The CLC family of chloride transport proteins comprises nine members in mammals (1). Although four of these are plasma membrane-residing chloride channels, the other five, CLC-3–7, localize to distinct, yet partially overlapping compartments of the endo-lysosomal pathway and to other specialized vesicles of the late biosynthetic pathway like synaptic vesicles (1–5). On these organelles, they are involved in enabling luminal acidification and/or chloride accumulation (6–10). Despite the pivotal role of CLC proteins in endo-lysosomal function and their involvement in diverse pathologies in mouse models and human genetic disease (1), little is known about the sorting steps by which they reach their subcellular destinations.

Sorting of endo-lysosomal transmembrane proteins is usually mediated by cytosolic motifs that are recognized by adaptor proteins. These recruit further components of the protein transport machinery, such as clathrin (11, 12). The dileucine motif (DE)XXLL(LI) and the tyrosine-based motif YXXΦ (with Φ being a bulky, hydrophobic amino acid) are recognized by the adaptor protein (AP) complexes AP-1–4. The cell uses different APs to recruit cargo proteins for specific transport routes such as AP-2 for endocytosis from the plasma membrane, AP-1 for transport between the trans-Golgi network and early endosomes, and AP-3 for sorting from early endosomes and the trans-Golgi network to late endosomes (13). The role of AP-4 is less understood. Another sorting signal, which is recognized by adaptors of the Golgi-localized, γ-ear containing, Arf-binding (GGA) family, is the dileucine motif DXXXLL (14). The three mammalian GGAs mediate sorting at the trans-Golgi network by binding to the DXXXLL motif of cargo and recruiting clathrin for transport to early or late endosomes. It is unclear whether different GGAs (GGA1, -2, -3) are involved in different sorting events (15).

For the predominantly plasma membrane-localized CIC-2, two sorting motifs have been identified, a dileucine motif in the C terminus that recruits AP-1B for sorting to the basolateral membrane of epithelial cells (16) and a tyrosine-based motif in a cytoplasmic loop between helices D and E that mediates recycling between endosomes and the plasma membrane (17). Bar-ttin, the β-subunit of CIC-K channels at the plasma membrane, carries an amino acid sequence (PQPPYVRL) likely to be involved in endocytosis (18). However, it is not clear whether the critical tyrosine belongs to a functional YXXΦ sorting motif or the sequence functions as a so-called PY motif for ubiquitination-regulated internalization as in the case of the sodium channel ENaC (19). So far no “conventional” AP or GGA binding motifs have been described for intracellular CLCs. CIC-3, which localizes to endocytic compartments (2, 4, 10, 20–23) as well as to synaptic vesicles and synaptic-like microvesicles (2, 3, 24), has been shown to interact via its N terminus with AP-1, AP-2, and clathrin (23). The interaction with clathrin was shown to be dependent on an acidic amino acid stretch with two dileucines, whereas that with AP-2 did not require this stretch. Targeting of CIC-3 to synaptic vesicles and synaptic-like microvesicles has been reported to require AP-3 (3). Various motifs different from AP or GGA interaction sequences...
have been shown to be important for the subcellular localization of endo-lysosomal CLCs. A splice variant of CIC-3, CIC-3B, exhibits a PDZ binding motif at its extreme C terminus (25) that recruits CIC-3B to the Golgi complex via an interaction with the PDZ domain-carrying Golgi protein GOPC (26). For CIC-4, which has mostly been reported to localize to endosomes (4, 27) but also to the endoplasmic reticulum (ER) (28), an N-terminal amino acid stretch is reportedly involved in ER retention when expressed heterologously (28). The predominantly endosomal CIC-5 (4, 27, 29–32), a small portion of which is endogenously found also in the plasma membrane (30, 33), bears a C-terminal-located PY motif (34). Although not required for in vivo CIC-5 function (33), studies in Xenopus oocytes and cultured opossum kidney cells revealed that ubiquitin ligases bind this motif and ubiquitylate CIC-5, stimulating its internalization from the cell surface (34, 35). Although endogenous CIC-6 localizes to late endosomes (36), heterologously expressed CIC-6 has been found on early and recycling endosomes (37). A basic amino acid stretch in CIC-6 seems to be involved in the recruitment of ClC-6 into detergent-resistant endosomes (37). A basic amino acid stretch in CIC-6 seems to explain the higher background in non-expressing cells in Figs. 4 and 5. Certain CLC sequences encoding the following amino acids of the respective human CLC were inserted into pGEX-5X-1 (GE Healthcare) resulting in an N-terminal GST tag: CIC-1-NT, aa 1–117; CIC-1-CT, aa 592–989; CIC-3-NT, aa 1–67; CIC-3-CT, aa 586–760; CIC-4-NT, aa 1–67; CIC-4-CT, aa 584–760; CIC-5-NT, aa 1–54; CIC-5-CT, aa 573–746; CIC-6-NT, aa 1–80; CIC-6-CT, aa 591–869; CIC-7-NT, aa 1–126. For the GST fusion protein of the Ostm1 C terminus, the sequence encoding aa 307–338 of mouse Ostm1 has been cloned into pGEX-5X-1.

For cell culture expression, Torpedo CIC-0 (42) was subcloned into pcDNA3 (Invitrogen). Constructs for human CIC-5, human CIC-6, and rat CIC-7 in this vector have been described previously (40, 41). For the generation of chimeric constructs containing parts of CIC-0 and either CIC-6 or CIC-7, the DNA sequences encoding the N-terminal part (aa 1–48 for CIC-0, aa 1–77 for CIC-6, and aa 1–123 for CIC-7), the transmembrane region (43) from the beginning of helix B until the end of helix R (aa 49–524 for CIC-0, aa 78–588 for CIC-6, and aa 124–614 for CIC-7), and the C-terminal region (aa 525–805 for CIC-0, aa 589–869 for CIC-6, and aa 615–805 for CIC-7) of the respective CLC were combined by recombinant PCR with overlapping primers and cloned into pcDNA3. For expression of fluorescently tagged Ostm1, the sequence encoding mouse Ostm1 was cloned into pEGFP-N3 (Clontech) linking Ostm1 with the C-terminal green fluorescent protein (GFP) by the sequence VDGTAGPSIAT.

To express hClC-5 in Xenopus oocytes, the cDNA was cloned into pTLN (44). An HA epitope was inserted between amino acids Glu107 and Val108 (extra-cytosolic loop between helices B and C) or at the C terminus by PCR mutagenesis. Point mutations were introduced by PCR with primers carrying the respective mutation. All constructs were confirmed by sequencing the complete ORF.

**GST Pulldown Assays**—GST fusion proteins were expressed in Escherichia coli (BL21, DE3) for 5–6 h at 25 °C after induction with 0.12 mg/ml isopropyl-β-D-thiogalactopyranoside before pelleting the cells by centrifugation at 5000 × g. Cells were lysed by sonication in PBS supplemented with 0.5 mg/ml AEBSF (Roche Applied Science), protease inhibitor mixture (Complete®, Roche Applied Science), and lysozyme (Sigma) and subsequent incubation with 1% (w/v) Triton X-100 on ice. GST fusion proteins were affinity-purified from a 20,000 × g supernatant by a 2-h incubation with glutathione-Sepharose (GE Healthcare) under constant agitation at 4 °C and subsequent washing with PBS. Purity and concentration was estimated by Coomassie staining after SDS-PAGE with BSA as standard.

For a single pulldown experiment, 1.0–1.5 ml of lysate of a 10-cm dish of confluent HeLa cells in PBS supplemented with 1% (w/v) Triton X-100, 0.5 mg/ml AEBSF (Pefabloc SC), protease inhibitor mixture (Complete®), and 1 mM Na3VO4 was centrifuged at 10,000 × g for 10 min, and the supernatant was incubated with roughly 50 μg of GST fusion protein coupled to Sepharose for 2 h under constant agitation at 4 °C. After 4 washes with PBS, supplemented with 0.1% (w/v) Triton X-100, 0.5 mg/ml AEBSF, and protease inhibitor mixture (Complete®), bound protein was eluted by incubation in SDS sample buffer at 55 °C for 15 min. After sedimenting the Sepharose beads, the

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eluate was separated by SDS-PAGE and probed by immunoblot. Pulldown assays from mouse brain or kidney lysate were performed equivalently, with PBS replaced by HEPES-buffered saline. For tissue lysate preparation, two brains or four kidneys of WT C57Bl/6 mice were homogenized in 10 ml of HEPES-buffered saline with 0.5 mg/ml AEBSF, protease inhibitor mixture (Complete/H23041), and 1 mM Na3VO4. The supernatant of a 10-min centrifugation at 10,000 g was supplemented with 1% final Triton X-100. 1.5 ml of the supernatant of 2 further centrifugation steps (15 min at 10,000 x g and 20 min at 50,000 x g) was used per pulldown as described above.

Expression in Cell Culture and Fluorescence Microscopy—Plasmid DNA encoding the respective construct was transfected using FuGENE6 (Roche Applied Science) according to the manufacturer’s instruction, and cells were grown in a humidified 5% CO2 incubator at 37 °C for further 24–48 h before fixation with 4% paraformaldehyde in PBS for 15 min. For immunostaining, cells were incubated with 30 mM glycine in PBS for 5 min and permeabilized with 0.1% saponin in PBS for 10 min. Both primary and AlexaFluor-coupled secondary (Molecular Probes) antibodies were applied in PBS, 0.05% saponin supplemented with 3% BSA. Images were acquired with an LSM510 laser scanning confocal microscope equipped with a 63 x 1.4 NA oil immersion lens (Zeiss).

Voltage Clamp Analysis and Surface Expression Assay in Xenopus laevis Oocytes—Capped cRNA was transcribed from constructs in pTLN linearized with MluI (extra-cytosolic HA) or HpaI (C-terminal HA) using the mMessage mMachine kit (Ambion) and SP6 polymerase. 20 ng of cRNA were injected into defolliculated oocytes. Oocytes were kept at 17 °C in ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 5 mM HEPES, pH 7.5) for 2 days before analysis. Two-electrode voltage clamp measurements were performed at room temperature using a TurboTec10C amplifier (npi electronic) and pClamp10 software (Molecular Devices) as described previously (34). Surface expression of HA-tagged ClC-5 protein was determined as described previously (34, 45).

RESULTS

Binding of Endosomal Sorting Machinery to N- and C-terminal Cytosolic Domains of Endosomal/Lysosomal CLCs—To screen for interactions of intracellular CLCs with the endosomal/lysosomal transport machinery, we generated recombinant GST fusion proteins of their cytosolic N- and C-terminal domains (NT and CT, respectively) and of the cytosolic CT of the ClC-7 subunit Ostm1, a protein with a single transmembrane span. In addition, we included the cytosolic domains of the plasma membrane-residing channel ClC-1 and GST alone. Pulldown experiments from HeLa cell lysate were performed with equal amounts of bait proteins and an immunoblot against α-adaptin (AP-2) are given in supplemental Fig. S1. D, shown is a summary of various pulldown experiments from HeLa cell, mouse brain, and kidney lysate as shown in A–C. ( ), no binding detected; (+), not detected in all experiments; +, binding always detected; + +, always strongly detected. The right column summarizes the published subcellular localization of the respective CLC.
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GGAs, however, could not be probed in those tissues as our antibodies did not recognize the mouse proteins.

ClC-1 interacted through its N terminus with AP-2 (Fig. 1A). ClC-3-NT bound clathrin, as reported previously (23). In contrast to this previous study, we did not detect binding of AP-1 or -2 to ClC-3 (Fig. 1A). Neither did ClC-3 fusion proteins bind AP-3, as would have been expected from the reported role of AP-3 in targeting ClC-3 (3). ClC-4 bound clathrin weakly with its N terminus (Fig. 1A), but no binding to AP or GGA proteins was found with either its N or C terminus (Fig. 1, A and B). ClC-5-NT pulled down AP-1 and AP-2 as well as clathrin (Fig. 1A), whereas none of the investigated proteins was bound by the ClC-5 C terminus (Fig. 1B). The N terminus of ClC-6 interacted weakly with AP-2 and more strongly with AP-3 (Fig. 1A). AP-3 also bound to the ClC-6 C terminus, albeit less strongly (Fig. 1B). Both the N and C termini of ClC-7 bound AP-2 and AP-3 (Fig. 1, A–C), and its N terminus also bound AP-1 (Fig. 1, A and C). AP-1 and AP-4 binding to the C terminus of ClC-7 appeared weak because it was not detected in all pulldown assays (compare Figs. 1B and 2G). The N terminus of ClC-7 also pulled down GGA2 more efficiently than GGA3 (Fig. 1A) and GGA1 (not shown). The C terminus of the ClC-7 β-subunit Ostm1 bound AP-2 and AP-3 but not GGA2 or GGA3 (Fig. 1C). These interactions were also found when pulldown experiments were performed with lysates of murine adult fibroblasts lacking CIC-7 (39) or Ostm1 (38) (not shown), excluding a possible indirect interaction of Ostm1-CT mediated by bound CIC-7 and vice versa.

Identification of the Sorting Motifs Mediating Clathrin and Adaptor Binding—To investigate the interactions in more detail, we set out to identify the respective binding motifs. The N and C termini of CLCs contain numerous potential tyrosine-based or dileucine sorting motifs that might mediate the binding of APs or GGAs (Fig. 2A). To test for the actual role of the potential motifs in intracellular CLCs, we replaced the tyrosines of tyrosine-based motifs and the leucines (or leucine-isoleucines) of dileucine motifs, respectively, by alanines in our GST fusion proteins and tested their impact on adaptor binding in pulldown experiments.

The N-terminal domain of ClC-3, which bound clathrin but not APs or GGAs, contains a potential tyrosine-based motif at Tyr27 (27YDFD, Fig. 2A). However, previous work (23) showed that clathrin did not bind to this motif but to a more N-terminal stretch containing acidic residues and two dileucines (13LLDLDE, Fig. 2A). Our pulldown experiments confirmed that the strong binding of ClC-3-NT to clathrin (Fig. 1A) was virtually abolished when all four leucines of this stretch were replaced by alanines (Fig. 2B).

Within the N terminus of ClC-5, which bound AP-1, AP-2 and clathrin, there is one potential tyrosine-based motif at Tyr14 (14YDDF). It is conserved in the N termini of ClC-3 and ClC-4 (Fig. 2A), which, however, lack detectable AP binding (Fig. 1A). We, therefore, initially tested another sequence in ClC-5-NT (ESTWALL48) that almost matches the (DE)XXX(LI) consensus sequence (Fig. 2A). However, mutating Leu68 and Ile68 to alanines did not significantly reduce the binding of APs and clathrin to ClC-5-NT, whereas, surprisingly, mutating Tyr14 to Ala strongly reduced these interactions (Fig. 2C). Combining Y14A with the L47A/I48A double mutation did not reduce binding further (Fig. 2C).

The N terminus of ClC-6, which bound AP-2 and AP-3 (Fig. 1A), contains three potential tyrosine-based motifs (855YEFL, 61YLEV, and 79YEA) (Fig. 2A). Because the crystal structure of the prokaryotic EcClC-1 protein suggests that the third motif might extend into the first membrane-spanning helix B (43), we focused on the first two motifs. Mutating Tyr65 to Ala strongly reduced AP-2 and AP-3 binding, with a weaker effect seen with the Y61A mutant (Fig. 2D). Combining both mutations virtually abolished binding (Fig. 2D). Recently, the basic amino acid stretch 871KKGRR (Fig. 2A) was implicated in the localization of heterologously expressed CIC-6 to early and/or recycling endosomes, an effect that may involve lipid rafts (37). When this stretch had been mutated to 71AAGAA, CIC-6 was trafficked to late endosomes/lysosomes (37). Introducing this mutation in our GST-CIC-6-NT seemed to affect binding of AP-2 and AP-3 only slightly (Fig. 2D).

Despite the low efficiency with which the C terminus of ClC-6 pulled down AP-3 (Fig. 1B), it displays many potential sorting motifs (Fig. 2A). The EKEDLL796 sequence conforms to the (DE)XXX(LI) consensus motif for AP binding, whereas DLTTL795 conforms to the DXXL consensus sequence for GGA binding. Both sequences are located in the stretch between the two cystathione β-synthetase domains (46, 47), which is particularly long in ClC-6. In this interdomain stretch there are also three tyrosine-based motifs (717YPNL, 774YAEM, and 784YDPD). Such a motif is also found at the very end of the protein (866YQTI). Another potential Tyr-based motif (855YEFL) is predicted to be positioned within an α-helix of the cystathione β-synthetase 2 domain and is not conserved in mouse (where the Tyr is substituted by Asn). We, therefore, did not investigate it further. We next replaced the key tyrosines and leucines, respectively, of the other motifs by alanines. Mutants in which the extreme C-terminal 866YQTI motif was disrupted (Y866A), either by itself or together with mutations of other potential sorting motifs, did not bind AP-3 anymore (Fig. 2E, supplemental Fig. 2A). In contrast, various mutants in which the 866YQTI motif was preserved (Fig. 2E), even those with all of Leu705, Leu706, Tyr717, Tyr774, Tyr784, and Leu794-Leu795 substituted with alanines (supplemental Fig. 2A), still pulled down AP-3 similar to the WT C terminus. We conclude that specific binding of AP-3 to the CIC-6 C terminus depends on the 866YQTI motif at the extreme C terminus. In comparison to the N-terminal fusion protein, however, AP-3 binding to CIC-6-CT is weak.

The N terminus of human ClC-7 contains a DXXL consensus sequence for GGA binding (DDEL77) (Fig. 2A) that might underlie the binding of GGA2 and GGA3 (Fig. 1A). The binding of AP-1, AP-2, and AP-3 might be mediated by the potential (DE)XXX(LI)-type motif EAAPLL24 and/or the tyrosine-based motif with 94YESL (Fig. 2A). Indeed, mutating leucines Leu68 and Leu69 to alanines specifically abolished the interaction with the GGA proteins, leaving AP binding unaffected (Fig. 2F). Conversely, replacing Leu23 and Leu24 by alanines abolished the pulldown of APs but not of GGAs. We, therefore, did not examine a putative role of the 94YESL motif in AP binding.
As expected, combined disruption of both dileucine motifs inhibited binding of both APs and GGAs (Fig. 2F). The only conventional AP binding motif in the C terminus of ClC-7 is a canonical YXXΦ motif at Tyr<sup>715</sup> (Y<sup>715</sup>PRF<sup>718</sup>, Fig. 2A). However, mutating this tyrosine to alanine did not reduce the binding of AP complexes to ClC-7-CT (Fig. 2G).

FIGURE 2. Identification of adaptor protein binding sites. A, shown is a comparison of the N- and C-terminal regions of human ClC-3–7. Residues shown in lowercase were not included in the GST fusion proteins. The two conserved cystathionine β-synthetase domains and the beginning of helix B and end of helix R are indicated. Potential AP binding (DE)<sub>X</sub><sub>3</sub>L(LI) and Y<sub>X</sub><sub>H</sub> sorting motifs are highlighted as white on a black background. Gray boxes indicate an N-terminal leucine-rich stretch in ClC-3 (23), the N-terminal ESTWALI<sub>48</sub> sequence, and the PY motif in ClC-5 (34), a basic amino acid stretch in the N terminus of ClC-6 (37), and a putative unconventional sorting motif (WE) (49) in the C termini of ClC-6 and -7, respectively. CHC, clathrin heavy chain. B–H, Western blots of eluates from pulldown experiments explore the binding of interactors identified in Fig. 1 to the respective fusion proteins, either WT or mutants in candidate binding motifs. Eluates from GST columns and 1% (0.5% in E) input are shown as controls. For Ponceau staining showing similar amounts of bait protein see supplemental Fig. S2.
Although it weakly bound AP-2 and AP-3, the cytosolic C terminus of Ostm1 does not display any consensus tyrosine-based or dileucine motifs. To narrow down the position of the binding site, we generated two overlapping constructs with the first part of the C terminus (Ser307-Thr328) and the extreme C-terminal part (Ile319-Thr338), respectively, both fused to GST. The sequence between Ser307 and Thr328 was sufficient to pull down AP-2 and AP-3 as efficiently as the full C terminus, whereas no binding to the C-terminal fragment Ile319-Thr338 was detected (Fig. 2H). The interaction was not dependent on the dipeptide LI319 (Fig. 2H).

Internalization of Heterologously Expressed ClC-5 Is Largely Independent of Its N-terminal AP-2 Binding Motif—The 14YDDF motif in the N terminus of ClC-5 binds AP-2 (and other adaptors, Figs. 1A and 2C) and might, therefore, be involved in its endocytosis from the plasma membrane. To test this hypothesis, we introduced the Y14A mutation into a full-length ClC-5 construct and expressed it in CHO cells, which have negligible endogenous levels of ClC-5 (48). Immuno-fluorescence microscopy did not reveal obvious differences in the subcellular localization between WT and Y14A ClC-5 (Fig. 3A). Both proteins displayed some plasma membrane localization in addition to intracellular vesicular staining. We tested whether we could detect stronger plasma membrane staining with a mutant in the C-terminal “PY-like” internalization motif (34) (Fig. 2A). This motif mediates E3 ubiquitin ligase-mediated internalization of ClC-5 in heterologous expression systems (34, 35). However, we could not observe an increase in plasma membrane expression of this Y672E mutant by immunofluorescence (Fig. 3A). Even 2-fold increases in plasma membrane expression are difficult to detect by immunofluorescence. For a more reliable, quantitative comparison of cell surface expression between WT and mutants, we
introduced an HA epitope into the extracytosolic loop between helices B and C of ClC-5 (34). As a negative control we appended an HA tag to the cytosolic C terminus. All constructs were expressed to similar extents in *Xenopus* oocytes (Fig. 3B).

The extracellular presence of the epitope was quantified using an HA antibody in a chemiluminescence assay (34, 45). These experiments revealed that the surface expression of ClC-5Y14A and WT ClC-5 were indistinguishable, whereas the PY mutant ClC-5Y672E showed a roughly 2-fold higher surface expression (Fig. 3C) as in our previous work (34). Likewise two-electrode voltage clamp measurements of oocytes expressing the Y672E mutant yielded about 2-fold higher currents than those expressing either WT or Y14A ClC-5 (Fig. 3D). We conclude that unlike the C-terminal PQPPYVRL675 motif, the N-terminal 14YDDF motif does not play a significant role in plasma membrane localization of ClC-5.

**ClC-6 Sorting through Its Cytosolic Domains**—To investigate the role of the sorting motifs identified in ClC-6, we compared the subcellular localization of full-length WT with mutant ClC-6 in transfected HeLa cells. As reported previously (37), heterologously expressed ClC-6 colocalized with the recycling-endosome marker transferrin receptor (TFR) (Fig. 4A) rather than with markers of late endosomes (not shown). This contrasts with the late endosomal localization of native ClC-6 (36). When the tyrosines of the confirmed AP3-binding 866YQTI motif and of the 48YESL and 61YLEV consensus sequences were changed to alanine either alone or in combination, the localization of the resultant mutant was not altered (not shown). We, therefore, mutated all potential “classical” sorting motifs in the N and C termini of ClC-6 (Fig. 2A) in combination. However, even this protein (ClC-6Y48A,Y61A,LL705/706AA,Y717A,Y774A,Y784A,LL795/796AA,Y866A) was targeted to TFR-positive endosomes of transfected cells like WT ClC-6 (Fig. 4A). We then introduced into this heavily mutated construct two other point mutations (W590A,E591A) to disrupt a potential unconventional sorting signal reported to mediate lysosomal targeting (49). These two residues are located at the end of the last intramembrane helix R and had not been included in the fusion proteins used for pulldown experiments (Fig. 2A). However, even these additional mutations did not change the co-localization of ClC-6 with the TFR (not shown).

We finally generated chimeric proteins in which portions of ClC-6 were replaced by equivalent segments of the plasma membrane Cl−/H+ channel ClC-0 from *Torpedo marmorata* (42). Cytoplasmic N-terminal and C-terminal domains and the central transmembrane domain were assembled in different combinations, and their subcellular localization was determined in transfected HeLa cells (Fig. 4B). When both the N and C termini of ClC-6 were replaced in chimeras 0-6-0 and 6-0-0 by those of ClC-0, predominant ER-like staining was observed in less than 50% of transfected cells. In the majority of cells the 0-6-0 chimera was strongly plasma membrane-localized just like ClC-0 itself (Fig. 4B), demonstrating the importance of the cytosolic domains for...
endosomal sorting. Unfortunately, chimeras possessing the ClC-6 C terminus and the N terminus of ClC-0 (0-6-6 and 0-0-6) did not leave the ER (Fig. 4B). The two chimeras with an N terminus of ClC-6 and the C terminus of ClC-0 (6-6-0 and 6-0-0) localized to intracellular punctate structures (Fig. 4B) where they colocalized with the TfR (not shown) just as heterologously expressed ClC-6. Hence, the N terminus of ClC-6 is sufficient for endosomal targeting. Unexpectedly, the chimera 6-0-6 displayed a perinuclear localization pattern (Fig. 4B) and colocalized with the Golgi protein GM130 (supplemental Fig. 3A). Disrupting the C-terminal 866YQTI AP-3 binding site of ClC-6 by the Y866A mutation did not affect the apparent ER localization of 0-6-6 and 0-0-6 (not shown) nor the perinuclear localization of 6-0-6 (supplemental Fig. 3B) even when combined with mutations Y48A and Y61A, which together virtually abolished binding of AP-2 and AP-3 to the ClC-6 N terminus (Fig. 2D).

Sorting Motifs Responsible for the Subcellular Localization of ClC-7/Ostm1—To investigate the role in lysosomal sorting of ClC-7 of identified AP and GGA binding motifs, we transfected HeLa cells with rat ClC-7 (rClC-7) or with chimeras between rClC-7 and ClC-0. We did not cotransfect the /H9252-subunit Ostm1 because Ostm1 bound APs in our pulldown experiments and, therefore, might have confounded our results. Transfected full-length rClC-7 nearly perfectly colocalized with the late endosomal/lysosomal marker protein LAMP-1 (Fig. 5A) as observed previously in native cells (40). When we replaced the cytosolic N- and C-terminal regions of rClC-7 by those of ClC-0, the resulting chimera 0-7-0 yielded a predominantly reticular staining pattern indicative of ER retention (Fig. 5A). However, a small proportion of 0-7-0 reached the plasma membrane. Unlike WT ClC-7, the chimera did not colocalize with LAMP-1 (Fig. 5A).

The N terminus of rClC-7 suffices to direct the plasma membrane Cl− channel to late endosomes and lysosomes, as revealed by the co-localization of 7-0-0 with LAMP-1 in transfected cells (Fig. 5B). When we disrupted in this construct the N-terminal EGAPLL24 and DDELL67 motifs (homologous to EAAPLL24 and DDELL69 in human (supplemental Fig. 4), which bind APs and GGAs, respectively (Fig. 2F)) either alone (supplemental Fig. 5) or in combination (Fig. 5B), the mutant 7-0-0 chimeras were still sorted to late endosomes/lysosomes. Additionally mutating tyrosine Tyr92 (homologous to human Tyr94 in the YESL94 motif that was not involved in AP binding (Fig. 2F)) did not alter this localization (supplemental Fig. 5).

Compared with human CIC-7, rCIC-7 exhibits an additional (DE)XXXL(LI) consensus motif (EETPLL37) that is a candidate site for AP protein binding (supplemental Fig. 4). Replacing both leucines of this motif by alanines did not affect the subcellular localization of 7-0-0, whereas the combined disruption of all three N-terminal dileucine motifs brought the 7-0-0 chimera to the plasma membrane (supplemental Fig. 5). Surprisingly, disruption of the GGA binding motif DDELL67 was not required for this effect, as also the mutant in which only both consensus sites for AP binding were disrupted (7LL23/24AA,LL36/37AA -0-0) reached the cell surface instead of colocalizing with LAMP-1 (Fig. 5B).

FIGURE 5. Lysosomal sorting determined by the ClC-7 N terminus. A, subcellular localization of rClC-7 (top), a chimera of rClC-7 with N- and C-terminal domains replaced by those of ClC-0 (0-7-0, below), and WT ClC-0 (bottom) after transient transfection of HeLa cells, in comparison to LAMP-1 as marker for late endosomes and lysosomes. ClC-7 colocalizes with LAMP-1, whereas ClC-0 shows plasma membrane expression. The 0-7-0 chimera shows weak plasma membrane expression in addition to strong ER-like staining. B, sorting determinants in the ClC-7 N terminus investigated in HeLa cells transfected with a chimeric protein (7-0-0) in which the N terminus of the plasma membrane channel CIC-0 was replaced by that of rCIC-7 (top panel) or by ClC-7 N termini carrying two combinations of mutations in the EGAPLL24, EETPLL37, and DDELL67 dileucine motifs (EETPLL37 present in rat, but not humans). The N terminus of ClC-7 sufficed to target ClC-0 to lysosomes, and combined disruption of the first two motifs in 7LL23/24AA,LL36/37AA -0-0 resulted in cell surface localization.
We next explored the role of these N-terminal dileucine motifs for the targeting of full-length rClC-7. Introducing those mutations that directed the 7-0-0 chimera to the plasma membrane (Fig. 5B, supplemental Fig. 5) into rClC-7 resulted in mutants that resided to some extent in the plasma membrane (rClC-7LL23/24AA,LL36/37AA (Fig. 6) and rClC-7LL23/24AA,LL36/37AA,YF713/716AA (supplemental Fig. 6)). In further agreement with results for the 7-0-0 chimera, neither the individual disruption of the three N-terminal dileucine motifs (not shown, supplemental Fig. 6) nor the combined disruption of the EGAPLL24 and DDELL67 motifs (Fig. 6) changed the localization of ClC-7.

The remaining partial colocalization of these mutants with LAMP-1 cannot be attributed to signals remaining in the mutated ClC-7 N terminus because these mutations completely abolished the late endosomal/lysosomal localization of the 7-0-0 chimera (Fig. 5B). Because the 0-7-0 chimera (Fig. 5A) suggests that this localization is not owed to the transmembrane part, it is probably the C terminus, which bound AP adaptors in our pulldown experiments (Fig. 1B), that provides additional cues for endosomal/lysosomal sorting. Although disruption of the only tyrosine-based consensus motif (YPRF715) in the C-terminal GST fusion protein of human ClC-7 did not interfere with binding of APs (Fig. 2G), we mutated the homologous Tyr713 and Phe716 in full-length rClC-7. This mutant (rClC-7Y713F/716AA) remained localized to late endosomes/lysosomes (supplemental Fig. 6). When this mutation was added on top of those combinations that already partially shifted the constructs to the cell surface, the resulting rClC-7LL23/24AA,LL36/37AA,YF713/716AA (not shown) and rClC-7LL23/24AA,LL36/37AA,LL66/67AA,YF713/716AA (supplemental Fig. 6) still displayed the partial colocalization with LAMP-1 in addition to its localization to their presence at the plasma membrane. Obviously the YPRF motif of CIC-7 is not responsible for the apparent ability of the CIC-7 C terminus to partially direct ClC-7 to lysosomes when N-terminal lysosomal trafficking signals have been disrupted.

CIC-7 is required for ER export of its β-subunit, Ostm1, but ClC-7 is targeted to lysosomes even in the absence of Ostm1 (38). As we found weak binding of APs to the CIC-7 β-subunit Ostm1 (Figs. 1C and 2H), we wondered whether Ostm1 could support lysosomal sorting of CIC-7 mutants whose dominant lysosomal targeting sequences had been disrupted. To this end, we transiently cotransfected Ostm1 bearing a C-terminal GFP tag (Ostm1-GFP) with either WT or sorting mutants of rClC-7. Coexpression of both WT rClC-7 and rClC-7LL23/24AA,LL36/37AA was sufficient to ensure ER export of Ostm1. In both cases Ostm1-GFP colocalized with the CIC-7 construct (Fig. 7). With WT CIC-7, Ostm1-GFP was sorted to late endosomes/lysosomes, whereas it strongly labeled the cell surface in addition to a partial lysosomal localization when cotransfected with rClC-7LL23/24AA,LL36/37AA. Thus, CIC-7 determines the localization of Ostm1.

DISCUSSION

Despite the pivotal role of CLC Cl−/H+ exchangers in endosomal/lysosomal function, it has remained enigmatic how their differential localization to the various endosomal/lysosomal compartments is achieved. We used GST fusion proteins of the N- and C-terminal cytosolic domains of all intracellular CLCs to systematically test and compare their interactions with clathrin and its adaptors, AP-1–4 and GGA proteins. The resulting interaction pattern did not depend on the source of cell lysates used for the pulldown assay (HeLa cells, mouse brain, or kidney) and agreed well with the subcellular localization of the various CLC proteins (Fig. 1D). For example, AP-3, which mediates cargo sorting for transport to late endosomes, interacted specifically with late endosomal CIC-6 and lysosomal CIC-7/Ostm1, whereas it was not bound by the other CLCs, which localize to earlier endosomal compartments or the plasma membrane. On the other hand, the AP-2 adaptor complex involved in endocytosis from the plasma membrane was strongly bound by CIC-5, which cycles between endosomes and the cell surface.

The amino acid sequences of cytoplasmic CLC domains suggested the presence of consensus binding motifs. In several cases, N- or C-terminal domains displayed more than one candidate binding site. For instance, the N terminus of CIC-7 contains sites for both AP and GGA binding, and the N terminus of
CIC-6 displays several sites for AP binding that may be functionally redundant to some degree. Candidate binding sites were validated experimentally by disrupting them through mutagenesis, either individually or in combination. When introduced into the respective fusion protein, these mutations often abolished or reduced binding of adaptor proteins or clathrin, thereby confirming these motifs as being functionally relevant (an overview of these motifs is given in supplemental Table 1). In some cases, however, such mutations failed to affect binding. This situation is not unusual because the candidate binding site might be sterically inaccessible or may require more amino acids than those specified in the consensus sequence. Moreover, even if a “real” binding site had been disrupted by mutagenesis, the functional consequence might be masked by an additional, unidentified binding site in the fusion protein. Indeed, unconventional binding sites do exist, and we were unable to identify the site(s) by which the C terminus of either CIC-7 or its β-subunit Ostm1 bound AP-2 and AP-3.

Whereas the binding of specific adaptor proteins to the various CLC transporters agreed well with their intracellular localization (Fig. 1D), it often proved difficult to demonstrate their involvement in the intracellular trafficking of CLC proteins. Even when all confirmed adaptor binding sites in the N and C termini of CIC-6 were disrupted by mutagenesis, the heavily mutated CIC-6 was still trafficked to TBR-positive recycling endosomes just like transfected WT CIC-6. One has to realize, however, that this localization is abnormal. Native CIC-6 is found in late endosomes of neurons, the only cells significantly expressing this CLC protein (36). Because the CIC-6 mRNA is rather ubiquitously expressed (50), CIC-6 might require a neuron-specific β-subunit for its stability similar to CIC-K CI− channels, which are unstable without their β-subunit barttin (51), or like CIC-7, which needs Ostm1 (38). In contrast to Ostm1, which is not needed for the lysosomal localization of CIC-7 (38), barttin plays a crucial role in targeting CIC-K channels to the plasma membrane (18). Likewise, a so far unknown β-subunit for CIC-6 might traffic CIC-6 to late endosomes. If agreeing with our previous work (38). Even when the disruption of lysosomal sorting signals in CIC-7 led to a partial mislocalization of the transporter to the plasma membrane, co-expression with Ostm1 did not increase the proportion of CIC-7/Ostm1 found in late endosomes/lysosomes. Likewise, and also agreeing with our previous work (38), co-transfection with WT CIC-7 trafficked Ostm1 to late endosomes/lysosomes. Importantly, CIC-7 mutants that mislocalized to the plasma membrane carried Ostm1 to that domain as well. Hence, the subcellular localization of Ostm1 seems to depend entirely on sorting signals in CIC-7, and Ostm1 lacks an effect on CIC-7 trafficking.

Both N and C termini of CIC-7 strongly bound AP-3, an adaptor involved in trafficking to late endosomes. Subsequent transport to lysosomes does not require further sorting. The CIC-7 N terminus also bound GGA proteins, which might direct CIC-7 to early endosomes from where it would be sorted to late endosomes by AP-3. The prominent role in lysosomal sorting of the CIC-7 N terminus was revealed by a chimera in which it replaced the N terminus of the plasma membrane Cl− channel CIC-0. The resulting chimera 7-0-0 was targeted to late endosomes/lysosomes rather than to the plasma membrane. Strong lysosomal targeting signals are provided by the two dileucine AP-binding motifs present in the rat CIC-7 N terminus. When these two motifs were disrupted together, the mutated 7-0-0 chimera was found in the plasma membrane like CIC-0. Somewhat surprisingly, the GGA binding site did not seem important for lysosomal sorting. Full-length CIC-7 could be partially directed to the plasma membrane by disrupting just those two AP binding motifs. However, a large proportion of the mutant remained in late endosomes/lysosomes to which it was probably directed by its AP-3 binding C terminus. As disruption of the only conventional candidate AP binding site in the C terminus had no effect, we were unable to fully direct CIC-7 to the plasma membrane with a few point mutations. Nonetheless, the partially plasma membrane localized CIC-7 mutant that carries just four point mutations in the cytoplasmic
N terminus should prove useful for characterizing its biophysical properties.

With the notable exception of the N terminus of ClC-5, which bound AP-2 (and clathrin) to a site that we confirmed by mutagenesis, the N and C termini of CIC-3 through CIC-5 did not bind APs or GGAs in our pulldown experiments. Although AP-2 binding to CIC-5 would fit well with the assumed recycling of CIC-5 over the plasma membrane, the disruption of its binding motif did not increase its abundance in the plasma membrane. Hence, other mechanisms must operate in directing these endosomal CLCs to their respective compartments. One such mechanism may be binding to clathrin as described previously for ClC-3 (23). In addition, there might be binding sites in the cytoplasmic aspect of the membrane-spanning parts of CLC proteins, an issue we could not investigate with our pulldown experiments. Indeed, a tyrosine-based motif between intramembrane helices D and E has recently been implicated in the rapid recycling of the Cl− channel CIC-2 between the plasma membrane and an endosomal compartment (17). On the other hand, CIC-3, -4, and -5 may heterodimerize (4, 52) akin to the previously described heteromer formation between plasma membrane CLC anion/proton exchangers. The known roles of confirmed binding partners in facilitating specific sorting steps agreed well with the native subcellular localization of the CLCs they bound to. In several cases, however, these interactions are not the only ones that direct vesicular CLCs to their normal destination, because no change in localization was observed when the respective binding sites were disrupted. Those cases where vesicular CLC proteins could be directed to the plasma membrane with a few point mutations, however, should provide excellent opportunities to study their biophysical properties in detail.

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