Delivery of Na^+,K^+-ATPase in Polarized Epithelial Cells

The sodium and potassium ion–stimulated adenosine triphosphatase (Na^+,K^+-ATPase) is an essential membrane protein found in all vertebrate cells that maintains ionic gradients, electrical potential, and osmotic balance (*1*). In nonpolarized cells the sodium pump is uniformly distributed, but in polarized epithelial cells it is found in either basolateral (*2*) (intestine, kidney, endocrine, and exocrine glands) or apical (*3*) (choroid plexus and retinal pigment epithelium) plasma membrane domains.

Two mechanisms may control polarized distribution of the sodium pump in epithelial cells. Caplan *et al.* (*4*) used a photosensitive ouabain probe and showed that newly synthesized active subunits of Na^+,K^+-ATPase were directly targeted to the basolateral surface of MDCK cells, which suggests that sorting in the Golgi apparatus is a major determinant of its polarized distribution. In contrast, R. W. Hammerton *et al.* (*5*), who used domain-selective biotinylation as an assay, demonstrated equal delivery of subunits to apical and basolateral surfaces and a longer half-life of this protein on the basal membrane. Because Na^+,K^+-ATPase forms a complex with the basolateral ankyrin-fodrin cytoskeleton (*6*), Hammerton *et al.* postulated that the main mechanism for polarization of the sodium pump was not Golgi sorting and vectorial delivery, but stabilization of the enzyme by interaction with the membrane cytoskeleton.

We studied the surface delivery of the Na^+,K^+-ATPase in the thyroid epithelial cell line FRT (Fischer rat thyroid) (*7*), a highly polarized epithelial cell line with well-characterized targeting patterns for endogenous and exogenous plasma membrane proteins (*8*). Studies were done after 1 or 7 days of culture on filters to determine how the asymmetric distribution of Na^+,K^+-ATPase develops during the polarization of the epithelium. Tight junctions appeared to be functional at both times, as the transepithelial resistances were about 1,000 and 10,000 to 12,000 ohm/cm^2, respectively. With the use of laser scanning confocal microscopy 1 day after plating, we found that both α and β subunits were exclusively localized within the lateral domain (Fig. 1, A and B). This restricted distribution was similar at 7 days of culture (Fig. 1, C and D).

We measured the surface distribution of Na^+,K^+-ATPase by domain-selective biotinylation and streptavidin-agarose precipitation after the monolayers were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine (Fig. 2). Both subunits were precipitated with a monoclonal antibody to the β subunit; reprecipitation of the supernatant with the same antibody or with a monoclonal antibody to the α subunit did not reveal any additional enzyme. In agreement with the immunofluorescence results, the α and β subunits were highly polarized (>95%) to the basolateral surface on the first and seventh days of filter culture (Fig. 2). The total amount of both subunits increased with the time of culture because of cell division.

To examine the kinetics of biosynthesis of the α and β subunits, we pulse-labeled cells with [^{35}S]methionine and [^{35}S]cysteine. Subsequently, the Na^+,K^+-ATPase was immunoprecipitated and analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE). At the earliest time tested (chase time 0), both subunits were precipitable with the monoclonal antibody to the β subunit (Fig. 3). The β subunit still had the mobility of the high mannose form (41 kD), rather than the lower mobility (47 to 50 kD) observed after it was processed in the Golgi complex, which indicated that assembly into a heterodimer had occurred in the endoplasmic reticulum immediately after synthesis (*9*).

We studied the delivery of the newly synthesized αβ complex to the apical and basolateral surfaces with a biotin targeting assay (Fig. 4) (*10*). After 7 days of culture, both Na^+,K^+-ATPase subunits were vectorially targeted to the basolateral surface with similar kinetics (Fig. 4A). A small amount (<1%) of Na^+,K^+-ATPase was detected at each time point on the apical surface. After 1 day of culture, both subunits also appeared to be sorted directly to the basolateral membrane (Fig. 4B). After reaching maximal amounts at 120 min (chase time), the expression of both subunits on both surfaces slowly decreased for the next 4 hours, which indicated no major differences in stability between the apical and basolateral protein pools. Densitometric analysis of two independent experiments indicated that over 95% of the α and β subunits was directly delivered to the basolateral surface in monolayers that were 1 or 7 days old. These results suggest that vectorial delivery of αβ dimers is established early in the development of FRT monolayers and contributes to the polarized distribution of Na^+,K^+-ATPase.

Our findings are different from those reported by Hammerton *et al.*, who observed unpolarized delivery of the α subunit

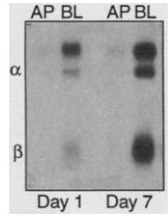

Fig. 2. Autoradiogram of the distribution of biotinylated α and β subunits of Na^+,K^+-ATPase after 1 and 7 days of filter culture. In both cases, filter-grown cells were metabolically labeled overnight with 200 μCi of [^{35}S]methionine and [^{35}S]cysteine, then labeled from the apical (AP) or the basolateral (BL) side with NHS-LC-Biotin [sulfosuccinimidyl 6-(biotinamido) hexanoate] (Pierce). Na^+,K^+-ATPase α and β subunits were co-immunoprecipitated with the use of β subunit–monoclonal antibody. The biotinylated (surface) fraction of the protein was recovered by subsequent precipitation with avidin-agarose beads (Pierce) and analyzed by SDS-PAGE and autoradiography.

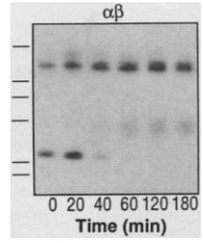

Fig. 3. Pulse chase analysis of the assembly and processing of α and β subunits of Na^+,K^+-ATPase. Cells were labeled for 20 min with [^{35}S]methionine and [^{35}S]cysteine, then incubated in regular medium for the indicated times. Both subunits were immunoprecipitated with the use of a monoclonal antibody to the β subunit and separated by SDS-PAGE. The α subunit was detected as a band of 105 kD; the β subunit was processed from a precursor of about 41 kD to the fully mature product of 47 kD in about 50 min. From the earliest time of chase, α and β subunits were co-immunoprecipitated. Molecular size markers (top to bottom) are 116, 82, 64, 58, 36, and 26 kD.

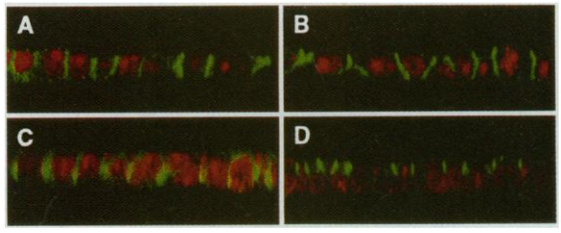

Fig. 1. Steady-state distribution of α and β subunits of Na^+,K^+-ATPase after 1 and 7 days of filter culture, indirect immunofluorescence and laser scanning confocal microscopy. After 1 (**A** and **B**) and 7 (**C** and **D**) days of culture on filters, cells were fixed in 2% paraformaldehyde, permeabilized with 0.075% saponin, and then processed for indirect immunofluorescence microscopy with monoclonal antibodies specific for α (A and C) or β (B and D) subunits of Na^+,K^+-ATPase and a fluorescein isothiocyanate (FITC)–coupled secondary antibody. The nuclei were stained with propidium iodide.

in Madin-Darby canine kidney (MDCK) cells (*5*). The different results may reflect cell-specific variations in the sorting machineries of the two cell types (*11*). Or, given that previous studies focused on the delivery of α subunits and did not explore the targeting of β subunits or heterodimers, it is possible that MDCK cells over produce α subunits that are subsequently delivered to the apical surface without the β subunit. However, this is unlikely because transport of α subunits appears to require early dimerization with β subunits (*9*).

The different results in targeting experiments might have been caused by the use of different experimental protocols. Hammerton *et al.* did targeting experiments after transferring cells from medium with low (< 5 μM) [Ca^{2+}] to medium with normal (1.8 mM) [Ca^{2+}] (*5, 12*) in order to study the surface delivery of the pump at short times after the induction of cell-cell contact. We performed identical experiments in FRT cells. The targeting of αβ heterodimers was polarized to the basolateral membrane of FRT cells at all times, with an initial increase in polarity between 6 and 12 hours after induction of cell-cell contact (Fig. 5). These findings confirm our earlier observations with cells plated under normal conditions and demonstrate that the disparity in results was not a result of this difference in the experimental protocol.

Nonetheless, we found that variations in the experimental protocol can lead to erroneous targeting results. Cells plated on Transwell (Costar) chambers grew not only on the filters, but also on the sides of the chamber. In the biotinylation procedure (*13*), cells were lysed only after excision of the filter from the chamber. If the cells were lysed in the chamber, the steady-state distribution of Na^+,K^+-ATPase measured by the biotinylation assay was relatively unpolarized (*14*). When targeting of Na^+,K^+-ATPase was studied in FRT cells that were lysed in situ, rather than from excised filters, the delivery of both α and β subunits was not polarized (Fig. 6). Such different targeting results might be a result of the labeling of basolateral proteins of cells grown on the sides of the chamber by biotin added apically; these cells would, however, be inaccessible to biotin added basolaterally. MDCK cells grown on plastic are less well polarized and have leakier junctions than cells grown on filters (*15*). With this condition we found variable results in different experiments, probably because of variations in the number and tightness of cells grown on the sides of the chamber.

Our results suggest that in the FRT cell lines the polarized distribution of the sodium pump is achieved by vectorial delivery of the heterodimer (which is assembled in the endoplasmic reticulum) from the Golgi apparatus to the cell surface. The polarized delivery is observed a few hours after functional tight junctions are detected. Thus this mechanism must participate not only in the maintenance, but also in the establishment of the polarized distribution of the enzyme during development of the epithelial monolayer. The vectorial delivery of the Na^+,K^+-ATPase may be mediated by basolateral signals that are recognized by the trans Golgi network sorting machinery.

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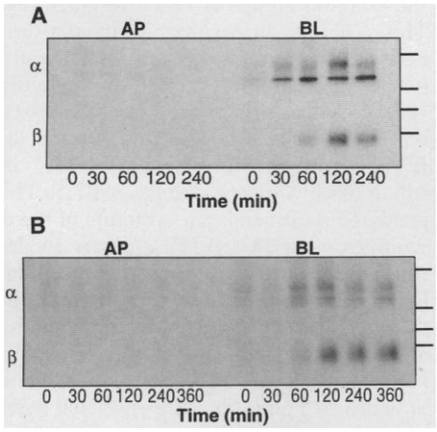

Fig. 4. Delivery of newly synthesized α and β subunits of Na^+,K^+-ATPase after 7 (**A**) and 1 (**B**) days of culture. Cells were incubated for 20 min with [^{35}S]methionine and [^{35}S]cysteine, then with regular medium for the times indicated. Biotinylation with NHS-LC-Biotin (Pierce) was applied to the apical (AP) or basolateral (BL) membrane to detect the protein at the cell surface. Cells were extracted, and α and β subunits of Na^+,K^+-ATPase were sequentially precipitated (as in Fig. 2) and processed for SDS-PAGE and fluorography. Molecular size markers (top to bottom) are 116, 82, 64, and 58.

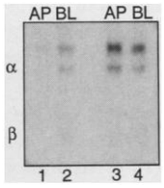

Fig. 6. Delivery of newly synthesized α and β subunits of Na^+,K^+-ATPase extracted from cells on intact Transwell (Costar) and excised filters. FRT cells were grown for 7 days on Transwell (Costar), then labeled for 1 hour with [^{35}S]methionine and [^{35}S]cysteine and surface proteins were labeled with biotin on the apical (AP) or from the basolateral (BL) sides. Cells were lysed from filters excised with a razor blade (lanes 1 and 2) as usual, or were directly lysed in the Transwell chambers (lanes 3 and 4). Na^+,K^+-ATPase was immunoprecipitated as in Fig. 5 and analyzed by SDS-PAGE and fluorography.

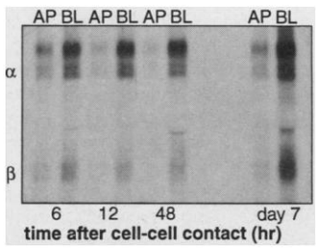

Fig. 5. Delivery of newly synthesized α and β subunits of Na^+,K^+-ATPase at different times after cell contact. Cells were plated at confluency on collagen coated filters in medium containing a low concentration of Ca^{2+} and, after 12 hours, transferred to medium containing normal concentration of Ca^{2+}. At different times after calcium–induced cell-cell contact (*5, 12*), cells were labeled for 1 hour with [^{35}S]methionine and [^{35}S]cysteine, then labeled with NHS-SS-Biotin [sulfosuccinimidyl-2-(biotinamide) ethyl-1,3′-dithiopropionate] (Pierce) on the apical (AP) or basolateral (BL) membrane to capture the first wave of newly synthesized protein to arrive at the cell surface. The cells were extracted, and subunits of Na^+,K^+-ATPase labeled with biotin were purified by successive precipitation with antibodies to the β subunit and avidin-agarose and processed for SDS-PAGE and fluorography. AP, apical biotinylation; BL, basolateral biotinylation. The two right lanes are control cells in which the delivery of α and β subunits of Na^+,K^+-ATPase was measured after 7 days of continuous filter culture.

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Earlier studies on sodium pump sorting in polarized renal epithelial cells have suggested that this enzyme, like most other basolateral proteins heretofore examined, is sorted intracellularly and delivered vectorially to the basolateral surface (*1*). Hammerton *et al.*. (*2*) used the same cell type but a different technique and arrived at the opposite conclusion. Their results suggested that Na^+,K^+-ATPase is randomly delivered to the apical and basolateral plasmalemmal surfaces (*2*), and that stabilizing interactions with cytoskeletal elements that underly the basolateral but not the apical cell surface (*3*) result in a much longer residence time for the pump inserted into the basolateral domain. Their study is consistent with a model in which the sodium pump is not sorted intracellularly, but instead achieves its basolateral distribution through a mechanism based on differential stabilization. Because these discrepant observations carry markedly different implications for our understanding of epithelial sorting processes, we have investigated further the pathways involved in the accumulation of the Na^+,K^+-ATPase at the basolateral cell surface.

Both of the studies mentioned above (*1*, *2*) examined the delivery of newly synthesized Na^+,K^+-ATPase to the surface of MDCK cells. This cell line, derived from the canine renal distal tubule, maintains its parent tissue's dramatic morphologic and biochemical polarity when grown in culture (*4*). In order to examine the targeting of the Na^+,K^+-ATPase in these cells, we use monoclonal antibodies raised against each of the sodium pump's two membrane protein subunits prepared from dog kidney (Fig. 1A). As expected (*5*), protein immunoblot analysis performed on membranes derived from MDCK cells revealed that the antibody directed against the α subunit of Na^+,K^+-ATPase reacts with a 100-kD polypeptide, while the β-subunit antibody reacts with a single broad band of molecular weight of about 55 kD. These antibodies can also be used to demonstrate the polarized distribution of the Na^+,K^+-ATPase in confluent MDCK cells. Confocal immunofluorescence microscopy showed that the α subunit is restricted in its distribution to the lateral membranes of MDCK cells that have grown as a confluent monlayer on a permeable filter support for 96 hours. (Fig. 1B and 1C). Similar results were obtained for the β subunit. We repeated the experiment of Hammerton *et al.* (*2*) using a similar protocol with minor modifications. Cells were pulse labeled and exposed to apical and basolateral surface biotinylation after the initiation of the chase period. Newly synthesized Na^+,K^+-ATPase was detected at the basolateral surface as early as the 30-min chase point (Fig. 2). We did not detect any radioactively labeled sodium pump available to apical biotinylation. Uvomorulin, another basolateral membrane protein (*6*), was also found to appear exclusively at the basolateral surface. In contrast, a 114-kD apical protein (gp114) (*7*) was only biotinylated from the apical surface, which demonstrated that our NHS-SS-Biotin reagent (Pierce) had access to this cell surface domain. These data are consistent with earlier studies on the time course and polarity of sodium pump surface delivery (*1*, *8*) and suggest that the Na^+,K^+-ATPase is sorted intracellularly and delivered vectorially to the appropriate plasmalemmal domain.

The NHS reagent reacts covalently with the ε amino groups of lysine residues (*9*). This reaction favors lysines in the uncharged NH_2 form over the NH_3^+ form and hence proceeds more rapidly at high pH (*10*). Consequently, we did our cell surface biotinylations at pH 9.0. Hammerton *et al.* (*2*) used an established protocol (*11*) in which biotinylation is done at pH 7.5. The predicted transmembrane structure of the α subunit of Na^+,K^+-ATPase places six lysine residues in this protein's extracellular loops (*12*). Furthermore, these lysines appear to be closely apposed to the membrane and may have limited access to NHS-SS-biotin present in the bulk solution. The presence of a filter, and the epithelial basement membrane at the basolateral surface, would likely limit this access further (*13*). If the Na^+,K^+-ATPase is a poor substrate for biotinylation at pH 7.5, differences in biotinylation efficiency at the apical and basolateral surface might influence the results of the experiments.

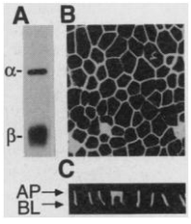

Fig. 1. Labeling of the basolateral surfaces of epithelial cells with monoclonal antibodies to the Na^+,K^+-ATPase subunit proteins. (**A**) Protein immunoblot analysis of a membrane fraction prepared from MDCK cells. Membranes prepared from confluent MCDK cells (*17*) were separated by SDS-PAGE (*18*) and transferred to nitrocellulose (*19*). The Na^+,K^+-ATPase subunit polypeptides were detected by incubating the blot with both subunit-specific monoclonal antibodies (*20*) and then with a goat antibody to mouse immunoglobin G (IgG) conjugated to horseradish peroxidase (HRP) (Sigma). HRP activity was detected by enzyme chemiluminescence (ECL) (Amersham). The positions of the α and β subunits are indicated. (**B** and **C**) Confocal immunofluorescence demonstrating that the sodium pump α and β subunits are basolateral in polarized MDCK cells. MDCK cells grown on 0.45-μm Transwell permeable filter supports (Costar) were fixed for 10 min in methanol at −20°C and processed for immunofluorescence (*20*) with the α-subunit–specific monoclonal antibody and a goat antibody to mouse IgG conjugated to FITC (Boehringer-Mannheim). Samples were analyzed with a Zeiss laser scanning confocal microscope. An image is presented in B. The x–z cross sections of the MDCK monolayers depicted in C averaged eight line scans at each z value with a motor step of 0.2 μm. The apical (AP) and basolateral (BL) surfaces of the monolayer are indicated by arrows.

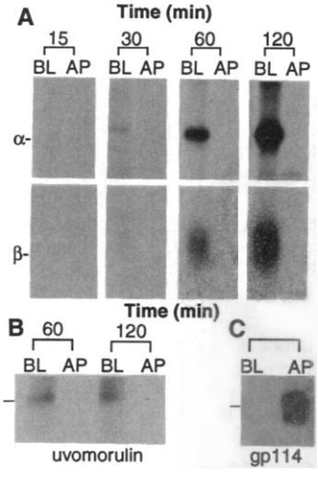

Fig. 2. Delivery of newly synthesized α and β subunits of Na^+,K^+-ATPase to the basolateral surface. Surface delivery of sodium pump subnits (**A**) and uvomorulin (**B**). MDCK monolayers grown to confluence for 5 days on 0.45-μm Transwell filters (Costar) were pulse-labeled for 20 min at 37°C with [^{35}S]methionine from their basolateral surfaces (*4*). At 15, 30, 60, and 120 min after the addition of nonradioactive methionine (10,000 times the amount of [^{35}S]methionine), monolayers were subjected to apical (AP) or basolateral (BL) labeling with NHS-SS-Biotin (Pierce). Biotinylation was done according to a modification of the standard method (*11*) in which the reaction took place at pH 9.0 (*10*) rather than 7.5. The filters were excised and the cells were collected. Cell lysates were subjected to immunoprecipitation with a monoclonal antibody to the α subunit, β subunit (A), or uvomorulin (B) (*19*), and then incubated with avidin-agarose beads (Pierce) (*11*). Proteins recovered were analyzed by SDS-PAGE, fluorography, and densitometric scanning. (**C**) Biotinylation of the apical surface. Unlabeled MDCK monolayers grown on filters were subjected to apical or basolateral biotinylation. Biotinylated proteins were recovered through incubation with avidin-agarose beads, separated by SDS-PAGE (*18*), and transferred to nitrocellulose membranes (*19*). The blot was probed with a monoclonal antibody to the apical protein gp114 (*7*).

We measured the efficiency of sodium pump subunit biotinylation at pH 7.5 or 9.0. MDCK cells grown on permeable filter inserts were biotinylated at their apical and basolateral surfaces under both conditions. Cells were solubilized, and biotinylated proteins were isolated from the cell extract through the addition of avidin-agarose beads. Proteins eluted from the beads were separated by SDS-PAGE and analyzed by protein immunoblotting with the monoclonal antibodies to the α and β subunits. Biotinylation efficiency was determined by comparing the quantity of biotinylated protein recovered to that in serial dilutions of total cell lysates. When biotinylation was done at pH 9.0, about 5% of the total amount of α subunit, in the cell was detected at the basolateral surface (Fig. 3) (*14*). At pH 7.5, less than 0.5% of the α subunit–sodium pump was available to basolateral labeling. Reaction efficiency was about 11% at pH 9.0 and about 1% at pH 7.5 for the β subunit, which contains 25 lysines in its ectodomain (*15*). The amount of sodium pump β subunits avaiable to apical biotinylation was not affected by the pH of the labeling reaction (Fig. 3). Thus, it appears that basolateral biotinylation of the Na^+,K^+-ATPase is inefficient at pH 7.5, whereas labeling at the apical surface (which lacks the impediments presented by the filter and the basement membrane) is no more efficient at pH 9.0 than at pH 7.5. Thus, it is possible that the observations of Hammerton *et al.* (*8*) were affected by the low and differential biotinylation efficencies of basolateral and apical Na^+,K^+-ATPase at pH 7.5.

Hammerton *et al.* solubilized biotinylated MDCK monolayers by adding detergent solutions directly to the apical media compartments of the filter cups (*2*). In our studies, filters were excised from the cups before solubilization. We have found (*10*) that epithlial cells plated on these permeable supports form a confluent monolayer on the filter at the base of the cup and on the walls of the cup facing the apical media compartment, as did Zurzolo and Rodriguez-Boulan in the comment above. The monolayer on the cup wall can extend from the filter to the meniscus of the media bathing the apical compartment. Under standard growing conditions, the monolayer adherent to the cup wall can account for about 40% of the total cell surface area. These cells on the cup wall are accessible to biotinylation only from the apical surface and, because they are growing on plastic rather than on a permeable support, are apt to have reduced biochemical polarity (*16*). By adding their solubilization buffer directly to the intact filter cup, Hammerton *et al.* probably included at least some of these cells from the cup wall in their analysis.

We have used biotinylation to examine the surface delivery of the sodium pump in monolayers harvested by solubilization either before or after excision of the filter from the cup (*10*). When biotinylation was performed at pH 7.5, newly synthesized α subunit of Na^+,K^+-ATPase was detected from the apical side if filters were harvested before excision (Fig. 4). The conditions in this experiment were similar to those of Hammerton *et al.* (*2*), and the experiment appears to reproduce their observation. Little, if any, apical Na^+,K^+-ATPase was observed when the filters were removed from the cup before the solubilization step. Thus, inclusion of the cup wall cells, which can only contribute to the apical signal, may effectively reduce the apparent fraction of sodium pump present at the basolateral surface. When biotinylation was done at pH 9.0, no apical sodium pump was observed, whether or not filters were harvested before excision (Fig. 4). Because we have shown that biotinylation is more efficient at pH 9.0, this result was not expected. However, it can be explained by the Ca^{2+} concentrations present in the biotinylation solutions used under the two different conditions. Biotinylation at pH 7.5 is usually done in the presence of phosphate-buffered saline with a nominal Ca^{2+} concentration of 0.1 mM. The actual concentration of Ca^{2+} may be lower, as a result of precipitation of $CaPO_4$ formed during the preparation of the solution. In contrast, pH 9.0 was obtained with triethanolamine buffer with 2.0 mM Ca^{2+}. It is possible that low Ca^{2+} concentrations disrupted the occluding junctions of the less polarized cells growing on the cup walls, thus allowing apically added NHS-Biotin access to the basolateral sodium pump. This would not occur in the presence of the high Ca^{2+} conditions that prevail during biotinylation with pH 9.0. The difference in accessibility of the basolateral surfaces of the cells on the cup wall under these two conditions might explain the observation that excision exerts an effect at a pH of 7.5, but not at pH 9.0. When biotinylation was done at pH 7.5 in triethanolamine buffer in the presence of 2 mM Ca^{2+}, we did not detect newly synthesized Na^+,K^+-ATPase apically, whether or not the filter was excised before the cells were harvested.

Our results are consistent with those of Caplan *et al.* (*1*) and of Zurzolo and Rodriguez-Boulan. Thus, it would appear that, at least in our strain of MDCK cells, the polarized distribution of the Na^+,K^+-ATPase is the product of biosynthetic sorting. Although cytoskeletal interactions may stabilize the Na^+,K^+-ATPase at the basolateral surface, they appear not to be the sole mechanism in producing the sodium pump's anisotropic distribution in our experiments.

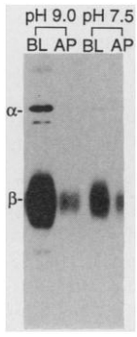

Fig. 3. Effect of pH on biotinylation of α and β membrane proteins. MDCK monolayers grown to confluence on filters for 5 days were labeled with biotin from their apical (AP) or basolateral (BL) surfaces at pH 7.5 or 9.0 (*10, 11*). Filters were excised, monolayers were harvested, and labeled proteins were recovered from cell lysates through incubation with avidin-agarose beads (*11*). Proteins recovered from the beads were separated by SDS-PAGE, transferred to nitrocellulose (*19*), and probed with the antibodies directed against the α and β subunits. Specific binding was visualized by ECL and measured by densitometry.

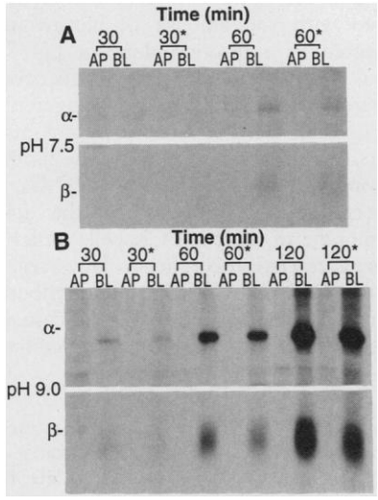

Fig. 4. Effect of filter excision on apparent polarity of sodium pump delivery. MDCK monolayers grown to confluence for 5 days on 0.45-μm Transwell filters (Costar) were pulse-labeled for 20 min at 37°C with [^{35}S]methionine from their basolateral surfaces (*1*) and subjected to chase incubation and surface biotinylation at (**A**) pH 7.5 or (**B**) 9.0 as in Fig. 2. After each chase interval, cells were either harvested from filters through the addition of 1 ml of solubilization buffer (1% NP-40, 150 mM NaCl, 5 mM EDTA, and 50 mM tris, pH 7.4) directly to the cup, or filters were excised from the cups and cells were harvested by suspending the filters in 1 ml of solubilization buffer. Immunoprecipitation and detection of biotinylated α and β proteins was done as in Fig. 2. Lanes corresponding to material prepared from excised filters are indicated by (*).

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Response: We welcome the opportunity to discuss in more detail technical and interpretive aspects of our report (*1*). During the course of our study, we noted that the relatively high amount of the α subunit of Na^+,K^+-ATPase delivered to the apical membrane could result from an underestimate of the amount of protein delivered to the basal-lateral membrane caused by inefficient biotinylation. Before submitting our original study (*1*), we undertook many control studies. We coat Transwell (Costar) filters with a dilute solution of type I collagen (*2*), but have found that tracer macromolecules, [^{35}S]methionine, [^{3}H]inulin, [^{3}H]ouabain, or proteins secreted from MDCK cells [for example, gp81 (*3*)], diffused across the filter and became equilibrated in the two compartments within 90 min regardless of the presence or absence of the collagen coating (*4*). In our initial studies, we labeled surface protein with biotin (*5*), such that the whole length of the lateral membrane from the base of the cell to the tight junction was accessible to the biotinylating reagent. Because cell-cell contacts in these cells are regulated by Ca^{2+}-dependent cell adhesion proteins (*6*), we added low concentrations of trypsin (0.04% w/v) or EGTA (5 mM) to the wash and biotinylation solutions. Under conditions in which tight junction integrity was maintained, we did not detect an increase in the amount of the α subunit of Na^+,K^+-ATPase that was delivered to the basal-lateral membrane. In another attempt, we vigorously stirred the biotinylating solution in the basal-lateral compartment of the filter assembly; again, we were unable to detect an increase in the amount of the α subunit of Na^+,K^+-ATPase on the basal-lateral membrane.

Gottardi and Caplan suggest that we may have underestimated the amount of Na^+,K^+-ATPase in the basal-lateral membrane because of inefficiency of biotinylation at pH 7.4. We have sought to improve the efficiency of biotinylation by increasing the pH of the biotinylating solution from 7.4 to 9.0 (Fig. 1). Under these conditions, we detected increased biotinylation of newly synthesized and steady-state α-subunit Na^+,K^+-ATPase at both the apical and basal-lateral membranes as compared with the amount of biotinylation detected in a reaction done at pH 7.4. However, the ratio of newly synthesized α subunit of Na^+,K^+-ATPase detected at these membrane domains was approximately 1:1, regardless of the pH of the biotinylation solution (*1*). Gottardi and Caplan detected Na^+,K^+-ATPase in the apical membrane of their MDCK cells under either biotinylation condition. However, we found (*1*) that the steady-state distribution of the α subunit of Na^+,K^+-ATPase was restricted to the basal-lateral membrane (>98%), although a slightly increased protein signal was detected on the apical membrane after biotinylation at pH 9 (Fig. 1). Using a procedure similar to that of Gottardi and Caplan, we found the efficiency of biotinylation of Na^+,K^+-ATPase in these experiments to be 30 to 40%. We conclude that our results showing that newly synthesized Na^+,K^+-ATPase is delivered to both the apical and basal-lateral membranes were not a result of low and differential biotinylation efficiencies of basal-lateral and apical Na^+,K^+-ATPase at pH 7.4.

Gottardi and Caplan and Zurzolo and Rodriguez-Boulan find that cells grow on the sides (collar) of the chamber, and suggest that this might create a misleading high estimate of the α subunit of Na^+,K^+-ATPase on the apical cell surface. In the protocol that we used to extract cells (*1*, *3*), only 400 μl of buffer was added to the apical compartment, which covers less than 0.5 mm of the cells on the collar (equivalent to less than 8% of the total surface area of cell growth on the filter); the height of cells covered was determined directly by staining cells in 400 μl of buffer that contained 1% methylene blue under conditions identical to those used for cell extraction.

Both the steady-state distribution and delivery of the newly synthesized α subunit of Na^+,K^+-ATPase were determined in cells that were extracted on filters either attached to the collar (in situ) or after excision from the collar (Fig. 2). There was little or no difference in the patterns of distribution of either newly synthesized or steady-state Na^+,K^+-ATPase (*1*). We also analyzed the amount of steady-state Na^+,K^+-ATPase that was biotinylated on the cells on the collar and found that it was low (Fig. 2, collar); presumably, Na^+,K^+-ATPase is polarized on the basal-lateral

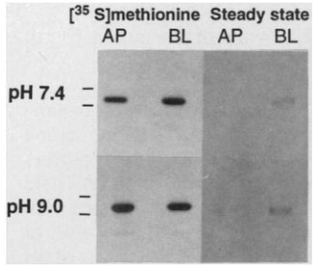

Fig. 1. Detection of [^{35}S]methionine and steady-state α subunit of Na^+,K^+-ATPase at the apical (AP) and basal-lateral (BL) membrane domains of MDCK cells after cell surface biotinylation at pH 7.4 or 9.0. Molecular size markers (top to bottom) are 116 and 97 kD. Confluent monolayers of "contact naïve" MDCK cells were established as in (*2, 4, 17*). Five days after induction of cell-cell contact, cells were metabolically labeled with [^{35}S]methionine for 1 hour (*1*). Cell surface protein was detected by biotinylation with NHS-SS-Biotin (Pierce) in either Hepes-buffered Ringer buffer (pH 7.4) containing 2.25 mM $CaCl_2$, or in a buffer (pH 90) containing 10 mM Na-borate, 154 mM NaCl, 12 mM KCl, and 2.25 mM $CaCl_2$ to capture the first wave of newly synthesized protein that arrived at the cell surface. Cells were extracted (*1*), and Na^+,K^+-ATPase was sequentially precipitated with specific antibody (*1*) and avidin-agarose (Pierce) (*5*). Precipitates were separated by SDS-PAGE and fluorography. For analysis of the steady-state distribution of the α subunit of Na^+,K^+-ATPase, cells on pairs of filters were treated with sulfosuccinimido biotin (Sulfo-NHS-Biotin) (Pierce) at pH 7.4 or 9 as described above [see also (*1*), (*4*)]. Cells were extracted, and Na^+,K^+-ATPase was immunoprecipitated with specific antibody (*1*), separated by SDS-PAGE, and transferred by electrophoresis to nitrocellulose. The biotinylated proteins were detected by probing the nitrocellulose with peroxidase conjugated secondary antibody (Vector Laboratory) and visualized by chemoluminescence (ECL) (Amersham). Autoradiograms were exposed for 18 hours at −70°C. The results represent three independent trails.

membrane of the collar cells, and little is accessible to biotinylated reagent added to the apical side.

In both experiments, we added 2.25 mM of $CaCl_2$ to the biotinylation buffer, and neither buffer contained phosphate-buffered saline that could chelate Ca^{2+}. Thus, our detection of Na^+,K^+-ATPase in the apical membrane appears not to have been the result of opening of tight junctions and subsequent labeling of Na^+,K^+-ATPase in the basal-lateral membrane. We demonstrated that the monolayers were impermeable at all time points by addition of [^{3}H]inulin to the apical or basal-lateral compartment of the filter assembly during the incubation with biotinylating reagent (*1*).

If cells on the collar were creating an artificially high apical membrane signal of the newly synthesized α subunit of Na^+,K^+-ATPase, they would also have contributed to the apical signal of both steady-state Na^+,K^+-ATPase and of other basal-lateral membrane proteins. However, under conditions identical to those used to detect newly synthesized Na^+,K^+-ATPase, we found that less than 1% of steady-state Na^+,K^+-ATPase was detected in the apical membrane after biotinylation at pH 7.4 (Figs. 1 and 2). In a parallel analysis of other basal-lateral proteins in 5-day cultures of cells, we did not detect proteins at steady or newly synthesized states on the apical membrane, even though cells were extracted on filters attached to the collar (*1, 3*). While we agree that cell growth on the filter collar is a possible source of error, our results suggest that it does not contribute to our finding of newly synthesized α subunit of Na^+,K^+-ATPase in the apical membrane of these MDCK cells.

Zurzolo and Rodriguez-Boulan also analyzed the distribution and delivery of Na^+,K^+-ATPase in cells after induction of cell-cell by a Ca^{2+} switch protocol. It is difficult to compare the results with those in (*1*). We have developed a protocol for establishing a preculture of "contact naïve" cells before initiating cell-cell contacts in confluent monolayers of cells (*4*). The generation of this population of contact naïve cells is important because the development of polarity occurs independently without interference from structures or protein complexes that exist in cells that were initially polarized but are degraded during the preculture step. Gottardi and Caplan studied only Na^+,K^+-ATPase sorting after 5 days, and therefore it is not possible to determine whether protein sorting played any role in the generation of cell-surface polarity.

Although our result that the α subunit of Na^+,K^+-ATPase is not sorted in these cells may be unexpected, we have shown (with protocols that are identical to those used in the analysis of Na^+,K^+-ATPase) that other membrane and secreted proteins are polarized at steady state and are targeted from the trans Golgi network (TGN) to the appropriate membrane domain in these MDCK cells. The analysis of these proteins serves as an important internal control and includes proteins restricted to the basal-lateral membrane [E-cadherin (*3*); desmoglein I (*3*); α_3 and β_1 integrins (*7*); laminin (*9*)], or to the apical membrane [gp135 (*3*); gp81 (*3*)]. Surface polarity of cadherins is established before polarized delivery of newly synthesized protein to the basal-lateral membrane; again, we showed that protein delivered to the apical membrane was rapidly removed, while protein delivered to the basal-lateral membrane was retained in a manner similar to that described for Na^+,K^+-ATPase (*1, 3*). These extensive analyses show that our results (*1*) were not a result of artifacts induced by our protocols for either cell surface biotinylation or protein extraction. We restate that one mechanism for generating cell surface polarity of Na^+,K^+-ATPase involves specific retention of protein at the cell surface by binding to the membrane-cytoskeleton (*1–3*). Another mechanism may involve cell type or clone-specific delivery of Na^+,K^+-ATPase to the basal-lateral membrane. Mechanisms of selective retention or sorting in the generation of Na^+,K^+-ATPase polarity are not mutually exclusive.

The two comments above state that sorting and targeting of Na^+,K^+-ATPase is sufficient to explain how the distribution of this protein becomes polarized. However, confocal immunofluorescence microscopy of Na^+,K^+-ATPase distributions in both cell types shows that Na^+,K^+-ATPase does not accumulate in the basal-lateral membrane (figure 1 in the comment by Zurzolo *et al.* and figure 1 in the comment by Gottardi *et al.*). Rather, Na^+,K^+-ATPase is restricted to the *lateral* membrane; this is unlikely to be a result of signal enhancement of the lateral membrane at cell-cell contacts, because incubation of MDCK cells with the fluorescent lipid analog DiI-C_{16} results in equivalent staining of all membranes. In contrast, note that the basal-laterally targeted G protein of vesicular stomatitis virus is distributed equally in both the lateral and basal membranes of these cells (*8*).

In addition, it is not clear whether Na^+,K^+-ATPase is "sorted and targeted" to the basal-lateral membrane in rat thyroid follicle cells, as suggested by Zurzolo and Rodriguez-Boulan (*9*). Other proteins and lipids are sorted differently in rat thyroid follicle cells as compared with MDCK cells (*9*). Although we cannot exclude the possibility that Na^+,K^+-ATPase is directly sorted in rat thryoid follicle cells, it remains a possibility that Na^+,K^+-ATPase is delivered to the cell surface by bulk flow that is predominantly directed to the basal-lateral membrane in these cells.

We suggested (*1*) that Na^+,K^+-ATPase might have a weak affinity for the sorting machinery in our clone of MDCK cells, which results in the delivery of newly synthesized protein to both membrane domains with a slight bias towards the apical membrane. The ratio of bulk flow to the apical and basal-lateral membranes in MDCK cells is 60:40 (*10*). Glycosphingolipids are poorly sorted in our clone of MDCK cells (*11*) compared with the strain II MDCK cell line used by others (*12*). Clustering of glycosphingolipids in the TGN has been

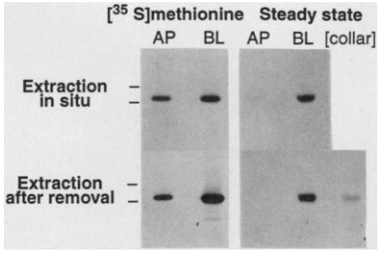

Fig. 2. Distribution of newly synthesized and steady-state Na^+,K^+-ATPase in MDCK cells after protein extraction from cells on filters either attached to the collar (sides of the chamber) (extraction in situ) or after excision of the filter from the collar (extraction after removal). Molecular size markers (top to bottom) are 116 and 97 kD. Confluent monolayers of "contact naïve" MDCK cells were established (*2, 4, 17*). Five days after induction of cell-cell contact, arrival of newly synthesized α subunit of Na^+,K^+-ATPase at the apical (AP) and basal-lateral (BL) membrane domains of cells on pairs of filters was determined as described in Fig. 1, except that cell surface biotinylation was performed at pH 7.4. After biotinylation, cells were extracted in the filter assembly with 400 μl and 900 μl of buffer applied to the apical and basal-lateral compartments, respectively (*1*); alternatively, the filter was removed from the collar with a scalpel, placed in a 35-mm petri dish, and extracted in 1.3 ml of buffer. Cell extracts were processed as described in Fig. 1. For analysis of the steady-state distribution of the α subunit of Na^+,K^+-ATPase at this time, cells on pairs of filters were treated with Sulfo-NHS-Biotin at pH 7.4 (*1, 4*). Filters were extracted either attached to the collar or after excision from the collar as described above; in the latter case, the cells on the collar were also extracted. Na^+,K^+-ATPase was immunoprecipitated with specific antibody (*1*), separated by SDS-PAGE, and electrophoretically transferred to nitrocellulose. The biotinylated proteins were detected by probing the nitrocellulose with peroxidase conjugated secondary antibody (Vector Laboratory) and visualized by chemoluminescence (ECL) (Amersham). Autoradiograms were exposed for 18 hours at −70°C. The results represent three independent trials.

proposed as an important mechanism for sorting apical membrane proteins in MDCK cells (*13*). It is possible that in cells that sort glycosphingolipids, Na^+,K^+-ATPase is excluded from the apical pathway and, hence, is diverted into the basal-lateral pathway; in the absence of glycosphingolipid sorting (such as in our clone of MDCK cells) Na^+,K^+-ATPase may enter both pathways. Side-by-side comparison of sorting of newly synthesized Na^+,K^+-ATPase showed that the α subunit is delivered to the basal-lateral membrane of strain II, but to both apical and basal-lateral membranes of our clone of MDCK cells (*11*).

Finally, analysis of the distribution of Na^+,K^+-ATPase in other polarized cells reveals that mechanisms for sorting it may be different from those described for other proteins. Recent studies by Caplan and co-workers have shown that, in hippocampal neurons, Na^+,K^+-ATPase is localized to both cell body and dendrites and to the axon, whereas other marker proteins are restricted to the axon or dendrites, which are thought to be comparable to the basal-lateral and apical membranes, respectively, of MDCK cells (*14*); Rodriguez-Boulan and co-workers have also shown that in retinal pigmented epithelium, Na^+,K^+-ATPase is localized to the apical membrane with ankyrin and fodrin, but that these cells sort viral glycoproteins in the same direction as in MDCK cells (*15*); in early kidney development, and in tubule cysts from *cpk* mice with congenital recessive polycystic kidney disease, Na^+,K^+-ATPase is localized to both the apical and lateral membranes at steady state (*16*). Taken together, these observations indicate that the mechanisms of generating polarized distributions of Na^+,K^+-ATPase may be more complex than those proposed for single membrane spanning proteins and may involve sorting and retention pathways.

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