Evidence for a Functional Interaction between the ClC-2 Chloride Channel and the Retrograde Motor Dynein Complex*

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The ClC-2 chloride channel has been implicated in essential physiological functions. Analyses of ClC-2 knock-out mice suggest that ClC-2 expression in retinal pigment epithelia and Sertoli cells normally supports the viability of photoreceptor cells and male germ cells, respectively. Further, other studies suggest that ClC-2 expression in neurons may modify inhibitory synaptic transmission via the γ-aminobutyric acid, type A receptor. However, complete understanding of the physiological functions of ClC-2 requires elucidation of the molecular basis for its regulation. Using cell imaging and biochemical and electrophysiological techniques, we show that expression of ClC-2 at the cell surface may be regulated via an interaction with the dynein motor complex. Mass spectrometry and Western blot analysis of eluate from a ClC-2 affinity matrix showed that heavy and intermediate chains of dynein bind ClC-2 in vitro. The dynein intermediate chain co-immunoprecipitates with ClC-2 from hippocampal membranes suggesting that they also interact in vivo. Disruption of dynein motor function perturbs ClC-2 localization and increases the functional expression of ClC-2 in the plasma membranes of COS7 cells. Thus, cell surface expression of ClC-2 may be regulated by dynein motor activity. This work is the first to demonstrate an in vivo interaction between an ion channel and the dynein motor complex.

ClC-2 is a member of the ClC family of chloride channels (1). Expression of recombinant ClC-2 in Xenopus oocytes (2) and in mammalian cells (3) confers the expression of a hyperpolarization and swelling-activated chloride conductance path exhibiting an inwardly rectifying current-voltage relationship. Recent studies in which ClC-2 expression was disrupted in model organisms support the claim that ClC-2 directly mediates this chloride conductance. For example, a chloride conductance path with the above biophysical properties was detected in studies of salivary gland cells obtained from normal mice whereas this function was absent in salivary gland cells obtained from ClC-2 knock-out mice (4). Similarly, depletion of the Caenorhabditis elegans ortholog of ClC-2, CLH-3, in mamma-
so that the reversed restriction sites on this vector would reverse the orientation of the open reading frame to create the antisense plasmid (10). The constructs for the expression of GFP-tagged dynamitin, and DSRed-tagged CC1 were kindly provided by Dr. T. Schroer (The Johns Hopkins University). For immunofluorescence analysis, endogenous CIC-2 was detected using a peptide-purified polyclonal antibody (8 μg/ml) directed against the N terminus of CIC-2. Dynein complex was labeled with a monoclonal antibody against the intermediate chain (IC) of dynein (1:100; Chemicon, Pittsburgh, PA), early endosomal compartments with monoclonal anti-EEA1 antibody (1:200; BD Biosciences), and tubulin with monoclonal anti-α tubulin (1:2000; Sigma). Fluorescein-conjugated donkey anti-mouse IgG (in 50% glycerol; 1:250; Jackson ImmunoResearch Laboratories) and donkey anti-rabbit IgG (1:1000; Molecular Probes, OR) secondary antibodies were employed. For transmission electron microscopy, sections were acquired with a JEOL TEM transmission electron microscope (JEOL USA, Peabody, MA) equipped with a digital camera (AMT Corp., Danvers, MA).

Imaged (NIH imaging) was employed for line scanning analysis to quantitatively assess CIC-2 subcellular distributions in COS7 cells. Lines were typically drawn from the periphery of the nucleus to the cell edge through the intracellular staining pattern for CIC-2. This line was then divided into four equal segments. The fluorescence intensity for each segment was quantified and expressed as a fraction of total fluorescence intensity.

Immunogold Electron Microscopy—Paraformaldehyde- and glutaraldehyde-fixed COS7 cells were infused with a solution of 10% gelatin in PBS at 37 °C, allowed to gel at 4 °C, and infused with 2.3 μm sucrose overnight. Ultrathin sections (100 nm) were mounted on Formvar-coated nickel grids. Sections were labeled with CIC-2 and/or dynein antibodies as described previously (9). Controls included the omission of the primary antibodies and the preabsorption of the primary antibodies with a CIC-2 antibody that was acquired with a JEOL TEM transmission electron microscope (JEOL USA, Peabody, MA) equipped with a digital camera (AMT Corp., Danvers, MA).

Protein Affinity Columns and Immunoblotting—1 ml Hit-Trap columns (Amersham Biosciences) were injected with either 10 mg/ml amylase (Sigma) or CIC-2, purified as described previously (14) in 0.2 M NaHCO₃, 0.5 M NaCl, pH 8.3 (supplemented with 4% pentadecaffluorooctanoic acid (Dakwood Products Inc., West Columbia, SC) for the application of purified CIC-2. After a 30-min incubation at room temperature, the column was washed with Buffer A (0.5 M ethanamine, 0.5 M NaCl, pH 8.0), followed by Buffer B (0.1 μM acetoate, 0.5 M NaCl, pH 4). The bound brain extract was eluted through the column, equilibrated previously with 1% Triton in PBS, pH 8.0. Briefly, tissue was homogenized in 0.1% Triton X-100 in PBS with protease inhibitors, 10 mg/ml aprotinin and leupeptin, 1 mM benzamidite, 2 mM dithiothreitol, 10 μM E64, 0.1 mM sodium orthovanadate. Homogenate was spun at 100,000×
g for 2 h to remove unsolubilized material before application to the column. The column was then washed twice in equilibration buffer, and bound protein was eluted with 4% pentadecaffluorooctanoic acid in 100 mM phosphate buffer, pH 4.0. 1-mL fractions were collected, and protein content was assayed by measuring absorbance at 280 nm. Fractions containing the highest levels of protein (18–22) were pooled and concentrated in an Amicon Centriprep 50 concentrator (Millipore Corpora- tion, Bedford, MA). Following determination of protein concentration using a modified Lowry protocol, eluant from all three columns was subjected to SDS-polyacrylamide gel electrophoresis (4–12% Tris-glycine, NOVEX) and silver staining.

For Western blotting analysis, antibodies were diluted to the required working concentrations in blocking buffer (5% milk, 0.1% Tween 20 in PBS). For sequential probing of the same samples, membranes were stripped by washing in SDS-stripping buffer at 55–60 °C for 40 min. Blots were first re-blocked and re-probed with secondary antibody to confirm effective stripping, before incubation with the desired antibody.

MALDI-TOF Mass Spectrometry and Identification of CIC-2-interacting Protein—Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry was performed by the Mass Spectrometry Laboratory at the Molecular Medicine Research Centre (University of Toronto). Briefly, the dominant protein band (100 kDa) eluted from the CIC-2 affinity column was excised from the silver-stained gel, washed twice with acetonitrile followed by 0.1 M ammonium bicarbonate, and digested with 6.25 ng of trypsin (Roche Molecular Biochemicals) in 4 mM CaCl₂, 50 mM ammonium bicarbonate at 37 °C overnight. Tryptic peptides were extracted from the gel slice with two washes of 100 μl of 0.1 M ammonium bicarbonate. Pooled peptides were acidified by addition of acetic acid to 1% and reverse phase extracted with a 1:1 suspension of C18 silica resin. Resin was washed with two 100 μl aliquots of 2% seawater, 1% acetic acid, and peptides were eluted with 5 μl of 65% acetonitrile, 1% acetic acid. A MALDI-TOF peptide mass fingerprint was obtained with an Applied Biosystems Voyager DE STR instrument using α-cyano hydroxycinnamic acid as a matrix.

Co-immunoprecipitation—Hippocampal slices (300-μm thickness) were prepared from adult male rats (Sprague-Dawley, 150–200 g Charles River) as described previously (13). A crude membrane preparation was obtained from tissue homogenate (in PBS with protease inhibitors by centrifugation at 100,000 ×
g for 1 h following a 10-min low speed spin at 2,000 ×
g to pellet nuclei and unlysed cells. For co-immunoprecipitations, 50 μg of protein from crude membrane preparation was solubilized in buffer containing 1% Igepal CA-630, 5 mM EDTA, pH 7.4, 150 mM NaCl, 1 mM DTT, 0.05% (w/v) SDS, 1% Triton X-100 with protease inhibitors) at 37 °C for 10 min. Samples were spun at 15,000 ×
g to sediment unsolubilized protein aggregates and incubated with CIC-2 antibody (2 μg/ml) at 16 h at 4 °C. The antibody-protein com-
plexes were adsorbed from solution with protein A-Agarose beads (Roche Molecular Biochemicals). Proteins were eluted from beads with SDS loading buffer containing 63 mM Tris-Cl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, and 25 mM dithiothreitol. Samples were reduced with 10 mM Tris (2-carboxyethyl) phosphine and analyzed for the presence of dynein and ClC-2.

Cell Surface Biotinylation—COST fibroblasts grown to 70% confluency on 15-cm dishes were incubated on ice for 30 min. Cells were washed with biotinylation buffer (1 mM MgCl₂, 1 mM CaCl₂, pH 7.8) and then incubated with 1.0 mg/ml sultosuccinimidyl-2-(biotinamido) ethyl-1,3-dithiopropionate (Pierce) for 30 min on ice. Biotinylation was quenched by rinsing cells with 1% bovine serum albumin in PBS. Cells were then lysed with modified RIPA buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% (w/v) SDS, 1% Triton X-100 with protease inhibitors). Solubilized cell extracts were collected by centrifugation at 15,000 × g for 10 min. Supernatants were incubated with streptavidin beads (Pierce) for 1 h at 4 °C, and biotinylated proteins were eluted from beads with 63 mM Tris-Cl, pH 6.8, 10% (v/v) glycerol, 0.5 mM EDTA, 2% (w/v) SDS, 2% β-mercaptoethanol, and 50 mM dithiothreitol.

Eluants were then analyzed for the presence of ClC-2.

Patch Clamp Studies of COS7 Cells—COS7 membrane currents were measured using conventional whole cell patch clamp technique as described previously (10). Briefly, currents were measured using an Axopatch 200A patch clamp amplifier (Axon Instruments, Foster City, CA) and were filtered at 100 Hz with a six-pole Bessel Filter. Sampling rate was 4 kHz for most data, and junction potentials were corrected. Voltage clamp protocols were generated using pCLAMP software (version 7; Axon Instruments). Current-voltage relationships were determined in a stepwise clamp protocol. From a holding potential of 30 mV, voltage pulses of 3.0 s were applied from −140 to +40 mV in 20-mV increments. The bath solution contained 140 mM NaCl, 2 mM CaCl₂, 2 mM MgCl₂, 2 mM EGTA, and 50 mM HEPES. Both pipette and bath solutions were adjusted to pH 7.4 and 280 milliosmoles.

Statistics—Patch clamp and immunofluorescence quantitation data are presented as the means ± S.E. Most statistical analyses were performed using the Student’s unpaired t test. Results obtained in patch clamp studies with drug-treated and -transfected cells were analyzed using the unpaired Student’s t test. Differences between multiple treatment groups were assessed by ANOVA, and differences between specific pairs were subsequently analyzed using the Bonferroni method.

RESULTS

CIC-2 Interacts with Rat Brain Dynein in vitro—As previous studies by Bali et al. (12) implicate a role for vesicular trafficking in CIC-2 regulation, we sought to identify proteins that may interact with CIC-2 and serve to mediate or regulate its translocation between the plasma membrane and intracellular vesicles. An affinity column containing anti-ClC-2 antibody was used to capture ClC-2-binding proteins. Recombinant rat CIC-2 protein, engineered to possess a polyhistidine tail at its N terminus, was expressed in Sf9 cells using the baculovirus expression system and purified by metal affinity using the methods described in our previously published work (14). Purified CIC-2 was conjugated to a Hi-Trap column, through which mouse brain homogenate was passed. Brain tissue was used as starting material as CIC-2 is abundantly expressed in this organ (Fig. 1A). Control experiments were conducted simultaneously using an unconjugated and an amylase-conjugated Hi-Trap column. Interacting proteins were eluted using a pH gradient, and fractions containing the highest levels of protein were pooled, concentrated, and analyzed by SDS-PAGE and silver staining. Multiple silver-stained bands were detected specifically in the eluant from the CIC-2 affinity column (Fig. 1B). We excised the prominent 100-kDa band for analysis by mass spectrometry. Briefly, a MALDI-TOF mass fingerprint of the excised band was obtained, and monoisotopic protonated peptide masses were used to search the NCBI sequence databases using PROFOUND. The tentative protein identification as a fragment of DHC subunit was based on the high number of matched peptide masses observed (25% sequence coverage) and low mass error (<0.5 atomic mass unit).

The dynein motor complex is comprised of several different polypeptide components; a functional complex is typically composed of two copies of one of the heavy chain subunits, together with multiple copies of intermediate, light intermediate, and light chains (15). Dissociated heavy chain (isolated from purified bovine brain cytoplasmic dynein) migrates with an apparent molecular mass slightly greater than 200 kDa (15). As shown in Fig. 1B, silver stain analysis of the CIC-2 column eluant detects a similarly sized protein. However, commercially available antibodies were not effective in detecting the putative DHC fragment in total brain lysate or in immunoblots of the eluant from the CIC-2 affinity, possibly because of digestion of the antibody epitope. Recent experiments conducted by King et al. (15) report unstable DHC-containing subcomplexes generated from potassium iodide-treated purified bovine dynein, which was attributed to a loss in stability conferred by interactions with DIC and dynein light chain subunits. In fact, SDS-PAGE analysis of the DHC-containing complex revealed several distinct bands with molecular masses of slightly greater than 100 kDa (15). It is therefore possible that fragmentation of DHC may have occurred in our CIC-2 column because of a loss in stabilizing contacts with other dynein subunits during gradient pH elution. We reasoned that other subunits of the dynein motor complex may have been specifically eluted as multiple silver-stained bands were seen in Fig. 1B. Indeed, immunoblotting revealed the presence of DIC specifically in the CIC-2 column eluant (Fig. 1C). Furthermore, DIC migrates as a 75–80-kDa protein, and inspection of the

FIG. 1. CIC-2 interacts with the dynein motor complex in vitro. A, CIC-2 expression in murine hippocampus. Western blotting reveals a dominant band at 97 kDa, which is abolished upon pre-incubation of the CIC-2 antibody with antigenic peptide (+). Lanes were probed for actin to assess equal loading. B, eluted proteins from columns to which purified CIC-2, amylase, or nothing was conjugated were subjected to silver stain analysis. The major protein band (100 kDa) specific to the CIC-2 eluant was excised and identified by MALDI-TOF mass spectrometry to be a fragment of DHC. C, Western blotting analysis confirms the presence of DIC (a single 75–80-kDa band, also seen in whole brain homogenate; see Fig. 2B) in CIC-2 column eluant but not the amylase-conjugated column eluant.

Lanes were probed for actin and dynein and ClC-2.

are presented as the means ± S.E. Most statistical analyses were performed using the Student’s unpaired t test. Results obtained in patch clamp studies with drug-treated and -transfected cells were analyzed using the unpaired Student’s t test. Differences between multiple treatment groups were assessed by ANOVA, and differences between specific pairs were subsequently analyzed using the Bonferroni method.
silver-stained gel in Fig. 1B reveals the presence of a protein of this mass. These in vitro findings suggest that ClC-2 protein can interact (directly or indirectly) with the multi-subunit dynein motor complex.

**CIC-2 Interacts with Dynein in in Vivo Studies**—To determine whether the interaction between CIC-2 and dynein exists in vivo, immunoprecipitation experiments were performed using rat hippocampal membranes and polyclonal antibodies directed against the N or the C terminus of CIC-2 or preimmune IgG as a control. As with the N-terminal antibody (Nt-ClC-2; see Fig. 1A), the C-terminal-directed antibody (Ct-ClC-2) is specific as Western blotting of membranes prepared from murine brain tissue reveals a single band that migrates at 97 kDa that is absent in the CIC-2 knock-out sample (generated by Melvin and co-workers (4); see Fig. 2C). Crude hippocampal membranes were prepared, and immunoprecipitations were performed in the presence of 1% Triton X-100 plus 0.05% SDS as described under “Materials and Methods.” Immunoprecipitates were subjected to SDS-PAGE and probed using our CIC-2 antibody. Wild-type (+/+), and CIC-2 knock-out (−/−) mouse brain tissue membranes (4) were probed with anti-Ct-ClC-2. Blots were also probed with anti-actin to assess equal protein loading.

To determine whether ClC-2 is trafficked via the endosomal pathway, we expressed ClC-2 in COS7 fibroblasts as this cell line endogenously expresses ClC-2 and can be readily transfected. Confocal micrographs of CIC-2-specific immunofluorescence in COS7 cells revealed a predominant perinuclear staining pattern with a fainter signal detected close to the cell surface (Fig. 3A, indicated by arrowheads). Electron micrographs of immunogold-labeled CIC-2 supported our claim that CIC-2 localizes to the plasma membrane of COS7 cells (Fig. 3B). Further confirmation of the presence of CIC-2 at the cell surface was achieved using cell surface biotinylation. Intact COS7 fibroblasts were incubated with sulfosuccinimidyl-2-(biotinamido) ethyl-1,3-dithiopropionate to selectively label cell surface-associated proteins. To ensure that biotin had not labeled intracellular protein, we also assessed biotinylation of the abundant intracellular protein, actin. As shown in Fig. 3D, Western blotting analysis of biotinylated proteins isolated from COS7 extracts shows the presence of CIC-2 and the absence of actin. Hence, this biochemical assay reports cell surface incorporation of CIC-2 endogenously expressed in COS7 cells.

Whole cell patch clamp studies confirmed the functional expression of CIC-2 at the cell surface. Currents typical of those conferred by CIC-2 expression, with activation at hyperpolarizing voltage steps of at least −60 mV and greater, and an inwardly rectifying current-voltage relationship was detected (Fig. 3E). The observed current density had a magnitude of −21.5 ± 1.3 pA/pF (mean ± S.E., n = 10) at a membrane potential of −140 mV. To ensure that these measured currents were in fact mediated by CIC-2, we employed an antisense strategy. We have previously used this approach to confirm the functional expression of endogenous CIC-2 in Caco-2 cells (10), as there are no known specific blockers of the CIC-2-mediated chloride conductance (16). In this previous study, we showed that antisense CIC-2 cDNA significantly suppressed CIC-2 protein expression and reduced hyperpolarization-activated currents. This effect was specific, as an endogenous depolarization-activated chloride conductance was unaffected by antisense CIC-2 cDNA transfection (10). As in our previous studies in Caco-2 cells, we found that antisense CIC-2 cDNA transfection of COS7 cells led to significant suppression of endogenous hyperpolarization-activated chloride currents (Fig. 3E, F). We observed a dramatic reduction in the magnitude of hyperpolarization-activated current densities (at −140 mV) in antisense-transfected cells (Fig. 3E; −7.2 ± 0.6 pA/pF, n = 10) relative to control (−21.5 ± 1.3 pA/pF; see Fig. 3F, n = 10; p < 0.0001). The abolition of hyperpolarization-activated chloride currents upon CIC-2 antisense (aCIC-2) transfection demonstrates that CIC-2 channels are functionally expressed in the cell surface of COS7 cells. Furthermore, as in the previous Caco-2 cell studies (10), antisense CIC-2 cDNA had no effect on endogenous depolarization-activated chloride currents (at +40 mV, 2.6 ± 0.1 pA/pF in control cells versus 2.0 ± 0.3 in antisense-transfected cells, n = 10; p > 0.1) arguing that the effect was specific. Therefore, CIC-2 channels are expressed in the plasma membrane of COS7 cells, and hence, this cell line is suitable for studies of vesicular trafficking to and from the plasma membrane.

**The Retrograde Trafficking of CIC-2 May Involve the Endosomal Pathway**—To determine whether CIC-2 is trafficked via
endosomes in COS7 cells, we examined the relative localization of CIC-2 with respect to a marker of early endosomal vesicles; EEA1 (Fig. 4, A–C) using confocal microscopy. We found that the pattern of CIC-2-specific staining in COS7 cells at 37 °C showed no appreciable steady-state co-localization with EEA1 in COS7 cells, shown in Fig. 4A (merged image). However, as CIC-2 channels may associate with this compartment only transiently, we imposed a temperature block, i.e. 20 °C for 2 h, a maneuver known to be permissive to internalization but restrictive to trafficking out of early endosomes. This treatment has been shown to result in the formation of swollen early endosomes (17, 18). When COS7 cells were analyzed immediately following the 20 °C incubation, CIC-2-specific immunofluorescence retained a perinuclear staining pattern but also acquired the appearance of swollen vesicular structures, which partially overlapped with EEA1-specific immunofluorescence (Fig. 4B). Co-localization of CIC-2 in EEA1-positive compartments was most clearly evident in cells at 20 °C, which had been treated previously with cycloheximide to minimize protein expression in the biosynthetic pathway (Fig. 4C).

Quantitative analysis of the percentage of overlapping CIC-2-specific and EEA1-specific immunofluorescence relative to total CIC-2-specific immunofluorescence was performed to provide a quantitative assessment of protein co-localization under different experimental conditions. In total, 71 cells exposed to the 20 °C treatment and 16 cells exposed to 20 °C with cycloheximide were compared with 64 untreated cells. Under control conditions, only 8.9 ± 0.9% of CIC-2 signal was found to co-localize with EEA1. Temperature arrest of vesicular exit from endosomes resulted in a statistically significant increase (p < 0.0001) in CIC-2 co-localization with EEA1 signal in the presence and in the absence of cycloheximide (22.0 ± 4.1% and 39.8 ± 4.4% respectively; see Fig. 4D). These results suggest that CIC-2 undergoes trafficking via endosomes and is retained in early endosomal compartments at 20 °C.

We would expect that the 20 °C trafficking block would decrease CIC-2 expression on the cell surface, as this temperature is permissive to internalization but inhibitory to trafficking out of early endosomes via recycling endosomes (19). As is shown in Fig. 4E, the level of CIC-2 expressed at the cell surface (assessed by biotinylation band densitometry) decreased by almost 63% upon incubation at 20 °C (n = 3). Together with the observation of increased co-localization of CIC-2 with EEA1 in cycloheximide-treated cells, these results support the notion that CIC-2 undergoes retrograde trafficking between the plasma membrane and endosomes.

CIC-2 Subcellular Distribution in COS7 Cells Is Dependent on Microtubule Integrity and Motor Function of the Dynein Complex—To determine whether our biochemical findings suggesting an in vivo interaction between CIC-2 and the dynein motor complex are physiologically significant, we assessed the effect of disrupting microtubules and dynein motor function on CIC-2 localization. We first compared the subcellular distribution of CIC-2 with that of dynein. As shown in Fig. 5B, dynein exhibited a perinuclear distribution similar to CIC-2 (Fig. 5A), with significant dynein-specific immunofluorescence close to the cell surface. At a higher magnification, co-localization of CIC-2 and dynein was observed in regions proximal to the plasma membrane (Fig. 5, A and B, insets; co-localization indicated by arrowheads). Co-localization of these two proteins at the plasma membrane was evident in electron micrographs of immunogold-labeled CIC-2 and dynein (Fig. 5C). CIC-2 and dynein were also found to co-localize on continuous intracellular membranes (data not shown). Collectively, these findings suggest a possible role for dynein in mediating retrograde CIC-2 trafficking.

The microtubule-depolymerizing agent nocodazole has been reported to inhibit dynein-based motility (20). If the dynein motor complex participates in the regulation of CIC-2 localization we predict that nocodazole treatment would perturb the...
Fig. 4. A sub-population of intracellular CIC-2 localizes to early endosomes. A, minimal co-localization of CIC-2 with EEA1 in COS7 cells at 37 °C increases significantly upon incubation at 20 °C (B). C, accumulation of steady state levels of stable CIC-2 in early endosomes in cycloheximide-treated (CHX) cells. Scale bar represents 10 μm. D, quantitative analysis of CIC-2/EEA1 co-localization. A statistically significant increase in CIC-2 co-localization with EEA1 (*) was observed under both conditions. E, 20 °C temperature block causes a significant decrease in CIC-2 biotinylated at the cell surface (*) , n = 3) reflecting endosomal sequestration.

normal subcellular distribution of CIC-2. To test this prediction, COS7 cells were therefore subjected to a 30-min incubation in serum-free growth medium supplemented with either 35 μM nocodazole in dimethyl sulfoxide (Me2SO; final concentration 0.01%) (v/v) or 0.01% Me2SO as control (Fig. 6, B and A, respectively). Following immediate fixation, cells were then processed for immunofluorescence analysis and labeled for CIC-2 and tubulin. As shown in Fig. 6B, nocodazole appeared to cause a dispersion of CIC-2. This effect was reversible as we found that CIC-2 localization was restored upon removal of the drug from the culture medium (data not shown), also ruling out the possibility of cytotoxic effects of the drug (21). When COS7 cells were subjected to nucodazole treatment at a reduced temperature (4 °C) at which microtubule depolymerization is still observed, but protein trafficking is halted, CIC-2 distribution was found to be unaltered (data not shown).

We employed Image J software to quantify the relative effects of nocodazole or Me2SO (vehicle) on CIC-2 distribution. Line scan analysis was conducted on 36 drug-treated cells, and 32 Me2SO-control cells (randomly chosen from three independent trials). Mean values and their corresponding standard errors were calculated and plotted as bar graph representations using Origin software (Fig. 6C; black bars represent nocodazole-exposed cells), and unpaired Student's t tests were performed to determine whether any differences observed were statistically significant. Typically 55.3 ± 1.6% (mean ± S.E.) of endogenous CIC-2 fluorescence signal was within the first quarter length of the cell, with 27.8 ± 1.3%, 8.7 ± 0.7%, and 8.1 ± 0.6% in the second, third, and fourth quarter lengths, respectively. Microtubule disruption was found to shift CIC-2 fluorescence signal away from the perinuclear region toward the periphery of the cell. CIC-2 signal was distributed more evenly throughout the cytosol, so that less than half the original signal was in the first quarter length (26.0 ± 1.1%, p < 0.00001). A concomitant increase in fluorescence signal was seen in the peripheral quadrant, (21.7 ± 0.9% versus 8.1 ± 0.6%, p < 0.00001), supporting our visual impression that CIC-2 distribution is dependent on microtubule integrity. Collectively, these findings suggest that CIC-2 localization is regulated in part by a microtubule motor-dependent process.

As an alternative approach toward studying the role of dynein in CIC-2 trafficking, we investigated the effect of impaired dynein motor activity on CIC-2 localization. This was achieved by the overexpression of a dynactin subunit, dynamitin (22). Dynamitin is believed to act as an intermediary or adaptor complex that facilitates dynein binding to its cargo (reviewed in Ref. 23). Overexpression of dynamitin results in the disruption of the dynactin complex by separating the p150glued subunit that interacts with dynein, from the Arp1 filament, which is thought to facilitate cargo/dynein protein interactions (24). The overexpression of dynamitin has been reported to disrupt dynein-dependent maintenance of membrane organelle distribution (22) and microtubule organization (25). The dynein-dynactin complex is also implicated in the movement of material within the endocytic pathway, as dynactin disruption by dynamitin overexpression was found to perturb endosomal trafficking (26). Similar effects can be seen by overexpressing the coiled-coil 1 domain of p150glued (CC1; amino acids 217–548), which binds dynein intermediate chain in vitro (27, 28) and is thus thought to be the dynein-binding domain of dynactin (25). To assess the effect of impaired dynein function on CIC-2 localization, we first tested the effectiveness of our system by labeling dynamitin-GFP-transfected COS7 cells with anti-EEA1 antibody. As expected on the basis of previous studies (22, 26), dynamitin-GFP-transfected cells were observed to have an altered EEA1 (early endosome) staining pattern (Fig. 6D, left panel). In contrast to its native compact juxtanuclear distribution, overexpression of dynamitin induced a redistribution of EEA1 into a more dispersed pattern, extending throughout the cytosol toward the periphery of the transfected cell (Fig. 6D, left panel; transfected cells indicated by GFP fluorescence, right panel). Similarly, CIC-2 distribution was disrupted in dynamitin overexpressing COS7 cells (Fig. 6E, left panel). CIC-2 lost its perinuclear localization and exhibited a more scattered distribution throughout the cytosol and toward the edge of the cell. We also observed a significant increase in CIC-2 localization at the plasma membrane in certain transfected cells (indicated by arrowheads; see Fig. 6E, left panel). Similar changes in CIC-2 distribution were seen upon transfection with the DRSredCC1 construct (data not shown), supporting the hypothesis that the dynein motor complex mediates trafficking of CIC-2 via the endosomal pathway.

Line scan analysis was performed to quantitatively assess the effect of dynamitin-GFP expression on CIC-2-specific immunofluorescence. 51 transfected cells (identified by GFP signal) and 54 untransfected cells were examined. The cells were randomly chosen from four independent transfections, and control cells were taken from transfected coverslips to minimize deviations in fluorescence signals attributed to experimental variation. Mean values and their corresponding standard errors were calculated and plotted as bar graph representations....
using Origin software (Fig. 6F; black bars represent transfected cells), and unpaired Student’s t tests were performed to determine whether any differences observed were statistically significant. On average, 62.8 ± 1.5% (mean ± S.E.) of endogenous ClC-2 fluorescence signal was within the first quarter length, with 24.7 ± 1.3%, 9.1 ± 0.7%, and 5.9 ± 0.2% in the second, third, and fourth quarter lengths, respectively. As with the nocodazole treatment, overexpression of dynamitin-GFP was found to shift this distribution away from the perinuclear compartments, so that only 38.0 ± 0.7% signal was in the first quarter length (p < 0.00001). A statistically significant increase in fluorescence signal was seen in the remaining three quarter lengths (28.8 ± 0.6%, p < 0.05; 21.0 ± 0.7%, p < 0.00001; and 12.7 ± 0.5%, p < 0.00001), suggesting that the overexpression of dynamitin resulted in a redistribution of ClC-2 toward the periphery of the cell.

**ClC-2 Functional Expression at the Cell Surface of COS7 Cells Is Dependent on Motor Function of the Dynein Complex**—The functional significance of disrupting ClC-2 localization by dynin inhibition was assessed in patch clamp studies. We measured the amplitude of hyperpolarization-activated currents in cells that were transfected with dynamitin-GFP. Consistent with our line scan analysis, we observed a significant increase in the amplitude of ClC-2-mediated current density in dynamitin-GFP-transfected cells (Fig. 7A, bottom trace). The current density measured at −140 mV increased 67%, from −21.5 ± 1.4 pA/pF (n = 10) to −35.9 ± 1.3 pA/pF (mean ± S.E., n = 14, p < 0.0001), suggesting that disruption of dynein function increases the number of ClC-2 channels in the plasma membrane (Fig. 7C). To ensure that the observed effect was specifically because of impaired dynein function at the level of vesicular trafficking and not because of impaired dynein bio-synthesis, we assessed the effect of erythro-9-(2-hydroxy-3-nonyl) adenine hydrochloride (EHNA), an inhibitor of the ATPase activity of the dynein complex (29, 30) (Fig. 7, B and D). Hyperpolarization-activated currents were recorded prior to and following treatment with 50 μM EHNA. ClC-2-mediated currents were typically recorded 10 min after addition of
EHNA (Fig. 7A, middle trace). Multiple trials indicated a 2-fold increase in current density (−43.2 ± 2.3 pA/pF, n = 10, p < 0.0001) at −140 mV (Fig. 7B). These data are summarized in the bar graph shown in Fig. 7E. Identical experiments performed on Caco2 intestinal epithelial monolayers revealed an even greater increase in ClC-2 channel function at the plasma membrane upon dynein inhibition with EHNA (50 μM). Hyperpolarization-activated chloride currents mediated by ClC-2 increased from −23.9 ± 2.5 pA/pF to −69.9 ± 12.9 pA/pF (n = 7, p < 0.01; see Fig. 7D). Collectively, these findings are consistent with our hypothesis that dynein may be important in the vesicular transport of ClC-2.

To determine whether the observed increase in ClC-2 channel function following disruption of dynein function is indicative of an increase in the amount of ClC-2 protein at the cell surface, we assessed the effect of EHNA on expression of ClC-2 in the plasma membrane by surface biotinylation. We assessed the effect of this pharmacological inhibitor of dynein function rather than the effect of dynamin overexpression to optimize the number of affected cells and enhance the gain of our biochemical assay. Inhibition of dynein by the addition of EHNA was found to increase the amount of biotinylated ClC-2 by ~4-fold (Fig. 8). These findings suggest that the increase in hyperpolarization-activated currents caused by disruption of dynein function reflects an increase in the amount of ClC-2 at the cell surface.

**DISCUSSION**

In the present report, we provide biochemical evidence for an *in vitro* and *in vivo* interaction between ClC-2 and dynein in brain tissue. First, we have shown that the dynein complex can interact with purified intact ClC-2 protein in our *in vitro* binding assay. This interaction likely also occurs *in vivo*, as dynein and ClC-2 can be co-immunoprecipitated from rat hippocampal slices. The functional consequences of this interaction were elucidated in the COS7 cell line using confocal microscopy and patch clamp electrophysiology. We demonstrate that dynein is important in ClC-2 trafficking, as disruption of dynein function not only causes dispersion of ClC-2 subcellular localization toward the plasma membrane but also increases ClC-2 expression and activity at the cell surface.

The molecular basis underlying the interaction between the dynein motor complex and ClC-2 remains to be determined. Hypothetically, the dynein motor complex may bind directly to ClC-2 as recent studies by Tai et al. (31) showed that the membrane protein, rhodopsin, can bind directly to the dynein light chain protein, Tctex. Interestingly, we could successfully co-immunoprecipitate dynein with ClC-2 using our C-terminal-directed antibody. As both antibodies are effective in immunoprecipitating similar amounts of protein, this result possibly implicates the N terminus of ClC-2 in mediating its interaction with the dynein complex. The N terminus of ClC-2 has been implicated previously in the regulation of the channel function of the protein (32). However, our preliminary *in vitro* binding experiments (including GST-fusion pull-down and enzyme-linked immunosorbent assay assays) failed to provide evidence supporting a specific, direct interaction between purified dynein (kindly provided by S. King and T. Schroer) and GST-fusion proteins containing ei-
ther the N terminus (residues 31–74) or C terminus (residues 869–907) of CIC-2. On the other hand, it is well known that dynactin interacts with and activates cytoplasmic dynein (33). Further, it has been shown that dynactin may recruit dynein to cargo in membrane vesicles directly (34) or indirectly via cytoskeletal proteins such as spectrin (35–37). Hence, in our future work, we will test the prediction that dynactin and spectrin may mediate the interaction between CIC-2 and dynein.

A role for the dynein motor complex in endocytosis has been implicated in previous studies. Burkhardt et al. (22) showed that disruption of the dynactin-dynein complex in COS7 cells by overexpression of dynamitin led to dispersion of endosomes toward the cell periphery. This observation was confirmed in the present work (Fig. 6D) and supports the putative role for this complex in retrograde endosomal trafficking (22, 26). Furthermore, cytoplasmic dynein has been shown to participate in phosphatidylinositol 3-kinase-regulated GLUT4 internalization in adipocytes (38). However, the present studies are the first to show that function of the dynein motor complex contributes to retrograde endosomal trafficking of an ion channel, thereby providing a novel molecular framework with which to study the regulated trafficking of CIC-2 channels and possibly other members of the CIC family of chloride channels. Two other members of this family, CIC-3 and CIC-5, have been localized to endosomal compartments (39–41). However, unlike CIC-2, which is thought to mediate its primary function at the cell surface, both CIC-3 and CIC-5 are stably expressed in endomembranes where they are thought to regulate the function of this organelle (39, 41). Hence, the molecular components mediating localization and trafficking of CIC-3 and CIC-5 may be distinct from those mediating CIC-2 trafficking.

In light of our current findings, we suggest that CIC-2 channel function at the cell surface can be regulated by modulation of vesicle retrieval from or insertion into the plasma membrane by dynein. In our future work, we plan to determine the role of dynein-mediated vesicular trafficking in CIC-2 activation by experimental maneuvers shown previously to regulate CIC-2 function at the cell surface. For example, activation of protein kinase C, cyclin-dependent kinase p34cdc2/cyclin B, and phosphatidylinositol 3-kinase have been shown to inhibit CIC-2 function at the cell surface (12, 32, 42, 43). Furthermore, Zheng et al. (43) showed that cyclin B-dependent phosphorylation of rabbit CIC-2 led to its enhanced ubiquitination and degradation. Finally, it will be important to determine how post-translational modification of CIC-2 regulates its interaction with molecular components within the CIC-2 trafficking pathway, such as the dynein motor complex.

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Evidence for a Functional Interaction between the CIC-2 Chloride Channel and the Retrograde Motor Dynein Complex
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