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). Hammer-тон 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 plasma-lamellar 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 monolayer 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 Hammer-ton 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 NH₂ form over the NH₃⁺ form and hence proceeds more rapidly at high pH (10). Consequently, we did our cell surface biotinylation at pH 9.0. Hammer-ton 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 base-ment 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 MDCK 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 immunoglobulin G (IgG) conjugated to horseradish peroxidase (HRP) (Sigma). HRP activity was detected by enzyme chemiluminescence (ECL) (Amer sham). 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 subunits (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 [35S]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 [35S]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 available 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 efficiencies 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 epithelial 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). In this condition the experiments 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 Ca2+ 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 Ca2+ concentration of 0.1 mM. The actual concentration of Ca2+ may be lower, as a result of precipitation of CaPO4 formed during the preparation of the solution. In contrast, pH 9.0 was obtained with triethanolamine buffer with 2.0 mM Ca2+. It is possible that low Ca2+ 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 Ca2+ 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 Ca2+, 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.

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REFERENCES AND NOTES
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 underestimation of the amount of protein delivered to the basolateral 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, [35S]methionine, [3H]linulin, [3H]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 Ca²⁺-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 basolateral membrane. In another attempt, we vigorously stirred the biotinylation solution in the basolateral 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 basolateral membrane.

Gottardi and Caplan suggest that we may have underestimated the amount of Na⁺,K⁺-ATPase in the basolateral 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 biotinylation 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 basolateral 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 basolateral 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 basolateral membranes were not a result of low and differential biotinylation efficiencies of basolateral and apical Na⁺,K⁺-ATPase at pH 7.4.

Gottardi and Caplan and Zuzolo 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 basolateral cell surface.
Delivery of Na+,K(+)‐ATPase in polarized epithelial cells
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