Membrane Trafficking of Large Conductance Calcium-activated Potassium Channels Is Regulated by Alternative Splicing of a Transplantable, Acidic Trafficking Motif in the RCK1-RCK2 Linker*

Received for publication, May 1, 2010. Published, JBC Papers in Press, May 17, 2010, DOI 10.1074/jbc.M110.139758

Lie Chen‡, Owen Jeffries‡, Iain C. M. Rowe§, Zhi Liang‡, Hans-Guenther Knaus‡, Peter Ruth‡, and Michael J. Shipston†‡

From the ‡Centre for Integrative Physiology, College of Medicine & Veterinary Medicine, University of Edinburgh, Edinburgh EH8 9XD, Scotland, United Kingdom, the §Division for Molecular and Cellular Pharmacology, Department of Medical Genetics, Molecular and Clinical Pharmacology, Medical University Innsbruck, Peter-Mayr Strasse 1, 6020 Innsbruck, Austria, and ¶Pharmacology and Toxicology, Institute of Pharmacy, University Tuebingen, 72076 Tuebingen, Germany

Trafficking of the pore-forming α-subunits of large conductance calcium- and voltage-activated potassium (BK) channels is a major determinant of BK channel surface expression, although the regulatory mechanisms are poorly understood (14). Alternative splicing that results in premature truncation of the BK channel α-subunit C terminus also results in intracellular retention of the BK channel as exemplified by the murine Δε23 (15) and rabbit rbSlo2 (16) as a result of a loss of putative C-terminal ER export signals as well as the RCK2 domain (15, 16).

Recent data also suggest that the intracellular C-terminal linker between the two predicted regulator of potassium conductance domains (see Fig. 1a, RCK1 and RCK2) is also an important determinant of BK channel surface expression. First, a rat splice variant SVCyt that has an ~80-amino acid in-frame deletion of the linker region is poorly expressed at the cell surface (17). Second, deletion of >30 amino acids in the linker regions produces nonfunctional channels that lack significant cell surface expression (18). However, the mechanism(s) re-
sponsible for the trafficking defect in these linker deletion mutants are not known.

In this report, we identify multiple trafficking motifs within the intracellular RCK1-RCK2 linker that control cell surface expression of BK channel α-subunits expressed in mammalian cells. Importantly, we reveal an acidic cluster-like motif (DDXXDDXXI) that is critical for cell surface expression of the channel that can be transplanted to a heterologous nonchannel protein to enhance membrane expression. Furthermore, we have isolated a widely expressed human BK channel splice variant (hSloΔ579–664), in which the exons encoding these trafficking motifs are excluded. Exclusion of these exons results in an in-frame 86-amino acid deletion that encodes a channel that is a dominant negative of cell surface expression. Taken together, our data reveal that alternative splicing of the RCK1-RCK2 linker region, resulting in inclusion/exclusion of multiple trafficking motifs, is an important determinant of BK channel cell surface expression.

EXPERIMENTAL PROCEDURES

Cloning of the hSloΔ579–664 Variant, Channel Mutagenesis, and GABA<sub>B</sub>R1a Constructs—A human tissue rapid scan cDNA pool (Origene) was screened for splice variants by PCR-amplifying a region between exons 15 and 25 (see Fig. 1a) of the human BK channel α-subunit with the forward and reverse primer pairs: 5′-TTggCCAACCTCTTCTCCC-3′ and 5′-gTgCT-TgAgCTCATGggTAAT-3′, respectively. PCR amplicons were cloned into the pCR II-TOPO vector (Invitrogen). To generate full-length human BK channel α-subunit cDNAs, the novel variant hSloΔ579–664 amplicon from pCR II-TOPO was subcloned into the murine BK channel α-subunit with an N-terminal FLAG tag and/or a C-terminal HA or eYFP tag described previously (15, 19–21). Site-directed mutations were generated using the QuikChange® site-directed mutagenesis kit (Stratagene) using standard procedures. All of the amino acid numbering is based on the human BK channel α-subunit (hSlo) with start methionine at MDALI (accession number AAD31173).

GABA<sub>B</sub>R1a receptor plasmids with N-terminal extracellular HA-GABA<sub>B</sub>R1a and HA-GABA<sub>B</sub>R1a-ASRR plasmids were kind gifts from Prof. Lily Jan (University of California at San Francisco) (22). To engineer the DDXXDDXXI sequence at the C terminus of both constructs, we PCR-amplified the C terminus with forward (5′-TTTTgCCAACggAggAACCAAg-3′) and reverse (5′-CTCTAgATCAATTTgTggATCTgTgA-GgTCATCCTTgTAAgCA-3′) primers. The reverse primer encodes a DDXXDDXXI sequence, and the resultant PCR amplicons were ligated into the GABA<sub>B</sub>R1a plasmids using Clal and Xbal restriction sites. All of the sequences were confirmed by automated sequencing on both strands (MGW-Biotech).

Quantitative Real-Time-PCR TaqMan<sup>TM</sup> Assay—Quantitative analysis of the human BK channel variant transcripts was performed using a TaqMan<sup>TM</sup> assay (15). The probes and primer sets of total hSlo and hSloΔ579–664 were designed with Primer Express v1.2. TaqMan<sup>TM</sup> probes, labeled at the 5′ end with 6-carboxyfluorescein and at the 3′ end with 6-carboxytetramethylrhodamine were synthesized by ABI (Applied Biosystems). The following TaqMan<sup>TM</sup> assays were used to screen cDNAs from human tissues: for total hSlo: forward, 5′-gTCTCATAATgTACACAgAATATCTCT-3′; reverse, 5′-gCAgACTTgTAICTCAAgCTATgCA-3′; and probe, 5′-CCCTgTgggCTCCTgCCTCCTAgCTCT-3′; and for hSloΔ579–664: forward, 5′-gCTCTAATgATgAgCTCATgAg-3′; reverse, 5′-TgATCATgCggATgAAATTCACA-3′; and probe, 5′-CCACCAgAgAgAgCggCATgA-3′. The efficiency, correlation coefficient (R<sup>2</sup>), and limit of detection for each TaqMan<sup>TM</sup> assay were: for total hSlo: 2.03, 0.97, and <0.3 fg of cDNA; and for hSloΔ579–664: 2.02, 0.99, and <0.3 fg of cDNA. All of the data were analyzed using ABI Prism 7000 SDS software version 1.0 (Applied Biosystems). Transcript expression was determined from standard curves generated using dilutions of the respective splice variant plasmid DNA, and variant expression is given as a percentage of total BK channel transcripts in each tissue.

**HEK293 Cell Culture and Immunofluorescence**—HEK293 cells were maintained and transfected as described (15, 21). Cell surface labeling of the N-terminal FLAG epitope of BK channels in nonpermeabilized HEK293 cells was performed (15) using mouse monoclonal anti-FLAG M2 antibody (50 μg/ml Sigma) and Alexa-594-conjugated anti-mouse rabbit IgG (Molecular Probes). The cells were subsequently fixed in 4% paraformaldehyde for 30 min, permeabilized with 0.3% Triton X-100 for 10 min, and blocked with phosphate-buffered saline containing 3% bovine serum albumin plus 0.05% Triton X-100 for 30 min. The intracellular C-terminal HA epitope tag was detected using 0.5 μg/ml anti-HA polyclonal rabbit antibody (Zymed Laboratories Inc.) and Alexa-488-conjugated anti-rabbit chicken IgG (Molecular Probes), and the cells were mounted using Mowiol.

Confocal images were acquired on a Zeiss LSM510 laser scanning microscope using a 63× oil Plan Apochromat (NA = 1.4) objective lens in multi-tracking mode to minimize channel cross-talk and analyzed as described (19). FLAG surface expression was quantified in two ways: (i) using a threshold method to detect the total number of all transfected cells that displayed FLAG surface expression in each group and (ii) using absolute measures based on ratios of surface FLAG (extracellular) fluorescence to intracellular signal (eYFP or HA as appropriate) in a random subset of all cells analyzed using Image J. The data were then normalized to the corresponding control group (100%) as indicated in the respective figure legend. In these experimental paradigms the data obtained for relative surface expression using the threshold method were quantitatively the same as using the absolute ratio measure, therefore these data were pooled. In Fig. 2b >90% of all of the transfected cells display surface expression of the respective e22 and zero variants; however, we could not detect surface expression of the hSloΔ579–664 variant in any cell examined. The same approach was used for the HA-tagged GABA<sub>B</sub>R1a receptor constructs except that distinct fluorescent second antibodies directed against the N-terminal HA tag were used in nonpermeabilized and permeabilized conditions.

To assay co-localization of the channels with the ER, HEK293 cells were co-transfected with the HA-tagged channels and the pdsRed-ER (Clontech) vector. The HA tag was detected as above, and confocal images taken at Nyquist sampling rates were collected and analyzed as described previously (23). The

TCAATgAAATgTACACAgAATATCTCT-3′; reverse, 5′-gCAgACTTgTAICTCAAgCTATgCA-3′; and probe, 5′-CCCTgTgggCTCCTgCCTCCTAgCTCT-3′; and for hSloΔ579–664: forward, 5′-gCTCTAATgATgAgCTCATgAg-3′; reverse, 5′-TgATCATgCggATgAAATTCACA-3′; and probe, 5′-CCACCAgAgAgAgCggCATgA-3′. The efficiency, correlation coefficient (R<sup>2</sup>), and limit of detection for each TaqMan<sup>TM</sup> assay were: for total hSlo: 2.03, 0.97, and <0.3 fg of cDNA; and for hSloΔ579–664: 2.02, 0.99, and <0.3 fg of cDNA. All of the data were analyzed using ABI Prism 7000 SDS software version 1.0 (Applied Biosystems). Transcript expression was determined from standard curves generated using dilutions of the respective splice variant plasmid DNA, and variant expression is given as a percentage of total BK channel transcripts in each tissue.

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images were deconvolved using Huygens software (Scientific Volume Imaging) and analyzed using ImageJ (National Institutes of Health) to obtain the Pearson’s correlation coefficient. Coefficients range from 1 to −1. A value of 1 indicates a complete positive correlation between the two channels, whereas −1 stands for a negative correlation.

**Immunoprecipitation and Western Blotting**—Immunoprecipitation (IP) and Western blotting were performed as previously described (15). HEK293 cells were solubilized at 4 °C in lysis buffer (NLB) containing 150 mM NaCl, 50 mM HEPES, pH 7.5, 1.5 mM MgCl2, 10 mM sodium pyrophosphate, 20 mM NaF, 1 mM EDTA, 5 mM EGTA, 10% (v/v) glycerol, 1% Triton X-100, and complete protease inhibitor mixture (Roche Applied Science). Before preclearing, the channels were immunoprecipitated with anti-HA (rabbit polyclonal; Zymed Laboratories Inc.) or anti-FLAG M2 monoclonal mouse antibody (Sigma). Negative control IPs included: (i) IP of mock transfected cells; (ii) IP from cells transfected with channels without the cognate epitope tag; (iii) beads alone; or (iv) irrelevant IP antibody. Bound complexes were separated through a 10% SDS-PAGE gel; thus the rest of the intracellular C terminus including RCK2, the calcium bowl, and other essential trafficking motifs for cell surface expression are retained. b, hSloΔ579-664 variant mRNA levels expressed as percentages of total BK channel mRNA transcripts in selected human tissues determined using TaqMan™ analysis. All of the data are the means ± S.E., n = 3/tissue region. MW, molecular mass.

**FIGURE 1.** Cloning and tissue mRNA expression of a human BK channel splice variant, hSloΔ579-664, with an in-frame 86-amino acid deletion in the RCK1-RCK2 linker region. a, schematic illustrating the topology of a BK channel α-subunit with an extracellular N terminus and a large intracellular C terminus. Exon (numbered open boxes) structure for the hSlo zero variant (with no alternatively spliced inserts in this region) and the hSloΔ579-664 variant are shown. The representative agarose gel illustrates multiple amplicons generated by the forward (fwd) and reverse (rev) primers in human colon cDNA. The upper and lower arrows indicate the human hSlo zero and hSloΔ579-664 variants, respectively, with the corresponding amino acid sequence. hSloΔ579-664 results from an 86-amino acid in-frame deletion; thus the rest of the intracellular C terminus including RCK2, the calcium bowl, and other essential trafficking motifs for cell surface expression are retained. b, hSloΔ579-664 variant mRNA levels expressed as percentages of total BK channel mRNA transcripts in selected human tissues determined using TaqMan™ analysis. All of the data are the means ± S.E., n = 3/tissue region. MW, molecular mass.
to mock transfected HEK293 cells, reflects membrane hyperpolarization (BK channel activation).

BK channel activation was fully blocked by 1–10 μM paxilline (not shown; see Ref. 23). The data were analyzed with SoftMax Pro and exported to Igor Pro, Microsoft Excel, and/or Prism for further analysis. To compare between mutants, the relative fluorescent units were determined 70 s into the assay. The response of each channel mutant was then normalized to the hyperpolarization response of the zero channel (100%).

**Statistics**—Statistical analysis was performed using Igor Pro v6.0 using a one-way ANOVA with a Dunnett’s post hoc test for significance between groups at \( p < 0.05 \).

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**FIGURE 2.** Lack of cell surface expression of the hSloΔ579–664 variant. 

**a**, representative confocal images of HEK293 cells expressing FLAG-hSloΔ579–664-HA (top panels), FLAG-e22-HA (middle panels), or FLAG-zero-HA (bottom panels). The extracellular FLAG epitope was labeled (red) under nonpermeabilized conditions (cell surface) with the C-terminal HA epitope tag (green) labeled following cell permeabilization. FLAG and HA labeling from the same cell are then overlaid (Merge). The FLAG-e22-HA construct is a splice variant with an alternatively spliced exon (exon 22) included between exons 19 and 23. The scale bars are 2 μm.

**b**, quantification of surface expression expressed as a percentage of the total number of transfected cells with detectable cell surface (FLAG) expression for experiments as performed in **a**. The data are the means ± S.E. from a minimum of three independent experiments with >600 cells analyzed/group. ***, \( p < 0.001 \), ANOVA with post hoc Dunnett’s test compared with FLAG-zero-HA construct.

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**FIGURE 3.** The hSloΔ579–664 variant is a dominant negative of cell surface expression. 

**a**, Western blot of total cell lysates from the FLAG-hSloΔ579–664-HA and FLAG-zero-HA variants expressed in HEK293 cells. **b**, representative blots from co-immunoprecipitation (IP) experiments from HEK293 cells co-expressing the zero-HA variant with either the FLAG-hSloΔ579–664 or FLAG-e22 variants. **Left panel**, channels were immunoprecipitated with mouse anti-FLAG M2 antibody, and blots were probed with rabbit anti-HA antibody. **Right panel**, channels were immunoprecipitated with rabbit anti-HA antibody and probed with mouse anti-FLAG M2 antibody. **c**, zero-HA subunits were co-expressed with either FLAG-hSloΔ579–664-eYFP, FLAG-e22-eYFP, or FLAG-zero-eYFP channels in HEK293 cells, and cell surface (FLAG) expression was quantified. **d**, FLAG-zero-eYFP subunits were co-expressed with either hSloΔ579–664-HA, e22-HA or zero-HA channels in HEK293 cells. In **c** and **d**, the data are the cell surface FLAG staining values expressed as percentages of control (surface FLAG-zero-eYFP levels when co-expressed with the zero-HA construct) under nonpermeabilized conditions. The data are the means ± S.E. from a minimum of six independent experiments with >700 cells analyzed/group in **c** and **d** and three independent experiments in **a**, **b**, and **e**. ***, \( p < 0.001 \), ANOVA with post hoc Dunnett’s test compared with other groups.
ant, hSloΔ_{579–664} was less than 10% (Fig. 1b). The proportional expression of the new variant hSloΔ_{579–664} was highest in those tissues, such as heart, thyroid, plasma blood cell, and fetal liver, which express the lowest total BK channel transcript levels.

**hSloΔ_{579–664} Is a Dominant Negative of Cell Surface Expression**—Fusion of green fluorescent protein to the C terminus of the rat SVcyt homolog resulted in trapping of the SVcyt variant into the cytoplasm of mammalian cells with no detectable current (17). In addition, recent data suggest that deletion of >30 amino acids in the RCK1-RCK2 linker results in nonfunctional channels (18). To address whether the hSloΔ_{579–664} splice variant could be expressed at the cell surface and form functional channels, we took two approaches.

First, we asked whether single channel/macropatch BK currents were detectable in HEK293 cells expressing the hSloΔ_{579–664} variant using both untagged constructs as well as channels with an N-terminal FLAG epitope and a C-terminal HA epitope. No identifiable BK channel currents were observed in either 39 cell attached patches or 29 excised inside out patches exposed over the potential range ±100 mV and with >100 μM free calcium. In contrast, using the zero variant under identical conditions, we observed multiple channels in >55% of patches in cell-attached or excised patch configurations (data not shown). A similar lack of functional expression was also observed in fluorescent membrane potential assays (e.g. see Fig. 8).

Second, to determine whether the hSloΔ_{579–664} was expressed at the cell surface, we transfected the FLAG-hSloΔ_{579–664}-HA construct in HEK293 cells (Fig. 2). In non-permeabilized cells, the extracellular N-terminal FLAG tag could not be detected in immunofluorescence assays in any cell (n > 1500 cells analyzed), suggesting that the channel could not insert into the plasma membrane (Fig. 2). In contrast, in the same experiments two distinct splice variants (FLAG-e22-HA and FLAG-zero-HA variants (15)) showed robust flag tag expression at the cell surface in >90% of nonpermeabilized, transfected cells (Fig. 2). To confirm that the lack of FLAG epitope detection with the FLAG-hSloΔ_{579–664}-HA variant was not due to a lack of protein expression, we analyzed expression of the HA epitope tag in the same cells under permeabilized conditions (Fig. 2a). The hSloΔ_{579–664} variant displayed robust expression in both immunocytochemical (Fig. 2a) and Western blot (Fig. 3a) assays. Indeed, total cellular protein levels of the hSloΔ_{579–664} construct were not significantly different from that observed with zero constructs, suggesting that the lack of cell surface expression does not result from decreased synthesis and/or increased degradation of the hSloΔ_{579–664}.
Trafficking Signals in the BK Channel RCK1-RCK2 Linker

(a) Putative trafficking motifs

(b) Cell surface

(c) Surface expression (% of zero)

(d) Total

(e) Surface expression (% of zero)
variant (Fig. 3a). As expected from the 86-amino acid deletion, the hSloΔ579–664 variant was detectable as an ~110-kDa immunoreactive band in Western blots (Fig. 3a). Probing for the hSloΔ579–664 variant in intact HEK293 cells revealed that the hSloΔ579–664 variant was retained in intracellular structures within the cytoplasm. Although a functional role for intracellular BK channels has been reported, for example in mitochondria (26), the hSloΔ579–664 variant did not co-localize with mitochondrial markers (data not shown) in HEK293 cells but was extensively trapped in the ER (Figs. 5 and 6). Taken together, these data suggest that a homomeric hSloΔ579–664 variant is trafficking-deficient, is trapped intracellularly, and is thus unable to form functional channels at the plasma membrane.

Because BK channels exist as tetramers, the hSloΔ579–664 variant may be able to assemble with other BK channel splice variant α-subunits. To test this idea, we first performed reciprocal co-immunoprecipitation assays by expressing a FLAG-tagged hSloΔ579–664 variant with HA-tagged zero subunits (Fig. 3b). Co-expression of the FLAG-hSloΔ579–664 variant with the zero-HA variant resulted in robust, reciprocal co-immunoprecipitation of both variants (Fig. 3b). Similar co-immunoprecipitation was observed using the e22 splice variant (Fig. 3b), which also shows robust cell surface expression (Fig. 2), or between hSloΔ579–664-HA and FLAG-zero (not shown). Thus hSloΔ579–664 can heteromultimerize with other BK channel α-subunits, suggesting that channel assembly per se is not compromised.

We next asked whether cell surface expression of the hSloΔ579–664 variant may be rescued upon co-expression with cell surface trafficking competent α-subunits. We thus expressed a FLAG-hSloΔ579–664-eYFP variant with the zero-HA construct to allow simultaneous monitoring of expression of both constructs in the same cell while assaying for the external FLAG epitope tag (Fig. 3c) in nonpermeabilized cell surface assays. However, no significant rescue of the hSloΔ579–664 variant was observed. As controls, the co-expression of zero-HA had no effect on either FLAG-e22-eYFP or FLAG-zero-eYFP surface expression (Fig. 3c). This was confirmed in cell surface biotinylation assays (Fig. 3e).

Because other BK channel α-subunit splice variants may act as dominant negative regulators of cell surface expression (15, 27), we thus asked whether the hSloΔ579–664 variant could control cell surface expression of other variants. Using a FLAG-tagged zero-eYFP construct (FLAG-zero-eYFP) co-expressed with the hSloΔ579–664-HA, zero-HA, or e22-HA variants allowed us to assay cells in which both constructs were co-expressed while independently assaying for cell surface expression using the FLAG epitope. Co-expression of FLAG-zero-eYFP and hSloΔ579–664-HA constructs resulted in a significant reduction (>60%) of cell surface expression of FLAG-zero-eYFP (Fig. 3d). The effect of hSloΔ579–664-HA was not due to an overexpression artifact because co-expression of FLAG-zero-eYFP with e22-HA was without effect on FLAG-zero-eYFP surface expression.

Identical data were obtained in cells co-expressing FLAG-zero channels lacking the eYFP tag with hSloΔ579–664-HA; surface FLAG expression in the presence of hSloΔ579–664-HA was 37.6 ± 4.2% of FLAG-zero, whereas co-expression with e22-HA resulted in FLAG surface expression that was 95.0 ± 5.6% of FLAG-zero channels. The residual cell surface expression of FLAG-zero channels in these immunofluorescence assays when co-expressed with hSloΔ579–664 most likely results from formation of homomultimers of FLAG-zero at the cell surface because in both imaging and cell surface biotinylation assays (Fig. 3e), we could not detect hSloΔ579–664 at the cell surface. As hSloΔ579–664 and zero channels express at similar levels in HEK293 cells (Fig. 3a); this may indicate that the efficiency of heteromultimerization is compromised when channel subunits incorporate the hSloΔ579–664 variant.

The dominant negative effects of hSloΔ579–664 data were recapitulated with biochemical assays of cell surface biotinylation (Fig. 3e). No significant surface expression could be detected of either zero-HA or FLAG-hSloΔ579–664-eYFP in cells expressing both constructs supporting the dominant negative role of hSloΔ579–664. In contrast, robust surface expression of both FLAG-e22-eYFP and zero-HA could be detected in cells co-expressing these constructs (Fig. 3e). These data suggest that the hSloΔ579–664 variant acts as a dominant negative of cell surface expression.

Exons 18 and 19, but Not Exon 23, Are Essential for Cell Surface Expression—Because the hSloΔ579–664 variant could heteromultimerize with other BK channel α-subunits and act as a dominant negative of cell surface expression, we hypothesized that the mechanism underlying the trafficking defect was not a result of incorrect channel assembly, because of the 86-amino acid deletion, but rather arose from the deletion of essential, discrete trafficking signals within the RCK-RCK2 linker upon exclusion of exons 18, 19, and 23 in the hSloΔ579–664 variant.

As a first step to test this idea, we assayed the contribution of the individual exons 18, 19, and 23, which are excluded in the hSloΔ579–664 variant, to cell surface expression by determining the percentage of nonpermeabilized cells expressing each construct with the presence of hSloΔ579–664-HA (Fig. 4). The results showed that the absence of exons 18 and 19 resulted in a significant reduction in cell surface expression (Fig. 4).

An acidic cluster-like motif in exon 19 is essential for cell surface expression. a, ClustalW sequence alignment of exons 18 and exon 19 from human (Homo sapiens, accession number AAD31173), mouse (Mus musculus, accession number AAL69971), chicken (Gallus gallus, accession number NP_989935), turtle (Trachemys scripta, accession number AAC41281), worm (Caenorhabditis elegans, accession number NP_00102459), and fly (Drosophila melanogaster, accession number NP_524486). The exons form the extreme C terminus of the computationally predicted RCK1 domain and the start of the unstructured RCK1-RCK2 linker. Three putative trafficking/sorting motifs predicted in this region are shown with only the acidic DDXXDXXI motif fully conserved across phyla. Amino acid numbering is based on the amino acid sequence of the human sequence AAD31173 that starts with MDALI. b, representative confocal sections from HEK293 cells transfected with the DDXXDXXI mutants (D617A/D618A and I625A) and zero channels with the N-terminal epitope eYFP (Fig. 3e). As controls, the co-expression of zero-HA had no effect on either FLAG-e22-eYFP or FLAG-zero-eYFP surface expression (Fig. 3e). This was confirmed in cell surface biotinylation assays (Fig. 3e).

FIGURE 5. An acidic cluster-like motif in exon 19 is essential for cell surface expression. a, ClustalW sequence alignment of exons 18 and exon 19 from human (Homo sapiens, accession number AAD31173), mouse (Mus musculus, accession number AAL69971), chicken (Gallus gallus, accession number NP_989935), turtle (Trachemys scripta, accession number AAC41281), worm (Caenorhabditis elegans, accession number NP_00102459), and fly (Drosophila melanogaster, accession number NP_524486). The exons form the extreme C terminus of the computationally predicted RCK1 domain and the start of the unstructured RCK1-RCK2 linker. Three putative trafficking/sorting motifs predicted in this region are shown with only the acidic DDXXDXXI motif fully conserved across phyla. Amino acid numbering is based on the amino acid sequence of the human sequence AAD31173 that starts with MDALI. b, representative confocal sections from HEK293 cells transfected with the DDXXDXXI mutants (D617A/D618A and I625A) and zero channels with the N-terminal epitope eYFP (Fig. 3e). As controls, the co-expression of zero-HA had no effect on either FLAG-e22-eYFP or FLAG-zero-eYFP surface expression (Fig. 3e). This was confirmed in cell surface biotinylation assays (Fig. 3e).
whether cell surface expression of the hSloΔ579–664 variant could be rescued by the reinsertion of single or double exons in combination (Fig. 4). We thus generated a number of chimaeras in which one or two exons were ligated in-frame between exons 16 and 24 in the hSloΔ579–664 variant. Inclusion of exons 18, 19, or 23 alone (constructs e18, e19, or e23) did not rescue any cell surface expression of the hSloΔ579–664 variant. Similarly, inclusion of exon 19 with exon 23 (e19 + e23) or exon 18 with exon 23 (e18 + e23) did not rescue cell surface expression of the hSloΔ579–664 variant. In contrast, inclusion of both exons 18 and 19 (e18 + e19) partially rescued cell surface expression in both quantitative immunofluorescence assays (Fig. 4a) as well as cell surface biotinylation assays (Fig. 4b). These data suggest that: (i) exon 23 is not essential per se for cell surface expression; (ii) the length of the amino acid insertion per se is not important for cell surface expression; and (iii) exons 18 and 19 are required for cell surface expression, and thus their exclusion is likely to result in loss of putative trafficking signals.

An Acidic Cluster Motif in the RCK1-RCK2 Linker Is Required for Cell Surface Expression—To further refine our analysis, we aligned exons 18 and 19 from the zero variants of BK channel orthologs from man to flies (Fig. 5a). This revealed the high conservation of this region that spans the very C terminus of the computationally predicted RCK1 domain and the start of the unstructured (NORS) RCK1-RCK2 linker region (18).

Examination of the amino acid sequence encoded by exons 18, 19, and 23 (Fig. 5a) revealed three regions that may act as putative trafficking motifs: (i) The junction of exon 16 and exon 18 encodes a putative TGN-endosome trafficking signal in vertebrate BK channels (EXXXLI) similar to the consensus (D/E)XXXLI(L/I). However, (D/E)XXXLI(L/I) motifs show considerable degeneracy (28) with an RXXXXL signal exploited in the Glut4 transporter (29) and an EXXXLI motif in AQP4 (30). (ii) Exon 19 encodes a putative acidic cluster signal DXXD that is important for trafficking of a number of potassium channels (22, 31–34). In addition, the acidic cluster may also form part of a DXXX-like motif (DXXXI) that is predicted (using the PredictProtein server; data not shown) to form a short α-helical structure. Intriguingly, such a short α-helical region is commonly observed in other ER exit signals with little primary sequence homology (16, 35) and is predicted to play an important role in the more C-terminal ER exit signal in BK channels (16). Furthermore, this short α-helical region represents the only computationally predicted structured region in the otherwise unstructured NORS (no regular secondary structure) RCK1-RCK2 linker (18) conserved from man to flies. (iii) The very 5′ start of exon 23 encodes another putative acidic motif (EDE) that is conserved in vertebrates.

We took a site-directed mutagenesis approach using the zero variant to examine the contribution of these putative trafficking signals in BK channel cell surface expression (Fig. 5, b and c). Mutation of Glu576 or of Leu580 and Ile581 to alanine to disrupt the EXXXLI motif at the exon 16-exon 18 junction, as well as alanine mutation of the EDE motif in exon 23, significantly reduced cell surface labeling of the zero variant in imaging assays but did not abolish it (Fig. 5c). Furthermore, a combination of mutations at both sites did not abolish cell surface labeling, because expression was still 20.1 ± 4.2% of the zero variant.

FIGURE 6. Trafficking-deficient BK channel mutants are trapped in the ER. a, representative single confocal sections from permeabilized cells co-transfected with the corresponding HA-tagged BK channel site-directed mutant (construct, green) and the endoplasmic reticulum marker expression plasmid pdsRed-ER (ER, red). The merged images are shown in the right-hand panels. b, summary bar graph of Pearson's correlation coefficient for quantitative co-localization of the respective HA-tagged channels with the pdsRed-ER marker (a value of 1.0 would indicate complete co-localization). *, p < 0.05, ANOVA with post hoc Dunnet's test compared with the zero channel.
In contrast, mutation of the DDXXDXXXI motif (D617A/D618A and I625A constructs) completely abolished cell surface labeling in imaging assays as with the hSlo\(_{D796-664}\) variant (Fig. 5, b and c). To overcome the lack of cell surface expression in these mutants, we also performed cell surface biotinylation assays (Fig. 5, d and e). Mutation of the DDXXDXXXI motif (D617A/D618A mutant) again abolished cell surface expression as for the hSlo\(_{D796-664}\) splice variant. In contrast, mutation of the EXXXLI or EDE motifs (E576A or E634A/D635A/E636A, respectively) significantly reduced but did not abolish cell surface expression of the channel (Fig. 5, d and e). However, because mutation of the EDE motif alone significantly reduced surface expression, this would suggest that the inability to fully rescue surface expression with the e18 + e19 construct in Fig. 4 is a result of the loss of the EDE sequence within exon 23 in the e18 + e19 construct. Mutation of the DDXXDXXXI motif also completely abolished cell surface labeling in the e18 + e19 construct, further confirming the essential requirement for this sequence (not shown).

The mutant channels were now predominantly ER-localized as determined by co-localization assays (Fig. 6, a and b). We determined the Pearson’s correlation coefficient in quantitative immunofluorescence imaging assays with the channel constructs upon co-expression with the ER marker pdsRed-ER (Clontech). For the zero variant the coefficient was 0.59 ± 0.05, which was significantly (ANOVA, post hoc Dunnett’s test, \(p < 0.01\)) increased with the hSlo\(_{D796-664}\) variant as well as the D617A/D618A and I625A mutants (Fig. 6b), demonstrating trapping of these mutants in the ER.

Because the DDXXDXXXI acidic-like motif plays a dominant role in determining cell surface expression, we thus asked whether this motif could function as a transplantable trafficking signal. We exploited the GABA\(_p\)R1a receptor (a nonchannel subunit of the G-protein-coupled receptor for the \(\gamma\)-aminobutyric acid neurotransmitter), which is normally retained in the ER by a RXRR-dependent ER retrieval and retention mechanism to examine whether the DDXXDXXXI motif could enhance cell surface expression of the receptor as for acidic trafficking motifs identified in other potassium channels (22). We engineered the DDXXDXXXI motif onto the intracellular C terminus of the GABA\(_p\)R1a receptor and monitored cell surface to intracellular expression by probing for the extracellular N-terminal HA tag under nonpermeabilized and permeabilized conditions using quantitative immunofluorescence (Fig. 7). In agreement with previous studies (22), the DDXXDXXXI motif could not rescue surface expression of the wild type GABA\(_p\)R1a receptor (Fig. 7b).



FIGURE 7. Acidic cluster sequence (DDXXDXXXI) is a transplantable ER export motif. a, representative single confocal sections from HEK293 cells expressing the GABA\(_p\)R1a receptor with (GABA\(_p\)R1a(ASRR)) and without (GABA\(_p\)R1a(ASRR)) the DDXXDXXXI motif engineered onto the C terminus. The N-terminal (extracellular) HA epitope tag was labeled under nonpermeabilized (left panels, red) and permeabilized (middle panels, green) conditions in the same cell with the merged images shown in the right-hand panels. The GABA\(_p\)R1a receptor contained an arginine to alanine mutation (ASRR) compared with the wild type GABA\(_p\)R1a receptor. The scale bars are 2 µm. b, summary bar graph of quantitative surface/intracellular HA expression normalized to the ratio for the GABABR1a(ASRR) expressing cells (100%). **, \(p < 0.01\), ANOVA with post hoc Dunnett’s test compared with the zero channel (n = 16–19/group).
BK channel that acts as a dominant negative for cell surface expression.

The DDXXDXXXI motif may comprise both an acidic cluster motif as well as a degenerate DXLL motif. Indeed in worms and flies, a DXLI motif is retained, whereas the third position is an arginine in vertebrates. Thus, although the motif does not share sequence conservation with other trafficking motifs, it is intriguing that the DXXXI motif is predicted to form a short α-helix at the very beginning of the predicted linker region that otherwise lacks a regular secondary structure (18). A short α-helical structure is a feature commonly associated with ER export signals that do not show sequence homology (16, 35) as suggested for the more C-terminal ER export sequence in BK channels (16). Acidic cluster motifs are also commonly used as ER export signals, including in other potassium channels (22, 31, 33, 34). These features also appear crucial for cell surface expression of BK channels. In contrast, the EDExacidic cluster in exon 23 is not essential for surface expression, but mutagenesis does significantly reduce it. Similar acidic clusters have been reported in other transmembrane proteins including inwardly rectifying and TASK3 potassium channels (22, 31, 33, 34). The EXXXL1 motif is most likely a member of the (D/E)XXXL (L/I) sorting motif that shows considerable degeneracy; in fact, in AQP4 channels an EXXLI motif is essential for correct trafficking (30).

The demonstration here of exclusion of the ER export DDXXDXXXI acidic motif by alternative splicing in the intracellular C-terminal linker of BK channels nicely contrasts with the inclusion of an hydrophobic (CVLF) ER retention signal through alternative splicing of the N-terminal intracellular S0-S1 loop of BK channels (12). Taken together, these data strongly support the hypothesis that alternative splicing plays a major role in controlling cell surface expression of ion channels and that this can be achieved in the same channel by diametrically opposite mechanisms: through either exclusion or inclusion of cognate trafficking motifs.

Why do BK channels have multiple trafficking motifs whose inclusion can be controlled by alternative splicing? Because BK channels have pleitropic functions in virtually all tissues of the body, it is likely that multiple trafficking and sorting signals are required to allow the correct surface expression and subcellular localization of BK channels relevant to the target tissue of interest. Furthermore, increasing evidence suggests that cell surface expression of BK channels is dynamically regulated, both through signals that may regulate splicing and through post-translational modifications and assembly with distinct regulatory β-subunits. Thus these multiple mechanisms are likely coordinated to expose or mask the correct complement of trafficking and sorting signals to allow appropriate distribution of BK channels within distinct cell types. Indeed, alternative splicing of the rat SVcyt ortholog of hSlo579–664 is dynamically regulated in corporeal tissue in models of diabetes (17). Whether the dominant negative function of hSlo579–664 is physiologically relevant in this or other model systems remains to be explored. Clearly the inclusion (13, 27) or exclusion (as observed with the hSlo579–664 Variant here) of trafficking motifs through alternative splicing most likely represents a fundamental mechanism for controlling BK channel cell sur-

**DISCUSSION**

We have identified three distinct motifs within the intracellular RCK1-RCK2 linker of BK channels that control their cell surface expression. In particular, an acidic cluster-like motif, DDXXDXXXI, is critical for cell surface expression and is highly conserved from flies to man. This acidic motif can be transplanted to nonchannel proteins to accelerate ER export but cannot override pre-existing ER retention signals, as described for other acidic motifs (22). Importantly, alternative splicing of a human BK channel splice variant hSlo579–664 that excludes the exons encoding this motif results in a trafficking-deficient
face expression under a variety of physiological and pathophysiological conditions.

Acknowledgments—We thank Heather McClafferty and Lijun Tian and other members of the respective laboratories for critical discussions during this work and Trudi Gillespie and the IMPACT imaging facility for assistance in confocal imaging assays. The GABA$_\text{R}$,La receptor wild type and ASRR constructs were generous gifts from Lily Jan (University of California at San Francisco).

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