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Specific mutations in mammalian P4-ATPase ATP8A2 catalytic subunit entail differential glycosylation of the accessory CDC50A subunit

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ABSTRACT

P4-ATPases, or flippases, translocate phospholipids between the two leaflets of eukaryotic biological membranes. They are essential to the physiologically crucial phospholipid asymmetry and involved in severe diseases, but their molecular structure and mechanism are still unresolved. Here, we show that in an extensive mutational alanine screening of the mammalian flippase ATP8A2 catalytic subunit, five mutations stand out by leading to reduced glycosylation of the accessory subunit CDC50A. These mutations may disturb the interaction between the subunits.

1. Introduction

P4-ATPases, or flippases, translocate phospholipids from cytoplasmic to exoplasmic membrane leaflets of eukaryotic biological membranes [1–3]. Flippases are essential for creating and maintaining the asymmetry between the two leaflets. Several of the 14 mammalian flippases have been causally linked to severe disorders [4–10], and human flippases as well as flippases of parasites are involved in resistance toward chemotherapy and anti-infectious drugs, respectively [11–15]. Hence, understanding the flippase structure and mechanism is of great interest.

The P4-ATPases belong to the family of P-type ATPases, which at the cost of ATP hydrolysis transport substrates across membranes during an enzymatic cycle that is well-studied for especially the P2-ATPases Na^+, K^+-ATPase and Ca^{2+}-ATPase [16–21]. P4-ATPases exhibit an enzymatic behavior similar to P2-ATPases, as they use ATP to undergo phosphorylation of a conserved aspartate residue and dephosphorylate upon substrate binding from the exoplasmic side [22–24]. However, the detailed molecular flippase mechanism has not been resolved.

The molecular structure of flippases is still unknown, but based on comparison of amino acid sequences, the P4-ATPases seem to exhibit a topology similar to the P2-ATPases, namely three distinct cytoplasmic domains and a membrane domain, which is composed of ten transmembrane helices [25,26]. Furthermore, these "α-subunits" of P4-ATPases in analogy with the αβ-complex of Na^+, K^+-ATPase form heterodimeric complexes with accessory subunits from the distinct CDC50 family of proteins, which consist of three members in mammals [23,27,28]. Based on the amino acid sequence, CDC50 proteins are generally predicted to be composed of two transmembrane helices, an exoplasmic domain, and short cytoplasmic terminals [26,29]. The exoplasmic domain contains several disulfide bridges and is heavily glycosylated [27,30,31]. For most P4-ATPases, interaction with CDC50 protein is required for exit from the endoplasmic reticulum and for chaperoning to their correct subcellular localization, and CDC50 proteins might

Abbreviations: PS, phosphatidylserine; MD, molecular dynamics; WT, wild type
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also play a role in the catalytic cycle [24,32–35]. Thus, they appear to functionally resemble the β-subunit of Na^+, K^+-ATPase, which likewise consists of a glycosylated exoplasmic domain, but linked to only one transmembrane span [36].

Structural homology models of P4-ATPase α-subunit have been presented, and various hypotheses on the detailed transport mechanism are emerging [37–39]. The most comprehensive model to date depicts the mammalian flippase ATP8A2, modeled in two conformations, E_2P and E_2, using Ca^{2+}-ATPase as the main template. In combination with functional data, this model strongly advocated a flippase mechanism where the phospholipid headgroup is transported through a membrane-spanning groove in the protein, being forwardly propelled by a "hydrophobic gate mechanism" [37]. However, in lack of appropriate templates, it is yet an enigma how CDC50 proteins bind to their P4-ATPase partners, and whether or not they participate directly in the binding and translocation of the phospholipids. We here present a novel observation on the influence of certain mutations in the ATP8A2 α-subunit on the glycosylation of the CDC50 β-subunit, which leads us to a proposal for the P4-ATPase–CDC50 interaction site.

2. Materials and methods

2.1. Expression and immuno-purification of bATP8A2-CDC50A

Bovine ATP8A2 fused to a C-terminal 1D4 tag was co-expressed with bovine CDC50A in HEK293T cells and immuno-purified using anti-1D4-coupled Sepharose beads as described previously [22,37]. The concentration of ATP8A2 was determined by comparison with a BSA standard dilution series using standard SDS gel electrophoresis and coomassie blue staining. Hence, protein mass refers to the amount of ATP8A2 (regardless of the mass of CDC50A) consistently through this paper.

2.2. PNGase treatment

Purified ATP8A2 in complex with CDC50A (60 ng protein) in Glycoprotein Denaturing Buffer (*New England Biolabs* (*NEB*)) was denatured at 95 °C for 5 min. The samples were chilled on ice, centrifuged for 10 s, and mixed with G7 Reaction Buffer (*NEB*) and 1% NP40 (*Sigma–Aldrich*). Finally, the samples were incubated with 250 units of PNGase F (*NEB*) at 37 °C for 30 min and lastly chilled on ice.

2.3. Immunoblotting

For immunoblotting, 30–100 ng of purified ATP8A2 in complex with CDC50A (in some cases pre-treated with PNGase F) were separated by SDS gel electrophoresis on TGX gels (*Bio-Rad*) and transferred to PVDF membranes for 10 min using the Trans-Blot Turbo system (*Bio-Rad*) according to the manufacturer's protocol. Membranes were blocked with 0.5% milk in PBS for 15 min, incubated with Cdc50-9C9 antibody [28] diluted 50-fold in PBS for 45 min, washed 3 × 5 min with PBS-T (PBS containing 0.05% Tween 20), incubated with secondary horse radish peroxidase (HRP)-coupled Polyclonal Rabbit Anti-Mouse Antibody (P 0260, *Dako*) diluted 1:2500 in PBS-T containing 0.5% milk for 90 min., and washed 3 × 5 min with PBS-T prior to development with Clarity Western ECL Substrate (*Bio-Rad*) and data collection on an ImageQuant LAS 4000 Mini (*GE Healthcare Life Sciences*).

2.4. ATPase activity assay

Measurements of ATPase activity were carried out as described previously [3,37] at 37 °C for 15 min in the presence of a saturating concentration of the activating lipid 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (PS) mixed with Egg PC (*Avanti Polar Lipids*) in the presence of CHAPS detergent. The ATP hydrolysis was terminated by addition of 6% SDS, and the released amount of phosphate was measured colorimetrically. The activity was normalized to the expression level by calculation of the activity per mg of ATP8A2 protein ("specific activity").

2.5. Assay for lipid flipping activity

Lipid uptake in whole cells was measured using the NC-3000TM fluorescence imaging cell analyzer (*Chemometec*). HEK293T cells were transfected with WT or mutant ATP8A2 DNA, and ATP dependent cellular uptake of fluorescently NBD-labeled PS was studied 48 h after transfection. Cells were washed in PBS, detached from the dish in PBS containing 5 mM EDTA, transferred to tubes and washed twice with serum free media at 4 °C. To measure internalization of NBD-PS (1-oleoyl-2-{6-[(7-nitro-2–1,3-benzoxadiazol-4-yl)amino]hexanoyl}-sn-glycero-3-phosphoserine (ammonium salt), *Avanti Polar Lipids*) into the internal plasma membrane leaflet, 100 μL cell suspension was thermo-equilibrated at 15 °C for 5 min, and an equal amount of serum free medium containing 10 μM NBD-PS was added to give a final concentration of 5 μM NBD-PS, followed by incubation for 2 min at 15 °C. To remove NBD-PS from the outer leaflet of the cell membrane, the cells were incubated in the presence of serum free medium containing 2.5% BSA (Bovine Serum Albumin, fatty acids free, *Sigma–Aldrich*) for 30 min on ice. The cells were washed twice with PBS and stained with Hoechst 33342 (*Chemometec*). Internalization of the NBD probe was determined for 5000 living single cells. All cells were gated based on DNA stain, and this gate was transferred into a histogram of green fluorescence (NBD). Internalization of NBD-PS was calculated as the fraction of cells showing higher NBD fluorescence intensity than cells transfected with the inactive mutant E198Q [22].

2.6. Structural models

The modeling based on a multiple sequence alignment of bovine ATP8A2 and 2782 homologous P-type ATPases and refinement using molecular dynamics simulations has been previously described [37]. The models have been deposited in the Model Archive and can be downloaded at http://www.modelarchive.org/project/index/doi/ma-ax7dd.

3. Results

3.1. Certain mutations of ATP8A2 lead to reduced glycosylation of CDC50A

The present study encompasses 141 point mutations consisting of alanine replacement of single amino acid residues in the predicted membrane domain of the bovine flippase ATP8A2 and three additional L367 mutants (Table 1). Mutated or WT bovine ATP8A2 and WT CDC50A were co-expressed in HEK293T cells, and the complexes were co-immunopurified as described previously [3,22].

On the immunoblots, CDC50A in most cases appeared as a smear of ~55–75kDa (Fig. 1A), a pattern which has previously been shown to represent various degrees of glycosylation of this protein [27,33]. However, for 5 out of the 141 alanine scan mutant complexes studied, CDC50A presented as a single band of ~50kDa, namely for mutations E126A, I305A, L361A, L366A, and Y878A. Fig. 1A presents these five mutant proteins and selected control mutant proteins comparable to these in terms of side chain type and position. When treated with PNGase F, all mutants in this