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The roles of the Na,K-ATPase beta 1 subunit in pump sorting and epithelial integrity

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Abstract In epithelial MDCK cells, the Na,K-ATPase is co-localized with adherens junctions in all stages of monolayer formation starting from initiation of cell–cell contact. The Na,K-ATPase and adherens junction proteins stay partially co-localized even after internalization due to disruption of intercellular contacts by Ca^{2+} deprivation. Similar to adherens junction proteins, the Na,K-ATPase is resistant to extraction with non-ionic detergent, suggesting pump association with the cytoskeleton. In contrast, the heterodimer formed by expressed unglycosylated Na,K-ATPase β_1 subunit and the endogenous α_1 subunit is easily dissociated from the adherens junctions and cytoskeleton by detergent extraction. The MDCK cells in which half of the endogenous β_1 subunits in the lateral membrane are substituted by unglycosylated β_1 subunits display a slower rate of cell-to-cell contact formation and decreased ability to both spread over the surface and migrate. The lack of N-glycans in the Na,K-ATPase β_1 subunit results in an impairment of mature cell–cell junctions as detected by an increase in the paracellular permeability of the MDCK cell monolayers and by a decrease in resistance of adherens junction proteins to extraction by a non-ionic detergent. Therefore the N-glycans of the Na,K-ATPase β_1 subunit are important for retention of the pump at the sites of cell–cell contact. Moreover, they are important for the integrity and stability of cell–cell junctions in mature epithelia. In addition, N-glycans contribute to the formation of cell–cell contacts between surface-attached dispersed cells by mediating lamellipodia formation and stabilizing the newly formed adherens junctions.

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The Na,K-ATPase is co-localized with adherens junctions in MDCK cells

A glycosylated β subunit of the Na,K-ATPase is obligatory for normal pump maturation and trafficking of the enzyme. The three isoforms of the Na,K-ATPase β subunit (β_1, β_2 and β_3) differ in the number of sites of N-glycosylation (3, up to 8 and 2, respectively). N-glycans linked to the β subunit isoforms of the Na,K-ATPase are important for stable membrane integration of the α subunit, folding, stability, subunit assembly and enzymatic activity of the pump. The roles of individual N-glycans of the β subunit isoforms in the post-ER trafficking, membrane targeting and plasma membrane retention of the Na,K-ATPase are isoform-specific. The β_1 subunit is the major β subunit isoform in most epithelial cells. This review presents recent data on the roles of the N-glycans of the Na,K-ATPase β_1 subunit in the lateral membrane retention of the pump and intercellular adhesion in epithelia.

Epithelial cells of multicellular organisms function as barriers to maintain the distinct molecular composition of apical and basal compartments. Within the epithelial cell monolayer, individual cells are linked to each other to maintain the structural integrity and to retard or prevent the diffusion of solutes through the intercellular space. The junctional complex in epithelial cells consists of three components: tight junctions, adherens junctions and desmosomes (Farquhar and Palade 1963). Adherens junctions and desmosomes mechanically link adjacent cells, whereas tight junctions are responsible for intercellular sealing

(Tsukita et al. 2001; Perez-Moreno et al. 2003). Each junction consists of integral lateral membrane proteins, cytoplasmic anchoring proteins and cytoskeletal proteins. The interaction between the extracellular domains of the integral lateral membrane proteins of two adjacent cells results in an intercellular junction. The junction is stabilized by the intracellular linkage of integral lateral membrane proteins to the cytoskeleton via the anchoring proteins present in each cell. Both intercellular and intracellular linkages are crucial for the stability of the junction.

Polarization of the Na,K-ATPase starts at the very early stages of the cell monolayer development (Vagin et al. 2007). Even in a single cell, the pump is distributed in a highly polarized manner. A minor fraction of the pump is localized in the perinuclear region of the cytoplasm, while the major fraction is present in the basal membrane (Vagin et al. 2006). It is interesting that none of the pumps are detected in the rest of the plasma membrane facing free space. Upon the inception of a cell–cell junction between two cells, the pump is rapidly accumulated in the newly formed lateral membrane connecting adjacent cells. This accumulation occurs in the case of cell division (Fig. 1a) or in the case of new cell–cell contact formation (Fig. 1b–c). In both cases, the Na,K-ATPase is precisely co-localized with the adherens junction marker, β-catenin, in the newly formed lateral membrane (Vagin et al. 2006). Live imaging shows that accumulation of the pump in the sites of cell–cell contact happens literally at the moment of its formation (Fig. 1c).

In the mature MDCK cell monolayer, the Na,K-ATPase resides exclusively on the lateral membranes (Shoshani et al. 2005; Vagin et al. 2006). Only a minor fraction of the pump is present in the basal membrane. The enzyme is co-localized with β-catenin, a marker of adherens junctions, throughout the lateral membrane from the basal to the apical surface. It resides just below the tight junctions as detected by co-staining for occludin, a marker of tight junctions. Formation and stability of the E-cadherin-based adherens junctions are Ca^{2+}-dependent. Incubation of MDCK cells in a Ca^{2+}-free medium results in disruption of intercellular junctions, separation of the cells and internalization of the Na,K-ATPase and most junctional proteins. The internalized Na,K-ATPase is still partially co-localized with β-catenin, suggesting that the enzyme is retrieved from the membrane together with the adherens junction proteins (Vagin et al. 2006).

Therefore, the Na,K-ATPase co-localizes with the adherens junctions from the moment of their formation and is retrieved from the membrane together with the

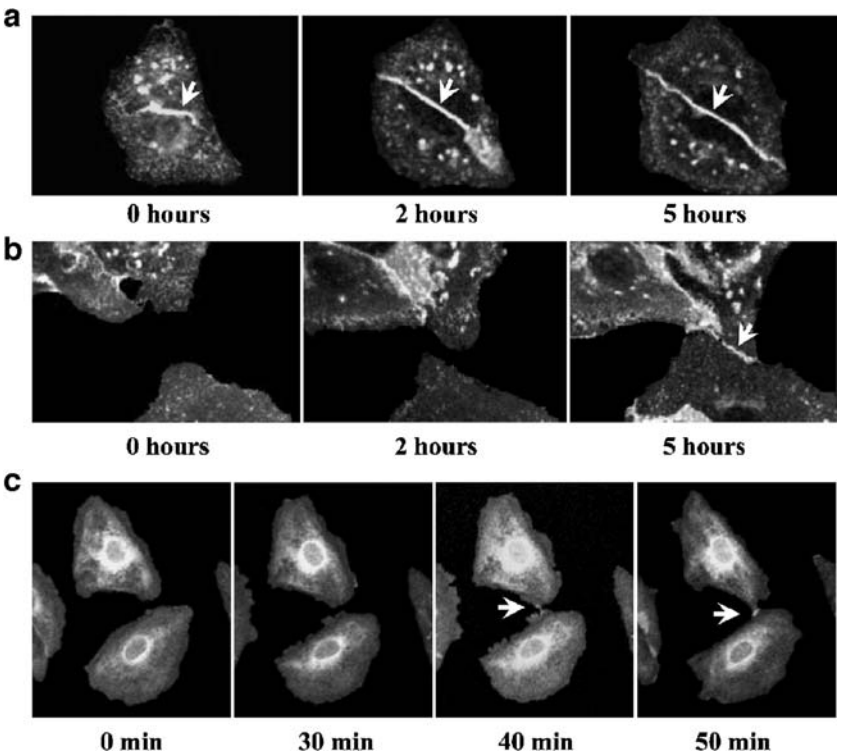

Fig. 1 Accumulation of YFP-linked β_1-subunit of the Na,K-ATPase (YFP-β_1) in the sites of adherens junctions during MDCK colony formation occurs simultaneously with the cell contact formation. MDCK cells were plated sparsely on the glass-bottom dishes. Formation of new lateral membranes was followed the next day by taking confocal microscopy images of live cells at indicated time points. Accumulation of YFP-β_1 (white arrows) in the newly formed lateral membrane is seen either after cell division (**a**) or as a result of formation of a new cell contacts (**b** and **c**). The figure is reproduced from (Vagin et al. 2006)

adherens junction proteins upon disruption of the cell contacts.

The N-glycans of the Na,K-ATPase β_1 subunit are important for retention of the pump at the sites of cell–cell contact and for stability of the adherens junctions

As for the proteins of the adherens and tight junctions, the Na,K-ATPase is resistant to extraction by a non-ionic detergent (Vagin et al. 2006). The treatment of cells with Triton X-100 is a simple test for protein association with the cytoskeleton: most soluble and membrane proteins are removed from the cells by this treatment, while the proteins associated with the cytoskeleton are preserved. The resistance of the pump to the detergent treatment is consistent with the ability of the pump to bind the membrane cytoskeletal protein spectrin via an anchoring protein, ankyrin, as detected in various cells (Nelson and Veshnock 1986; Nelson et al. 1990). However, in MDCK cells, spectrin is detected not only in the lateral membranes but also in the cytoplasm as a mesh-like structure (Vagin et al. 2006). In contrast, the Na,K-ATPase is found exclusively in the lateral membrane. Therefore, there must be other cell components, in addition to spectrin and ankyrin, that restrict the pump location at the sites of cell–cell contact. It is possible that the Na,K-ATPase associates with the components of the adherens junctions. However, immunoprecipitation studies have not detected a direct interaction between the Na,K-ATPase α_1 subunit and E-cadherin (Nelson et al. 1990). Alternatively, the pump could be stabilized at the sites of the cell–cell contact due to the interaction between Na,K-ATPase molecules residing in the lateral membranes of adjacent cells. Recent data suggest that such an interaction could be mediated by the Na,K-ATPase β_1 subunits (Shoshani et al. 2005). MDCK cells co-cultured with non-polarized CHO cells expressed the β_1 subunit only on the borders between two MDCK cells, but not on the borders between MDCK and CHO cells that do not contain a β_1 subunit (Shoshani et al. 2005). However, the β_1 subunit was detected on the borders between MDCK and CHO cells when CHO cells were transfected with the β_1 subunit.

How do the β_1 subunits of neighboring cells interact with each other? It is known that N-glycans are important for various types of intercellular interactions (Akama et al. 2002; Crean et al. 2004; Otto et al. 2004; Rosen 2004; Comelli et al. 2006; van Die and Cummings 2006). To test whether the N-glycans of the β_1 subunit are important for intercellular interaction between the β_1 subunits of neighboring cells and retention of the pump at the sites of cell–cell junctions, the mutant lacking all three N-glycosylation sites in the Na,K-ATPase β_1 subunit was expressed in MDCK cells in order.

Quantification of the endogenous Na,K-ATPase subunit expression levels on the plasma membranes of non-transfected cells and cells expressing the unglycosylated mutant showed that the amounts of the α_1 subunits in the lateral membranes are similar in these two cell lines. However, the level of the endogenous β_1 subunit is decreased by half in the transfected cells (Vagin et al. 2006). Since the α_1 subunit can not be present in the membrane alone, the other half must be assembled with the unglycosylated mutant. It has been shown that N-glycans are not required for the Na,K-ATPase activity. Therefore, the mutant cell line has the normal number of active pumps on the membrane but half of them lack N-glycans.

In contrast to the wild type Na,K-ATPase β_1 subunit, the unglycosylated mutant was detected in both the plasma membrane and intracellular vesicles (Vagin et al. 2006). Expression of the mutant resulted in partial re-distribution of the endogenous α_1 subunit from the plasma membrane to the intracellular vesicles. Co-staining for the markers of intracellular vesicular compartments showed that the mutant-containing vesicles are early endosomes (Vagin et al. 2006). Therefore, the removal of glycosylation sites from the β_1 subunit does not impair the plasma membrane delivery of the pump but increases its susceptibility to endocytosis. This suggests that N-glycans of the β_1 subunits are important for lateral membrane retention of the pump.

The unglycosylated mutant of the Na,K-ATPase β_1 subunit is also partially removed from the basolateral membrane after the treatment of the cells with Triton X-100 (Fig. 2). Moreover, the mutant removes a fraction of the endogenous α_1 subunit from the membrane. Furthermore, the adherens junction proteins, E-cadherin and β-catenin, also become less resistant to the detergent treatment in the mutant-expressing cell line (Fig. 2). Therefore, N-glycans of the Na,K-ATPase β_1 subunit are important for stability of the complex that is comprised of the Na,K-ATPase, E-cadherin, catenins and the cytoskeleton (Fig. 3).

It is known that the cytoplasmic linkage of E-cadherin to the cytoskeleton is not sufficient to ensure stability of the adherens junctional complex. The Ca^{2+}-dependent interaction between the extracellular domains of the two E-cadherin molecules of two neighboring cells is another necessary linkage. Similar to E-cadherin, the Na,K-ATPase is stabilized in the lateral membrane by attachment at both cytoplasmic and extracellular sites (Fig. 3) and not just the cytoplasmic site as previously postulated. A cytoplasmic region of the α_1 subunit is linked to spectrin via ankyrin, while the β_1 subunit stabilizes the pump at the sites of cell contact due to glycosylation-dependent interaction with the β_1 subunit of neighboring cells and/or with the extracellular

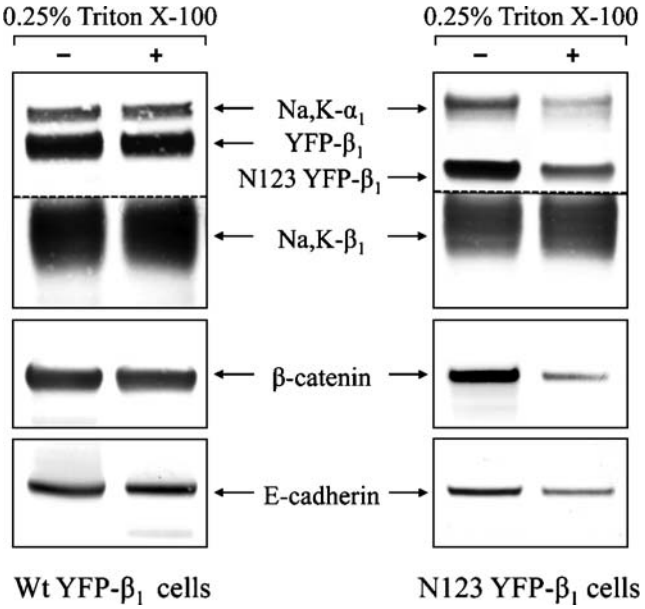

Fig. 2 Prevention of N-glycosylation of the Na,K-ATPase β_1 subunit increases the detergent solubility of the pump and adherens junction proteins. The tight monolayers of MDCK cells grown on transwell inserts were biotinylated from the basolateral surface, incubated with or without 0.25% Triton X-100 in PBS for 15 min, and washed twice with PBS. Then cellular proteins were solubilized using the lysis buffer containing 1% Triton X-100. Biotinylated proteins were isolated using streptavidin–agarose beads and analyzed by a Western blot analysis using specific antibodies against the Na,K-ATPase subunits and the proteins of the adherens junctions. The endogenous and expressed Na,K-ATPase subunits were analyzed using the same blot that was cut in half as shown. The upper part of the blot was probed with the antibodies against the α_1 subunit and against YFP, while the lower part–with the antibody against the β_1 subunit. In the control MDCK cell monolayers expressing the YFP-linked Na,K-ATPase β_1 subunit (YFP-β_1), the basolateral YFP-β_1, the Na,K-ATPase α_1 and β_1 subunits, E-cadherin and β-catenin were resistant to the treatment of cells with 0.25% Triton X-100 (*left panel*). In contrast, in the cell line expressing the YFP-linked unglycosylated mutant of the Na,K-ATPase β_1 subunit (N123-YFP-β_1), the amounts of the basolateral N123-YFP-β_1, the Na,K-ATPase α_1 subunit, E-cadherin and β-catenin were reduced after the detergent treatment, indicating these proteins are more susceptible to detergent extraction in the absence of N-glycans on the Na,K-ATPase β_1 subunit. Consistently, the amount of the endogenous normally glycosylated Na,K-ATPase β_1 subunit did not change after the detergent treatment in this mutant-expressing cell line (*right panel*). Na,K-α_1-the Na,K-ATPase α_1 subunit; Na,K-β_1-the Na,K-ATPase β_1 subunit

domain of E-cadherin directly or via a putative multivalent lectin that links two proteins by binding to their N-glycans (Fig. 3). Association of the Na,K-ATPase and adherens junctions is probably maintained by the linkage between spectrin and F-actin as shown on the model (Fig. 3). The recent finding that E-cadherin can directly bind ankyrin/spectrin (Kizhatil et al. 2007) suggests that the Na,K-ATPase and E-cadherin can be linked to each other via the spectrin cytoskeleton.

The N-glycan-mediated interaction between the β_1 subunits of the neighboring cells can explain why highly polarized distribution of the Na,K-ATPase and accumulation at the sites of cell contact is observed with inception of cell-to-cell contact and also why the Na,K-ATPase $\alpha_1\beta_1$ complex localizes predominantly on the lateral but not on the basal membranes in mature MDCK cell monolayers. The intracellular linkage between the Na,K-ATPase and adherens junction proteins through spectrin can explain why the pump and β-catenin internalized after cell detachment co-localize. Consistent with the dependence of the stability of adherens junctions on the presence of N-glycans on the Na,K-ATPase β_1 subunit, over-expression of the Na,K-ATPase β_1 subunit in MSV-MDCK cells increased the detergent-resistance of E-cadherin (Rajasekaran et al. 2001).

The N-glycans of the Na,K-ATPase β_1 subunit are important for the formation of initial cell–cell junctions

Association of the Na,K-ATPase with the adherens junctions might suggest that the pump contributes to the intercellular adhesion in epithelia. In support of this assumption, the antibody that binds to the extracellular domain of the Na,K-ATPase β_1 subunit significantly decreases the rate of cell–cell contact formation between collagen-attached dispersed MDCK cells. The degree of inhibition is similar to that observed in the presence of the anti-E-cadherin antibody (Vagin et al. 2006). Furthermore, the cell line expressing the unglycosylated mutant of the β_1 subunit displays significantly slower progression of cell-to-cell adhesion than non-transfected cells and cells transfected with wild type β_1 subunit. Therefore, the N-glycans of the β_1 subunit are important for the formation of the initial cell–cell junctions.

Instability of the newly formed adherens junctions is probably one of the reasons for the lower rate of the cell–cell contact formation in the mutant-expressing cells. However, the slower formation of intercellular contacts might also depend on the capacity of cells to adhere to the surface and to spread over the surface. The rate of the cell-matrix adhesion is not changed in the mutant-expressing cells. However, the rate of cell spreading over the surface is decreased by 30%. Consistently, the rate of the directional migration of the cell monolayer is reduced in the mutant-expressing cell line compared to that of the control cells by 31% as quantified using the "wound healing" assay. It is known that the ability of epithelial cells to spread over the surface and migrate depend on the formation of cell protrusions, usually large broad lamellipodia. Protrusions are driven by actin polymerization and are stabilized by adhesion to the extracellular matrix. These adhesions are formed at the front edge of the cell and are disassembled at the cell rear, allowing directional cell migration (Ridley et al. 2003). Thus, the decreased rate in cell spreading or

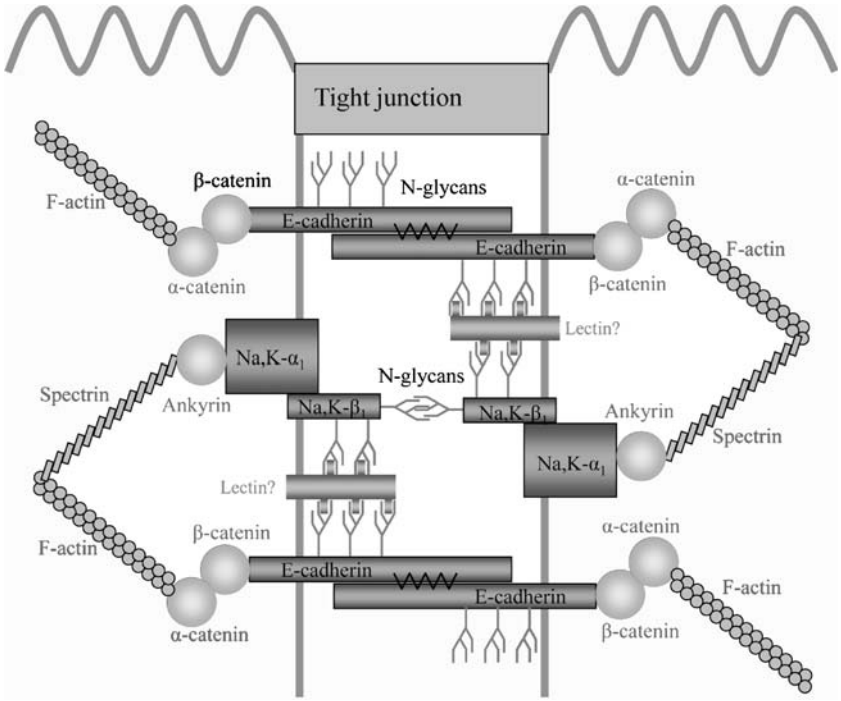

Fig. 3 A model showing contribution of N-glycans of the Na,K-ATPase β_1 subunit to retention of the pump at the sites of cell–cell contact and to stabilization of adherens junctions. Na,K-α_1-the Na,K-ATPase α_1 subunit; Na,K-β_1-the Na,K-ATPase β_1 subunit. The figure is reproduced from (Vagin et al. 2006)

directional migration must be related to the impaired ability of the mutant cells to form protrusions. Therefore, N-glycans of the Na,K-ATPase β_1 subunit are important for the lamellipodia formation. In agreement with these results, an increase in the ability to form lamellipodia was demonstrated as a result of over-expression of the Na,K-ATPase β_1 subunit in MSV-MDCK cells (Barwe et al. 2006).

The N-glycans of the Na,K-ATPase β_1 subunit are important for the integrity of the mature cell–cell junctions

To test whether the lack of N-glycans affects the integrity of the mature epithelia, the paracellular permeability of the cell monolayers for the membrane-impermeable dye, BCECF free acid was measured. The paracellular permeability of the cell monolayers formed by the mutant cells is higher by 26% than in non-transfected cells, indicating that N-glycans of the Na,K-ATPase β_1 subunit are important for the integrity of the tight junctions. This result is unexpected, since the Na,K-ATPase is located below the tight junctions (Vagin et al. 2006). The lack of N-glycans probably affects the tight junctions through the destabilization of the adherens junctions. The assembly and maintenance of the tight junctions are dependent on the formation and maintenance of the adherens junctions (Miyoshi and Takai 2005). Disruption or weakening of the adherens junctions by loss-of-function mutations in E-cadherin and catenins or by chelating Ca^{2+} from the culture medium causes partial or complete disassembly of the tight junctions. Therefore, the integrity of the tight junctions depends on the intactness and stability of the adherens junctions. Even though the adherens junctions and the tight junctions are clearly different intercellular structures, their components interact with each other. The integrity of the tight junctions is maintained and regulated by a variety of signaling pathways that are initiated by the cytoplasmic components of the adherens junctions such as β-catenin and afadin, and the tight junctions such as ZO-1 and PAR3. Therefore, these cytoplasmic partners of the cell adhesion molecules have dual functions. They mechanically link the adherens and tight junctions to the cytoskeleton, and also trigger signaling events that involve many signal transducers, including G-proteins (Rho, Rac, Cdc42, Ras and ARF6) and protein kinases (PKC, PKA and c-Src) (Miyoshi and Takai 2005).

This is consistent with the conclusion that the lack of N-glycans on the Na,K-ATPase β_1 subunit increases the paracellular permeability of the monolayers formed by the unglycosylated mutant-expressing cells through destabilization of the adherens junctions.

Conclusions

The N-glycans of the Na,K-ATPase β_1 subunit are important for retention of the pump at the sites of cell–cell contact. Moreover, normal glycosylation of the Na,K-ATPase β_1 subunit is required for stability of the adherens

junctions and for the integrity of the tight junctions in mature epithelia. N-glycans of the β_1 subunit are also important for the formation of the initial cell–cell junctions between surface-attached dispersed cells by mediating lamellipodia formation, which is necessary for cell spreading over the surface prior to the formation of cell–cell contacts, and by stabilizing the newly formed adherens junctions.

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