We demonstrate that the C-terminal truncation of hIK1 results in a loss of functional channels. This could be caused by either (i) a failure of the channel to traffic to the plasma membrane or (ii) the expression of non-functional channels. To delineate among these possibilities, a hemagglutinin epitope was inserted into the extracellular loop between transmembrane domains S3 and S4. Surface expression and channel function were measured by immunofluorescence, cell surface immunoprecipitation, and whole-cell patch clamp techniques. Although deletion of the last 14 amino acids of hIK1 (L414STOP) had no effect on plasma membrane expression and function, deletion of the last 26 amino acids (K402STOP) resulted in a complete loss of membrane expression. Mutation of the leucine heptad repeat ending at Leu408 (L399A/L406A) completely abrogated membrane localization. Additional mutations within the heptad repeat (L385A/L392A, L392A/L406A) or of the a position (I396A/L403A) resulted in a near-complete loss of membrane-localized channel. In contrast, mutating individual leucines did not compromise channel trafficking or function. Both membrane localization and function of L399A/L406A could be partially restored by incubation at 27 °C. Co-immunoprecipitation studies demonstrated that leucine zipper mutations do not compromise multimer formation. In contrast, we demonstrated that the leucine zipper region of hIK1 is capable of co-assembly and that this is dependent upon an intact leucine zipper. Finally, this leucine zipper is conserved in another member of the gene family, SK3. However, mutation of the leucine zipper in SK3 had no effect on plasma membrane localization or function. In conclusion, we demonstrate that the C-terminal leucine zipper is critical to facilitate correct folding and plasma membrane trafficking of hIK1, whereas this function is not conserved in other gene family members.
Membrane Trafficking of hIK1

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of channel function, as assessed by patch clamp techniques (20). This loss of function could result from either a failure of hIK1 to traffic to the plasma membrane or the expression of non-functional channels. To delineate among these possibilities an HA epitope was inserted into the extracellular loop between transmembrane domains S3 and S4 such that cell surface expression could be monitored by immunofluorescence (IF) and whole-cell patch-clamp techniques. The function of hIK1 could be measured using the whole-cell patch clamp technique. We demonstrate that a C-terminal leucine zipper, distal to the calmodulin-binding domain, is required for the trafficking of hIK1 to the plasma membrane and that mutations of the leucine zipper alter the assembly of the distal C-terminal tail of hIK1. In contrast, this conserved leucine zipper is not required for the correct trafficking of another gene family member, SK3, to the plasma membrane.

EXPERIMENTAL PROCEDURES

Molecular Biology—pBF plasmid containing the cDNAs for full-length hIK1 and rSK3 were kindly provided by J. P. Adelman (Vollum Institute, Oregon Health Sciences University). These cDNAs were subcloned into pcDNA1. (+) (Invitrogen) using the EcoRI and Xhol restriction sites. A hemagglutinin (HA), VPVDVPVDA epitope was inserted into hIK1 (Ha-hIK1) between Gly132 and Ala133, i.e. the extracellular loop between transmembrane domains S3 and S4, by sequential overlinear extension PCR using plasmid-specific primers in conjunction with the primers: forward, 5'-TATCCGTAGCAGCTGCAGTACGCTG-GCCGCTGACCTCCCCGCAG-3' and reverse, 5'-GGCTGATTCGGCGAC-ACGTGCTACGGATAACCATAATCTCGACACGACGGCGGG-3', where the HA epitope is highlighted in boldface. The PCR product was subcloned into pcDNA3.1 (+) using EcoRI and Xhol restriction sites. All mutations in Ha-hIK1, L414STOP, K402STOP, L378A/L385A (ZIP1,2), (Val369 GLDST) epitope tag to the last 59 amino acids of the C terminus of hIK1 (Invitrogen) by utilizing subcloned into pcDNA3.1( myc) restriction sites. For co-immunoprecipitation of HA- and Myc-tagged hIK1 constructs, whole-cell current densities were obtained on at least 10 cells to account for any variation in expression across the stable cell lines.

Antibodies and Immunofluorescence Labeling—To detect HA-hIK1 and rSK3 in immunofluorescence (IF), immunoprecipitation (IP), and immunoblotting (IB), experiments antibodies were obtained from the following sources (dilutions used are indicated): V5 and Xpress (XP) (1:5,000; Invitrogen), polyclonal HA (1:150) and monoclonal (1:1,000) HA (HA.11, Covance, Richmond, CA), c-myc (clone 9E10, 1:1,000; Roche Molecular Biochemicals), and polyclonal rSK3 antibody directed against residues 2–21 of human SK3 (1:1,000; Chemicon International, Temecula, CA). Secondary antibodies were obtained from various sources as follows: biotin-conjugated goat anti-mouse IgG (1:200; Molecular Probes, Eugene, OR), streptavidin conjugated to Alexa Fluor® 488 (1:500; Molecular Probes), Cy3.18-conjugated goat anti-mouse IgG (1:1,300; Amersham Biosciences). HRP-conjugated goat anti-mouse IgG (1:2,000; Kirkegaard & Perry Laboratories, Gaithersburg, MD), and HRP-conjugated goat anti-rabbit IgG (1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA). Antibodies were dissolved in PBS before use.

For IF 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 in ice-cold PBS, blocked in 1% BSA (5 × 5 min) followed by goat serum (10% for 20 min). HA-hIK1 was then labeled sequentially by incubating in 1× (primary) monoclonal HA antibody (1:1,000; 90 min) and 2× (secondary) biotin-conjugated goat anti-mouse IgG (1:200; 90 min) followed by streptavidin conjugated to Alexa-488 (1:500) for 90 min. Each labeling step was followed by 3× washes with 1% BSA (5 min each) to remove unbound Ab. All steps were performed at 4 °C to prevent endocytosis of the channel. Following cell surface labeling, the cells were again washed in ice-cold PBS, fixed with 2% formaldehyde for 10 min, and 0.1% Triton X-100/2% paraformaldehyde/PBS, blocked with 1% BSA and 100 μM extracellular free Ca2+ as above, and then intracellular localized HA-hIK1 was labeled sequentially with 1× monoclonal HA antibody (1:1,000; 90 min), and 2× Cy3.18 conjugated goat anti-mouse IgG (1:5,000) antibody. Finally, nuclei were labeled with Hoechst 33258 (Sigma). 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 TCSNT 3 laser 4 PMT system. To ensure maximal X-Y spatial resolution, sections were scanned at 1024 × 1024 pixels, using sequential 2-color image collection to minimize cross-talk between the channels imaged. All images shown in a single figure were scanned on the same day. The images were adjusted for contrast and brightness using Adobe Photoshop, combined into a single figure, and RGB brightness/contrast adjustment contrast adjusted for all panels.

Immunoprecipitation (IP)—C59 constructs were translated from cDNA in the presence of [35S]methionine using the TNT T7-Coupled Bicistronic Lysate System (Promega, Madison, WI). Aliquots of the translation reaction were chased in the presence of either 100 or 10-fold excess of unlabelled cystine. The reactions were resolved on a 10% gel, dried, and subjected to autoradiography. For co-immunoprecipitation of HA- and Myc-tagged hIK1 constructs,
HEK293 cells were transiently transfected in 60-mm dishes using Li- 
fectAMINE 2000 and 5 μg of each plasmid (total, 10 μg of DNA and
20 μl of lipid). When only a single construct was transfected (HA or 
Myc), empty pcDNA3.1(+), was included (5 μg) to keep the final con-
centration of plasmid and lipid the same in all dishes. 18–24 h post-
transfection, cells were washed three times with ice-cold PBS and then 
lysed with IP buffer. Protein concentrations were determined and nor-
malized to achieve equivalent loading. Crude lysates were then pre-
cleared with protein A-Sepharose beads (Sigma) and incubated with 
rabbit polyclonal anti-HA antibodies. Immune complexes were precipi-
tated with protein A-Sepharose beads, followed by sequential washes in 
IP buffer containing 500, 300, and 150 mM NaCl, supplemented with 
1× radioimmunoprecipitation assay (RIPA) 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 immuno blot 
analysis as described below.

For CS-IP, cells were grown to confluence in a 100-mm dish and then 
wasched in ice-cold PBS, blocked in 1% BSA/PBS, and labeled with 
polyclonal HA.11 Ab (1:500) for 90 min at 4 °C. Unbound Ab was 
removed by extensive washing in 1% BSA followed by washes in PBS.
As above, all steps were performed at 4 °C to prevent endocytosis of 
the channel and/or Ab. The cells were then lysed, and protein concen-
trations were normalized and the immune complexes directly subjected to 
IP as described above. Following trypsinization to nitrocellulose, the blot 
was probed using monoclonal HA Ab (1:1,000) as described below. In 
addition to the IP, 15 μg of IP was set aside following cell lysis for 
an IB. 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, lys-
ed with IP buffer, separated by SDS-PAGE, and transferred to nitro-
cellulose. Blots were blocked for 1 h at room temperature using TBS-
blocking solution containing 5% v/v milk powder, 0.1% (v/v) Tween 20,
and washed in ice-cold PBS, blocked in 1% BSA/PBS, and labeled with 
HCOOH (pH 7.5, 150 mM NaCl, supplemented with 1× radioimmunoprecipita-
tion assay (RIPA) 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.

For CS-IP, cells were grown to confluence, lysised with IP buffer, separated by SDS-PAGE, and transferred to nitro-
cellulose. Blots were blocked for 1 h at room temperature using TBS-
blocking solution containing 5% v/v milk powder, 0.1% (v/v) Tween 20,
and washed in ice-cold PBS, blocked in 1% BSA/PBS, and labeled with 
HCOOH (pH 7.5, 150 mM NaCl, supplemented with 1× radioimmunoprecipita-
tion assay (RIPA) 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.

We previously demonstrated that truncation of hIK1 at 
Lys6102 resulted in a complete loss of functional channels at the 
cell surface (20). This result could be caused by either a failure of 
channels to correctly traffic to the plasma membrane or from 
channels that traffic normally but are non-functional. To dis-
inguish between these possibilities, an HA epitope was in-
serted into the extracellular loop between S3 and S4 (see “Ex-
perimental Procedures”) such that cell surface expression could 
be evaluated by IF and CS-IP techniques, whereas function 
was assessed by the whole-cell patch clamp technique. Initially, 
we confirmed that HA-hIK1 could be detected at the cell sur-
face by IF. As shown in Fig. 1A, wild-type HA-hIK1 is highly 
expressed at the cell surface (green) as well as being expressed 
intracellularly (red), as expected. Cells expressing hIK1 (no HA 
epitope) exhibited no fluorescence when labeled using an iden-
tical protocol (data not shown). We next determined whether 
insertion of the HA epitope had any effect on the biophysical 
and pharmacological characteristics of hIK1 as assessed by the 
excised, inside-out patch clamp technique. We and others dem-
strated previously that hIK1 is activated by the pharmacologi-
cal agent, DCEBIO (15), and inhibited by clotrimazole (2, 4, 
7, 11). In excised patch clamp studies, DCEBIO activated HA-
hIK1 with an apparent K_s of 3.8 ± 0.8 μM (n = 3), whereas 
clotrimazole inhibited HA-hIK1 with an apparent K_s of 65 ± 5 
μM (n = 3), values similar to those reported previously for hIK1 
(2, 4, 7, 11, 15). In addition, insertion of the HA epitope had no 
significant effect on either the single channel I-V relationship 
of the channel (chord conductance of 31 ± 1 pS at −100 mV and 
11 ± 1 pS at +100 mV, n = 4) or Ca2+–dependent gating (K_s 
= 831 ± 29 nm, Hill coefficient of 2.1; n = 3), as these are values 
similar to what we and others have reported previously (7, 11, 
12, 14, 23, 24) for endogenously and heterologously expressed 
hIK1. In total, these results indicate that insertion of an HA 
epitope into hIK1 did not affect channel function. Thus, we 
utilized this construct to define the role of the cytoplasmic C terminus in the cell surface expression of hIK1.

Based on the observation that the HA epitope did not alter 
the function of hIK1 channel activity in excised patches, we 
confirmed HA-hIK1 could be activated by the pharmacological 
 opener, DCEBIO (10 μM), and inhibited by clotrimazole (3 μM) 
in whole-cell patch clamp studies. Following establishment of 
the whole-cell configuration, current averaged 224.0 ± 38.5 pA 
at −20 mV, indicative of very few active channels (Fig. 1E). 
DCEBIO increased the whole-cell current to 5,013.4 ± 597.0 
PA, and this was inhibited by clotrimazole to 930.5 ± 176.4 
(n = 19). As the average capacitance for these 19 cells was 
26.6 ± 1.4 pF, this yielded an average current density of 
166.6 ± 19.4 pA/pF, as shown in Fig. 1E. Furthermore, as seen 
in Fig. 1D, HA-hIK1 displays no significant time- or voltage-
dependent activation during whole-cell recording as reported 
previously for hIK1 (11, 12).

Truncation of hIK1 C Terminus Compromises Cell Surface 
Expression—We demonstrated previously (20) that functional 
hIK1 channels could be recorded in excised, inside-out patches 
following truncation at Lys1414, whereas truncation at Lys6102 
resulted in a complete loss of channel function. Thus, we ini-
itially introduced these stop mutations into HA-hIK1 to deter-
mine whether K402STOP and L414STOP traffic to the plasma 
membrane. As shown in Fig. 1, in contrast to HA-hIK1 (A), 
K402STOP (C) failed to express at the cell surface as assessed 
by IF (no green), although intracellular channel is prevalent in 
the cell surface expression of the channel (Fig. 1B). To provide a quantitative 
estimate of cell surface expression of these truncated 
channels, we utilized the whole-cell patch clamp technique. Representative 
whole-cell recordings for HA-hIK1, K402STOP, and L414STOP are shown in Fig. 1D with average current density data shown in 
Fig. 1E. Whereas HA-hIK1 was highly expressed (166.6 ± 19.4 
pA/pF, n = 19), K402STOP failed to express functional channels 
at the cell surface (0.6 ± 0.2 pA/pF, n = 12). In contrast to this 
complete loss of functional expression, L414STOP resulted in a 
more modest decrease in current density (95.8 ± 20.8 pA/pF, n 
= 17). These data suggest that amino acid residues within the 
distal C terminus, between Lys6102 and Lys1414, are critical for the
Mutation of the C-terminal Leucine Zipper Abrogates Membrane Trafficking of hIK1—Sequence gazing of the amino acids between Lys 402 and Leu 414 reveals two potential structural motifs that may be required for correct trafficking of hIK1: (i) a di-leucine motif (Leu 409/Leu 410), and (ii) the terminal leucine (Leu 406) of a proposed leucine zipper motif. The last 59 amino acids of the hIK1 C terminus (Val 369–Lys 427), encompassing the entire leucine zipper, are shown in Fig. 2. Di-leucine motifs are known to play a critical role in both Golgi exit as well as endocytic recycling of a wide range of proteins (25), including ion channels (26), whereas leucine zippers are important in protein-protein interactions (27). Therefore, we used site-directed mutagenesis to define the role of the di-leucine and leucine zipper motifs in the trafficking of hIK1. As shown in Fig. 3, mutation of Leu 409/Leu 410 to alanines (DI-LEU) did not prevent trafficking of HA-hIK1 to the cell surface. Whole-cell patch clamp analysis confirmed functional expression of di-leucine at the cell surface (Fig. 4B), although the current density was significantly reduced (87.0 ± 26.1 pA/pF; n = 18) compared with HA-hIK1. Although these results suggest this di-leucine motif may play some role in the trafficking of hIK1, they cannot explain the complete loss of surface expression observed following truncation at Lys 402 (Fig. 1).

To assess the role of the leucine zipper in the trafficking of HA-hIK1, we initially mutated the 4th (Leu 399) and 5th (Leu 406) positions in the leucine zipper to proline (L399P) and phenylalanine (L406F). This mutation resulted in a complete abrogation of plasma membrane expression, as assessed by both IF (Fig. 3A, ZIP4,5) and cell surface immunoprecipitation (CS-IP, Fig. 3B). However, as shown in Fig. 3, this loss of function was not due to a loss of protein, as shown by both the IF labeling of intracellular channel (Fig. 3A) as well as the similar levels of protein upon immunoblot (Fig. 3B). Note that hIK1 runs as a doublet under the conditions utilized in our immunoblot and immunoprecipitation studies. Although the reasons for this micro-heterogeneity are unclear, it is a consistent finding for all of the constructs studied (Fig. 3B) and is also observed following in vitro translation in the presence of [35S]methionine (data not shown). We confirmed the role of this C-terminal leucine zipper in the trafficking of HA-hIK1 by introducing additional double mutations in the 2nd and 3rd leucines (L385A/L392A; ZIP2,3), the 3rd and 5th leucines (L392A/L406A; ZIP3,5), and the 1st and 2nd leucines (L378A/L385A; ZIP1,2). As shown in Fig. 3, ZIP2,3 and ZIP3,5 failed to traffic to the plasma membrane, although the channel was expressed intracellularly. In contrast, the ZIP1,2 mutation was clearly expressed at the cell surface as assessed by both IF (not shown) and CS-IP (Fig. 3B).

In coiled-coil domains, the hydrophobic pocket is determined by both the leucines in the d position, as well as hydrophobic...
amino acids in the α position (27). Thus, we determined whether the α position amino acids corresponding to Leu
399
 and Leu
406
 would similarly affect channel localization. Therefore, we mutated Ile
396
 and Leu
403
 to alanines (α-POS). As was true with the ZIP4,5 mutation, mutation of I396A/L403A resulted in a channel that failed to traffic to the plasma membrane as determined by both IF (Fig. 3A) and CS-IP (Fig. 3B). In an additional series of studies, we determined whether individual Leu/Ala mutations in the leucine zipper would affect trafficking of HA-hIK1. As shown in Fig. 3, A and B, mutating each of the leucine residues within the leucine zipper individually failed to affect surface expression of HA-hIK1.

To confirm the functional expression of these leucines zipper mutations, we utilized the whole-cell patch clamp technique to determine current densities for each of these constructs. As shown in Fig. 4, ZIP4,5P failed to express functional channels at the cell surface (0.2 ± 0.1 pA/pF, n = 14), consistent with our IF and CS-IP data. As this mutation (L399P/L406F) would be expected to cause a significant alteration in protein structure, we also generated an alanine-substituted mutant (L399A/L406A; ZIP4,5A). As shown in Fig. 4B, this resulted in a similar reduction in current density (4.8 ± 2.4 pA/pF, n = 10). Current density measurements confirmed that the ZIP2,3, ZIP3,5 and the α position (α-POS) mutations were expressed at very low levels at the plasma membrane (ZIP2,3 = 18.4 ± 7.0 pA/pF, n = 16; ZIP3,5 = 1.1 ± 0.6 pA/pF, n = 14; α-POS; 13.0 ± 5.2 pA/pF, n = 16; α-POS).

**Fig. 2. Identification of potential C-terminal structural motifs in hIK1.** Primary amino acid sequence of the distal C terminus of hIK1 (C59; Val
369
–Lys
427
) highlighting important structural motifs, including (i) a leucine zipper (residues 378–406, α positions underlined, α positions indicated by asterisks, and (ii) a di-leucine motif (residues 409–410, indicated by a bracket below the LL). An arrow indicates sites of C-terminal truncations at Lys
402
 and Leu
114
.
ZIP1 leucine mutations with the heptad repeat are denoted as leucine zipper mutants L378A/L385A, L385A/L392A, L392A/L406A, L399P/L406F, L399A/L406A and I396A/L403A, respectively. Individual order to highlight expression at the cell surface. This partial label the intracellular channel following incubation at 27 °C. Currents are shown for mutations in the C terminus of hIK1. Rectified of plasma membrane expression of ZIP4,5A-HA-hIK1. As shown in Fig. 5A, reducing the temperature to 27 °C resulted in a partial restoration of plasma membrane expression of ZIP4,5A-HA-hIK1 (green, right panel). Note that we did not label the intracellular channel following incubation at 27 °C in order to highlight expression at the cell surface. This partial correction of cell surface expression was confirmed by CS-IP measurements. As shown in Fig. 5B, Zip4,5A-HA-hIK1 was not detected at the cell surface when grown at 37 °C, although protein expression was not compromised (IB in Fig. 5B). However, growing the cells at 27 °C for 24 h resulted in a partial restoration of cell surface expression. Whole-cell patch clamp studies (Fig. 5, C and D) confirmed that, following incubation at 27 °C for 24 h, DCEBIO stimulated a significant CLT-sensitive whole-cell current in ZIP4,5A-HA-hIK1 expressing cells compared with cells grown at 37 °C (27 °C, 46.9 ± 13.5 pA/pF, n = 17; 37 °C, 4.8 ± 2.4 pA/pF, n = 10). Note that this channel displayed normal macroscopic gating kinetics following correction of the trafficking defect by incubation at 27 °C suggesting this leucine zipper does not play a fundamental role in channel gating.

**Mutation of the C-terminal Leucine Zipper Does Not Affect Assembly of hIK1 Subunits**—The inability of HA-hIK1 to correctly traffic to the plasma membrane following mutation of the C-terminal leucine zipper could be caused by either a gross structural change, thereby precluding tetramer formation, or by a more subtle effect that affects only the distal C terminus of hIK1. To investigate the role of the C-terminal leucine zipper in tetramer formation, we generated a myc-tagged hIK1 and performed co-immunoprecipitation experiments. HA-hIK1 and myc-hIK1 were transiently transfected either alone or in combination into HEK293 cells, immunoprecipitated using anti-HA Ab, separated by SDS-PAGE, and immunoblotted with anti-myc Ab. As shown in Fig. 6, we were able to co-immuno-

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**Fig. 4. Current density measurements of leucine zipper and di-leucine motif mutations in HA-hIK1.** Whole-cell currents were recorded from stably transfected HEK cells as described under “Experimental Procedures.” A, representative DCEBIO-stimulated whole-cell currents are shown for mutations in the C terminus of hIK1. B, current density (pA/pF) for each construct at −20 mV (mean ± S.E.; number of experiments is indicated in parentheses). hIK1 currents that were significantly different from wild-type hIK1 are indicated (*, p < 0.05, Student’s t test). Control denotes untransfected HEK cells. DI-LEU denotes L409A/L410A. ZIP1,2, ZIP2,3, ZIP3,5, ZIP4,5F, ZIP4,5 and α-POS denote leucine zipper mutants L378A/L385A, L385A/L392A, L392A/L406A, L399P/L406F, L399A/L406A and L396A/L403A, respectively. Individual leucine mutations with the heptad repeat are denoted as ZIP1 (L378A), ZIP2 (L385A), ZIP3 (L392A), ZIP4 (L396A), and ZIP5 (L403A).
precipitate myc-hIK1 with an HA antibody (3rd lane), confirming assembly of hIK1 into minimally dimers and likely trimers. Following mutation of the C-terminal leucine zipper in the HA-hIK1 backbone (ZIP4,5A-hIK1), a co-immunoprecipitate pulled down quantitatively similar amounts of Myc-tagged wild-type hIK1 (5th lane), suggesting that a heterotetrameric complex between wild-type and mutated subunits assembles correctly. Finally, when the leucine zipper was mutated in both myc-hIK1 and HA-hIK1, quantitatively similar amounts were also detected on co-immunoprecipitates (6th lane) demonstrating that mutation of the C-terminal leucine zipper does not affect channel tetramerization.

**Fig. 5.** Trafficking of ZIP4,5-hIK1 can be partially restored by incubation at reduced temperature. A, confocal microscopy images are shown for wild-type HA-hIK1 at 37°C (left panel), ZIP4,5-hIK1 at 37°C (middle panel), and ZIP4,5-hIK1 at 27°C (in intracellular channel was not labeled for clarity, right panel). Plasma membrane and intracellular hIK1 are shown in green and red, respectively. B, top panel, cell surface immunoprecipitation (CS-IP) of HA-hIK1 (lane 1), ZIP4,5 grown at 37°C (lane 2), and ZIP4,5 grown at 27°C (lane 3). Bottom panel, immunoblot (IB) showing similar levels of expression for each of the constructs. The immunoblots shown are representative of 3 separate experiments. C, representative examples of DCEBIO-stimulated whole-cell currents elicited by applying voltage pulses from −100 to +80 mV in 20-mV increments for 250 ms every 2 s, from a holding potential of −60 mV. D, current density (pA/pF) for each construct at −20 mV (mean ± S.E.; number of experiments is indicated in parentheses). hIK1 currents that were significantly different from wild-type hIK1 are indicated (*, p < 0.05, Student’s t test).
Assembly of the Distal C Terminus of hIK1 Is Dependent Upon an Intact Leucine Zipper—Our co-immunoprecipitation studies demonstrate that the C-terminal leucine zipper of hIK1 is not required for channel tetramerization. Thus, we considered the possibility that leucine zipper mutations might modify more subtle sub-domain interactions within the C terminus. To address this question we utilized a differentially epitope-tagged (either V5 or Xpress) 59-amino acid C-terminal domain of hIK1 (Fig. 2; Val365-Lys427; C59), encompassing the leucine zipper, but not the calmodulin-binding domain, to examine the role of the leucine zipper in C-terminal self-assembly. As shown in Fig. 7A, following in vitro translation in the presence of [35S]methionine and IP, the V5 and Xpress (XP) epitope-tagged constructs run at different apparent molecular masses of –11 and 10 kDa, respectively (lanes 1 and 4), thereby allowing co-assembly to be evaluated via IP. Upon co-translation of wild-type V5 and XP constructs, V5 and XP antibodies pulled down products corresponding to both V5-C59 and XP-C59 (lanes 2 and 3, respectively), demonstrating co-assembly of these C-terminal domains. However, when the Xpress epitope-tagged C-terminal leucine zipper was mutated (L399A/L406A; XP-ZIP4,5), co-assembly with V5-C59 was abrogated (lanes 6 and 7), demonstrating a critical role for the leucine zipper in C-terminal self-assembly. Interestingly, mutation of either L399A (XP-ZIP4; lanes 8 and 9) or L406A (XP-ZIP5; lanes 10 and 11) alone was not sufficient to disrupt co-assembly of this C-terminal domain. Thus, our results with the C terminus of hIK1 exactly mirror our results on full-length hIK1, i.e. double leucine zipper mutations are required to disrupt assembly/trafficking. The specificity of these antibodies was confirmed by demonstrating that V5 Ab failed to pull down XP-C59 (lane 12) and XP Ab failed to pull down V5-C59 (lane 13) when either V5- or XP-C59 was expressed alone. Furthermore, as shown in Fig. 7B, co-assembly of epitope-tagged C59 fragments occurred only when the fragments were translated together (co; 1st and 3rd lanes) and not when the fragments had been synthesized separately and then mixed afterward (mix; 2nd and 4th lanes).

The C-terminal Leucine Zipper Is Not Required for Membrane Trafficking of rSK3—hIK1 belongs to a gene family, KCNN, containing three additional members, the small conductance, apamin-sensitive, Ca2+-dependent K+ channels, SK1–3. Whereas hIK1 shares only 40–42% identity with the SK channels, the C-terminal leucine zipper is conserved in SK1 and SK3 (the 2nd leucine position is replaced by phenylalanine in SK2). Thus, we determined whether the function of this leucine zipper as a trafficking determinant was conserved in rSK3. For these studies we mutated the 3rd and 4th leucines (L660A/L667A) of the C-terminal leucine zipper in rSK3 to alanines. Although the leucine zipper is conserved between hIK1 and rSK3, it is out of register by a single heptad repeat when sequence alignments are compared. Thus, the 3rd and 4th leucines of rSK3 correspond to the 4th and 5th leucines of hIK1. Cell surface expression and function were determined by proteinase K digestion and whole-cell patch clamp studies, respectively. Stable cell lines expressing either rSK3 or the mutant rSK3-ZIP3,4 were treated with proteinase K, a nonspecific serine protease that when applied externally cleaves peptide bonds adjacent to the carboxylic group of aliphatic and aromatic amino acids. Cell lysates prepared from proteinase K-treated and control cells were then analyzed by SDS-PAGE and immunoblotting using a commercially available rSK3 antibody. As shown in Fig. 8A, lysates prepared from untreated cells expressing wild-type rSK3 (lane 1) and mutant rSK3-ZIP3,4 (lane 3) exhibit a single product with an apparent molecular mass of ~80 kDa, representative of full-length rSK3. Enzymatic digestion with externally applied proteinase K eliminated the bulk of the 80-kDa form and induced the appearance of a novel lower molecular mass product of ~50 kDa in both wild-type rSK3 (lane 2) and rSK3-ZIP3,4 (lane 4)-expressing cells. This lower molecular mass product is indicative of proteolytic digestion of rSK3. These data demonstrate that both wild-type rSK3 and mutant rSK3-ZIP3,4 are expressed predominantly on the cell surface. Functional expression of this mutated rSK3 channel was confirmed utilizing whole-cell patch clamp current density measurements. As shown in Fig. 8B, mutation of ZIP3,4 in rSK3 did not significantly affect current density (rSK3 = 26.8 ± 9.9 pA/pF, n = 4; rSK3-ZIP3,4 = 19.7 ± 7.6 pA/pF, n = 3). To further confirm that an intact leucine zipper is not required for efficient trafficking of rSK3, we introduced the additional double mutations, L667A/L674A (rSK3-ZIP3,4), and L653A/L660A (rSK3-ZIP2,3), as these are the same mutations, with regard to leucine zipper position, made in hIK1. Cell surface expression was evaluated by proteinase K digestion. As shown in Fig. 8C, similar to the ZIP3,4 mutation, these additional leucine zipper mutations in rSK3 did not abrogate cell surface expression. Collectively these data suggest that, in contrast to hIK1, the C-terminal leucine zipper of rSK3 is not critical for correct trafficking to the plasma membrane.

**DISCUSSION**

Members of the KCNN gene family, including SK1–3 and IK1 are, as a family, widely expressed in both brain and peripheral tissues where they play critical roles in a host of...
physiological processes. However, the molecular motifs involved in assembly and trafficking of these channels to the plasma membrane, both in general and to specific subdomains (e.g. the basolateral membrane of epithelial cells), have been little evaluated. To begin to address these questions we inserted an HA epitope into the extracellular domain between S3 and S4 of hIK1. We demonstrate that this epitope insertion can be utilized for the detection of cell surface-localized hIK1 by both IF and cell surface IP techniques while having no effect on either the single channel properties (single channel conductance, Ca\(^{2+}\) dependence), macroscopic gating kinetics, as assessed by whole-cell patch clamp, or pharmacological regulation (activated by DCEBIO, inhibited by clotrimazole) of hIK1. As such, we have utilized this construct to define the role of the distal C terminus in the trafficking of hIK1 to the plasma membrane.

Members of the KCNN gene family share a conserved leucine zipper motif in their distal C termini. Previous studies (33–35) have demonstrated a role for leucine zippers in the voltage-dependent gating of K\(^{+}\) and Ca\(^{2+}\) channels. In contrast to these voltage-gated channels, mutation of the C-terminal leucine zipper in hIK1 had no apparent effect on macroscopic channel gating, i.e. following correction of the trafficking defect by incubating the cells at 27 °C, the resultant DCEBIO-induced current displayed apparently normal gating characteristics (Fig. 5). Also, we demonstrate that an intact leucine zipper is not required for the assembly of hIK1; mutation of the C-terminal leucine zipper in all four subunits of hIK1 does not diminish their ability to assemble, as assessed by co-immunoprecipitation studies (Fig. 6). Rather, we demonstrate that an intact leucine zipper is required for the correct trafficking of hIK1 to the plasma membrane (Fig. 3), suggesting a more subtle folding defect as opposed to a complete loss of subunit assembly. The ability to correct this apparent folding defect by growing the cells at 27 °C (Fig. 5) also argues for a more subtle folding effect. Our results are similar to data previously reported (36) for the GABA\(_A\) receptor where the coiled-coil interaction of receptor subunit C termini stabilizes the active conformation but are not necessary for functional dimerization.

Although our studies have focused on the distal C terminus of hIK1, Joiner et al. (37) recently demonstrated that the assembly of calmodulin with the proximal C-terminal CAMBD is required for the targeting of hIK1 to the plasma membrane and that this was due to an enhanced assembly of hIK1 into tetramers in the presence of calmodulin. It was further demonstrated that the overexpression of the distal C terminus of hIK1, including the leucine zipper, inhibited the cell surface expression of full-length hIK1 (37). Here we demonstrate that the distal C terminus of hIK1 self-assembles in a leucine zipper-dependent manner and that this is required for the correct targeting of hIK1 to the plasma membrane.

The small conductance Ca\(^{2+}\)-activated K\(^{+}\) (SK) channels, SK1–3, share 42–44% identity with hIK1. Whereas the majority of this conservation occurs in the backbone (S1–S6) region of the channels, the C termini also demonstrate regions of high homology. Indeed, each of these channels possesses a CAMBD in their proximal C termini as well as a conserved leucine zipper in their distal C termini. Despite this conservation of a leucine zipper in SK1, SK3, and hIK1, our results demonstrate a lack of conservation in function for this motif, i.e. whereas mutation of the leucine zipper of hIK1 results in a dramatic loss of cell surface localized channel (Fig. 3), a similar mutation in SK3 had no effect on cell surface expression (Fig. 8). In this regard, it is interesting to note that Xia et al. (16) demonstrated...
that truncation of rSK2, distal to the CAMBD, did not affect expression of functional channels. In total, these results suggest that the distal C terminus in general, and the leucine zipper in particular, does not play a critical structural role in the SK members of the KCNN gene family, whereas it is absolutely required for the more distantly related IK gene family member.

Whereas our results clearly point to a role for the leucine zipper in the trafficking of hIK1, we further demonstrate that the most proximal leucine (Leu378, ZIP1) can be mutated, either alone or in combination with Leu385 (ZIP1,2), with no deleterious effects on channel trafficking (Figs. 3 and 4). Interestingly, distinct studies have demonstrated that the CAMBD of IK and SK channels extends 95–98 amino acids from the S6 transmembrane domain (amino acids R287 to N384 in IK1) with the Ca\(^{2+}\)-dependent binding domain being at the distal end of this motif (13, 17). Thus, Leu378 would be predicted to overlap with the Ca\(^{2+}\)-dependent CAMBD such that it may not be accessible for protein-protein interactions.

We demonstrate that the distal C terminus of hIK1 (C59, Fig. 2) co-assembles in a leucine zipper-dependent manner (Fig. 7). Similar to the trafficking of full-length HA-hIK1, the co-assembly of C59 is only disrupted by the introduction of a double mutation (ZIP4,5), whereas single mutations (ZIP4 or ZIP5) have no effect on the assembly process. These results suggest that, in full-length hIK1, the distal C terminus self-assembles and that this is required for correct trafficking of the channel. Schumacher et al. (18) recently used x-ray crystallography to identify the structure of calmodulin bound to the proximal C terminus CAMBD of the rSK2 channel (18). Unfortunately, this crystal structure consists of only the CAMBD; it does not incorporate the C-terminal leucine zipper of rSK2. Therefore, it will be of particular interest to obtain crystals of the entire C terminus for both IK and SK channels and to determine the alignment of the leucine zipper \(\alpha\)-helices within the context of the CAMBD-Ca\(^{2+}\)-calmodulin complex so that we can envisage how mutations within the zipper compromise membrane trafficking of hIK1 but not the SK channels.

Whereas the C-terminal leucine zipper of hIK1 may associate with itself there are at least two alternative possibilities that should be considered. First, leucine zippers are known to be involved in protein-protein interactions (27). Therefore, the C-terminal leucine zipper in hIK1 may be required for interactions with additional proteins necessary for the trafficking of the channel to the plasma membrane. In this regard, if the SK channels do not share these protein-protein interactions then mutation of the leucine zipper in these channels would not have the same effect on channel expression. A second possibility is that the C-terminal leucine zipper interacts with another domain within hIK1 itself. For example, hIK1 has a second potential leucine zipper extending from the cytosolic NH\(_{3}\) terminus into the first transmembrane domain (Leu\(^{35}\)/Leu\(^{25}\)/Leu\(^{22}\)/Leu\(^{29}\), a domain not conserved in SK1–3. Thus, the NH\(_{3}\) and C termini of hIK1 may assemble in order to form a channel that can traffic efficiently to the plasma membrane. This association of cytosolic domains is known to be important in the proper assembly and trafficking of other ion channels (38).

In conclusion, we demonstrate that the trafficking of hIK1 is dependent upon an intact C-terminal leucine zipper. Although this leucine zipper is not required for channel tetramerization, it appears to be crucial for the assembly of the C terminus of hIK1 into a trafficking competent conformation. Interestingly,
whereas this motif is conserved across the KCNN gene family it is not functionally conserved, suggesting a clear divergence in the structural requirements for the correct folding and trafficking of IK and SK gene family members.

REFERENCES

1. Devor, D. C., Singh, A. K., Frizzell, R. A., and Bridges, R. J. (1996) Am. J. Physiol. 271, L775–L784
2. Devor, D. C., Singh, A. K., Gerlach, A. C., Frizzell, R. A., and Bridges, R. J. (1997) Am. J. Physiol. 273, C531–C540
3. Devor, D. C., Singh, A. K., Lambert, L. C., DeLuca, A., Frizzell, R. A., and Bridges, R. J. (1999) J. Gen. Physiol. 113, 743–760
4. Logsdon, N. J., Kang, J., Togo, J. A., Christian, E. P., and Aiyar, J. (1999) J. Biol. Chem. 274, 32723–32726
5. Khanna, R., Chang, M. C., Joiner, W. J., Kaczmarek, L. K., and Schlichter, L. C. (1999) J. Biol. Chem. 274, 14838–14849
6. Ghanashani, S., Wulf, H., Miller, M. J., Rohm, H., Neben, A., Gutman, G. A., Cahalan, M. D., and Chandy, K. G. (2000) J. Biol. Chem. 275, 37137–37149
7. Vandorpe, D. H., Shmulder, B. E., Jiang, L., Lii, B., Maylie, J., Adelman, J. P., de Franceschi, L., Cappellini, M. D., Brugnara, C., and Alper, S. L. (1998) J. Biol. Chem. 273, 21542–21553
8. Neylon, C. B., Lang, R. J., Fu, Y., Bobik, A., and Reinhart, P. H. (1999) Circ. Res. 85, 33–43
9. Köhler, R., Degenhardt, C., Kuhn, M., Runkel, N., Paul, M., and Hoyer, J. (2000) Circ. Res. 87, 496–503
10. Edwards, G., Gardener, M. J., Feletou, M., Brady, G., Vanhoutte, P. M., and Weston, A. H. (1999) Br. J. Pharmacol. 128, 1064–1070
11. Ishii, T. M., Silva, C., Hirschberg, B., Bond, C. T., Adelman, J. P., and Maylie, J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11651–11656
12. Joiner, W. J., Wang, L. Y., Tang, M. D., and Kaczmarek, L. K. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11013–11018
13. Fanger, C. M., Ghanashani, S., Logsdon, N. J., Rauer, H., Kalman, K., Zhou, J., Beckingham, K. D., and Chandy, K. G., and Aiyar, J. (1999) J. Biol. Chem. 274, 5746–5754
14. Syme, C. A., Gerlach, A. C., Singh, A. K., and Devor, D. C. (2000) Am. J. Physiol. 278, C570–C581
15. Singh, S., Syme, C. A., Singh, A. K., Devor, D. C., and Bridges, R. J. (2001) J. Pharmacol. Exp. Ther. 296, 600–611
16. Xia, X., Falkaer, B., Rivard, A., Wayman, G., Johnson-Pais, T., Keen, J. E., Ishii, T., Hirschberg, B., Bond, C. T., Lutsenko, S., Maylie, J., and Adelman, J. P. (1998) Nature 395, 503–507
17. Keen, J. E., Khawaled, R., Farrens, D. L., Neelands, T., Rivard, A., Bond, C. T., Janowsky, A., Falkaer, B., Adelman, J. P., and Maylie, J. (1999) J. Neurosci. 19, 8850–8868
18. Schumacher, M. A., Rivard, A. F., Bachinger, H. P., and Adelman, J. P. (2001) Nature 410, 1120–1124
19. Gerlach, A. C., Gangopadhyay, N. N., and Devor, D. C. (2000) J. Biol. Chem. 275, 585–596
20. Gerlach, A. C., Syme, C. A., Ghanshani, S., Adelman, J. P., and Devor, D. C. (2001) J. Biol. Chem. 276, 10963–10970
21. Wulf, A., and Schwab, A. (2000) J. Membr. Biol. 187, 71–79
22. Manganas, L. N., and Trimmer, J. S. (2000) J. Biol. Chem. 275, 29685–29693
23. Devor, D. C., and Frizzell, R. A. (1993) Am. J. Physiol. 265, C1271–C1280
24. Nielsen, M. S., Warth, R., Bleih, M., Weigand, B., and Greger, R. (1998) Pfluegers Arch. 435, 267–272
25. Le Borgne, R., and Hoflack, B. (1998) Biochim. Biophys. Acta 1404, 195–209
26. Sharma, N., Crane, A., Clement, J. P., IV, Gonzalez, G., Babenko, A. P., Bryan, J., and Aguilar-Bryan, L. (1999) J. Biol. Chem. 274, 20628–20632
27. Kobe, B., and Deisenhofer, J. (1994) Trends Biochem. Sci. 19, 415–421
28. Brown, C. R., Hong-Brown, L. Q., and Welch, W. J. (1997) J. Clin. Invest. 99, 1432–1444
29. Furutani, M., Trudeau, M. C., Hagiwara, N., Seki, A., Gong, Q., Zhou, Z., Imamura, S., Nagashima, H., Kasamuk, H., Takao, A., Momma, K., January, C. T., Robertson, G. A., and Matsuoka, R. (1999) Circulation 99, 2290–2294
30. Kagan, A., Yu, Z., Fishman, G. I., and McDonald, T. V. (2000) J. Biol. Chem. 275, 11241–11248
31. Zhou, Z., Gong, Q., Epstein, M. L., and January, C. T. (1998) J. Biol. Chem. 273, 21061–21066
32. Zhou, Z., Gong, Q., and January, C. T. (1999) J. Biol. Chem. 274, 31123–31128
33. McCormack, T., Tannoye, M. A., Iverson, L. E., Lin, J., Ramawat, M., McCormack, T., Campanelli, J. T., Mathew, M. K., and Rudy, B. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 2931–2935
34. Garcia, J., Nakai, J., Imoto, K., and Beam, R. G. (1997) Biophys. J. 72, 2515–2523
35. Judge, S. I. V., Yeh, J. Z., Goolsby, J. E., Monteiro, M. J., and Bever, C. T., Jr. (2002) Mol. Pharmacol. 61, 915–920
36. Grunewald, S., Schupp, B. J., Ikeda, S. R., Runer, R., Steigerwald, F., Kornau, H., and Kühr, G. (2002) Mol. Pharmacol. 61, 1070–1080
37. Joiner, W. J., Khanna, R., Schlichter, L. C., and Kaczmarek, L. K. (2001) J. Biol. Chem. 276, 37890–37895
38. Tucker, S. J., and Ashcroft, F. M. (1999) J. Biol. Chem. 274, 33393–33397
Trafficking of the Ca$^{2+}$-activated K$^+$ Channel, hIK1, Is Dependent upon a C-terminal Leucine Zipper
Colin A. Syme, Kirk L. Hamilton, Heather M. Jones, Aaron C. Gerlach, LeeAnn Giltinan, Glenn D. Papworth, Simon C. Watkins, Neil A. Bradbury and Daniel C. Devor

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