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A sequence motif responsible for ER export and surface expression of Kir2.0 inward rectifier K^+ channels

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Abstract **Integral membrane proteins are sorted via the secretory pathway. It was proposed that this pathway is non-selective provided that the cargo protein is properly assembled and lacks an endoplasmic reticulum (ER) retention signal. However, recent experimental evidence suggests that efficient export of proteins from the ER to the Golgi complex is not simply a default pathway. Here we demonstrate a novel sequence motif (FxYENEV) in the cytoplasmic C-terminus of mammalian inward rectifier potassium (Kir) channels which determines ER export. This motif is found to be both necessary and sufficient for efficient export from the ER that eventually leads to efficient surface expression of Kir2.1 channels.** **© 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Endoplasmic reticulum; Golgi; Potassium channel; PSD-95

1. Introduction

Protein trafficking between different intracellular compartments is achieved by transport vesicles. Their formation is driven by coat proteins recruited to the site where they bud off from the donor membrane [1]. While clathrin coats vesicles for intracellular traffic between the Golgi complex and the plasma membrane and within the endocytic pathways [2], vesicles for the transport between the endoplasmic reticulum (ER) and the Golgi compartment are enveloped in coat–protein complexes (COPs) [3]. Thus, in the early secretory pathway, transport of membrane proteins such as ion channels to the cell surface is mediated by COP-II-coated vesicles, which package them after synthesis in the ER for anterograde transport to the Golgi complex (for review: [4]).

Experimental evidence suggests that export of membrane proteins from the ER is a selective rather than a default process [5]. The selectivity may be accomplished by sequence information within the cytoplasmic domains of the cargo proteins that leads to preferential coat protein recruitment. To date, only a few such sequence motifs for promoting ER export have been suggested. A di-phenylalanine motif binding COP-II components has been found in p24 and ERGIC-53, which are proteins known to cycle between ER and Golgi [6–8]. In addition, a di-acidic motif depending on an upstream tyrosine residue has been proposed to signal for efficient ER export of the non-cycling vesicular stomatitis virus glycoprotein [9,10].

In polarized epithelial cells, potassium channels of the Kir family are differentially sorted to specific subcellular destinations such as the apical or basolateral membrane domain [11–13]. It has been proposed that subcellular sorting of ion channels depends at least partially upon associated proteins like auxiliary β subunits or PDZ domain scaffold proteins such as members of the PSD-95 family [14–16]. In addition, these associated proteins can control surface expression of ion channels influencing ER export and the formation of surface clusters [17–19]. Yet there has been no evidence that Kir channel subunits themselves would carry differential sequence information, which promotes export from the ER and thereby controls surface expression.

2. Materials and methods

2.1. Gene construction

N-terminal fusion constructs of Kir channel subunits with enhanced green fluorescent protein (EGFP) were designed by inserting the respective cDNA in-frame into the commercially available EGFP-C1 eukaryotic expression plasmid (Clontech Laboratories GmbH, Heidelberg, Germany). Translation of the fusion proteins was tested by anti-GFP immunoblot analysis (monoclonal anti-GFP, Clontech Laboratories GmbH) that detected bands at predicted molecular weights of 75 and 65 kDa for $_{GFP}$Kir2.1 and $_{GFP}$Kir4.1, respectively (data not shown). To estimate cell surface expression, the Flag epitope (DYKDDDDK) was introduced into the extracellular domain of Kir2.1 at amino acid position 116 by PCR. Point and deletion mutants were also constructed by PCR with oligonucleotides carrying the desired mutations. All PCR-derived products were verified by sequencing.

2.2. Cell culture and transfection

Opossum kidney (OK) cells (American Type Culture Collection, USA) were grown in DMEM-F12 supplemented with 10% fetal calf serum and 1% penicillin/streptomycin (10 000 IU) (Gibco Life Technologies GmbH, Karlsruhe, Germany) at 37°C and 5% CO_2. At ~80% confluence, OK cells were transfected with the respective cDNAs using Effectene Reagent (Qiagen, Hilden, Germany), following the supplier's protocols. The Golgi complex was identified by staining living cells with a Texas red-conjugated BODIPY-ceramide complexed with defatted bovine serum albumin following the supplier's protocols (Molecular Probes, Leiden, The Netherlands). In addition, cells were incubated with 10 μg/ml brefeldin A (BFA, Sigma, Deisenhofen, Germany), which reversibly fuses the Golgi compartment with the endoplasmic reticulum.

2.3. Immunocytochemistry

The anti-Kir4.1 antiserum was raised in rabbit against a synthetic peptide corresponding to the following amino acid sequence in the Kir4.1 C-terminus: SPGGLRDSTVRYGDPEKLKL (338–357). The antiserum was affinity-purified by antigenic peptide-coupled CH Sepharose 4B (Amersham Pharmacia Biotech AB, Uppsala, Sweden). It

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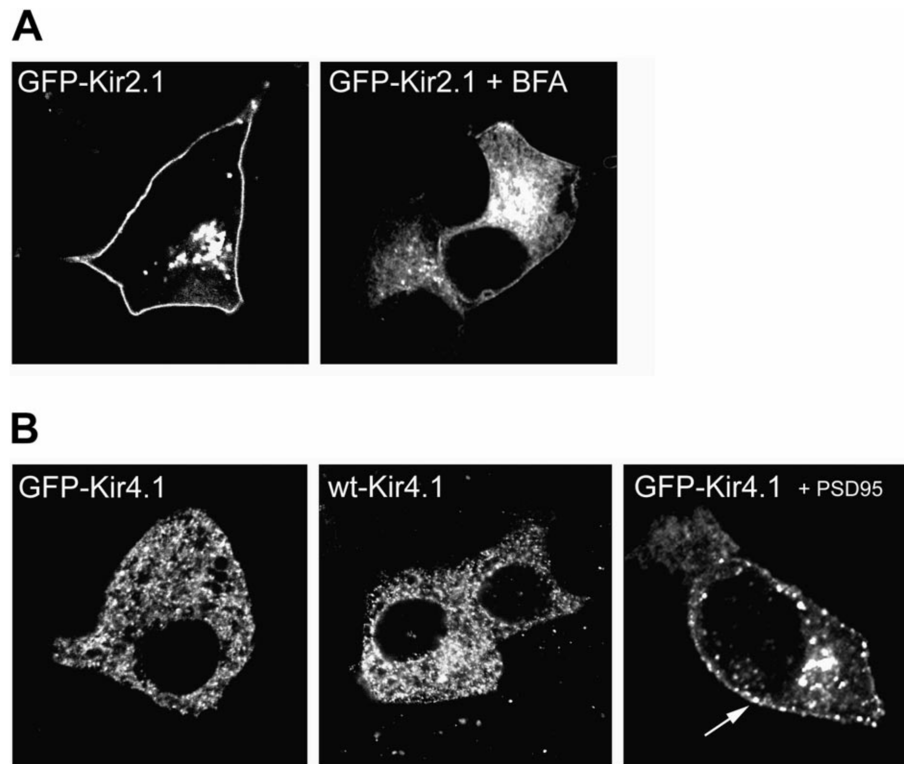

Fig. 1. Subcellular distribution of Kir2.1 and Kir4.1 in epithelial cells. A: Representative confocal images of OK cells expressing $_{GFP}$Kir2.1 imaged before (left) and after incubation (right) with the fungal Golgi toxin BFA. Note the BFA-induced redistribution of $_{GFP}$Kir2.1 from the Golgi compartment to the ER. B: Representative confocal images of OK cells expressing $_{GFP}$Kir4.1 (left) and wt Kir4.1 (middle) detected by a polyclonal antiserum. Coexpression with PSD-95 (right) increased surface expression and induced clustering (arrow) of $_{GFP}$Kir4.1 (expression of PSD-95 was verified by immunocytochemistry, data not shown).

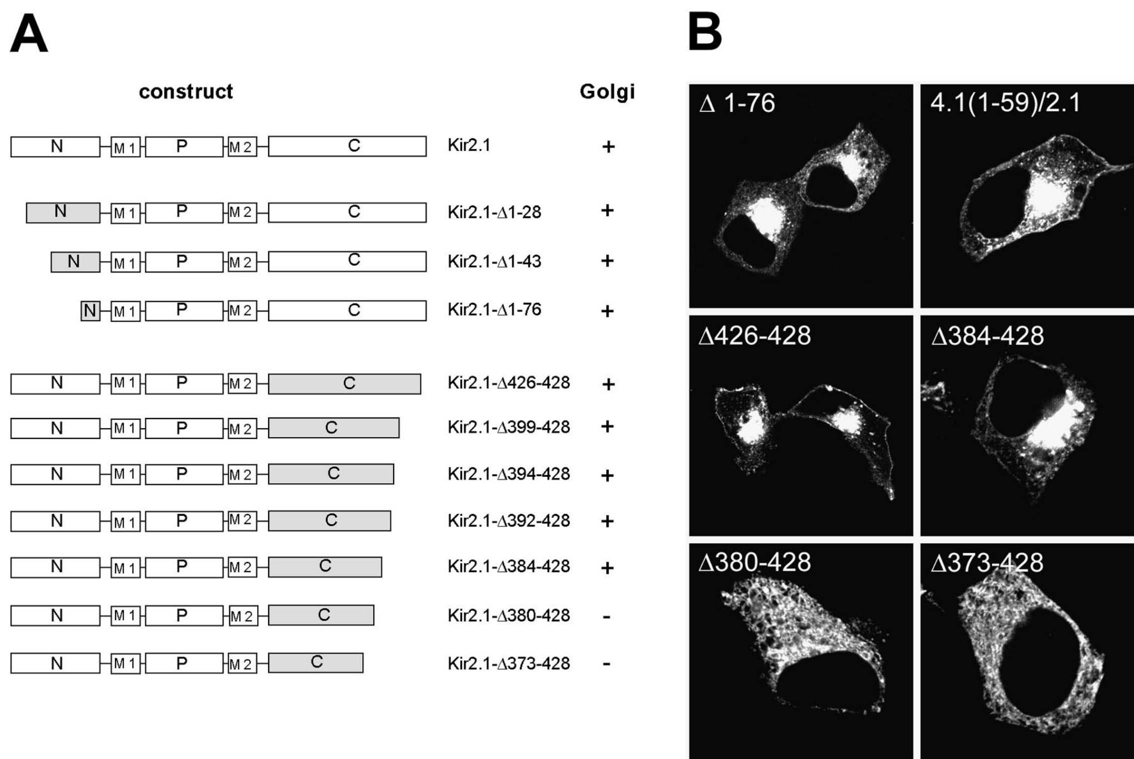

Fig. 2. Kir2.1 contains a C-terminal motif necessary for ER export. A: Schematic drawing of N- and C-terminal deletion constructs of Kir2.1 that were tested for ER export. +/− indicates efficient Golgi concentration/ER retention, respectively. B: Representative confocal images of the deletion constructs indicated that were expressed as GFP fusion proteins in OK cells.

proved to be subtype-specific among Kir channels, being not cross-reactive with Kir2.1 or Kir1.1 (data not shown). The following commercially available primary antibodies were used: monoclonal anti-PSD-95 (MA1-046; Affinity Bioreagents, Inc.) and monoclonal anti-Flag M2 (Sigma). For immunocytochemistry, cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at 4°C and pretreated with 10% normal goat serum (NGS) in PBS with or without (extracellular detection of the Flag epitope) 0.05% Triton X-100 (PBS-T) for 1 h at room temperature (RT) to block unspecific antibody binding. Then they were incubated with the respective primary antibody diluted 1:200 in 2% NGS/PBS-T for 1 h at RT. Immunoreactivity was visualized by a goat anti-rabbit IgG or goat anti-mouse IgG secondary antibody conjugated to cy-3 (1:1000 in 10% NGS/PBS).

2.4. Imaging

Cells were imaged with a confocal laser scanning microscope (LSM510, Zeiss, Göttingen, Germany) using the following excitation wavelengths and filter settings: EGFP: ex 488 nm Ar laser/em BP505–530 nm; cy-3: ex 543 nm He laser/em LP560 nm.

3. Results and discussion

As depicted in Fig. 1, the two Kir channels Kir2.1 and Kir4.1 showed differential subcellular segregation upon expression in OK cells. GFP-fused Kir2.1 ($_{GFP}$Kir2.1) was expressed in the plasma membrane and juxtanuclear vesicles

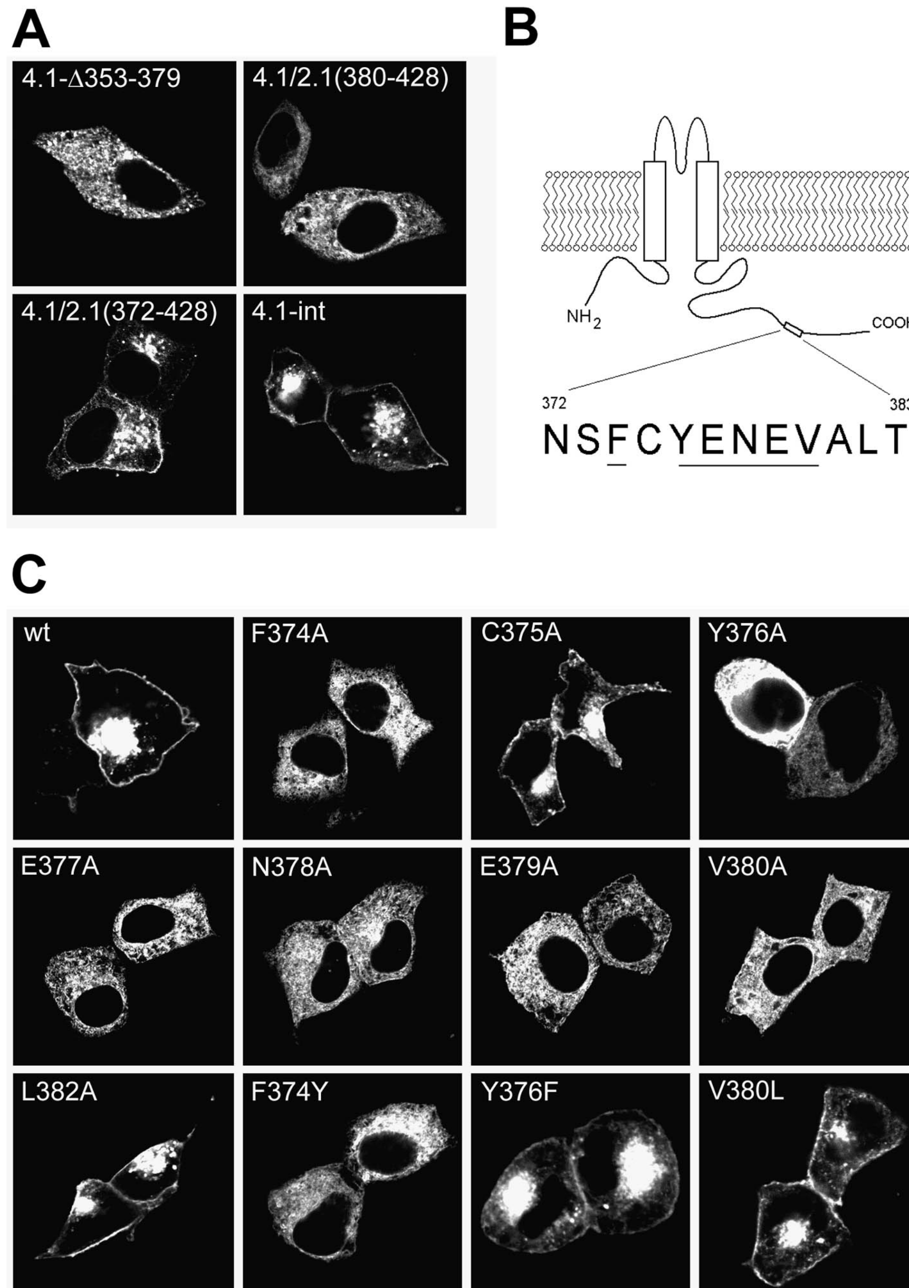

Fig. 3. The Kir2.1 C-terminal motif is sufficient for ER export. A: Representative confocal images of OK cells expressing GFP fusions of the chimeric constructs indicated (Kir4.1-int: aa 372–383 of Kir2.1 inserted into Kir4.1 at amino acid position 353). B: Localization of the putative ER export signal within the Kir2.1 C-terminus. Amino acid residues critical for ER export as defined by site-directed mutagenesis are underlined. C: Representative confocal images of OK cells expressing the point mutations of $_{GFP}$Kir2.1 indicated. Note that only C375A, L382A, and the conservative mutations Y376F and V380L leave the signal functional as determined by efficient ER export.

which represent the Golgi complex (Fig. 1A, left), as (i) incubation of transfected cells with the fungal Golgi toxin BFA resulted in a reversible redistribution of $_{GFP}$Kir2.1 to the ER (Fig. 1A, right) and (ii) we found colocalization of $_{GFP}$Kir2.1 with a Golgi marker (data not shown). In contrast, $_{GFP}$Kir4.1 was homogeneously distributed within the ER, showing neither a concentration within the Golgi apparatus nor unequivocally detectable plasma membrane fluorescence (Fig. 1B, left). Missorting of $_{GFP}$Kir4.1 due to GFP fusion was excluded as wt Kir4.1 detected with a polyclonal antiserum exhibited a distribution identical to that of $_{GFP}$Kir4.1 (Fig. 1B, middle). The distinct subcellular expression patterns of $_{GFP}$Kir2.1 and $_{GFP}$Kir4.1 did not vary with the period of time after transfection nor with the cDNA concentrations used for transfection (data not shown). However, co-expression with PSD-95 increased surface membrane expression of $_{GFP}$Kir4.1, suggesting that Kir4.1, unlike Kir2.1, requires a partner protein in order to achieve sufficient surface expression (Fig. 1B, right).

The molecular basis for the subcellular segregation of Kir2.1 and Kir4.1 was further investigated by constructing various N- and C-terminal deletion mutants of Kir2.1 (Fig. 2A). Truncation of up to 76 N-terminal and 45 C-terminal amino acids only reduced plasma membrane fluorescence but failed to affect ER export of $_{GFP}$Kir2.1 to the Golgi complex (Fig. 2A,B). In contrast, deletion of 49 or more C-terminal amino acids abolished both plasma membrane fluorescence and the distinct localization of $_{GFP}$Kir2.1 to the Golgi compartment. Thus, $_{GFP}$Kir2.1Δ380–428 and $_{GFP}$Kir2.1Δ373–428 displayed homogeneous distribution in the ER, identical to that observed with $_{GFP}$Kir4.1. These results suggested that the C-terminus of Kir2.1 contains a sequence motif that is necessary for efficient export of the protein from the ER. The reduced plasma membrane fluorescence observed with the N-terminal deletion mutants of Kir2.1 was restored by substitution with the respective N-terminal sequence of Kir4.1, demonstrating that the N-terminus does not contribute to the differential sorting properties of the two channel subunits (Fig. 2B).

To investigate whether the putative C-terminal motif is also sufficient for ER export, we designed a set of chimeric constructs between Kir4.1 and Kir2.1. As shown in Fig. 3A, replacing the Kir4.1 C-terminus by the last 57 amino acids of Kir2.1 was indeed sufficient for its translocation into the Golgi complex. This was not due to deleting a putative ER retention signal in the Kir4.1 C-terminus, because (i) truncation of the C-terminus alone did not affect the subcellular distribution, (ii) replacing the Kir4.1 C-terminus by only 49 amino acids of Kir2.1 did not enhance ER export, but (iii) insertion of amino acids 372–383 of Kir2.1 into the Kir4.1 C-terminus was sufficient for efficient translocation of Kir4.1 to the Golgi complex. Thus, we hypothesized a sequence motif between amino acid positions 372 and 383 to be both necessary and sufficient for ER export of Kir channel subunits (Fig. 3B). Site-directed mutagenesis revealed FxYENEV as amino acid residues critical for its function as determined by efficient ER export to the Golgi compartment (Fig. 3C). Within this motif, some residues could be conservatively exchanged such as tyrosine 376 for phenylalanine and valine 380 for leucine, whereas phenylalanine 374 could not be replaced by tyrosine without rendering the motif non-functional. Alignment of Kir channel C-termini shows conservation of the FCYENEV motif in the Kir2.0 subfamily. Accordingly, we found $_{GFP}$Kir2.3 to have a similar subcellular distribution as $_{GFP}$Kir2.1 (data not shown).

Since ER export to the Golgi is a necessary intermediate step in the secretory pathway, we finally investigated whether the identified ER export signal also controls surface expression of Kir2.1. As shown by extracellular epitope tagging, efficient ER export was required for efficient plasma membrane expression (Fig. 4). Disruption of the export signal reduced surface expression below the level of immunocytochemical detection.

In summary, we demonstrate an ER export motif that is differentially expressed within the family of the Kir channels. Our data are in good agreement with the findings of a very recent study by Ma et al., who isolated FCYENE as the functional export motif in Kir2.1 [20]. In line with their observation that efficient anterograde transport of Kir2.1 mutants which lack the export signal could be restored by coexpression of wt Kir2.1, we hypothesize that the identified sequence acts not merely by promoting channel maturation in the ER but rather as a recognition signal for preferential incorporation into COP-II-coated transport vesicles [4]. Recognition seems to be general in eukaryotic cells, as we observed the subcellular segregation of $_{GFP}$Kir2.1 and $_{GFP}$Kir4.1 not only in epithelial cell lines (OK, MDCK, HEK 293) but also in yeast (unpublished results). Such universality is further supported by the fact that the motif can improve ER export of other membrane proteins. While we demonstrate this for a different Kir subfamily, the Kir4.1 channel, Ma et al. successfully transferred the motif to a member of a different potassium

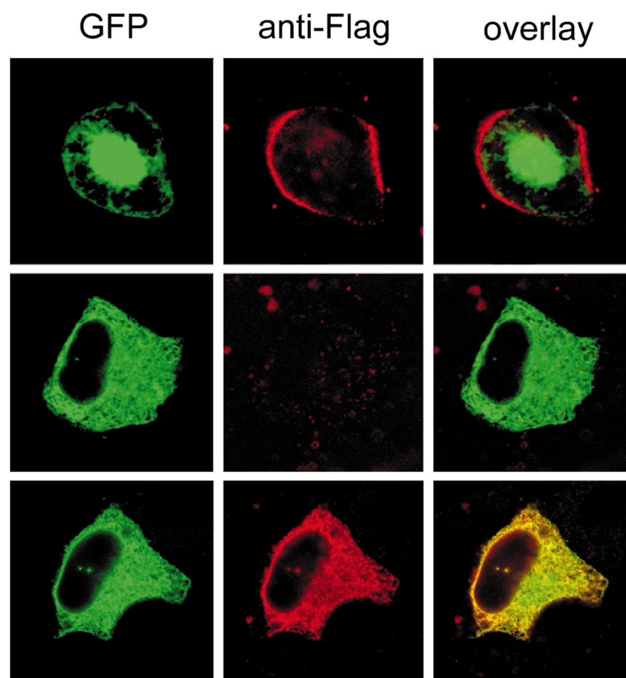

Fig. 4. Efficient surface expression of Kir2.1 requires efficient ER export. Shown are representative confocal images of OK cells expressing $_{GFP}$Kir2.1 (upper panel) and $_{GFP}$Kir2.1-E377A (middle/lower panel) which carry a Flag epitope within the extracellular H5 loop (see Section 2). Anti-Flag immunocytochemistry of non-permeabilized cells revealed unequivocal surface expression for $_{GFP}$Kir2.1 but not for $_{GFP}$Kir2.1-E377A. The latter was only detected after membrane permeabilization (lower panel).

channel family (Kv1.2) and a G protein-coupled receptor [20]. In a physiological setting, the identified motif will enable Kir2.0 channels to efficiently reach the cell surface on their own, without the need for interacting partners. Other Kir channel subunits such as Kir4.1, which in native tissue do efficiently localize to the plasma membrane [11,13,21], might require interacting cofactors carrying ER export sequences themselves or prolonging channel half-life within the surface membrane. First candidates would be other heteromerizing Kir channel subunits [22,23] and PDZ domain scaffold proteins, known to be coexpressed with Kir4.1 in native tissue [18,21,24].

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