The ClC-3 Chloride Transport Protein Traffics through the Plasma Membrane via Interaction of an N-terminal Dileucine Cluster with Clathrin*

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CIC-3 is a ubiquitously expressed chloride transport protein that is present in synaptic vesicles and endosome/lysosome compartments. It is largely intracellular but has been observed at the plasma membrane as well. The aim of this study was to identify the pathways and regulation of CIC-3 trafficking to intracellular sites. At the steady state, ~94% of transfected CIC-3 was localized intracellularly, and only 6% was at the plasma membrane. Pulse labeling with [35S]methionine and biotinylation demonstrated that about 25% of newly synthesized CIC-3 traffics through the plasma membrane. We used both immunofluorescence microscopy and biotinylation assays to assess the trafficking of CIC-3. Plasma membrane CIC-3 was rapidly endocytosed (t1/2 ~ 9 min); a portion entered a recycling pool that returned to the cell surface after internalization, and the remainder trafficked to more distal intracellular compartments. CIC-3 associated with clathrin at the plasma membrane. Coimmunoprecipitation and glutathione S-transferase pulldown assays demonstrated that the N terminus of CIC-3 binds to clathrin. Alanine replacement of a dileucine acidic cluster within the cytosolic N terminus (amino acids 13–19) resulted in a molecule that had decreased endocytosis and increased surface expression. This replacement also abolished interaction with clathrin as assessed both by coimmunoprecipitation and glutathione S-transferase pulldown assays. We conclude that CIC-3 is primarily an intracellular transport protein that is transiently inserted into the plasma membrane where it is rapidly endocytosed. Internalization of CIC-3 depends on the interaction between an N-terminal dileucine cluster and clathrin.

The CIC chloride transport proteins are ubiquitously expressed and highly conserved. They participate in housekeeping processes such as pH regulation, trans-epithelial transport, and regulation of electrical excitability. Nine mammalian proteins that function either as Cl− ion channels or H+/Cl− exchangers have been identified (1–3). They are localized either at the plasma membrane or in intracellular membrane sites and can have a ubiquitous or a more restricted tissue distribution. CIC-3, CIC-4, and CIC-5 are members of one branch of this family that functions primarily intracellularly. They have similar physiological properties, can form heterodimers with other family members, and contribute to endosomal acidification (4, 5). CIC-4 and CIC-5 are electrogenic Cl−/H+ exchangers (2, 3), similar to the bacterial ClC channel (6), and thus couple Cl− flux to vesicular pH gradients. CIC-3 has not been specifically shown to be an antiporter, but it has similar biophysical properties to CIC-4 and CIC-5 (7) and thus may function in this way as well.

The trafficking pathways for CIC-3 are not well defined, and in different experimental systems CIC-3 has been observed both at the plasma membrane and in intracellular compartments. Whether this dichotomy results from independent trafficking pools or whether CIC-3 moves between these pools is unknown. Intracellular targeting of membrane proteins is accomplished by the presence of specific short cytoplasmic motifs, which bind to adaptor proteins specific for individual compartments. Most sorting motifs characterized to date exist within the cytosolic domains of transmembrane proteins. CIC-3 contains several potential trafficking motifs, but their significance has not been determined.

The endosome/lysosome compartment can be accessed through a biosynthetic direct pathway from Golgi to endosome/lysosome. It can also be accessed through an indirect pathway in which proteins traffic first to the plasma membrane and are subsequently internalized and routed to endosomes/lysosomes. Clathrin-based systems play important roles in targeting membrane proteins from both the plasma membrane and the trans-Golgi network to the endosomal/lysosomal compartment. Several adaptor protein complexes direct clathrin assembly into curved lateral arrays and couple it to cargo recruitment (8, 9). Currently, very little is known about the mechanisms underlying CIC-3 trafficking and the adaptor proteins responsible for CIC-3 trafficking.

In this study, we have used biotinylation, pulse labeling, and a novel CIC-3 construct with an externally facing HA2 epitope to follow both steady-state distribution and trafficking of the molecule. These studies show that CIC-3 is primarily an intracellular channel, but it localizes to the plasma membrane as well. Plasma membrane CIC-3 is rapidly endo-

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1 The abbreviations used are: HA, hemagglutinin; GST, glutathione S-transferase; GFP, green fluorescent protein; PBS, phosphate-buffered saline; MesNa, sodium 2-mercaptoethanesulfonate; DMEM, Dulbecco’s modified Eagle’s medium; WT, wild type.
cytosed where it undergoes both recycling as well as delivery to a distal compartment. Endocytosis requires a specific clathrin-interacting dileucine acidic cluster within the cytosolic N-terminal domain.

**EXPERIMENTAL PROCEDURES**

*Reagents and Antibodies—* General reagents were obtained from Sigma or Fisher. PfuTurbo® DNA polymerase (catalog number 600252) was from Stratagene (La Jolla, CA); FuGENE 6 (catalog number 1 814 443) was from Roche Applied Science; EZ-link sulfo-NHS-SS-biotin (catalog number 21331), immunopure immobilized streptavidin gel (catalog number 20349), and protein A/G (catalog number 20421) were from Pierce; cycloheximide (catalog number 01810) was from Sigma; Alexa Fluor® 594-conjugated transferrin (catalog number T-13343) was from Molecular Probes (Eugene, OR); [35S]methionine (catalog number AG1094) and bulk GST purification module (catalog number 27-4570-01) were obtained from Amersham Biosciences. Antibodies were purchased as indicated. Mouse monoclonal anti-GFP antibody (catalog number 632381) was from Clontech; mouse monoclonal anti HA antibody (catalog number MMS-101R) was from Covance (Princeton, NJ); rabbit polyclonal anti-HA antibody (catalog number H6908) was from Sigma; mouse monoclonal anti-transferrin receptor antibody (catalog number 13-6800) was from Zymed Laboratories Inc.; mouse monoclonal anti sodium potassium ATPase antibody (catalog number ab7671), rabbit polyclonal anti caveolin-1 antibody (catalog number ab2910), and goat polyclonal clathrin heavy chain antibody (catalog number ab6316) were from Abcam (Cambridge, MA); mouse monoclonal clathrin heavy chain antibody (catalog number 610500) and mouse monoclonal anti β2 subunit of AP-2 antibody (catalog number 610382) were from BD Transduction Laboratories; goat polyclonal anti-actin antibody (catalog number sc-1616) was from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor® 488 goat anti-mouse IgG (H+L) (catalog number A-11001), Alexa Fluor® 594 goat anti-mouse IgG (H+L) (catalog number A-11005), Alexa Fluor® 488 donkey anti-mouse IgG (H+L) (catalog number A-21202), and Alexa Fluor® 594 chicken anti-goat IgG (H+L) (catalog number A-21468) were from Molecular Probes. Donkey anti-rabbit IgG horseradish peroxidase linked whole antibody (catalog number NA934V) and sheep anti-mouse IgG horseradish peroxidase-linked whole antibody (catalog number NA 931V) were from Amersham Biosciences.

*Constructions and Cell Lines—* CIC-3 GFP and CIC-3 HA, both constructs with epitopes fused to the C terminus of CIC-3, were constructed in pEGFP-N1 as described previously (10, 11). CIC-3 1217HA, a CIC-3 construct with an extracellular/lumen side HA epitope between the B and C helices (12), CIC-3 Δ12–30, CIC-3 Δ13–19, and CIC-3 13–19A were generated from the parent constructs using the QuikChange® site-directed mutagenesis kit (Stratagene). GST-CIC-3 N was prepared by subcloning bases 1–210 (corresponding to amino acids 1–70) of CIC-3 into pGex-4T-3 (a kind gift from Dr. Guillermo Alt-enberg) between the BamHI and XhoI sites. GST-CIC-3 N Δ12–30 and GST-CIC-3 13–19A were generated with the QuikChange site-directed mutagenesis kit (Stratagene). All constructs were confirmed by sequencing in the Protein Chemistry Laboratory of the University of Texas Medical Branch. COS-7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. COS-7 cells were transiently transfected with the different cDNAs using FuGENE 6.

*Yeast Two-hybrid Screening—* Two-hybrid screening was performed in the budding yeast, Saccharomyces cerevisiae, using the Matchmaker yeast two-hybrid system (BD Biosciences). Plasmid pGBK7 contained the N or C terminus of CIC-3 fused to the DNA binding domain of GAL4. Several adaptor proteins expressed as fusion proteins with the activation domain of GAL4 were kindly provided by Dr. Juan Bonifacio. Positive interactions were identified by screening on tryptophan-, leucine-, and histidine-deficient media.

*Immunofluorescence—* COS-7 cells, growing on 18-mm coverslips, were incubated with HA antibody (1:400) at 37 °C in PBS for 45 min as indicated. After incubation and washing, the cells were rinsed, fixed with 3% paraformaldehyde in phosphate-buffered saline, and labeled with secondary antibody. In some experiments cells were permeabilized with 0.25% saponin after fixation but before secondary antibody incubation. For double-labeling of CIC-3 and clathrin, CIC-3 was visualized as described above using Alexa Fluor® 488-conjugated donkey anti-mouse IgG (1:500) as secondary antibody. Clathrin was identified by incubation with anti-clathrin antibody (1:100) and subsequently with Alexa Fluor® 594 chicken anti-goat IgG (H+L) for 1 h at room temperature.

Transferrin receptor was visualized in living cells by incubation with Alexa Fluor® 594-conjugated transferrin ligand (5 μg/ml) in DMEM at 37 °C. The cells were then fixed, permeabilized, and visualized. Immunofluorescence was visualized with a Nikon Eclipse E800 upright epifluorescent microscope as described previously (10) or a Zeiss LSM 510 confocal microscope with a 63× (1.4 NA) oil immersion objective. For the confocal images, samples were visualized with the 488 and 543 nm laser lines and emission filter set at 505–530 nm for GFP and Alexa Fluor® 488 detection or 585–615 nm for Alexa Fluor® 594 detection.

*Biotinylation Assays—* COS-7 cells were grown in 6-cm dishes and transfected with different constructs. At 48 h after transfection, cells were washed twice in ice-cold PBS and incubated with 1 ml of sulfo-NHS-SS-biotin diluted in PBS (pH 8) for 30 min at 4 °C. Unreacted biotin was quenched with 50 mM glycine in PBS. After three washes at 4 °C in PBS, cells were immediately lysed. For experiments in which surface biotin was cleaved, cells were washed as described and then incubated in DMEM for various times at either 4 or 37 °C prior to a 2.5-h incubation with cleavage solution (150 mM NaCl, 20 mM Tris, 50 mM sodium 2-mercaptoethanesulfonate (MesNa) (pH 8.6)). Cells were subsequently lysed in ice-cold lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% IGEPAL CA-630 with protease inhibitor mixture (Sigma P8340)). The lysates were incubated with streptavidin-agarose beads and pelleted at 3000 × g for 2 min. Lysates and pellets were subjected to SDS-PAGE on 8–16% polyacrylamide gels (13). The proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Bio-Rad), blocked overnight at 4 °C with 5% nonfat dried milk, 0.1% Tween 20 in Tris-buffered saline, and subse-
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quantitatively incubated with anti-HA antibody (1:1000) or anti-GFP antibody (1:1000). The membranes were then washed and incubated with appropriate secondary antibodies, and detected with the ECLplus chemiluminescence system (Amersham Biosciences). Immunoreactive bands were quantified by densitometry using TotalLab software (Ultralum Inc., Claremont, CA). Statistical significance was tested using paired t test.

Percent of surface expression was calculated by comparing total lysate ClC-3 with the biotinylated fraction as $100 \times P/(T \times F)$, where $P$ is the density of the CIC-3 band in the streptavidin pellet; $T$ is the density of the CIC-3 band in the total lysate aliquot; and $F$ is the ratio of total lysate used for streptavidin precipitation compared with the quantity applied directly to the gel, i.e. lysate volume used for streptavidin precipitation/lysate volume applied to the gel. Percent endocytosis was calculated from experiments in which cells were biotinylated, incubated at 37 °C, and then exposed to the biotin cleavage solution at 37 °C for different periods of time. After rinsing cells into ice-cold PBS solution containing glycine to quench the excess biotin, biotinylated proteins were extracted and subjected to streptavidin precipitation and Western blot. An alternative, two-step cleavage method was also used. COS-7 cells were transfected with CIC-3 HA. After 2 days they were washed twice in ice-cold PBS and incubated with 1 ml of sulfo-NHS-SS-biotin diluted in PBS (pH 8) for 30 min at 4 °C. After quenching and washing as above, cells were incubated in DMEM for 15 min at 37 °C followed by incubation with cleavage solution at 4 °C. The cleavage solution was removed, and cells were then re-incubated in DMEM at 4 or 37 °C for 30 min. A second incubation with cleavage solution at 4 °C was then performed. The cells were then lysed and subjected to streptavidin precipitation and Western blot.

**Metabolic Labeling and Immunoprecipitation—CIC-3**

COS-7 cells were grown in 10-cm dishes to a confluence of 90%. They were then metabolically labeled by incubation for 20 min with 0.8 mCi/ml of [35S]methionine in methionine-free DMEM. Monolayers were washed three times with PBS before adding sulfo-NHS-SS-biotin at a concentration of 0.5 mg/ml for 4 h at 37 °C. After quenching with 50 mM glycine in PBS, cells were lysed with ice-cold lysis buffer with protease inhibitor mixture. Biotinylated proteins were obtained by streptavidin precipitation of postnuclear supernatants, obtained after centrifugation at 10,000 × g for 15 min. Streptavidin precipitation was sequentially performed four times to remove all biotinylated CIC-3 HA from the supernatant. The streptavidin pellets were combined, and biotinylated proteins were released from the beads by incubation in cleavage solution (see above) at 4 °C twice in 12 h. The final streptavidin supernatant and the cleavage product of the streptavidin pellets were then separately immunoprecipitated with polyclonal HA antibody (Sigma H908 1:200) after removal of proteins to the protein A/G-agarose beads in the absence of specific antibody. The precleared supernatants were incubated at 4 °C overnight on a rotator with the specific HA antibody. Fresh protein A/G-agarose beads (200 μl of 50% slurry) were added and then incubated for 6 h at 4 °C. After centrifugation at 3,000 × g for 2 min, supernatants were aspirated; beads were washed, and antibody-antigen complexes were dissociated with 2× sample buffer. SDS-PAGE was then performed as described. Dried gels were exposed to x-ray film, and protein bands were quantitated by autoradiography.

**GST Pulldown and Immunoprecipitation—GST and GST fusion proteins were produced in Escherichia coli strain BL-21 according to procedures described for the glutathione S-transferase gene fusion system (Amersham Biosciences). GST or GST fusion protein was incubated with glutathione-Sepharose 4B beads for 0.5 h at room temperature followed by extensive washing in phosphate-buffered saline. It was then incubated with COS-7 cell lysates prepared as described above. After overnight incubation at 4 °C, the beads were pelleted at 3000 × g for 2 min and washed three times with lysis buffer. The bound proteins were eluted by incubating the beads in SDS loading buffer containing β-mercaptoethanol at 95 °C for 5 min and subjected to SDS-PAGE and immunoblotting with anti-AP-2 β-subunit (1:5000), anti-clathrin (1:1000), or anti-caveolin (1:1000) antibodies.

For coimmunoprecipitation, whole cell lysates from CIC-3 HA-transfected COS-7 cells were used. The lysates were first pre-cleared by 6 h of incubation at 4 °C with protein A/G beads. The beads were subsequently removed by centrifugation for 2 min at 3,000 × g. The precleared supernatant was then incubated at 4 °C overnight on a rotator with either the nonimmune IgG or specific antibody for the HA epitope or clathrin (Sigma H908 1:200 or Santa Cruz Biotechnology sc-12734 1:100). Fresh protein A/G-agarose beads were then added and incubated for 6 h at 4 °C. After centrifugation at 3,000 × g for 2 min, supernatant was aspirated, and SDS-PAGE sample buffer was added to the beads. The sample was heated and separated by SDS-PAGE. Immunoblotting was subsequently performed.

**RESULTS**

CIC-3 Partially Localizes to the Plasma Membrane—To investigate the trafficking path of CIC-3, we transiently transfected COS-7 cells and determined the extent of its presence at the plasma membrane. GluR6, a known plasma membrane receptor, was used as a control (14). As reported previously (10), CIC-3 demonstrated a predominant intracellular distribution and was present in large intracellular vesicles. This contrasted with the obvious plasma membrane distribution of GluR6 (Fig. 1A). To further determine whether CIC-3 was present at the plasma membrane, we used surface biotinylation. CIC-3 GFP was transiently expressed, and cell surface proteins were labeled at 4 °C with sulfo-NHS-SS-biotin, precipitated with streptavidin-coated beads, and analyzed by SDS-PAGE and immunoblotting. GluR6 showed a strong band both in total lysate and in the streptavidin pellet. Biotinylated CIC-3
precipitation procedure completely cleared biotinylated ClC-3 that had been present in the plasma membrane during this 4-h incubation period. Cells were subsequently lysed; biotinylated protein pellets containing biotinylated proteins (SA Pt) were separated from non-biotinylated fractions (SA Spt) and in each fraction ClC-3 was immunoprecipitated with (+ lane) or without (− lane) anti-HA antibody. D, densitometry analysis of relative ClC-3 immunoprecipitation band density from three independent experiments performed as in C. PM, biotinylated plasma membrane fraction; non-PM, non-biotinylated fraction representing ClC-3 that had not been inserted into the plasma membrane. n = 3. E, COS-7 cells expressing ClC-3 HA were pretreated with cycloheximide, lysed, and subjected to immunoblotting for HA epitope at the indicated times to assess the stability of the ClC-3 protein.

The fate of plasma membrane ClC-3 was assessed by antibody binding in live, nonpermeabilized cells. Specificity of this protocol was confirmed by using a combination of pulse labeling and surface biotinylation to directly determine the fraction of newly synthesized ClC-3 that had undergone internalization. ClC-3 HA-transfected cells were pulse-labeled with [35S]methionine and incubated with biotinylation reagent for 4 h at 37 °C. Streptavidin precipitates containing biotinylated proteins (SA Pt) were separated from non-biotinylated proteins remaining in the supernatant (SA Spt), and each fraction was immunoprecipitated with (+ lane) or without (− lane) anti-HA antibody. D, densitometry analysis of relative ClC-3 immunoprecipitation band density from three independent experiments performed as in C. PM, biotinylated plasma membrane fraction; non-PM, non-biotinylated fraction representing ClC-3 that had not been inserted into the plasma membrane. n = 3. E, COS-7 cells expressing ClC-3 HA were pretreated with cycloheximide, lysed, and subjected to immunoblotting for HA epitope at the indicated times to assess the stability of the ClC-3 protein.

The above analysis could be confounded if nascent ClC-3 were differentially degraded in plasma membrane and non-plasma membrane fractions to a significant extent. We thus determined the stability of ClC-3 by immunoblotting after inhibition of protein synthesis with cycloheximide. Fig. 1E demonstrates that ClC-3 is not detectably degraded over a 2-h period.

Plasma Membrane ClC-3 Undergoes Rapid Internalization—The above results show that ~25% of ClC-3 is thus initially inserted into the plasma membrane but only 6% remains there at steady state. To test whether this discrepancy results from endocytosis, we examined internalization of surface-biotinylated ClC-3. Cell surface proteins were biotinylated with sulfo-NHS-SS-biotin (Fig. 2A, lane 1), a derivatization that could be cleaved with an impermeant reducing agent, MesNa, either immediately after biotinylation (Fig. 2A, lane 2) or after a 15-min incubation at 37 °C (Fig. 2A, lane 3). Although immediate cleavage of the biotin label was very effective, ClC-3 demonstrated substantial retention of biotinylation after the 15-min incubation, representing endocytosis during the incubation period. In contrast, Na-K-ATPase, a protein that does not undergo internalization, was not protected by a period of post-biotinylation incubation (Fig. 2A). We next measured the time course of ClC-3 internalization. After biotinylation at 4 °C with sulfo-NHS-SS-biotin, the cells were incubated at 37 °C for different times prior to cleavage and then subjected to streptavidin precipitation and Western blot for ClC-3. Fig. 2B shows an example of the biotinylation status of ClC-3 according to this protocol, and Fig. 2C demonstrates results from six similar experiments. A portion of the ClC-3 molecule undergoes internalization with a t1/2 of ~9 min.

Traffic of Plasma Membrane ClC-3—We generated ClC-3 constructs with either an intracellular or external HA epitope to follow the trafficking of the molecule. The external epitope allowed specific antibody labeling of surface-expressed ClC-3 channels on living cells. We first expressed these constructs in COS-7 cells to evaluate their steady-state distribution. ClC-3 with a C-terminal (intracellular) HA epitope, ClC-3 with an external HA epitope and a C-terminal GFP, and ClC-3 with the external HA epitope but without an internal GFP epitope all resulted in the formation of intracellular vesicles. This steady-state distribution was the same as that seen previously for ClC-3 C-terminal GFP and unmodified ClC-3 (10). The fate of plasma membrane ClC-3 was assessed by antibody binding in live, nonpermeabilized cells. Specificity of this approach was first assessed by immunofluorescence microscopy. For cells expressing ClC-3 with the intracellular HA
epitope, antibody labeling was observed only after permeabilization and showed a largely intracellular distribution (Fig. 3, A–D). When cells expressed CIC-3 with the extracellular HA epitope, antibody labeling was observed in both permeabilized and nonpermeabilized cells (Fig. 3, E–H). Permeabilized cells (Fig. 3F) displayed the same intracellular pattern as did the internal epitope, but nonpermeabilized cells demonstrated labeling specific for the plasma membrane (Fig. 3H).

To follow trafficking, COS-7 cells were transfected with the CIC-3 derivative containing both an extracellular HA epitope and internal GFP. Live cells were incubated for 45 min at 37 °C with anti-HA antibody, rinsed in PBS to wash away the extra antibody, and then chased in DMEM at 37 °C for different times. Cells were subsequently fixed, permeabilized, and incubated with fluorescent secondary antibodies. Before the chase, HA fluorescence (Fig. 3I, red) was clearly defined at the plasma membrane. The green GFP fluorescence was primarily seen intracellularly because the majority of cellular CIC-3 is intracellular, and with this background, the visualization of the relatively dim plasma membrane fluorescence was limited by the dynamic range of the imaging camera. Amplification of the green GFP signal confirmed colocalization between GFP and the red plasma membrane distribution of antibody binding to the external HA epitope (data not shown).

We next observed the effect of different chase times on the trafficking of the antibody bound to the extracellular HA epitope. After a 30-min chase, plasma membrane resident CIC-3 moved to small endocytic vesicles (Fig. 3J) and by 2 h had accumulated into the large central vesicles (Fig. 3K) that are the major site of total cellular CIC-3 at the steady state (10). The plasma membrane can therefore serve as an intermediate trafficking site.

**Internalized CIC-3 Undergoes Recycling**—To determine whether internalized cell surface CIC-3 enters a recycling pool, we biotinylated the cell surface pool, allowed it to internalize for 15 min, and then cleaved the remaining surface biotin. These cells were then further incubated at either 0 or 37 °C for 30 min. At the end of this period they underwent a second round of cleavage. Fig. 4A demonstrates that the first post-biotinylation incubation resulted in endocytosis and protection of a fraction of the biotinylated CIC-3 from cleavage. A second incubation period at 0 °C did not result in any sensitivity to a second round of cleavage. However, a second incubation at 37 °C rendered an additional fraction of CIC-3 sensitive to cleavage. In this regard CIC-3 behaved similarly to the transferrin receptor, an endogenous protein known to undergo recycling (13). The loss of biotinylation of CIC-3 by a second round of cleavage following a 37 °C incubation did not result from degradation of the CIC-3 molecule because incubation at 37 °C alone, without MesNa cleavage, did not reduce CIC-3 biotinylation (Fig. 4B). Fig. 4C summarizes...
data from six experiments performed as in Fig. 4A and shows that CIC-3 recycles similarly to transferrin receptor.

To further confirm that CIC-3 is recycled, COS-7 cells were treated with cycloheximide to inhibit de novo protein synthesis and incubated under trafficking-permissive conditions (37 °C) in the presence of the biotinylation reagent. We compared the time course of CIC-3 biotinylation with that of two control proteins, transferrin receptor, which is known to participate in membrane recycling, and the α-subunit of the Na-K-ATPase, which is a negative control for recycling (16). As shown in Fig. 4D (upper panel) and Fig. 4E (closed circles), transferrin receptor is only partially biotinylated at time 0, and its biotinylation increases over 2-fold until reaching a maximum by ~30 min. Biotinylation of Na-K-ATPase (Fig. 4, D, lower panel, and E, triangles) is much more stable and increases only minimally over this period. CIC-3 (Fig. 4, D, middle panel, and E, open circles) behaves nearly identically to transferrin receptor and is unlike Na-K-ATPase. Therefore, like transferrin receptor, it undergoes recycling.

A tyrosine motif, showed a primarily intracellular distribution pattern, similar to the wild type CIC-3 (Fig. 5E). Biotinylation showed that the amount of endocytosis was also similar to the wild type CIC-3 (Fig. 5, G and H). This result suggests that the tyrosine motif is not responsible for the impaired endocytosis seen with CIC-3 Δ12–30. We next targeted the dileucine acidic cluster at position 13–19 that represent important CIC-3 endocytosis signal is present between amino acids 12 and 30.

We next tested the tyrosine motif in this segment. CIC-3 27A30A, a mutant that specifically ablated the

**FIGURE 4. CIC-3 recycles from the plasma membrane.** A, representative blot of a two-step cleavage of biotinylated CIC-3. CIC-3 HA-transfected COS-7 cells were surface-biotinylated (lane 1) and either immediately cleaved with MesNa (lane 2) or incubated for 15 min at 37 °C prior to cleavage (lane 3). Cells treated as for lane 3 were then incubated for an additional 30 min at either 4 °C (lane 4) or 37 °C (lane 5) prior to a second round of cleavage with MesNa. The cells were then lysed and subjected to streptavidin precipitation. Upper panel, HA immunoblot of streptavidin pellets; lower panel, same protocol, blotted with antibody against the transferrin receptor. B, HA immunoblot is displayed for an experiment performed as in A except that after the first cleavage cells were incubated for an additional 30 min at 37 °C either without (lane 4) or with (lane 5) a second round of cleavage with MesNa. C, relative band intensity after single cleavage as in A, lane 3 (black bars), a second cleavage after 4 °C incubation as in A, lane 4 (light gray bars), or a second cleavage after 37 °C incubation as in A, lane 5 (dark gray bars). n = 6 independent experiments. *, p < 0.05 compared with 4 °C incubation. D, COS-7 cells expressing CIC-3 HA were treated with cycloheximide for 2 h, biotinylated at 4 °C, and then further incubated with biotinylation reagent at 37 °C. At the indicated times, cell lysates were streptavidin-precipitated and subject to Western blotting with antibodies for HA epitope, transferrin, or α-subunit of the Na-K-ATPase. A representative blot is shown. E, average percentage of biotinylation determined by densitometry analysis of four independent experiments from D. Closed circles, transferrin receptor; open circles, CIC-3 HA; triangles, Na-K-ATPase. The lines represent best fits to a single exponential rise to a maximum.
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**FIGURE 5.** The CIC-3 N terminus contains an internalization signal. A, yeast two-hybrid assays demonstrate interaction between the N-terminal cytosolic part of CIC-3 but not the C-terminal segment with μ subunits of AP-1 and AP-2. Neither interacted with AP-3, B, N-terminal sequence of CIC-3, C–F, confocal images of COS-7 cells transfected with WT CIC-3 GFP (C), CIC-3 Δ12–30 GFP (D), CIC-3 27A30A GFP (E), and CIC-3 13–19A GFP (F). Bar, 10 μm. Similar images were observed in six independent preparations (G). Endocytosis was determined by biotinylation and assessment of protection from cleavage as shown in Fig. 2. Immunoblots of streptavidin pellets with HA antibody are shown. Lane 1 streptavidin pellets before cleavage; lane 2, streptavidin pellets performed after immediate cleavage; lane 3, after biotinylation, cells were first incubated for 15 min at 37 °C followed by cleavage and streptavidin precipitation. H, average percent protection from cleavage after 15 min of incubation was determined by densitometry analysis of five experiments as shown in G. Data are presented as mean ± S.E. % endocytosis = 100 × (lane 3 – lane 2)/lane 1. *, p < 0.05. J–K, trafficking path of CIC-3 Δ13–19. COS-7 cells were transfected with CIC-3 Δ13–19 with internal GFP and external HA, incubated with HA antibody for 45 min, and chased at 37 °C for 0 min (I), 30 min (J), and 2 h (K). Bar, 10 μm. Similar images were observed in eight independent experiments.

Association of CIC-3 with Clathrin during Endocytosis—To determine whether endocytosed CIC-3 associates with clathrin, we expressed the external HA epitope construct of CIC-3, incubated live cells with anti-HA antibody, and examined sequential immunofluorescence images dually stained for CIC-3 and clathrin. Clathrin was detected in a punctate labeling pattern at the plasma membrane as well as in small intracellular vesicles (Fig. 6, A, D, and G). Immunolabeling of the external epitope of CIC-3 (Fig. 6, B, E, and H) showed considerable colocalization at zero chase time (Fig. 6, A–C) and after 30 min of chase (Fig. 6, D–F), at which time endocytosed CIC-3 was located in peripherally localized small vesicles. At 120 min of chase (Fig. 6, G–I), the internalized CIC-3 was present in large vesicles in a more central area of the cell and no longer associated with clathrin. We further examined the association between endocytosed CIC-3 and transferrin, a protein known to internalize via clathrin-coated pits (17). After surface labeling of CIC-3 for 45 min, we incubated the cells at 37 °C for 15 min with the fluorescent derivative, transferrin-594. As shown in Fig. 6, J–L, CIC-3 and transferrin were both endocytosed and frequently colocalized in the same population of endocytic vesicles.

Interactions between the N-terminal segment of CIC-3 and proteins involved in endocytosis were assessed with GST-pull-down assays. GST fusion constructs of the N termini of either wild type or the endocytosis-defective constructs, CIC-3 Δ12–30 and CIC-3 N-terminal 13–19A, were used to precipitate proteins derived from COS-7 cell lysates. As shown in Fig. 7A, the WT N terminus of CIC-3 bound to both clathrin and AP-2β but not caveolin-1. CIC-3 N-terminal Δ12–30 and CIC-3 N-terminal 13–19A also bound to AP-2β but did not pull down clathrin. These data demonstrate that the dileucine acidic cluster at positions 13–19 is required for clathrin binding.

To further confirm the interaction between the clathrin and CIC-3 molecules in vivo, we performed communoprecipitation experiments. Immunoprecipitation with an anti-HA antibody was able to pull down clathrin when WT CIC-3 HA was expressed but not in the absence of CIC-3 expression or with CIC-3 13–19A HA. Conversely, immunoprecipitation with an anti-clathrin antibody was also able to pull down WT CIC-3 HA (Fig. 7B). Taken together, these results indicate that CIC-3 physically associates with clathrin in COS-7 cells and that this association depends on the presence of the N-terminal dileucine acidic cluster.

**DISCUSSION**

CIC-3 is a member of the CIC-3/CIC-4/CIC-5 subfamily of chloride transport proteins. These proteins are present in diverse membrane sites and are required for acidification of intracellular vesicles. CIC-3 has been shown to be involved in acidification of synaptic vesicles and endosomes (18–20), but its deficiency is associated with lysosomal defects as well (21). It exists in several different isoforms that result from splicing variations (22, 23), and the factors that control CIC protein localization are incompletely understood.

In this study we have examined the trafficking of the short form of CIC-3. The molecule is primarily intracellular, but a small fraction is present on the plasma membrane, and this component is rapidly internalized in association with clathrin. Endocytosed CIC-3 either enters a recycling pool or slowly traffics to a distal compartment with the characteristics of late endosomes or lysosomes (10). Internalization is dependent on the presence of a 7-amino acid dileucine acidic cluster in the cytosolic N terminus of CIC-3. The ability of CIC-3 N-terminal variants to undergo endocytosis corresponds to the ability of these N-terminal segments to bind clathrin. These findings are consistent with our previous work showing that expressed CIC-3 localizes to a population of large intracellular vesicles that have the properties of late endosomes/lysosomes and contributes to chloride entry and exit in these compartments (10). Therefore, at steady-state...
CIC-3 is present in plasma membrane, recycling early endosomes as well as late endosomes/lysosomes.

CIC-3 reaches its intracellular sites both by a direct pathway, where it is sorted directly from the Golgi to more distal intracellular sites, and an indirect pathway in which it is first inserted into the plasma membrane and subsequently endocytosed. Simultaneous pulse labeling and biotinylation demonstrated that about 25% of newly synthesized CIC-3 reaches the plasma membrane, whereas about 75% is never inserted into plasma membrane. This result is consistent with the data from other lysosomal membrane proteins such as Lgp-A and Lamp proteins (24, 25). The importance of the direct pathway is consistent with the earlier work of Faundez and co-workers (19) who demonstrated that AP-3-dependent mechanisms control the targeting of CIC-3 in both neuronal and non-neuronal cells. AP-3 is known to be involved in sorting of lysosomal membrane proteins from the trans-Golgi network and is important in the biogenesis of lysosome-related organelles (26). However, the indirect pathway also appears to be important as it is the mechanism by which CIC-3 enters the recycling compartment and is specifically controlled by targeting sequences within the N terminus.

In contrast, a dileucine acidic cluster at positions 13–19 was required both for endocytosis and clathrin binding but not for AP-2 binding. This suggests that AP-2 binding is not sufficient for normal trafficking, and the link to clathrin involves another mechanism. The LLDLLDE sequence is similar to the previously identified clathrin binding sequence LLDDL (30) and is shared by the γ2-subunit of AP-1, another clathrin-binding adaptor protein. We have not determined whether CIC-3 binds directly to clathrin or whether the dileucine acidic cluster on CIC-3 interacts with clathrin via an as yet unidentified adaptor protein. However, although the molecular details of the CIC-3 clathrin interaction have not yet been defined, this interaction is required for endocytosis. Future experiments will be required to determine whether AP protein interactions are involved in CIC-3 endocytosis.

This study was performed in cell culture systems overexpressing CIC-3. It is thus important to consider whether plasma membrane insertion and endocytosis of CIC-3 is specific to this system. Several considerations lead to the conclusion that plasma membrane insertion and endocytosis are important for native CIC-3 as well. First, recent findings in hippocampal neurons show the presence of CIC-3 at the plasma membrane of

Several lines of evidence demonstrate an association between CIC-3 and clathrin and suggest that CIC-3 is internalized via a clathrin-dependent pathway. First, immunofluorescence shows that endogenous clathrin and CIC-3 colocalize in a punctate pattern at the plasma membrane, and this colocalization is retained for the first 30 min after internalization (Fig. 6). Second, internalized CIC-3 colocalizes with internalized transferrin receptor, a protein that is known to be internalized via clathrin-mediated endocytosis. Third, CIC-3 binds to clathrin in cell homogenates, and the ability of N-terminal mutants of CIC-3 to undergo endocytosis correlates with their ability to bind clathrin in both communoprecipitation and GST pulldown assays. The CIC-3 interaction with clathrin could be either direct or mediated by another adaptor protein.

Because clathrin binding generally occurs via adaptor proteins, particularly AP-2, we searched for classical motifs implicated in this interaction. Although the N terminus of CIC-3 has a putative tyrosine motif (24, 27–29), this appears to be unrelated to the ability of the molecule to bind either AP-2 or clathrin and is not required for endocytosis.
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native cells (31), and impaired acidification in recycling endosomes has been observed (20) in CIC-3−/− cells. Second, the presence of defined internalization motifs in CIC-3 and its entry into a recycling compartment suggests that the molecule has the inherent capacity for entry into the endocytic pathway. Third, our data shows that 25% of newly synthesized CIC-3 is initially inserted into the plasma membrane, whereas only 6% remains there at steady state. This confirms that the plasma membrane fraction is indeed an intermediate location.

The extent to which the epitope tags alter CIC-3 trafficking is also an issue. We previously published studies in which we compared native CIC-3 with C-terminal FLAG, and C-terminal GFP derivatives. All three molecules were similarly distributed at the plasma membrane as measured by whole cell patch clamp experiments, and all three similarly formed the enlarged central vesicular structures (7). We are therefore confident that the molecule tolerates these C-terminal additions without changing its steady-state distribution. Similarly, in this study CIC-3 tolerated the insertion of C-terminal GFP, C-terminal HA, or an external loop HA without any change in steady-state distribution assessed by microscopy. We were only able to perturb distribution by substitution of the dileucine acidic cluster. We therefore conclude that in our system these epitope additions do not alter trafficking of the molecule.

Although we were able to use the external epitope construct of CIC-3 to demonstrate its presence on the plasma membrane and to follow its internalization, we were not able to use this construct for kinetic studies. This is because the HA antibody did not bind to the epitope at temperatures lower than 37 °C. It was therefore necessary to label the HA epitope by incubation at 37 °C for 45 min prior to initiating chases with antibody-free external solution. Internalization had presumably already occurred prior to the initial fixation of the cells. In practice, we found that under this condition, most cells showed primarily plasma membrane staining. With further chase times, the plasma membrane CIC-3 moved to the distal compartments. Therefore, although we do not have a pure initial labeling state, it was possible to follow the subsequent trafficking events of plasma membrane-derived CIC-3.

It is now understood that CIC-4 and CIC-5 are electrogenic Cl−−H+ exchangers and not chloride channels. This is likely the case for CIC-3 as well (15), and this information requires a reevaluation of the role of the CIC proteins in vesicular acidification. Currently, one can only speculate about how these molecules function, but CIC-3 may play different roles at different intracellular sites. For example, in the nascent endocytic vesicles, luminal chloride would be greater than cytosolic chloride, and Cl−−H+ exchange would be expected to augment the H-ATPase in vesicular acidification. In more distal compartments, CIC antiporter may play a greater role in chloride entry and volume expansion of intracellular vesicles whereby the proton gradients produced by the ATPase would drive concentrative entry of osmotically active chloride ions.

This latter possibility is supported by our previous observation (10) that manipulation of chloride gradients had profound and rapid effects on the size of CIC-3-containing vesicles. Reduction of intracellular Cl− resulted in dramatic shrinkage of the vesicles, and a sudden increase in cytosolic Cl− resulted in rapid expansion, sometimes to volumes that increased more than 3-fold. These are unexpected findings for an ion conductance where solute movement is limited by the generation of an opposing diffusion potential, but it is not unexpected for a coupled transport process, the electrogenicity of which can be compensated by the activity of the proton ATPase.

In summary, our studies demonstrate that CIC-3 is largely an intracellular protein. It takes both direct and indirect pathways to its final destinations that include both early recycling vesicles and late endosomes/lysosomes. The protein associates with clathrin, and both clathrin binding and endocytosis require the presence of a dileucine acidic cluster in the N terminus of CIC-3. The presence of multiple binding motifs, multiple AP protein interactions, and the possibility that CIC channels form heterodimers with other family members (5) suggests that these proteins may be multifunctional and might play a scaffolding role in addition to their functions in chloride and proton translocation.

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