Transmembrane domain length of viral K+ channels is a signal for mitochondria targeting

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Transmembrane domain length of viral K⁺ channels is a signal for mitochondria targeting


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K⁺ channels operate in the plasma membrane and in membranes of organelles including mitochondria. The mechanisms and topogenic information for their differential synthesis and targeting is unknown. This article describes 2 similar viral K⁺ channels that are differentially sorted; one protein (Kev) is imported by the Tom complex into the mitochondria, the other (Kcv) to the plasma membrane. By creating chimeras we discovered that mitochondrial sorting of Kcv depends on a hierarchical combination of N- and C-terminal signals. Crucial is the length of the second transmembrane domain; extending its C terminus by ≥2 hydrophobic amino acids redirects Kev from the mitochondrial to the plasma membrane. Activity of Kev in the plasma membrane is detected electrically or by yeast rescue assays only after this shift in sorting. Hence only minor structural alterations in a transmembrane domain are sufficient to switch sorting of a K⁺ channel between the plasma membrane and mitochondria.

algal viruses | dual targeting | K⁺ channel sorting | PBCV-1 | EsV-1

Viruses often serve as tools to solve basic questions in biochemistry and structure biology. For example, many biochemical pathways have been discovered because viruses commandeered the cellular machinery for transcription, translation, and protein targeting and use these pathways for their own purposes. Thus, analyses of these viral pathways have helped to uncover many basic cellular mechanisms, which otherwise would have been difficult to study (1). Likewise, because of their small size, structural studies on virus proteins have often served to understand the basic architectural features of more complex homologous proteins (2).

A current topic in cell biology that can be studied with viral proteins is the question of how structurally similar membrane proteins or even the same protein are targeted to either the endoplasmic reticulum (ER) or the mitochondria (3, 4). Hydrophobic membrane proteins such as ion channels, which end up in the plasma membrane, are generally cotranslationally targeted into the ER and then shuttled through the secretory pathway to their final destination. Targeting nascent polypeptide chains to the ER is mediated by a hydrophobic signal sequence, which eventually guides the protein to the translocon (5). However, the same type or very similar proteins, which are functional in the plasma membrane, are also located in other membrane-enclosed compartments, such as mitochondria or chloroplasts (3, 4, 6). One example of such dual localization is K⁺ channels; for example the KvL1.3 channel is present in both the plasma membrane and in the inner membrane of the mitochondria (7). At present it is not understood how these proteins are targeted to the mitochondria. One possibility is that they are synthesized in the cytoplasm and sorted directly to the mitochondria. The mechanisms and topogenic information in the proteins, which are responsible for the differential synthesis and targeting of similar membrane proteins, are unknown.

We have recently identified a protein (Kcv) encoded by Paramecium bursaria chlorella virus (PBCV-1) (family Phycodnaviridae) that forms a functional K⁺ channel in heterologous cells (8). Kcv has a monomer size of 94 aa and is the smallest protein known to form a functional K⁺ channel. Nonetheless, Kcv is predicted to have all of the structural features typical of eukaryotic and prokaryotic K⁺ channels (8, 9). Heterologous expression of Kcv, as well as reconstitution in planar lipid bilayers, results in a characteristic K⁺-selective, Ba²⁺-sensitive, and moderately voltage-dependent conductance (8–10). This means that this small viral protein contains all of the information for targeting the protein to the plasma membrane. The present study compares Kcv with another viral K⁺ channel, Kev. The Kcv channel is coded by virus EsV-1 (Ectocarpus siliculosus virus-1); like PBCV-1, EsV-1 is a member of the family Phycodnaviridae. The Kev protein is 124 aa long, and it is predicted to form a 2-transmembrane domain (TMD) K⁺ channel that is structurally similar to Kcv and to eukaryotic K⁺ channels (11). Data presented in this report show that, despite their structural similarity, the 2 viral K⁺ channels are sorted to different cellular compartments. Kcv enters the secretory pathway and reaches the plasma membrane, where it produces a measurable conductance. The second channel, Kev, is sorted to the mitochondria. Mutational analyses and domain exchange experiments between the 2 channels resulted in the identification of 2 sorting signals in Kcv: a mitochondrial targeting sequence located at the N terminus, and structural features in the downstream end of the second TMD. Modifications of the latter allow switching of channel sorting between mitochondria and the secretory pathway. These results suggest that a competition between sorting signals determines the destination of a K⁺ channel protein.

Results

K⁺ Channel Gene in EsV-1. The genome of the phycodnavirus EsV-1 contains a 124-codon ORF (ORF 223) with all of the elements (e.g., 2 TMDs, selectivity filter) of a eukaryotic K⁺ channel [11; supporting information (SI) Fig. S1A]. Overall, the protein, named Kev (12), has 29% amino acids identity with the prototype K⁺ channel protein Kcv from virus PBCV-1; however, the C-terminal portion of the 2 proteins has 41% amino acids identity.


The authors declare no conflict of interest.

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their endogenous Kchannel, transformed with the homologous TRK1, the human Kir2.1, or different Kchannels or a Kesv-GFP chimera (Kesv:GFP) were expressed in either HEK293 cells or X. laevis oocytes we observed, in contrast to the results from a previous study (12), only an occasional up-regulation of endogenous currents but no appreciable K+ conductance (Fig. S2).

Kesv Is Sorted to the Mitochondria. Figs. 2A and B show the cellular distribution of Kcv:GFP and Kesv:GFP in HEK293 cells, respectively. Kcv:GFP has a tubular-like distribution, indicating that it is located in membranes (Fig. 2A). A prominent ring-like staining always occurs around the nucleus owing to staining of the ER. The location of Kcv in the secretory pathway and in the plasma membrane is consistent with the ability to record a Kcv-mediated conductance in transfected HEK293 cells (10) and to rescue K+ transport–deficient mutants (Fig. 1).

To test the bioinformatic prediction that Kesv is targeted to the mitochondria (Table S1) HEK293 cells were incubated with MitoTracker red (Invitrogen). Fig. 2Aa–c and 2Ba–c show typical images of HEK293 cells transfected with either Kcv:GFP or Kesv:GFP, respectively, and stained with the fluorescent mitochondrial marker. The red channel shows that the MitoTracker stains internal structures with the typical hallmarks of mitochondria. An overlay of the images with MitoTracker red and GFP shows a pronounced colocalization of the colors, indicating that Kesv:GFP is located in the mitochondria (Fig. 2Bc and d, colocalization coefficient: 0.36 ± 0.09 [n = 16]). A similar analysis of Kcv:GFP-expressing HEK293 cells shows that GFP and MitoTracker fluorescence are different (Fig. 2Ac and d). The occasional colocalization at the edges of the mitochondria is due to limited resolution of the mitochondria from adjacent GFP-containing structures (Fig. 2Ac, Inset). The mean colocalization coefficient in Kcv:GFP-expressing cells is 0.14 ± 0.08 (n = 16) and significantly (P < 0.0001) lower than that obtained for Kesv:GFP-expressing cells [0.36 ± 0.09 (n = 16)]. Kesv:GFP differs as predicted from Kcv:GFP; it is not targeted to the secretory pathway but to the mitochondria. However, the images also reveal a high GFP background in the cytoplasm. The reason for this is not known, but the results indicate that any signal recognition machinery involved in mitochondrial sorting is probably not perfect.

Fig. 1. Growth phenotype of yeast Δtrak1Δtrak2 mutants transformed with different K+ channels or a K+ transporter. Yeast cells were transformed with genes encoding either homologous TRK1, human Kir2.1 channel, or the viral Kcv or Kesv, as well as with mutants in which the second TMD of Kesv was extended at position 113 by 3 (Kesv113VVV) or 2 valines (Kesv113V). In addition, yeast cells were transformed with a chimera in which the N terminus of Kcv was replaced with the N terminus of Kesv (Kcv(NT)Kesv), or vice versa (Kesv(NT)Kcv). All yeasts were grown on medium containing either 100 mM or 0.5 mM K+. Only yeast transformed with Kesv and Kesv(NT)Kcv failed to grow on low-K+ medium.

Fig. 2. Differential colocalization of Kcv:GFP and Kesv:GFP with MitoTracker in HEK293 cells. (A) (Upper) Color-coded structural elements of Kcv:GFP chimera comprising TM1 and TM2, N-terminal domain (NT) (all in light gray), pore (orange), and GFP (green). Confocal image of exemplary HEK293 cell expressing Kcv:GFP (Aa) and staining of the same cell with MitoTracker red (Ab). Overlay of the 2 images in which colocalization of the 2 colors is shown in white (Ac). Inset magnifies a region of the cell and shows that the green and red fluorescence are well separated. The apparent colocalization only results from an insufficient resolution of the red-stained mitochondria and the green-stained perinuclear ring. (Ad) Scatter plot of green and red pixels from region of interest (borders of green fluorescent cell). The yellow bars show thresholds for both colors. The dashed line provides linear regression solution. Pearson’s colocalization coefficient in the present example is 0.12. (B) (Upper) Color-coded structural elements of Kesv:GFP chimera comprising TM1 and TM2, N-terminal domain (NT) (all in dark gray), pore (blue), and GFP (green). Confocal image of exemplary HEK293 cell expressing Kesv:GFP (Ba) and staining of the same cell with MitoTracker red (Bb). Overlay of the 2 images in which colocalization of the 2 colors is shown in white (Bc). Inset magnifies a region of the cell and shows that the green and red fluorescence colocalize. (Bd) Scatter plot of green and red pixels as in Fig. 2A. Pearson’s colocalization coefficient in this example is 0.36.
Kesv Is Actively Imported Into Mitochondria. To examine the mitochondrial localization of Kesv further, the Kesv:GFP chimera and GFP alone (as a control) were translated in vitro and incubated with isolated mitochondria from yeast. The 35S-labeled Kesv:GFP and GFP proteins are detected separately (Fig. 3A, lane 1). After re-isolation of the mitochondria only Kesv:GFP remained associated with the mitochondria (Fig. 3A, lane 2). Further incubation of the isolated mitochondria with 600 mM NaCl did not disrupt the association of the protein with the mitochondria (Fig. 3A, lane 3), indicating a tight association.

To test whether Kesv is imported into mitochondria, in vitro–translated and 35S-labeled Kesv protein was incubated with isolated yeast mitochondria in the presence and absence of a membrane potential. The data in Fig. 3B show that Kesv is only imported in the presence of a membrane potential and that after uptake into mitochondria, Kesv is protected against externally added protease K. The results of these experiments suggest that the K⁺ channel is imported into the mitochondria in a voltage-dependent manner.

To examine the oligomeric state of the imported channel the 35S-labeled Kesv protein was imported into isolated mitochondria as reported above. After removal of externally associated protein by incubation with protease K and re-isolation, the mitochondria were lysed by digitonin and the mitochondrial proteins separated by blue native PAGE (Fig. 3C). A specific macromolecular complex of ∼40 kDa was detected only under conditions that favored uptake of Kesv. The molecular weight of this complex is similar to that predicted for a Kesv tetramer. The results of these experiments suggest that the K⁺ channel is present in the mitochondria as a functional tetramer.

To examine the import pathway of Kesv into the mitochondria we performed the same kind of import experiments with isolated mitochondria from either WT yeast or from yeast mutants without Tom22 (tom22Δ) (14). The Tom22 protein is an important component of the canonical mitochondrial import machin-

cery; the absence of this protein destabilizes the TOM complex (Fig. S3) and greatly reduces protein uptake by this pathway (14). A quantification of the import of radiolabeled Kesv into the mitochondria shows that the mitochondria from the mutant yeast fail to accumulate Kesv (Fig. 3D). The results of these experiments indicate that Kesv is actively imported into mitochondria and that the outer membrane TOM complex is involved in the uptake of the protein.

The N Terminus of Kesv Is Not Essential for Mitochondrial Sorting. Kesv has a hydrophobic N-terminal domain, which is longer than its Kcv counterpart (12; Fig. S1). This N terminus reveals several positive amino acids that, together with the predicted α-helical structure, are typical properties of a signal peptide for mitochondrial targeting (15). Predictions indicated that Kesv has a high (P = 0.8) and Kcv only a low (P = 0.4) probability of being targeted to the mitochondria (Table S1). Indeed, a chimera (NT-Kesv:GFP) comprising the Kesv N terminus (M1-T36) plus GFP was found to accumulate in the mitochondria of HEK293 cells; this apparent accumulation in the mitochondria, which occurs on a large background of GFP signal in the cytoplast, did not occur when the 2 critical arginines (R², R⁶) in NT-Kesv:GFP were mutated to glutamines (Fig. S4A and B). Collectively, these experiments support the prediction that the Kesv N-terminal domain could promote mitochondria import; the weak mitochondrial accumulation over a large cytoplasmic background nonetheless implies that this signal is not very strong.

To further test the signal peptide nature of the Kesv N terminus, we prepared 3 plasmid constructs to examine its significance in targeting the entire Kesv:GFP protein to the mitochondria: (i) mutations were produced in key amino acids in the putative Kesv signal peptide domain, (ii) the signal peptide was fused to Kcv, and (iii) the Kesv N terminus was replaced by that of Kcv.

The results with all 3 constructs in transfected HEK293 cells (Fig. 4B and C and Fig. S4C; see above) established that the Kesv N-terminal signal peptide is neither required for mitochondrial targeting nor strong enough to impose mitochondrial targeting. Fig. 4A shows an example of the distribution of Kesv:GFP containing the critical mutations (R²E, R⁶E) in the signal peptide. Despite the low predicted probability (Table S1), the construct still accumulates in the mitochondria (Fig. 4A and C).
The same result was obtained after mutating the 2 remaining positively charged amino acids (R16 or K25) in the signal peptide, which might be involved in mitochondrial sorting (Table S1). Finally, when the entire N terminus of Kesv (M1–T36) was replaced with the Kcv N terminus (M1–E23), the protein still accumulated in the mitochondria (Fig. 4C).

These results established that the Kesv N-terminal signal peptide was able to direct GFP to the mitochondria (Fig. S4A and B). However, when the signal peptide was fused to Kcv, the protein sorted to the secretory pathway; thus the signal peptide failed to impose sorting to the mitochondria. This fusion protein still enters the secretory pathway (Fig. 4B and C).

Collectively, these experiments establish that the N terminus of Kesv is neither sufficient nor essential to determine the sorting of the channel proteins; other signals must be present in the 2 channels, which determine their destination.

A Sorting Signal Is Associated with the Inner Transmembrane Domain. To identify other relevant sorting signals, we constructed a chimera protein [Kcv(P)Kesv:GFP] in which the pore of Kcv (E30–K72) was replaced with the Kesv pore (D60–L92). This chimera protein should reveal whether the sorting information is associated with the pore or with the transmembrane domains. The results show that this chimera is no longer targeted to the mitochondria but enters the secretory pathway when it is expressed in HEK293 cells (Fig. S4A). This result implies that at least some sorting information is contained in the TMDs.

Interestingly, program TMHMM2.0, an algorithm that predicts the length of TMDs (16), indicates that the first and second TMD of eukaryotic and prokaryotic K+ channel proteins with 2 TMDs are 23 aa long. However, the second TMD in the 2 viral channels is significantly shorter than those of all other K+ channels with the same predicted architecture. In particular, the length of Kesv TM2 is predicted to be 2 aa shorter than Kcv TM2 (Table S2). Also, TM2 of Kesv is predicted to start further downstream than that of Kcv (Fig. S1A). These predicted differences in TM length and in position occur despite the high amino acids identity in this region for the 2 proteins.

To examine the relevance of the TMD length to channel localization and function, we extended the C-terminal portion of the Kesv TMD2 by adding 2–6 hydrophobic amino acids in position 113 of Kesv:GFP. The different constructs and their predicted effects on TM2 length and on the energy for partitioning into the membrane (17) are presented in (Table S2).

Fig. 5B shows an example of the cellular distribution of a Kesv:GFP protein in which TM2 was extended by 3 hydrophobic amino acids (IVL) in position 113. The protein no longer colocalizes with MitoTracker red but mimics the distribution of Kcv:GFP (Fig. 5B and D). This is apparent from the perinuclear distribution of GFP and the apparent separation of GFP and MitoTracker red fluorescence, as well as the low colocalization coefficient (Fig. 5D). This same qualitative result was obtained if the extension consisted of the amino acid motif VVV rather than IVL (Fig. 5D). Similar localization results were obtained if 2, 3, or 6 hydrophobic amino acids were inserted in this position (Fig. 5D). However, visual inspection of the images, as well as colocalization analyses, indicate that sorting of the Kesv:GFP mutant to the secretory pathway is strongest when 6 aa are added to TM2.

After determining that the length of TM2 was important for sorting, we examined whether length was solely responsible for sorting. Therefore, the Kesv:GFP protein was extended by the 3-amino acids IVL at amino acids positions 108, 110, and 115. The resulting cellular distribution of these constructs was compared with that obtained with the extension in position 113. Inspection of the image (e.g., Fig. 5C), as well as colocalization analyses (Fig. 5D), reveals that the TM2 extension is position dependent and not determined by the physicochemical properties of the TMDs (Table S2). Only extension of TM2 to the downstream end (≥ position 113) shifted sorting from the mitochondria to the secretory pathway. The 2 upstream extensions had no impact on sorting; a representative image of a HEK293 cell expressing Kesv:GFP with a 3-amino acids extension in position 108 indicates that this construct primarily colocalizes with the mitochondria (Fig. 5C and D).

Kesv with Modified Targeting Signals Generates K+ Conductance. The results suggest that redirection of a channel protein from sorting to the mitochondria to the secretory pathway leads to active channels in the plasma membrane. These results also predict that only those constructs that reach the secretory pathway should rescue the Δtrk1, Δtrk2 yeast mutant in low K+ (Fig. 1). Worth noting is that this general conclusion is supported by the imaging data using GFP-tagged proteins as well as by the yeast rescue assay and the mitochondria import study (Fig. 3), which both rely on untagged proteins. This means that the tag has no major impact on the general distribution of the proteins.

To further examine the ability of Kesv to function as a K+ channel we also tested 2 chimeras in HEK293 cells (Figs. 4 and 5) that support yeast growth on low-K+ concentrations. Channel mutants that maintained their mitochondrial sorting did not rescue the yeast mutants on low-K+ medium (Fig. 1). Worth noting is that this general conclusion is supported by the imaging data using GFP-tagged proteins as well as by the yeast rescue assay and the mitochondria import study (Fig. 3), which both rely on untagged proteins. This means that the tag has no major impact on the general distribution of the proteins.
The sorting of viral channels in heterologous systems is advantageous in understanding their function. Viruses PBCV-1 and EsV-1 are both members of the family Phycodnaviridae and clearly have a common evolutionary ancestor. However, they have different hosts, habitats, and life cycles, suggesting that a long time has elapsed since they diverged (11). Despite these differences, they both code for proteins that form K⁺ channels. One protein, Kcv, has previously been shown to form a functional K⁺ channel (8). The present results show that a second virus-encoded channel, Kesv, has the functional properties of a canonical K⁺ channel in the plasma membrane of mammalian cells. However, channel activity only occurs after the protein is targeted to the plasma membrane. In contrast to a previous report (12), we found no Kesv-mediated plasma membrane conductance.

The discovery of 2 functional K⁺ channels in different viruses is interesting from a virology viewpoint. Both viruses have large genomes, >330 kb, and encode 231 (EsV-1) or 366 (PBCV-1) proteins. However, they only encode 33 proteins in common, including the 2 K⁺ channels (11). Intuitively, one would predict that common conserved genes between the 2 viruses would encode proteins with a similar function(s) in the life cycles of the 2 viruses. The present experiments do not directly address the question of the function of the 2 K⁺ channels, but the fact that the 2 proteins have different targeting properties in heterologous systems suggests that they also have different functions in their hosts. The Kesv protein probably performs in the mitochondria of its host. Because of the central function of mitochondria in cells, these organelles seem to be a preferred target of viral channels. For example, the PB1-F2 protein from influenza A virus and the channel-forming p7 protein from hepatitis C virus are targeted to this organelle (18, 19).

The most interesting finding in the present study is that both channel proteins are similar in terms of their predicted primary structure. However, in heterologous systems one channel is targeted to the plasma membrane through the secretory pathway, whereas nearly identical proteins travel to the mitochondria. For example, the PB1-F2 protein from influenza A virus and the channel-forming p7 protein from hepatitis C virus are targeted to this organelle (18, 19).

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The fact that the decision for sorting relies on a structure near the C terminus of the 2 viral channel proteins is surprising. This result means that the sorting does not occur during the synthesis of these proteins because typically, direct translocation into the mitochondria or the ER proceeds in a cotranslational manner beginning with the N terminus of the protein. The C-terminal localization of the signal domain in the 2 viral proteins means that structural features, which affect protein folding/unfolding are probably essential for targeting the small viral K⁺ channel proteins (18, 23).

The overall similarity in the architecture of the viral channels to eukaryotic K⁺ channels implies that similar mechanisms may
also be relevant in the sorting of these eukaryotic channel proteins to different organelles.

Materials and Methods

Constructs and Mutagenesis. For expression in HEK293 cells, Kcv or Kesv genes were cloned into the BglII and EcoRI sites of the pEGFP-N2 vector (Clontech) in frame with the downstream EGFP gene by deleting the Kcv and Kesv stop codon. Point mutations were created by the QuickChange method (Stratagene) and confirmed by DNA sequencing. Chimeras of Kesv and Kcv were created by sequential PCRs according to strategies described in ref. 24. The chimeras comprised the following elements: (i) Kesv(NT)Kcv: the Kesv N terminus (NT) was replaced with the Kcv N terminus [Kcv (M1–E15) and Kesv (S15–K32)]; (ii) Kcv(NT)Kesv: the Kcv NT was replaced with the Kesv N terminus [Kesv (M1–T90) plus Kcv (P13–L94)]; (iii) Kcv(TM2)Kesv: the Kcv TM2 was replaced with the Kesv TM2 [Kcv (M1–D97) plus Kesv (L92–K135)]; (iv) Kesv(TM2)Kcv: the Kesv TM2 was replaced with the Kcv TM2 [Kesv (M1–D97) plus Kcv (E95–L94)]; (v) Kcv(P)Kesv: the Kcv P was replaced with Kesv P [Kcv (M1–E95) plus Kesv (D90–L92) plus Kcv (T74–L94)].

For expression in yeast, K+ channels and mutants were cloned into a derivative of pYES2 vector (Invitrogen) as described in ref. 25.

Expression of Channel Proteins and Electrophysiology. Cell culture, transfection protocols and methods for recording membrane currents in HEK293 cells were performed as described elsewhere (10).

Confocal Microscopy. HEK293 cells were investigated approximately 24 h after transfection with a Leica TCS SP spectral confocal microscope equipped with an argon/krypton laser (Leica Microsystems). Images were acquired with an HCC PL APO 63×/1.2wv objective. EGFP was excited with a 488-nm argon laser line, and confocal sections were collected using a 505–530-nm emission setting. MitoTracker red CMXRos (Invitrogen) was excited with a 543-nm helium–neon laser and detected with a 590–640-nm bandpass filter. Images and colocalization were analyzed with ImageJ software (National Institutes of Health).

Fig. S1.  K⁺ channel homologues coded by 2 algal viruses. (A) Alignment of predicted amino acid sequences of K⁺ channel proteins Kcv from chlorella virus PBCV-1 and Kesv from virus EsV-1. Both sequences have an overall 55% similarity (-) and 29% amino acid identity (asterisk). The position of the predicted selectivity filter is boxed. The positions of TM domains predicted by TMHMM-2.0 algorithm are shown by bars (Kesv) and dashed lines (Kcv). Four different structure prediction programs (see Materials and Methods) indicate the presence of an α-helix (double line) in the N terminus of Kesv. The positive amino acids highlighted in the N terminus of Kesv are critical for the signal peptide nature of this domain. (B) Hydrophobicity profile of the 2 proteins (Kesv, black line; Kcv, gray line) calculated according to the Kyle and Doolittle algorithm with a moving window of 15 aa and plotted against amino acid number. The two sequences were normalized (amino acid position 0) to the common selectivity filter motive GYG/GFG.
Fig. S2. Expression of Kesv:GFP generates no appreciable K⁺ conductance in HEK293 cells. (A) Steady-state I/V relation of HEK293 cells expressing only GFP (open square, n = 20) or Kesv:GFP (filled square, n = 23). The Kesv:GFP-expressing cells exhibit no significant increase in conductance. Only the SD is higher compared with the control. Occasionally we observed some cells with more current than control cells; one example is shown in (B). In these cases, however, the currents were insensitive to changes in the K⁺ concentration in the bath medium (data not shown) or to a replacement of K⁺ by Na⁺ (B). The steady-state I/V relation of the data in B reveals no appreciable difference between the currents recorded in K⁺ (open circles) or Na⁺ (filled circles) (C).
Fig. S3.  Tom 22D mutant has a corrupted Tom complex.  WT and *tom22Δ* mitochondria, respectively, were lysed in the presence of digitonin and analyzed by blue native PAGE as described in Materials and Methods. The proteins were blotted on a PVDF membrane, and Tom40 was visualized using a polyclonal antiserum.
The N-terminus of a channel does not determine localization. (A) Confocal images of exemplary HEK293 cell expressing chimera comprising N-terminal domain (NT) of Kesv (Mt-T36) plus GFP. The green channel shows the distribution of GFP (a) the red channel the fluorescence of mitotracker red (b). Intensity plot (c) from line scan of GFP (green) and mitotracker (red) fluorescence in region of the white line in b. Note coincidence of fluorescent maxima. (B) Same as in A but with mutation (R3E, R4E) of charges in N-terminal domain. Note the absence of distribution maxima of GFP in a and c. (C) Confocal image of exemplary HEK293 cell expressing chimera of Kesv:GFP in which the N-terminus was replaced by that of Kcv (a) and staining of the same cell with mitotracker red (b). Intensity plot (c) from line scan of GFP (green) and mitotracker (red) fluorescence in region of the white line in a. Note the alternating position of red and green intensity peaks. Scale bars: 10 μm.
Fig. S5. Kesv mutants with altered sorting signals generate conductance with moderate K⁺ selectivity in plasma membrane of HEK293 cells. Representative current responses to standard clamp protocol (holding voltage: 200 ms, 0 mV; test voltages: 600 ms + 80 mV to ≥ −120 mV; post voltage: ≥100 ms, 0 mV) in a HEK293 cell transfected with only GFP (A), with Kcv(P)Kev:GFP (B), or a mutant of Kesv:GFP with an extension of TM2 by 3 aa (Kesv113IVL:GFP) (C). The symbols in A–C indicate the point of data collection and cross-reference with symbols in (D), which shows the corresponding steady-state current/voltage (I/V) relations. Inset in D shows the mean steady-state currents (± SD) at −100-mV test voltage from cells transfected with Kcv(P)Kev:GFP (open bar, n = 19) or Kesv113IVL:GFP (gray bar, n = 6) compared with mock GFP-transfected or untransfected HEK293 cells (solid bar, n = 25). (E) Nernst plots of mean reversal voltages (± SD, n ≥ 3) obtained from instantaneous I/V relations in bath solutions with different K⁺ concentrations from HEK293 cells transfected with Kcv(P)Kev:GFP (filled circle), Kesv:GFP113IVL (open triangle), or mock-transfected cells (open circle).
Table S1. Prediction for targeting and experimental localization of Kcv:GFP, Kesv:GFP, and its mutants

<table>
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<tr>
<th>Channel</th>
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<td>0.30</td>
<td>mi</td>
</tr>
<tr>
<td>KesvR3ER4ER16E</td>
<td>0.1</td>
<td>0.04</td>
<td>mi</td>
</tr>
<tr>
<td>KesvR3ER4ER16S</td>
<td>0.1</td>
<td>0.04</td>
<td>mi</td>
</tr>
<tr>
<td>GFP</td>
<td>0.06</td>
<td>0.03</td>
<td>ud</td>
</tr>
<tr>
<td>Kesv(NT):GFP</td>
<td>0.8</td>
<td>0.50</td>
<td>mi</td>
</tr>
<tr>
<td>Kesv(NT)R3ER4E:GFP</td>
<td>0.2</td>
<td>0.06</td>
<td>ud</td>
</tr>
<tr>
<td>Kcv(NT)Kesv:GFP</td>
<td>0.8</td>
<td>0.60</td>
<td>sp</td>
</tr>
<tr>
<td>Kesv(NT)Kcv:GFP</td>
<td>0.5</td>
<td>0.02</td>
<td>mi</td>
</tr>
</tbody>
</table>

The predictions were calculated using Mitoprot (1) and TargetP (2) prediction algorithms. The localization of the proteins either in mitochondria (mi) or the secretory pathway (sp) or in an undefined location (ud) was determined from confocal images.

Table S2. Physicochemical properties and cellular localization of viral channels Kcv and Kesv

<table>
<thead>
<tr>
<th></th>
<th>oct (ΔG(kcal/mol))</th>
<th>int</th>
<th>TM</th>
<th>Destination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kcv</td>
<td>7.3</td>
<td>3.0</td>
<td>20 (20)</td>
<td>sp</td>
</tr>
<tr>
<td>Kesv</td>
<td>11.4</td>
<td>4.1</td>
<td>18 (23)</td>
<td>mi</td>
</tr>
<tr>
<td>KesvV</td>
<td>12.3</td>
<td>4.2</td>
<td>23</td>
<td>sp</td>
</tr>
<tr>
<td>KesvVV</td>
<td>12.3</td>
<td>4.2</td>
<td>23</td>
<td>sp</td>
</tr>
<tr>
<td>Kesv108IVL</td>
<td>13.8</td>
<td>5.2</td>
<td>23</td>
<td>mi</td>
</tr>
<tr>
<td>Kesv110IVL</td>
<td>13.8</td>
<td>5.2</td>
<td>23</td>
<td>mi</td>
</tr>
<tr>
<td>Kesv113IVL</td>
<td>13.8</td>
<td>5.2</td>
<td>23</td>
<td>sp</td>
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<tr>
<td>Kesv113IVLIVL</td>
<td>14.9</td>
<td>5.7</td>
<td>23</td>
<td>sp</td>
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</tbody>
</table>

The data reveal the calculated Gibbs free energy [ΔG (kcal/mol)] for partitioning of TM2 from Kcv, Kesv, and mutants from bilayer into water. Data obtained by using the Membrane Explorer Program and the Wimley and White scale (1) for a 19-aa-long transmembrane domain with amino acids 110 for Kesv and 86 for Kcv in the center. The data provide values for transition energy from the octonal face (oct) and for the interface (int) to water. Also shown is the predicted number of amino acids in TM2 of the respective channels as calculated by the TMHMM2.0 algorithm. The data calculated, including the GFP and linker, are given in parentheses. sp, secretory pathway; mi, mitochondria.