

The PDZ-binding Chloride Channel ClC-3B Localizes to the Golgi and Associates with Cystic Fibrosis Transmembrane Conductance Regulator-interacting PDZ Proteins*

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ClC chloride channels are widely distributed in organisms across the evolutionary spectrum, and members of the mammalian family play crucial roles in cellular function and are mutated in several human diseases (Jentsch, T. J., Stein, V., Weinreich, F., and Zdebik, A. A. (2002) *Physiol. Rev.* 82, 503–568). Within the ClC-3, -4, -5 branch of the family that are intracellular channels, two alternatively spliced ClC-3 isoforms were recognized recently (Ogura, T., Furukawa, T., Toyozaki, T., Yamada, K., Zheng, Y. J., Katayama, Y., Nakaya, H., and Inagaki, N. (2002) *FASEB J.* 16, 863–865). ClC-3A resides in late endosomes where it serves as an anion shunt during acidification. We show here that the ClC-3B PDZ-binding isoform resides in the Golgi where it co-localizes with a small amount of the other known PDZ-binding chloride channel, CFTR (cystic fibrosis transmembrane conductance regulator). Both channel proteins bind the Golgi PDZ protein, GOPC (Golgi-associated PDZ and coiled-coil motif-containing protein). Interestingly, however, when overexpressed, GOPC, which is thought to influence traffic in the endocytic/secretory pathway, causes a large reduction in the amounts of both channels, probably by leading them to the degradative end of this pathway. ClC-3B as well as CFTR also binds EBP50 (ERM-binding phosphoprotein 50) and PDZK1, which are concentrated at the plasma membrane. However, only PDZK1 was found to promote interaction between the two channels, perhaps because they were able to bind to two different PDZ domains in PDZK1. Thus while small portions of the populations of ClC-3B and CFTR may associate and co-localize, the bulk of the two populations reside in different organelles of cells where they are expressed heterologously or endogenously, and therefore their cellular functions are likely to be distinct and not primarily related.

Chloride channels mediate a broad range of cellular functions. Among the several different families of proteins that constitute these channels, the neurotransmitter ligand-gated γ -aminobutyric acid (GABA) and glycine and voltage-gated ClC classes are the largest and best characterized (1). The ClC family is especially diverse with members in all organisms

including at least nine in mammals. Mutations in several of these are disease-causing (2). ClC-1, ClC-2, and ClC-K channels within one branch of the mammalian family reside in the plasma membrane of cells in different tissue types whereas ClC-3 through ClC-7 form another large branch and reside primarily in membranes of intracellular organelles with the highly homologous ClC-3, -4, -5 channels occupying the endosomal/lysosomal compartments. These three may all provide negative charge shunts that increase the rate of acidification of these compartments by proton ATPases (1, 3, 4). A similar function has been suggested for bacterial ClC channels in extreme acid resistance response (5).

Recently, a splice variant of human ClC-3 was discovered that has a PDZ¹ domain-binding C terminus (6). When heterologously expressed, this form, termed ClC-3B, was detectable mainly in a general intracellular localization. However, when co-expressed with the PDZ protein, EBP50, a small amount of the channel protein was detected on the surface at the leading edge of spreading cells. Furthermore, EBP50-mediated association with the other known PDZ-binding chloride channel, CFTR, was reported as was an ability of CFTR to activate ClC-3B. The CFTR chloride channel is distinct from the other families of chloride channels and belongs to the large family of ABC transport proteins (7). CFTR resides in the apical membrane of epithelial cells where it is crucial to ion and fluid secretion and reabsorption (8). Hence the possibility of ClC-3B trafficking to the plasma membrane and either being influenced by CFTR when it is present or potentially substituting for it when it is absent or dysfunctional in cells of CF patients is of great importance to understanding or influencing the disease. For this reason we have carefully examined the subcellular localization of heterologously and endogenously expressed ClC-3B as well as its possible PDZ protein-mediated interaction with CFTR. We found that ClC-3B is localized to the Golgi and differs in its location from the late endosomal isoform ClC-3A and from most CFTR, present at the plasma membrane. Both ClC-3B and CFTR interact with PDZ domains of GOPC,² EBP50, and PDZK1 and PDZK1 promotes the asso-

¹ The abbreviations used are: PDZ, PSD-95/discs large/ZO-1; EBP50; ERM-binding phosphoprotein 50; CFTR, cystic fibrosis transmembrane conductance regulator; GIPC, GAIP-interacting protein, C terminus; GOPC, Golgi-associated PDZ and coiled-coil motif-containing protein; GRASP, Golgi reassembly stacking protein; GST, glutathione S-transferase; Mint, Munc18-interacting protein; NKCC, sodium potassium chloride cotransporter 1; HA, hemagglutinin; mAb, monoclonal antibody; SNARE, soluble NSF attachment protein receptors.

² GOPC or Golgi-associated PDZ coiled-coil protein was discovered and named by Yao *et al.* (27). Homologous cDNAs were independently isolated by Neudauer *et al.* (11) and termed PIST for PDZ domain protein interacting specifically with TC10; by Charest *et al.* (20) and termed FIG for Fused In Glioblastoma; and by Cheng *et al.* (19) and

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ciation of the two PDZ-binding chloride channels. Despite this the only co-localization of CFTR and ClC-3B that we detected is at the Golgi where a small amount of CFTR resides. ClC-3A and ClC-3B were found to interact with each other, most likely as heterodimers as described for other ClC channels.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Full-length cDNAs of ClC-3A and ClC-3B were amplified by PCR using a human pancreas cDNA library (Clontech) as template. PCR fragments were first introduced into the EcoRV site of pBluescript and then subcloned into pcDNA3. A c-Myc tag was added to the ClC-3B open reading frame at the N terminus; ClC-3A was tagged at its N terminus with amino acid residues 723–732 of the MRP1 protein as an epitope recognized by mouse monoclonal antibody 42.4 (9).

Partial cDNA fragments encoding different PDZ domains of EBP50, PDZK1, and GOPC were subcloned into pGEX-5X-3 (Amersham Biosciences) to generate glutathione S-transferase (GST) fusion proteins. The boundaries of the expressed polypeptides containing different PDZ domains were amino acid residues 1–129 for PDZ domain 1 of EBP50, amino acid residues 133 to the C-terminal end for PDZ domain 2 of EBP50, amino acid residues 1–140 for PDZ domain 1 of PDZK1, amino acid residues 127–237 for PDZ domain 2 of PDZK1, amino acid residues 231–371 for PDZ domain 3 of PDZK1, amino acid residues 368 to the C-terminal end for PDZ domain 4 of PDZK1, and amino acid residues 281–363 for the PDZ domain of GOPC. A cDNA fragment encoding the C-terminal amino acid residues 1440–1480 of CFTR was subcloned into pGEX-5X-3 vector to produce a fusion of GST and the C terminus of CFTR.

Cell Culture and Transfection—BHK-21, HEK293T, Calu-3, CaPan-1, CFPAC-1, T84, Caco-2, MDCK, and PANC-1 cells were obtained from the American Type Culture Collection (ATCC) and grown at 37 °C in 5% CO₂. BHK-21 cells were transiently transfected with LipofectAMINE Plus Reagent (Invitrogen) or stably transfected using calcium phosphate (10). For transient expression cells were transfected with cDNAs of CFTR, ClC-3A, ClC-3B, EBP50, or PDZK1 subcloned into pcDNA3. GOPC was transiently expressed using the plasmid pKH₃-PIST (11). For transient cotransfection experiments equivalent amounts of individual plasmid DNAs were employed. For stable transfections pNUT vector was cotransfected with ClC-3A in pcDNA3 or ClC-3B in pcDNA3. Stably transfected cells were selected with 500 μM methotrexate in the growth medium, individual clones were isolated and ClC-3A or ClC-3B expression was analyzed by Western blotting. HEK293T cells were transiently transfected using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions.

Cell Lysis and Membrane Preparation—Cells were washed in ice-cold phosphate-buffered saline and lysed with Nonidet P-40 lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris, pH 7.4, 10 mM NaMoO₄) at 4 °C for 30 min. Protease inhibitors were added to Nonidet P-40 lysis buffer prior to use as described earlier (12). Cell lysates were centrifuged at maximal speed in an Eppifuge at 4 °C, and supernatants were collected for further experiments. Scrapings from human bronchus were extracted with 2× Nonidet P-40 buffer and sonicated briefly before centrifugation. Scrapings from colon were first ground in a homogenizer in extraction buffer and then processed exactly as the bronchial scrapings. The preparation of membranes from cultured cells was performed exactly as described earlier (13).

Primary Antibodies—Mouse monoclonal antibodies recognizing ClC-3A and ClC-3B were raised against conjugated peptides derived from C-terminal sequences of ClC-3A (RHMAQQTANQDPASIMFN) and ClC-3B (KQHVEPLAPPWHYNKKR). Initial screens of hybridomas were performed by peptide ELISA, and followed by secondary screening by Western blotting of lysates or membranes from cells heterologously expressing ClC-3A or ClC-3B. The derived monoclonal antibodies 34.1 and 69.16 recognized ClC-3A and ClC-3B, respectively. Additionally, ClC-3A and ClC-3B were detected with antibodies 42.4 and 9E10 directed against their N-terminal tags. CFTR was detected with antibody 596, 528, or 570, FLAG-tagged PDZK1 with antibody anti-FLAG M2 (Sigma), HA-tagged GOPC, and HA-tagged EBP50 with the antibody 16B12 (Babco). The antibodies from the following manufacturers were used to detect proteins by immunofluorescence: Giantin, Babco; Golgin, Molecular Probes, GM130, BD Transduction Laboratories; γ-Adaptin, Santa Cruz Biotechnology; Calnexin, StressGen; EEA1, BD Transduc-

termed CAL for CFTR-associated ligand. We have used the term GOPC, which seems most descriptive and does not derive from one of the proteins with which it interacts.

tion Laboratories; ZO-1, Zymed Laboratories Inc.; NaK-ATPase, ABR. Antibodies against MUC1 and NKCC were generously provided by Sandra Gendler and Christian Lytle, respectively.

Immunoblotting, Immunoprecipitation, and Pull-down Experiments—Immunoblotting and immunoprecipitations were performed as described earlier (14). For immunoblots and immunoprecipitations either cell lysates or membranes solubilized in 1% Nonidet P-40 buffer were used. For pull-down assays GST fusion proteins were overproduced in bacterial strain BL-21 and immobilized on glutathione-Sepharose 4B beads (Amersham Biosciences). After incubation with cell lysates or solubilized membranes, bound proteins were washed three or four times and eluted with electrophoresis sample buffer.

Deglycosylation—Proteins were deglycosylated with endoglycosidase H (Roche Molecular Biochemicals) or N-glycosidase F (Glyko) according to the instructions of the manufacturers.

Confocal Immunofluorescence—BHK-21 and HEK293T cells were grown on collagen-coated chamber slides (BD PharMingen) and fixed in 4% paraformaldehyde for 10 min, washed with phosphate-buffered saline, permeabilized in 0.1% saponin in phosphate-buffered saline, and blocked with 1% bovine serum albumin and 5% normal goat serum in phosphate-buffered saline. Primary antibodies were added for at least 1 h in the same buffer. Secondary antibodies were either goat anti-mouse or goat anti-rabbit IgG conjugated to Alexa 488 or Alexa 568. In addition anti-ClC-3A and ClC-3B antibodies were directly labeled with Alexa 488 or Alexa 568 according to the instructions of the manufacturer (Molecular Probes). Directly labeled anti-HA (Alexa 594 or 488) and anti-FLAG antibodies (fluorescein isothiocyanate or Cy3) were purchased from Babco and Sigma, respectively.

Calu-3 cells were grown in a liquid air interface on Transwell Clear inserts (Costar) for several weeks. Cell layers were fixed in 4% paraformaldehyde for 10 min. Frozen sections were thawed and treated with 1% sodium borohydride for 10 min and exposed to 1% SDS for 5 min to improve antibody labeling. Detection of endogenously expressed ClC-3B was facilitated using the Alexa 488 Signal-Amplification kit for mouse antibodies from Molecular Probes. RhoB was expressed as a GFP fusion using pEGFP-Endo (Clontech). Cells were examined on a LSM510 confocal microscope from Zeiss.

RESULTS

Cloning and Expression of ClC-3A and B cDNAs—Our interest was attracted by the report by Ogura *et al.* (6) of a PDZ-binding isoform of the ClC-3 chloride channel because of its potential relation to CFTR. To be able to investigate this we cloned cDNAs corresponding to ClC-3A and ClC-3B by PCR from a total human pancreas cDNA pool. The protein sequences coded for by these cDNAs with their principal features are shown in Fig. 1A. As originally shown by Ogura *et al.* (6), the two forms are identical except at their C-terminal ends where insertion of an additional exon makes ClC-3B longer than ClC-3A by 47 residues and makes their sequences differ at several other positions before the end of ClC-3A. The final four amino acids of ClC-3B fit the consensus for binding to Class I PDZ proteins (15). Consensus extracytoplasmic N-glycosylation sites are indicated between helices B and C, and L and M, assigned by alignment with the bacteria ClCs for which three-dimensional structures have been obtained (16).

pcDNA3 plasmids with different epitope tags at the N termini of ClC-3A and ClC-3B were transfected into BHK-21 and HEK293 cells so that the proteins could be detected with antibodies to the tags. Both transient and stable BHK transfectants exhibited a heterogeneous larger band of ~130 kDa and a more distinct smaller band of ~90 kDa (Fig. 1B). The ClC-3A bands detected with the mAb 42.4 to an MRP1 epitope (9) and ClC-3B bands with mAb 9E10 to the c-Myc tag appear very similar as expected since they differ in size by only 47 amino acids. Comparison of transient and stable expression in BHK cells revealed that the ratio of the smaller to the larger bands was higher in the former as if complete maturation had not yet occurred. This appeared also to be the case in HEK293 cells where both bands appeared nearly identical to those in transiently expressing BHK-21 cells.

Endoglycosidase digestion provided further characterization

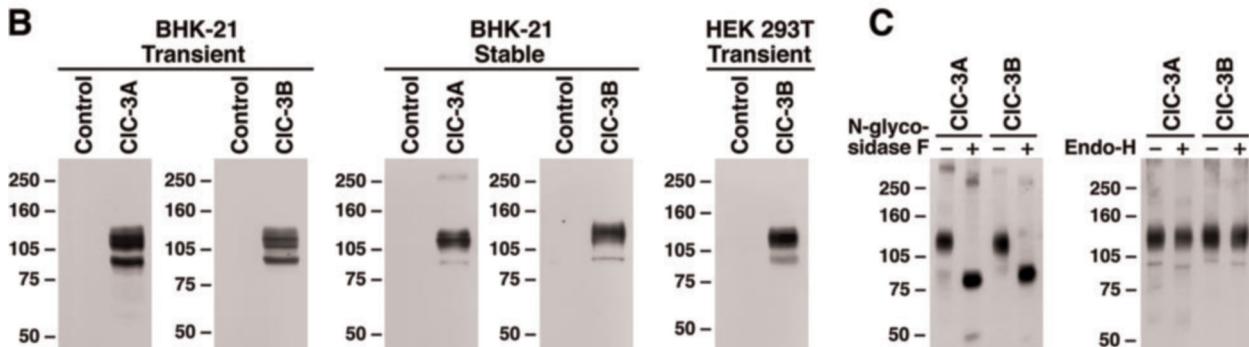
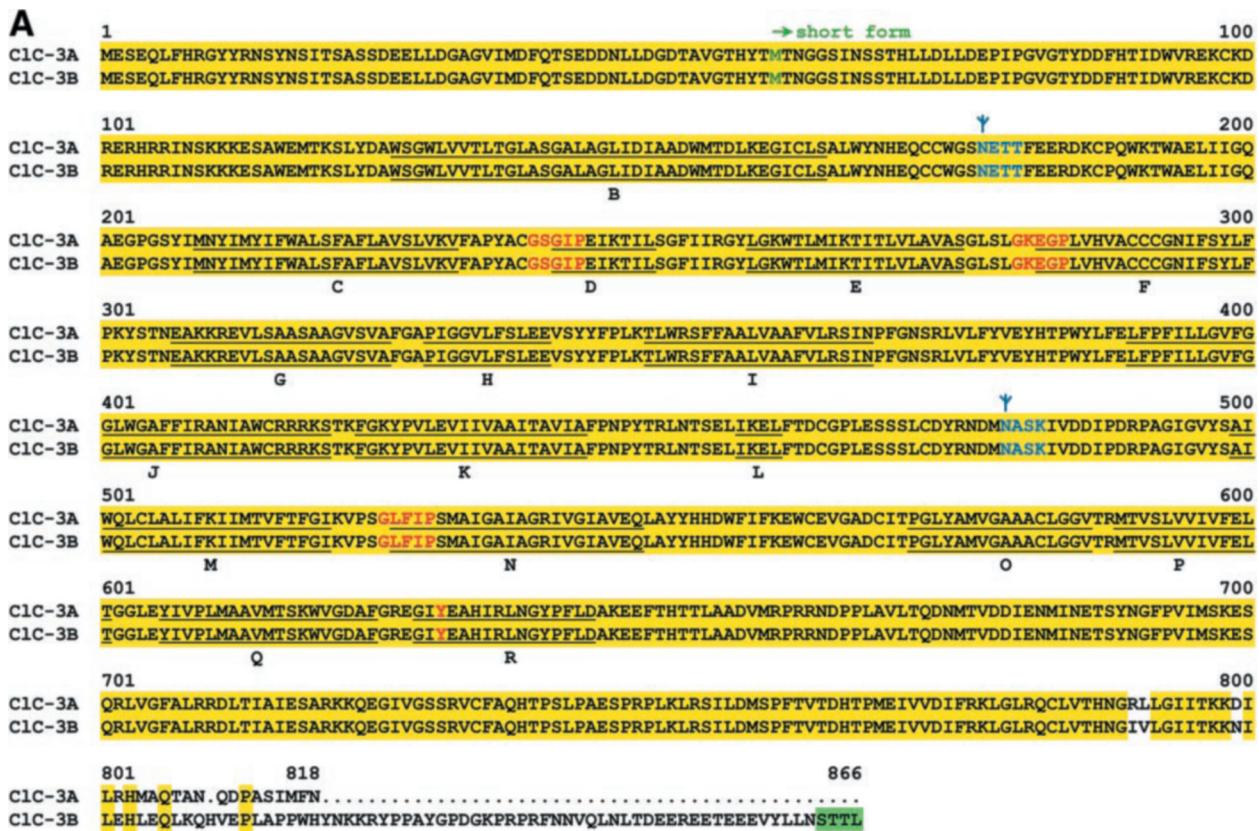


Fig. 1. Expression of ClC-3A and ClC-3B. *A*, sequence alignment of ClC-3A and ClC-3B. ClC-3A and ClC-3B share identical sequences with the exception of the last 47 amino acids. ClC-3B has at its C-terminal end a PDZ-binding motif highlighted in green. α -Helices crossing the membrane partially or completely are underlined (*B* to *R*), and residues important for chloride selectivity are shown in red. These predictions were derived from an alignment with prokaryotic CIC channels, whose structures have been determined (16). Two potential *N*-glycosylation sites are shown in blue. A shorter form of ClC-3A has been described (37). The N-terminal end of the short form is indicated with an arrow. *B*, heterologous expression of ClC-3A and ClC-3B in BHK-21 and HEK293T cells. Cells were transiently or stably transfected as described under “Experimental Procedures.” ClC-3A was detected with antibody 42.4, which recognizes the N-terminal MRP1 tag (BHK-21 transient) or with antibody 34.1, which recognizes the C-terminal tail (BHK-21 stable). ClC-3B was detected with antibody 9E10, which recognizes the N-terminal c-Myc-tag or with antibody 69.16, which recognizes the C-terminal tail (BHK-21 stable and HEK293T stable). In the first lane of each panel, the same amount of protein was loaded from cells not expressing ClC-3A or ClC-3B (*control*). *C*, endoglycosidase digestion of ClC-3A and ClC-3B. Membranes were prepared from BHK cells stably expressing ClC-3A or ClC-3B, treated with *N*-glycosidase F or endoglycosidase H, separated by SDS-PAGE, and ClC-3A or ClC-3B were detected by immunoblotting.

of these electrophoretic bands (Fig. 1*C*). *N*-glycosidase F caused the 130-kDa and 90-kDa bands to collapse to a band slightly smaller than the latter in the case of both isoforms. Endoglycosidase H had a similar effect on the 90-kDa bands, indicating they are glycosylated species. Their decrease in size corresponds to ~5 kDa, consistent with the removal of two core oligosaccharide chains, probably from the two consensus *N*-glycosylation sites in the protein sequence (Fig. 1*A*). The 130-kDa bands were unaffected by endoglycosidase H as expected if they had acquired complex oligosaccharide chains. Thus both forms of ClC-3 are *N*-glycosylated membrane proteins that would be synthesized on membrane-bound ribosomes and

transported to the Golgi where the glycosyl transferases that assemble complex oligosaccharide chains are located.

Differential Localization of ClC-3A and ClC-3B—In previous work, ClC-3A has been most definitely localized to late endosomes or lysosomes (4) and to synaptic vesicles in the brain (3). These same studies provided evidence that its presence contributes to the rate of acidification of these compartments (3, 4). When expressed in BHK cells ClC-3A co-localizes extensively with the late endosomal marker RhoB, and to a lesser extent with the early endosomal marker EEA-1 (Fig. 2*A*). This localization is distinct from that of calnexin in the ER and several Golgi markers including giantin, GM130, and γ -adaptin. In

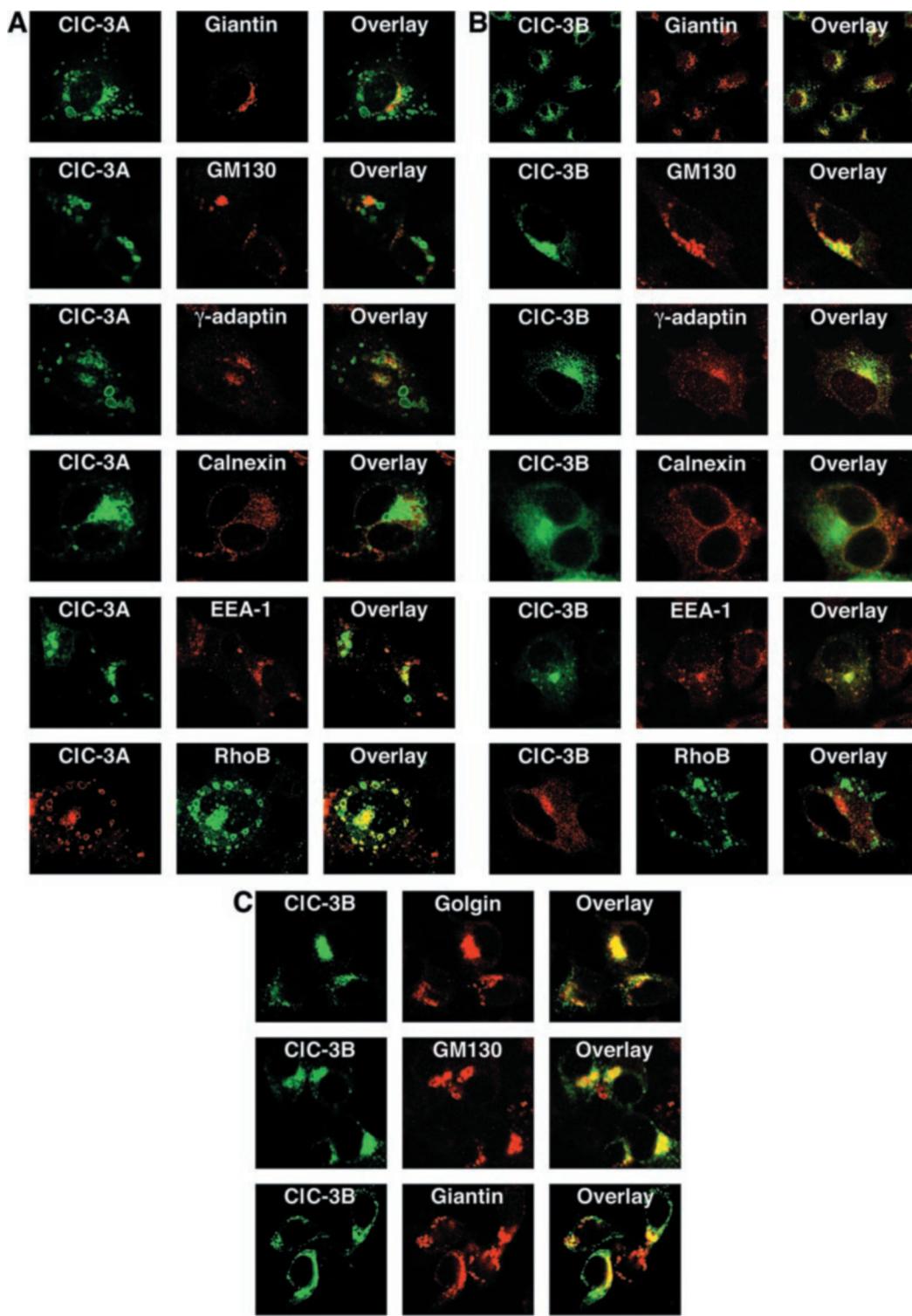


FIG. 2. Localization of ClC-3 proteins relative to organelle markers. *A*, ClC-3A heterologously expressed in BHK-21 cells. Cells were transiently transfected with ClC-3A. ClC-3A was detected by immunofluorescence as described under “Experimental Procedures” using antibody 42.4. *B*, ClC-3B heterologously expressed in BHK-21 cells. Cells were transiently (rows 2–6) or stably (Giantin, *first row*) transfected with ClC-3B and ClC-3B was detected by immunofluorescence using antibody 69.16 or 9E10. *C*, ClC-3B heterologously expressed in HEK293T cells. Cells were transiently transfected with ClC-3B. ClC-3B was detected by immunofluorescence as described under “Experimental Procedures” using antibody 69.16.

distinct contrast ClC-3B did co-localize with these markers, especially giantin, GM130, and γ -adaptin (Fig. 2*B*). Of the endosomal markers there was some co-localization only with EEA-1, suggesting that some of both isoforms of the ClC-3 channel may occupy the early endosome compartment, possibly even as heterodimers (see below). The primarily distinct local-

ization of the two channels; however, is emphasized when each is compared with the same marker. With giantin, for example, there is nearly complete correspondence with ClC-3B but very little with ClC-3A, even though some of it is situated contiguous with that compartment. This is not surprising of course since there is extensive traffic between the *trans*-Golgi network

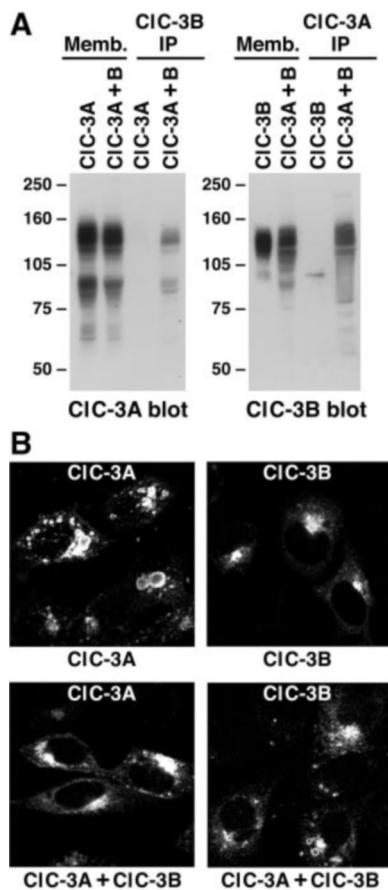


FIG. 3. CIC-3A and CIC-3B interact with each other. *A*, co-immunoprecipitation of co-expressed CIC-3A and CIC-3B. Membranes from BHK-21 cells transiently expressing CIC-3A and CIC-3B were subjected to immunoprecipitation of CIC-3A or CIC-3B using antibodies 42.4 or 9E10, respectively. Co-immunoprecipitated CIC-3A and CIC-3B were detected by Western blotting using the same antibodies. *B*, influence of CIC-3A and CIC-3B co-expression on the localization of each. CIC-3A and CIC-3B were transiently co-expressed in BHK-21 cells. CIC-3A was visualized with antibody 42.4 conjugated to Alexa 568, and CIC-3B was detected using antibody 69.16 conjugated to Alexa 488.

and endosomes. The Golgi localization of ClC-3B is emphatically confirmed in Fig. 2C where its staining in HEK293 cells coincides with that of three Golgi proteins. Thus while Ogura *et al.* (6) showed that most heterologously expressed ClC-3B was intracellular, our results further resolve this primarily to the Golgi indicating that the two ClC-3 isoforms reside in virtually continuous but clearly distinct organelles.

Heterodimerization of ClC-3A and ClC-3B—Since ClC-3A and B are identical except for their C termini and all ClC channels are dimers (1), heterodimerization might be expected. To assess whether this occurred, we determined if they could be co-immunoprecipitated from BHK cells in which they were co-expressed. Fig. 3A shows that each isoform was present in an immunoprecipitate of the other member of the pair. Interestingly, in these cells the intracellular localizations of both were partially shifted toward that of the other (Fig. 3B). Thus the characteristic endosomal pattern of ClC-3A is also exhibited by a small amount of ClC-3B when the two are expressed together. Conversely, the Golgi pattern of ClC-3B alone is displayed by ClC-3A in many cells on co-expression of the two. Localization to the Golgi seems to dominate over the endosomal location when heterodimerization occurs since there are clearly more cells with a predominant Golgi localization of ClC-3A than cells showing an endosomal location of ClC-3B (not shown). Homodimers of both ClC-3 proteins are expected to also form and retain their individual locations.

Endogenous ClC-3B Expression and Localization in Epithelia—Since we were primarily interested in its possible relationship to CFTR and relevance to cystic fibrosis, we examined the presence of ClC-3B in epithelial cells. First epithelial scrapings from human bronchus and colon were homogenized in detergent (Nonidet P-40) and immunoprecipitated with a mAb to ClC-3B, 69.16. After SDS-PAGE analysis under fully reducing conditions, a Western blot was probed with the same antibody (Fig. 4A). Distinct ClC-3B bands appeared in colonic samples from all three individuals tested and one bronchial sample; the other two bronchial samples gave weak signals but these observations clearly confirm ClC-3B expression in epithelial tissue affected in cystic fibrosis. We then examined expression in a number of epithelial cell lines and found positive signals of variable intensity in Western blots of lysates of all (Fig. 4B). ClC-3B appears most highly expressed in two of the lines, which also contain large amounts of CFTR viz. Calu-3 derived from submucosal glands and Capan-1, a pancreatic ductal cell line. Confocal immunofluorescence of Calu-3 cells grown on permeable supports at an air-liquid interface revealed ClC-3B in intracellular vesicular structures (Fig. 4C) as when it was heterologously expressed. This distinct population of vesicles is similar to those stained with the Golgi markers, giantin and golgin and entirely distinct from several apical and basolateral markers which clearly demarcate these portions of the cell surface. Thus, as when heterologously expressed, the principal residences of ClC-3B (Golgi) and CFTR (apical) are different.

ClC-3B Interactions with PDZ Proteins—The PDZ binding capacity of ClC-3B led to the idea that it might associate with and even be influenced by CFTR, possibly via EBP50 (6). We tested the ability of ClC-3B to bind to the individual PDZ domains of three different PDZ proteins, EBP50, PDZK, and GOPC, known to interact with CFTR (17–19). GST fusions with each of the PDZ domains were incubated with detergent lysates of BHK cells expressing CFTR and ClC-3B. Both channel proteins bound preferentially to the first PDZ domain of EBP50 and to a much lesser extent to the second (Fig. 5A). This result is different from that of Ogura *et al.* (6) who reported that ClC-3B preferred PDZ2 of EBP50 and hence that ClC-3B and CFTR might be coupled by EBP50. The C-terminal sequences of both channels indicate they should bind class I PDZ domains (15). When similar experiments were performed with the four PDZ domains of PDZK1, ClC-3B appeared to bind only the first (Fig. 5B). CFTR, however, bound strongly to both domains 1 and 3. Hence at least in principle, a ternary complex could form with ClC-3B at PDZ1 and CFTR at PDZ3 of PDZK1. Since we found that ClC-3B resides primarily in the Golgi it seemed reasonable that it might bind to the Golgi-associated coiled-coil PDZ protein, GOPC, which binds CFTR (19). Fig. 5C confirms that the single PDZ domain of GOPC binds ClC-3B as well as CFTR.

Since the three PDZ proteins interact with both channel proteins, we examined the influence of each of the PDZ proteins on the localization of ClC-3B and CFTR (Fig. 6). Consistent with the results of Cheng *et al.* (19), EBP50 overexpression had little effect on the expression of CFTR, which is already at the cell surface. ClC-3B, however, was shifted from its presence just in the Golgi to somewhat more peripheral locations in the cell but was not detectable in the plasma membrane. PDZK1 overexpression similarly had little effect on CFTR, which is reasonable because PDZK1 and most CFTR are located in nearly the same place even when not co-expressed. On ClC-3B, however, PDZK1 had a stronger impact than EBP50, causing even more of that channel to move toward the cell periphery, although apparently not entirely to the plasma membrane. GOPC overexpression has a much more dramatic effect on both channels, causing them to become condensed with it in a focal

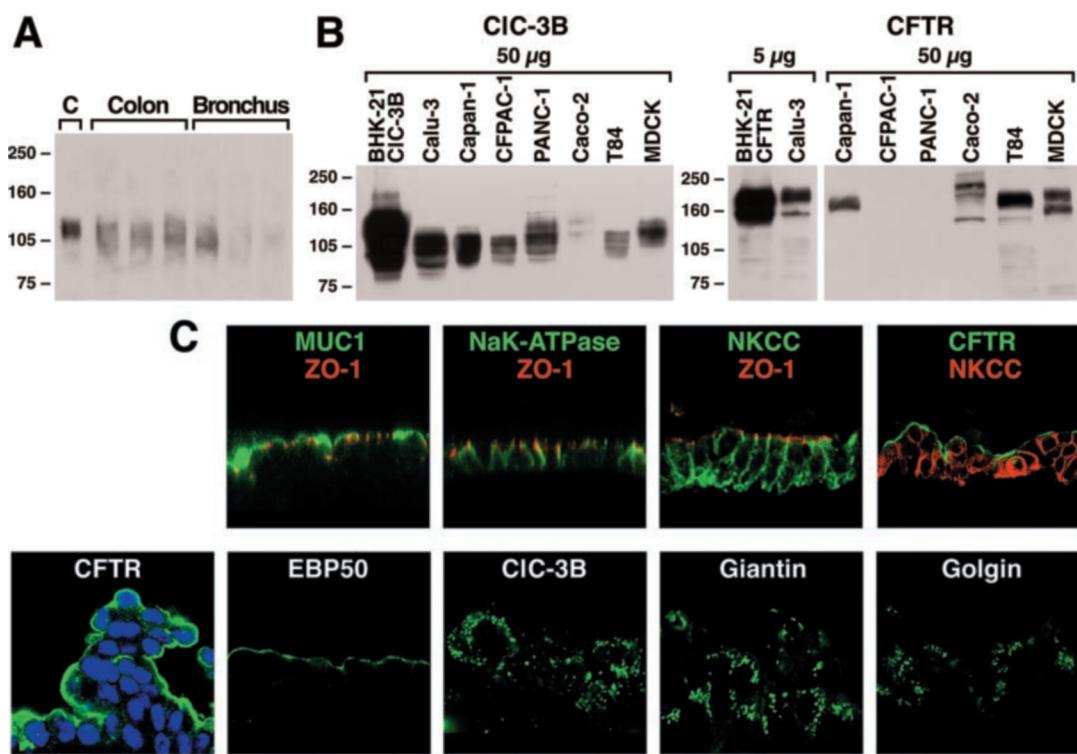


FIG. 4. Endogenous expression of ClC-3B in epithelial cells. *A*, ClC-3B was immunoprecipitated from lysates of human colonic and bronchial epithelia using antibody 69.16 and detected with the same antibody by Western blotting. *B*, ClC-3B and CFTR in epithelial cell lines. Membranes were prepared from epithelial cell lines and separated by 6% SDS-PAGE. ClC-3B was detected using antibody 69.16, and CFTR was detected using antibody 596. *C*, localization of ClC-3B in polarized Calu-3 cells. Calu-3 cells were grown at an air-liquid interface and stained for immunofluorescence microscopy as described under “Experimental Procedures.” MUC1, EBP50, and CFTR localize to the apical membrane. Immunofluorescence of sodium, potassium ATPase (*NaK-ATPase*), sodium potassium chloride cotransporter 1 (*NKCC*), and Zonula occludens protein 1 (*ZO-1*) demonstrate polarization of the cells. Nuclei were stained with propidium iodide and are shown in *blue* in the CFTR panel.

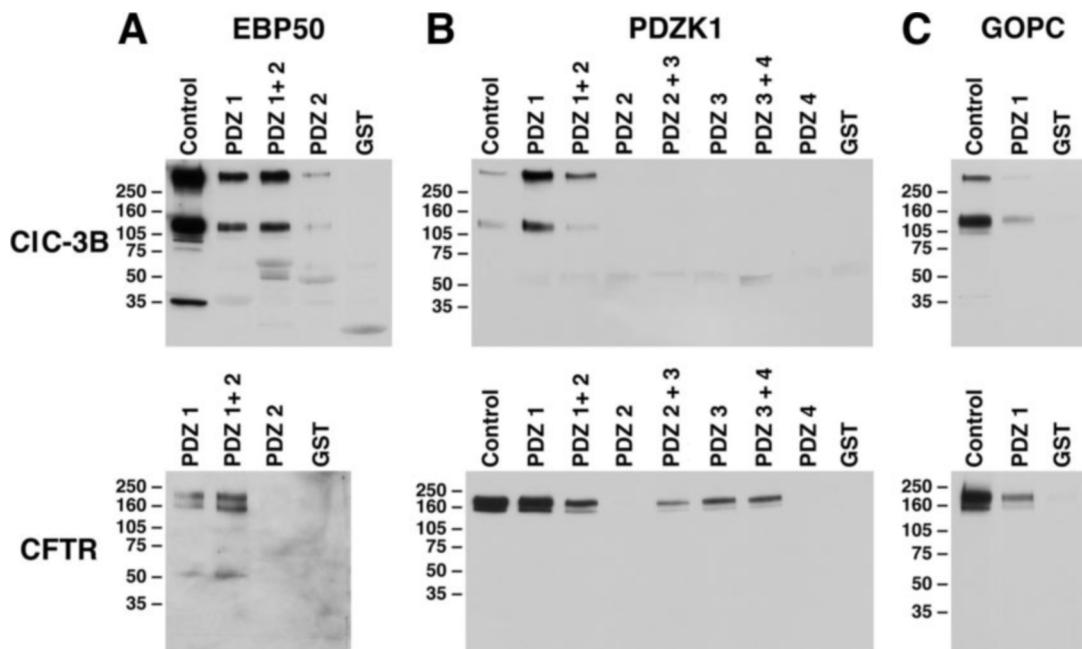


FIG. 5. Abilities of GST fusions of PDZ domains of EBP50 (A), PDZK1 (B), and GOPC (C) to interact with ClC-3B and CFTR. GST fusions with PDZ domains of EBP50, PDZK1, and GOPC were bound to glutathione-Sepharose beads and incubated with lysates from BHK-21 cells expressing ClC-3B or CFTR. The beads were washed, and bound proteins eluted. Lysates from cells expressing ClC-3B or CFTR were loaded as a positive control. Individual or combined PDZ domains employed are indicated above each lane. The high molecular weight ClC-3B bands near the top of the gels reflect strong aggregation in non-ionic detergent.

Golgi location. The apparent large reduction in the amount of both channel proteins on co-expression with GOPC but not EBP50 or PDZK1 is confirmed by Western blots of whole cell lysates (Fig. 7). While these findings clearly show that the

Golgi PDZ protein, GOPC, interacts strongly with the Golgi chloride channel, ClC-3B, this interaction cannot be entirely responsible for their co-localization since CFTR also is bound by GOPC but resides mostly at the cell surface.

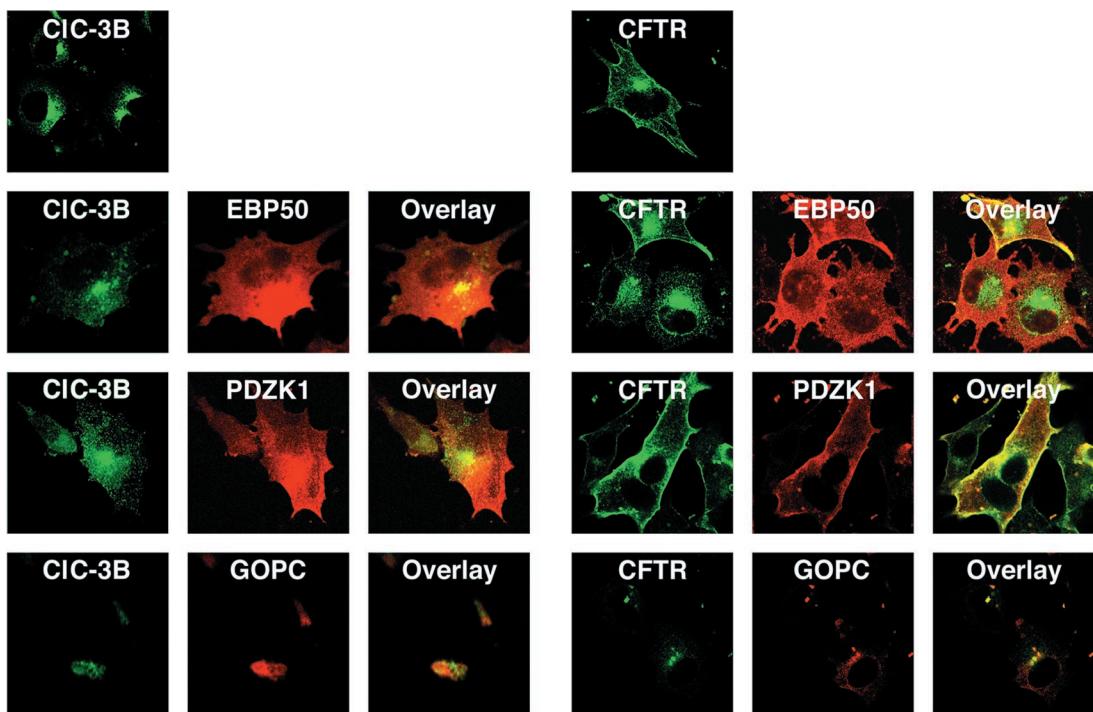


FIG. 6. Effect of EBP50, PDZK1, and GOPC on the localization of ClC-3B and CFTR. ClC-3B or CFTR were transiently co-expressed with EBP50, PDZK1, or GOPC, and immunofluorescence microscopy was performed as described under “Experimental Procedures.” Individual ClC-3B and CFTR panels at top indicate localization of proteins expressed alone; below are their localization on co-expression with the PDZ protein indicated.

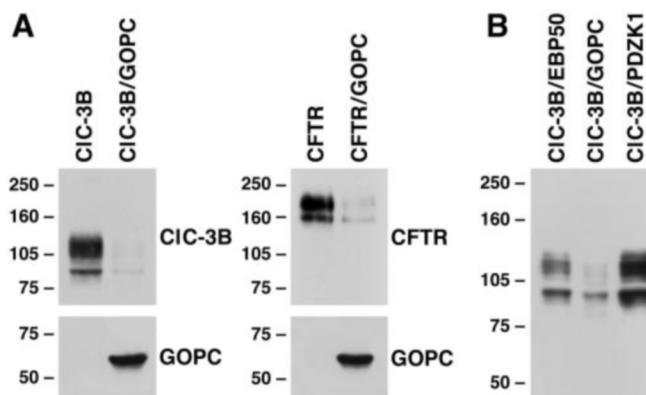


FIG. 7. Influence of overexpression of PDZ proteins on the amount of ClC-3B and CFTR. *A*, co-expression of GOPC with ClC-3B or CFTR. ClC-3B or CFTR were transiently overexpressed in BHK-21 cells without or with GOPC as described under “Experimental Procedures.” Cell lysates were analyzed by Western blotting using the antibodies 69.16 and 596 for ClC-3B and CFTR, respectively. *B*, co-expression of EBP50, GOPC, or PDZK1 with ClC-3B. EBP50, GOPC, or PDZK1 were transiently cotransfected with ClC-3B as described under “Experimental Procedures.” ClC-3B was visualized by Western blotting using antibody 69.16.

ClC-3B Interaction with CFTR—Since PDZK1 bound the two channel proteins at different PDZ domains it seemed possible that it might couple them. To test this possibility two types of experiments were performed. In the first, both ClC-3B and CFTR could be co-immunoprecipitated with PDZK1 (Fig. 8A). Second, we compared the ability of a GST fusion with the C-terminal 40 amino acids of CFTR to pull-down ClC-3B from lysates of cells in which it was overexpressed alone or together with PDZK1 or one of the other PDZ proteins. Fig. 8B shows that interaction between the CFTR tail and ClC-3B is greatly enhanced by PDZK1. The low level of association without overexpressed PDZK1 may reflect the action of endogenous PDZ proteins or interaction between these two integral membrane

proteins mediated by other means. In this assay, GOPC did not appear to cause increased association (Fig. 8B) despite its strong self-association via its C-terminal coiled-coil domain (11, 20). Similarly, EBP50, which also self-associates, did not appear to mediate interaction of ClC-3B with the tail of CFTR (Fig. 8B). This result is consistent with the fact that both channel proteins are bound primarily by the same PDZ domain of EBP50 (Fig. 5A). Overall, these results show that PDZK1 can mediate interactions between ClC-3B and CFTR. However, in our experiments the two channels are seen to co-localize only in the Golgi where a very small portion of cellular CFTR resides (Fig. 9). Although PDZK1 seems to cause some movement of ClC-3B from the Golgi, the co-localization with CFTR at the surface shown by Ogura *et al.* (6) was not detected in our experiments. This does not exclude the possibility that it may occur under different conditions. Nevertheless, the most consistent observation is of the co-localization of much of the ClC-3B pool with a small proportion of the total CFTR pool in a Golgi compartment.

DISCUSSION

Since CFTR and ClC-3B, members of every different protein families, are both chloride channels with C termini that bind class I PDZ domains, it is reasonable to ask what may be the significance, if any, of this common feature. This is of particular interest from the perspective of the proposal of Ogura *et al.* (6) that the two channel proteins may interact via the PDZ protein, EBP50, enabling CFTR to regulate the ClC-3B channel. The sites of localization of CFTR in cells are reasonably well established (21–23) as are certain aspects of its trafficking (22, 23). The bulk of the protein resides at the apical plasma membrane of epithelial cells in which it is endogenously expressed with small but detectable amounts associated with intracellular membranes in the secretory pathway (22). When expressed heterologously in non-polar mammalian cells, most of the mature protein is at the plasma membrane but significant amounts are intracellular, most in the ER (14). These major

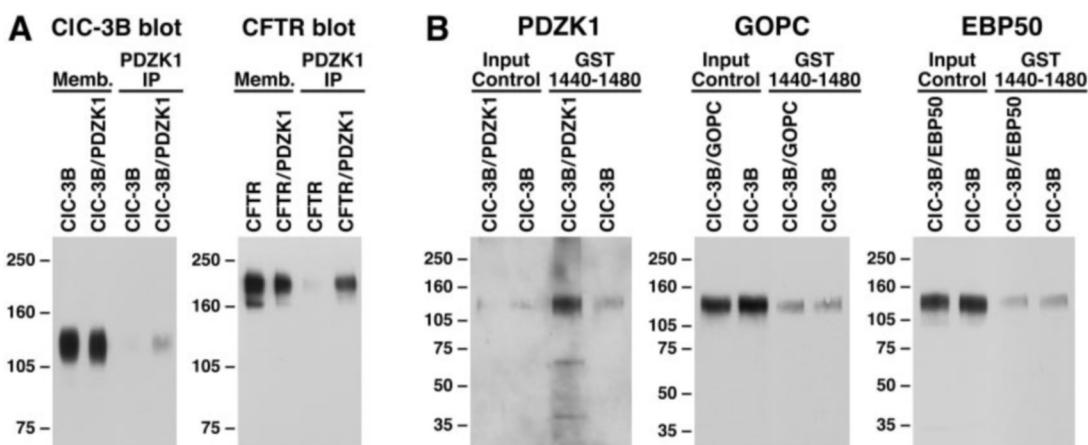


FIG. 8. Interaction of ClC-3B and CFTR. *A*, co-immunoprecipitation of PDZK1 and ClC-3B or CFTR. PDZK1 was immunoprecipitated from solubilized membranes prepared from BHK-21 cells expressing ClC-3B or CFTR alone or with PDZK1. ClC-3B or CFTR were detected by immunoblotting. *B*, influence of overexpression of PDZK1, GOPC, and EBP50 on the association of ClC-3B with the C-terminal tail of CFTR. A fusion of the last 40 amino acids of CFTR (1440–1480) and GST was bound to glutathione-Sepharose beads and incubated with lysates or solubilized membranes from BHK-21 cells expressing ClC-3B with or without PDZK1 or EBP50. To avoid the down-regulation of ClC-3B in the presence of co-expressed GOPC two different membrane preparations from cells overexpressing ClC-3B or GOPC were combined and solubilized to test the influence of GOPC on the association of ClC-3B with the C terminus of CFTR.

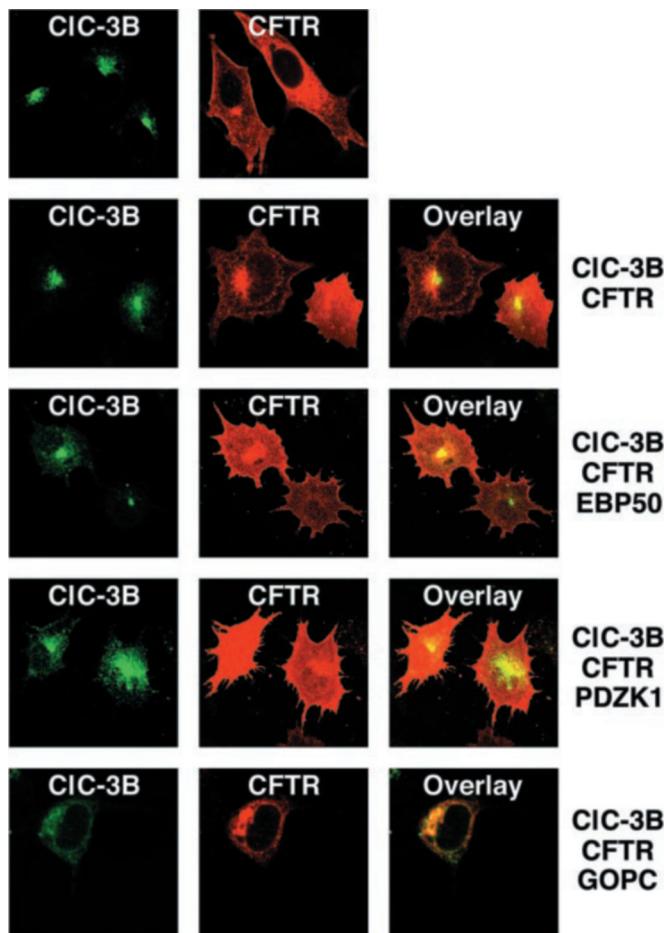


FIG. 9. Co-localization of a small pool of CFTR with ClC-3B. ClC-3B, CFTR, CIC-3B, and EBP50, PDZK1, or GOPC and were transiently expressed in BHK-21 cells. CFTR and ClC-3B were visualized by immunofluorescence as described under “Experimental Procedures.”

features of CFTR localization have been observed by many investigators and are amply illustrated in Figs. 4C, 6, and 9. The only novel feature of CFTR localization arose from our focus on the Golgi because of the finding that ClC-3B is present primarily at the Golgi. A small but readily detectable amount of CFTR, when heterologously expressed, co-localized with

ClC-3B at the Golgi (Fig. 9). This is worthy of note only because most investigators have not reported on CFTR in the Golgi and those who have (22) emphasized a lesser amount there than in the ER. Indeed, in Calu-3 epithelial cells in which CFTR is endogenously expressed, only weak signals are detected in any intracellular compartment (Fig. 4).

In contrast to CFTR, localization of the newly discovered ClC-3B has not previously been extensively characterized. The original work of Ogura *et al.* (6) showed only that most of the protein was intracellular with a small amount appearing at the leading surface of spreading cells in which EBP50 was also overexpressed. We have now shown that virtually all of the ClC-3B either heterologously expressed in BHK or HEK 293 cells or endogenously expressed in Calu-3 epithelial cells resides in the Golgi, separate from but nearly contiguous with ClC-3A, which is in late endosomes (Fig. 2). Both ClC-3 isoforms must at least transit the Golgi since they acquire complex N-linked oligosaccharide chains (Fig. 1C). ClC-3A has been found to reside in endosomal compartments (3, 4). The fact that ClC-3B remains Golgi-associated whereas ClC-3A does not would seem likely due to the PDZ-binding capacity of the former. Several Golgi-associated PDZ proteins are known including the GRASPs (24), Mints (25), GIPC (26), and GOPC (27). We focused on GOPC since Cheng *et al.* (19) recently have shown that it binds and influences the localization and turnover of CFTR. We confirmed these findings with CFTR and found that overexpression of GOPC had a similar influence on ClC-3B (Figs. 6 and 7). Although the mechanisms involved are not yet clear, these responses of the two PDZ-binding channel proteins is consistent with a growing body of evidence that GOPC plays an important role in vesicular trafficking in the secretory and endocytic pathways. It was discovered by its binding to the C terminus of a member of the frizzled family of cell surface WNT receptors and co-localized with a portion of the population of these receptors at the Golgi (27). Independently, the second coiled-coil domain of GOPC was found to bind TC-10, a member of the Rho-GTPase family involved in the regulation of the endocytic pathway (11). The same domain also interacts with the Golgi membrane protein, syntaxin 6, suggesting a relationship of GOPC with SNARE-mediated membrane recognition and fusion (20). Very recently the PDZ domain of GOPC was found to interact with a neurodegenerative mouse mutant of the PDZ-binding GluRδ2 glutamate ion channel, and its C-terminal coiled-coil domain with Beclin1, a factor

promoting autophagy where cellular constituents are trafficked to lysosomes and degraded (28). Hence, GOPC appears capable of more generally shifting the balance of PDZ-binding membrane proteins in the degradative direction. The other important clue to GOPC function comes from the knock-out of its gene in mice which results in failure of acrosome formation, a Golgi-dependent function, during spermatogenesis without detectable effect on other tissues (29).

While GOPC, which is known to self-associate via its coiled-coil domains (11, 27) binds and co-localizes with some CFTR and ClC-3B molecules, we did not find evidence that it promoted association between them (Fig. 8). Under special circumstances favoring dimerization instead of interactions with components of the trafficking machinery, GOPC could theoretically promote association of ClC-3B and CFTR. However, its influence seems more likely to be on the fate of each individually under normal circumstances, in that both are severely knocked down by its overexpression. Other Golgi PDZ proteins, including those mentioned above, also have been proposed to play roles in the trafficking and biosynthetic sorting of other PDZ-binding membrane proteins (24–26) and may also with these chloride channels. How such a multiplicity of potential interactions within a specific organelle are regulated is not yet understood.

Similarly, PDZ protein binding alone clearly does not entirely determine the major localizations of CFTR and ClC-3B in cells since, in addition to GOPC, they both bind EBP50 and PDZK1, which reside primarily at the cell surface. Interactions of CFTR with these promotes its endocytic recycling and, hence, residence in the apical membrane of epithelial cells (30). CFTR and ClC-3B bind to different PDZ domains of PDZK1 and it is able to promote association of the two channels (Fig. 8). PDZK1, which normally has a very similar localization as CFTR, causes some redistribution of ClC-3B toward more peripheral regions of cells (Figs. 6 and 9), but we have been unable to detect any in the plasma membrane on co-expression with either PDZK1 or EBP50. Neither PDZK1 nor EBP50 knocked down the amount of ClC3B or CFTR as GOPC did.

As mentioned above, while promotion of cell surface retention of CFTR is attributed to interaction with PDZ proteins such as EBP50 at that location (30), this clearly does not occur with ClC-3B. Additional factors must be at play in determining the primary steady-state localization of the two channel proteins.

Although ClC-3A was not the primary focus of our experiments it was necessary to precisely correlate its localization with that of a number of markers of different intracellular membrane compartments to clearly distinguish it from ClC-3B. This result (Fig. 2) showed a late endosomal localization in excellent agreement with the findings of Stobrawa *et al.* (3) and Li *et al.* (4). This is significant for at least two reasons. First, there have been several claims of heterologously expressed ClC-3A at the cell surface (31, 32). However, these findings when made with functional assays of channel activity may have reflected endogenous channels (33) and when made by immunofluorescence in some cases used a commercial antibody that recognized proteins other than ClC-3A (1). Li *et al.* (4) have reported that small amounts of an N-terminally truncated version of ClC-3A does reach the cell surface. Both swelling (31) and calcium/calmodulin kinase (32) activated chloride channels attributed to ClC-3A in some of these studies are not altered in cells from mice in which the *ClC-3* gene was knocked out (3). Second, since there is now evidence that ClC-3A functions as an anion shunt to increase the rate of endosomal acidification (3, 4), ClC-3B, which is identical in sequence except at the C terminus, probably has a similar function in Golgi membranes.

Overall, our present study has clearly established that the

ClC-3B isoform is a Golgi channel where it may function as an anion shunt during acidification as ClC-3A does in late endosomes (3, 4). While this possibility remains to be rigorously tested, if confirmed it would also fit well with this general function of the other members of the ClC-3, -4, -5 branch of the mammalian ClC family (1). Our findings do not preclude the possibility that a small amount of ClC-3B might traffic to the cell surface and perhaps even be influenced by CFTR under some circumstances as suggested by Ogura *et al.* (6). Mohammad-Panah *et al.* (34) have reported immunolocalization of ClC-4 to the apical surface of epithelial cells in intestinal crypts. Appearance of ClC-3B at the cell surface could require the involvement of a β -subunit as is the case with ClC-Ka, where barttin plays this role (35) or interference with recognition of a PY internalization and degradation motif, as is the case with ClC-5 (36). However, our findings do not support the idea that interactions with subplasma membrane PDZ proteins such as EBP50 (or PDZK1) bring about either cell surface localization or association with CFTR at the plasma membrane.

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