Identification of γ-Aminobutyric Acid Receptor-interacting Factor 1 (TRAK2) as a Trafficking Factor for the K⁺ Channel Kir2.1*

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To identify proteins that regulate potassium channel activity and expression, we performed functional screening of mammalian cDNA libraries in yeast that express the mammalian K⁺ channel Kir2.1. Growth of Kir2.1-expressing yeast in media with low K⁺ concentration is a function of K⁺ uptake via Kir2.1 channels. Therefore, the host strain was transformed with a human cDNA library, and cDNA clones that rescued growth at low K⁺ concentration were selected. One of these clones was identical to the protein of unknown function isolated previously as γ-aminobutyric acid receptor-interacting factor 1 (GRIF-1) (Beck, M., Brickley, K., Wilkinson, H., Sharma, S., Smith, M., Chazot, P., Pollard, S., and Stephenson, F. (2002) J. Biol. Chem. 277, 30079–30090). GRIF-1 specifically enhanced Kir2.1-dependent growth in yeast and Kir2.1-mediated ⁴⁶Rb⁺ efflux in HEK293 cells. Quantitative microscopy and flow cytometry analysis of immunolabeled surface Kir2.1 channel showed that GRIF-1 significantly increased the number of Kir2.1 channels in the plasma membrane of COS and HEK293 cells. Physical interaction of Kir2.1 channel and GRIF-1 was demonstrated by co-immunoprecipitation from HEK293 lysates and yeast two-hybrid assay. In vivo association of Kir2.1 and GRIF-1 was demonstrated by co-immunoprecipitation from brain lysate. Yeast two-hybrid assays showed that an N-terminal region of GRIF-1 interacts with a C-terminal region of Kir2.1. These results indicate that GRIF-1 binds to Kir2.1 and facilitates trafficking of this channel to the cell surface.

Electrical activity in excitable tissues largely relies on tight control of K⁺ channel expression. The physiological relevance of changes in K⁺ channel expression has been demonstrated in a variety of normal and pathological states in heart, brain, and pancreas. It has been found that K⁺ channel protein synthesis, assembly, trafficking, and turnover all contribute to the steady state density of K⁺ channels on the cell surface (reviewed in Ref. 1). The molecular mechanisms underlying this regulation are just beginning to emerge.

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3 The abbreviations used are: GABA, γ-aminobutyric acid; GRIF, γ-aminobutyric acid receptor-interacting factor; HA, hemagglutinin; GFP, green fluorescent protein; PBS, phosphate-buffered saline; NGS, normal goat serum; FITC, fluorescein isothiocyanate; AD, activation domain; BD, DNA-binding domain; TRAK2, trafficking protein, kinesin binding 2.
EXPERIMENTAL PROCEDURES

Yeast Strains—Saccharomyces cerevisiae strain SGY1528 with disrupted native K⁺ transporters TRK1 and TRK2 originates from Ref. 16 and has the following genotype: MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trpl-1 ura3-1 trk1::HIS3 trk2::TRP1. The Kir2.1 channel was transformed into SGY1528 strain using a multicopy vector pYMet-Kir2.1 (17), a derivative of pYES (Invitrogen). Kir2.1 is transcribed under control of methionine-repressible promoter. For library screening we used the SGY1528 derivative named iKir2.1 that expresses chromosomally integrated Kir2.1 fused to Met25 promoter and URA3 selective marker. To construct this strain, pYMet-Kir2.1 plasmid was excised of 2-micron DNA by EcoRV digestion, self-ligated, and transformed into SGY1528. Ura⁺ transformants were selected, and Kir2.1 integration was verified using the following criteria: 1) growth on the medium with 2 mM KCl, but not on less then 1 mM KCl; 2) methionine repression of Kir2.1-dependent growth; and 3) stability of the growth rescue after cultivation in nonselective conditions (rich YEPD medium, supplemented to 100 mM KCl). The presence of Kir2.1, URA3, and Met25 promoter after nonselective growth was verified by PCR.

Yeast Cultivation—K⁺-free medium contained 2% glucose, 0.16% yeast nitrogen base without amino acids, sodium, and ammonia, 8 mM H₃PO₄, 2 mM MgSO₄, 0.2 mM CaCl₂, 0.1% vitamin mix, 0.1% trace elements mix, 0.2% adenine, de-ionized water (adapted from Ref. 16). The media were adjusted to pH 6.5 with arginine base and sterilized by filtration. KCl was added to desired concentration. For preparation of plates with low [K⁺]一道 agarose instead of agar was used. Yeast medium ingredients were from Qbiogene. Yeast transformation was performed using the alkaline cation method (Qbiogene). For growth assay, logarithmic cultures with starting density of OD₆₀₀ = 0.05 were incubated at 30 °C in a shaker incubator for 48 h. Culture growth was monitored spectrophotometrically at λ = 600 nm.

Screening of cDNA Library—Host strain iKir2.1 was transformed with a library of human brain cDNA in pGAD vector (Stratagene). Transformants were selected on medium lacking uracil and leucine supplemented to 100 mM KCl and replica plated on medium without uracil, leucine, and methionine supplemented to 0.5 mM KCl. cDNA clones from growing colonies were extracted and tested for a connection with the Kir2.1 channel.

Yeast Two-hybrid Assay—C-terminal fragments of Kir2.1 were fused to GAL4 activation domain in pGADT7 vector (BD Biosciences Clontech). GRIF-1 fragments were fused to the GAL4 DNA-binding domain on pGBK7 vector (BD Biosciences Clontech). Kir2.1 and GRIF-1-containing plasmids were co-transformed into YRG-2 strain (Stratagene) of the following genotype: MATα ura3-52 his3-200 ade2-101 lys2-801 trpl-901 leu2-3 112 gal4-542 gal80-538 LYS2::UASGAL4. Transformants were selected on medium lacking leucine and tryptophan. Growth of transformants on the plates without histidine was interpreted as interaction of the test proteins.

Cell Lines—COS and HEK293 cells were grown in Dulbecco’s modified Eagle’s medium with 4.5 g/liter glucose and 0.02 glutamine, supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, 100 μg/ml streptomycin at 37 °C and 5% CO₂. Kir2.1 and GRIF cDNA in pCDNA3 vector were transfected into COS and HEK293 cells using Lipofectamine reagent. HEK293 cell line stably expressing Kir2.1 channel tagged with the HA epitope was a gift of Dr. Min Li. Stable HEK293 transfectants were maintained in the presence of 200 μg/ml G418.

68Rb⁺ Flux Assay—HEK293 cells stably expressing Kir2.1-HA channels were plated on polylysine-coated plates and transfected with GRIF-containing and control plasmids. 24–48 h after transfection cells were loaded with 68Rb⁺ for 4 h by incubation with 1 μCi/ml 86RbCl. Prior to 86Rb⁺ measurements cells were washed two times with 86Rb⁺-free medium. Then medium was collected at 1–40 min time points, and the amount of released 86Rb⁺ was measured by scintillation counting. At 40 min, the cells were lysed with 1% SDS, and residual radioactivity in cells was determined.

Quantitative Microscopy—COS cells were co-transfected with Kir2.1 channel tagged with GFP at the N terminus and extracellular HA epitope (GFP-Kir2.1-HA, gift from Dr. Nikolaj Klocker) and GRIF-containing or control plasmids. Twenty-four hours after transfection, live cells were blocked with 10% normal goat serum in PBS (NGS) and incubated with primary rat anti-HA antibody 3F10 (Roche Applied Science) diluted 1:500 in 2% NGS. Incubations with antibodies were performed at room temperature for 30 min; PBS washings were performed three times for 5 min each. The cells were visualized at 40X magnification with inverted microscope (Olympus IX70). The cells were illuminated with 488- and 543-nm light. GFP and Texas Red signals, corrected to background, were quantified in captured images of individual cells using SimplePCI software.

Flow Cytometry—HEK293 cells stably expressing Kir2.1 channel tagged with extracellular HA epitope were transiently transfected with GRIF-containing or control plasmids. Twenty-four hours after transfection, the cells were washed with PBS and harvested by incubation with 0.5 mM EDTA in PBS for 5–10 min at room temperature. Following wash with PBS cells were incubated with the goat anti-rabbit antibody conjugated with Texas Red (Jackson Immunoresearch Labs) diluted 1:1000 in 2% NGS. Incubations with antibodies were performed at room temperature for 30 min; PBS washings were performed three times for 5 min each. The cells were visualized at 40X magnification with inverted microscope (Olympus IX70). The cells were measured with 488- and 543-nm light. GFP and Texas Red signals, corrected to background, were quantified in captured images of individual cells using SimplePCI software.
incubated with 30 μl anti-FLAG resin (Sigma) for 1 h at 4 °C. The supernatants were aspirated, and the pellets were gently rinsed three times with 1 ml of lysis buffer. The immune complexes were eluted with 50 μl of 100 μg/ml FLAG peptide in lysis buffer, mixed with Laemmli buffer, boiled for 10 min, and analyzed by immunoblotting. Following electrophoresis in 10% SDS-polyacrylamide gel proteins were transferred to polyvinylidene difluoride membrane. The membranes were blocked with 5% nonfat dry milk in PBS with 0.5% Tween (PBST) for 1 h at room temperature and incubated overnight at 4 °C with polyclonal anti-Kir2.1 antibody (Alomone) diluted 1:1000. After double washing with PBST, the membranes were incubated with 5% nonfat dry milk in PBS with 0.5% Tween (PBST) for 1 h at room temperature and incubated overnight at 4 °C with horseradish peroxidase-conjugated secondary antibody. Western blots were developed using high sensitivity Pierce ECL kit. Complexes of GRIF-FLAG with the Kir2.1 C terminus tagged with Myc were precipitated using anti-FLAG resin as above. Clarified lysates were incubated with 2 μl of rabbit polyclonal anti-X-press antibody (Invitrogen) overnight at 4 °C, and immunocomplexes were captured on 30 μl of protein A/G Plus-agarose reagent (Santa Cruz Biotechnology) for 2 h at 4 °C. The beads were washed four times with 1 ml of lysis buffer, mixed with Laemmli buffer, and incubated for 10 min at 95 °C for elution. The eluates were subjected to 8% SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting with monoclonal anti-Myc antibody (Abcam) diluted 1:3000.

For immunoprecipitation of native Kir2.1 and GRIF proteins, the brain was harvested from two adult mice and immediately homogenized in 5 ml of Buffer D (20 mM Hepes, pH 7.6, 125 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) (15) supplemented with protease inhibitors (Pierce). Homogenate was lysed in buffer D supplemented with 1% Triton X-100 for 1 h at 4 °C and clarified with centrifugation at 25,000 × g for 20 min. For immunoprecipitation, the supernatant was incubated with 5 μl of affinity-purified anti-Kir2.1/2.2 antibodies or control preimmune rabbit IgG (kindly provided by Dr. Carol Van denberg) overnight at 4 °C. Immune complexes captured on 30 μl of protein A-agarose were collected by sedimentation and dried by aspiration. The pellets were gently washed three times in buffer D using centrifugation at 800 × g at room temperature and subjected to immunoblotting. Anti-GRIF-1 antibody (a gift from Prof. Anne Stephenson) was used as in Ref. 21.

**RESULTS**

**Isolation of GRIF-1 as an Enhancer of Kir2.1-dependent Yeast Growth**—Growth of a yeast Δtrk1 Δtrk2 double mutant at low K⁺ concentration is rescued by expression of mammalian Kir2.1 channels (Fig. 1A). Kir2.1 expressed from a single chromosomal copy restores growth at 2 mM KCl but not at 0.5 mM KCl (Fig. 1A, sector 3). In contrast, transformation with a 2 micron-based multicopy Kir2.1 plasmid enables growth at
0.5 mM KCl (Fig. 1A, sector 2). Using this dosage effect, we sought to identify mammalian cDNAs that enable single copy Kir2.1-dependent growth at 0.5 mM KCl and thus are likely to encode positive regulators of Kir2.1. The single copy Kir2.1 strain, iKir2.1, was transformed with a human cDNA yeast expression library, and transformants able to grow at 0.5 mM KCl were selected. To confirm cDNA dependence of the growth phenotype, library plasmids were rescued from yeast expressing Kir2.1 were transiently transfected with GRIF-1 construct or control vector (vect.). The bars represent the mean release ± S.E. in 22 and 11 independent transfections for GRIF-1 and hGRIF samples, respectively. p < 0.001 as determined by paired t test.

**FIGURE 2.** GRIF clones enhance Kir2.1-mediated 86Rb+ efflux in HEK293 cells. A, time course of 86Rb+ efflux from stable Kir2.1 cell line transiently transfected with GRIF-1, hGRIF497, and control plasmid. The cells were loaded with 86Rb and washed, and 86Rb release was measured at the indicated time points. B, amount of 86Rb released during the first 3 min after washing from cells co-expressing Kir2.1 with GRIF clones or control vect. The bars represent the mean release ± S.E. in 22 and 11 independent transfections for GRIF-1 and hGRIF497, samples respectively. p < 0.001 as determined by paired t test.

**GRIF-1 Enhances Kir2.1 Surface Expression**

into the iKir2.1 strain. One of the confirmed cDNAs, KP39, promoted growth at 0.5 mM KCl (Fig. 1, A, compare sector 4 with sector 3; and B, left panel). However, KP39 did not increase growth at 0.5 mM KCl of the Δtrk1 trk2 strain in the absence of Kir2.1 (Fig. 1A, sector 5). Likewise, it did not improve growth at a high, nonselective K+ concentration (Fig. 1B), a condition in which Kir2.1 is not necessary for growth. Therefore, the KP39-encoded protein does not generally increase growth. Rather, the facilitation of growth by KP39 is coupled to Kir2.1 channel function.

Sequencing of KP39 identified a 1491-bp cDNA fragment exactly corresponding to the 5′ half of the open reading frame of a known gene ALS2CR3. The ALS2CR3 gene maps to the 2q33–q34 human chromosome locus associated with human juvenile amyotrophic lateral sclerosis (22). A causal relationship between ALS2CR3 and amyotrophic lateral sclerosis has not been established, and the function of this gene in humans remains unknown (22). A rat ortholog of ALS2CR3 protein has been identified in yeast two-hybrid system as GABA-receptor interacting factor-1 (GRIF-1, also named TRAK2) (21). KP39 contains the first 497 codons of the ALS2CR3 open reading frame, and its deduced amino acid sequence is 86.6% identical with the N-terminal half of rat GRIF-1. Thus, we now refer to KP39 as hGRIF497. Functionally, the full-length rat GRIF-1 cDNA was indistinguishable from hGRIF497 in its ability to enhance Kir2.1-dependent yeast growth (Fig. 1, A, sector 6, and C).

**GRIF-1 Increases Kir2.1-mediated 86Rb+ Efflux in Mammalian Cells**

To examine effects of GRIF-1 expression on Kir2.1 function in mammalian cells, we used the 86Rb+ flux assay. Because hydrated Rb+ ion is similar in permeability to K+, 86Rb+ flux can be used as an indicator of K+ channel activity. HEK293 cells stably expressing Kir2.1 were transiently transfected with GRIF-1 constructs or corresponding empty vectors. The cells were then loaded with 86Rb+ and washed, and the time course of 86Rb+ release was determined. In these assays, channel activity is revealed in the initial rate of release. As seen in Fig. 2A, initial efflux of 86Rb+ from cells transfected with full size GRIF-1 or hGRIF497 fragment
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GRIF-1 Increases Surface Expression of Kir2.1—To address the mechanism of GRIF-enhanced K⁺ flux, we examined surface expression of Kir2.1 with or without ectopic expression of GRIF constructs. HEK293 cells stably expressing Kir2.1 channel with HA epitope at the first extracellular loop were transiently transfected with full size GRIF-1, hGRIF₄₉₇ or empty vectors, immunostained for surface HA epitope, and analyzed by flow cytometry. Fig. 3A shows that GRIF-1 and hGRIF₄₉₇ each increase mean surface channel immunofluorescence. With both GRIF constructs, surface density of Kir2.1 channels was elevated by ~30% (Fig. 3B), which agrees with the increase of ⁸⁶Rb⁺ efflux in this cell line. Additionally, we examined the GRIF effect on Kir2.1 surface expression using quantitative microscopy. COS cells were transiently co-transfected with GRIF-1 or corresponding empty vector, as well as Kir2.1 tagged with extracellular HA epitope and GFP at the N terminus. Live transfected cells were immunolabeled with anti-HA antibody and Texas Red-conjugated secondary antibody to mark surface channels. Red and green fluorescence corresponding to surface and total Kir2.1 signals, respectively, in individual cells were measured using quantitative fluorescence microscopy. The Texas Red signal was then normalized by the GFP signal to yield a measure of Kir2.1 surface expression. As shown in Fig. 3C, cells transfected with GRIF-1 had a significantly stronger surface Kir2.1 signal. Notably, total cellular Kir2.1 channel protein expression, measured by GFP-Kir2.1 fluorescence (Fig. 3D) and Western blot (Fig. 4A), was not affected by GRIF. Thus, full-length GRIF-1 and the N-terminal half of GRIF-1 each increase surface localization of Kir2.1 in two different cell lines without affecting total Kir2.1 protein expression. Taken together, these results demonstrate that

occurs faster than from vector-transfected cells. Measuring ⁸⁶Rb⁺ efflux from multiple independent transfections proved the statistical significance of this difference (Fig. 2B). Overall, GRIF-1 expression in 40% of the cells increased total Kir2.1-mediated ⁸⁶Rb⁺ flux by 12% ± 1.7%. Thus, channel activity was increased by ~30% by GRIF-1. In contrast, transfection of GRIF-1 constructs into HEK293 cells that do not express Kir2.1 had no effect on the background ⁸⁶Rb⁺ efflux, which was ~10% of that in Kir2.1-transfected cells (data not shown). Therefore, we conclude that GRIF expression increases Kir2.1 channel activity.

GRIF-1 is physically associated with the Kir2.1 channel. A, co-immunoprecipitation (IP) of GRIF-1-FLAG and Kir2.1 from HEK293 lysates. Lysates were precipitated on anti-FLAG resin and probed with anti-Kir2.1 antibody. B, co-immunoprecipitation of GRIF-1 with C terminus of Kir2.1. HEK293 cells were co-transfected with Kir2.1 (180–428)-Myc and GRIF-1-FLAG. The lysates were precipitated on anti-FLAG resin and immunoblotted (IB) with anti-Myc antibody. C, co-immunoprecipitation of GRIF-1 N-terminal fragment with C terminus of Kir2.1. Kir2.1 (180–428)-Myc was co-transfected with GRIF (1–497)-X-press. The lysates were precipitated using anti-X-press antibody and immunoblotted with anti-Myc antibody. D, yeast two-hybrid analysis of Kir2.1-GRIF interaction. Yeast strain YRG-2 was co-transformed with C terminus of Kir2.1 fused to GAL4 AD and GRIF-1 and hGRIF₄₉₇ fused to GAL4 BD. Control fusions SV40-AD and p53-BD interact in yeast two-hybrid assay. Growth of transformants on media without histidine indicates protein-protein interaction. E, co-immunoprecipitation of native brain Kir2 channels and GRIF-1. Detergent-solubilized protein from whole murine brain was precipitated with anti-Kir2.1/Kir2.2 antibody or control preimmune IgG and probed with anti-GRIF-antibody.
GRIF-1 increases K⁺ transport through Kir2.1 by increasing surface expression of this channel.

**GRIF-1 and Kir2.1 Associate Physically**—The effects of GRIF-1 on Kir2.1-dependent yeast growth, Kir2.1 activity and surface expression may result from physical association between these two proteins. To test this possibility, we examined GRIF-1/Kir2.1 association with co-precipitation and yeast two-hybrid assays. For the co-precipitation assay, HEK293 cells were transiently transfected with GRIF-1/FLAG and Kir2.1 fragments tagged with Myc epitope. The lysates were precipitated on anti-FLAG agarose and immunoblotted with anti-Myc antibody. The lysates were affinity-purified or immunoprecipitated from cell lysates, and both sets of data indicate specific association of the C-terminal cytoplasmic domain of Kir2.1 channel with GRIF497. Consistent with these results, the cytoplasmic domain of Kir2.1 associates with full-size GRIF-1 as well as with its N-terminal half. This association is similar in strength to that between the positive control pair, SV40-AD and p53 BD. None of the GRIF constructs showed association with SV40-AD. Similarly, the Kir2.1 C terminus fused to AD did not confer histidine-independent growth alone or in combination with p53 BD, demonstrating the specificity of Kir2.1-GRIF-1 association (Fig. 4D). Thus, yeast two-hybrid results confirm biochemical results, and both sets of data indicate specific association of the C-terminal cytoplasmic domain of Kir2.1 with the N-terminal half of GRIF-1.

Finally, to determine whether the Kir2.1-GRIF-1 interaction occurs in vivo, we attempted co-immunoprecipitation of these two proteins from murine brain. Fig. 4E (IP Kir2) shows that precipitate collected with anti-Kir2.1/Kir2.2 antibodies contains GRIF-1 protein. In contrast, control precipitate collected with nonspecific preimmune serum gave no signal (Fig. 4E, mock IP). Thus, native Kir2 and GRIF proteins associate in vivo, indicating the physiologic relevance of the interaction originally discovered with heterologous expression.

**Localization of GRIF-binding Segment in Kir2.1**—To identify Kir2.1 regions responsible for association with GRIF-1, we constructed deletions of Kir2.1 C terminus (180 – 428 amino acids) and examined their association with GRIF-1. Co-immunoprecipitation showed that full-length GRIF-1 or its N-terminal half (amino acids 1 – 497) was inserted into the GAL4 DNA-binding domain (BD) vector. AD and BD constructs were then co-transformed into the YRG-2 yeast strain whose growth in the absence of histidine depends on functional Gal4 transcriptional activator. If Kir2.1 and GRIF-1 associate, this should bring AD and BD portions of Gal4 in close proximity, thus reconstructing functional Gal4 and allowing growth of YRG-2 in the absence of histidine. As shown in Fig. 4D, the cytoplasmic domain of Kir2.1 associates with full-size GRIF-1 as well as with its N-terminal half. This association is similar in strength to that between the positive control pair, SV40-AD and p53 BD. None of the GRIF constructs showed association with SV40-AD. Similarly, the Kir2.1 C terminus fused to AD did not confer histidine-independent growth alone or in combination with p53 BD, demonstrating the specificity of Kir2.1-GRIF-1 association (Fig. 4D). Thus, yeast two-hybrid results confirm biochemical results, and both sets of data indicate specific association of the C-terminal cytoplasmic domain of Kir2.1 with the N-terminal half of GRIF-1.

GRIF-1 binding domain localizes to the C terminus of Kir2.1 channel. A, co-immunoprecipitation (IP) of GRIF-1 with fragments of Kir2.1 C terminus. HEK293 cells were transiently transfected with GRIF-1/FLAG and Kir2.1 fragments tagged with Myc epitope. The lysates were precipitated on anti-FLAG agarose and immunoblotted (IB) with anti-Myc antibody. B, yeast two-hybrid mapping of GRIF-binding domain in C terminus of Kir2.1. The black lines indicate Kir2.1 fragments that interact with GRIF497. C, sequence of Kir2.1 348 – 396 fragment required for Kir2.1-GRIF interaction. The coiled coil is underlined, and the trafficking signals are in bold type. The asterisk indicates the predicted O-GlcNAc modification site.
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381–428) abrogated the yeast two-hybrid interaction. These data indicate that the region of Kir2.1 responsible for association with GRIF497 is located between amino acids 348 and 396, with the 360–381 sequence being essential (Fig. 5C).

Kir2.1 and GRIF-1 Co-localize in COS Cells—Co-immunoprecipitation and yeast two-hybrid interaction experiments indicate that Kir2.1 and GRIF-1 associate in vitro and in yeast cells. To examine association between these two proteins in mammalian cells, we transiently transfected COS cells with HA-tagged Kir2.1 and FLAG-tagged GRIF-1 and examined intracellular localization of both proteins by fluorescence microscopy with anti-HA and anti-FLAG antibodies. In cells transfected with GRIF-1 alone, this protein was diffusely localized throughout the cell, as reported previously by Beck et al. (21) (Fig. 6A). Kir2.1 immunofluorescence was observed routinely in perinuclear aggregates and punctate structures in the cytosol, consistent with localization to different compartments of the secretory pathway (Fig. 6B). Co-expression with GRIF-1 did not cause noticeable change in the pattern of Kir2.1 localization (data not shown). However, the subcellular distribution of GRIF-1 did change in the presence of Kir2.1. In addition to diffuse localization, GRIF-1 immunoreactivity was concentrated in punctate structures characteristic of Kir2.1 pattern (Fig. 6C). In these punctate structures, GRIF-1 often co-localized with Kir2.1 (Fig. 6D). Therefore, co-expression of Kir2.1 changed the pattern of subcellular distribution of GRIF-1. Moreover, Kir2.1 and GRIF-1 co-localized, consistent with association in mammalian cells.

DISCUSSION

Here we report a novel protein partner of the mammalian K+ inwardly rectifying channel Kir2.1. This protein is encoded by the ALS2CR3 gene mapping in the human juvenile amyotrophic lateral sclerosis critical region, 2q33–2q34. The rat ortholog of human ALS2CR3 protein, GRIF-1, was identified recently as a binding partner of the β-subunit of GABA receptor (21). Based on GRIF-1 binding to kinesin and its homology with proteins implicated in vesicular and organelle motility, a role for GRIF-1 as a trafficking factor has been proposed (23). We identified a direct effect of GRIF-1 to promote K+ channel surface expression and activity.

Human and rat GRIF proteins enable growth at low K+ concentration of a Δtrk1 trk2 yeast strain expressing a single copy of Kir2.1. Enhancement by GRIF-1 of Kir2.1-dependent K+ flow is not a yeast-specific phenomenon because similar enhancement occurs in mammalian cells (HEK293), as indicated by 86Rb+ flux data. In mammalian cells, ectopic expression of full-length or the N-terminal half of GRIF-1 increases surface expression but not total Kir2.1 protein expression. This suggests that GRIF-1 increases K+ currents via increased delivery of Kir2.1 to the plasma membrane. Kir2.1 and GRIF-1 not only functionally interact, they also co-precipitate from mammalian cell lysates, associate in the yeast two-hybrid assay, and co-localize when expressed in mammalian cells, all indicating specific physical association. Furthermore, co-immunoprecipitation from brain indicates that native Kir2.1 and GRIF-1 proteins associate in vivo. The site responsible for association with GRIF-1 is located in the cytoplasmic tail of Kir2.1 between residues 348 and 396. This 49-amino acid-long region contains signals critically important for Kir2.1 trafficking from endoplasmic reticulum to plasma membrane, as well as putative coiled coil, amphipatic helix, and O-GlcNAc modification sites. Based on these findings, we propose that GRIF-1 acts as a binding partner of Kir2.1 that facilitates trafficking of this K+ channel through the secretory pathway.

GRIF-1 belongs to the TRAK family of coiled coil domain-containing proteins implicated in trafficking of vesicles and mitochondria. The family includes the O-GlcNAc transferase-interacting protein OIP (24); Milton, a protein transporting mitochondria to neuronal synapses in Drosophila (25); and TRAK1, a protein required for GABA receptor homeostasis in mice (26). All of these proteins specifically bind the heavy chain of the microtubule-associated motor protein kinesin (23, 25). It has been proposed that GRIF/TRAK proteins provide a link between specific cargo proteins or organelles and kinesin motors (23, 25, 26). A trafficking adaptor role for GRIFs finds further support in the fact that the N-terminal half of GRIF-1 contains a stretch of 300 amino acids that is 47% homologous to the huntingtin-associated protein HAP-1 (27), which plays a role in trafficking of proteins and organelles in neurons by linking them to kinesin (28). Therefore, it is possible that GRIF-1 (TRAK2) links Kir2.1 to kinesin heavy chain, which is important for delivery of K+ channels to the cell surface (29).

The role of GRIF-1 as a trafficking factor for Kir2.1 is also supported by its binding to a segment of Kir2.1 that contains structural features relevant for protein-protein interaction and Kir2.1 trafficking signals (Fig. 5C). First, the 350–380 region of Kir2.1 contains regularly spaced hydrophobic amino acids that may form an amphipatic α-helix, and the 376–389 region is predicted to assume a coiled coil structure (29). A coiled coil structure is also located in the N-terminal half of GRIF-1 and is
essential for interaction with GABA receptor (21). Thus, it is possible that coiled coil domain in Kir2.1 interacts with a similar structure in the N-terminal half of GRIF-1 and so contributes to Kir2.1-GRIF-1 association. Second, threonine-353 in the GRIF-binding region of Kir2.1 is a putative site of O-GlcNac modification (30). The addition of O-GlcNac to serine or threonine residues is a labile modification that plays signaling role and may be an alternative to phosphorylation at the same site (reviewed in Ref. 31). Several proteins known to be modified by O-GlcNac have functions that are relevant to the secretory pathway, including Golgi reassembly stacking protein 2 (GRASP55), cytoskeletal proteins, and microtubule assembly proteins MAP1B and MAP2B (32). This suggests that O-GlcNac plays a role in protein trafficking. In light of the reported association of GRIF-1 with proteins MAP1B and MAP2B (32), this suggests that GRIF-1 may be an alternative to phosphorylation at the same threonine residues.

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