The Polarized Distribution of Na\(^+\),K\(^+\)-ATPase: Role of the Interaction between β Subunits

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The very existence of higher metazoans depends on the vectorial transport of substances across epithelia. A crucial element of this transport is the membrane enzyme Na\(^+\),K\(^+\)-ATPase. Not only is this enzyme distributed in a polarized manner in a restricted domain of the plasma membrane but also it creates the ionic gradients that drive the net movement of glucose, amino acids, and ions across the entire epithelium. In a previous work, we have shown that Na\(^+\),K\(^+\)-ATPase polarity depends on interactions between the β subunits of Na\(^+\),K\(^+\)-ATPases located on neighboring cells and that these interactions anchor the entire enzyme at the borders of the intercellular space. In the present study, we used fluorescence resonance energy transfer and coprecipitation methods to demonstrate that these β subunits have sufficient proximity and affinity to permit a direct interaction, without requiring any additional extracellular molecules to span the distance.

INTRODUCTION

Most epithelia, including cells cultured as monolayers that are used as model systems (Cereijido et al., 1978), express Na\(^+\),K\(^+\)-ATPase in a polarized manner toward the basolateral side of the cell (Cereijido et al., 1981) and more specifically at cell borders facing the intercellular space (Contreras et al., 1995; Cereijido et al., 2001; Shoshani et al., 2005). This position is so resilient that when cells are harvested with trypsin/EDTA, although Na\(^+\),K\(^+\)-ATPase is first randomized and redistributed over the whole plasma membrane, it recovers its polarity in a few hours upon replating at confluence (Cereijido et al., 1980). During this recovery, even pumps that were trapped on the apical (incorrect) surface because of the quick formation of the tight junction (TJ) are removed and relocated to the correct side (Contreras et al., 1989). The generation of cell surface polarity of most membrane proteins involves sorting signals encoded in their amino acid sequence (Matter and Mellman, 1994; Matter, 2000; Rodriguez-Boulan et al., 2005; Weisz and Rodriguez-Boulan, 2009), trafficking routes that include apical or basolateral recycling endosomes (Gonzalez and Rodriguez-Boulan, 2009), and interactions with epithelial-specific protein complexes such as AP-1B and clathrin, which may be regulated by small GTPases (Ellis et al., 2006; Gravotta et al., 2007; Tanos and Rodriguez-Boulan, 2008; Deborde et al., 2008; Mellman et al., 2008; Gonzalez and Rodriguez-Boulan, 2009).

However, previous efforts to identify an amino acid sequence that functions as a basolateral polarity signal in Na\(^+\),K\(^+\)-ATPase have failed (Muth et al., 1998; Dunbar and Caplan, 2000). Moreover, the basolateral localization of the pump is independent of AP-1B because the pump localizes to the basolateral surface in the μ1B-deficient cell line LLC-PK1 (Duffield et al., 2004) and in Madin-Darby canine kidney (MDCK) cells in which μ1B expression has been suppressed via RNA interference (Gravotta et al., 2007). In epithelial cells, newly synthesized Na\(^+\),K\(^+\)-ATPase is sent directly to the basolateral membrane (Caplan et al., 1986; Contreras et al., 1989; Gottardi and Caplan, 1993a; Mays et al., 1995, Zurzolo and Rodriguez-Boulan, 1993; Gottardi and Caplan 1993b; Mays et al., 1995, Zurzolo and Rodriguez-Boulan, 1993). A recent study that used a SNAP tag system (New England Biolabs, Ipswich, MA) to determine the trafficking itinerary of the newly synthesized Na\(^+\) pump revealed that basolateral delivery of the Na\(^+\),K\(^+\)-ATPase does not involve passage through recycling endosomes. Furthermore, because it is an AP-1B–independent cargo, it follows a different pathway en route to the basolateral domain of the epithelial plasma membrane, involving a distinct (and as yet unidentified) post-Golgi transport intermediate of the cellular sorting machinery (Farr et al., 2009). Once at the target domain, the asymmetric distribution of membrane proteins is reinforced by selective retention (Cereijido et al., 2003). Thus, most cell–cell adhesion proteins anchor to components of the cytoskeleton. Nevertheless, it has been shown that retention can also be achieved by cis (in the same plasma membrane) and/or trans (between adjacent cells) homotypic or heterotypic molecular interactions (Yoshida and Takeichi, 1982; Gallin et al., 1983; Nose et al., 1988; Contreras et al., 1995; Nagar et al., 1996; Nusrat et al., 2000, 2005; Arrate et al., 2001; Kostrewa et al., 2001; Niessen and Gumbiner 2002; Momose et al., 2002; Chen et al., 2005; Blasig et al., 2006; Patel et al., 2006). Na\(^+\),K\(^+\)-ATPase has been shown to be retained at the basolateral membrane domain through binding to the ankyrin-fodrin cytoskeleton (Hammer et al., 1991). Independent of the mechanisms that contribute to Na\(^+\),K\(^+\)-ATPase targeting and based on the identification of the β2

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isoform as an adhesion molecule on glia (AMOG; Gloor et al., 1990), we hypothesized that the β subunit is involved in Na⁺,K⁺-ATPase polarization (Contreras et al., 1995). Several lines of evidence suggest that the β subunit anchors the pump at the lateral borders of epithelial cells by a homotypic β–β interaction at the intercellular space: A given cell only expresses Na⁺,K⁺-ATPase at the cell border provided the neighboring cell expresses its own Na⁺,K⁺-ATPase at the contacting border (Contreras et al., 1995). MDCK cells (from dog kidney) cocultured with Chinese hamster ovary (CHO) cells only express Na⁺,K⁺-ATPase at contacting homotypic MDCK/MDCK borders, but not at heterotypic MDCK/CHO borders. However, if CHO cells are transfected beforehand with dog β subunits (CHO-β), then the dog Na⁺,K⁺-ATPase is expressed both at homo- and heterotypic contacts. This result is even more surprising considering that the Na⁺,K⁺-ATPases of these CHO-β cells contain the transfectomized dog α subunit and a hamster α subunit, which is dragged along to its correct position in the plasma membrane (Shoshani et al., 2005). However, it is presently unknown whether β subunits of neighboring cells are sufficiently close to allow for direct β–β interaction across the intercellular space, or if this is instead established through intermediate extracellular molecules. In the present work, we specifically addressed this question using in vitro and in vivo protein–protein interaction assays.

MATERIALS AND METHODS

Cell Culture

MDCK and normal rat kidney (NRK)-E52 cells were grown in DMEM containing 10% fetal calf serum (FCS), and CHO cells were cultured in a mixture of F-12/DMEM. Cells were harvested by trypsin-EDTA and plated on dishes with or without glass coverslips, or on glass-bottom dishes for live imaging (MatTek, Ashland, MA). Cells were transfected with plasmid DNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer’s protocol. After 5 d, cells were sorted by fluorescence-activated cell sorting by using a MoFlo Cell Sorter (Beckman Coulter, Fullerton, CA) for yellow- and red-β subunit and for a hamster α subunit, which is dragged along to its correct position in the plasma membrane (Shoshani et al., 2005). However, it is presently unknown whether β subunits of neighboring cells are sufficiently close to allow for direct β–β interaction across the intercellular space, or if this is instead established through intermediate extracellular molecules. In the present work, we specifically addressed this question using in vitro and in vivo protein–protein interaction assays.

Constitutes and Recombinant Proteins

The full-length cDNA of the canine Na⁺-K⁺-ATPase β subunit was N-terminally tagged with a His6 epitope by polymerase chain reaction (PCR) amplification using forward primer 5’-GGCGCGCGAGAATCTGCATATTATATCTTATGACTCTTATAGTTTTG–3’ and reverse primer 5’-GGCGGGCCGACGCGCTTACGCATATTATATCTTATGACTCTTATAGTTTTG–3’. PCR products were cloned into a pCR 2.1 TOPO vector (Invitrogen) and sequenced with a capillary-based electrophoresis sequence ABI Prism 310 (Genetic Analyzers, Applied Biosystems, Foster City, CA). Positive clones were digested with NotI, and the fragment was ligated to a similarly digested pHIS6neo vector (Clontech, Palo Alto, CA) to generate the expression vector pRlSNeo-his6β. The chimera DPP(1-96) was a gift from Dr. D. M. Fambrough and was generated as described previously (Hamrick et al., 1993) for the chicken β1 subunit. To generate the fusion proteins (β1-cyan fluorescent protein [CFP] and β1-yellow fluorescent protein [YFP]), the full cDNA of the rat kidney Na⁺,K⁺-ATPase β subunit was amplified with forward primer 5’-TGACCTGAGCAGAATCTGCATATTATATCTTATGACTCTTATAGTTTTG–3’ and reverse primer 5’-GAATCTGCATTATATATCTTATGACTCTTATAGTTTTG–3’. The PCR products were cloned into a plasmid vector (Stratagene, La Jolla, CA). The cDNA of the rat β1 subunit was digested with XhoI and Kpnl and inserted into mammalian expression vectors pAmCyan-N1 and pEYFP-N1 (Clontech) that had been previously digested with KpnI and XhoI. Plasmids were transfected into 293T cells to generate stable clones. Cell mixtures for immunofluorescence (IF) analysis were made as described previously (Contreras et al., 1995). In brief, NRK cells were transfected with pEYFP-N1 and pAmCyan-N1 plasmid DNA to coexpress CFP/YFP and YFP/CFP. After 48–72 h, cells from the mixed monolayer were lysed with lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate). To obtain cDNA expression. Western blot analysis was done using the following primary antibodies: mouse anti pan-species β subunit antibody (1:1000 dilution; Santa Cruz, CA); a mouse monoclonal antibody against β1 subunit donated by Dr. M. Caplan (1:200 dilution; Yale University); and a mouse monoclonal anti-rat β subunit antibody (1:4,800 dilution) purchased from American Diagnostica (Cranbury, NJ). Anti-mouse peroxidase-conjugated secondary antibodies were used to detect the primary antibodies (1:5000 dilution; Amersham, Buckinghamshire, United Kingdom). After washing with 10 and 20 mM imidazole, bound proteins were eluted with 500 mM imidazole. Eluates were loaded on a 10% SDS-PAGE gel and analyzed by immunoblotting using an anti-dog β subunit antibody and a SuperSignal West HRP substrate (Pierce Chemical, Rockford, IL). For these assays, CHO SDβ cells were cultured in serum-free media for 4 d. The conditioned medium was then collected and further purified by fast-performance liquid chromatography chromatography.

Immunoprecipitation

MDCK cells stably expressing rat β1-YFP were cocultured with NRK-E52 cells. For 48–72 h, cells from the mixed monolayer were lysed with lysis buffer (10 mL of 3× SDS-PAGE gel and analyzed by immunoblotting using an anti-dog β subunit antibody and a SuperSignal West HRP substrate (Pierce Chemical, Rockford, IL). For these assays, CHO SDβ cells were cultured in serum-free media for 4 d. The conditioned medium was then collected and further purified by fast-performance liquid chromatography chromatography.

Immunofluorescence

The β subunit and its recombinant variants were assayed by immunofluorescence as described previously (Shoshani et al., 2005). For blocking, cells were incubated with primary antibodies against the β1 subunit, washed six times with PBS-Tween (0.05%), and then incubated with the secondary antibodies (1:1000 for 1 loaded from room temperature. The following secondary antibodies were used: Alexa 495-conjugated goat anti-mouse immunoglobulin G (Invitrogen) and cyanine 5-conjugated goat anti-mouse. Specimens were mounted with VECTASHIELD medium (Vector Laboratories, Burlingame, CA) and observed with a confocal microscope (Leica, Bannockburn, IL) or a Fluoview FV1000 confocal microscope (Olympus, Center Valley, PA).

Immunoblotting

Western blot analysis of whole cell protein extract was performed as described previously (Shoshani et al., 2005). In brief, MDCK, NRK-E52, and CHO cells were washed with PBS and solubilized with radiolabeled immunoprecipitation assay (RIPA) buffer (10 mM piperazine-N,N,N’-tris(2-ethanesulfonic acid), pH 7.4, 150 mM NaCl, 2 mM ethylenediamine-tetraacetic acid (EDTA), 1% Triton X-100, 0.5% sodium deoxycholate, and 10% glycerol) containing protease inhibitors (Complete Mini; Roche Diagnostics, Indianapolis, IN). The protein content of the cell lysate was measured (BCA protein assay reagent; Pierce Chemical, Rockford, IL) and prepared for SDS-polyacrylamide gel electrophoresis (PAGE) by boiling in sample buffer. The resolved proteins were electrophoretically transferred onto a polyvinylidene difluoride membrane (Hybnd-Blot; GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). The proteins of interest were then detected with the specific polyclonal or monoclonal antibodies indicated in each case, followed by species-appropriate peroxidase-conjugated antibodies (Zymed Laboratories, South San Francisco, CA) and chemiluminescence detection (ECL PLUS; GE Healthcare).

Pull-Down Assay

CHO cells stably expressing β1-His6 were cultured for 48 h and then lysed with RIPA buffer containing protease inhibitors and the protein content was determined. pHotase bait was immobilized on nickel-nitrotriacetic acid beads (His Trap FF column; GE Healthcare) previously equilibrated with 10 ml of RIPA containing protease inhibitors. In general, 4 mg of total protein extract was loaded and allowed to interact for at least 1 h at 4°C with gentle shaking. After 10 washes with 10 ml of 20 mM imidazole, the supernatant of SDβ-expressing CHO cells was loaded as prey, and interaction was allowed to occur overnight at 4°C. After washing with 10 and 20 mM imidazole, bound proteins were eluted with 500 mM imidazole. Eluates were loaded on a 10% SDS-PAGE gel and analyzed by immunoblotting using an anti-dog β subunit antibody and a SuperSignal West HRP substrate (Pierce Chemical, Rockford, IL). For these assays, CHO SDβ cells were cultured in serum-free media for 4 d. The conditioned medium was then collected and further purified by fast-performance liquid chromatography chromatography.

Fluorescence Resonance Energy Transfer (FRET) assay by Acceptor Photobleachung

For analyzing FRET efficiency, live MDCK cells stably expressing β1-CFP or β1-YFP were cocultured on glass-bottom dishes (MatTek) for 48–72 h. The plates were washed with freshly prepared buffer (10% glycerol, 50 mM Tris-HCl, pH 6.8, and 1 M NaCl), followed by removal of the agarose beads by centrifugation. The supernatant was then collected on 8% SDS-PAGE gels. Western blot analysis was done using the following primary antibodies: mouse anti pan-species β subunit antibody (1:1000 dilution; Santa Cruz, CA); a mouse monoclonal antibody against β2 subunit donated by Dr. M. Caplan (1:200 dilution; Yale University, New Haven, CT); and a mouse monoclonal anti-rat β subunit antibody (1:4,800 dilution) purchased from American Diagnostica (Cranbury, NJ). Anti-mouse peroxidase-conjugated secondary antibodies (1:5000 dilution; Zymed Laboratories) were then applied.
percentage increase of postbleach donor intensity compared with prebleach donor intensity. FRET efficiency (E) was calculated from the ratio of CFP fluorescence evaluated before (D_{pre}) and after (D_{post}) photobleaching, using FV10-ASW version 1.7 (Olympus) and the following formula:

\[
E = \left(\frac{D_{pre} - D_{post}}{D_{pre}}\right) \times 100
\]

Cell cultures expressing only β₉-CFP or β₉-YFP were used for correction and determination of the laser intensity, as well as to determine the gain and offset settings. Emission intensities in several membrane regions were recorded at 475–525 nm for β₉-CFP and at 540–575 nm for β₉-YFP.

Statistical Analyses
Statistical analyses were performed with Prism 4 software (GraphPad Software, San Diego, CA). Results are expressed as means ± SE. The groups were compared by one-way analysis of variance (ANOVA) using the Bonferroni multiple-comparison test. Differences were considered significant if p < 0.05.

RESULTS

The β-β Interaction Is Species Specific
Interactions between adjacent cells from different animal species are not unique or universal but depend on the type of cell–cell junction involved. TJs are promiscuous, because those established between cells belonging to different animal species have a degree of sealing that can be predicted from the transcellular electrical resistance of each cell type and their proportions in the mixed monolayer (González-Mariscal et al., 1989). In contrast, adherens junctions are homotypic and can be seldom, if ever, established between epithelial cells from different animal species (Contreras et al., 2002; Halbleib and Nelson, 2006). We have found previously that the homotypic requirement for cell–cell junctions extends to the distribution of Na⁺,K⁺-ATPase (Contreras et al., 1995). In keeping with our previous observations (Shoshani et al., 2005), Figure 1A shows that MDCK cells present a typical “chicken fence” pattern of Na⁺,K⁺-ATPase distribution in monolayers of pure MDCK cells; however, when the MDCK cells are cocultured with NRK-E52 epithelial cells from NRK (Figure 1B), Na⁺,K⁺-ATPase is only detected in homotypic interactions. In contrast, when the NRK cells are transfected with dog β₁ (NRKβ₁), MDCK cells express Na⁺,K⁺-ATPase in all cell–cell borders (Figure 1C).

Dog β₁ Subunits Interact In Vitro
The β-β interaction in vitro was studied with pull-down assay using the wild-type dog β₁ subunit (βWT), its extra- cellular domain (SDβ₁) and the complete dog β₁, tagged with a hexahistidine repeat epitope tag (βHis₆) (Figure 2A). Wild-type CHO cells express a Na⁺,K⁺-ATPase that is barely recognized by a specific anti-dog β₁ subunit antibody (Figure 2B). These cells were transfected with the CDNA for dog SDβ₁ and βHis₆ to generate stable clones. Figure 2, C and D, shows CHO cells expressing cytoplasmic SDβ₁ in transit to be secreted and βHis₆ in the plasma membrane (green), respectively. Western blots of lysates of βHis₆-expressing CHO cells present a band of ~50 kDa corresponding to the fully glycosylated form of the dog β₁ subunit detected with an antibody against this species (Figure 2E, lane 1). As shown in Figure 2E (lane 2), this band corresponds to βHis₆, because it is also detected using a His6 detection kit (His probe). We then immobilized purified βHis₆ on a nickel matrix column, and after extensive washing, loaded it with SDβ₁ supernatant (SN SDβ₁). Figure 2E (lane 3) shows a broad band of soluble β₁ ectodomain that, as expected, is lighter because it lacks the cytoplasmic and transmembrane domains. Notably, a His probe does not recognize any protein when applied to SN SDβ₁ (lane 4). After an incubation period, bound proteins were eluted with imidazole buffer, separated on SDS-PAGE, and analyzed by Western blotting using an antibody against dog β₁ subunit or His probe. The pattern of bands observed in Figure 2E (lane 5) corresponds to the in vitro mixture of the soluble extracellular domain of the dog β₁ subunit and βHis₆ and is similar to the pattern observed in lane 6, which corresponds to the eluted (pulled down) proteins, indicating that the soluble extracellular domain of dog β₁ subunit and βHis₆ were interacting. For comparison, lane 7 shows a negative pull-down assay in which wild-type CHO cell supernatant was used. Lanes 8 and 9 are analogous to lanes 6 and 7, except that this time the membrane was blotted with His probe. As expected, bands corresponding to the β₁ subunit are observed only where βHis₆ is present. Together, these results suggest that β-β interaction can be reconstituted in vitro. This interaction probably involves the extracellular domains, although this experiment does not show that the interaction is direct.

Molecular Tools to Study β-β Interactions In Vivo
As mentioned in the Introduction, the aim of this work was to study the interaction of the β subunit of a given Na⁺,K⁺-ATPase with that of another Na⁺,K⁺-ATPase in the plasma membrane of a cell across the intercellular space. Our first approach, using an in vitro protein–protein interaction assay (Figure 2), suggested that such an interaction is possible, although it did not confirm a direct interaction. To test this possibility in vivo, we constructed recombinant rat β₁ subunits fused to the fluorescent proteins CFP or YFP, which we will refer to as Rβ CFP and Rβ YFP, respectively (Figure 3A). These constructs were expressed in MDCK cells. Using a specific antibody against the dog β₁ subunit in a Western blot assay, the only band detected is, of course, the band representing the endogenous β₁ subunit of MDCK cells (Figure 3B, lanes 1–3). Whereas this antibody detects no band at all in NRK cells (Figure 3B, lane 4), a specific antibody against the rat β₁ subunit shows a single band of 95 kDa in cell extracts of MDCK cells transfected with either Rβ CFP or Rβ YFP (MR βYFP and MR βCFP, Figure 3C, lanes 1 and 2). No band is observed in wild-type MDCK cells (Figure 3C, lane 3). An antibody against GFP that recognizes all GFP-derived fluorescent proteins confirms that the 95-kDa band corresponds to the rat β₁ YFP (Figure 3D, lane 1) and rat β₁ CFP (Figure 3D, lane 2). As expected, this high-molecular-weight band is not detected in nontransfected MDCK cells (Figure 3D, lane 3). To make sure that the fluorescent β₁ subunit is expressed in a membrane position that would allow us to study β-β interactions in cells, we used immu-
noffluorescence and confocal microscopy to visualize the localization of Rβ CFP and Rβ YFP. Figure 3E shows the typical chicken fence pattern of the β1 subunit as seen in nontransfected MDCK cells using an antibody against the dog β1 subunit. This pattern is not observed if the anti-rat β1 subunit antibody is used (Figure 3F). As shown in Figure 3, G and H, both fluorescent proteins are expressed at the lateral plasma membrane of transfected MDCK cells. This fluorescent signal colocalizes with the rat β1, as seen by anti rat β1 antibody staining (Supplemental Figure S1, A and B) and staining of the endogenous dog β1 subunit (Supplemental Figure S1, C and D). Moreover, nontransfected MDCK cells are excited in the same conditions as the YFP-transfected ones (Supplemental Figure S2, A and C), and when observed with confocal microscopy, they do not exhibit a fluorescent signal. Nonetheless, cadherin expression, as assayed by an anti-pan-cadherin antibody, is well conserved in MDCK cells expressing Rβ YFP (Supplemental Figure S2, B and D). Therefore, the molecular tools we have developed to study the interaction between β subunits (Rβ CFP and Rβ YFP) are localized to the plasma membrane and do not perturb the distribution of other membrane molecules.

**β–β Interactions Are Strong and Specific**
To analyze whether the β1 subunits of Na+/K+-ATPases of neighboring cells interact with each other (transinteraction), we applied an immunoprecipitation (IP) assay. For this strategy, MDCK cells transfected with Rβ YFP and wild-type NRK cells were cocultured. Cell lysates of nontransfected MDCK cells and of wild-type NRK cells were separated on SDS-PAGE gels and immunoblotted with a mAb that recognizes the β1 subunit of various animal species. A clear band corresponding to the endogenous β1 subunit was detected in MDCK cell extract (Figure 4A, lane 3) and in NRK cell extract (Figure 4A, lane 2). To analyze the possible β–β interaction between the rat β1 subunits of neighboring cells, immunoblotting of the cell lysate obtained from cocultures (Figure 4A, lane 3) and immunoprecipitation with an anti-β1 subunit antibody (Figure 4A, lane 4) were performed. Two bands were observed: a band of high molecular weight (95 kDa), corresponding to the exogenous rat β1 subunit (Rβ YFP) and a lower weight band (55 kDa), corresponding to the endogenous dog and rat β1 subunits (which cannot be distinguished by molecular weight). Conversely, immunoprecipitation of the transfected Rβ YFP by using an antibody against GFP produced a 55-kDa coimmunoprecipitated protein (Figure 4A, lane 5), indicating that these two proteins strongly interact. Nevertheless, the possibility exists that the immunoprecipitated recombinant rat β1 subunit could coprecipitate the dog β1 subunit of a neighboring Na+/K+-ATPase present in the same cell membrane (cis interaction). To rule out this possibility, we analyzed the anti-GFP immunoprecipitates by immunoblotting, using specific antibodies against either the dog...
β₁ subunit or the rat β₁ subunit. As shown in Figure 4B, the dog-specific antibody detected neither the low-molecular-weight (WT) nor the high-molecular-weight (recombinant) rat β₁ subunits (lane 1) when cocultures were IPed with an anti-GFP antibody, whereas the rat-specific antibody recognized both proteins (lane 2). On the other hand, the antibody against the dog subunit barely recognized the endogenous dog β₁ in immunoprecipitates obtained with an antibody against β₁ from several species (lane 3), whereas the anti rat-β₁ antibody recognized both the endogenous and YFP-tagged proteins (lane 4). Immunoprecipitates of pure MRβ YFP cells immunoblotted with antibodies against both the rat β₁ and dog β₁ subunits show a band corresponding to the dog β₁ subunit (lane 5) and a band corresponding to the rat recombinant protein (lane 6), suggesting that β-β interaction between dog subunits may occur naturally in MDCK cells. As negative controls for immunoprecipitation, we used cocultures of WT MDCK and NRK and blotted them with an anti-β₁ antibody (Figure 4C). As expected, dog and rat β₁ are detected in the immunoprecipitates for the β₁ subunit (Figure 4C, lanes 1 and 2) but are not detected in GFP immunoprecipitates (lane 3). The overall conclusion from this group of experiments is that rat β₁ subunits of a given cell interact with the homotypic β₁ subunits of neighboring cells.

Rat β₁ Subunits Located in Neighboring Cells Interact In Vivo

To further demonstrate that rat β₁ subunits of neighboring cells interact in situ, two stable clones of MDCK cells, one expressing Rβ CFP and the other Rβ YFP, were generated (Figure 3). Cocultures of MDCK cells expressing CFP or YFP fusion proteins were used to perform FRET by acceptor photobleaching assays. FRET quenches donor fluorescence; therefore, the rebound of donor fluorescence after photodestruction of acceptors provides a straightforward means to measure the apparent FRET efficiency, Eapp. The raw data consist of two images: donor fluorescence taken before (D PRE) and after (D POST) acceptor photobleaching. A representative confocal image of an experiment in which photobleaching of YFP was performed on a selected domain of cell membrane is shown in Figure 5. Merged images of CFP and YFP fluorescence are shown in Figure 5, A and B. A strong laser line of 515 nm fades YFP but not CFP (Figure 5B). Photobleaching of YFP increased the fluorescence intensity of CFP in the region of interest (ROI) 1 (Figure 5B) by 26.6% relative to its intensity before photobleaching and the calculated energy transference efficiency (%E) of 10 experiments was 21.3%. This %E suggests that the β₁ subunits tagged with CFP and YFP must be located at <10 nm from each other (Kenworthy, 2005). As an internal control, fluorescence intensity was measured in ROI 2 (Figure 5B), where CFP does not have YFP in its proximity; therefore, its intensity does not change (Supplemental Table 1). As a positive control, a fused CFP-YFP molecule was expressed in the cytoplasm of MDCK cells. In this construct, the two proteins are located close enough to transfer resonance energy (Supplemental Figure S3, A–C). Photobleaching of YFP increased the fluorescence intensity of CFP (ROI 3 in Supplemental Figure S3C) by 13.4% relative to its intensity before photobleaching, representing a %E equal to 11.5% (Supplemental Table 1). Figure 5C summarizes these results. The first bar represents the energy transfer between two β₁ subunits placed in the plasma membrane of neighboring (living) MDCK cells. The shaded bar corresponds to the positive control (fused CFP-YFP). The third bar shows the endogenous negative control (ROI 2). The %E values of
bar 1 and 2 are significantly higher (p < 0.001) than that of bar 3. To validate the system, a region was chosen in which CFP-expressing cell was separated from YFP-expressing cell by an MDCK cell that did not express either protein (ROI 4 in Supplemental Figure S3F). Photobleaching of this area showed no energy transfer effect (Supplemental Table 1). As an additional negative control, MDCK cells expressing the CFP plasmid alone (without the β1 subunit) in contact with cells expressing Rβ YFP were analyzed. When region ROI 5 was photobleached (Supplemental Figure S3I), there was no energy transfer effect, as expected (Supplemental Table 1). Together, these results suggest that the β1 subunits of Na+/K+-ATPases in neighboring MDCK cells are close enough to directly interact. This was a basic assumption of the model proposed by Shoshani et al. (2005) to account for the maintenance of the polarized distribution of the Na+/K+-pump in epithelial cells.

**DISCUSSION**

Half a century ago, Koefoed-Johnsen and Ussing (1958) put forward a plausible explanation for the transepithelial transport of Na+ (Figure 6A) that was subsequently used as a model for the transport of substances across all epithelia (Figure 6B). Ironically, although a central element of the KJ-U model is the asymmetric distribution of Na+/K+-ATPase and this asymmetry was later supported by the fact that ouabain inhibits Na+ transport when added to the inner bathing solution but not to the outer one, the intrinsic mech-
Figure 6. Polarized expression of epithelial Na\textsuperscript{+},K\textsuperscript{+}-ATPase. (A) Seminal model of Koe-foed-Johnsen and Ussing (1958), in which the Na\textsuperscript{+},K\textsuperscript{+}-ATPase (reinforced in magenta) was assumed to occupy the basal side of the cell, which constitutes the inner-facing barrier. (B) From this position, the pump transports Na\textsuperscript{+} toward the interstitial side of the cell, producing a net decrease in the cytoplasmic concentration of this ion and setting up an electrochemical force that drives counter- and cotransporters of sugars, amino acids, and ions and possible the existence of net currents. (C) Confocal transverse section of a monolayer of MDCK cells. The nuclei are stained with propidium iodide (red) and the β subunits of Na\textsuperscript{+},K\textsuperscript{+}-ATPase are stained with a specific antibody (green), showing that this subunit is localized to the lateral surfaces of cells, but not to the apical (left) or basal sides. (D) Na\textsuperscript{+},K\textsuperscript{+}-ATPase molecules of two neighboring epithelial cells with interacting β subunits (green), as shown previously (Shoshani et al., 2005) and in the present work. (E) Na\textsuperscript{+},K\textsuperscript{+}-ATPase molecules anchored to the lateral membranes. Because of the tight junction, Na\textsuperscript{+} ions pumped into the intercellular space can only diffuse inwards, generating vectorial transport across the epithelium.
a role in the apical sorting of the corresponding α/β complex.
More, it has been demonstrated that the polarized distribution of Na⁺,K⁺-ATPase is correlated with the β subunit isoform expressed by a given cell. Hence, β₁ is directed to the lateral membrane in epithelial MDCK cells (Shoshani et al., 2005; Vagin et al., 2005) and β₂ is delivered to the apical membrane in ADPKD (Wilson et al., 2000) and HGT-1 cells (Vagin et al., 2005). In agreement with the idea that the multiple N-glycosylation sites in the β₂ isoform function as an apical localization determinant, Vagin et al. (2005) have shown that the introduction of extra N-glycosylation sites into the β₁ isoform results in apical delivery of the mutated β₁ in HGT-1 cells. Accordingly, Wilson et al. (2000) transfected MDCK cells with the β₂ isoform and demonstrated that the α₁/β₂ complex is delivered to the apical domain. In contrast, Liang et al. (2006) have shown that deglycosylation treatments in well-polarized hepatic cells by deglycosylation drugs, or by site-directed mutagenesis of the N-linked-glycosylation residues, cause the β₁ subunit to traffic from the native basolateral domain to the apical/canalicular domain.

The polarized delivery of Na⁺,K⁺-ATPases to the membrane has been shown to be strictly dependent on the assembly of αβ dimers in the endoplasmic reticulum (Geering et al., 1996, Tokhtaeva et al., 2009). Nevertheless, it is not clear whether these dimers can disassemble and their subunits persist as functional independent proteins once they are inserted in the plasma membrane. In this respect, Moreno et al. (2002) have shown that the removal of a postsynaptic density 95/disc-large/zona occludens group from the cytoplasmic C-terminal end of the Shaker K⁺-channel does not prevent polarization but dramatically reduces the protein’s residence time in the cell membrane. Likewise, binding between β subunits might anchor the two Na⁺,K⁺-ATPases to which they belong to the cell membrane.

Sodium transport across epithelia is important, not only because of the physiological role of this ion, but also because the Na⁺ pumped out of the cytoplasm by Na⁺,K⁺-ATPase creates an asymmetry between cytoplasmic Na⁺ and extracellular Na⁺. This asymmetry establishes an electrochemical gradient across the plasma membrane that provides the driving force and increases the substrate affinity for a wide variety of Na⁺/glucose, Na⁺/amino acid, Na⁺/K⁺, and Na⁺/Ca²⁺ cotransporters (usually, carriers only acquire an affinity for the cotransported substance once they combine with Na⁺) (see Cereijido and Rotunno, 1971). The net movement of substances such as glucose, amino acids and ions across epithelia is, in turn, a key requirement for the existence of metazoan life. Not surprisingly, Na⁺ transport is closely modulated in response to a multitude of physiological, pharmacological, and pathological conditions.

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