THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 285, NO. 51, pp. 40088–40096, December 17, 2010

Heteromeric Interactions Required for Abundance and Subcellular Localization of Human CDC50 Proteins and Class 1 P_4-ATPases*[S]

Received for publication, April 28, 2010, and in revised form, September 16, 2010 Published, JBC Papers in Press, October 14, 2010, DOI 10.1074/jbc.M110.139006

Lieke M. van der Velden[‡], Catharina G. K. Wichers[‡], Adriana E. D. van Breevoort[‡], Jonathan A. Coleman[§], Robert S. Molday[§], Ruud Berger[‡], Leo W. J. Klomp[‡], and Stan F. J. van de Graaf[‡1]

From the [‡]Department of Metabolic and Endocrine Diseases, Universitair Medisch Centrum Utrecht, and the Netherlands Metabolomics Centre, 3508 AB Utrecht, The Netherlands and the [§]Departments of Biochemistry and Molecular Biology and Ophthalmology and Visual Sciences, Centre for Macular Research, University of British Columbia, Vancouver V6T 1Z3, Canada

Members of the P_4 family of P-type ATPases (P_4-ATPases) are believed to function as phospholipid flippases in complex with CDC50 proteins. Mutations in the human class 1 P_4-ATPase gene *ATP8B1* cause a severe syndrome characterized by impaired bile flow (intrahepatic cholestasis), often leading to end-stage liver failure in childhood. In this study, we determined the specificity of human class 1 P_4-ATPase interactions with CDC50 proteins and the functional consequences of these interactions on protein abundance and localization of both protein classes. ATP8B1 and ATP8B2 co-immunoprecipitated with CDC50A and CDC50B, whereas ATP8B4, ATP8A1, and ATP8A2 associated only with CDC50A. ATP8B1 shifted from the endoplasmic reticulum (ER) to the plasma membrane upon coexpression of CDC50A or CDC50B. ATP8A1 and ATP8A2 translocated from the ER to the Golgi complex and plasma membrane upon coexpression of CDC50A, but not CDC50B. ATP8B2 and ATP8B4 already displayed partial plasma membrane localization in the absence of CDC50 coexpression but displayed a large increase in plasma membrane abundance upon coexpression of CDC50A. ATP8B3 did not bind CDC50A and CDC50B and was invariably present in the ER. Our data show that interactions between CDC50 proteins and class 1 P_4-ATPases are essential for ER exit and stability of both subunits. Furthermore, the subcellular localization of the complex is determined by the P_4-ATPase, not the CDC50 protein. The interactions of CDC50A and CDC50B with multiple members of the human P_4-ATPase family suggest that these proteins perform broader functions in human physiology than thus far assumed.

P-type ATPases are ATP-powered pumps transporting a large variety of substrates present in bacteria, archaea, and eukaryotes. Based on sequence similarity, they are divided into five families or types: P_1–P_5. Members of the P_4 family of P-type ATPases (P_4-ATPases) are expressed exclusively by eukaryotes and are considered prime candidates to serve a role as (amino)phospholipid translocases (also called flippases) (1, 2). Through knockdown, knock-out, or gene mutation studies, P_4-ATPase function has been investigated in different organisms. These studies demonstrated that P_4-ATPase function is important in membrane stability, vesicle biogenesis, and vesicular trafficking (3–10). Mutations in members of the Lem3/Cdc50 (CDC50) protein family lead to identical phenotypes as mutations in P_4-ATPases in yeast, as association with CDC50 proteins is required for P_4-ATPase function (11–15).

In yeast, the Cdc50 family consists of three members: Crf1p, Cdc50p, and Lem3/Ros3p (16). All three mediate endoplasmic reticulum (ER)[2] exit of (specific) yeast P_4-ATPases and enable these proteins to reach their correct subcellular location (11, 15, 17). In humans, little is known about the function of CDC50 proteins in relation to P_4-ATPases. Humans have three *CDC50* genes: *CDC50A*, *CDC50B*, and *CDC50C* (18). Full-length transcripts of human *CDC50A* and *CDC50B* are found in multiple tissues. *CDC50C* is a testis-specific expressed gene, but no full-length transcripts have been found in hominoids (19). Fourteen P_4-ATPase genes have been identified in the human genome (20, 21). This suggests that CDC50 proteins may associate with more than one P_4-ATPase or that there are P_4-ATPases that operate on their own. At present, it is not clear why the human genome harbors so many P_4-ATPases. It is unknown if these proteins have specialized functions at the cellular level or if P_4-ATPase family members perform similar functions but are expressed in a tissue-specific manner.

ATP8B1 is the only P_4-ATPase so far that is unequivocally linked to a human disease (22). Mutations in the *ATP8B1* gene cause ATP8B1 deficiency, a severe autosomal recessive syndrome characterized by a variety of symptoms (23–25). The most prominent presentation is intrahepatic cholestasis (impairment of bile flow), which can lead to fatal liver damage. Next to liver, ATP8B1 is among others highly expressed in the intestine and cochlear hair cells. Impaired ATP8B1

* This work was supported by Netherlands Organization for Scientific Research (NWO) SFJvdG Project 016.096.108 and by the Wilhelmina Children's Foundation, the Utrecht University High Potential Program, and the Dutch Digestive Disease Foundation (to L. W. J. K.).

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental "Experimental Procedures" and Figs. S1 and S2.

[1] To whom correspondence should be addressed: UMC Utrecht, KC-02.069.1, P. O. Box 85090, 3508 AB Utrecht, The Netherlands. Tel.: 31-88-755-4293; Fax: 31-88-755-4295; E-mail: s.vandegraaf-4@umcutrecht.nl.

[2] The abbreviations used are: ER, endoplasmic reticulum; VSV-G, vesicular stomatitis virus G; TfR, transferrin receptor.

function in these organs can lead to diarrhea and impaired hearing (24, 25). Paulusma *et al.* (26) investigated the role of CDC50 family members and human ATP8B1. ATP8B1 showed a functional interaction with CDC50A, and it was subsequently suggested that CDC50A might play a role in ATP8B1-related disease. P_4-ATPases are divided into subclasses based on sequence homology (21, 27), and ATP8B1 belongs to the class 1 P_4-ATPases. Other prominent members of this class are ATP8A1 (previously called ATPase II), which is the first cloned mammalian flippase (28), and ATP8B3, a testis-specific member important for male fertility in mice (29, 30). Little is known about the other class 1 P_4-ATPases, especially in humans. In this study, we determined the specificity of class 1 P_4-ATPase interactions with CDC50 proteins. Furthermore, we demonstrate the functional consequences of these interactions on cellular location and plasma membrane abundance of both protein classes.

EXPERIMENTAL PROCEDURES

DNA Constructs—pcDNA3.1-CDC50A-V5, pcDNA3-ATP8A2-Rho1D4, and pCB7-VSV-ATP8B1 were described previously (1, 31). The open reading frame of human ATP8B1 was subcloned into pcDNA3.1 with a C-terminal HA tag. Open reading frames of human CDC50B, ATP8B1, ATP8B2, and ATP8B4 (German Resource Center for Genome Research, Berlin, Germany; and Kazusa DNA Research Institute, Chiba, Japan) subcloned in pcDNA3.1 with a C-terminal HA tag were generously provided by Dr. J. Holthuis (Utrecht University). Human testis and hippocampus RNAs (Invitrogen) were treated with Superscript II reverse transcriptase (Invitrogen) and used to clone ATP8B3 and ATP8A1, respectively. ATP8B3 was cloned into pCB7 with an N-terminal vesicular stomatitis virus G (VSV-G) tag, and ATP8A1 was cloned into the pEBB vector (32) with a C-terminal HA tag. The SNAP open reading frame was cloned at the C terminus of CDC50A. All constructs were verified by sequence analysis.

Fluorescence Microscopy—Human bone osteosarcoma (U2OS) cells were cultured in Dulbecco's modified Eagle's medium/GlutaMAX (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 100 units/ml penicillin/streptomycin (Invitrogen) under 5% CO_2 at 37 °C. Cells were transiently transfected with polyethyleneimine. After 2 days, cells were fixed with paraformaldehyde, and P_4-ATPase proteins were visualized using rabbit anti-VSV-G (for ATP8B1 and ATP8B3), mouse anti-Rho1D4 (for ATP8A2), mouse anti-HA (for ATP8B2, ATP8B4, and ATP8A1) or rabbit anti-ATP8A1 antibody (supplemental Fig. S1). Co-labeling of ER marker proteins was performed either with rabbit anti-calreticulin antibody (for ATP8B2, ATP8B4, ATP8A1, and ATP8A2; Alexis Biochemicals) or mouse anti-protein-disulfide isomerase antibody (for ATP8B1 and ATP8B3; Abcam, Cambridge, United Kingdom). Co-labeling with the Golgi markers mouse anti-p58 antibody (Sigma) and rabbit anti-GM130 antibody (Abcam) was performed for ATP8A1. The secondary antibodies used were goat anti-rabbit Alexa 568, goat anti-rabbit Alexa 488, and goat anti-mouse Alexa 488, and goat anti-mouse Alexa 568 (Invitrogen) and donkey anti-mouse Cy5 (Jackson ImmunoResearch Laboratories, West Grove, PA). CDC50 proteins were visualized using FITC-conjugated mouse anti-V5 antibody (Invitrogen). Images were acquired using an LSM 710 Meta confocal microscope equipped with a ×63/1.40-numerical aperture Plan-Apochromat objective (Carl Zeiss, Jena, Germany) by sequential excitation at 488 and 561 nm.

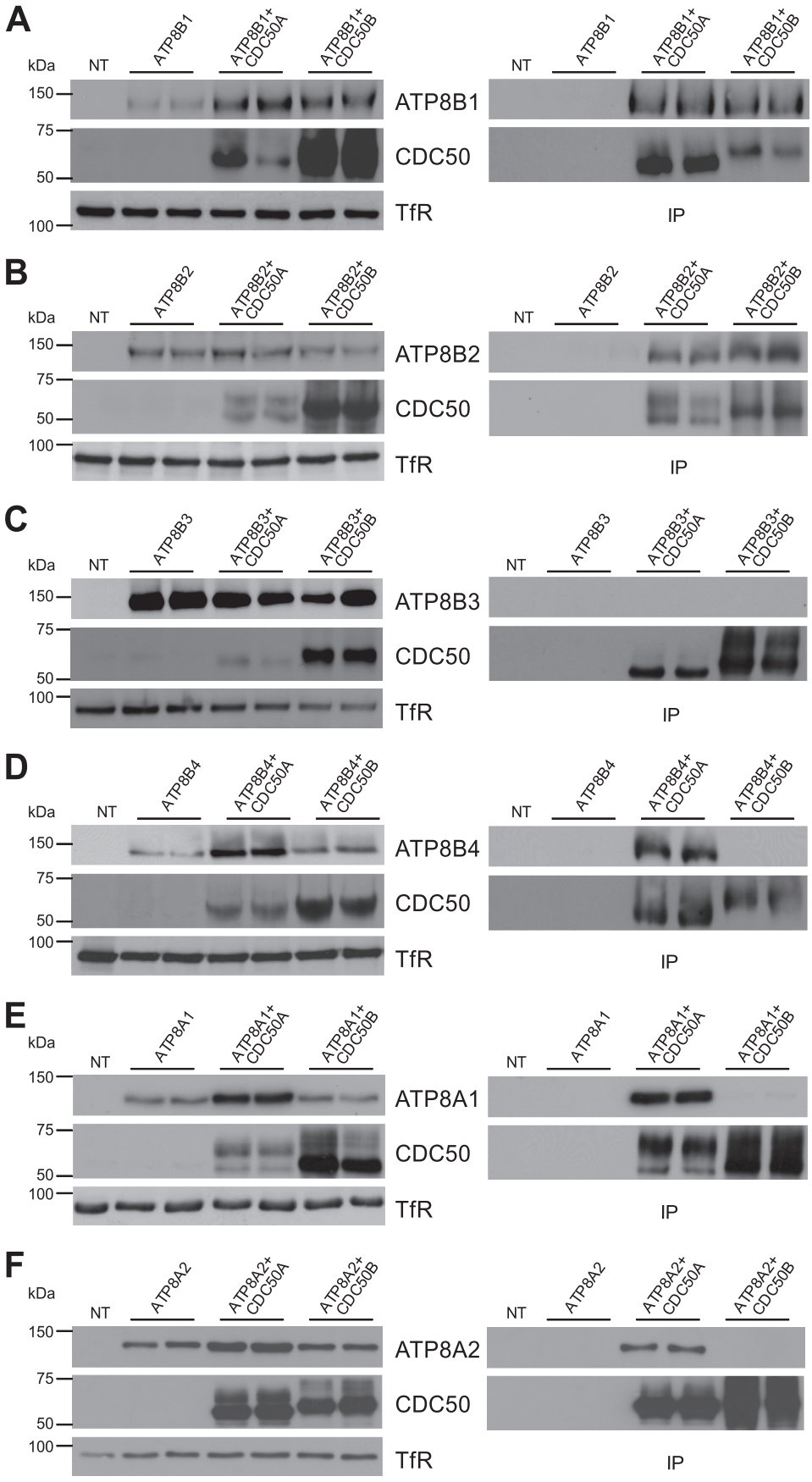
Cell-surface Biotinylation—U2OS cells were transfected using polyethyleneimine. Two days after transfection, proteins at the cell surface were biotinylated and isolated as described (31). P_4-ATPase and CDC50 proteins in cell lysates and in the NeutrAvidin precipitates were analyzed by immunoblot analyses using rabbit anti-VSV-G (Abcam), mouse anti-HA (PD7 clone, Sigma), mouse anti-Rho1D4 (Sigma), and mouse anti-V5 (Invitrogen) antibodies. Incubation of the blots with mouse anti-transferrin receptor antibody (Invitrogen) was routinely performed as a loading control. Mouse anti-HSP70 antibody (Cell Signaling Technologies, Beverly, MA) was used to probe for cytosolic proteins and was consistently absent from the NeutrAvidin precipitate. The secondary antibodies used for detection were goat anti-mouse HRP (Pierce) and goat anti-rabbit HRP (DAKO, Carpinteria, CA).

Co-immunoprecipitation—Co-immunoprecipitation analysis was performed as described previously (31). Precipitated proteins were analyzed by immunoblot analysis using rabbit anti-V5 antibody (Sigma) to detect CDC50 proteins and rabbit anti-VSV-G (for ATP8B3) or HRP-conjugated mouse anti-HA (for ATP8B1, ATP8B2, ATP8B4, and ATP8A1; Sigma). Co-immunoprecipitation analysis of ATP8A2 and CDC50 proteins was performed by incubating lysates with rabbit anti-V5 antibody immobilized on protein A-agarose beads, followed by immunoblotting with mouse anti-V5 and mouse anti-Rho1D4 antibodies.

Carbohydrate Analysis—U2OS cells were transiently transfected with CDC50A or cotransfected with CDC50A and a P_4-ATPase. Two days after transfection, total cell lysates were treated with endoglycosidase H (Westburg, Leusden, The Netherlands) or *N*-glycosidase F (Westburg) or left untreated and subjected to immunoblotting.

RESULTS

Protein/Protein Interactions of P_4-ATPases and CDC50 Proteins—The interaction of CDC50A and CDC50B with ATP8B1 was investigated by co-immunoprecipitations using transiently transfected U2OS cells. Upon coexpression of ATP8B1 and CDC50A or CDC50B, a specific anti-V5 tag signal was detected as broad bands at ~50 kDa in cell lysates (Fig. 1*A*, *left panels*) and V5 precipitates (*right panels*). ATP8B1 efficiently co-immunoprecipitated with both CDC50A and CDC50B. No signal was detected in the precipitated fraction using lysates of non-transfected cells or cells transfected with ATP8B1 only. Similarly, the interactions of other human class 1 P_4-ATPases with CDC50A and CDC50B were determined. ATP8B2 was detected as a single band at ~140 kDa, in line with its predicted molecular mass (Fig. 1*B*, *left panels*). ATP8B2 co-precipitated with both CDC50A and CDC50B (Fig. 1*B*, *right panels*). ATP8B3 was well detected at ~145 kDa (Fig. 1*c*, *left panels*) and did not co-precipitate with


A
kDa
NT
ATP8B1
ATP8B1+ CDC50A
ATP8B1+ CDC50B
150
75
50
100
ATP8B1
CDC50
TfR
IP
B
ATP8B2
ATP8B2+ CDC50A
ATP8B2+ CDC50B
ATP8B2
CDC50
TfR
IP
C
ATP8B3
ATP8B3+ CDC50A
ATP8B3+ CDC50B
ATP8B3
CDC50
TfR
IP
D
ATP8B4
ATP8B4+ CDC50A
ATP8B4+ CDC50B
ATP8B4
CDC50
TfR
IP
E
ATP8A1
ATP8A1+ CDC50A
ATP8A1+ CDC50B
ATP8A1
CDC50
TfR
IP
F
ATP8A2
ATP8A2+ CDC50A
ATP8A2+ CDC50B
ATP8A2
CDC50
TfR
IP

CDC50A or CDC50B (*right panels*). Finally, ATP8B4, ATP8A1, and ATP8A2 associated specifically with CDC50A (Fig. 1, *D–F*). In all cases, the P_4-ATPase signal in immunoprecipitates was not detected when V5-tagged CDC50A or CDC50B was not coexpressed, indicating the specificity of the procedure.

Expression Levels of P_4-ATPases Are Enhanced upon CDC50A or CDC50B Coexpression—From the lysate blots of the co-immunoprecipitation experiments, we noticed that the P_4-ATPase signal in the total lysate was dependent on CDC50 expression. Coexpression of CDC50A increased the total cellular expression level of ATP8B1, ATP8B2, ATP8B4, ATP8A1, and ATP8A2 (Fig. 1, *A*, *B*, and *D–F*; see Fig. 3, *A*, *B*, and *D–F*, and Fig. 4) compared with the expression levels of these P_4-ATPases when transfected alone. Cotransfection of CDC50B increased the expression levels of only ATP8B1 (Fig. 1*A*), whereas none of the other P_4-ATPases showed this effect (Fig. 1, *B–F*). In contrast, cotransfection of CDC50 proteins had no effect on the total cellular expression of ATP8B3 (Fig. 1*C*).

Subcellular Translocation of Class 1 P_4-ATPase Proteins upon Coexpression of CDC50 Proteins—We investigated the effect of CDC50A or CDC50B coexpression on the subcellular localization of class 1 P_4-ATPases by confocal microscopy. In the absence of CDC50A protein coexpression, ATP8B1, ATP8B3, ATP8A1, and ATP8A2 localized to the ER in transiently transfected U2OS cells, illustrated by the significant colocalization with the ER marker proteins calreticulin and protein-disulfide isomerase. ATP8B2 and ATP8B4 showed plasma membrane localization and additional minor colocalization with ER markers (Fig. 2, *A–F*, *upper panels*). Upon coexpression of CDC50A or CDC50B, ATP8B1 localization shifted to the plasma membrane, where it colocalized with the CDC50 proteins (Fig. 2*A*). Such dramatic translocation upon coexpression of CDC50A or CDC50B was less obvious for ATP8B4, which already resided at the plasma membrane (Fig. 2*D*). Significant colocalization with CDC50A was observed for ATP8B2 and ATP8B4 at the plasma membrane (Fig. 2, *B* and *D*). ATP8B2 resided predominantly in the ER in the presence of CDC50B (Fig. 2*B* and supplemental Fig. S2). Upon coexpression with CDC50A, ATP8A1 and ATP8A2 translocated to the Golgi complex and plasma membrane (Fig. 2, *E–H*, and supplemental Fig. S2). CDC50A colocalized with ATP8A1 and ATP8A2 at the Golgi complex and plasma membrane. In contrast, upon CDC50B coexpression, ATP8A1 and ATP8A2 remained in the ER (Fig. 2, *E* and *F*, and supplemental Fig. S2, *E* and *F*). No effect of CDC50 coexpression was seen on the subcellular localization of ATP8B3, which resided in all cases at the ER (Fig. 2*C*).

Coexpression of CDC50A and/or CDC50B Enhances Plasma Membrane Expression of P_4-ATPases—The P_4-ATPase translocation to the plasma membrane upon coexpression of CDC50 proteins was investigated by cell-surface biotinylation. Coexpression of CDC50A strongly enhanced the cell-surface expression of ATP8B1, ATP8B2, ATP8B4, ATP8A1, and ATP8A2 (Fig. 3, *A*, *B*, and *D–F*). ATP8B3 was undetectable in the biotinylated fraction in both the presence and absence of CDC50 proteins (Fig. 3*C*). Furthermore, ATP8B1 was the only class 1 P_4-ATPase with markedly enhanced plasma membrane expression when CDC50B was coexpressed (Fig. 3*A*). Although this P_4-ATPase co-precipitated with CDC50B, ATP8B2 abundance at the plasma membrane was unaltered or even decreased upon CDC50B coexpression. Expression of CDC50B even reversed the stimulatory effect of CDC50A on ATP8B2 plasma membrane abundance in a dose-dependent manner (Fig. 4). CDC50A was detected in the biotinylated fraction in the presence of ATP8B1, ATP8B2, ATP8B4, ATP8A1, and ATP8A2, whereas CDC50B was detected mainly in this fraction upon coexpression of ATP8B1. Neither CDC50A nor CDC50B was present in the biotinylated fraction upon coexpression of ATP8B3. The transferrin receptor (TfR) signal was similar under all conditions, assuring equal loading. Omission of sulfo-NHS-SS-biotin (no biotin) during the cell-surface labeling precluded binding of P_4-ATPases, CDC50A, and CDC50B to the NeutrAvidin beads, and no signal for these proteins was detected in non-transfected cells (Fig. 3, *A–F*). Furthermore, we could not detect cytosolic HSP90 in the NeutrAvidin precipitates, demonstrating the specificity of the assay for cell-surface proteins (data not shown).

CDC50 Proteins Are Dependent on P_4-ATPases for ER Exit and Expression—Figs. 1–4 show that CDC50 proteins are determinants of the expression and localization of several of the P_4-ATPases under investigation in this study. We also investigated whether the expression and localization of CDC50A and CDC50B were dependent on the coexpression of a P_4-ATPase. CDC50A transiently transfected in U2OS cells localized at the ER. CDC50B resided in the ER and showed partial plasma membrane localization (Fig. 5*A*). CDC50A translocated from the ER to the plasma membrane when coexpressed with ATP8B1, ATP8B2, ATP8B4, ATP8A1, or ATP8A2 and colocalized with the P_4-ATPase at the plasma membrane or Golgi complex. Cell-surface biotinylation experiments further demonstrated that CDC50A and CDC50B plasma membrane expression was strongly enhanced when ATP8B1 was cotransfected (Fig. 5*B*). Limited amounts of CDC50A and CDC50B were detectable in the biotinylated fraction in the absence of ATP8B1 coexpression upon longer exposures of the film (data not shown). Furthermore, the expression of both CDC50A and CDC50B was enhanced upon coexpression of ATP8B1, as shown in Fig. 5*B* (*upper panels*). Finally, coexpression with ATP8B1, ATP8B2, ATP8B4, ATP8A1, or ATP8A2 resulted in the appearance of an endoglycosidase H-insensitive high molecular mass CDC50A

FIGURE 1. **Protein/protein interactions of P_4-ATPases and CDC50 proteins.** U2OS cells were transiently transfected with P_4-ATPase only or cotransfected with P_4-ATPase and CDC50A or CDC50B. Protein expression of P_4-ATPases and CDC50 proteins in total lysates was determined by immunoblotting using anti-HA antibody (*A*, *B*, *D*, and *E*), anti-VSV-G antibody (*C*), or anti-Rho1D4 antibody (*F*). CDC50 proteins were visualized by anti-V5 antibody, and TfR was used as a loading control. Total cell lysates were incubated with anti-V5 antibody immobilized on agarose beads. Immunoprecipitates (*IP*) were subsequently immunoblotted for P_4-ATPase and CDC50 protein expression. ATP8B1 and ATP8B2 associated with CDC50A and CDC50B, whereas ATP8B4, ATP8A1, and ATP8A2 associated only with CDC50A. ATP8B3 bound neither CDC50A nor CDC50B. *NT*, non-transfected cells.

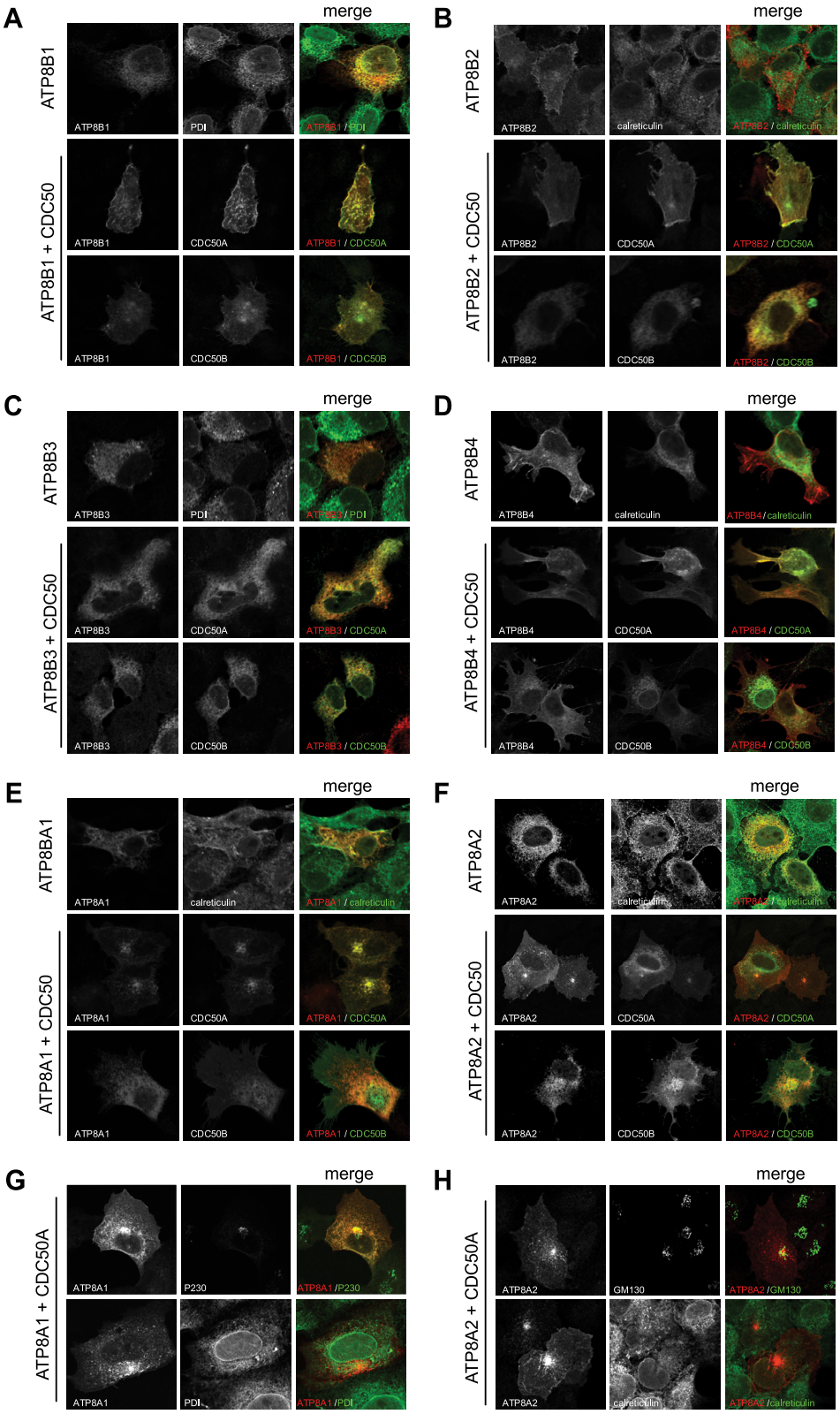


FIGURE 2. **Subcellular localization of P_4-ATPases and CDC50 proteins in U2OS cells.** Cells were transiently transfected with P_4-ATPase alone (*A–F*, *upper panels*) or cotransfected with P_4-ATPase and CDC50A (*A–F*, *middle panels*, and *G* and *H*) or CDC50B (*A–F*, *lower panels*). Using confocal microscopy, P_4-ATPases were visualized with mouse anti-HA antibody, followed by goat anti-mouse Alexa 568 (*B*, *D*, and *E*), rabbit anti-VSV-G and goat anti-rabbit Alexa 568 antibodies (*A* and *C*), rabbit anti-ATP8A1 and goat anti-rabbit Alexa 568 antibodies (*G*), or mouse anti-Rho1D4 and goat anti-mouse Alexa 568 antibodies (*F* and *H*). CDC50A and CDC50B were visualized using FITC-conjugated mouse anti-V5 antibody. P_4-ATPases transfected alone were co-stained with goat anti-rabbit calreticulin antibody (*B* and *D–F*) or mouse anti-protein-disulfide isomerase antibody (*PDI*; *A* and *C*) to visualize the ER. Cells cotransfected with ATP8A1 and CDC50A were co-stained with rabbit anti-ATP8A1, FITC-conjugated mouse anti-V5, and mouse anti-p230 antibodies (Golgi marker; *G*, *upper panels*) or mouse anti-protein-disulfide isomerase antibody (ER marker; *G*, *lower panels*). To visualize the Golgi localization of ATP8A2 upon coexpression of CDC50A, co-staining with rabbit anti-GM130 antibody (Golgi marker) was performed (*H*, *upper panels*).

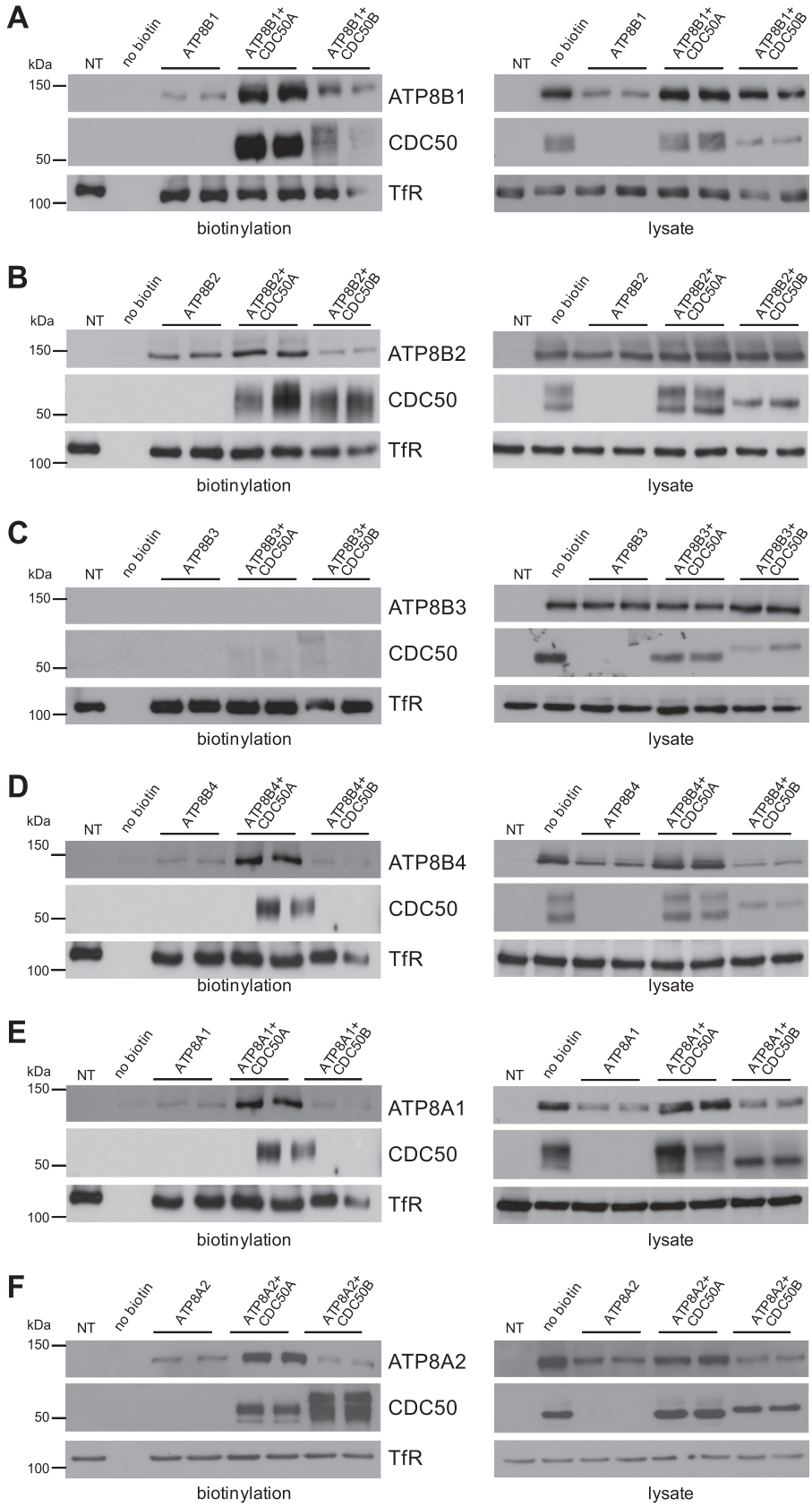


FIGURE 3. **Coexpression of CDC50A and/or CDC50B enhances plasma membrane expression of P_4-ATPases.** U2OS cells were transiently transfected with P_4-ATPase only or cotransfected with P_4-ATPase and CDC50A or CDC50B. After 48 h of transfection, cell surface-resident proteins were labeled with sulfo-NHS-SS-biotin and precipitated from cell lysates using NeutrAvidin beads. Precipitates (biotinylation) and total cell lysates were immunoblotted for P_4-ATPases, CDC50 proteins, and TfR. The signal of P_4-ATPases, CDC50, or TfR was not detected when biotin was omitted (*no biotin*) in the cell-surface labeling or in non-transfected cells (*NT*).

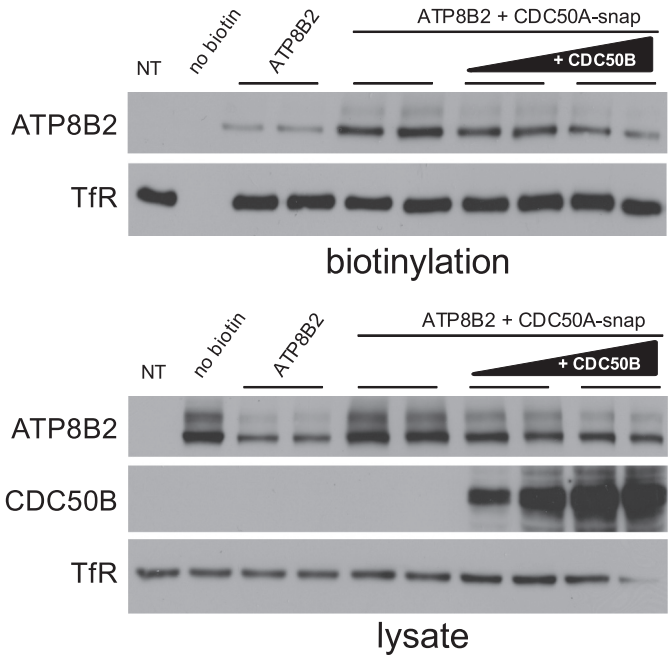


FIGURE 4. **CDC50B coexpression prevents ATP8B2 translocation to the plasma membrane.** U2OS cells were transiently transfected with ATP8B2 only or cotransfected with CDC50A-SNAP or with CDC50A-SNAP and CDC50B-V5. Surface-resident ATP8B2 was quantified by biotinylation, followed by immunoblotting. CDC50B coexpression resulted in a dose-dependent decrease in ATP8B2 plasma membrane localization, despite the presence of CDC50A. *NT*, non-transfected cells.

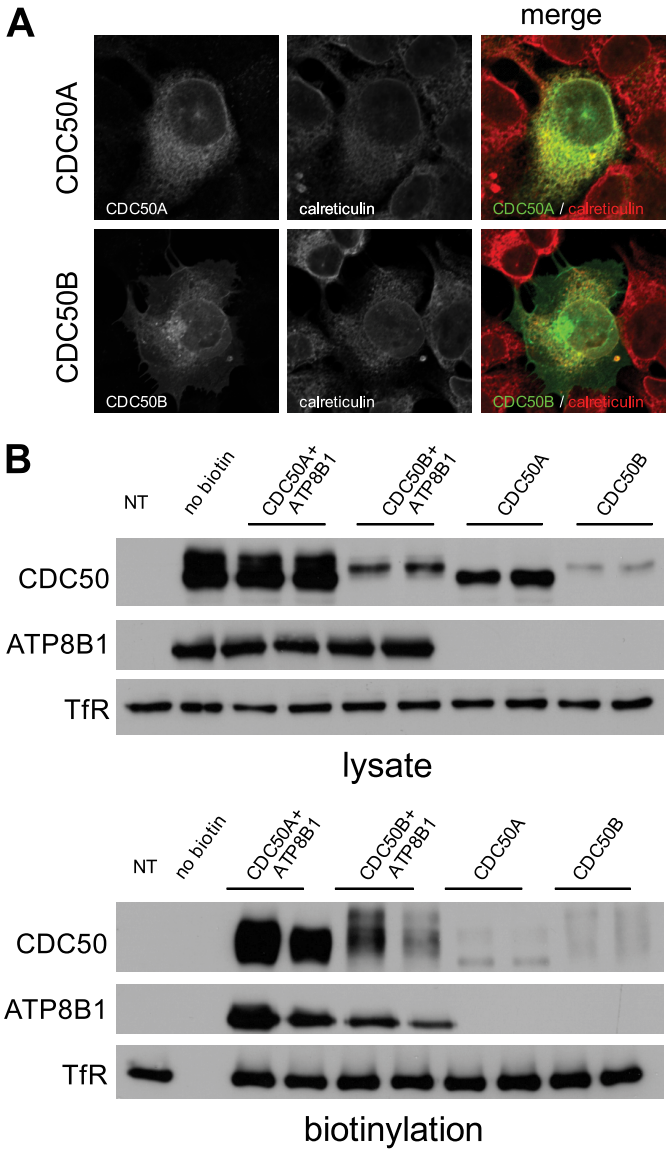


FIGURE 5. **CDC50 proteins are dependent on P_4-ATPases for ER exit and expression.** U2OS cells were transiently transfected with CDC50A (*upper panels*) or CDC50B (*lower panels*) and visualized with FITC-conjugated anti-V5 antibody using confocal microscopy. The ER was visualized with goat anti-rabbit calreticulin antibody (*A*). U2OS cells were transiently transfected with CDC50A or CDC50B only, with CDC50A and ATP8B1, or with CDC50B and ATP8B1 (*B*). Forty-eight hours after transfection, cell-surface proteins were labeled with sulfo-NHS-SS-biotin and precipitated from lysates using NeutrAvidin beads. Total lysates (*upper panels*) and precipitates (biotinylation; *lower panels*) were subsequently immunoblotted for CDC50 proteins and ATP8B1. TfR was used as a loading control. Plasma membrane expression of CDC50 proteins, ATP8B1, or TfR was not detected when biotin was omitted (*no biotin*) in the cell-surface labeling. *NT*, non-transfected cells.

glycoprotein (Fig. 6, *A*, *B*, and *D–F*). This species of CDC50A was not observed in the absence of P_4-ATPases or in the presence of ATP8B3 (Fig. 6*C*). Here, CDC50A migrated as a single band, which was endoglycosidase H-sensitive. Both the high and low molecular mass species of CDC50A were sensitive to *N*-glycosidase F (Fig. 6).

DISCUSSION

To date, 14 *P_4-ATPase* and three *CDC50* genes have been identified in humans (18, 20, 21, 27). This suggests that each CDC50 protein binds multiple P_4-ATPases or that some P_4-ATPases function without CDC50. For the majority of the human P_4-ATPases, no information on the interaction with CDC50 proteins or consequence of this has been described. In this study, we systematically determined the association of class 1 P_4-ATPases with CDC50 proteins and the functional consequence on subcellular location and protein abundance. We demonstrated that CDC50 proteins are pivotal for the expression and localization of human class 1 P_4-ATPases. This relationship is dual, as P_4-ATPases are equally important for the expression and localization of CDC50 proteins. There are three lines of evidence that support this conclusion.

First, the class 1 P_4-ATPases and CDC50 proteins promiscuously interact, as demonstrated with co-immunoprecipitations (Fig. 1). CDC50A interacts with five of the six investigated P_4-ATPases, whereas CDC50B interacts with two P_4-ATPases. CDC50A and CDC50B were previously identified as interaction partners of ATP8B1 (26). Here, we demonstrated that CDC50A interacts not only with ATP8B1 but also with ATP8B2, ATP8B4, ATP8A1, and ATP8A2 (Fig. 1). Furthermore, CDC50B also interacts with ATP8B2. However, the relevance of this interaction remains unclear, as CDC50B does not enhance ATP8B2 expression or plasma membrane abundance. The interaction of CDC50 proteins with several P_4-ATPase family members was previously reported in other species. The *Saccharomyces cerevisiae* CDC50 protein Lem3p interacts with both Dnf1p and Dnf2p (15, 17). In addition, it was recently shown that three *Arabidopsis thaliana* CDC50 proteins, ALIS2, ALIS3, and ALIS4, all interact with ALA2 and ALA3, two plant P_4-ATPases (14, 33).

Second, most P_4-ATPase/CDC50 interactions considerably enhance the total cellular expression levels of the P_4-ATPases.

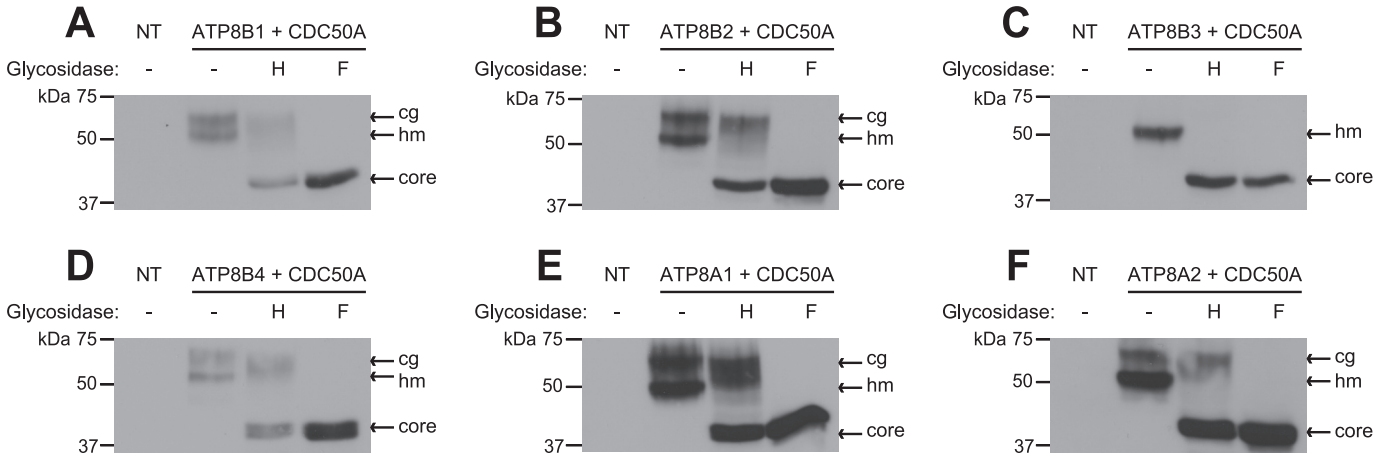


FIGURE 6. **Coexpression of associated P_4-ATPases results in complex glycosylation of CDC50A.** Lysates of U2OS cells coexpressing CDC50A with P_4-ATPases were treated with endoglycosidase H (*H*) or *N*-glycosidase F (*F*) or left untreated (−). Complex glycosylated CDC50A (*cg*) and high-mannose CDC50A (*hm*) proteins are indicated, and the protein band labeled *core* represents the non-glycosylated core CDC50A protein. *N*-Glycosidase F cleaves all *N*-linked sugars from glycoproteins, whereas endoglycosidase H sensitivity is limited mainly to ER-resident glycoproteins. *NT*, non-transfected cells.

The total cellular expression levels of all of the P_4-ATPases strongly increase upon coexpression of an interaction-competent CDC50 protein (except ATP8B2/CDC50B) (Fig. 1). This relation is interdependent because coexpression of an interacting P_4-ATPase with CDC50A or CDC50B also results in increased total cellular expression of the CDC50 proteins (Fig. 5). Stimulation of protein expression as a functional consequence of interactions with CDC50 proteins was previously observed for yeast Cdc50 family members and P_4-ATPases (34) but was not investigated in detail. The P_{2C}-ATPases (Na^+/K^+-ATPases and H^+/K^+-ATPases) are the only other members of the P-type ATPase superfamily known to interact with a β-subunit (35). Interestingly, also P_{2C}-ATPases and their β-subunits mutually depend on each other to adopt a stable configuration and to be exported out of the ER (36).

Third, the P_4-ATPase/CDC50 protein interactions have functional consequences for the subcellular localization of P_4-ATPases. As observed by confocal microscopy and cell-surface biotinylation, P_4-ATPases translocate to the plasma membrane and/or Golgi complex upon coexpression of an interacting CDC50 protein (Figs. 2 and 3). In the absence of an associated CDC50 protein, a large fraction of P_4-ATPase protein is present at the ER, in particular ATP8B1, ATP8A1, and ATP8A2. ATP8B2 and ATP8B4 partially reside at the plasma membrane in U2OS cells without exogenous coexpression of CDC50 proteins, as shown by confocal microscopy. However, with cell-surface biotinylation experiments, we showed that ATP8B1, ATP8B2, ATP8B4, ATP8A1, and ATP8A2 plasma membrane expression is strongly enhanced upon coexpression of CDC50A. Again, this relationship of P_4-ATPase and CDC50 proteins is interdependent; CDC50 proteins expressed individually localize mainly at the ER (Fig. 5*A*). Coexpression of an interacting P_4-ATPase results in significant CDC50 protein translocation, as was demonstrated by confocal microscopy and cell-surface biotinylation (Fig. 5). Association with ATP8A1 and ATP8A2 results in translocation to the Golgi complex and plasma membrane (Fig. 2, *E–H*), whereas association with ATP8B1, ATP8B2, and ATP8B4 targets CDC50A to the plasma membrane (Fig. 2, *A*, *B*, and *D*). ER exit of CDC50A upon coexpression of ATP8B1, ATP8B2, ATP8B4, ATP8A1, and ATP8A2 is further reflected by the appearance of a higher molecular mass CDC50A species, which was endoglycosidase H-insensitive/*N*-glycosidase F-sensitive, indicative of complex glycosylation, a post-translational modification that takes place in the Golgi complex. In the absence of an associated P_4-ATPase, CDC50A migrates as a single lower molecular mass species that is sensitive to endoglycosidase H, in line with localization in the ER. Different P_4-ATPases localize to different subcellular compartments, whereas they all interact with CDC50A. This suggests that the subcellular localization signals reside within the human P_4-ATPase. Similarly, López-Marqués *et al.* (33) recently showed that, in *Arabidopsis*, the subcellular localization of P_4-ATPases after ER exit is independent of the nature of the associated CDC50 protein. Furthermore, localization of ALIS proteins (*Arabidopsis* CDC50 proteins) is different when coexpressed with distinct plant P_4-ATPases.

It is unclear if the ER localization of ATP8B3 represents its functional localization or is the result of an inability of ATP8B3 to interact with CDC50A or CDC50B. Mouse ATP8B3 and its close homolog FetA both localize to the acrosomal ridge in sperm cells (a Golgi complex-derived organelle) (29, 30, 37). In contrast to the situation in mouse, which displays *Cdc50c* expression in testes, no full-length transcripts of *CDC50C* are found in humans, providing a possible rationale for this difference (19). The linkage between ATP8B1 mutations and human disease suggest that this P_4-ATPase displays a unique biological function. In that respect, it is remarkable that, with the exception of ATP8B3, all class 1 P_4-ATPases are (partially) present at the plasma membrane.

In yeast, the P_4-ATPase Drs2p cannot compensate for loss of Dnf1p and Dnf2p, two P_4-ATPases that localize to the plasma membrane (9). Interestingly, Drs2p shows the highest homology to ATP8A1 and is also localized predominantly to the Golgi complex. Furthermore, it is doubtful whether ATP8B2 and ATP8B4 are coexpressed with ATP8B1 at the apical plasma membrane of polarized cells. At least in intesti-

nal cells, ATP8B2 and ATP8B4 are virtually not expressed, even after knockdown of ATP8B1(38).

Taken together, we have demonstrated that human CDC50 proteins are essential for ER exit of most class 1 P_4-ATPases and that P_4-ATPases are equally important for ER exit of CDC50 proteins. Furthermore, our data indicate that subcellular localization of the complex is determined by the P_4-ATPases and that they are likely to operate at the plasma membrane, except for ATP8A1 and ATP8A2 (Golgi complex) and ATP8B3 (ER or unknown). Finally, the interactions of CDC50A and CDC50B with multiple members of the human P_4-ATPase family suggest that these proteins perform broader functions in human physiology than thus far assumed.

REFERENCES

1. Coleman, J. A., Kwok, M. C., and Molday, R. S. (2009) *J. Biol. Chem.* **284,** 32670–32679
2. Zhou, X., and Graham, T. R. (2009) *Proc. Natl. Acad. Sci. U.S.A.* **106,** 16586–16591
3. Alder-Baerens, N., Lisman, Q., Luong, L., Pomorski, T., and Holthuis, J. C. (2006) *Mol. Biol. Cell* **17,** 1632–1642
4. Chen, C. Y., Ingram, M. F., Rosal, P. H., and Graham, T. R. (1999) *J. Cell Biol.* **147,** 1223–1236
5. Devaux, P. F., Herrmann, A., Ohlwein, N., and Kozlov, M. M. (2008) *Biochim. Biophys. Acta* **1778,** 1591–1600
6. Farge, E., Ojcius, D. M., Subtil, A., and Dautry-Varsat, A. (1999) *Am. J. Physiol.* **276,** C725–C733
7. Gall, W. E., Geething, N. C., Hua, Z., Ingram, M. F., Liu, K., Chen, S. I., and Graham, T. R. (2002) *Curr. Biol.* **12,** 1623–1627
8. Graham, T. R. (2004) *Trends Cell Biol.* **14,** 670–677
9. Pomorski, T., Lombardi, R., Riezman, H., Devaux, P. F., van Meer, G., and Holthuis, J. C. (2003) *Mol. Biol. Cell* **14,** 1240–1254
10. Ruaud, A. F., Nilsson, L., Richard, F., Larsen, M. K., Bessereau, J. L., and Tuck, S. (2009) *Traffic* **10,** 88–100
11. Kato, U., Emoto, K., Fredriksson, C., Nakamura, H., Ohta, A., Kobayashi, T., Murakami-Murofushi, K., Kobayashi, T., and Umeda, M. (2002) *J. Biol. Chem.* **277,** 37855–37862
12. Noji, T., Yamamoto, T., Saito, K., Fujimura-Kamada, K., Kondo, S., and Tanaka, K. (2006) *Biochem. Biophys. Res. Commun.* **344,** 323–331
13. Pérez-Victoria, F. J., Sánchez-Cañete, M. P., Castanys, S., and Gamarro, F. (2006) *J. Biol. Chem.* **281,** 23766–23775
14. Poulsen, L. R., López-Marqués, R. L., McDowell, S. C., Okkeri, J., Licht, D., Schulz, A., Pomorski, T., Harper, J. F., and Palmgren, M. G. (2008) *Plant Cell* **20,** 658–676
15. Saito, K., Fujimura-Kamada, K., Furuta, N., Kato, U., Umeda, M., and Tanaka, K. (2004) *Mol. Biol. Cell* **15,** 3418–3432
16. Radji, M., Kim, J. M., Togan, T., Yoshikawa, H., and Shirahige, K. (2001) *Yeast* **18,** 195–205
17. Furuta, N., Fujimura-Kamada, K., Saito, K., Yamamoto, T., and Tanaka, K. (2007) *Mol. Biol. Cell* **18,** 295–312
18. Katoh, Y., and Katoh, M. (2004) *Oncol. Rep.* **12,** 939–943
19. Osada, N., Hashimoto, K., Hirai, M., and Kusuda, J. (2007) *Gene* **392,** 151–156
20. Axelsen, K. B., and Palmgren, M. G. (1998) *J. Mol. Evol.* **46,** 84–101
21. Halleck, M. S., Pradhan, D., Blackman, C., Berkes, C., Williamson, P., and Schlegel, R. A. (1998) *Genome Res.* **8,** 354–361
22. Bull, L. N., van Eijk, M. J., Pawlikowska, L., DeYoung, J. A., Juijn, J. A., Liao, M., Klomp, L. W., Lomri, N., Berger, R., Scharschmidt, B. F., Knisely, A. S., Houwen, R. H., and Freimer, N. B. (1998) *Nat. Genet.* **18,** 219–224
23. Knisely, A. S., Agostini, R. M., Zitelli, B. J., Kocoshis, S. A., and Boyle, J. T. (1997) *Arch. Dis. Child.* **77,** 276–277
24. Lykavieris, P., van Mil, S., Cresteil, D., Fabre, M., Hadchouel, M., Klomp, L., Bernard, O., and Jacquemin, E. (2003) *J. Hepatol.* **39,** 447–452
25. Stapelbroek, J. M., Peters, T. A., van Beurden, D. H., Curfs, J. H., Joosten, A., Beynon, A. J., van Leeuwen, B. M., van der Velden, L. M., Bull, L., Oude Elferink, R. P., van Zanten, B. A., Klomp, L. W., and Houwen, R. H. (2009) *Proc. Natl. Acad. Sci. U.S.A.* **106,** 9709–9714
26. Paulusma, C. C., Folmer, D. E., Ho-Mok, K. S., de Waart, D. R., Hilarius, P. M., Verhoeven, A. J., and Oude Elferink, R. P. (2008) *Hepatology* **47,** 268–278
27. Halleck, M. S., Lawler, J. F., Jr., Blackshaw, S., Gao, L., Nagarajan, P., Hacker, C., Pyle, S., Newman, J. T., Nakanishi, Y., Ando, H., Weinstock, D., Williamson, P., and Schlegel, R. A. (1999) *Physiol. Genomics* **1,** 139–150
28. Tang, X., Halleck, M. S., Schlegel, R. A., and Williamson, P. (1996) *Science* **272,** 1495–1497
29. Gong, E. Y., Park, E., Lee, H. J., and Lee, K. (2009) *Reproduction* **137,** 345–351
30. Wang, L., Beserra, C., and Garbers, D. L. (2004) *Dev. Biol.* **267,** 203–215
31. van der Velden, L. M., Stapelbroek, J. M., Krieger, E., van den Berghe, P. V., Berger, R., Verhulst, P. M., Holthuis, J. C., Houwen, R. H., Klomp, L. W., and van de Graaf, S. F. (2010) *Hepatology* **51,** 286–296
32. Mizushima, S., and Nagata, S. (1990) *Nucleic Acids Res.* **18,** 5322
33. López-Marqués, R. L., Poulsen, L. R., Hanisch, S., Meffert, K., Buch-Pedersen, M. J., Jakobsen, M. K., Pomorski, T. G., and Palmgren, M. G. (2010) *Mol. Biol. Cell* **21,** 791–801
34. Chen, S., Wang, J., Muthusamy, B. P., Liu, K., Zare, S., Andersen, R. J., and Graham, T. R. (2006) *Traffic* **7,** 1503–1517
35. Geering, K. (2001) *J. Bioenerg. Biomembr.* **33,** 425–438
36. Ackermann, U., and Geering, K. (1990) *FEBS Lett.* **269,** 105–108
37. Xu, P., Okkeri, J., Hanisch, S., Hu, R. Y., Xu, Q., Pomorski, T. G., and Ding, X. Y. (2009) *J. Cell Sci.* **122,** 2866–2876
38. Verhulst, P. M., van der Velden, L. M., Oorschot, V., van Faassen, E. E., Klumperman, J., Houwen, R. H., Pomorski, T. G., Holthuis, J. C., and Klomp, L. W. (2010) *Hepatology* **51,** 2049–2060