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An Extracellular Loop of the Human Non-Gastric H,K-ATPase α-subunit is Involved in Apical Plasma Membrane Polarization

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Key Words
ATP1AL1 • Sorting signals • P-type-ATPase • Epithelial cell polarity • Proton pump • MDCK cell

Abstract
The human non-gastric H,K-ATPase, ATP1AL1, belongs to the gene family of P-type ATPases. Consistent with their physiological roles in ion transport, members of this group, including the Na,K-ATPase and the gastric and non-gastric H,K-ATPases, are differentially polarized to either the basolateral or apical plasma membrane in epithelial cells. However, their polarized distribution is highly complex and depends on specific sorting signals or motifs which are recognized by the subcellular targeting machinery. For the gastric H,K-ATPase it has been suggested that the 4th transmembrane spanning domain (TM4) and its flanking regions induce conformational sorting motifs which direct the ion pump exclusively to the epithelial apical membrane. Here, we show in transfected Madin-Darby canine kidney (MDCK) cells that the related non-gastric H,K-ATPase, ATP1AL1, does contain similar sorting motifs in close proximity to TM4. A short extracellular loop between TM3 and TM4 is critical for this pump's apical delivery. A single point mutation in the corresponding region redirects ATP1AL1 to the basolateral membrane. In conclusion, our work provides further evidence that the cellular distribution of P-type ATPases is determined by conformational sorting motifs.

Introduction

The structural and functional polarisation of epithelial cells is a prerequisite for their highly specialized vectorial solute and ion transport capabilities. The generation of this asymmetry is a dynamic process, being dependent on both extracellular and intracellular cues. It requires cell-cell and cell-matrix contacts, integrates different pathways, and requires the segregation and retention of proteins and lipids in either the apical or the basolateral plasma membrane domains. Special sorting signals and a sorting machinery with a high degree of reliability have to be established to guarantee the generation and maintenance of the necessary differences between the two plasma membrane domains. In light of their importance, therefore, interest in defining the sorting

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signals associated with many proteins has grown within the last several years. It has been shown that for a growing number of proteins dileucine and tyrosine based signals, mostly found at intracellular C-terminal tails, mediate basolateral distribution in various epithelial cell types [1, 2]. On the other hand, there is some evidence that one principle of apical transport is the association of apical proteins with glycosphingolipid enriched plasma membrane domains (GEM), via glycophosphatidyl inositol (GPI) anchors. In addition, N- and O-type glycosylation can serve as an apical sorting signal [3, 4]. However, there are other apical proteins, like the gastric H,K-ATPase alpha-subunit or CD3-ε for which there is no evidence of any association with GEM nor glycosylation influenced targeting [5, 6]. The gastric H,K-ATPase embeds relevant apical sorting information within its fourth transmembrane domain [6, 7]. Based upon a chimera expression approach employing the gastric H,K-ATPase and the Na,K-ATPase sequences it was deduced that a short stretch of amino acids of the H,K-ATPase catalytic subunit predicts its subcellular distribution. The responsible region is located in the 4th membrane spanning domain. However, it was shown that TM4 is sufficient but not necessary for apical targeting. Adjacent amino acid sequences proximal to TM4 located in the second extracellular loop and sequences distal to TM4 located in a large intracellular loop induce or expose conformational sorting motifs which mediate apical distribution of the ion pump. Interestingly, the functionally and structurally highly related non-gastric H,K-ATPases do not seem to contain the same TM4 localized sorting motif. Like the Na,K-ATPase and the gastric H,K-ATPase, the human non-gastric H,K-ATPase (ATP1AL1) belongs to the P-type ATPase gene family. It appears to be restricted primarily to the luminal plasma membranes of epithelial cells [8-10]. Although the 4th transmembrane domain of ATP1AL1 and its surrounding region is highly homologous to the corresponding Na,K-ATPase sequence (86 %), these two proteins are sorted to different surfaces. There is some evidence, however, that other P-type ATPases, including the Na,K-ATPase and the non-gastric H,K-ATPase (ATP1AL1), possess targeting signals that are based on conformationally induced motifs. For the Na,K-ATPase, the basolateral signal has not been clearly identified but appears to be encoded within the N-terminal region of the corresponding α-subunit (position 1-519) [11]. To identify the sorting motif of the non-gastric H,K-ATPase we focused on the human ATP1AL1 gene product. This alpha-subunit shares a homology of ~63% [12] with the alpha subunits of the Na^+,K^+- and gastric H,K-ATPases and has a molecular weight of 110 kDa. Like its relatives, ATP1AL1 is a heterodimeric protein composed of a non-glycosylated alpha-subunit (ATP1AL1) and a heavily glycosylated beta-subunit. The alpha-subunits, which span the plasma membrane 10 times, contain the ATP binding and ion translocation sites [13]. They interact with a beta-subunit, which spans the membrane once in a type II orientation [14]. The dimerization of alpha and beta is essential for posttranslational processing and transport of the newly synthesized ion pump from the endoplasmic reticulum (ER) to the plasma membrane [15].

Although the ATP1AL1 α-subunit was shown to functionally assemble with the Na,K-beta1 subunit in prostate cells [16], other studies using heterologues cell expression show that the ion pump preferentially assembles with the gastric H,K-ATPase beta subunit [10, 17]. Chimera studies suggest that any potential sorting information associated with either the gastric H,K-ATPase or the Na,K-ATPase β-subunit is dominated by α-subunit encoded signals [10, 18].

In our present work we propose that the non-gastric ATP1AL1 protein is similar to the gastric ion pump in employing α-subunit encoded sorting information to reach the plasma membrane. We took advantage of a chimera strategy to fuse Na,K-ATPase and ATP1AL1 domains in order to identify the apical signals. Our results, obtained with stably transfected MDCK cells, show that indeed the 6 amino acids, comprising the ectodomain close to TM4 of ATP1AL1, play a crucial role in ion pump distribution. Furthermore, a single point mutation within this area changes the exclusive apical distribution of ATP1AL1, supporting the view that ATP1AL1 embodies conformation-dependent information.

Materials and Methods

Construction of chimeras and point mutations

Chimeras were constructed between the rat Na,K-ATPase α_1- subunit (Acc.No. NM_012504) and the human nongastric H,K-ATPase α-subunit, ATP1AL1 (NM_001676) Corresponding cDNAs were subcloned into Bluescript for mutagenesis and pcDNA 3.1, pCB6 and pJB20 for generation of stable transfected cells. Chimeras I-III were created by introducing corresponding silent AccI, HpaI and AflII restriction-sites into the ATP1AL1 and Na,K-ATPase cDNAs (for details see Tab. 1) using the QuikChange™ XL Site-Directed Mutagenesis Kit (Stratagene). The introduced point mutations in Chimera I (Chimera I-E→K) and II (Chimera II-K→E), affected the first amino acid of the second extracellular loop of both ATPases. In Chimera II (ATP1AL1-Na,K construct) a lysine from ATP1AL1 was changed to a glutamic acid (Chimera II-K→E) and in chimera I

Table 1. Description of chimera I-III in detail. All chimeras are composed partly of ATP1AL1 and Na,K-ATPase-α1. The amino acid numbers of the different fragments and their fusion sites are shown.

Construct	Protein fragments and restriction sites (RS)				
	Na^+,K^+-ATPase	RS	ATP1AL1	RS	Na^+,K^+-ATPase
Chimera I	1-314	Acc I	315-346	Hpa I	347-1023
Chimera II			1-329	Acc I	330-1038
Chimera III	1-312	Afl II	313-1023		

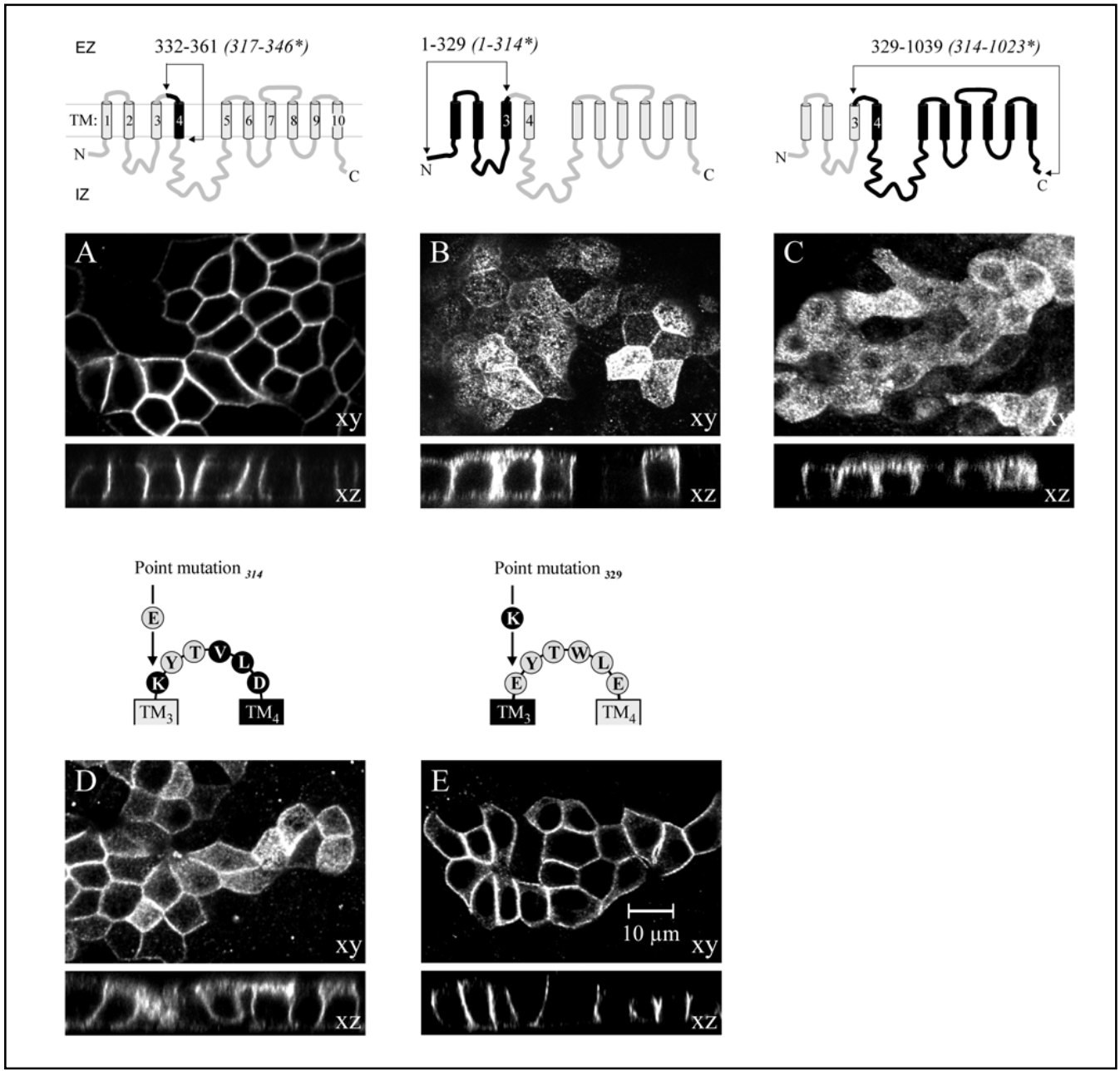

Fig. 1. Figure 1 shows confocal immunofluorescence images (en face and in xz cross-section) of stably transfected MDCK cells, which express the chimeras I (A), II (B), their point mutated forms I-E→K (D) and II-K→E (E) and Chimera III (C). Schematic diagrams (above each image) illustrate the chimeras' structures. Chimeras are composed of the parent proteins ATP1AL1 (black) and Na^+,K^+-ATPase-α_1 (grey). Because the 1st and 5th intracellular domains of ATP1AL1 and Na,K-ATPase-α1 differ in size, structurally matching protein parts have different aa-position numbers. For instance, amino acid sequence 1-329 of ATP1AL1 coincides with *1-314* of Na,K-ATPase-α1(*). Point mutations performed on the second extracellular loop (ECL2) of Chimera I and II are shown in detail. Chimera I-E→K (D), II (B) and III (C) appear to be located in both the apical and basolateral plasma membrane while Chimeras I (A) and II- II-K→E (E) are restricted to the basolateral plasma membrane.

(Na,K + TM4 of ATP1AL1 -construct) the original glutamic acid from the Na,K-ATPase was changed to a lysine (Chimera-I-E→K; Tab. 1, Fig.1). Finally, corresponding point mutations affecting the first amino acid of ECL2 were introduced into ATP1AL1 (ATP1AL1- K329E) and into Na,K-ATPase (Na,K-ATPase-E314K). To distinguish the transfected Chimera III and the Na,K-ATPase-E314K construct from the endogenously expressed Na,K-ATPase in MDCK cells, a hemagglutinine epitope (HA-tag, Roche) was fused to their respective N-termini. Similarly Chimeras I and Chimera-I-E→K contain a small peptide of the gastric H,K-ATPase at their N-terminus. This N-terminal tag was previously shown not to influence protein sorting and can be recognized by an anti-gastric-H,K-ATPase antibody [7]. All constructs were sequenced on both strands. Chimeras I and Chimera-I-E→K were subcloned into the mammalian expression vector pCB6 (Geneticin resistance). Chimeras II, II-K→E, III and Na,K-ATPase-E314K were subcloned into pcDNA3.1/Hygro$^+$ (Invitrogen). ATP1AL1 and ATP1AL1-K329E were subcloned into pJB20 (Geneticin resistance). Finally all plasmids were stably transfected in Madin Darby Kidney (MDCK) wild type cells (ATCC).

Tissue culture and transfection

MDCK wild type cells (ATCC #CCL34) were cultivated in minimal essential medium (MEM) containing 10 % foetal bovine serum (Sigma), 2mM L-glutamine, 50 U/ml penicillin and streptomycin (Life Technologies, Germany) at 37°C, 5 % CO_2 in a humified incubator. For immunofluorescence and biotinylation experiments $0.1x10^5$-$1x10^5$ cells were seeded onto 12mm or 24 mm polycarbonate (PC) filter inserts (Corning), respectively. All experiments were carried out with fully confluent cell layers after 3-5 days. Medium was changed every two days.

Transfection was performed with the liposomal reagent DOTAP (Roche). ATP1AL1, ATP1AL1-K329E and chimera III containing the C-terminal portion of ATP1AL1 were cotransfected together with the gastric H,K-β-subunit. Stably transfected cells were selected in the presence of 0.9 g/l geniticin (PAA Laboratories, Linz) and 6mg/l hygromycin (Invitrogen). For each transfection several independent clones derived by single cell dilution were raised.

Immunofluorescence

Transfected MDCK cells were grown on 12 mm polycarbonate (PC) transwell filter inserts for 5 days. 12 h before fixation the medium was changed and supplemented with Na-butyrate (10 mM final concentration). Cells were washed with PBS$^+$ (0.1 mM $CaCl_2$, 1mM $MgCl_2$, 4°C) and fixed with ice-cold methanol for 7 min. For the anti-HA-antibody cells were fixed with freshly prepared paraformaldehyde (PFA; 4%, 2 h at 4°C). Blocking (30min) and antibody dilution was performed with GSDB (16 % goat serum (Sigma), 0.3 % Triton X-100, 0.1 % bovine serum albumin (Sigma), 0.45 M NaCl, 20 mM NaPi, pH 7.4 [19]. Samples were incubated with anti-ATP1AL1 antibodies (1:500;[10]) or anti-gastric H,K α-subunit antibody (1:500) for recognition of Chimeras I and I-E→K (Laboratory of M. Caplan), and anti-HA antibody (1:100; Roche) for 3 h at room temperature (RT). After washing with PBS$^+$ samples were incubated with secondary anti-mouse fluorescein-labeled and anti-rabbit rhodamine red-labeled antibodies (1:100, Sigma) for 1 h at RT. Washed PC filters were mounted with Mowiol (Calbiochem, Germany). Confocal images were obtained with a Laser Scanning Fluorescence Microscope (Olympus, Fluoview). The images shown (Fig.1 and 2), taken in the XY or XZ directions, are representative (n=5) for each stably transfected cell line. All experiments were repeated with at least two different isolated cell clones.

Cell surface biotinylation and Western blotting

Cell surface biotinylation and Western blotting were performed as previously described [20]. In brief: Transfected MDCK cells were plated (1×10^5 cells) on 24 mm PC filter inserts (0.4 µm pores, Costar) and grown to confluency for 5 days. Expression of the transfected cDNAs was enhanced with Na-butyrate treatment (10 mM) for 12 h. Cells were cooled down on ice and washed two times with cold HEPES buffer (122.5 mM NaCl/ 5.4 mM KCl/ 0.8 mM $MgCl_2$/ 1.2 mM $CaCl_2$/ 1 mM NaH_2PO_4/ 5.5 mM glucose/ 10 mM HEPES, 4°C). Thereafter, cells were selectively biotinylated from the apical and basolateral side (30 min, pH 9.0, 4 °C) with NHS-SS-Biotin (Pierce, Rockford, IL) as previously described [21]. Protein concentration of whole cell lysates was determined as previously described [20] and similar protein amounts were used for streptavidin-agarose bead precipitation of the biotinylated fraction. Biotinylated proteins were separated from the beads (Sigma, Germany) by heating (10 min, 95 °C) in SDS sample buffer (80 mM dithiothreitol, 5.6 % SDS, 0.008 % bromophenol blue, 0.24 M Tris, pH 8.9, 16 % glycerol). The concentration of biotinylated proteins was measured as described [22]. Briefly, aliquots of biotinylated proteins in SDS sample buffer were spotted on nitrocellulose membranes, air-dried, and stained with amido-black solution (0.5 % amido black (wt/vol), 45 % (vol/vol) methanol, 45 % (vol/vol) H_2O, 10 % (vol/vol) acetic acid). Afterwards, the nitrocellulose membrane was incubated in destaining solution (47.5 % (vol/vol) methanol, 47.5 % (vol/vol) H_2O, 5 % (vol/vol) acetic acid), which removes unspecific amido black binding. The membrane was dissolved in solubilization buffer (80 % (vol/vol) formic acid, 10 % (vol/vol) acetic acid, 10 % (wt/vol) trichloroacetic acid), and the protein concentration was measured with a photometer. Similar amounts of biotinylated proteins were analysed by SDS-PAGE and Western blotting using the anti-ATP1AL1 antibody (1:1,000) and the anti-HA antibody (1:250). Detection was performed using goat anti-rabbit (1:1,000; Sigma) and anti-mouse (1:4,000; Roche) antibodies conjugated to Horseradish Peroxidase and developed with the Enhanced Chemiluminescence technique (Amersham Pharmacia Biotech).

Results

Cellular distribution of the H,K-ATPase / Na,K-ATPase chimeras and point mutants in stably transfected MDCK cells

It was previously shown that the human non-gastric H,K-ATPase, ATP1AL1, is functionally polarized to the

Table 2. Amino acid sequence of the 2nd extracellular loop (ECL2) of ATP1AL1, Na,K-ATPase and chimeric constructs, including their cellular localization according to the indirect immunofluorescence results (bl = basolateral, ap = apical). The assembled or cotransfected β-subunit is also shown.

Protein	ECL2						Sorting	beta-subunit
	1	2	3	4	5	6		
ATP1AL1	K	Y	Q	V	L	D	ap	gH,K
Na,K-ATPase	E	Y	T	W	L	E	bl	endogenous Na,K
ATP1AL1-K329E	E	Y	Q	V	L	D	ap/bl	gH,K
Na+,K+-ATPase-E314K	K	Y	T	W	L	E	ap/bl	endogenous Na,K
Chimera I	E	Y	T	V	L	D	bl	endogenous Na,K
Chimera I-E K	K	Y	T	V	L	D	ap/bl	endogenous Na,K
Chimera II	K	Y	T	W	L	E	ap/bl	endogenous Na,K
Chimera II-K E	E	Y	T	W	L	E	bl	endogenous Na,K
Chimera III	K	Y	Q	V	L	D	ap/bl	gH,K

Black (amino acid) boxes indicate ATP1AL1 amino acids, grey ones Na,K-ATPase amino acids. Cotransfection of the gH,K β-subunit was only necessary if the sequences of the chimeras distal from TM4 corresponded to the ATP1AL1 sequence which is not able to interact with the endogenously expressed Na,K β-subunit. In contrast, chimeras with Na,K-ATPase α-subunit sequences distal from TM4 can assemble with the endogenously expressed Na,K β-subunit. The respective coexpressed β-subunits do not contain dominant sorting motifs [6, 7, 10].

apical plasma membrane of stably transfected MDCK cells [10, 23]. Like the related gastric H,K-ATPase (gH,K) from stomach parietal cells, ATP1AL1 is targeted by an unknown mechanism to its physiological site of action. A detailed analysis to determine the apical sorting information of the gH,K-ATPase revealed that the fourth transmembrane domain (TM4) of the α-subunit is essential for apical targeting of the enzyme. Eight amino acids from TM4 were shown to be sufficient to guide the gastric ATPase and a respective Na,K-ATPase chimera to the apical membrane [7]. In contrast, the non-gastric ATP1AL1 α-subunit does not contain similar amino acids sequences in TM4 but shares a greater homology (86%) with the TM4 of the basolaterally targeted Na,K-ATPase over the 29 aa which form the putative TM4. Hence, it is likely that these related H, K-ATPases do not use the same amino acid motifs to generate the sorting information that they require to reach the apical plasma membrane. To test this hypothesis, we took advantage of the chimera strategy previously established to identify the gastric H,K-ATPase specific sorting signals. We constructed and stably expressed several chimeras between ATP1AL1 and the Na,K-ATPase in MDCK cells. Because the chimeric α-subunits need to assemble with a β-subunit for maturation and plasma membrane delivery [10, 14, 18, 20] either the gastric H,K-β-subunit had to be coexpressed or the endogenously expressed Na,K-ATPase β-subunit served as the assembly partner (Tab.2).

For chimera I we used as a backbone construct the tagged Na,K-ATPase-α_1 subunit and replaced the TM4 (aa 317-346) by TM4 of ATP1AL1 (aa 332-361). The confocal XY and XZ images of chimera I (Fig. 1A) showed that the protein only accumulated at the basolateral plasma membrane. Hence, chimera I proved that the signal that drives the apical sorting of ATP1AL1 is not embedded in TM4, in contrast to the related gastric enzyme [7]. However, sequence alignments between the α-subunits of ATP1AL1 (aa 1-329) and the Na,K-ATPase (aa 1-314) showed that their N-terminal regions are only 58 % identical and could play a role in apical targeting of ATP1AL1. In addition it was proposed that the Na,K-ATPase contains basolateral information in the N-terminal region (aa 1-519) of the α-subunit [11]. Accordingly a new construct, chimera II, containing these first N-terminal 329 amino acids of ATP1AL1 fused to the corresponding Na^+,K^+-ATPase C-terminal part (709 amino acids) was tested. Interestingly, chimera II could be found in both the apical and basolateral plasma membranes (Fig. 1B). Hence, the N-terminal region of ATP1AL1 was either able to redirect the Na,K-ATPase protein at least partially to the apical domain or the protein is sorted by default to both membranes because sorting signals were destroyed. As a consequence, the reversal of chimera II was constructed and stably expressed together with the gastric β-subunit. This chimera III was built from the first 313 amino acids derived from the Na^+,K^+-ATPase fused to the C-terminal portion of ATP1AL1 (711 amino acids). Chimera III showed a similar distribution like chimera II with both apical and basolateral staining (Fig. 1C). The surprising result that both conversely constructed chimeras were sorted in the same manner made us focus on the 2nd extracellular loop (ECL2) of both ATPases.

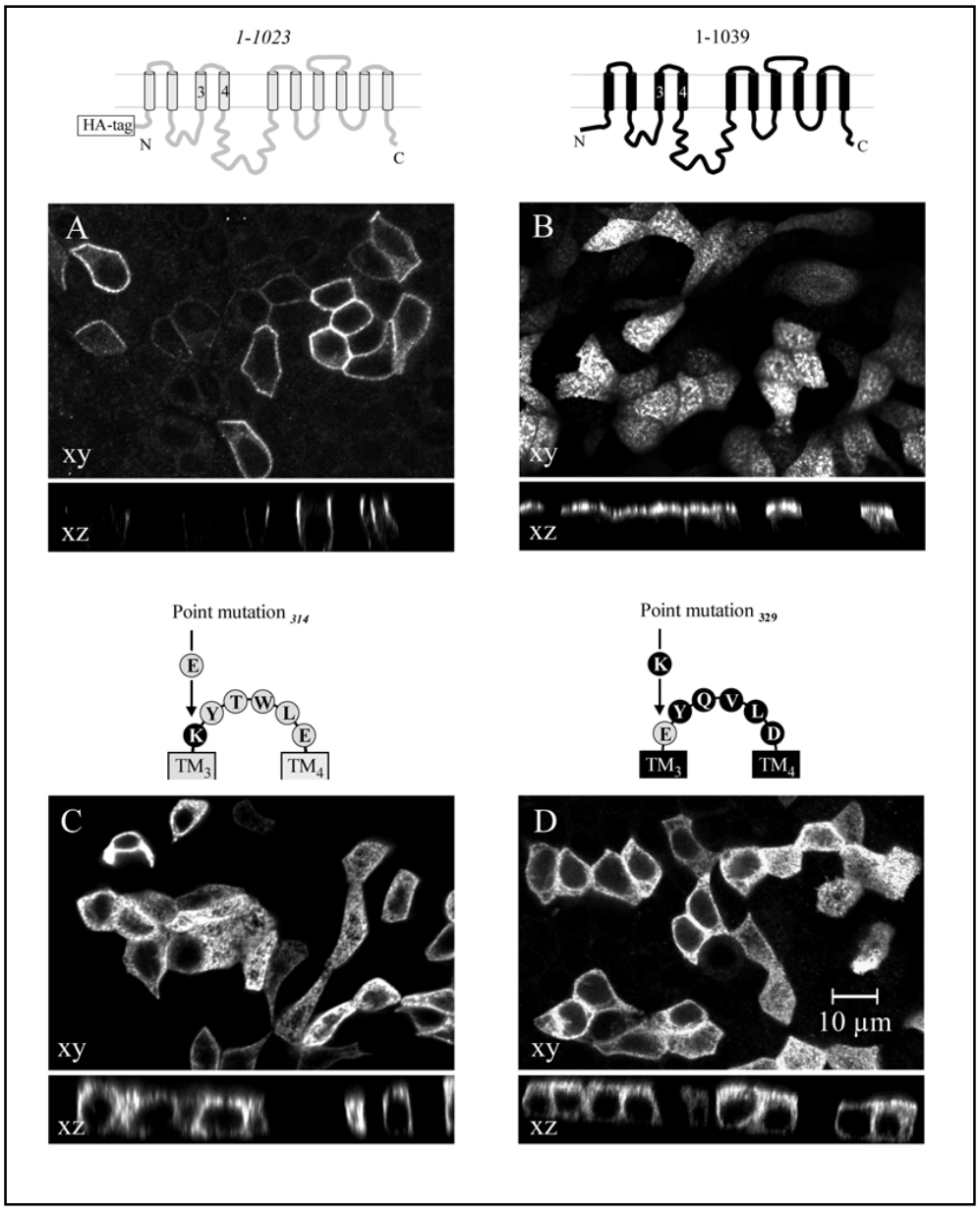

Fig. 2. Localizations of the Na,K-ATPase-α1 (A, grey) and ATP1AL1 (B, black) and their respective point mutated constructs (C, D). In order to distinguish the recombinant expressed Na,K-ATPase from its endogenous form, the sequence was tagged with a HA-epitope. The HA-tag does not influence the typical basolateral localization of Na,K-ATPase-α1 (A). The point mutations of the first amino acid in the second ectodomain (C, D) alter the distribution of Na,K-ATPase-α1 (A) and ATP1AL1 (B). Both Na,K-ATPase-E314K and ATP1AL1-K329E appear to be located in both the apical and basolateral plasma membrane domains.

The ECL2's of ATP1AL1 and NaK-ATPase show a striking similarity except with respect to the charge of the first amino acid 329, a positively charged lysine residue in ATP1AL1 and respectively 314, a negatively charged glutamic acid residue in the Na,K-ATPase (Tab. 2). According to hydropathy plots the two amino acids of both ATPases are the first putative residues of the 6 amino acid long ECL2. In chimera II and its reversal, chimera III, the lysine residue was not substituted by the corresponding NaK-ATPase glutamic acid and hence could perhaps play a role in the delivery of both chimeras to the apical membrane. To elucidate the importance of this lysine for plasma membrane delivery of the chimeras, point mutated versions of chimera I (basolateral) and II (apical and basolateral) were generated. In the first mutant, the glutamic acid (pos. 314) of the basolaterally sorted chimera I was substituted by a lysine (Chimera I-E→K). Indeed, this single point mutation changed the sorting characteristics of chimera I, since chimera I-E→K could be localized in the apical and basolateral plasma membranes (Fig. 1D). The second mutant was generated by replacing the lysine (pos. 329) of chimera II by a glutamic acid (Chimera II-K→E). In contrast to Chimera II, the mutant form II-K→E was now strictly basolaterally delivered (Fig.1E).

As a consequence, the importance of the positively charged lysine 329 in the ECL2 of ATP1AL1 was tested by its substitution with the negatively charged glutamic

Table 3. Amino acid sequence comparison between ECL2s of identified Na,K-ATPase and ATP1AL1 α-subunits. Note, that, with Bufo marinus as an exception, all of the non-gastric H,K-ATPase α subunits identified to date show a conserved lysine as a first residue in the predicted ECL2 domain. Also, the aspartate residue in position 6 of ECL2 is conserved. The Na,K-ATPase α1-subunits all show a negatively charged glutamic acid in position 6 of ECL2 and only three α1-subunits show a conserved negatively charged residue (glutamic acid) in position 1 of ECL2.

		TM3				ECL2						TM4			
						1	2	3	4	5	6				
Human, Na^+,K^+-ATPase-α1	309	S	L	I	L	**E**	Y	T	W	L	**E**	A	V	I	F
Human, Na^+K^+-ATPase-α2	307	S	L	I	L	**G**	Y	S	W	L	**E**	A	V	I	F
Human, Na^+,K^+-ATPase-α3	299	S	L	I	L	**G**	Y	T	W	L	**E**	A	V	I	F
Oryctolagus cuniculus , Na^+,K^+-ATPase-α1	309	S	L	I	L	**E**	Y	T	W	L	**E**	A	V	I	F
Rattus norvegicus , Na^+,K^+-ATPase-α1	309	S	L	I	L	**E**	Y	T	W	L	**E**	A	V	I	F
Bufo marinus , Na^+,K^+-ATPase-α1	309	S	L	I	L	**H**	Y	T	W	L	**E**	A	V	I	F
Xenopus laevis , Na^+,K^+-ATPase-α1	311	S	L	I	L	**Q**	Y	T	W	L	**E**	A	V	I	F
Consensus		S	L	I	L		Y		W	L	**E**	A	V	I	F
Human, ATP1AL1	325	A	V	S	L	**K**	Y	Q	V	L	**D**	S	I	I	F
Cavia porcellus , ng H^+,K^+-ATPase-α	319	A	V	S	L	**K**	Y	R	V	L	**D**	S	I	I	F
Oryctolagus cuniculus , ng H^+,K^+-ATPase-α	319	A	V	C	M	**K**	Y	H	V	L	**D**	A	I	I	F
Rattus norvegicus , ng H^+,K^+-ATPase-α	322	A	V	C	M	**K**	Y	Y	V	L	**D**	A	I	I	F
Mus musculus , ng H^+,K^+-ATPase-α	321	A	V	C	M	**K**	Y	Y	V	L	**D**	A	I	I	F
Bufo marinus , ng H^+,K^+-ATPase-α	328	A	I	C	M	**G**	Y	S	A	L	**N**	S	I	I	F
Consensus		A					Y			L			I	I	F

acid. The cellular distribution of the stably transfected ATP1AL1-point mutant (ATP1AL1-K329E) was compared with the polarization of the original wildtype ATP1AL1 in MDCK cells. Both cell clones are cotransfected with the gastric β-subunit in order to guarantee plasma membrane delivery as previously described and to rule out potential β-subunit specific effects [10]. The confocal analysis in Fig. 2B and D show that the single amino acid exchange of lysine 329 to glutamic acid (Fig. 2D) redirects the formerly strictly apically localized ATP1AL1 (Fig. 2B) to intracellular compartments as well as to what appears to represent both the apical and the basolateral plasma membranes. The point mutated ATP1AL1 protein was always detected in both membranes by means of confocal analysis, independent of expression level, which varied from clone to clone. A similar result was found when we expressed a point mutated Na,K ATPase. The glutamic acid 314 of the α-subunit was exchanged by a lysine residue (Na,K-ATPase-E314K). The formerly strictly basolaterally localized Na,K-ATPase (Fig. 2A) also showed intracellular and apparently apical plasma membrane staining in stably transfected MDCK cells (Fig. 2C). Hence, the confocal analysis indicates a key role of the lysine amino acid in ECL2.

To prove the plasma membrane incorporation of the point mutated ATP1AL1 and Na,K-ATPase α-subunits, selective surface biotinylations and Western blotting experiments were performed (Fig. 3). In contrast to the impression derived from the confocal analysis of ATP1AL1-K329E (Fig. 2C), the protein could not be detected in the apical biotinylated protein fraction. ATP1AL1-K329E could only be detected in basolaterally biotinylated plasma membranes. Although the expression level of the ATP1AL1 and ATP1AL1-K329E clones were different, the mutated α-subunit was dominantly found in the basolateral fraction whereas the wild type protein is restricted to the apical compartment (Fig. 2B). The respective protein concentrations were carefully controlled and two different cloned cell lines were examined, showing the same result with a prominent basolateral signal. The basolateral detection of the ATP1AL1-K329E mutant strongly supports the important role of this lysine residue. In contrast to the apparent apical membrane appearance of the Na,K-ATPase-E314K mutant, only a basolateral signal was detected by Western blotting . The biotinylated Na,K-mutant could not be found among the apical biotinylated proteins, but was restricted to the basolateral plasma membrane. Hence, the apical immunofluorescence signal for the Na,K-ATPase could be explained by a subapical but not membrane incorporated pool of α-subunit proteins.

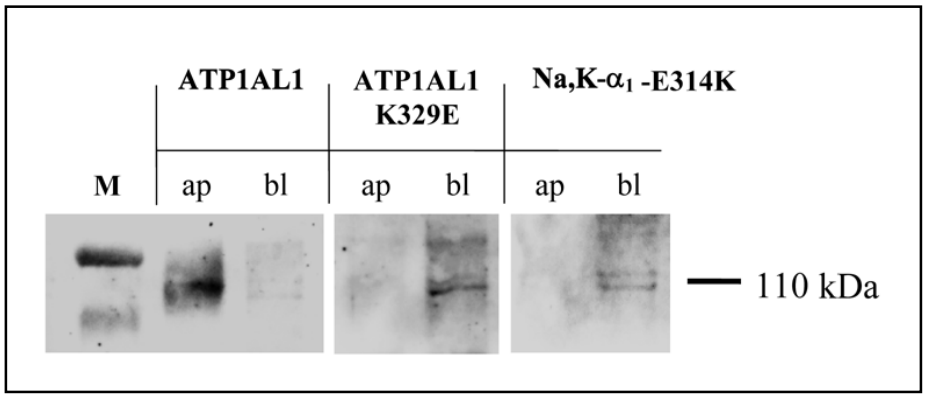

Fig. 3. Western blot analysis of surface biotinylated ATP1AL1, ATP1AL1-K329E and Na,K-α1- E314K transfected MDCK cells. Filter grown cells were selectively biotinylated from the apical or basolateral surface. Corresponding Western blots of streptavidin recovered proteins were probed with the ATP1AL1 α-subunit specific antibody. Na,K-α1-E314K, marked by a HA-tag, was probed with an anti HA-antibody. ATP1AL1 is predominantly located to the apical plasma membrane (ap), while ATP1AL1-K329E can only be detected in the basolateral (bl) compartment. Na,K-α1-E314K was also exclusively found in the basolateral protein fraction. M indicates the molecular weight marker lane.

Discussion

It has been previously shown that a chimera composed of the Na,K-ATPase-α1, equipped with TM4 of the gastric H,K-ATPase, was redirected to the apical plasma membrane. Though the region was sufficient for apical targeting it was not the only choice. The ectodomain 2 (6 amino acids), proximal to TM4, together with distal intracellular residues was also efficient in redirecting the Na,K-ATPase to the apical membrane in transfected LLC-PK1 cells. The authors concluded, therefore, that TM4 alone or its flanking sequences are able to create conformational sorting motifs for the gastric H,K-ATPase [6].

In contrast, the non gastric H,K-ATPase does not contain an apical sorting motif in TM4. The corresponding sequence is almost identical to that found in the basolaterally sorted Na,K-ATPase, and chimera I proved that TM4 of ATP1AL1 is not able to route the Na,K-ATPase to the apical membrane. It has also been noted that the high degree of sequence similarity between the TM4 (86 %) is the reason for both ion pumps' shared ability to transport Na^+ ions [15, 23, 24]. Expression of chimera II and its reversal, chimera III, indicated that the N-terminus proximal to TM4 could be involved in apical targeting. First, the related Na,K-ATPase was shown to express a dominant sorting signal in the N-terminal region [11] and second, both non-gastric H,K/Na,K- and Na,K/non-gastric H,K- chimeras were found in the apical and basolateral plasma membrane by means of indirect immunofluorescence (Fig. 1B, 1C).

However, the apical and basolateral distribution of both reverse chimeras could also be explained by the existence of hierarchically organized sorting signals. This was shown for the polymeric immunoglobulin receptor or the lactase-phlorizin hydrolase [25, 26], by a default localization due to the destruction of specific sorting signals [27, 28] or by the expression of nonsense chimera proteins.

Because the chimeric proteins must assemble with their respective β-subunits (Na,K-β-subunit for chimera II, gH,K-β-subunit for chimera III) in order to reach the plasma membrane, the delivery of incorrectly folded α/β proteins is unlikely. A closer analysis of chimera II and III revealed that both chimeras possess a positively charged lysine residue as the first amino acid of the second extracellular loop (ECL2), which is highly conserved among the class of the apical non-gastric H,K-ATPases (Tab. 3). In contrast, the basolateral Na,K-ATPase-α1 subunits possess a less highly conserved glutamic acid at the corresponding position. A point mutation of chimera II (II-L→E) converting the lysine to a glutamic acid redirected the protein exclusively to the basolateral membrane also arguing against a default protein sorting. The importance of the lysine residue for targeting of ATP1AL1 was proven by three additional constructs. First, the point mutant of chimera I, chimera I-E→K, which is built from the Na,K-ATPase but includes TM4 of ATP1AL1 (Fig. 1A) was redirected from only the basolateral membrane to both the basolateral and apical membranes after substitution of glutamic acid by lysine. Second, the apically and basolaterally located non-gastric H,K/Na,K chimera (chimera II) was delivered exclusively to the basolateral membrane after the lysine → glutamic acid substitution. Most importantly, the lysine 329 of the ATP1AL1 sequence was exchanged by a glutamic acid. The single point mutant ATP1AL1 was directed to the basolateral membrane, in marked contrast to the behaviour of the wild type parent protein. The Western blot results

provide clear evidence that the single point mutated non-gastric H,K-ATPase is predominantly delivered to the basolateral membrane.

Since the identification of apical proteins by surface biotinylation may be more difficult compared to the identification of basolateral proteins [21], we concluded that the apparent apical position of ATP1AL1-K329E seen with confocal imaging, probably represents a subapical localization rather than a steady state integration of ATP1AL1-K329E into the apical plasma membrane. It is conceivable that the mutated ion pump remains in a subapical (SAC) compartment [29] and either cannot enter the apical plasma membrane or cannot be stabilized there. Colocalization with respective SAC markers could help identifying the corresponding intracellular compartments.

A subapical accumulation of α-subunits could also explain the different results from indirect immunofluorescence and surface biotinylation with respect to the Na,K-ATPase point mutant. The single point mutation of Na,K-ATPase-α1 (E314K) directed the protein to an apparent apical and basolateral localization, as judged by confocal microscopy (Fig. 2C). However, its integration into the lipid bilayer could only be confirmed for the basolateral membrane. The results, presented here, suggest that the basolateral sorting of the Na,K-ATPase may be also influenced by glutamic acid 314, the first amino acid of its ECL2, since all chimeras which contain this glutamic acid residue are at least partially sorted to the basolateral plasma membrane. On the other hand, chimera I-E→K, II and III are also distributed to the basolateral membrane domain without having a glutamic acid in the first position of ECL2. Muth et al [11] showed that the Na,K-ATPase makes use of basolateral sorting information located in the first 519 amino acids. In combination with our results, there is evidence that the responsible signal could be located between position 314 and position 519.

Although our results clearly demonstrate that the subcellular distribution of ATP1AL1 strongly relies on the presence of lysine 329 in the 2nd ectodomain, we do not know how the signal is created or communicates with the responsible sorting mechanism. We assume that lysine 329 is able to interact with other close amino acids. At physiological pH, the ectodomain shows two exposed and charged amino acids. Lysine 329 (pKa 10.4) is positively charged whereas the aspartic acid 334 (pKa 3.9) is negatively charged. Hence, it is possible that a salt bridge between both residues is formed. This salt bridge could induce a conformational sorting motif which is recognized by an apical sorting pathway. If lysine 329 is mutated, the protein could possibly also enter the basolateral pathway. Although salt bridges are known to be responsible for diverse conformational effects like receptor activation [30-33] or protein/protein interaction in the secretory pathway [34], their potential role in protein sorting is still unclear. Structural modelling of the ectodomain and the surrounding region could help to test this hypothesis.

Taken together our results demonstrate that the 2nd ectodomain of the catalytic ATP1AL1 subunit plays an important role in targeting of the ion pump in transfected MDCK cells. The expression of chimeric proteins and point mutants indicate that a charged lysine residue in the ectodomain contributes to the plasma membrane localization of ATP1AL1. Hence, sorting information for the non-gastric ATPase is also located close to the 4th transmembrane spanning domain, similar to the proposed conformational sorting motifs for the related gastric H,K-ATPases. Future studies involving mutations of the entire 2nd ectodomain and the surrounding region will help to elucidate the role of the polar residues in sorting of ATP1AL1 and the Na,K-ATPase.

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