

# Sorting of H,K-ATPase $\beta$ -Subunit in MDCK and LLC-PK<sub>1</sub> Cells is Independent of $\mu$ 1B Adaptin Expression

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**The cytoplasmic tail of the H,K-ATPase  $\beta$ -subunit contains a putative tyrosine-based motif that directs the  $\beta$ -subunit's basolateral sorting when it is expressed in Madin-Darby Canine Kidney (MDCK) cells. When expressed in LLC-PK<sub>1</sub> cells, however, the  $\beta$ -subunit is localized to the apical membrane. Several proteins that contain tyrosine-based motifs, including the low-density lipoprotein and transferrin receptors, show a similar sorting 'defect' when expressed in LLC-PK<sub>1</sub> cells. For low-density lipoprotein and transferrin receptors, this behavior is due to the differential expression of the  $\mu$ 1B subunit of the AP-1B clathrin adaptor complex.  $\mu$ 1B is expressed by MDCK cells, but not LLC-PK<sub>1</sub> cells, and transfection of  $\mu$ 1B into LLC-PK<sub>1</sub> cells restores basolateral localization of low-density lipoprotein and transferrin receptors. For the  $\beta$ -subunit, however,  $\mu$ 1B expression in LLC-PK<sub>1</sub> cells does not induce its basolateral expression. We found that the  $\beta$ -subunit interacts with both  $\mu$ 1B and  $\mu$ 1A *in vitro* and *in vivo*. The capacity to participate in a  $\mu$ 1B interaction therefore is not sufficient to program the  $\beta$ -subunit's basolateral localization in MDCK cells. Our data suggest that the H,K-ATPase  $\beta$ -subunit's basolateral sorting signal is either masked in certain epithelial cells, or requires an interaction with sorting machinery other than AP-1B for delivery to the basolateral plasma membrane.**

**Key words:** adaptor protein-1 complex, epithelia, H, K-ATPase  $\beta$ -subunit, polarity, sorting

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The plasma membranes of polarized epithelial cells are divided into apical and basolateral domains. These membrane surfaces are functionally distinct and each contains a different complement of proteins and lipids (1). Protein targeting to the basolateral membrane in polarized epithelial cells is mediated by various sorting signals that are embedded within the cytoplasmic domains of the basolateral proteins themselves (2).

Tyrosine-based motifs constitute a class of sorting signals that are represented in the cytoplasmic domains of many membrane proteins. These motifs are recognized by clathrin adaptor complexes and play important signaling roles in endocytosis, lysosomal targeting and basolateral sorting (3). The tyrosine-based motif is defined by a four-amino acid sequence, YXX $\emptyset$ , where X can be any amino acid and  $\emptyset$  is an amino acid with a bulky hydrophobic side chain (4). The tyrosine in these motifs is crucial for the function of the targeting signal; however, the amino acids that flank the YXX $\emptyset$  domain also participate in interactions with adaptor complexes (5,6).

The intracellular trafficking of the heterodimeric gastric H,K-ATPase is affected by a tyrosine residue in a putative tyrosine-based motif. The gastric H,K-ATPase is composed of two subunits,  $\alpha$  and  $\beta$ . The cytoplasmic tail of the  $\beta$ -subunit includes the sequence 'FRHY'. Although this motif presents the canonical YXX $\emptyset$  amino acid sequence in reverse orientation, and the requirement for the phenylalanine residue has not been verified, previous work has demonstrated that the tyrosine residue is necessary for the regulated endocytosis of the H,K-ATPase  $\beta$ -subunit in both the stomach and the kidney (7,8). The tyrosine residue in this putative tyrosine-based motif also plays a role in protein sorting when the H,K-ATPase  $\beta$ -subunit is expressed in polarized epithelial cells (9). Heterologously expressed H,K-ATPase  $\beta$ -subunit accumulates at the basolateral membrane in the Madin-Darby canine kidney (MDCK) cell line; however, when the tyrosine is mutated to an alanine, a significant percentage of the  $\beta$ -subunit is also localized to the apical membrane (9). This result demonstrates that this tyrosine residue influences not only the internalization but also the basolateral distribution of the H,K-ATPase  $\beta$ -subunit in at least some polarized epithelial cell types.

Although tyrosine-based motifs specify basolateral sorting in MDCK cells, not all epithelial cells are capable of interpreting the sorting information that these motifs contain. MDCK cells exhibit properties more consistent with cells of the renal distal tubule, whereas the LLC-PK<sub>1</sub> renal cell line exhibits properties more consistent with those of renal proximal tubule cells. The H,K-ATPase  $\beta$ -subunit is sorted to the basolateral membrane of MDCK cells, but it is localized to the apical membrane of LLC-PK<sub>1</sub> cells (10). A similar phenomenon is seen with several proteins that contain tyrosine-based motifs, including the low-density lipoprotein (LDL) and transferrin receptors, both of which sort to the basolateral membrane when expressed in

MDCK cells and to the apical membrane when expressed in LLC-PK<sub>1</sub> cells (11–13).

A large body of biochemical work has demonstrated that the information encoded in tyrosine-based motifs can be interpreted by adaptor proteins. Adaptor proteins are heterotetrameric complexes that link clathrin to membrane proteins and play an important role in membrane invagination and protein trafficking (14). The  $\mu$  subunit interacts with tyrosine-based motifs (15). The adaptor protein-1 complex (AP-1) has been implicated in basolateral sorting, based on the observation that vesicles targeted to the basolateral membrane bud from endosomal tubules that are coated with clathrin and AP-1 (16). The AP-1 complex is capable of associating with at least two  $\mu$  subunits,  $\mu$ 1A and  $\mu$ 1B. The  $\mu$ 1A protein is ubiquitously expressed; however,  $\mu$ 1B has a more limited distribution and is primarily expressed in polarized epithelial or exocrine cell-types (17). While  $\mu$ 1B is expressed in several epithelial cell lines, including MDCK cells, it is not expressed in LLC-PK<sub>1</sub> cells (17). These findings suggested that  $\mu$ 1B could be involved in sorting, and that the presence or absence of this protein may account for the differential sorting behaviors exhibited by various polarized epithelial cells (17). In fact, stable expression of  $\mu$ 1B in LLC-PK<sub>1</sub> cells causes a dramatic redistribution of the LDL and transferrin receptors from the apical to the basolateral membrane in these cells (13). This result led to the conclusion that  $\mu$ 1B is an important mediator of basolateral targeting for proteins containing tyrosine-based motifs (13).

To test whether expression of  $\mu$ 1B in LLC-PK<sub>1</sub> cells would alter the apical targeting of the H,K-ATPase  $\beta$ -subunit, we stably transfected LLC-PK<sub>1</sub> cells that express functional levels of  $\mu$ 1B or  $\mu$ 1A with a cDNA encoding the H,K-ATPase  $\beta$ -subunit. Additionally, we tested for possible interactions between the tyrosine-containing motif of the H,K-ATPase  $\beta$ -subunit and  $\mu$ 1B. We found that  $\mu$ 1B expression is not sufficient to alter the distribution of the H,K-ATPase  $\beta$ -subunit polypeptide.

## Results

LLC-PK<sub>1</sub> cell lines that stably express  $\mu$ 1B or  $\mu$ 1A were stably transfected with a cDNA encoding the H,K-ATPase  $\beta$ -subunit. We found that the LLC-PK<sub>1</sub> cell lines that express  $\mu$ 1A do not typically form a well-differentiated monolayer, whereas the LLC-PK<sub>1</sub> cell lines that express  $\mu$ 1B are less likely to grow in multiple layers. These observations are in agreement with the observations of Fölsch et al. and Ohno et al. (13,18). When the  $\beta$ -subunit of the H,K-ATPase is expressed by transfection in cultured cell lines, it is delivered to the cell surface, even in the absence of the H,K-ATPase  $\alpha$ -subunit (19). Confocal immunofluorescence microscopy demonstrates that the expression of  $\mu$ 1B does not induce the basolateral accumulation of the

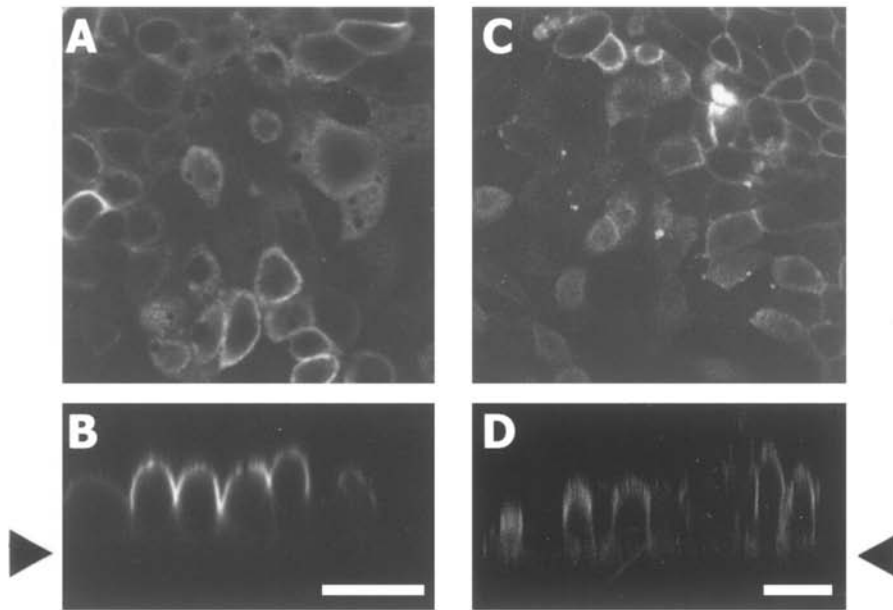
H,K-ATPase  $\beta$ -subunit (Figure 1A, B). Additionally, the H,K-ATPase  $\beta$ -subunit remains localized to the apical membrane in the LLC-PK<sub>1</sub> cells that stably express  $\mu$ 1A (Figure 1C,D). The data presented in Figure 1 suggest that, unlike the LDL and transferrin receptors, the expression of  $\mu$ 1B is not sufficient to redirect the H,K-ATPase  $\beta$ -subunit to the basolateral membrane in LLC-PK<sub>1</sub> cells.

In order to ensure that the cells that are transfected with both  $\mu$ 1B and the H,K-ATPase  $\beta$ -subunit are expressing functional levels of the  $\mu$ 1B adaptor subunit polypeptide, we utilized a defective adenovirus system to super-transfect both cell lines with a cDNA encoding the LDL receptor. Confocal immunofluorescence microscopy indicates that the LDL receptor accumulates at the basolateral membrane of LLC-PK<sub>1</sub> cells that express both  $\mu$ 1B and the H,K-ATPase  $\beta$ -subunit (Figure 1E,F), but the receptor remains at the apical surface of LLC-PK<sub>1</sub> cells that express  $\mu$ 1A and the H,K-ATPase  $\beta$ -subunit (Figure 1G,H). This result confirms that the cell line that is transfected with both  $\mu$ 1B and the H,K-ATPase  $\beta$ -subunit is expressing functional levels of  $\mu$ 1B.

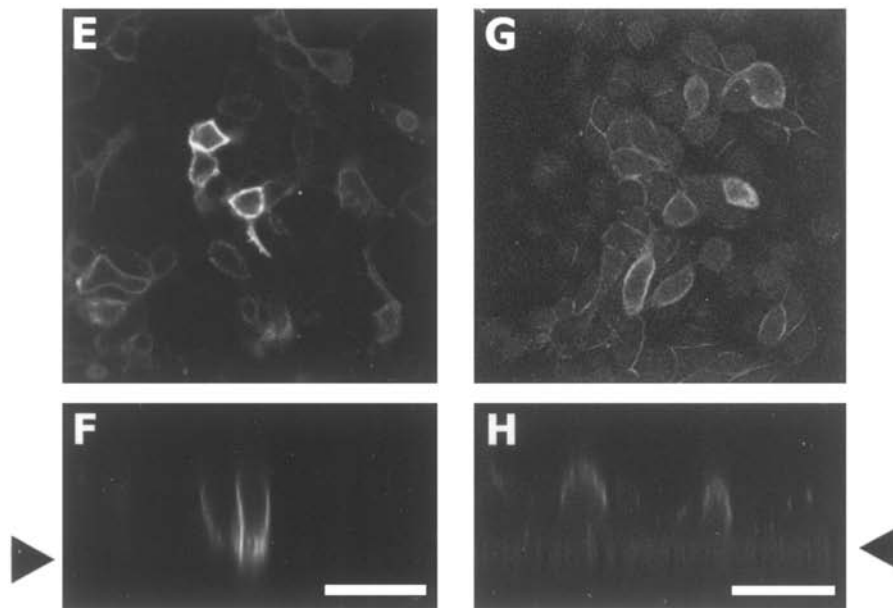
The fact that stable expression of  $\mu$ 1B does not influence the distribution of the H,K-ATPase  $\beta$ -subunit raises the question of whether the  $\mu$ 1B subunit and the H,K-ATPase  $\beta$ -subunit cytoplasmic tail are capable of interacting at all. To facilitate the examination of this issue, we prepared a series of FLAG-tagged  $\mu$ -subunits. The adaptor subunits  $\mu$ 1A,  $\mu$ 1B and  $\mu$ 2 were all tagged internally to ensure that the  $\mu$  subunit can be incorporated into functional adaptor protein complexes (Figure 2A) (20,21). When FLAG-tagged  $\mu$ 1A and  $\mu$ 1B are transiently transfected into COS-7 cells, immunoprecipitation with anti-FLAG antibody precipitates  $\mu$ -subunits as well as the associated  $\gamma$ -subunit of the AP-1 complex (Figure 2B). As expected, immunoprecipitation of lysates of cells transfected with  $\mu$ 2 with anti-FLAG antibody brings down the  $\mu$ 2 subunit, but does not precipitate the  $\gamma$ -subunit since this protein is not present in the adaptor protein-2 complex into which  $\mu$ 2 incorporates. These data demonstrate that immunoprecipitations with the anti-FLAG antibody are specific, and that the tagged  $\mu$ 1A and  $\mu$ 1B subunits are incorporated into fully assembled AP-1 complexes.

To assess the potential for interaction between the cytoplasmic tail of the H,K-ATPase  $\beta$ -subunit and the  $\mu$ -subunits, we employed GST fusion proteins for *in vitro* binding assays (Figure 2C). The  $\beta$ -subunit is a Type II membrane protein; therefore, we generated a construct that contains the N-terminal cytoplasmic residues of the H,K-ATPase  $\beta$ -subunit fused to the N-terminus of GST. Lysate from untransfected COS-7 cells and COS-7 cells transiently transfected with  $\mu$ 1A and  $\mu$ 1B were incubated with the GST fusion protein, and precipitated material was subjected to SDS-PAGE analysis. The resulting blots were probed with anti-FLAG antibody. These data indicate that the  $\mu$ 1A and  $\mu$ 1B proteins are capable of interaction with

LLC-PK<sub>1</sub>:: $\mu$ 1B::HK $\beta$     LLC-PK<sub>1</sub>:: $\mu$ 1A::HK $\beta$



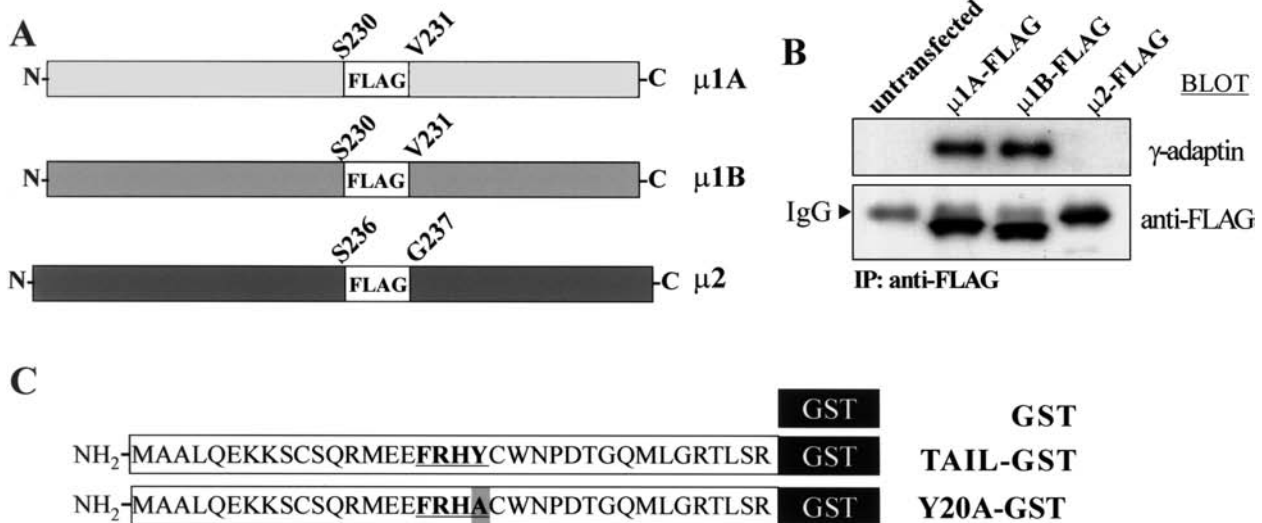
LLC-PK<sub>1</sub>:: $\mu$ 1B::HK $\beta$     LLC-PK<sub>1</sub>:: $\mu$ 1A::HK $\beta$   
LDLr                                    LDLr



**Figure 1: The H,K-ATPase  $\beta$ -subunit is localized to the apical membrane in LLC-PK<sub>1</sub> cells that express functional levels of  $\mu$ 1B or  $\mu$ 1A.** The H,K-ATPase  $\beta$ -subunit is predominantly detected in the apical membranes of LLC-PK<sub>1</sub> cells that stably express  $\mu$ 1B, viewed *en face* and in X/Z cross-section (A and B). The H,K-ATPase  $\beta$ -subunit is also predominantly localized to the apical membrane of LLC-PK<sub>1</sub> cells that stably express  $\mu$ 1A, viewed *en face* and in X/Z cross-section (C and D). LDL receptor is primarily localized to the basolateral membrane of LLC-PK<sub>1</sub> cells that stably express  $\mu$ 1B, viewed *en face* and in X/Z cross-section (E and F). In contrast, LDL receptor is predominantly localized to the apical membrane of LLC-PK<sub>1</sub> cells that stably express  $\mu$ 1A, viewed *en face* and in X/Z cross-section (G and H). Scale bars are equal to 20 $\mu$ m.

the H,K-ATPase  $\beta$ -subunit cytoplasmic tail GST construct (Figure 3A). A  $\gamma$ -adaptin signal is also observed when the tail constructs are incubated with lysate from untransfected cells, demonstrating that endogenous intact AP-1A complex is also capable of interaction with these GST constructs (Figure 3B).

Previous work has demonstrated that the tyrosine residue in the H,K-ATPase  $\beta$ -subunit cytoplasmic tail is required for the appropriate sorting of the  $\beta$ -subunit in MDCK cells (9). To determine whether the tyrosine residue is required for interaction between the  $\beta$ -subunit cytoplasmic tail and the AP-1 complex  $\mu$ -subunits, we generated the Y20A tyrosine



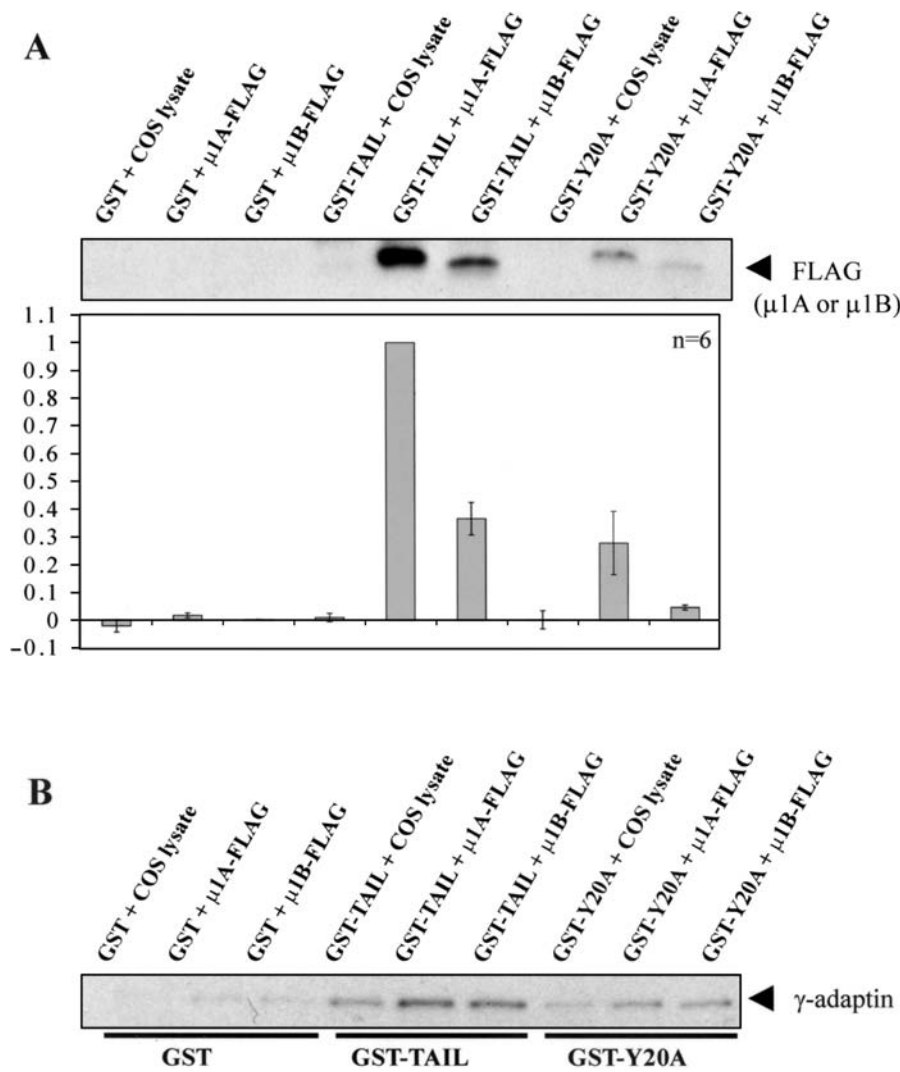
**Figure 2: Schematic depiction of constructs utilized in GST-pulldowns and immunoprecipitations.** A.  $\mu$ 1A,  $\mu$ 1B and  $\mu$ 2 were epitope tagged within the polypeptide chain to ensure that the  $\mu$  subunits can assemble into functional adaptor complexes. B. The FLAG-tagged  $\mu$ 1A,  $\mu$ 1B and  $\mu$ 2 constructs were transfected into COS-7 cells and the cells were lysed in 1% Triton X-100. A postnuclear supernatant was incubated with anti-FLAG antibody, and all transfected  $\mu$ -subunits were immunoprecipitated. Endogenous  $\gamma$ -adapting precipitates only with the  $\mu$ 1A and  $\mu$ 1B constructs. C. N-terminal GST-fusion constructs were generated to investigate whether the AP-1 complex is capable of interacting with the H,K-ATPase  $\beta$ -subunit cytoplasmic tail. The tyrosine-based motif is in bold type.

to alanine mutant tail construct Y20A-GST (Figure 2C). As expected, mutating the tyrosine in the  $\beta$ -subunit cytoplasmic tail markedly reduced its interaction with the AP-1 subunits  $\mu$ 1A and  $\mu$ 1B (Figure 3A). Thus, the tyrosine residue in H,K-ATPase  $\beta$ -subunit cytoplasmic tail is important for the *in vitro* interaction between the  $\beta$ -subunit and the AP-1 complex. The fact that some interaction can be detected between AP-1 proteins and Y20A-GST suggests that this tyrosine based motif is not the only determinant that mediates this interaction. These data indicate that the altered sorting behavior of the  $\beta$ -subunit Y20A mutant in MDCK cells may be attributable in part to its reduced capacity for interaction with the AP-1B complex (9).

In order to test whether the  $\beta$ -subunit and  $\mu$ 1B interact in LLC-PK<sub>1</sub> cells, we prepared cell lines that stably express both the H,K-ATPase  $\beta$ -subunit and  $\mu$ 1A-FLAG or  $\mu$ 1B-FLAG. Immunofluorescence localization experiments performed on subconfluent cells reveal that the H,K-ATPase is found in both intracellular structures and at the cell surface (Figure 4A, a,d,g). As previously shown by Fölsch et al. (20) both  $\mu$ 1A and  $\mu$ 1B are found throughout the cytoplasm and are also localized to the perinuclear region (Figure 4A, e,h). Little overlap between the H,K-ATPase  $\beta$ -subunit and the  $\mu$ -subunits is observed, except for an area of weak colocalization in a perinuclear region (Figures 4A,f,i). Strong colocalization of these proteins is not expected, since the interaction between the  $\beta$ -subunit and the  $\mu$ -subunits is likely to be transient. Similar results are obtained for the tyrosine to alanine mutant H,K-ATPase  $\beta$ -subunit Y20A and  $\mu$ 1A-FLAG or  $\mu$ 1B-FLAG (data not shown).

We also determined the polarity of the  $\beta$ -subunit in LLC-PK<sub>1</sub> cells expressing the FLAG-tagged  $\mu$ 1 subunit constructs. Confocal immunofluorescence microscopy demonstrates that the H,K-ATPase  $\beta$ -subunit remains localized to the apical membrane (Figure 4B, a,b). As expected, the H,K-ATPase  $\beta$ -subunit is also localized to the apical membrane in a control cell line that expresses a  $\mu$ 1A-FLAG (Figures 4B, c,d).

We tested whether the H,K-ATPase  $\beta$ -subunit and the  $\mu$ 1 subunits interact in LLC-PK<sub>1</sub> cells by performing immunoprecipitation experiments. The H,K-ATPase  $\beta$ -subunit exhibits two major forms in Western blot analysis as a result of post-translational modifications. The H,K-ATPase  $\beta$ -subunit has seven N-glycosylation sites, all of which are subject to modification with carbohydrate chains (22). Asano et al. have demonstrated that in HEK cells transfected with the  $\beta$ -subunit, bands with a molecular mass of approximately 40–50 kDa ( $\beta_c$ ) can be digested with Endo H, and are thus likely to represent H,K-ATPase  $\beta$ -subunit bearing core high mannose modification of its N-glycosylation sites (22). Asano et al. also demonstrated that the mature higher molecular weight form of the H,K-ATPase  $\beta$ -subunit ( $\beta_m$ ) is modified with complex carbohydrates. We confirmed these data for LLC-PK<sub>1</sub> cells by treating cell lysate with endoglycosidase H and peptide:N-glycosidase F (data not shown). LLC-PK<sub>1</sub> cells stably transfected with both the  $\beta$ -subunit and FLAG-tagged  $\mu$ 1A or  $\mu$ 1B subunits were lysed, and the lysates were immunoprecipitated with anti-FLAG antibody. When the immunoprecipitated material was blotted with antibody



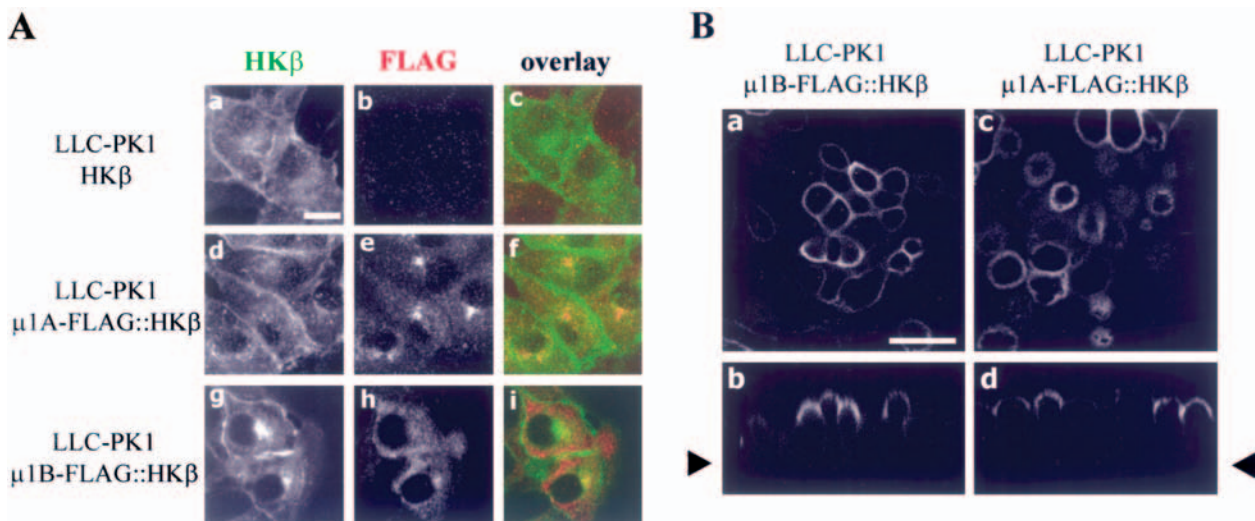
**Figure 3: Motifs within the cytoplasmic N-terminal tail of the H,K-ATPase  $\beta$ -subunit are capable of interacting with AP-1  $\mu$ -subunits.**

A, Untransfected COS-7 cells as well as COS-7 cells transiently transfected with  $\mu$ 1A and  $\mu$ 1B were lysed in 1% Triton X-100. Glutathione beads charged with equal amounts of GST, TAIL-GST or Y20A-GST were incubated with lysate from untransfected COS-7 cells, COS-7 cells transfected with  $\mu$ 1A-FLAG or COS-7 cells transfected with  $\mu$ 1B-FLAG. Proteins were eluted with reducing sample buffer and subjected to SDS-PAGE analysis. The resulting blots were probed with anti-FLAG antibody, and signal intensity from each band was quantified by densitometry. FLAG-tagged  $\mu$ 1A and FLAG-tagged  $\mu$ 1B interact with the TAIL-GST construct. Substitution of the tyrosine residue in TAIL-GST with an alanine residue in Y20A-GST reduces but does not prevent its interaction with the  $\mu$ 1 subunits. B, Blots were also probed with anti- $\gamma$ -adapatin antibody. The native AP-1 complex interacts with the TAIL-GST construct and, to a lesser extent, Y20A-GST.

directed against the H,K-ATPase  $\beta$ -subunit, we observed that only a relatively small amount of mature  $\beta$ -subunit coprecipitates with the  $\mu$ 1 subunits (Figure 5A). The observation that  $\mu$ 1B interacts fairly modestly *in vivo* with fully glycosylated  $\beta$ -subunit is consistent with the interpretation that  $\mu$ 1B does not participate in an extensive stable interaction with the H,K-ATPase  $\beta$ -subunit during or after the late stages of its postsynthetic processing. We also observed that the  $\mu$ 1A subunit demonstrates a pronounced interaction with the  $\beta_c$  subunit. The interaction between the H,K-ATPase  $\beta$ -subunit and  $\mu$ 1A appears to be more extensive than the interaction between the  $\beta$ -subunit and  $\mu$ 1B. It must be noted, however, that these experiments do not allow us to draw conclusions concerning the relative affinities of these interaction partners. In agreement with our GST-pulldown data, we find that mutation of the tyrosine residue in the H,K-ATPase  $\beta$ -subunit cytoplasmic tail reduces, but does not completely prevent, the association between the  $\beta$ -subunit and  $\mu$ 1A or  $\mu$ 1B. Immunoprecipitated material was also blotted with anti-FLAG antibodies in

order to visualize precipitated  $\mu$ 1B and with anti- $\gamma$ -adapatin antibody to ensure that FLAG-tagged  $\mu$ 1B subunit is incorporated into the AP-1 complex (Figure 5B).

The influence of AP-1B on the steady-state basolateral distribution of the LDL receptor may in part be associated with a potential role for this adaptor subunit in postendocytic sorting (23). Thus, the form of the  $\beta$ -subunit which undergoes postendocytic recycling may need to interact with AP-1B for expression of the  $\mu$ 1B subunit to affect the subcellular localization of the  $\beta$ -subunit. We biotinylated the surface population of H,K-ATPase  $\beta$ -subunit, allowed the protein to undergo internalization, and subsequently stripped the biotin remaining at the surface of the cell by treatment with the membrane-impermeant reducing agent 2-mercaptoethanesulfonic acid (MesNa). We then precipitated the biotinylated material with streptavidin beads to isolate surface proteins that were internalized and protected from the MesNa reagent. This experiment was performed on LLC-PK<sub>1</sub> cell lines stably transfected with the H,K-ATPase  $\beta$ -subunit alone or doubly transfected



**Figure 4: Localization of the H,K-ATPase  $\beta$ -subunit,  $\mu$ 1A and  $\mu$ 1B in stably transfected subconfluent LLC-PK<sub>1</sub> cells.** A, LLC-PK<sub>1</sub> cells were stably transfected with the H,K-ATPase  $\beta$ -subunit alone (a-c), the H,K-ATPase  $\beta$ -subunit and  $\mu$ 1A-FLAG (d-f) or the H,K-ATPase  $\beta$ -subunit and  $\mu$ 1B-FLAG (g-i). The  $\beta$ -subunit was detected with anti-H,K-ATPase  $\beta$ -subunit monoclonal antibody (green), and  $\mu$ 1 constructs were detected with an anti-FLAG polyclonal antibody (red). The H,K-ATPase  $\beta$ -subunit is localized to the plasma membrane and to intracellular vesicles. Both  $\mu$ 1 constructs are distributed diffusely in an intracellular pattern and are also found in the perinuclear region. Some overlap is seen between the staining patterns of the  $\beta$ -subunit and the  $\mu$ 1 subunits in this perinuclear region. A similar pattern is observed for the tyrosine to alanine H,K-ATPase  $\beta$ -subunit construct (data not shown). Scale bar is equal to 10  $\mu$ m. B, The H,K-ATPase  $\beta$ -subunit is also detected in the apical membranes of LLC-PK<sub>1</sub> cells that stably express  $\beta$ -subunit and  $\mu$ 1B-FLAG, viewed *en face* and in X/Z cross-section (a and b). Similarly, the H,K-ATPase  $\beta$ -subunit is predominantly localized to the apical membranes of LLC-PK<sub>1</sub> cells that stably express  $\beta$ -subunit and  $\mu$ 1A-FLAG, viewed *en face* and in X/Z cross-section (c and d). Scale bar is equal to 20  $\mu$ m.

with the  $\beta$ -subunit and  $\mu$ 1A or the  $\beta$ -subunit and  $\mu$ 1B (Figure 6). These experiments demonstrate that only the fully glycosylated form of the  $\beta$ -subunit is delivered to the cell surface and is subsequently internalized in these cell lines. Therefore, it is only the mature  $\beta_m$  form of the H,K-ATPase  $\beta$ -subunit that is available to participate in post-endocytic recycling. Thus, the fact that relatively little  $\beta_m$  coprecipitates with  $\mu$ 1B suggests that  $\mu$ 1B is not likely to play a substantial role in H,K-ATPase  $\beta$ -subunit recycling.

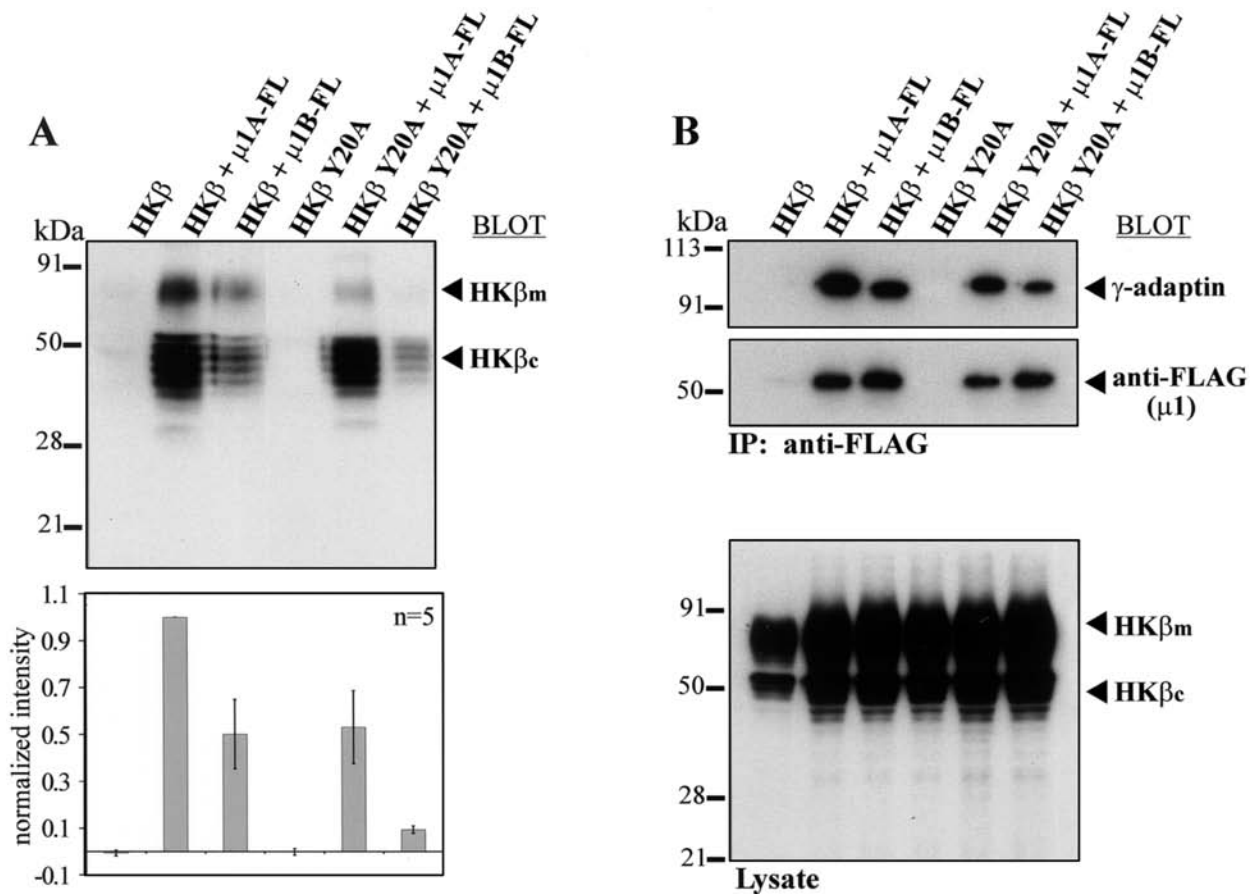
We also performed experiments to further characterize the effect of  $\mu$ 1B expression on the postendocytic recycling of the H,K-ATPase  $\beta$ -subunit. We utilized LLC-PK<sub>1</sub> cell lines that express the H,K-ATPase  $\beta$ -subunit alone and cells lines that express both the  $\beta$ -subunit and  $\mu$ 1A or the  $\beta$ -subunit and  $\mu$ 1B. We surface biotinylated the cells and one dish from each cell line was reserved. The remaining dishes were incubated at 37°C for 30 min and biotin remaining at the cell surface was stripped with MesNa. One dish from each cell line was reserved at this point, and the remaining dishes were returned to 37°C for either 10 or 30 min, followed by a second treatment with MesNa. In these dishes, any  $\beta$ -subunit that was initially internalized and protected from the MesNa and subsequently returned to the cell surface is stripped of its biotin. Very little  $\beta$ -subunit is precipitated by streptavidin beads after the second incubation at 37°C; therefore, much of the internalized H,K-ATPase  $\beta$ -subunit is rapidly returned to the cell sur-

face. The same result was obtained for all cell lines. Thus, expression of  $\mu$ 1B does not appear to affect the rate of cell surface recycling of the H,K-ATPase  $\beta$ -subunit.

## Discussion

Previous work has demonstrated that the H,K-ATPase  $\beta$ -subunit is sorted differentially in MDCK and LLC-PK<sub>1</sub> cells (9). Additionally, it has been established that the H,K-ATPase  $\beta$ -subunit contains a tyrosine residue that is necessary for its basolateral sorting in MDCK cells (9). In the current study we found that, in contrast to the behaviors of the LDL and transferrin receptors, expression of  $\mu$ 1B does not alter sorting of the H,K-ATPase  $\beta$ -subunit in LLC-PK<sub>1</sub> cells. This lack of influence on H,K-ATPase  $\beta$ -subunit sorting is observed despite the fact that  $\mu$ 1B is capable of interacting with the H,K-ATPase  $\beta$ -subunit in this cell line. Interestingly, the  $\beta$ -subunit tyrosine residue that is critical for this protein's basolateral localization in MDCK cells is important, but not absolutely required for, the interaction between the  $\beta$ -subunit cytoplasmic tail and AP-1B (9).

One possible explanation for the insensitivity of H,K-ATPase  $\beta$ -subunit sorting to  $\mu$ 1B expression is that the steady-state colocalization of the H,K-ATPase  $\beta$ -subunit may be effectively determined by clathrin-independent pathways. Such clathrin-independent pathways could



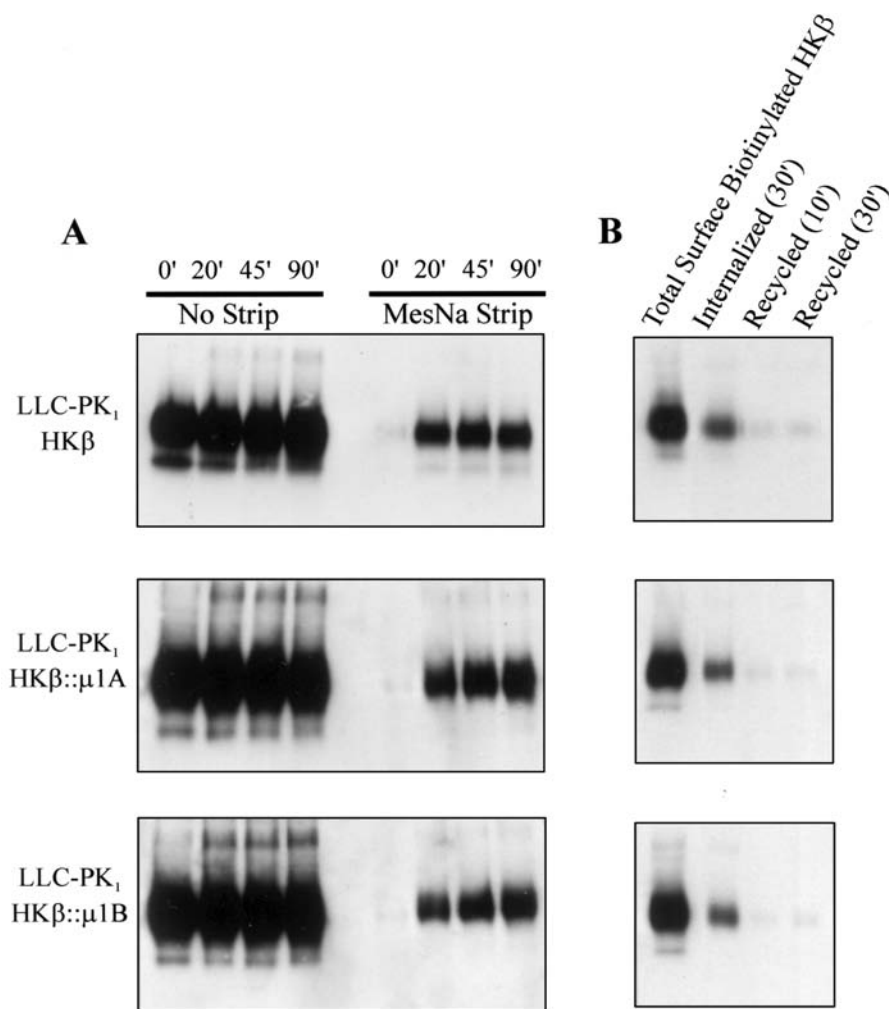
**Figure 5: The H,K-ATPase  $\beta$ -subunit interacts with  $\mu$ 1B in LLC-PK<sub>1</sub> cells.** A, LLC-PK<sub>1</sub> cells stably expressing the H,K-ATPase  $\beta$ -subunit alone or the tyrosine to alanine mutant  $\beta$ -subunit alone (HK $\beta$  alone, HK $\beta$ Y20A alone), the  $\beta$ -subunit constructs and  $\mu$ 1A-FLAG (HK $\beta$  +  $\mu$ 1A-FLAG, HK $\beta$ Y20A +  $\mu$ 1A-FLAG) or the  $\beta$ -subunit constructs and  $\mu$ 1B-FLAG (HK $\beta$  +  $\mu$ 1B-FLAG, HK $\beta$ Y20A +  $\mu$ 1B-FLAG) were lysed in 1% Triton X-100. Lysate was immunoprecipitated with anti-FLAG antibody. Precipitated proteins were blotted with anti-H,K-ATPase  $\beta$ -subunit antibody. The H,K-ATPase  $\beta$ -subunit coprecipitates with anti-FLAG antibody in cells that express either of the  $\mu$ -subunits along with the H,K-ATPase  $\beta$ -subunit. The H,K-ATPase  $\beta$ -subunit is not immunoprecipitated by anti-FLAG antibody in cells that express  $\beta$ -subunit alone. The results of five independent experiments were quantified by densitometry. B, Precipitated proteins were also blotted with anti- $\gamma$ -subunit and anti-FLAG antibodies. Endogenous  $\gamma$ -adaptin and the  $\mu$ 1-FLAG adaptins precipitate with anti-FLAG antibody in cells that express either of the  $\mu$ -subunits. Endogenous  $\gamma$ -adaptin and both  $\mu$ 1-FLAG adaptins are not immunoprecipitated by anti-FLAG antibody in cells that express  $\beta$ -subunit alone. 0.5% of the total lysate from each immunoprecipitation was subjected to SDS-PAGE and blotted with anti-H,K-ATPase  $\beta$ -subunit antibody. All cell lines express  $\beta$ -subunit.

potentially include selective retention of the H,K-ATPase  $\beta$ -subunit by cytoskeletal elements associated with distinct domains of the plasma membranes of MDCK and LLC-PK<sub>1</sub> cells. For instance, the H,K-ATPase  $\beta$ -subunit may be initially targeted to the basolateral membrane via  $\mu$ 1B-mediated interactions, but may then become stabilized at the apical membrane via cytoskeletal-mediated interactions following transcytosis.

Apical sorting of the H,K-ATPase  $\beta$ -subunit in LLC-PK<sub>1</sub> cells may also be attributable to the presence of active apical sorting information in this protein that could override the effects of  $\mu$ 1B expression. The endocytic receptor megalin contains a YXX $\Phi$  motif, but is sorted to the apical membrane in MDCK cells, presumably due to a dominant apical

sorting signal in its cytoplasmic tail (24). This scenario seems unlikely to apply to the H,K-ATPase  $\beta$ -subunit, since when the  $\beta$ -subunit is coexpressed with its normal interaction partners, it does not exert any dominant effects upon polarized sorting. For example, an Na,K/H,K-ATPase  $\alpha$ -subunit chimera accumulates at the basolateral surface of LLC-PK<sub>1</sub> cells. The H,K-ATPase  $\beta$ -subunit assembles with and follows this chimera to the basolateral membrane. Thus, the basolateral signal in the Na,K-ATPase  $\alpha$ -subunit masks or is dominant over any apical sorting information contained in the H,K-ATPase  $\beta$ -subunit (25).

The H,K-ATPase  $\beta$ -subunit associates with a number of proteins that may affect its sorting, and it is possible that the signals present in these partners could prevail over the



**Figure 6: H,K-ATPase  $\beta$ -subunit modified with complex carbohydrates is present at the plasma membrane and is endocytosed from the cell surface.** A, LLC-PK<sub>1</sub> cells stably transfected with the H,K-ATPase  $\beta$ -subunit alone, the  $\beta$ -subunit and  $\mu$ 1A-FLAG or the  $\beta$ -subunit and  $\mu$ 1B-FLAG were surface-labeled with NHS-SS-biotin. After biotinylation, cells were incubated at 37 °C to allow internalization of surface proteins for the indicated lengths of time. The cells were then cooled to 4 °C and one dish from each time-point was subjected to a MesNa strip. All cells were lysed in 1% Triton X-100 and incubated overnight with streptavidin-conjugated agarose beads. Samples were analyzed by SDS-PAGE and probed with anti-H,K-ATPase  $\beta$ -subunit monoclonal antibody. Only the  $\beta_m$  form of the  $\beta$ -subunit is endocytosed from the cell surface. B, LLC-PK<sub>1</sub> cells stably transfected with the H,K-ATPase  $\beta$ -subunit alone, the  $\beta$ -subunit and  $\mu$ 1A-FLAG or the  $\beta$ -subunit and  $\mu$ 1B-FLAG were surface-labeled with NHS-SS-biotin. Four dishes of each cell type were biotinylated. One dish of each cell type was reserved (Total Surface Biotinylated HK $\beta$ ) and the remaining cells were incubated at 37 °C to allow internalization of surface proteins for 30 min. The dishes were then cooled to 4 °C and subjected to a MesNa strip. One dish of each cell type was reserved (Internalized (30')) and the remaining dishes were incubated at 37 °C for either 10 min or 30 min and treated with MesNa for a second time (Recycled (10') or Recycled (30')). All cells were lysed in 1% Triton X-100 and incubated overnight with streptavidin-conjugated agarose beads. Samples were analyzed by SDS-PAGE and probed with anti-H,K-ATPase  $\beta$ -subunit monoclonal antibody. In all cell lines the majority of the  $\beta$ -subunit that was internalized in the first 37 °C incubation is returned to the cell surface after a second 37 °C incubation.

sorting influence exerted through the  $\mu$ 1B interaction. For instance, our immunoprecipitation data suggest that the H,K-ATPase  $\beta$ -subunit may associate more robustly with the  $\mu$ 1A AP-1 subunit than with the  $\mu$ 1B AP-1 subunit. This is not the case for the LDL receptor, which exhibits a greater propensity for association with AP-1B than with AP-1A (20). These data suggest that mere association with AP-1B is not sufficient for basolateral distribution of a

protein, and that differential affinities for the two AP-1 complexes may be important in determining the ultimate distributions of their associated cargoes.

Another possibility is that a different adaptor complex could direct the intracellular sorting of the H,K-ATPase  $\beta$ -subunit. One potential suspect is adaptor complex-4. Simmen et al. demonstrated that this adaptor complex is involved in the

basolateral sorting of LDL receptor and MPR46 in MDCK cells (26). It is not known whether the adaptor complex-4 is functionally expressed in LLC-PK<sub>1</sub> cells. Were it to be absent from this cell line, such a difference could conceivably account for the distinct sorting properties of the H,K-ATPase  $\beta$ -subunit in MDCK and LLC-PK<sub>1</sub> cells.

We have found that the H,K-ATPase  $\beta$ -subunit associates with proteins other than adaptor complexes, and these partners may also influence the sorting of the  $\beta$ -subunit. The  $\beta$ -subunit forms a complex with the tetraspanin CD63, most likely through interactions involving the transmembrane or extracellular domains of these proteins (27). CD63 has a tyrosine-based motif in its cytoplasmic tail that directs its sorting to the late endosomal/lysosomal compartment through interactions with the adaptor protein-2 and adaptor protein-3 complexes (28). We found that  $\beta$ -subunit that is associated with CD63 is also delivered to a CD63-positive intracellular compartment (27). Since CD63 is capable of recruiting adaptor complexes through its own tyrosine-based motif, the association between CD63 and the H,K-ATPase  $\beta$ -subunit may modify the interpretation of the  $\beta$ -subunit's tyrosine signal. Thus, the sorting consequences of the H,K-ATPase  $\beta$ -subunit's capacity to assemble with  $\mu$ 1B may not be simple and straightforward. The presence of two tyrosine motifs and multiple adaptor complexes in association with the H,K-ATPase  $\beta$ -subunit/CD63 pair might suppress or alter the basolateral targeting directive conferred by  $\mu$ 1B assembly.

It is also possible that  $\mu$ 1B expression does not direct basolateral distribution of the  $\beta$ -subunit because  $\mu$ 1B does not appear to manifest a substantial interaction with the mature, fully glycosylated  $\beta$ -subunit. The fully glycosylated  $\beta_m$  forms of the H,K-ATPase  $\beta$ -subunit reach the plasma membrane of stably transfected LLC-PK<sub>1</sub> cells, and are subsequently endocytosed from cell surface. It has been shown that  $\mu$ 1B may contribute to the basolateral steady-state localization of the LDL receptor by facilitating its basolateral recycling following endocytosis (23). Thus, the form of the  $\beta$ -subunit that undergoes postendocytic recycling may not be functionally available for interaction with AP-1B, thereby preventing  $\mu$ 1B expression from influencing at least this component of the processes that determine the  $\beta$ -subunit's localization.

Finally, additional and as yet unidentified molecular mechanisms may participate in interpreting basolateral sorting signals associated with tyrosine-based motifs. The tyrosine-based motif YDEV directs the basolateral targeting of a CD8/kidney anion exchanger chimera in LLC-PK<sub>1</sub> cells, despite the fact that these cells lack  $\mu$ 1B expression (29). Other cells which do not express  $\mu$ 1B, including neurons and hepatocytes, are capable of sorting proteins that contain tyrosine-based motifs, suggesting that molecular mechanisms other than the  $\mu$ 1B pathway can and must participate in basolateral sorting (17). The availability of a diverse set of signaling and trafficking

mechanisms is almost certainly beneficial to epithelial cells, given the variety of physiologic tasks that these cells must accomplish (2). Thus, a single mechanism for sorting proteins which contain a tyrosine-based motif to the basolateral membrane in polarized epithelial cells is unlikely to meet the needs of all such cell types.

## Materials and Methods

### Antibodies and reagents

Rabbit gastric H,K-ATPase  $\beta$ -subunit cDNA was kindly provided by G. Sachs (University of California, Los Angeles). Mouse adaptor complex  $\mu$ 1A and  $\mu$ 2 cDNA, as well as human  $\mu$ 1B cDNA were the generous gifts of J. Bonifacino (NIH). The H,K-ATPase  $\beta$ -subunit tyrosine to alanine mutant was prepared as described previously (9). The pEBG vector was obtained from L. Ptaszek and D. Schatz (Yale University). Adenovirus encoding for LDL receptor was the kind gift of J. Wilson (University of Pennsylvania). Anti-H,K-ATPase  $\beta$ -subunit monoclonal antibody was kindly provided by J. Forte (University of California, Berkeley). Anti-LDL receptor antibody hybridoma C7 was purchased from the ATCC. Monoclonal and polyclonal anti-FLAG antibodies, as well as anti  $\gamma$ -adaplin antibodies (clone 100/3) were purchased from Sigma (St. Louis, MO).

### Molecular biology

H,K-ATPase  $\beta$ -subunit cDNA was subcloned into the mammalian expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA) using the *Xba* I and *Kpn* I restriction sites. This construct was generated in both hygromycin and neomycin resistance vectors.

FLAG-tagged  $\mu$ 1A,  $\mu$ 1B and  $\mu$ 2 were generated by sequential polymerase chain reactions (PCR). The 5' terminus of  $\mu$ 1A was amplified using  $\mu$ 1A as a template with the N-terminal primer 5'-AAAAGGATCCGCGATGTCCGCCAGCGCCGTC-3' and the '3' internal FLAG' primer 5'-CTTGTCGTATCGTCTTTGTAGTCTGACTTGCTCTCCCTCGGC-3'. The 3' terminus of  $\mu$ 1A was amplified using  $\mu$ 1A as a template with the C-terminal primer 5'-ACCGCTCGAGCTTACTACTGGGTCCGGAGCTGATAATCTC-3' and a '5' internal FLAG' primer 5'-GACTACAAAGACGATGACGA-CAAGGTGGAGCTGGAGGATGTGAAATTC-3'. The amplified products of these two PCR reactions were mixed together in a 1:1 ratio and used as a template in a final PCR reaction. In this final reaction, the complete FLAG-tagged  $\mu$ 1A construct was generated using the previously described mixture as template with the N-terminal primer 5'-AAAAGGATCCGCGATGTCCGCCAGCGCCGTC-3' and the C-terminal primer 5'-ACCGCTCGAGCTTACTACTGGGTCCGGAGCTGATAATCTC-3'. The PCR fragment was cloned into pcDNA3.1 (hygromycin resistance) using *Bam* HI and *Xho* I. FLAG-tagged  $\mu$ 1B was generated using the same sequential PCR procedure, using  $\mu$ 1B as the template in the first

PCR reaction with the N-terminal primer 5'-CCCAAGC-TTGGGATGTCGCCCTCGGCTGTCTTCATTCTGGACGTTA-AGG-3', the C-terminal primer 5'-ACCGCTCGAGCTTACT-AGCTGGTACGAAGTTGGTAATCGC-3', the '3' internal FLAG' primer 5'-CTTGTCGTCATCGTCTTTGTAGTCTGA-TTTGTTCTTGCTGCGGCC-3' and the '5' internal FLAG' primer 5'-GACTACAAAGACGATGACGACAAGGTAGAGC-TGGAGGATGTAAAATTCCAC-3'. The PCR fragment was cloned into pcDNA3.1 (hygromycin resistance) using the *Hind* III and *Xho* I restriction sites. FLAG-tagged  $\mu 2$  was also generated using the same sequential PCR procedure, using  $\mu 2$  as the template in the first PCR reaction with the N-terminal primer 5'-CCCAAGCTTGGGAT-GATCGGAGGCTTATTCATCTATAATC-3', the C-terminal primer 5'-ACCGCTCGAGCTTACTAGCAGCGGGTTCA-TAAATGC-3', the '3' internal FLAG' primer 5'-CTTGTCG-TCATCGTCTTTGTAGTCACTCTTGCTTGTTCATCAGCTGT-GC-3' and the '5' internal FLAG' primer 5'-GACTA-CAAAGACGATGACGACAAGGGTAAGCAGTCGATCGCCA-TTGA-3'. The PCR fragment was cloned into pcDNA3.1 (hygromycin resistance) using the *Hind* III and *Xho* I restriction sites.

The N-terminal glutathione S-transferase (GST) fusion protein vector was generated by amplifying GST from the pEBG vector (30) with the N-terminal primer 5'-GCATGCGGCCG-GATGGCCCCTATACTAGGTTAT-3' and the C-terminal primer 5'-CTAGCTCGAGTCACTATTAACGCGGAACCCAGATTT-3'. The product was cloned into pET22b<sup>+</sup> (Novagen/EMD Biosciences, La Jolla, CA) using *Not* I and *Xho* I. In order to create the GST fusion proteins, oligonucleotides were annealed and the double-stranded product was ligated into the modified pET22b<sup>+</sup> vector using *Nde* I and *Not* I. The constructs utilized the following pairs of oligonucleotides: H,K-ATPase  $\beta$ -subunit tail, 5'-TATGGCCGCTTGCAGGAGAAGAAGTCGTGCAGCCA-GCGCATGGAGGAGTCCGCCACTACTGCTGGAACCCGG-ACACGGGGCAGATGCTGGGCCGACCCCTGTCTAGAGC-3' and 5'-GGCCGCTCTAGACAGGGTGCAGGCCAGCATCTGC-CCCGTGTCCGGGTTCCAGCAGTAGTGCGGAACTCCTCC-ATGCGTGGCTGCACGACTTCTTCTCCTGCAAGGCGGC-CA-3'; H, K-ATPase  $\beta$ -subunit tail tyrosine to alanine mutant 5'-TATGGCCGCTTGCAGGAGAAGAAGTCGTGC-AGCCAGCGCATGGAGGAGTCCGCCACGCCTGTGGAA-CCCGACACGGGGCAGATGCTGGGCCGACCCCTGTCTA-GAGC-3' and 5'-GGCCGCTCTAGACAGGGTGCAGGCCAG-CATCTGCCCCGTGTCCGGGTTCCAGCAGGCGTGCGGGA-ACTCCTCCATGCGCTGGCTGCACGACTTCTTCTCCTGCA-AGGCGGCCA-3'.

All constructs and PCR products were sequenced to ensure that errors were not introduced during amplification or other manipulations.

#### **GST fusion protein preparation**

The *Escherichia coli* strain BL21(DE3) (Novagen/EMD Biosciences) was transformed with GST constructs. A single colony was grown overnight in 50 ml of LB media

with ampicillin (100  $\mu$ g/ml). This culture was used to inoculate 500 ml of LB with ampicillin to an A600 of 0.1. The bacterial cells were grown at 37 °C until an A600 of 0.8–1.0 was attained, protein synthesis was induced with 0.1 mM isopropyl- $\beta$ -D-thiogalactopyranoside (American Bioanalytical, Natick, MA), and the cells were grown for an additional 4 h at 30 °C. Bacteria were then collected by centrifugation (5000  $\times g$  for 15 min) and resuspended in ice-cold phosphate buffered saline (PBS; 150 mM NaCl, 15 mM NaH<sub>2</sub>PO<sub>4</sub>, 85 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4) to a final volume of 28.5 ml. The cells were lysed by sonication in the presence of a protease inhibitor mixture (100  $\mu$ g/ml phenylmethylsulfonyl fluoride and 1  $\mu$ g/ml each of aprotinin, leupeptin and pepstatin A) until the suspension cleared. To the suspension was added 1.5 ml 20% Triton X-100 in PBS, and the mixture was allowed to incubate for 30 min at 4 °C. Soluble proteins were separated from cellular debris by centrifugation (10 000  $\times g$  for 30 min). The amount of GST fusion protein in each preparation was determined by incubating 20  $\mu$ l glutathione-Sepharose 4B (Amersham Biosciences, Piscataway, NJ) with a dilution series of the cleared lysate, followed by SDS-PAGE analysis and visualization with a Coomassie stain.

To perform the GST fusion protein pulldown experiments, glutathione-Sepharose beads were charged with equivalent amounts of the various fusion proteins by incubating 10  $\mu$ l beads with bacterial cell lysate overnight at 4 °C. The beads were washed 3 times with PBS to remove unbound material. To prepare COS-7 cell lysate, transiently transfected COS-7 cells were incubated in lysis buffer (1% Triton X-100, 5 mM MgCl<sub>2</sub>, 150 mM NaCl, 25 mM HEPES, pH 7.4) for 30 min. Insoluble material was removed by centrifugation at 10 000  $\times g$  for 30 min at 4 °C. The amount of protein present in the COS-7 cell lysate was determined using a Bradford protein assay. Equivalent amounts of COS-7 cell lysate were incubated with the charged glutathione-Sepharose beads overnight at 4 °C. The beads were then washed 3 times in cold lysis buffer and once in cold PBS. SDS-PAGE sample buffer containing 100 mM dithiothreitol was added to the immunoprecipitates, and samples were heated to 65 °C for 10 min. SDS-PAGE analysis of the samples was conducted as described below.

Data from six independent GST pulldown assays were quantified using a GS-800 Densitometer (Bio-Rad Laboratories, Hercules, CA). All values obtained in a given experiment were normalized to the intensity of the band obtained in the GST-TAIL/ $\mu$ 1A-FLG pulldown.

#### **Biochemical procedures**

For Western blot analysis, proteins were resolved with 10% SDS-PAGE using standard protocols. The protein was electrophoretically transferred to nitrocellulose membranes (Bio-Rad Laboratories) and blocked with milk solution (150 mM NaCl, 20 mM Tris, 5% milk (w/v), 0.1%

Tween (v/v), pH 7.5) to quench nonspecific protein binding. The blocked membranes were probed with monoclonal antibodies diluted in the milk solution. Horseradish peroxidase-conjugated goat antimouse IgG antibodies (Jackson ImmunoResearch, West Grove, PA) were used at a 1:50 000 dilution and bands were visualized with the Enhanced ChemiLuminescence kit (Amersham Biosciences).

For coimmunoprecipitations, a 10-cm dish of cells was incubated in 1 ml lysis buffer (1% Triton X-100, 5 mM MgCl<sub>2</sub>, 150 mM NaCl, 25 mM HEPES, pH 7.4) supplemented with protease inhibitor mixture for 30 min at 4 °C. Insoluble material was removed by centrifugation at 10 000  $\times$  *g* for 30 min at 4 °C. Lysate was incubated with 0.5  $\mu$ g (Figure 2B) or 3  $\mu$ g (Figure 5) anti-FLAG polyclonal antibody and immobilized Protein A agarose beads (Pierce, Rockford, IL) overnight at 4 °C. Beads were then washed 3 times with cold immunoprecipitation buffer supplemented with detergent, and 3 times with cold PBS. SDS-PAGE sample buffer containing 100 mM dithiothreitol was added to the immunoprecipitates, and samples were heated to 65 °C for 10 min. SDS-PAGE analysis of the samples was conducted as described above. Data from five independent immunoprecipitations were quantified using a GS-800 Densitometer.

#### **Internalization assays**

All manipulations shown in Figure 6A were performed at 4 °C unless otherwise noted. LLC-PK<sub>1</sub> cells were grown to confluence in tissue culture treated dishes. Cells were washed 3 times in ice-cold PBS<sup>++</sup> (PBS supplemented with 100  $\mu$ M CaCl<sub>2</sub> and 1 mM MgCl<sub>2</sub>) and incubated with 2.5 mM Sulfo-NHS-SS-biotin (Pierce) in biotinylation buffer (10 mM triethanolamine, 2 mM CaCl<sub>2</sub>, 125 mM NaCl, pH 7.4) 2  $\times$  20 min. Unreacted biotin was quenched 3  $\times$  5 min with 100 mM glycine in PBS<sup>++</sup>. For internalization assays, cells were placed in media heated to 37 °C and allowed to incubate at 37 °C for the appropriate length of time. For MesNa stripping, cells were removed from the incubator, washed with ice-cold PBS<sup>++</sup>, and incubated 3  $\times$  20 min at 4 °C in freshly mixed MesNa solution (100 mM MesNa, 100 mM NaCl, 1 mM EDTA, 0.2% bovine serum albumin (BSA), 50 mM Tris, pH 8.6). Excess MesNa was quenched by incubating the cells in freshly mixed 120 mM iodoacetic acid in PBS<sup>++</sup> 3  $\times$  5 min. Cells not subjected to the MesNa strip were removed from the incubator, washed with ice-cold PBS<sup>++</sup>, incubated 3  $\times$  20 min at 4 °C in buffer (100 mM NaCl, 1 mM EDTA, 0.2% BSA, 50 mM Tris, pH 8.6) and incubated 3  $\times$  5 min in 120 mM iodoacetic acid diluted in PBS<sup>++</sup> at 4 °C. Samples were then incubated in lysis buffer for 30 min and cleared by centrifugation at 10 000  $\times$  *g* for 30 min at 4 °C. The supernatant was rotated overnight at 4 °C with streptavidin-conjugated agarose beads. The beads were then washed and eluted as described for coimmunoprecipitations.

#### **Recycling assays**

All manipulations for experiments shown in Figure 6B were performed at 4 °C unless otherwise noted. LLC-PK<sub>1</sub> cells were grown to confluence in tissue culture treated dishes. Cells were biotinylated as described above. One dish of each cell line was maintained at 0 °C and the remaining cells were placed in media heated to 37 °C and allowed to incubate at 37 °C for 30 min. All dishes were stripped with MesNa and quenched with iodoacetic acid, as described above. Dishes that were not subjected to the strip were incubated in buffer without added MesNa and then incubated with iodoacetic acid solution. One dish of each cell line that was subjected to the MesNa strip was maintained at 0 °C and the remaining cells were placed in media heated to 37 °C and allowed to incubate at 37 °C for either 10 or 30 min. These dishes were then treated with MesNa solution for a second time. The reserved dishes were treated with buffer that did not contain MesNa, and all dishes were treated with iodoacetic acid. Dishes were then incubated in lysis buffer for 30 min and cleared by centrifugation at 10 000  $\times$  *g* for 30 min at 4 °C. The supernatant was rotated overnight at 4 °C with streptavidin-conjugated agarose beads. The beads were washed and eluted as described for coimmunoprecipitations.

#### **Cell culture**

LLC-PK<sub>1</sub> cells were maintained in  $\alpha$ -MEM (Gibco/Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (Sigma), 50 Units/ml penicillin, 50  $\mu$ g/ml streptomycin and 2 mM L-glutamine. All cells were grown in a humidified incubator at 37 °C and 5% CO<sub>2</sub> atmosphere. Transfection of COS-7 cells was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Assays were performed 48 h after transfection.

#### **Stable Transfection & Immunofluorescence**

cDNA was transfected into LLC-PK<sub>1</sub> cells as described previously (31). Transfected colonies were selected in 800  $\mu$ g/ml hygromycin (American Bioanalytical) or 1.8 mg/ml geneticin (Gibco), and screened by immunofluorescence and Western blot. Cell lines were maintained with 1.8 mg/ml geneticin and/or 800  $\mu$ g/ml hygromycin added to the media.

LLC-PK<sub>1</sub> cells were seeded on glass coverslips and allowed to grow for 2–3 days. Subconfluent cells were fixed in ice-cold methanol for 7 min. Fixed cells were washed 3 times in PBS<sup>++</sup> and then permeabilized and blocked in dilution buffer (PBS containing 10% goat serum (v/v), 2% saponin (w/v), 10 mM glycine) for 30 min. Coverslips were incubated in primary antibody diluted in dilution buffer (1:100 for HK $\beta$  monoclonal antibody, 1:200 for FLAG polyclonal antibody) for 1 h at room temperature. Cells were immersed 3 times in a wash buffer (0.1% BSA, 2% saponin in PBS<sup>++</sup>), and incubated for 1 h in fluorescein isothiocyanate-conjugated goat antimouse IgG (Sigma)

and rhodamine-conjugated goat antirabbit IgG (Chemicon) antibody diluted 1 : 100 in dilution buffer. Cells were then rinsed 3 times in wash buffer and once in water before mounting in Vectashield (Vector Laboratories, Burlingame, CA). Cells were visualized by confocal microscopy using an extended depth of focus macro to combine images from planes of focus separated by 1  $\mu\text{m}$  each. All images are the product of 4-fold line averaging.

For surface immunofluorescence, cells were grown to confluence on Transwell porous polycarbonate cell culture filters (Corning Costar, Corning, NY; 0.4  $\mu\text{m}$  pore size) for 3–5 days. Filters were moved to 4 °C, blocked with PBS<sup>++</sup> + 0.1% BSA 3  $\times$  5 min, and incubated in H,K-ATPase  $\beta$ -subunit monoclonal antibody diluted 1 : 100 in PBS<sup>++</sup> + 0.1% BSA for 2 h. Antibody was present on both the apical and basolateral sides of the filter. Cells were washed with PBS<sup>++</sup> in 3  $\times$  5 min, and were fixed in 2% paraformaldehyde/PBS<sup>++</sup> for 1 h. Filters were again washed with PBS<sup>++</sup> 3  $\times$  5 min. Cells were then permeabilized and incubated with a goat antimouse fluorescein isothiocyanate-conjugated secondary antibody as described previously (10).

#### **Adenovirus transfection/surface immunofluorescence**

Cells were grown to confluence on Transwell porous polycarbonate cell culture filters for 3–5 days. Cultures were washed once in serum-free medium from the apical side, and 50–100 colony-forming units of adenovirus encoding LDL receptor diluted in serum-free medium was added to the apical side of the filter. After 1 h of incubation at 37 °C in a 5% CO<sub>2</sub> atmosphere, the media in the apical chamber was removed and replaced with serum-containing media. Surface immunofluorescence was performed 2 days postinfection. Cells were washed and blocked with PBS<sup>++</sup> + 0.1% BSA at room temperature, and were then incubated for 3–5 min in supernatant from an anti-LDL receptor hybridoma cell line diluted 1 : 1 with blocking solution. Filters were washed twice in PBS<sup>++</sup> and fixed in 3% paraformaldehyde/PBS<sup>++</sup> for 15 min at room temperature. Cells were then permeabilized and incubated with a goat antimouse fluorescein isothiocyanate-conjugated secondary antibody as described previously (10).

#### **Confocal immunofluorescence microscopy**

Confocal sections were taken on a Zeiss LSM 410 laser scanning confocal microscope. Images are the product of 8-fold line averaging. X/Z cross-sections were generated with a 0.2 micron motor step. Contrast and brightness were set so that all pixels were in the linear range.

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#### **References**

1. Ikonen E, Simons K. Protein and lipid sorting from the trans-Golgi network to the plasma membrane in polarized cells. *Semin Cell Dev Biol* 1998;9:503–509.
2. Muth TR, Caplan MJ. Transport protein trafficking in polarized cells. *Ann Rev Cell Dev Biol* 2003;19:333–366.
3. Robinson MS, Bonifacino JS. Adaptor-related proteins. *Curr Opin Cell Biol* 2001;13:444–453.
4. Thomas DC, Roth MG. The basolateral targeting signal in the cytoplasmic domain of glycoprotein G from vesicular stomatitis virus resembles a variety of intracellular targeting motifs related by primary sequence but having diverse targeting activities. *J Biol Chem* 1994;269:15732–15739.
5. Geffen I, Fuhrer C, Leitinger B, Weiss M, Huggel K, Griffiths G, Spiess M. Related signals for endocytosis and basolateral sorting of the asialoglycoprotein receptor. *J Biol Chem* 1993;268:20772–20777.
6. Owen DJ, Setiadi H, Evans PR, McEver RP, Green SA. A third specificity-determining site in mu 2 adaptin for sequences upstream of Yxx phi sorting motifs. *Traffic* 2001;2:105–110.
7. Courtois-Coutry N, Roush D, Rajendran V, McCarthy JB, Geibel J, Kashgarian M, Caplan MJ. A tyrosine-based signal targets H/K-ATPase to a regulated compartment and is required for the cessation of gastric acid secretion. *Cell* 1997;90:501–510.
8. Wang T, Courtois-Coutry N, Giebisch G, Caplan MJ. A tyrosine-based signal regulates H-K-ATPase-mediated potassium reabsorption in the kidney. *Am J Physiol* 1998;275(5 Part 2):F818–F826.
9. Roush DL, Gottardi CJ, Naim HY, Roth MG, Caplan MJ. Tyrosine-based membrane protein sorting signals are differentially interpreted by polarized Madin-Darby canine kidney and LLC-PK1 epithelial cells. *J Biol Chem* 1998;273:26862–26869.
10. Gottardi CJ, Caplan MJ. An ion-transporting ATPase encodes multiple apical localization signals. *J Cell Biol* 1993;121:283–293.
11. Matter K, Hunziker W, Mellman I. Basolateral sorting of LDL receptor in MDCK cells: the cytoplasmic domain contains two tyrosine-dependent targeting determinants. *Cell* 1992;71:741–753.
12. Odorizzi G, Trowbridge IS. Structural requirements for basolateral sorting of the human transferrin receptor in the biosynthetic and endocytic pathways of Madin-Darby canine kidney cells. *J Cell Biol* 1997;137:1255–1264.
13. Folsch H, Ohno H, Bonifacino JS, Mellman I. A novel clathrin adaptor complex mediates basolateral targeting in polarized epithelial cells. *Cell* 1999;99:189–198.
14. Dell'Angelica EC, Ohno H, Ooi CE, Rabinovich E, Roche KW, Bonifacino JS. AP-3: an adaptor-like protein complex with ubiquitous expression. *EMBO J* 1997;16:917–928.
15. Bonifacino JS, Dell'Angelica EC. Molecular bases for the recognition of tyrosine-based sorting signals. *J Cell Biol* 1999;145:923–926.
16. Futter CE, Gibson A, Allchin EH, Maxwell S, Ruddock LJ, Odorizzi G, Domingo D, Trowbridge IS, Hopkins CR. In polarized MDCK cells basolateral vesicles arise from clathrin-gamma-adaptin-coated domains on endosomal tubules. *J Cell Biol* 1998;141:611–623.
17. Ohno H, Tomemori T, Nakatsu F, Okazaki Y, Aguilar RC, Foelsch H, Mellman I, Saito T, Shirasawa T, Bonifacino JS. Mu1B, a novel adaptor medium chain expressed in polarized epithelial cells. *FEBS Lett* 1999;449:215–220.
18. Sugimoto H, Sugahara M, Folsch H, Koide Y, Nakatsu F, Tanaka N, Nishimura T, Furukawa M, Mullins C, Nakamura N, Mellman I, Ohno H. Differential recognition of tyrosine-based basolateral signals by AP-1B

- subunit  $\mu$ 1B in polarized epithelial cells. *Mol Biol Cell* 2002;13:2374–2382.
19. Gottardi CJ, Caplan MJ. Molecular requirements for the cell-surface expression of multisubunit ion-transporting ATPases. Identification of protein domains that participate in Na,K-ATPase and H,K-ATPase subunit assembly. *J Biol Chem* 1993;268:14342–14347.
  20. Folsch H, Pypaert M, Schu P, Mellman I. Distribution and function of AP-1 clathrin adaptor complexes in polarized epithelial cells. *J Cell Biol* 2001;152:595–606.
  21. Nesterov A, Carter RE, Sorkina T, Gill GN, Sorkin A. Inhibition of the receptor-binding function of clathrin adaptor protein AP-2 by dominant-negative mutant  $\mu$ 2 subunit and its effects on endocytosis. *EMBO J* 1999;18:2489–2499.
  22. Asano S, Kawada K, Kimura T, Grishin AV, Caplan MJ, Takeguchi N. The roles of carbohydrate chains of the beta-subunit on the functional expression of gastric H (+),K (+) -ATPase. *J Biol Chem* 2000;275: 8324–8330.
  23. Gan Y, McGraw TE, Rodriguez-Boulan E. The epithelial-specific adaptor AP1B mediates post-endocytic recycling to the basolateral membrane. *Nat Cell Biol* 2002;4:605–609.
  24. Takeda T, Yamazaki H, Farquhar MG. Identification of an apical sorting determinant in the cytoplasmic tail of megalin. *Am J Physiol Cell Physiol* 2003;284:C1105–C1113.
  25. Muth TR, Gottardi CJ, Roush DL, Caplan MJ. A basolateral sorting signal is encoded in the alpha-subunit of Na-K-ATPase. *Am J Physiol* 1998;274(3 Part 1):C688–C696.
  26. Simmen T, Honing S, Icking A, Tikkanen R, Hunziker W. AP-4 binds basolateral signals and participates in basolateral sorting in epithelial MDCK cells. *Nat Cell Biol* 2002;4:154–159.
  27. Duffield A, Kamsteeg E-J, Brown AN, Pagel P, Caplan MJ. The tetraspanin CD63 enhances the internalization of the H,K-ATPase  $\beta$ -subunit. *Proc Natl Acad Sci U S A* 2003;100:15560–15565.
  28. Rous BA, Reaves BJ, Ihrke G, Briggs JA, Gray SR, Stephens DJ, Banting G, Luzio JP. Role of adaptor complex AP-3 in targeting wild-type and mutated CD63 to lysosomes. *Mol Biol Cell* 2002;13: 1071–1082.
  29. Devonald MA, Smith AN, Poon JP, Ihrke G, Karet FE. Non-polarized targeting of AE1 causes autosomal dominant distal renal tubular acidosis. *Nat Genet* 2003;33:125–127.
  30. Spanopoulou E, Zaitseva F, Wang FH, Santagata S, Baltimore D, Panayotou G. The homeodomain region of Rag-1 reveals the parallel mechanisms of bacterial and V (D) J recombination. *Cell* 1996; 87:263–276.
  31. Gottardi CJ, Pietrini G, Roush DL, Caplan MJ. Sorting of ion transport proteins in polarized cells. *J Cell Sci* 1993;17(Suppl):13–20.