Role of an S4-S5 Linker Lysine in the Trafficking of the Ca\(^{2+}\)-activated K\(^+\) Channels IK1 and SK3*  

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We have investigated the role of the S4-S5 linker in the trafficking of the intermediate (human (h) IK1) and small (rat SK3) conductance K\(^+\) channels using a combination of patch-clamp, protein biochemical, and immunofluorescence-based techniques. We demonstrate that a lysine residue (Lys197) located on the intracellular loop between the S4 and S5 domains is necessary for the correct trafficking of hIK1 to the plasma membrane. Mutation of this residue to either alanine or methionine precluded trafficking of the channel to the membrane, whereas the charge-conserving arginine mutation had no effect on channel localization or function. Immunofluorescence localization demonstrated that the K197A mutation resulted in a channel that was primarily retained in the endoplasmic reticulum, and this could not be rescued by incubation at 27 °C. Furthermore, immunoblot analysis revealed that the K197A mutation was overexpressed compared with wild-type hIK1 and that this was due to a greatly diminished rate of channel degradation. Co-immunoprecipitation studies demonstrated that the K197A mutation did not preclude multimer formation. Indeed, the K197A mutation dramatically suppressed expression of wild-type hIK1 at the cell surface. Finally, mutation of this conserved lysine in rat SK3 similarly resulted in a channel that failed to correctly traffic to the plasma membrane. These results are the first to demonstrate a critical role for the S4-S5 linker in the trafficking and/or function of IK and SK channels.

The human (h)\(^3\) intermediate conductance Ca\(^{2+}\)-activated K\(^+\) channel IK1 is a member of the KCNN gene family, which also includes SK1–3, the small conductance Ca\(^{2+}\)-activated K\(^+\) channels. Within this channel family, there is ~40% amino acid sequence homology, with the greatest level of identity occurring in the pore and proximal C terminus, a region known to constitutively bind calmodulin (1, 2), thereby conferring Ca\(^{2+}\) sensitivity to these channels. The hIK1 channel is now known to be the Gardos channel involved in red blood cell volume regulation (3). hIK1 is also known to be involved in the Ca\(^{2+}\)-dependent regulation of Cl\(^–\) secretion across intestinal and airway epithelia (4–8). More recent work has demonstrated that the progression of breast cancer cells through the cell cycle is dependent on membrane hyperpolarization resulting from the activation of hIK1 channels (9). hIK1 has also been shown to play a role in both macrophage activation and T-lymophocyte proliferation (10–12). The critical role that pharmacological modulation of hIK1 may play has been revealed by the demonstration that blockers of hIK1 reduce the autoimmune response to experimental encephalomyelitis in mice (13), reduce brain edema following traumatic brain injury (14), and prevent restenosis following balloon angioplasty (15). Also, openers have been shown to alter vascular tone and hence may modulate peripheral blood pressure (16, 17).

A great deal of research has focused on both the biophysical and pharmacological properties of hIK1 (18–20) and more recently on the assembly and second messenger activation (21–24). In this study, we have begun to investigate the role that the S4-S5 linker region plays in the function and trafficking of IK and SK channels. Cytoplasmic protein domains have been shown to play a role in ion channel regulation through interactions with the nearby pore region. For example, the S4-S5 region in the Shaker K\(^+\) channel is believed to be involved in the “ball-and-chain”-dependent inactivation (25–27). Recent studies have also demonstrated that the S4-S5 linker is directly involved in channel gating via an interaction with the distal S6 segment (28). This interaction has been confirmed by the recent crystal structure of the Kv1.2 channel (29, 30). In the inwardly rectifying K\(^+\) (Kir) ROMK channels, this region surrounding the K\(^+\)-selective pore is necessary for the proper assembly of homomeric channel subunits (31). In addition, the arginine and lysine residues located juxtaposed to the pore are involved in channel gating by intracellular pH (32, 33). We originally demonstrated that endogenously expressed hIK1 is inhibited by acidic cytoplasmic pH (6), and this was later confirmed in heterologously expressed hIK1 (34). As hIK1 expresses a lysine just prior to the S5 domain at a position nearly equivalent to that of the lysine expressed just proximal to the M1 domain in the Kir channels known to be involved in pH-dependent gating, we speculated that this region was a strong candidate for a regulatory domain. The growing importance of this region in all aspects of ion channel function and traffic prompted our investigation of this region in hIK1.

Here, we demonstrate that Lys197 is critical for proper trafficking of the hIK1 channel. This trafficking mutation was able to assemble into a multimeric structure and acted as a dominant-negative when paired with the wild-type channel, precluding the movement of wild-type channels to the surface. The K197A mutation was also resistant to protein degradation, thereby accumulating in the endoplasmic reticulum. The homologous amino acid substitution in rat (r) SK3 (K453) similarly resulted in loss of rSK3 channel expression at the cell surface. These results are the first to demonstrate an integral role for the S4-S5 linker in the trafficking of the IK/SK channels to the plasma membrane.

**MATERIALS AND METHODS**

*Cell Culture*—Human embryonic kidney (HEK) 293 cells were obtained from American Type Culture Collection (Manassas, VA) and

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3 The abbreviations used are: h, human; r, rat; HEK, human embryonic kidney; HA, hemagglutinin; PBS, phosphate-buffered saline; BSA, bovine serum albumin; pF, picofarad; DCEBIO, 5,6-dichloro-1-ethyl-1,3-dihydro-2H-benzimidazol-2-one; ER, endoplasmic reticulum.
cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in a humidified 5% CO₂ and 95% O₂ incubator at 37 °C. Cells were transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. Stable cell lines were generated for all constructs by subjecting cells to antibiotic selection (1 mg/ml G418) 48 h post-transfection. Selection was typically complete within 14 days post-transfection. Following selection, the concentration of G418 was reduced to 0.2 mg/ml. Note that clonal cell lines were not subsequently selected from this stable population of cells to avoid clonal variation.

**Molecular Biology**—PBPlasmids containing the cDNAs for hIK1 and rSK3 were provided by Dr. J. P. Adelman (Vollum Institute, Oregon Health Sciences University). The channel was then subcloned into pCDNA3.1(+)/myc epitope (EQKLISEEDL) by PCR amplification of hIK1 in pCDNA3.1(−) and then subcloned in-frame into pCDNA4/myc (Invitrogen) using EcoRI and BamHI restriction sites. The fidelity of all constructs utilized in this study was confirmed by sequencing (ABI PRISM 377 automated sequencer, University of Pittsburgh) and subsequent sequence alignment (NCBI BLAST) with hIK1 (GenBankTM accession numbers AF022150 (hIK1) and U69884 (rSK3)).

**Antibodies**—Antibody HA.11 for use in immunofluorescence, immunoprecipitation, and immunoblotting was obtained from Covance Inc. (Richmond, CA). Anti-c-Myc antibody (clone 9E10) was obtained from Roche Applied Science. Anti-rSK3 polyclonal antibody was obtained from Chemicon International, Inc. (Temecula, CA). Antibody against giantin was a gift from Dr. Ora Weisz (University of Pittsburgh), and the anti-calnexin primary antibody was acquired from Stressgen Biotechnologies Corp. (Victoria, British Columbia, Canada). Secondary antibodies were obtained from the indicated sources: biotin-conjugated goat anti-mouse IgG (Molecular Probes, Inc., Eugene, OR), Cy3.18-conjugated goat anti-mouse IgG (Amersham Biosciences), and horseradish peroxidase-conjugated goat anti-mouse IgG and horseradish peroxidase-conjugated goat anti-rabbit IgG (Pierce). Alexa Fluor® 488-conjugated streptavidin was obtained from Molecular Probes, Inc. (Eugene, OR).

**Immunofluorescence**—For immunofluorescence labeling, HEK293 stable cell lines were grown on poly-l-lysine (Sigma)-coated glass coverslips for 24 h prior to labeling. For detection of cell-surface HA-hIK1, the cells were washed with ice-cold phosphate-buffered saline (PBS) and blocked with 1% bovine serum albumin (BSA; 3 × 5 min), followed by goat serum (10% for 20 min). HA-hIK1 was then labeled sequentially by incubation in anti-HA monoclonal primary antibody (1:1000 dilution, 90 min), goat anti-mouse IgG secondary antibody (1:1000, 90 min). This approach allowed us to detect both cell-surface and intracellular HA-hIK1 in the same cells. Cells were then subjected to laser confocal microscopy using a Leica TCS NT3 laser 4 photomultiplier tube system. To ensure maximal x-y spatial resolution, sections were scanned at 1024 × 1024 pixels using sequential two-color image collection to minimize cross-talk between the channels imaged. Alternatively, the cells were subjected to standard epifluorescence using a Nikon Microphot-FXL microscope, with images captured using an Olympus 0701121-01A digital camera coupled to MagnaFire 2.0 software (Optronix, Goleta, CA). All images shown in a single figure were acquired on the same day using identical settings. The images were then imported into Adobe Photoshop and combined into a single figure, and RGB brightness/contrast was adjusted identically for all panels.

**Electrophysiology**—All electrophysiological recordings were carried out as described previously (21, 22, 35). During whole-cell patch-clamp experiments, the bath contained 140 mM NaCl, 4 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, and 10 mM HEPES (pH adjusted to 7.4 with NaOH). The pipette solution contained 130 mM KCl, 5 mM NaCl, 0.12 mM CaCl₂, 4 mM MgCl₂, 10 mM HEPES, and 0.2 mM EGTA (pH adjusted to 7.2 with KOH; free [Ca²⁺] of 200 nM). All experiments were performed at room temperature (22 °C) using HEK293 cells plated the previous day. Electrodes were fabricated from thin-walled borosilicate glass (World Precision Instruments, Inc., Sarasota, FL), were pulled on a vertical puller (Narishige International USA, Inc., Long Island, NY), and had a resistance of 1–4 MΩ. Currents were recorded using an Axopatch 200B amplifier (Axon Instruments, Union City, CA) interfaced to a computer using a Digidata 1322A digitizer (Axon Instruments). Following establishment of the whole-cell configuration, voltage steps were applied using pCLAMP 8.2 software (Axon Instruments) from a holding potential of −60 mV at 250-ms pulses every 2 s from −100 to +80 mV in 20-mV increments to generate a current-voltage relationship. Current was sampled at steady state (100 ms) to evaluate current density. Current density (pA/picoFarad (pF)) at 0 mV was calculated by dividing the current by the whole-cell capacitance.

**Immunoprecipitation**—For co-immunoprecipitation of HA- and Myc-tagged hIK1 constructs, HEK293 cells were transiently transfected in 60-mm dishes using Lipofectamine 2000 and 5 µg of each plasmid (total of 10 µg of DNA and 20 µl of lipid) as described previously (22, 24). When only a single construct was transfected (HA or Myc), the pCDNA3.1(−) empty vector was included (5 µg) to maintain the plasmid and lipid at the same final concentrations in all dishes. 18–24 h post-transfection, cells were washed three times with ice-cold PBS and then lysed with immunoprecipitation buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1% (w/v) Triton X-100, Complete EDTA-free protease inhibitor (Roche Applied Science, pH 7.4). Protein concentrations were determined and normalized to achieve equivalent loading. Crude lysates were then precleared with protein A-Sepharose beads (Sigma) and incubated with anti-HA polyclonal antibody. Immune complexes were precipitated with protein A-Sepharose beads, followed by sequential washes with immunoprecipitation buffer containing 500, 300, and 150 mM NaCl (twice each) and supplemented with 1× radioimmunoprecipitation assay buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% (w/v) Triton X-100, 1% (w/v) sodium deoxycholate, and 0.1% (w/v) SDS). After the final wash, the pellet was resuspended in Laemmli sample buffer, and proteins were resolved by SDS-PAGE (12% gel) and transferred to nitrocellulose for immunoblot analysis as described below.
Cell-surface immunoprecipitation experiments were carried out as described previously (22). Briefly, cells were grown to confluence in a 100-mm dish and then washed with ice-cold PBS, blocked with 1% BSA in PBS, and labeled with anti-HA polyclonal antibody (1:500 dilution) for 90 min at 4 °C. Unbound antibody was removed by extensive washing with 1% BSA, followed by three washes with PBS. As described above, all steps were performed at 4 °C to prevent endocytosis of the channel and/or antibody. The cells were then lysed; protein concentrations were normalized; and the immune complexes were directly subjected to immunoprecipitation as described above. Following transfer to nitrocellulose, immunoblotting was performed using anti-HA monoclonal antibody (1:1000 dilution) as described below. In addition to the immunoprecipitation, 20 μg of protein was set aside following cell lysis for immunoblotting. In this way, we were able to confirm similar levels of protein expression in cells failing to correctly traffic HA-hIK1 to the cell surface.

**Immunoblot Analysis**—HEK293 cells were grown to confluence, lysed with immunoprecipitation buffer, separated by SDS-PAGE, and transferred to nitrocellulose. Blots were blocked for 1 h at room temperature using Tris-buffered saline blocking solution containing 5% (w/v) milk powder, 0.1% (v/v) Tween 20, and 0.005% (v/v) antifoam A. Subsequently, blots were incubated with primary antibody (mouse anti-HA monoclonal (1:1000 dilution), anti-rSK3 polyclonal (1:2000 dilution), or mouse anti-c-Myc monoclonal (1:2000 dilution)) in Tris-buffered saline blocking solution for 1 h at room temperature, followed by incubation with secondary antibody (horseradish peroxidase-conjugated goat anti-mouse IgG (1:10,000 dilution) or horseradish peroxidase-conjugated goat anti-rabbit IgG (1:20,000 dilution)). The blot was then extensively washed, and detection was performed using West Pico chemiluminescent substrate (Pierce).

For the protein degradation time course, 400 μg/ml cycloheximide was added to cells plated in 35-mm culture dishes. Cells were then lysed and collected at the various time points, and the immunoblot protocol described above was followed.

**Proteinase K Digestion**—Proteinase K digestion was performed as described previously (24). Briefly, HEK293 cells stably transfected with wild-type, K453A, or K453R rSK3 were washed three times with ice-cold PBS. Each 60-mm dish was incubated with 10 mM HEPES, 150 mM NaCl, and 2 mM CaCl2 (pH 7.4) with or without 200 μg/ml proteinase K (Sigma) at 37 °C for 30 min. Proteinase K digestion was quenched by adding ice-cold PBS containing 6 mM phenylmethylsulfonyl fluoride and 25 mM EDTA. This treatment was followed by three washes with ice-cold PBS. Cleared lysates were prepared and analyzed by immunoblotting as described above.

**Chemiluminescence Assay**—HEK293 cells were transiently transfected with the Myc- and/or HA-tagged wild-type or K197A hIK1 channel 24 h prior to the experiment. The cells were washed twice with ice-cold PBS and blocked three times with 1% BSA in PBS. The cells were then incubated with polyclonal antibody HA.11 (1:100 dilution) for 90 min at 4 °C and washed three times with PBS containing 1% BSA. The incubates were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (1:2000 dilution) for 1 h at 4 °C. The cells were subsequently washed 10 times with PBS containing 1% BSA and then washed twice with ice-cold PBS. The horseradish peroxidase-labeled proteins were detected using 5 ml of West Pico chemiluminescent substrate for 5 min. The absorbance from each dish was measured using a TD-20/20 luminometer (Turner Designs, Inc., Sunnyvale, CA), and data are presented as a percent of the wild-type absorbance.

**Chemicals**—All chemicals were obtained from Sigma unless stated otherwise. 5,6-Dichloro-1-ethyl-1,3-dihydro-2H-benzimidazol-2-one (DCEBIO) was synthesized in the laboratory of Dr. R. J. Bridges (University of Pittsburgh) as described previously (36). Both DCEBIO and clotrimazole were made as 10,000-fold stock solutions in Me2SO. Complete EDTA-free protease inhibitor mixture was obtained from Roche Applied Science.

**Statistics**—All data are presented as means ± S.E., where n indicates the number of experiments. Statistical analysis was performed using Student’s t test. A p value <0.05 was considered statistically significant and is reported. The immunofluorescence and other biochemical assays were repeated at least three times to ensure the fidelity of our results.

**RESULTS**

We previously defined regions in both the N (22) and C (24) termini of hIK1 that are important for proper channel trafficking to the plasma membrane. Recently, we demonstrated that these regions of the channel are closely associated and that this association may modulate how these separate domains of hIK1 interact to regulate channel function (35). As the S4-S5 linker region has been shown to be important in the regulation and function of numerous K+ channels, including Shaker (28, 37) and human ERG, KvLQT, and HCN (32, 33, 38–41), we chose to investigate the role of this domain in the trafficking of the IK/SK channels.

In our initial studies, we performed an alanine scan of Arg<sup>189</sup>–Met<sup>200</sup>, comprising the distal portion of the proposed S4–S5 linker. For these experiments, single- or double-point mutations were made within the HA epitope-tagged hIK1 channel plasmid. We have previously demonstrated that insertion of the HA epitope within the second extracellular loop does not alter channel trafficking or function (24). We have also shown that the extracellular placement of this tag allows us to successfully detect a channel that has trafficked properly to the cell surface (22, 24).

Initial examination of the amino acids within the S4-S5 linker showed that all of the alanine-substituted channels were expressed at the cell surface except at position 197 (K197A) (Fig. 1A), although immunoblot analysis confirmed abundant steady-state expression of all channel constructs (Fig. 1B). Indeed, it should be noted that this alanine-substituted channel was expressed at significantly higher steady-state levels than wild-type hIK1. The additional substitution of methionine at Lys<sup>197</sup> (K197M) similarly led to a channel that failed to express at the cell surface (Fig. 1A). In contrast, maintaining a positive charge by substituting arginine for Lys<sup>197</sup> (K197R) resulted in a correctly localized channel (Fig. 1A). These results demonstrate a critical role for Lys<sup>197</sup> in the trafficking of hIK1 to the plasma membrane and further show that this is dependent upon a basic amino acid at this position.

To confirm a lack of functional channels at the cell surface following mutation of Lys<sup>197</sup>, we utilized the whole-cell patch-clamp technique. For these experiments, the hIK1 channels were first stimulated with DCEBIO (10 μM), an activator of hIK1 (36), followed by inhibition with clotrimazole (3 μM), a known blocker of hIK1 channels (42, 43). This DCEBIO-sensitive, clotrimazole-dependent current was then corrected for cell capacitance to obtain the average current density (pA/pF). For HA-tagged wild-type hIK1, the current density averaged 133 ± 14 pA/pF (n = 35) (Fig. 1C). Consistent with our cell-surface immunoprecipitation data, neither the K197A nor K197M channel exhibited any DCEBIO-dependent, clotrimazole-sensitive current: 0.3 ± 0.1 pA/pF (n = 6) and 0.2 ± 0.1 pA/pF (n = 3), respectively (Fig. 1C). However, the current density of the arginine-substituted channel (K197R) was not different from that of the wild-type channel (126 ± 29 pA/pF, n = 7). These results confirm that a basic amino acid is required at position 197 for the functional expression of hIK1 at the plasma membrane.
4-The above data demonstrate that K197A failed to traffic to the plasma membrane. To initially determine the cellular localization of wild-type hIK1 compared with K197A hIK1, we labeled cell-surface HA-tagged hIK1 at 4 °C, followed by labeling the intracellularly localized channel as described under “Materials and Methods.” As shown in Fig. 2A (left panel), HA-tagged wild-type hIK1 was expressed at the cell surface (green) as well as intracellularly (red), as described previously (22, 24). In contrast, no cell surface-localized K197A was detected (Fig. 2A, middle panel), consistent with our cell-surface immunoprecipitation data (Fig. 1A). However, the channel accumulated at high levels in an intracellular compartment (red), consistent with the increased protein expression detected by immunoblotting (Fig. 1B). As predicted from our above studies, the K197R mutation was expressed at the cell surface (green) (Fig. 2A, right panel).

Based on these preliminary data, we used immunofluorescence-based co-localization studies with antibodies against endoplasmic reticulum (calnexin)- and Golgi (giantin)-resident proteins to determine the intracellular compartment in which K197A was retained. Calnexin has been shown to be strictly localized to the rough endoplasmic reticulum (ER) as well as the contiguous nuclear envelope and the intermediate compartment, where it functions as a molecular chaperone and aids in protein folding (44–46). Giantin has been shown to be localized to the membrane of the Golgi complex and participates in the docking and movement of vesicles within the Golgi stacks (47, 48). As shown in Fig. 2B, the K197A mutation resulted in a channel that was localized throughout the cytoplasmic compartment in a fishnet-like pattern, consistent with ER localization. Indeed, comparison with the calnexin labeling (Fig. 2B, upper panels) demonstrates a high degree of co-localization, indicating that the K197A channel is retained in the ER. However, it should be noted that, in some cells, we observed a clear co-localization with giantin as well (Fig. 2B, lower panels), indicating that a fraction of the K197A hIK1 channels are capable of exiting the ER before being retained in the Golgi.

We previously demonstrated that mutation of the C-terminal leucine zipper in hIK1 results in a trafficking defect such that the channel is retained in the ER and that this can be corrected by incubating the cells at 27 °C (24). Thus, we determined whether the K197A mutation leads to a similar misfolding of the hIK1 protein that can be corrected at reduced temperature (27 °C). As shown in Fig. 3, lowering the incubation temperature to 27 °C did not increase cell-surface expression of either the K197A or K197M mutation as assessed by cell-surface immunoprecipitation. These results indicate that the elimination of a positive charge at amino acid 197 results in a channel that is retained intracellularly and that this mislocalization cannot be corrected by reduced temperature.

As is apparent from the above data, K197A hIK1 was expressed at much higher steady-state levels than wild-type hIK1. These data suggest that, despite accumulating in the ER, K197A is not a substrate for ER-associated degradation. To study the difference in protein half-life between the K197A and wild-type hIK1 channel, cycloheximide (400 μg/ml) was used to arrest protein synthesis. The total cell protein was then collected at various time points over a 24-h period and quantified by immunoblotting as described under “Materials and Methods.” As shown in Fig. 4, in the presence of cycloheximide, wild-type hIK1 exhib-
ited an exponential decline in protein expression with a half-life of 13.8 h, although ~30% of the hIK1 channel was resistant to degradation in the presence of cycloheximide. In contrast, K197A showed abundant intracellular labeling (red), whereas no green surface label was apparent. The K197R mutation showed an expression pattern similar to that of the wild-type channel with both cell-surface and intracellular labeling. B, subcellular localization of K197A. Cells were fixed; permeabilized; and labeled for HA-hIK1 (upper and lower left panels), giantin (upper middle panel), or calnexin (lower middle panel). The overlays for co-localization are shown (upper and lower right panels). There was little overlay between the K197A channel protein and the Golgi marker giantin (lower right panel). The K197A channel protein localized predominantly to the ER, as shown by the large amount of yellow when merged with the anti-calnexin antibody (upper right panel).

We previously demonstrated that mutation of the C-terminal leucine zipper results in channels that assemble into multimeric complexes, but fail to traffic to the plasma membrane, whereas mutation of the N-terminal leucine zipper results in channels that fail to correctly assemble (22, 24). To determine whether the trafficking-incompetent K197A mutation assembles, we performed co-immunoprecipitation studies using HA- and Myc-tagged hIK1 constructs as described under “Materials and Methods.” As shown in Fig. 5A (third lane), we were able to co-immunoprecipitate HA- and Myc-tagged wild-type hIK1, indicating that these channels co-assemble as expected for a tetrameric channel. Similarly, we were able to co-immunoprecipitate HA-K197A hIK1 and Myc-tagged wild-type hIK1 (Fig. 5A, fifth lane). Finally, when both HA-hIK1 and Myc-hIK1 channels were mutated to K197A, we were able to co-immunoprecipitate these channels (Fig. 5A, sixth lane), indicating that this mutation does not affect channel multimerization. It should also be noted that we were able to co-immunoprecipitate quantitatively more of the mutant channel than the wild-type channel, as expected for a channel with a prolonged half-life (Fig. 5A, compare third and sixth lanes).
S4-S5 Linker Controls Trafficking of hIK1

The above experiments demonstrate that mutation of Lys197 resulted in a channel that correctly assembled, but failed to traffic to the plasma membrane. Thus, we determined whether the interaction of K197A and wild-type channels would preclude wild-type hIK1 from trafficking to the plasma membrane. For these studies, we utilized a cell-surface chemiluminescence assay as described under "Materials and Methods." The chemiluminescence obtained following cotransfection of HA- and Myc-tagged wild-type hIK1 was normalized to 100% (Fig. 5B). When both the HA- and Myc-tagged channels contained the K197A mutation, there was a drastic reduction in cell-surface expression (1.1 ± 2.5%, n = 3), consistent with the observation that this mutation resulted in a trafficking-incompetent channel. Cotransfection of HA-tagged wild-type hIK1 with Myc-K197A hIK1 resulted in a decrease in surface expression of HA-hIK1 to 32.4 ± 8.8% (n = 3) of that seen in the absence of a mutant channel. These results further demonstrate that the K197A mutation does not preclude assembly with wild-type channels, and indeed, this interaction inhibits expression of wild-type channel subunits at the cell surface.

As hIK1 is a member of the KCNN gene family, which includes the small conductance Ca2+-activated potassium channels SK1–3, and as sequence alignment confirms that Lys197 is conserved across the gene family, we determined whether the homologous mutation in rSK3 would result in a similar phenotype. For these studies, Lys453 was mutated to either alanine (K453A) or arginine (K453R), and the current density was determined using the whole-cell patch-clamp technique, in which apamin, an inhibitor of SK channels (49), was used to block the DCEBIO-stimulated current. Similar to our results with hIK1, the K453A mutation in rSK3 resulted in a dramatically reduced current density of 2.4 ± 1.9 pA/pF (n = 10) compared with 79.1 ± 20.0 pA/pF (n = 12) for wild-type rSK3, whereas the charge-conserving mutation (K453R) had no effect on current density (73.9 ± 13.5 pA/pF, n = 3) (Fig. 6A).

To confirm that the decreased current density observed for the K453A mutation was due to a lack of plasma membrane-localized channel rather than to a defect in channel gating, we assessed cell-surface protein expression using a proteinase K cleavage assay as described previously (22). In the absence of proteinase K, rSK3 ran at an apparent molecular mass of ~80 kDa, consistent with the full-length protein (Fig. 6B, first lane). Following proteinase K treatment, the majority of this 80-kDa band was converted to a product with an apparent molecular mass of ~45 kDa (Fig. 6B, second lane), demonstrating that the majority of wild-type rSK3 is expressed at the cell surface at steady state. In contrast, the K453A rSK3 channel ran with an apparent molecular mass of ~80 kDa in the presence (Fig. 6B, third lane) or absence (fourth lane) of proteinase K, demonstrating that this mutation results in a channel that fails to traffic to the plasma membrane and is therefore protected from proteinase K degradation. Similar to our results for hIK1, the charge-conserving mutation in rSK3 (K453R) trafficked to the plasma membrane (Fig. 6B, fifth and sixth lanes). These results demonstrate a critical role for this lysine in the trafficking of both IK and SK members of the KCNN gene family.
Finally, we confirmed expression of the K453A rSK3 channel in an intracellular compartment by immunofluorescence localization (Fig. 6C). As expected, wild-type rSK3 showed a clear cell-surface pattern of labeling (Fig. 6C, upper panel). In contrast, the K453A mutation resulted in a channel that was clearly localized within the cell in a pattern consistent with ER localization (Fig. 6C, middle panel). Similar to our results with hIK1, the charge-conserving mutation (K453R) trafficked to the plasma membrane (Fig. 6C, lower panel). These findings suggest that the S4-S5 linker lysine control of membrane trafficking is conserved among the KCNN gene family members and is pivotal to the proper localization of the channel to the plasma membrane.

DISCUSSION

Our laboratory has previously shown that hIK1 trafficking and assembly are dependent on both N- and C-terminal leucine zippers (22, 24). Recently, we utilized fluorescence resonance energy transfer to demonstrate a close association between the N and C termini of hIK1, consistent with both leucine zippers playing a crucial role in channel assembly/trafficking (35). Joiner et al. (23) have also shown that the constitutive binding of calmodulin to the C terminus is required for the proper localization of hIK1 to the cell surface. Apart from the N and C termini, the roles of other domains of hIK1 in plasma membrane localization have not previously been investigated. In this study, we have demonstrated that a lysine (Lys197) within the predicted intracellular S4-S5 loop is required for the trafficking of hIK1 to the plasma membrane. We have shown that mutation of Lys197 to alanine or methionine in hIK1 results in the steady-state accumulation of hIK1 in the ER and that this is due to a greatly diminished rate of channel degradation. We extended our findings by demonstrating that the trafficking of another member of the KCNN gene family, rSK3, is similarly dependent upon the homologous lysine (Lys453) in the S4-S5 linker for its proper trafficking to the plasma membrane. These results represent the first demonstration of a role for the intracellular loop between the S4 and S5 domains in the trafficking of the IK/SK channels.

The role of the intracellular S4-S5 linker region in channel regulation and function has been well documented in many other ion channels. For example, examination of the Shaker channel inactivation gate showed that residues within the S4-S5 linker are part of the receptor region involved in the ball-and-chain-dependent inactivation (25). More recently, it has been shown that the S4-S5 linker, in conjunction with the distal portion of the S6 domain, controls the voltage-dependent gating of Shaker channels (28, 37). In these studies, it was proposed that the S4-S5 linker acts as the coupling device linking voltage to channel opening. Recent work from MacKinnon and co-workers (29, 30) have similarly demonstrated a significant role for the S4-S5 linker region in coupling channel voltage sensing with activation in the HCN pacemaker channels. Finally, in the KvLQT channel family, mutations within the S4-S5 linker region cause a decrease in current and modify channel interactions with the minK β-subunit, increasing the likelihood of long QT syndrome (52). We have demonstrated here for the first time that this region is also crucial for the trafficking of a class of non-voltage-gated K⁺ channels, the Ca²⁺-activated IK and SK channels.

We previously demonstrated that mutation of the C-terminal leucine zipper in hIK1 results in a channel that is trapped in the ER and that this trafficking deficiency can be rescued by incubating the cells at 27 °C (24). The ability to correct trafficking at a permissive temperature has also been shown for human ERG (53) and the cystic fibrosis transmembrane conductance regulator (54) and is generally taken as an indication
of a misfolded protein. In contrast to these results, the K197A mutation in hIK1 was insensitive to reduced temperature (Fig. 3), indicative of a more profound trafficking deficiency. One possible explanation for the retention of K197A hIK1 in the ER is that an ER retention signal becomes unmasked by this mutation. hIK1 possesses a potential RKR motif-based ER retention signal in its N terminus. However, we have previously shown that mutation of this RKR motif dramatically influences the ATP dependence of channel gating while having no significant effect on channel trafficking (35). These results argue against a role for an RKR motif-based signal in the retention of K197A hIK1. Whether additional, yet to be described ER retention motifs exist in hIK1 and whether these are responsible for the observed results remain to be determined.

Another possibility is that the K197A mutation results in an ER export signal being destroyed either directly or via a change in protein structure. However, our co-localization studies revealed that a fraction of the K197A channel escaped the ER and localized in the Golgi (Fig. 2B). This localization in the Golgi may be explained by Golgi export being disrupted in hIK1 following mutation of the S4-S5 linker lysine. In this regard, it is interesting to note that, in the inwardly rectifying K⁺ channels, this type of Golgi-selective export regulation has been observed, and channel protein sequence information for this exit is contained within a single basic amino acid within an intracellular domain of the channel (55). It must be stressed, however, that we did not see the same level of hIK1 accumulation in the Golgi as was reported for the Kir channel.

We have demonstrated that, in all experimental paradigms, K197A hIK1 accumulates at higher steady-state protein levels than wild-type hIK1. These results indicate that, in addition to being trafficked inappropriately, this mutation also alters the degradation fate of the channel. We have demonstrated that the K197A mutation correctly assembles into a multimeric complex and indeed interacts with and inhibits plasma membrane expression of wild-type hIK1 (Fig. 5). These results indicate that it is the fully assembled channel that is sequestered intracellularly rather than being targeted for degradation. Consistent with the increased steady-state level of the protein, we observed no effect of proteasomal (lactacystin) or lysosomal (leupeptin/pepstatin) inhibitors on total steady-state K197A hIK1 protein levels (data not shown). Based on these observations, we speculate that the protein machinery involved in protein folding and/or targeting for degradation fails to recognize the K197A mutation.

One possible explanation for the accumulation of the K197A mutation is that the channel fails to be released from one of the ER chaperones involved in protein folding, such as Hsp70/Hsc70 or Hsp90/Hsc90. The Hsp90 and Hsp70 chaperones are known to be involved in protein stabilization of multimeric subunits and in the folding processes necessary to allow proteins to reach their native states required for proper protein trafficking with the cell (56, 57). For instance, the human ERG channel-containing vesicles to the cell surface (59). Interestingly, recent studies on the binding domains within the Hsp70 and Hsp90 proteins have shown that there may be charge interactions between the various cargo proteins and chaperones (60, 61), suggesting a possible role for the charged lysine residue at position 197 of hIK1 in chaperone-mediate protein trafficking.

Another possibility is that the K197A mutation disrupts a non-classical chaperone interaction. Recently, the idea of chaperones assisting in the processing and trafficking of K⁺ channels has been examined in the voltage-dependent Kv channels, in which the chaperone KChAP has been shown to be essential for proper cell-surface expression. This novel chaperone will bind to the N and C termini of the various Kv subtypes and facilitate their trafficking to the plasma membrane (62). Whether an alternative chaperone-like protein binds to hIK1 has yet to be determined. However, recent evidence has suggested that the endocytosis of SK3 is dependent on the association with SH3GL3, a member of the endophilin family of proteins (63), leaving open the possibility that the trafficking of hIK1 is dependent on a yet to be described protein interaction.

In conclusion, this work has demonstrated an essential role for a lysine residue located within the intracellular S4-S5 linker of hIK1 (Lys197) and SK3 (Lys453) in protein trafficking. The mutant protein has a prolonged half-life compared with the wild-type protein and accumulates in the ER, suggesting that a protein-protein interaction required for channel degradation and/or trafficking is disrupted. This represents the first demonstration of a role for the S4-S5 linker in the processing and trafficking of the IK/5K family of ion channels.

REFERENCES

1. Schumacher, M. A., Crum, M., and Miller, M. C. (2004) Structure (Camb.) 12, 859–860
2. Schumacher, M. A., Rivard, A. F., Bachinger, H. P., and Adelman, J. P. (2001) Nature 410, 1120–1124
3. Hoffman, J. F., Joiner, W., Nehrke, K., Potapova, O., Foye, K., and Wickrema, A. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 7366–7371
4. Devor, D. C., and Frizzell, R. A. (1998) Am. J. Physiol. 274, C138–C148
5. Devor, D. C., and Frizzell, R. A. (1998) Am. J. Physiol. 274, C149–C160
6. Devor, D. C., and Frizzell, R. A. (1993) Am. J. Physiol. 265, C1271–C1280
7. Dharmanathpohona, K., and Pandol, S. J. (1986) J. Clin. Investig. 77, 348–354
8. Szkotak, A. J., Murthy, M., MacVinish, L. J., Duszky, M., and Cuthbert, A. W. (2004) Br. J. Pharmacol. 142, 531–542
9. Ouaddi-Abdouch, H., Le Bourhis, X., Roudbaraki, M., Toilson, R. A., Degourt, P., and Previrsaykaya, N. (2001) Receptors Channels 7, 345–356
10. Khanna, R., Chang, M. C., Joiner, W. J., Kaczmarek, L. K., and Schlüchter, L. C. (1999) J. Biol. Chem. 274, 14838–14849
11. Begenisich, T., Nakamoto, T., Ovitt, C. E., Nehrke, K., Brugnara, C., Alper, S. L. J., and Melvin, J. E. (2004) J. Biol. Chem. 279, 47681–47687
12. Hanley, P. J., Musset, B., Reigny, V., Limberg, S. H., Dalpke, A. H., Sus, R., Heeg, K. M., Preisig-Muller, R., and Daut, J. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 9479–9484
13. Reich, E. F., Cui, L., Lang, Y., Pugliese-Sivo, C., Golovko, A., Petro, M., Masuaga, G., Liu, I., Nomis, A. A., Zhang, L. K., Liang, X., Kozlowski, J. A., Narula, S. K., Zavodny, P. J., and Chow, C. C. (2005) Eur. J. Immunol. 35, 1027–1036
14. Mauler, F., Hinz, V., Horvath, E., Schuhmacher, J., Hofmann, H. A., Wirtz, S., Hahn, M. G., and Urbahn, H. (2004) J. Eur. Neurol. 20, 1761–1768
15. Kohler, R., Wulff, H., Eichler, I., Kneifel, M., Neumann, D., Knorr, A., Grigic, L., Kämpfle, D., Sti, H., Wibawa, J., Reusch, H. P., Paul, M., and Hoyer, J. (2003) Circ. Res. 87, 496–503
16. Adeagbo, A. S. (1999) Eur. J. Pharmacol. 379, 151–159
17. Ishii, T. M., Maylie, J., and Adelman, J. P. (1997) J. Biol. Chem. 272, 23195–23202
18. Joiner, W. J., Wang, L. Y., Tang, M. D. and Kaczmarek, L. K. (1999) Proc. Natl. Acad. Sci. U. S. A. 94, 11013–11018
19. Wulff, H., Guttman, G. A., Cahanal, M. D., and Chandy, K. G. (2001) J. Biol. Chem. 276, 32040–32045
20. Hamilton, K. L., Syme, C. A., and Devor, D. C. (2003) J. Biol. Chem. 278, 16690–16697
21. Jones, H. M., Hamilton, K. L., Papworth, G. D., Syme, C. A., Watkins, S. C., Bradbury, N. A., and Devor, D. C. (2004) J. Biol. Chem. 279, 15531–15540
22. Joiner, W. J., Khanna, R., Schlüchter, L. C., and Kaczmarek, L. K. (2001) J. Biol. Chem. 276, 37980–37985
23. Syme, C. A., Hamilton, K. L., Jones, H. M., Gerlach, A. C., Gilman, L., Papworth, G. D., Watkins, S. C., Bradbury, N. A., and Devor, D. C. (2003) J. Biol. Chem. 278, 8476–8486
24. Holmgren, M., Jurman, M. E., and Yellen, G. (1996) J. Gen. Physiol. 108, 195–206
