The large conductance, voltage- and Ca\(^{2+}\)-activated K\(^+\) (MaxiK) channel is expressed in several renal segments and functions in cell volume regulation and flow-mediated K\(^+\) secretion. Previously, we cloned two MaxiK channel isoforms, named rbslo1 and rbslo2, from rabbit renal cells. rbslo1 has a 58-amino acid insertion after the S8 hydrophobic domain, whereas rbslo2 is truncated and cannot be activated. Here we use the sequence differences between the two variants to examine their plasma membrane processing. Plasma membrane localization of rbslo1 and 2 expressed in HEK293 cells was assayed by electrophysiology, immunocytochemistry, and biochemistry studies. Consistent with its functional silence, rbslo2 localized primarily within the cytoplasm, presumably in the endoplasmic reticulum and Golgi region. Coexpression with MaxiK β subunits did not alter the cellular localization of either rbslo1 or rbslo2. When rbslo1 and 2 are cotransfected in non-polarized cells, they colocalized primarily within the cell with only rbslo1 detected at the plasma membrane. When transfected into polarized, medullary-thick ascending limb (mTAL) cells, rbslo1 is expressed at the apical membrane whereas the majority of rbslo2 localized throughout the cytoplasm. Given the high degree of similarity between the two isoforms, we conclude that the cytoplasmic tail of rbslo1 is important for the cell surface expression of MaxiK channels.

Large conductance, voltage-dependent, and Ca\(^{2+}\)-activated K\(^+\) (MaxiK, or BK)\(^+\) channels are widely distributed in neurons (1, 2), smooth muscle (3–5), and several nephron segments (6–9), where they play roles in regulating the neurotransmitter release, cell contractility, and volume decrease under hypotonic stress (10–14). The expression level of MaxiK channels varies from tissue to tissue and even within the same tissue (15).

The MaxiK channels are composed of the pore-forming α-subunit and modulatory β-subunit (16–20). A single gene encodes the α-subunit with the S1–S6 region homologous to the tetrameric voltage-gated K\(^+\) channels, especially in the pore-forming domain, S5-P-S6, and the voltage-sensing element, S4 (21, 22). Structures unique to the MaxiK channel α-subunit include an additional N-terminal transmembrane domain, S0, and a large C terminus. There are four hydrophobic segments (S7–S10) in the C-terminal region of the channel protein (21, 23). These segments were previously thought to be additional transmembrane domains, but now it is agreed that they are located inside the cell (21). The MaxiK C-terminal region has Ca\(^{2+}\) sensors and also affects the channel’s gating (24–27).

MaxiK channels play a fundamental role in many physiological processes, including action potential repolarization, modulation of calcium signals, hormone release, and regulation of vascular tone (28–31). MaxiK channels from different cell types vary in their calcium sensitivity, unitary conductance, and gating kinetics (5). The interaction of the MaxiK α-subunit with the modulatory β-subunits or other accessory proteins partially explains their overall functional diversity. More importantly, the function variation of MaxiK channels comes from the alternative splicing of its α-subunit (17, 22, 32). So far, ten splice sites have been reported with most of them localized in the intracellular C terminus.

The renal MaxiK channel is distributed in glomeruli, thin limbs of Henle’s loop, cortical and medullary ascending limbs of Henle’s loop, cortical collecting tubules, and outer and inner medullary collecting ducts (9). Renal MaxiK channels function in cell volume regulation and flow-mediated K\(^+\) secretion (12). Previously, we reported the cloning of two isoforms of the MaxiK channel α-subunit from a rabbit medullary thick ascending limb (mTAL) cell line (9). The longer one, rbslo1 (NCBI accession number AF201702), is highly homologous to MaxiK channels from other species such as mslo, hslo, and eslo and has an additional 58-amino acid (aa) exon insert between the S8 and S9 regions. The other isoform, rbslo2, is truncated at position Gly\(^{781}\) with a unique 15-aa sequence appended at the end (see Fig. 1 below). Using electrophysiological techniques, we have shown that rbslo1 functions in a voltage- and Ca\(^{2+}\)-dependent way, whereas rbslo2 cannot be activated at all.

The objectives of the present study were to determine at the protein level why rbslo2 is functionally silent. To this end we examined the electrophysiology, assembly, and intracellular localization of rbslo1, rbslo2, and several deletion mutants in cultured mammalian cell lines. Using whole cell current recordings, confocal microscopy, and cell surface biotinylation, we report here that the cytoplasmic tail present in rbslo1 but missing in rbslo2 is critically involved in apical cell membrane expression of the MaxiK channel in renal cells.

**EXPERIMENTAL PROCEDURES**

DNA Constructs of rbslo1, rbslo2, and MaxiK β Subunits—The MaxiK channel has seven transmembrane domains (S9–S6, Fig. 1) with an extracellular N terminus and a large cytoplasmic tail. The wild type
inverted fluorescence microscope intrinsic to the current recording system. GFP-positive cells were superimposed with an intracellular ion containing (in mM): 135 sodium aspartate, 10 NaCl, 5 KCl, 1 MgCl₂, 1 CaCl₂, 0.1 niflumic acid, and 10 Hepes (pH 7.4 with Tris). Aspartate-rich solution and niflumic acid were used to prevent the activation of endogenous currents. The pipette solution contained (in mM): 135 potassium aspartate, 15 KCl, 1 MgCl₂, 10 Hepes (pH 7.4 with Tris). Extracellular solution containing 10 or 30 mM glucose was further applied using a nearby pipette of 30- to 35-μm diameter. pClamp8 software (Axon Instruments) was used to sample and analyze data. The access conductance was monitored in the trace of capacitate surge. To minimize the voltage errors due to the lower access conductance, results that showed the amplitude of the current above 1 nA were obtained from the access conductance corrected and the single-channel. Current-voltage (I-V) relationships were obtained at 100 ms of each voltage step from a holding potential of ~80 mV.

Antibodies and Immunofluorescent Staining—Rabbit polyclonal antibody against C terminus of rbslo1 was from Alomone Laboratories; mouse monoclonal antibody (9E10) for myc and rabbit polyclonal antibody for ZO-1 were from Zymed Laboratories Inc.; antibody for GFP (rabbit) was from Clontech; antibody for CD4 (mouse) was from Calbiochem; chicken anti-myc antibody was from Aves Laboratories Inc.; chicken anti-KDEL antibody was from StressGen; mouse anti-β-actin antibody was from Affinity Bioreagents Inc. The lowest concentration of antibody giving the highest signal was used in these studies. In all experiments, negative controls were performed by either: 1) transfecting cells with DNA vectors or no DNA or 2) pre-absorbing each antibody with the corresponding antigenic peptide or both as appropriate.

For double-labeling experiments, single-stained specimens were examined under the other fluorescent channel to assure minimized bleed-through. Moreover, the intensity of the two dyes in double-stained specimens was balanced to reduce artifacts. No antibodies from the same species were used in any of the double-stained experiments.

For immunostaining, the fixed cells were blocked with 5% nonfat dry milk in PBS for 1 h. The cells were then incubated with the primary antibody for 1 h, washed with PBS three times, and incubated with the corresponding secondary antibody conjugated to Cy2 fluorescent dye (Sigma) for 1 h. After staining, the cover-slip (for HEK293) or Transwells (for mTAL) were washed in PBS and mounted using Prolong Antifade kit (Molecular Probes).

Western Blotting Analysis—Cells transiently transfected with DNA constructs encoding rbslo1 or rbslo2 fusion proteins were lysed in 20 mM Hepes, pH 7.5, 120 mM NaCl, 5.0 mM EDTA, 1.0% Triton X-100, 0.5 mM dithiothreitol, and 1.0 mM phenylmethylsulfonyl fluoride containing Complete protease inhibitor (Roche Molecular Biochemicals, 1 tablet per 50 ml of solution). The lysates were spun at 6000 × g for 5 min to pellet the insoluble material, and the proteins from supernatant were quantified using a Bio-Rad Protein Assay kit. For immunofluorescence experiments, the lysates were separated by SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes (Amersham Biosciences). The probing with specific antibodies and subsequent detection with ECL plus system (Amersham Biosciences) was performed according to standard procedure as described previously (33).

Confocal Laser Microscopy—The fluorescence label was examined with UltraView confocal imaging system (PerkinElmer Life Sciences). Images were generated using the manufacturer’s software suite. Contrast and brightness settings were chosen to ensure that all pixels were within the linear range. To obtain three-dimensional images of the cell, each XY plane of the sample with a depth of 0.4 μm in Z direction was scanned. The confocal laser line (488 nm for 150 μW) on sample was separated by SDS-PAGE and electrokoretically transferred to polycylinide difluoride membranes (Amersham Biosciences). The probing with specific antibodies and subsequent detection with ECL plus system (Amersham Biosciences) was performed according to standard procedure as described previously (33).

Cell Surface Biotinylation—HEK293 cells were grown on six-well plates for 48–72 h after DNA transfection. Cell surface biotinylation protocols were described by Overall et al. (2000). Biotinyl-phalloidin (Pierce) (1.0 mg/ml in PBS buffer) on the cells. The sample was incubated for 45 min on ice with gentle shaking. Cold PBS buffer (1 volume, 1.0 ml) containing 100 mM glycine was then added to stop the reaction. After an additional 20-min incubation, the cells were collected and lysed as described above. The biotinylated proteins from the cell lysate were recovered from the samples by incubating beads with immobilized streptavidin-agarose beads (Pierce). Bound proteins were eluted from the beads in Laemmli buffer, and the unbound samples were also saved. All the protein samples were quantified and then analyzed by SDS-PAGE and Western blotting as described above.

**Surface Expression of MaxiK Requires the Entire C Terminus**

**Fig. 1. rbslo1 and rbslo2.** Wild type rbslo1 has seven predicted transmembrane domains (TMD), S0–S6; four hydrophobic domains, S7–S10; and a 58-aa insertion between S8 and S9. myc-rbslo1 contains a myc tag at the N terminus. myc-rbslo1-Del, rbslo1 with N-terminal myc tag and truncated at Gly879, wt-rbslo2 is missing 332 aa of the C terminus and has a unique 15-aa C terminus. GFP-rbslo1 has a GFP tag at the N terminus, myc-rbslo2 has an N-terminal cDNA and is truncated at Gly879. The myc-rbslo1-Del and myc-rbslo2-Del have the same C terminus. The unique 15-aa C terminus in wt-rbslo2, GFP-rbslo2, and myc-rbslo2 is shown as a closed circle.

rbslo1 and rbslo2 cDNAs, i.e. wt-rbslo1 and wt-rbslo2 (Fig. 1), were prepared as described before (9). All the myc-tagged constructs were created by PCR amplification using the wild type rbslo1 or 2 as the template. The PCR primers were synthesized to flank the desired DNA sequence with a BamHI (for forward primer) or SalI (for reverse primer) and digestion site at the end. The PCR products were purified, double-digested by restriction enzymes BamHI and SalI, and ligated into pCMV-TAG3 (Stratagene) vector. To make the GFP-tagged rbslo2 construct, the coding region of wt-rbslo2 was isolated by double digestion using restriction enzymes HindIII and XhoI. The fragment was subcloned into pEGFP-C3 (Clontech) vector. All the rbslo1 and 2 constructs were co-transfected with CD4 (mouse) and higher expression level in mammalian cells. The wt-rbslo1 was investigated by a rabbit polyclonal antibody against its C terminus. For other experiments, we chose to add myc or GFP tag-EGFP-C3 (Clontech) vector and sequenced as described above.
RESULTS

Electrophysiology of rbslo1 and -2—HEK293 cells were co-transfected with GFP and wt-rbslo1 (A and B), wt-rbslo2 (C and D), myc-rbslo1-1 (E and F), myc-rbslo1-Del (G and H), or myc-rbslo2 (I and J). Typical recordings measured before (left panels) and after (middle panels) application of ionomycin (10 μM). The relationship of the steady-state current to membrane voltage is shown in the right panels. The cell was held at −80 mV, and 100-ms voltage steps were applied (from −100 through +100 mV at increments of 20 mV). Open circles indicate basal currents, and closed circles indicate ionomycin-stimulated currents.

In contrast to wt-rbslo1 channel, when cells were transfected with wt-rbslo2, no time-, voltage-, and Ca2+-dependent current activities were observed (10 cells tested, Fig. 2, C and D). Similar results were obtained using myc-rbslo2 (five cells tested, Fig. 2, I and J), GFP-rbslo2 (eight cells tested, data not shown), or the G781X mutant, myc-rbslo2-Del (four cells tested, data not shown), which lacks the C-terminal unique tail of 15 aa in rbslo2. These data suggest the cytoplasmic tail of the rbslo1 is essential for the generation of plasma membrane currents.

The rbslo1 and -2 Exist as Oligomers—Like the other potassium channels, functional MaxiK channels formed by the tetramerization of monomers (35, 36). Therefore, we asked whether both rbslo1 and 2 exist as oligomers using non-reduced electrophoresis and immunoblotting techniques. HEK293 cell lysates containing the exogenously expressed Xenopus oocytes and Chinese hamster ovary cells (9). As expected, cells expressing GFP only did not show any similar current activity (five cells, data not shown).

When wt-rbslo1 was tagged with myc at the N terminus (myc-rbslo1) and cotransfected with GFP into HEK293 cells, current activities similar to those with wt-rbslo1 were observed in 9 cells tested (Fig. 2, E and F). These data suggested that the myc tag at the N terminus does not affect the channel function of rbslo1. A G839X truncation mutant, myc-rbslo1-Del, which lacks the cytoplasmic tail of rbslo1, did not generate any time-, voltage-, and Ca2+-dependent currents when coexpressed with GFP (Fig. 2, G and H).
membrane and soluble proteins have different hydrodynamic properties, soluble molecular weight markers may not provide an accurate estimate of the molecular mass of a hydrophobic protein (37). Therefore, we further tested our proposed multimeric state of each band by plotting the relative migration (Rf) of each band against the putative number of subunits they represent (Fig. 3B). The linear relationship in each of the four plots indicates that the relative mobility of each band corresponds to its assigned subunit stoichiometry, suggesting that rbslo1 and 2 monomer, dimer, trimer, and tetramer existed (38, 39). These results in Fig. 3 indicate that both rbslo1 and 2 proteins retain the ability to assemble as oligomers and that the cytoplasmic tail of rbslo1 is not necessary for rbslo1 or 2 channel assembly.

Comparing the Distribution of rbslo1 and 2 in HEK293 Cells—To see if the lack of rbslo2 channel currents was due to its misdistribution in the cell, we transiently transfected HEK293 cells with various rbslo1 or 2 constructs. After 48 h,
the cells were examined under indirect immunofluorescent confocal microscopy using rabbit polyclonal anti-rbslo1 antibody for wt-rbslo1 and mouse monoclonal anti-myc antibody for myc-rbslo1 (Fig. 4, A and B). Both wt-rbslo1 and myc-rbslo1 displayed a clear cell surface distribution. Therefore, as suggested by our electrophysiological studies, rbslo1 protein traffics to the cell surface and the N terminus myc-tag does not interfere with the localization of the channel proteins. We also compared the effects of myc and GFP tags by cotransfecting both myc-rbslo2 and GFP-rbslo2 into the HEK293 cells (Fig. 4, C–E). We found that when coexpressed in one cell, myc-rbslo2 (C, Cy3-labeled secondary antibody) and GFP-rbslo2 (D) proteins have the same distribution as indicated by the yellow in the merged image (E). Thus neither N-terminal myc nor GFP tag disturbs the characteristics of rbslo channels.

**rbslo2 Does Not Traffic to the Cell Surface**—Fig. 4 suggests that the majority of rbslo2 is localized inside the cell. To see if some of the rbslo2 proteins are able to reach the cell surface, we compared the expression of rbslo2 constructs with the endogenous or transfected cell membrane markers ZO-1 (40) and CD4 (41), respectively. Fig. 5 shows either membrane marker, ZO-1 (A) or CD4 (D), in red and rbslo2 in green (B and E). A merge of the images (C and F) shows no regions of overlap (yellow) at the cell surface, indicating that rbslo2 was almost exclusively confined to the cytoplasmic region of the cell.

In a separate experiment to verify the surface presentation of rbslo1 and rbslo2 proteins, we conducted cell surface biotinylation experiments (Fig. 6). The data showed that a large amount of rbslo1 proteins was biotinylated, indicating its strong cell surface expression. On the contrary, for rbslo2, only a negligible amount of the protein was biotinylated. The Western blotting results for rbslo2 were similar to GFP, a control protein that does not express at the cell surface. Taken together with our immunofluorescent data, these results confirm that the majority of rbslo1, but not rbslo2, is delivered to the cell surface region.

**Intracellular Distribution of rbslo1 and rbslo2**—To further pursue the intracellular localization of rbslo1 and 2 proteins, we double-labeled rbslo1- or rbslo2-expressing cells with antibodies against channel proteins and markers of intracellular organelles. Both rbslo1 and 2 appear (Fig. 7, A and D, respectively) in green, and the ER, as detected by an anti-KDEL antibody, appears in red. Regions of overlap appear in yellow.

**Coexpression of MaxiK β Subunits Does Not Change the Subcellular Distribution of Either rbslo1 or rbslo2**—To study whether the MaxiK β subunits play a role in the intracellular trafficking of rbslo1 and 2, we cotransfected either rbslo1 or rbslo2 with the MaxiK β3 or β4 subunit (Fig. 8). In the presence of the β subunit in HEK293 cells, rbslo1 was still located at the cell surface (Fig. 8, A–C and G–I), whereas rbslo2 remained...
cell lysate/H11003 gels. Bars
Procedures. (1:1000) and rabbit anti-GFP (1:1000) antibodies, respectively. Surface biotinylation studies were performed as described under “Experimental Procedures.” For all three samples, cell lysates containing 30 μg of protein were loaded in the first lane. The unbound (second lane) and biotinylated (third lane) samples come from cell lysates originally containing 30 and 50 μg of protein, respectively. B, densitometry measurements of the three gels. Bars depict the percentage of total cell lysate at the cell surface (ratio of the amount of surface-biotinylated protein/to the total protein in the cell lysate × 100%). The star shows a significant difference between rbslo1 and rbslo2 surface biotinylation.

Fig. 6. Surface biotinylation study of rbslo1 and -2 channel proteins. A, DNA construct of wt-rbslo1 (top), GFP-rbslo2 (middle), or GFP (bottom) was transfected into HEK293 cells, and the exogenously expressed proteins were examined in Western blot using rabbit anti-rbslo1 (1:1000) and rabbit anti-GFP (1:1000) antibodies, respectively. Surface biotinylation studies were performed as described under “Experimental Procedures.” For all three samples, cell lysates containing 30 μg of protein were loaded in the first lane. The unbound (second lane) and biotinylated (third lane) samples come from cell lysates originally containing 30 and 50 μg of protein, respectively. B, densitometry measurements of the three gels. Bars depict the percentage of total cell lysate at the cell surface (ratio of the amount of surface-biotinylated protein/to the total protein in the cell lysate × 100%). The star shows a significant difference between rbslo1 and rbslo2 surface biotinylation.

mostly in the cytoplasm (Fig. 8, D–F and J–L). Comparing these results with those in Figs. 5 and 7, it can be seen that the subcellular distribution of rbslo2 did not change after coexpression with MaxiK β subunits.

rbslo1 Does Not Facilitate the Surface Expression of rbslo2—To study the possible effect of rbslo1 on the intracellular trafficking of rbslo2, we cotransfected both rbslo1 and 2 into HEK293 cells. When coexpressed, rbslo1 and 2 colocalized most intensely within the cytoplasmic region of the cell (Fig. 9A, panels A–F). Similar to what was observed with rbslo1 alone, only the red but not the yellow color can be seen at the cell surface, indicating the presence of only rbslo1 but not rbslo2. This is more clearly seen under non-permeabilized conditions. In a merged image of wt-rbslo1 (in red) and GFP-rbslo2 (in green), no yellow color can be detected (Fig. 9A, panels G–I). Thus rbslo1 did not promote the surface expression of rbslo2. In addition, rbslo2 did not exert a strong dominant-negative effect on the surface expression of rbslo1.

Fig. 9B confirms these results with surface biotinylation experiments. As shown in the top panel, rbslo1 protein was biotinylated when either expressed alone or coexpressed with rbslo2. In contrast, rbslo2 was not biotinylated when expressed alone in HEK293 cells (bottom panel). Coexpression with rbslo1 did not result in the biotinylation of rbslo2, indicating that rbslo2 remained primarily inside the cell. Interestingly, after coexpression with rbslo2, surface biotinylation of rbslo1 was weaker, implying that less rbslo1 reaches the cell surface (Fig. 9C). When transfected together, rbslo2 may reduce surface expression of rbslo1 somewhat, perhaps by competing with the same intracellular trafficking machinery.

Distribution of rbslo1 and rbslo2 in Polarized Renal Epithelia—Endogenous MaxiK channel proteins are expressed in renal epithelia (9, 12). In our Western blot experiments we also detected a specific band in cultured medullary thick ascending limb cell (mTAL) lysates using anti-rbslo1 antibody (data not shown). The band is very weak and has an apparent molecular mass of about 70 kDa, corresponding to another isoform of the MaxiK channel (42). To examine how rbslo1 and 2 channels are trafficked in polarized epithelial cells, we transfected myc-rbslo1 and myc-rbslo2 constructs into cultured mTAL cells. Use of the myc-tagged fusion construct allowed detection of only exogenously expressed rbslo1 and 2. At 72 h post-transfection,
polarized mTAL cells were fixed and permeabilized, and antibodies were added at both the apical and basolateral sides of the cell. After the immunostaining procedure, the sample was examined by confocal microscopy with ZO-1, the tight junction protein, as apical membrane marker. Pictures in Fig. 10 (A and B) are a reconstruction of serial sections of immunofluorescent images along the Z direction (see “Experimental Procedures”). The en face pictures depict signals from all confocal sections. The X-Z (top) or Y-Z cross-section (left) pictures show that myc-rbslo1 was almost exclusively localized at the apical surface of mTAL cells (Fig. 10A). In contrast, myc-rbslo2 did not achieve a polarized distribution. It spread diffusely in the cytoplasm of the cell (Fig. 10B). The location of rbslo2 was further determined by cotransfection of rbslo1 and 2 in mTAL cells. In Fig. 10C, rbslo1 was shown in red and rbslo2 in green. Although some small amount of apical membrane location of rbslo2 may occur, the results indicate that the majority of rbslo2 did not reach the plasma membrane of polarized mTAL cells. Both truncation mutants, i.e. myc-rbslo1-Del myc-rbslo2-Del, showed intracellular distributions similar to myc-rbslo2 (data not shown).

DISCUSSION

MaxiK channels exist in several nephron segments (6–9). Although their exact physiological roles in renal epithelia are unclear, the existing literature suggests that they may function in cell volume regulation and flow-mediated K⁺ secretion (12). We previously cloned two MaxiK channel splicing isoforms, rbslo1 and rbslo2, from rabbit mTAL cells. Although rbslo1 expression leads to functional currents in either mammalian cell lines or Xenopus oocytes, rbslo2 cannot be activated in any of the systems. Therefore, we asked whether the problem arises from a failure of assembly or one of cell surface delivery. In addition, because complex renal function relies upon the correct sorting, targeting, and restriction of ion channel proteins to specific regions of the nephronal epithelial cells (43, 44), it is important to know the detailed intracellular distribution of renal MaxiK channels at the protein level.

We used myc- and GFP-tagged fusion protein in some of our experiments. To verify that the tags did not have an effect, the channel’s behavior was carefully checked in the experiments shown in Figs. 2 and 4. Bravo-Zehnder et al. (45) also reported that myc-tagged MaxiK channel has characteristics similar to those of wild type. N terminus GFP tagging of hslol changes the channel’s electrophysiological properties (46). In our case, the GFP-tagged rbslo2 did not generate currents, like the wild type, and its intracellular distribution was not changed. Therefore, we used these constructs for our further immunofluorescence and Western blotting experiments.

In the present study we demonstrated, using Western blotting methods, that both rbslo1 and rbslo2 have the ability to form oligomers, including tetramers. The missing part in the C-terminal region of rbslo2 does not play an important role in the assembly of the channel. There is an additional 58-aa insertion in rbslo1 and a unique C terminus of 15 aa long in rbslo2. Because the MaxiK channel can still be assembled as oligomers without either of the two domains, we further conclude that neither of these two domains is important for channel assembly. For voltage-gated K⁺ channels, which are homologous to MaxiK channels in the N-terminal region, the structural element facilitating subunit assembly is localized in the hydrophilic N terminus (47). Recently, Quirk et al. (36) showed that a region between S6 and S7 in hslol is capable of self-association and required for functional channel expression. They concluded that this region mediates the tetramerization of hslol. Elucidation of the detailed sequence that is required for MaxiK channel assembly needs further investigation.

The mechanism underlying the renal MaxiK channel sorting remains unknown. We demonstrate here, using two separate methods, i.e. immunofluorescent microscopy and surface biotinylation, that the cytoplasmic domain of the MaxiK channel is necessary for its surface delivery. The rbslo2, which lacks this cytoplasmic domain, remained predominately within an intracellular pool and did not appear abundantly at the cell surface in either HEK293 or mTAL cells.
Using anti-KDEL and other antibodies as cell organelle markers, we showed localization of rbslo2 within the ER and Golgi apparatus. Little rbslo2 proteins localized to endosomes. Thus, the majority of rbslo2 is unable to process further beyond the ER or Golgi. Interestingly, the unique 15-aa C terminus of rbslo2 is a potential PSD-95, Dlg, zo-1 (PDZ) binding domain that has been reported to be involved in ion channel trafficking (33, 48, 49). However, the similar behavior of myc-rbslo2-Del, which has this unique domain deleted, compared with myc-rbslo2 suggests that this domain does not inhibit the function or surface expression of the channel.

At present, four MaxiK β subunits have been found in human and other mammals (19, 20, 50–52). Results in Fig. 8 showed that, when coexpressed either with the MaxiK β3 or β4 subunit, neither rbslo1 nor rbslo2 acquired a different subcellular distribution compared with that observed in the absence

Fig. 9. Immunocytochemistry and surface biotinylation studies on HEK293 cells coexpression of rbslo1 and -2. A, panels A–C, HEK293 cells cotransfected with wt-rbslo1 and myc-rbslo2 were immunolabeled under permeabilized conditions. Shown are confocal images of wt-rbslo1 (A, using rabbit anti-rbslo1 antibody (1:500)), myc-rbslo2 (B, using mouse anti-myc antibody (1:1000)), and the overlaid image with wt-rbslo1 in red and myc-rbslo2 in green (C). D–I, HEK293 cells cotransfected with wt-rbslo1 and GFP-rbslo2 were immunolabeled under permeabilized (D–F) or non-permeabilized (G–I) conditions. Shown are confocal images of wt-rbslo1 (D and G) and GFP-rbslo2 (E and H) stained with rabbit anti-rbslo1 antibody or examined using intrinsic GFP fluorescence, respectively. F and I, the overlay images with wt-rbslo1 in red and GFP-rbslo2 in green. B, surface biotinylation study on HEK293 cells transfected with wt-rbslo1 (left two lanes), GFP-rbslo2 (middle two lanes), or cotransfected with both wt-rbslo1 and GFP-rbslo2 (right two lanes). Upper gel, rabbit anti-rbslo1 antibody (1:1000) was used to show the signal of rbslo1 as indicated by the arrow; lower gel, rabbit anti-GFP antibody (1:1000) was used to show the signal of rbslo2 as indicated by the arrow. L, cell lysates samples. 15 µg of protein was loaded for rbslo1 sample (lane 1), 30 µg of protein for rbslo2 (lane 3), and cotransfection sample (lane 5); B, surface-biotinylated samples. All samples (lanes 2, 4, and 6) from cell lysates containing 150 µg of protein. C, densitometry study on gels in B. Bars depict the percentage of total cell lysate at the cell surface (ratio of the amount of surface-biotinylated protein/the total protein in the cell lysate × 100%). The left three bars come from data in the upper gel; the right three bars come from data in the lower gel. The star shows the significant difference in protein amount for surface-biotinylated rbslo1 before and after coexpression with rbslo2.
There are three differences between rbslo1 and rbslo2: 1) rbslo1 has a 58-aa insertion in the cytoplasmic domain between S8 and S9, 2) rbslo2 is truncated at Gly781, missing a 332-aa cytoplasmic tail, and 3) has a unique 15-aa C terminus. Our experiments here and previously (34) showed that neither the 58-aa insertion in rbslo1 nor the unique 15-aa C terminus in rbslo2 is likely to play a major role in plasma membrane trafficking. On the other hand, we present compelling data to show that the cytoplasmic tail of rbslo1 does play a role.

Many other MaxiK splice variants exist in different tissues. At least one of these can act as a dominant negative one that, when coexpressed with other subunits, inhibits the surface expression of the other subunits (53). We also examined whether rbslo2 can influence the surface expression of coexpressed rbslo1. Our experiments in Fig. 9 showed that both isoforms colocalized in the cytoplasm but only rbslo1 presented at the cell surface. Although rbslo2 still did not reach the cell surface, the amount of rbslo1 on the cell surface was partially reduced. These results, together with our previous electrophysiological data (9), indicate that rbslo2 has some effect on the trafficking and function of rbslo1, but it does not fully prevent the surface expression of rbslo1. However, this effect may also result from transfection of more than one plasmid and may not reflect an actual physiological reduction of rbslo1 by rbslo2.

Localization of ion channels and pumps to the correct membrane of polarized epithelia is important for vectorial ion movement (54, 55). Several prior studies have shown the distribution of MaxiK channel in polarized epithelial cells. Using single channel patch recording, currents from MaxiK channels were recorded at apical or basolateral membranes of different cell types (23, 56–63). Bravo-Zehnder et al. (45) reported that when transfected into Madin-Darby canine kidney cells, hslo protein traffics to the apical membrane. Endogenous rabbit MaxiK channel localizes at the apical membranes of collapsed thick ascending limb of the loop of Henle, as shown using indirect immunofluorescence microscopy (12). Because the rbslo1 and rbslo2 are different MaxiK splice variants having distinct molecular weights and sequences, we examined using confocal microscopy the reconstructed three-dimensional pictures of rbslo1 and rbslo2 expressed in mTAL cells. The representative images in Fig. 10 clearly indicate that rbslo1 localized abundantly at the apical membrane of the renal epithelia. In contrast, very little rbslo2 colocalized with rbslo1 at the plasma membrane (Fig. 10C), suggesting that most rbslo2 proteins remain in the cytoplasm. The results from polarized epithelial cells are fundamentally consistent with those from non-polarized HEK293 cells. That rbslo1 proteins exist at the apical membrane of renal epithelial cells is consistent with a role for these channels in K+ secretion in kidney.

In summary, we demonstrated that rbslo1 is localized at the cell surface in non-polarized cells and at the apical surface of polarized renal epithelial cells. Whereas rbslo2 retains the ability to assemble into oligomers, the majority does not reach the cell surface, remaining trapped in the ER and Golgi. Considering the high degree of similarity between the two isoforms,
we conclude that the cytoplasmic tail of rbslo1, missing in rbslo2, is critically involved in the cell surface expression of MaxiK channels.

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