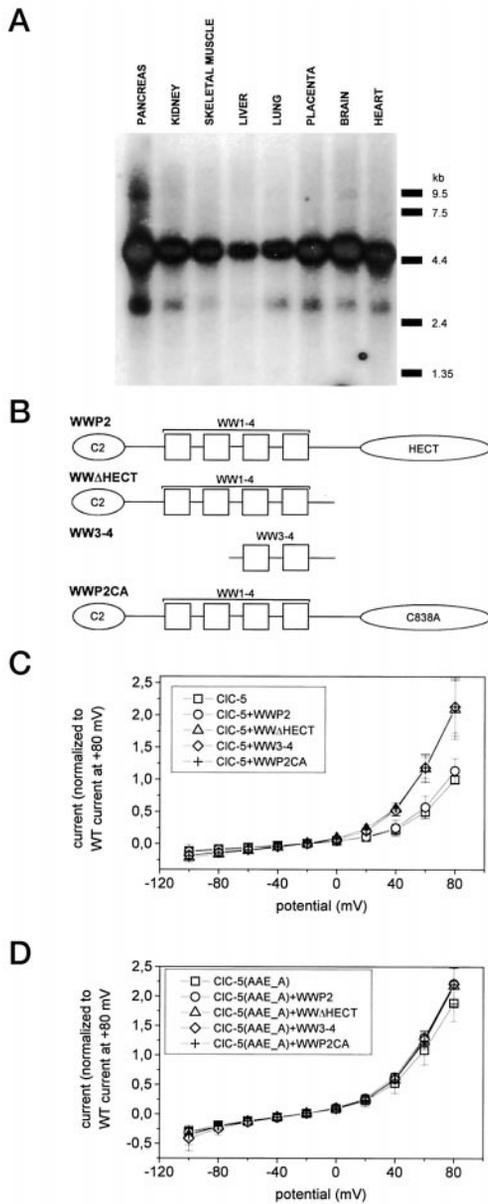
CIC-5 is the first chloride channel with a proven role in endocytosis. It is located predominantly in the membranes of vesicles of the (early) endocytotic pathway (7, 8), and its mu-



**FIG. 4. Effect of coexpressing rab5 constructs on currents mediated by CIC-5 or CIC-5(AAE\_A).** A, I-V relationship of CIC-5 expressed alone in oocytes compared with oocytes expressing CIC-5 with rab5, rab5(Q79L), or rab5(S34N). Coexpressing the dominant negative rab5(S34N) increased CIC-5 current approximately 2-fold, whereas rab5 and rab5(Q79L) decreased currents to the background level. The background level was determined by measuring oocytes expressing the three rab5 constructs without CIC-5. B, the same experiment as in A performed with the CIC-5(AAE\_A) mutant. There were no effects on the CIC-5(AAE\_A) mutant. All I-V relationships represent three different batches of oocytes with at least five oocytes per batch and combination. Currents were normalized to the CIC-5 WT current at +80 mV. (Error bars in A and B indicate  $\pm$  S.E.)

tational inactivation in man (6) and mice (9) leads to a severe defect in proximal tubular endocytosis. At least upon heterologous expression, a fraction of CIC-5 also resides in the plasma membrane (7, 10). In this work, we have identified an internalization signal in the carboxyl terminus of CIC-5, which, most likely by interacting with ubiquitin-protein ligases, limits its stability in the plasma membrane. This motif and its function closely resembles the PY motif identified in the ENaC (13-17).

The ENaC plays a crucial role in the regulation of distal tubular sodium reabsorption in the kidney. This process is exquisitely regulated by aldosterone and requires a short half-life of the channel in the plasma membrane. All three different subunits ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) carry proline-rich PY motifs in their carboxyl termini. These motifs are important for the ubiquitination, internalization, and degradation of the channel. The physiological importance of these motifs is impressively illustrated by the fact that human mutations that either delete or mutate PY motifs in Liddle's syndrome (13, 17, 30) lead to hypertension. This is a consequence of enhanced renal sodium



**FIG. 5. Tissue distribution of WWP2 and its effect on ClC-5 currents.** *A*, Northern blot analysis of WWP2 expression in several human tissues. *kb*, kilobases. *B*, schematic representation of WWP2 constructs. C2 indicates the amino-terminal domain that is thought to mediate Ca-dependent binding to membranes; the squares represent the four different WW domains; and HECT denotes the carboxyl-terminal ubiquitin-protein ligase domain. *C*, I-V relationships of currents measured for WT ClC-5 that was coexpressed with the various WWP2 constructs. *D*, the same as in *C*, but using the ClC-5(AAE\_A) mutant. All I-V relationships show mean currents from three different batches of oocytes with at least five oocytes per batch and combination. Currents were normalized to the ClC-5 WT current at +80 mV. (Error bars in *C* and *D* indicate  $\pm$  S.E.)

reabsorption resulting from an increased surface expression of ENaC. However, an increase in open probability was also reported (31). Using a yeast two-hybrid screen, Staub *et al.* (14) identified the ubiquitin-protein ligase Nedd4 as a protein that interacts with the PY motif via its WW domains. Subsequent work showed that the regulation of ENaC by Nedd4 involves ubiquitination of the channel protein (15, 28).

The finding that ClC-5 carries a signal resembling the ENaC PY motif in its carboxyl terminus prompted us to investigate whether it performs a similar role in this largely endosomal chloride channel. Indeed, similar to the findings with ENaC, currents of ClC-5 were increased when the PY motif was mu-

tated. This roughly 2-fold increase in currents correlated with an increased protein level in the plasma membrane, whereas the overall amount of the channel protein was unaffected. This strongly suggested that changes in trafficking, probably in the endocytotic removal from the plasma membrane, underlie the current increase. Indeed, in experiments in which the plasma membrane insertion of newly synthesized channels was prevented by brefeldin A, the apparent half-time of ClC-5 plasma membrane residence was drastically increased by mutations in the PY motif. The half-time of WT ClC-5 in the plasma membrane (20–24 h) was much longer than the 1–3.5 h described for ENaC (15, 16). However, ENaC may be a special case because it needs to be tightly and rapidly regulated in response to changes in salt and fluid balance. Because brefeldin A does not prevent the recycling of endocytosed ClC-5 back into the plasma membrane, the increased half-life of the PY motif mutant at the surface could also be interpreted in terms of an altered trafficking from endosomes to lysosomes. In this model, the mutants would undergo endocytosis at a rate comparable with WT, but the lack of ubiquitin conjugation allows them to evade targeting to the lysosomes. Channels lacking a functional PY motif could then be recycled back to the plasma membrane.

The notion that the PY motif in ClC-5 plays a role in endocytosis is strongly supported by experiments investigating the effects of rab5. Stimulating endocytosis by coexpressed WT or rab5(Q79L) or inhibiting endocytosis by rab5(S34N) decreased or increased ClC-5 currents, respectively. Importantly, this effect was virtually abolished when the PY motif on ClC-5 was inactivated. This suggests that rab5-dependent endocytosis of ClC-5 does not occur via an unspecific retrieval of membranes containing ClC-5 but depends on specific interactions with the PY motif.

Using a homology screen, Pirozzi *et al.* (18) have previously isolated several cDNAs encoding proteins with multiple WW domains. Glutathione *S*-transferase fusion proteins containing these domains were tested *in vitro* for binding to synthetic peptides containing PY-like motifs from several proteins, including ClC-5. The strongest binding to the ClC-5 peptide occurred with the fourth WW domain of a protein dubbed WWP2. WW domains of the other two WWP proteins also displayed some binding. In contrast, at least the first WW domain of rat Nedd4 was unable to bind to a synthetic peptide encompassing the PY motif of ClC-5 (32), showing the specificity of the interaction between WW domains and PY motifs. We have shown here that the most strongly interacting protein, *i.e.* WWP2, is coexpressed with ClC-5 in kidney. Therefore we chose this protein for our studies. However, we cannot exclude the possibility that ClC-5 shows physiologically more important interactions with other proteins containing WW domains. Similar to Nedd4, WWP2 has an amino-terminal C2 domain that is thought to mediate Ca-dependent lipid binding, four WW domains, and an amino-terminal HECT domain that is predicted to function in ubiquitin conjugation.

Coexpressing the WT form of WWP2 with ClC-5 in *Xenopus* oocytes had no discernible effects on chloride currents. This may be due to the fact that *Xenopus* oocytes express a Nedd4 homolog (33) and that its activity on ClC-5 is already near maximal. This contrasts with the recent observation (27, 28) that overexpressing Nedd4 reduced ENaC currents in *Xenopus* oocytes. The reason for this difference is currently unclear and may reflect *e.g.* different binding affinities. On the other hand, the stimulation of ClC-5 currents and surface expression by *bona fide* inactive WWP2 mutants strongly suggest that WW domain proteins interact with the PY motif of ClC-5 and that the mutants act by competing with endogenous *Xenopus* oocyte

homologs. Mutants lacking an HECT domain or carrying a point mutation that interferes with ubiquitin binding (28, 29) increased currents in the presence of an intact PY motif on CIC-5. This strongly suggests that ubiquitination plays a role in the PY motif-dependent endocytosis of CIC-5. Similar experiments also suggested that ENaC is ubiquitinated by Nedd4 (14, 28), and this could also be shown directly (15). Despite many attempts, however, we were unable to show that CIC-5 is ubiquitinated. Ubiquitinated CIC-5 may be short-lived and may therefore have escaped detection, a problem compounded by the fact that many proteins targeted for endocytosis carry only a single ubiquitin moiety (34). Alternatively, ubiquitination may occur on a third protein that might be associated with CIC-5. The unexpected voltage dependence of its currents has previously led to speculations that there may be an additional, as yet unknown, subunit that modifies its voltage dependence (10, 11).

In summary, we have shown that a PY motif in the carboxyl terminus of CIC-5 modulates its retention in the plasma membrane. Similar to Nedd4-mediated endocytosis of ENaC, this very likely involves the interaction with the WW domain of a ubiquitin-protein ligase. Because CIC-5 is mainly present in endosomal compartments where it is essential for endocytotic trafficking, its partial presence in the plasma membrane may be a side effect of recycling. It will be important to determine whether the interaction of its PY motif with WW domain-containing proteins is important for its cellular function, namely its crucial role in both receptor-mediated and fluid-phase endocytosis (9), and whether this interaction determines sorting processes further downstream in the endosomal pathway as well.

*Acknowledgment*—We thank Willy Günther for constructing the CIC-5(AAE\_A) mutant.

#### REFERENCES

- Jentsch, T. J., Friedrich, T., Schriever, A., and Yamada, H. (1999) *Pfluegers Arch. Eur. J. Physiol.* **437**, 783–795
- Steinmeyer, K., Klocke, R., Ortland, C., Gronemeier, M., Jockusch, H., Gründer, S., and Jentsch, T. J. (1991) *Nature* **354**, 304–308
- Koch, M. C., Steinmeyer, K., Lorenz, C., Ricker, K., Wolf, F., Otto, M., Zoll, B., Lehmann-Horn, F., Grzeschik, K. H., and Jentsch, T. J. (1992) *Science* **257**, 797–800
- Simon, D. B., Bindra, R. S., Mansfield, T. A., Nelson-Williams, C., Mendonca, E., Stone, R., Schurman, S., Nayir, A., Alpay, H., Bakkaloglou, A., Rodriguez-Soriano, J., Morales, J. M., Sanjad, S. A., Taylor, C. M., Pilz, D., Brem, A., Trachtman, H., Griswold, W., Richard, G. A., John, E., and Lifton, R. P. (1997) *Nat. Genet.* **17**, 171–178
- Matsumura, Y., Uchida, S., Kondo, Y., Miyazaki, H., Ko, S. B., Hayama, A., Morimoto, T., Liu, W., Arisawa, M., Sasaki, S., and Marumo, F. (1999) *Nat. Genet.* **21**, 95–98
- Lloyd, S. E., Pearce, S. H., Fisher, S. E., Steinmeyer, K., Schwappach, B., Scheinman, S. J., Harding, B., Bolino, A., Devoto, M., Goodyer, P., Rigden, S. P., Wrong, O., Jentsch, T. J., Craig, I. W., and Thakker, R. V. (1996) *Nature* **379**, 445–449
- Günther, W., Luchow, A., Cluzeaud, F., Vandewalle, A., and Jentsch, T. J. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 8075–8080
- Devuyt, O., Christie, P. T., Courtoy, P. J., Beauwens, R., and Thakker, R. V. (1999) *Hum. Mol. Genet.* **8**, 247–257
- Piwon, N., Günther, W., Schwake, M., Bösl, M. R., and Jentsch, T. J. (2000) *Nature* **408**, 369–373
- Friedrich, T., Breiderhoff, T., and Jentsch, T. J. (1999) *J. Biol. Chem.* **274**, 896–902
- Steinmeyer, K., Schwappach, B., Bens, M., Vandewalle, A., and Jentsch, T. J. (1995) *J. Biol. Chem.* **270**, 31172–31177
- Bateman, A. (1997) *Trends Biochem. Sci.* **22**, 12–13
- Schild, L., Lu, Y., Gautschi, I., Schneeberger, E., Lifton, R. P., and Rossier, B. C. (1996) *EMBO J.* **15**, 2381–2387
- Staub, O., Dho, S., Henry, P., Correa, J., Ishikawa, T., McGlade, J., and Rotin, D. (1996) *EMBO J.* **15**, 2371–2380
- Staub, O., Gautschi, I., Ishikawa, T., Breitschopf, K., Ciechanover, A., Schild, L., and Rotin, D. (1997) *EMBO J.* **16**, 6325–6336
- Shimkets, R. A., Lifton, R. P., and Canessa, C. M. (1997) *J. Biol. Chem.* **272**, 25537–25541
- Snyder, P. M., Price, M. P., McDonald, F. J., Adams, C. M., Volk, K. A., Zeiher, B. G., Stokes, J. B., and Welsh, M. J. (1995) *Cell* **83**, 969–978
- Pirozzi, G., McConnell, S. J., Uveges, A. J., Carter, J. M., Sparks, A. B., Kay, B. K., and Fowlkes, D. M. (1997) *J. Biol. Chem.* **272**, 14611–14616
- Sudol, M. (1996) *Prog. Biophys. Mol. Biol.* **65**, 113–132
- Harvey, K. F., and Kumar, S. (1999) *Trends Cell Biol.* **9**, 166–169
- Lorenz, C., Pusch, M., and Jentsch, T. J. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 13362–13366
- Zerangue, N., Schwappach, B., Jan, Y. N., and Jan, L. Y. (1999) *Neuron* **22**, 537–548
- Pelham, H. R. (1991) *Cell* **67**, 449–451
- Wood, S. A., Park, J. E., and Brown, W. J. (1991) *Cell* **67**, 591–600
- Mulner-Lorillon, O., Belle, R., Cormier, P., Drewing, S., Minella, O., Poulhe, R., and Schmalzing, G. (1995) *Dev. Biol.* **170**, 223–229
- Mukhopadhyay, A., Barbieri, A. M., Funato, K., Roberts, R., and Stahl, P. D. (1997) *J. Cell Biol.* **136**, 1227–1237
- Goulet, C. C., Volk, K. A., Adams, C. M., Prince, L. S., Stokes, J. B., and Snyder, P. M. (1998) *J. Biol. Chem.* **273**, 30012–30017
- Abriel, H., Loffing, J., Rebhun, J. F., Pratt, J. H., Schild, L., Horisberger, J. D., Rotin, D., and Staub, O. (1999) *J. Clin. Invest.* **103**, 667–673
- Huibregtse, J. M., Scheffner, M., Beaudenon, S., and Howley, P. M. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 2563–2567
- Schild, L., Canessa, C. M., Shimkets, R. A., Gautschi, I., Lifton, R. P., and Rossier, B. C. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 5699–5703
- Firsov, D., Schild, L., Gautschi, I., Merillat, A. M., Schneeberger, E., and Rossier, B. C. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 15370–15375
- Irusta, P. M., and DiMaio, D. (1998) *EMBO J.* **17**, 6912–6923
- Rebhun, J. F., and Pratt, J. H. (1998) *DNA Seq.* **9**, 295–306
- Strous, G. J., and Govers, R. (1999) *J. Cell Sci.* **112**, 1417–1423