AP-2-dependent Internalization of Potassium Channel Kir2.3 Is Driven by a Novel Di-hydrophobic Signal*

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The localization and density of Kir2.3 channels are influenced by the balance between PDZ protein interaction at the cell surface and routing into the endocytic pathway. Here, we explore mechanisms by which the Kir2.3 channel is directed into the endocytic pathway. We found that Kir2.3 channels are constitutively internalized from the cell surface in a dynamin-dependent manner, indicative of vesicle-mediated endocytosis. The rate of Kir2.3 endocytosis was dramatically attenuated following RNA interference-mediated knockdown of either α adaptin (AP-2 clathrin adaptor) or clathrin heavy chain, revealing that Kir2.3 is internalized by an AP-2 clathrin-dependent mechanism. Structure-rationalized mutagenesis studies of a number of different potential AP-2 interaction motifs indicate that internalization of Kir2.3 is largely dependent on a non-canonical di-isoleucine motif (II413) embedded within the C terminus. Internalization assays using CD4-Kir2.3 chimeras demonstrate that the di-isoleucine signal acts in an autonomous and transplantable manner. Kir2.3 co-immunoprecipitates with α adaptin, and disruption of the di-isoleucine motif decreased internalization of the channel with AP-2. Replacement of the di-isoleucine motif with a canonical di-leucine internalization signal actually blocked Kir2.3 endocytosis. Moreover, in yeast three-hybrid studies, the Kir2.3 di-isoleucine motif does not bind the AP-2 e2C-αr2 hemi-complex in the way that has been recently observed for canonical di-leucine signals. Altogether, the results indicate that Kir2.3 channels are marked for clathrin-dependent internalization from the plasma membrane by a novel AP-2-dependent signal.

Inwardly rectifying potassium (Kir)3 channels play pivotal roles in a wide array of physiological processes, including the control of membrane excitability, heart rate, hormone secretion, and salt balance (1). In recent years, it has become evident that physiological activity of Kir channels is not simply controlled by gating mechanisms (2) but also strongly influenced by surprisingly complex intracellular trafficking processes (3, 4). Individually tailored for specific physiological demands, different members of the Kir family are thought to contain unique repertoires of trafficking signals, directing traffic in the biosynthetic pathway as well as between the endosomal compartments and plasma membrane. In comparison to recent developments about trafficking processes that control sorting and export of channel proteins from the endoplasmic reticulum (3, 5–9) and Golgi (10, 11), the endocytic mechanisms responsible for controlling the density of channels at the plasma membrane still remain poorly understood.

Routing into the endocytic pathway can, in fact, influence the subcellular localization and surface density of Kir channels. Consider, for example, the polarized targeting of the inwardly rectifying potassium channel, Kir2.3, important for regulation of kaliuresis and potassium homeostasis (12, 13). Present evidence indicates that a hierarchy of trafficking processes, coordinated by several distinct signals, controls the number and location of Kir2.3 channels at the basolateral membrane. One signal at the C terminus of Kir2.3 directs the channel to the basolateral membrane in the biosynthetic pathway (14), preventing apical mis-sorting. Another signal, an adjacent PDZ interaction motif (15), offers a different localization mechanism. By facilitating Kir2.3 interaction with Lin-7, a component of an evolutionary conserved PDZ scaffolding complex at the basolateral membrane, the PDZ binding motif effectively retains the channel at the polarized locale (15–17). In fact, mutation of the PDZ motif or expression of TIP-1, a negative regulator of the PDZ complex, disrupts Kir2.3 interaction with the Lin-7 scaffold and causes a dramatic shift in the localization of the channel from the basolateral membrane to an endosomal compartment (15, 17). Thus, the polarized localization and surface density of Kir2.3 is not simply dependent on sorting in the biosynthetic pathway but is also controlled by the balance between PDZ scaffolding protein interaction and internalization from the basolateral membrane.

The cytoplasmic Kir2.3 C terminus contains several sites that resemble clathrin-dependent trafficking signals, raising the possibility that Kir2.3 channels may be endocytosed by a clathrin-mediated process. Typically recognized by canonical tyrosine-based sequences (“YYYYΦ” or “NPXY,” where Φ represents any amino acid and Φ represents bulky hydrophobic amino acids) or di-leucine-containing structures ((D/E)XXX(L/I)), these degenerate trafficking signals serve as binding sites for the different heterotetrameric clathrin-adaptor complexes (AP1–4) (18, 19). Although the AP complexes generally engage clathrin and simultaneously mark interacting proteins as cargo for incorporation into clathrin-coated vesicles, each AP sub-
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Type differently controls the routing and destination of vesicular cargo. Endocytosis from the plasma membrane is mediated by AP-2 (20–22). The other clathrin-adaptor complexes coordinate delivery of proteins from the trans-Golgi to endosomal-lysosomal system (AP-1A) (23), control trafficking between the endosomes and lysosomes (AP-3) (24–26), or possibly mediate delivery of cargo to the basolateral membrane in the biosynthetic pathway (AP-4) (27). The precise mechanisms controlling specificity of cargo interactions with appropriate AP subtypes are still not well understood but are likely to be governed by subtype-specific requirements for distinct phosphatidylinositol lipids at donor membranes (28–31), as well as subtype-specific preferences for particular residues within and neighboring the canonical clathrin-dependent trafficking signals (18, 32–37). In addition, the type of internalization motif (i.e. tyrosine- or di-leucine-based) present on a particular protein can have functional consequences for trafficking (38). Our effort to elucidate the mechanism by which Kir2.3 is controlled by endocytosis expands the repertoire of signals known to interact with AP complexes.

In the present study, we found Kir2.3 channels are targeted for AP-2 clathrin-dependent endocytosis by an unusual di-isoleucine containing motif, sharing resemblance to the canonical di-leucine signals. The discovery of this motif provides reason to suggest that there is a broader spectrum of clathrin-dependent trafficking signals than previously recognized.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Antibodies were purchased from commercial suppliers: rabbit anti-Kir2.3 antibody (Alomone, Jerusalem, Israel), rat anti-HA 3F10 (Roche Applied Science), mouse anti-adaptin α, mouse anti-clathrin heavy chain, and mouse A.v. monoclonal JL-8 for GFP detection (BD Transduction Laboratories, Lexington, KY), horseradish peroxidase-conjugated donkey anti-mouse or anti-rat antibody (Jackson Laboratories, Bar Harbor, ME), horseradish peroxidase-conjugated goat anti-mouse (Amersham Biosciences), and mouse anti-human CD4 Alexa fluor 488 (Caltag-Invitrogen).

**Molecular Biology**—Studies were performed using modified Kir2.3 (14), containing either an external hemagglutinin (HA) or FLAG epitope tag. The constructs used for the *Xenopus* oocyte studies were subcloned into the modified pSD6 vector between the 5′- and 3′-untranslated region of the *Xenopus* β-globin gene to increase expression efficiency (39). A polyadenylate sequence is also present in the 3′-untranslated region of this vector (dA23cD30). CD4-Kir2.3 chimeras were constructed using a PCR-based approach and contained the entire extracellular and transmembrane domains of the human CD4 protein. The Kir2.3 C-terminal end (amino acids 340–445) was fused at amino acid 422 of CD4. The control CD4 construct (CD4 stop) was obtained by introducing a stop codon at the fusion site, resulting in truncation of most of the native cytoplasmic C terminus. All constructs used for mammalian expression were subcloned into pcDNA 3.1+ (Invitrogen). Several constructs for the yeast three-hybrid experiments were a generous gift from the Bonifacino laboratory. These include the pBridge plasmid containing wild-type Nef fused to the Gal4 DNA binding domain with σ2 inserted in the second multiple cloning site and the pGAD17 containing αC fused to the activation domain. For the DNA binding domain-only negative control, the Nef protein was removed with the EcoRI and SalI restriction enzymes and the ends were blunted and ligated back together. Alternatively, the Nef protein was removed and a PCR-amplified terminal portion of Kir2.3 containing amino acids 398–445 was inserted in the pBridge plasmid. The short fragment of Kir2.3 was preferentially used in the yeast three-hybrid assay, because a longer fragment contained an acidic cluster, which resulted in auto-activation. A PCR-based strategy with MfeI Turbo DNA polymerase (QuikChange, Stratagene) was employed for site-directed mutagenesis. Dye termination DNA sequencing was used to verify the sequence of all modified cDNAs (University of Maryland School of Medicine Biopolymer Core).

**cRNA Synthesis**—*In vitro* transcription with SP6 RNA polymerase (mMessage Machine, Ambion Inc.) was used to produce complementary RNA from linearized plasmids containing the desired cDNA. Purification of the cRNA by spin column chromatography (MEGAclear, Ambion Inc.) followed. Yield was determined spectrophotometrically and verified using agarose gel electrophoresis.

**Oocyte Isolation and Injection**—Oocytes from female *Xenopus laevis* (Xenopus Express, Homosassa, FL) were isolated and maintained using the standard procedures as described previously (13). Briefly, frogs were anesthetized using 0.15% ethyl 3-amino-benzoate methanesulfonate salt, and a partial oophorectomy was performed through an abdominal incision. Oocyte aggregates were manually dissected from the ovarian lobes and then incubated in OR-2 medium (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl2, and 5 mM HEPES, pH 7.4) containing collagenase (type 3, Worthington) for 2 h at room temperature to remove the follicular layer. After extensive washing with collagenase-free OR-2, oocytes were stored at 19 °C in OR-3 medium (50% Leibovitz medium, 10 mM HEPES, pH 7.4, containing penicillin/streptomycin). 12–24 h later, healthy looking Dumont stage V–VI oocytes were pneumatically injected with 50 nl of diethyl pyrocarbonate-treated water containing 0.25 ng of channel cRNA with or without 1 ng of dynamin cRNA. Oocytes were then stored in OR-3 medium at 19 °C.

**Electrophysiology**—Whole cell currents in *Xenopus* oocytes were measured using a two-microelectrode voltage clamp as described previously (13, 40). Briefly, oocytes were bathed in a 45 mM K+ solution (45 mM KCl, 45 mM N-methyl-d-glucamine-Cl, 1 mM MgCl2, 1 mM CaCl2, 5 mM HEPES, pH 7.4). Voltage-sensing and current-injecting microelectrodes had resistances of 0.5–1.5 MΩ when backfilled with 3 M KCl. Once a stable membrane potential was attained, oocytes were clamped to a holding potential of −20 mV, and currents were recorded during 500-ms voltage steps, ranging from −100 mV to +100 mV in 20-mV increments. Data were collected using an ITC16 analo-to-digital, digital-to-analogue converter (Instrutech Corp.), filtered at 1 kHz, and digitized on-line at 2 kHz using Pulse software (HEKA Electronik) for later analysis. Data shown are based on the average maximum current, measured at −100 mV.

**Surface Expression**—Plasmalemma expression of the external HA-tagged Kir2.3 channel was measured in single oocytes.
following procedures outlined by Zerangue and colleagues (8) with slight modifications as before (41). In these studies, oocytes were fixed with 4% formaldehyde in OR-2 for 15 min at 4 °C and washed four times in OR-2. To block spurious antibody binding, oocytes were then incubated for 1 h at 4 °C in OR-2 containing 1% m bovine serum albumin (BSA). Exposed HA epitopes on the surface of intact oocytes were labeled with a rat monoclonal anti-HA antibody (0.5 µg/ml, Roche 3F10, 1% BSA, 4 °C, overnight), and then oocytes were washed with OR-2 containing 1% BSA, and incubated with horseradish peroxidase-coupled goat anti-rat (1 µg/ml, Jackson Laboratories, 1% BSA, 1.5 h). Cells were washed for 1 h with OR-2 containing 1% BSA and again for 10 min in OR-2 medium without BSA. Individual oocytes were placed in 50 µl of Enhanced Chemiluminescence Substrate (Pierce) and incubated for 7 min at room temperature. Luminescence from single oocytes was measured for 10 s in a Sirius luminometer and reported as relative light units (RLUs) per second.

Oocyte Membrane Protein Purification—Oocytes were processed following the protocol described by Kamsteeg and Deen (42) to isolate total membrane proteins. In brief, oocytes were washed twice in homogenization buffer (80 mM sucrose, 5 mM MgCl2, 5 mM NaH2PO4, 1 mM EDTA, 20 mM Tris, pH 7.4) containing a protease inhibitor mixture (5 µg/ml leupeptin, 1 µg/ml phenylmethylsulfonyl fluoride, and 5 µg/ml pepstatin A) and then broken by trituration with a 25-gauge syringe. To pellet yolk proteins and nuclei, homogenates were spun twice at low speed (100 × g) at 4 °C for 10 min. Supernatants were then spun at high speed (16,100 × g) for 20 min at 4 °C to collect the total membrane fraction. Pellets were washed once in the homogenization buffer and spun at top speed again for 15 min and then placed in solubilization buffer (4% sodium deoxycholate, 20 mM Tris, pH 8.0, 5 mM EDTA, 10% glycerol, containing the protease inhibitors) and rocked at room temperature for 15 min. Particulate material was pelleted (16,100 × g for 20 min at 4 °C), and the solubilized proteins in the supernatant were resolved by SDS-PAGE electrophoresis and transferred to nitrocellulose membranes.

Oocyte Surface Biotinylation Assay—The extracellular FLAG (DYKDDDDK)-tagged Kir2.3 channel was utilized for all biotinylation experiments. The lysine residues in the external epitope tag provide free amino-group substrates for biotinylation. Oocytes were washed with OR-2 and then incubated with 1.5 mg/ml sulfo-NHS-SS-biotin (Pierce) in OR-2 for 40 min. Free biotin reagent was quenched with OR-2 containing 50 mM Tris for 10 min. Oocytes were then washed three times with OR-2, and the membranes were purified as described. Protein concentrations were determined, and 100 µg of sample was added to 50 µl of prewashed NeutrAvidin-agarose beads (Pierce), and the volume was brought up to 325 µl using homogenization buffer. Samples were then rotated overnight at 4 °C. Beads were centrifuged at high speed (16,100 × g) for 5 min and washed three times with phosphate-buffered saline. Biotinylated protein was eluted from the beads at room temperature with 3-fold SDS sample buffer containing 50 mM dithiothreitol (1-h incubation). A Bio-Rad dot blot apparatus facilitated precise sample migration into nitrocellulose membrane using gravity.

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Cell Culture, Transfection, and Stable Cell Line Generation—Human embryonic kidney 293 (HEK 293) cells (ATCC) or COS-7 (ATCC) cells were cultured in a humidified atmosphere at 37 °C in 5% CO2 and then grown in DMEM supplemented with 10% fetal bovine serum, penicillin/streptomycin, and glutamine. For stable cell lines, HEK 293 cells were plated at ~75% confluence in 6-well dishes. Cells were transfected with 6 µg of recombinant pcDNA3.1+ plasmid using Lipofectamine 2000 (Invitrogen). Transfection medium was replaced with growth medium 5 h after transfection. Cells were detached 24 h after transfection with citric saline and plated at a low density in 100-mm Petri dishes. Selection medium containing 600 µg/ml Geneticin (Invitrogen) was added 48 h post transfection. Control HEK cell clones were produced by transfecting cells with the pcDNA3.1+ plasmid alone. COS-7 cells were transfected with FuGENE (Roche Applied Science).

Cell Lysis—HEK 293 cells were washed once in Ringer’s solution, harvested in HEENG buffer (20 mM HEPES, pH 7.6, 25 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol), and resuspended in HEENG containing 1% Triton and protease inhibitors. Cell lysates were resuspended by pipetting, incubated on ice for 45 min, and centrifuged at max speed to pellet insoluble material. Protein concentrations were assessed by Bradford Assay Reagent (Bio-Rad).

Biotinylation Internalization Assay—Cells were washed with ice-cold Ringer’s solution (5 mM HEPES, 144 mM NaCl, 5 mM KCl, 1.2 mM Na2HPO4, 5.5 mM glucose, 1 mM MgCl2, 1 mM CaCl2, pH 7.4), and the surface proteins were biotinylated using EZ-link sulfo-NHS-SS-biotin (0.3 mg/ml, Pierce) in Ringer’s solution at 5 °C for 45 min. Remaining biotin was quenched with 50 mM Tris, pH 7.5, in Ringer’s solution for 20 min at 5 °C. Cells were then placed at room temperature for the indicated amount of time (0–15 min) to allow internalization. The membrane-impermeant reducing agent MesNa (100 mM MesNa, 100 mM NaCl, 1 mM EDTA, 0.2% BSA, 50 mM Tris, pH 8.8) was added three times for 20 min at 4 °C to cleave biotin linked to the cell surface. Cells were then washed four times with Ringer’s solution and lysed. 15 µg of total protein was added to NeutrAvidin beads (Pierce) in a solution containing phosphate-buffered saline and 0.1% SDS and rotated overnight at 5 °C. Beads were washed four times with phosphate-buffered saline plus 0.1% SDS, and biotinylated protein was eluted from the beads with 3× SDS sample buffer (1 h at room temperature). A Bio-Rad dot blot apparatus facilitated precise sample migration into nitrocellulose membrane using gravity.

Internalization Rate Calculations—The amount of MesNa-resistant channel was quantified by densitometry for each time point and normalized to the total surface pool of channel. Internalization rates were then calculated using best fit linear regression for each experiment, and statistics were performed by comparing the rates of individual experiments. In preliminary studies, endocytic rates calculated with values measured every 3 min over a 15-min time course yielded identical results as those measured from three time points (0, 5, and 15 min). Therefore, the 0-, 5-, and 15-min time points were examined for the majority of the experiments to reduce the required number of samples.
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**Biotinylation Recycling Assay**—Cell surface was biotinylated and quenched as above, then cells were placed at room temperature for 30 min to allow surface-biotinylated channels to internalize. Cells were subjected to MesNa treatment (4 °C) as above to strip biotin from proteins remaining at the cell surface, resulting in a biotin-labeled internalized pool of channel. The cells were returned to room temperature again for variable amounts of time to permit recycling to the cell surface in either Ringer’s medium (no MesNa control groups to correct for degradation) or MesNa solution (to measure recycling). The MesNa-treated samples were placed in additional MesNa solution at 4 °C (two times, 20 min each) to ensure complete removal of biotin from channel that returned to the cell surface. The cells were then washed again four times with Ringer’s solution and lysed.

**Immunoprecipitation and Immunoblotting**—Cell lysates were precleared with Protein-G Plus Protein-A-agarose beads (Calbiochem) for 2 h at room temperature. The supernatants were then rotated overnight with the appropriate antibodies and new Protein-G Plus Protein-A beads. Beads were washed four times with phosphate-buffered saline and then eluted for 30 min at room temperature with SDS sample buffer. Eluates were separated by SDS-PAGE and transferred to nitrocellulose (Amer sham Biosciences). Nitrocellulose membranes were blocked in Tris-buffered saline with Tween 20 (0.1%) (TBS-T) containing 5% nonfat dry milk for 1 h at room temperature. Membranes were then incubated in 5% nonfat dry milk containing primary antibody at 4 °C overnight, washed in TBS-T for 10 min, incubated in 5% nonfat dry milk containing a horseradish peroxidase-conjugated secondary antibody, and then washed extensively for 20 min in TBS-T. Bound antibodies were then revealed using enhanced chemiluminescence reagent (Pierce) and fluorography (HyBlot CL, Denville). Increased levels of Kir2.3 II413AA protein expression relative to wild-type channel were consistently observed, possibly as a result of slower internalization and degradation. Consequently, we transfected less Kir2.3 II413AA DNA to achieve approximately equal expression of wild-type and mutant channels relative to total Kir2.3 II413AA DNA to achieve approximately equal expression of wild-type and mutant channels relative to total cellular protein. Densitometric measurements were made using Image (National Institutes of Health).

**siRNA Knockdown**—The AP-2 complex and clathrin heavy chain were targeted with sequences previously published (43). The α subunit of the AP-2 complex target sequence was NNGAGCAUGUCACGCUGGCCA, and the clathrin heavy chain target sequence was NNUCCAAUUCGAAGAC-CAAUU. siRNAs were synthesized as option A4 (2’-deprotected, duplexed, and desalted) by Dharmaco, Inc. Scrambled siRNA was used for controls (AllStars Negative Control siRNA, Qiagen). Cells were transfected with 50 nm final concentration of siRNA using TransIT-TKO transfection reagent (Mirus) and analyzed 72 h after transfection.

**Chimera Internalization Assay**—48 h following transfection with DNA encoding the CD4-Kir2.3 chimeras, COS-7 (ATCC) cells were washed with low glucose DMEM and labeled with Alexa Fluor 488-conjugated anti-CD4 antibody for 1 h at 4 °C. Excess antibody was removed by washing with DMEM, and cells were either incubated at 37 °C for 15 min to permit internalization or kept at 4 °C. Cells were then returned to 4 °C to prevent further trafficking. Antibody remaining at the cell surface was stripped with an acidic wash buffer (DMEM supplemented with 100 mm glycine, pH 3.0). Cells were then washed with DMEM, fixed with 4% paraformaldehyde, mounted on slides with Vectashield glycerol mounting medium (Vector Laboratories, Burlingame, CA), and sealed with nail polish.

**Imaging Methods and Quantification**—Cells were visualized using the Zeiss 410 confocal laser-scanning microscope (Carl Zeiss) under a 63× oil immersion lens with a numerical aperture of 1.40. Images of cells from individual experiments were acquired at the same contrast and brightness. The amount of endocytosed channel was estimated by quantifying the internalized Alexa 488-CD4 signal using Velocity version 3.0 (Improvision Inc., Lexington, MA). The reported values for the 15-min time point represent the number of pixels of internalized antibody multiplied by intensity and normalized to the cell surface area.

**Yeast Three-hybrid Assays**—The Saccharomyces cerevisiae strain AH109 (Clontech) was maintained on YPDA medium (YPD medium (Clontech) + 0.003% adenine hemisulfate). Transformation was performed with a lithium acetate-based protocol using the Yeastmaker Yeast Transformation System 2 (Clontech), and transformants were selected on dropout agar plates lacking tryptophan, methionine, and leucine. For colony growth assays, individual colonies were grown while shaking at 30 °C in 10 ml of this dropout media until they reached an optical density of 1.0. To test for interaction, aliquots of culture were then spotted on dropout plates with and without histidine and grown at 30 °C for 48 h. For quantification, 3 μl of the culture was added to 10 ml of dropout media lacking histidine, and the optical density (A600) was measured over a 72-h time course.

**Statistics**—Data are presented as means ± S.E. Statistical analysis was performed using GraphPad Prism version 4. Statistical significance was determined by t test when comparing two groups and by one-way randomized analysis of variance followed by Bonferroni’s post hoc test when comparing multiple groups or Dunnett’s post hoc test when test groups were compared with the control. *p* < 0.05 was considered significant.

**RESULTS**

**Kir2.3 Channels Are Constitutively Internalized from the Cell Surface**—Previous studies strongly suggest the density of Kir2.3 channels at the cell surface is significantly influenced by endocytic retrieval (14–17). To directly test whether this is, in fact, the case, the abundance of extracellular hemagglutinin (HA) epitope-tagged Kir2.3 channels at the plasmalemma was quantified in Xenopus oocytes by cell surface HA-antibody binding and luminometry before and after the trafficking of membrane proteins from the endoplasmic reticulum to the Golgi apparatus was blocked with brefeldin A (BFA). As depicted in Fig. 1A, the luminescence response is linear and related to the functional current density over a wide range of expression levels, permitting an accurate appraisal of relative Kir2.3 density at the cell surface.

In the absence of delivery of new channel proteins to the cells surface, luminescence measurements provide an estimate of the lifetime of Kir2.3 on the plasma membrane. In these studies, cells were permitted to achieve steady-state expression of the
Kir2.3 channels are constitutively internalized from the cell surface. A, extracellular HA-tagged Kir2.3 displays a linear relationship between the relative surface density of channel measured in arbitrary units of RLUs using the chemiluminescence assay and potassium current (I) as recorded by two-electrode voltage clamp (mean ± S.E., n = 3–5 oocytes/dose for electrophysiology, n = 9–10 oocytes/dose for luminescence). B, relative amounts of surface HA-tagged Kir2.3 in Xenopus oocytes were quantified by HA-antibody binding and luminometry over time in the absence and presence of BFA, a fungal metabolite that blocks forward trafficking of newly synthesized channels without effects on clathrin-mediated endocytosis. Surface expression is reported in RLUs as normalized to time zero (RLUs) (mean ± S.E., n = 26–30 oocytes/time point, three frogs). The decay of cell surface Kir2.3 in BFA is described by a two-endocytic compartment model such that the internalization rate (1.17% min⁻¹) is slightly faster than the recycling rate (0.92% min⁻¹), and the recycling rate is 18 times faster than entry into the non-recycling compartment. C, representative Kir2.3 biotinylation study in HEK 293 cells stably expressing extracellular HA-tagged Kir2.3. In these studies, surface channels were labeled with sulfo-NHS-SS-biotin in the cold and then incubated at room temperature for 0–15 min to permit trafficking. Biotin remaining at the cell surface was cleaved with MesNa, and internalized (biotinylated) proteins were recovered with NeutrAvidin-conjugated beads and detected along with the total surface pool in dot-immunoblots (IB) with anti-Kir2.3 antibodies. No proteins are detected in vector only transfected cells (first lane). Capture on NeutrAvidin beads requires prior biotinylation (second lane). D, quantification of channel internalized relative to the surface pool at each time point by densitometry (mean ± S.E., n = 3).

channel at the cell surface, and then were incubated for variable times in BFA (10 μM, 22 °C) or vehicle. The density of immunodetectable Kir2.3 at the plasmalemma decayed with a biphasic time course after exposure to BFA (Fig. 1B), first rapidly decreasing by ~55% in the first 3 h and then progressively declining by an additional 14% over the next 21 h. The response is indicative of endocytic internalization, similar to clathrin-dependent endocytosis of other channels heterologously expressed in Xenopus oocytes (44, 45). The biphasic time course and the presence of a significant residual surface pool of Kir2.3 in BFA is consistent with the idea that internalized channels preferentially recycle back to the cell surface rather than enter a compartment that is completely inaccessible to the plasmalemma.

To measure internalization directly, channels residing on the cell surface were first labeled with the impermeant, cleavable biotin analog, sulfo-NHS-SS-biotin, in the cold and then incubated at room temperature for variable times to initiate endocytosis. Cells were returned to 4 °C, and biotin was stripped from the proteins that remained at the cell surface with an impermeable reducing agent, MesNa. Internalized channel proteins remain biotinylated after MesNa cleavage and, therefore, can be recovered on NeutrAvidin beads and detected together with the total surface pool (not treated with MesNa) in immuno-dot blots probed with anti-Kir2.3 antibodies. As shown in a representative experiment (Fig. 1, C and D), increasing amounts of biotinylated channel became resistant to MesNa cleavage over the time course. The measured internalization rate of Kir2.3 (1.5%/min) is consistent with the estimates of internalization in oocytes (1.17%/min) and constitutive endocytic rates of other membrane proteins (46).

Kir2.3 Endocytosis Is Dynamin-dependent—In principle, Kir2.3 may be internalized from the cell surface by phagocytosis, pinocytosis, or vesicle-mediated endocytosis (i.e. clathrin-mediated, caveolae-mediated, or clathrin- and caveolae-independent endocytosis) (47). To test for the involvement of vesicle-mediated endocytosis, we evaluated the effect of dominant-negative dynamin on Kir2.3 internalization. Dynamin, a
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large GTPase, is required for clathrin-mediated endocytosis, caveolae-mediated endocytosis, and possibly some types of clathrin- and caveolae-independent endocytosis (47), where it coordinates endocytic vesicle fission from the plasma membrane. The ability of a mutant form of dynamin, K44A, to block clathrin-mediated endocytosis has been demonstrated extensively (48–51); substitution of alanine for the lysine 44 residue in the catalytic domain of dynamin disrupts GTPase activity preferentially (48–51); substitution of alanine for the lysine 44 residue in the catalytic domain of dynamin disrupts GTPase activity preferentially (48–51); substitution of alanine for the lysine 44 residue in the catalytic domain of dynamin disrupts GTPase activity preferentially (48–51); substitution of alanine for the lysine 44 residue in the catalytic domain of dynamin disrupts GTPase activity.

Consistent with the prediction that a small fraction of the channel is engaged in endocytosis at any given time, a relatively small proportion of Kir2.3 immunoprecipitates with AP-2. Therefore, the presence of Kir2.3 channels was evaluated in the absence and presence of BFA (3 h) by two independent methods in Xenopus oocytes co-injected with external FLAG epitope-tagged Kir2.3 and GFP-tagged dynamin cRNAs (wild-type (dyn, wt) or dominant-negative (dyn, K44A)). A, exogenous EGFP-dynamin detected in the different oocytes treatment groups by immunoblotting (IB) with an anti-GFP antibody; B, functional expression of the channel was assessed by two-microelectrode voltage clamp before and after BFA treatment. Shown are the mean ± S.E. of relative macroscopic current (I/Imax) in the absence and presence of BFA (12 oocytes/group, 2 frogs) for oocytes injected with Kir2.3 alone or co-injected with wild-type or dominant negative dynamin. C, representative surface biotinylation study of Kir2.3 in oocytes injected with Kir2.3 alone (endogenous dynamin), or co-injected with dynamin (wt or dominant negative) in the absence and presence of BFA. Cell surface channels were labeled with NHS-SS-biotin, recovered on NeutrAvidin-agarose beads, and then detected in dot blots with an anti-Kir2.3 antibody. Dot blot analysis was performed to facilitate accurate quantification of channel proteins. Shown are Kir2.3 at the plasma membrane (PM), captured on NeutrAvidin beads, relative to the total cellular input of Kir2.3. No proteins are detected in un.injectected oocytes (first lane) and unbiotinylated channels are not captured on NeutrAvidin beads (second lane). D, quantification of the surface biotinylation dot blots by densitometry. The bars represent the mean ± S.E. density of four independent surface biotinylation experiments.

RNase Studies Reveal That Kir2.3 Endocytosis Is AP-2 and Clathrin-dependent—To examine the functional significance of the interaction, we tested whether AP-2 and clathrin are actually required for Kir2.3 endocytosis by measuring the rates of channel internalization in Kir2.3 + HEK-293 cells following RNAi-mediated knockdown of either α adaptin (AP-2) or clathrin heavy chain protein. As shown in Fig. 4A, the expression of the target proteins was specifically and significantly attenuated of Kir2.3 potassium current following BFA treatment (3 h) in oocytes co-injected with wild-type dynamin, no change in Kir2.3 channel activity was observed in oocytes co-injected with the dominant negative dynamin, K44A (Fig. 2B). The response was verified by cell surface biotinylation. In these studies, external FLAG epitope-tagged Kir2.3 channels were labeled at the plasma membrane with a cell-impermeant form of biotin (sulfo-NHS-SS-biotin) before and after BFA treatment. Biotinylated proteins were then recovered on NeutrAvidin-agarose beads and detected by dot blot using an anti-Kir2.3 antibody. As shown in a representative experiment (Fig. 2C) and summarized in Fig. 2D, the significant attenuation of cell surface-biotinylated channel following BFA treatment was completely inhibited by dominant negative dynamin K44A. Taken together, these data demonstrate that a dynamin-dependent process governs Kir2.3 channel internalization, indicative of vesicular endocytosis.

Kir2.3 Interacts with the AP-2 Clathrin Adaptor—The presence of canonical clathrin-adaptor (AP-2) interaction sites in Kir2.3 raises the possibility that Kir2.3 might be internalized via a clathrin-dependent mechanism. As a first step to explore this idea, we examined whether the channel associates with AP-2 by co-immunoprecipitation in HEK-293 cells, stably transfected with wild-type Kir2.3 or vector alone. Recovered immunoprecipitates on anti-AP-2 α subunit antibody-coated beads were resolved and immunoblotted with either anti-Kir2.3 or anti-clathrin antibodies. As shown in Fig. 3, both the Kir2.3 channel and clathrin co-purified with the AP-2 complex (Fig. 3, lane 1). Co-immunoprecipitation of Kir2.3 was only observed in cells transfected with channel, and reactions without the AP-2 α subunit antibody failed to immunoprecipitate AP-2 α, clathrin, or Kir2.3, verifying specificity. Consistent with the prediction that a small fraction of the channel is engaged in endocytosis at any given time, a relatively small proportion of Kir2.3 immunoprecipitates with AP-2.
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**FIGURE 3.** Kir2.3 co-immunoprecipitates with AP-2 and clathrin. HEK293 cells were transiently transfected with Kir2.3 or vector alone (pcDNA3.1+), and solubilized lysates were immunoprecipitated (IP) with anti-α adaptin antibody and immunoblotted (IB) with anti-Kir2.3, anti-α adaptin, and anti-clathrin heavy chain antibodies. Kir2.3 is specifically immunoprecipitated by the anti-α adaptin antibody.

directly attenuated by RNA interference. In contrast to the absence of response to scrambled siRNA probes, the α adaptin RNAi reduced α adaptin by ∼67.8% (S.E. 4.3%) without effects on clathrin. Similarly, the clathrin siRNA probe reduced clathrin by ∼49.4% (S.E. 17%) without effects on α adaptin. As quantified from the cell surface biotinylation experiments (Fig. 4B), knockdown of either α adaptin or clathrin heavy chain was paralleled by a dramatic reduction in Kir2.3 endocytosis. Less MesNa-resistant, biotin-labeled channel was detected at each time point in cells depleted of α adaptin or the clathrin heavy chain by RNAi (Fig. 4B), reflecting a dramatic and statistically significant attenuation of the internalization rate (Fig. 4C). Thus, Kir2.3 is internalized from the plasma membrane by an AP-2 and clathrin-dependent process.

**Kir2.3 Endocytosis Is Mediated by a Novel Signal**—To determine whether the endocytic process is signal-dependent, potential AP-2 binding sites in Kir2.3 were identified and systematically studied. Analysis of the primary structure of the Kir2.3 cytoplasmic domains revealed three canonical AP-2 interaction sites, identified by YXXΦ or di-leucine motifs. Four non-canonical di-hydrophobic signals, conforming to the model sequence (D/E/R)XXXΦΦ, were also identified. As mapped on the recently solved crystal structure of the closely related channel, Kir2.1 (54), four of these appear to be accessible to AP-2 binding (RAQTLL, YLPL, YSPE, and EEAGII). To examine their roles, the penultimate tyrosine or di-hydrophobic pairs were individually mutated to alanine, and mutant channel function and endocytosis were evaluated in Xenopus oocytes. As measured by two-microelectrode voltage clamp and cell surface antibody binding, the LL185AA and the Y332A mutants exhibited biophysical properties that were indistinguishable from control (data not shown). Although the mutant channels were detected at the cell surface, the absence of channel activity in the surface pool in BFA, presumably reflecting a subtle impairment of trafficking in the post-endocytic pathway. By contrast, the II413AA mutation dramatically and specifically reduced channel internalization (Fig. 5C).

To more carefully characterize the role of the novel di-isoleucine motif in trafficking, the time course of endocytosis and recycling were measured directly by surface biotinylation in HEK293 cells stably expressing Kir2.3 II413AA or the wild-type channel. Initial rates of internalization were measured as described above (Fig. 1C) by quantifying the amount of surface-biotinylated channel protein that becomes resistant to MesNa cleavage over a relatively rapid chase period. As shown in a representative experiment (Fig. 6A), the accumulation of internalized Kir2.3 II413AA channel (surface-biotin labeled, MesNa-resistant channel) was attenuated at each time point relative to the wild-type channel, reflecting a significant reduction in the initial rate of internalization (Fig. 6B).

Direct measurements of recycling in HEK-293 cells were also performed. In these studies, surface-biotinylated channels were incubated at 22 °C for 30 min, allowing accumulation of biotinylated Kir2.3 in the endocytic compartment. After biotin was stripped from the surface with MesNa at 4 °C, cells were again warmed to stimulate recycling. Channels that recycle to the plasmalemma regain MesNa sensitivity, and the recycling rate can be inferred from the decay of biotin labeling. Indeed, a decline in the biotin-labeled Kir2.3 channel (WT or II413AA) was observed in the presence but not absence of MesNa (Fig. 7A). As derived from these data, the endocytic recycling rates of the wild-type Kir2.3 and II413AA channels were not statistically different (Fig. 7, B and C). Taken together, the results indicate that the di-isoleucine motif determines the rate of Kir2.3 endocytosis without directly affecting trafficking in the recycling pathway.

**The Di-isoleucine Signal Is Transplantable**—To ascertain whether the di-isoleucine motif was acting as a *bona fide*
signal, a chimeric CD4-Kir2.3 protein was generated containing the extracellular and transmembrane domains of CD4 and a C-terminal fragment of Kir2.3 that includes the di-isoleucine motif and lacks any canonical tyrosine- or di-leucine-based motifs. Unfortunately, the extensively biotinylated CD4 extracellular domain prevented efficient biotin removal. Therefore, internalization was studied in a dynamic antibody feeding assay (55). In these studies, chimeric proteins on the cell surface were labeled with an Alexa Fluor 488-conjugated CD4 antibody at 4 °C. Cells were then transferred to 37 °C to permit trafficking. Antibody remaining at the cell surface was stripped with an acidic wash buffer after cells were returned to 4 °C. The 15-min time point was chosen to allow the maximal amount of chimera endocytosis, while restricting measurements to the linear phase of internalization. As shown in Fig. 8 (A and B), coupling the Kir2.3 C terminus to the extracellular and transmembrane domains of CD4 caused a dramatic (9-fold) increase in internalization relative to CD4 alone. Mutation of the di-isoleucine motif to alanine suppressed internalization to levels of CD4 alone. Significantly, a similar response was observed when di-isoleucine was mutated to the canonical di-leucine. Thus, the di-isoleucine signal is transplantable, autonomous, and different than di-leucine internalization motifs.

The Di-isoleucine Motif Is a Novel AP-2 Interaction Site—To verify that the unique di-isoleucine motif affects endocytosis through an interaction with AP-2, co-immunoprecipitation analysis was employed. In these studies, HEK293 cells were transiently transfected with either Kir2.3 wild-type or II413AA mutant channels. Immunoprecipitates were recovered after incubation of the cell lysates with anti-AP-2 α adaptin antibody-coated beads. As described above (Fig. 3), Kir2.3 wild-type channel was specifically recovered with the AP-2 complex.
The II413AA mutation reduced AP-2 interaction (Fig. 9, A and B), providing a mechanism for di-isoleucine-mediated internalization of Kir2.3.

Di-leucine motifs were recently shown to interact with AP-2 via direct binding to the αC-σ2 hemicomplex (56, 57). Although the di-isoleucine motif on Kir2.3 shares an interesting degree of similarity with these di-leucine motifs, the chimera internalization experiment suggests that leucine residues cannot substitute for the isoleucine residues in this signal and raises the possibility that Kir2.3 interacts with AP-2 by a different mechanism. To probe for an interaction between the di-isoleucine motif and the αC-σ2 hemicomplex, we employed a refinement of the yeast interaction assay pioneered by Bonifacino and colleagues (56) to study how clathrin adaptors recognize di-leucine-based sorting signals. In this assay, often called yeast three-hybrid, the traditional DNA-binding domain vector is replaced with a vector, such as pBridge, that contains two multiple cloning sites for expression of a DNA-binding domain fusion and another protein under the control of a separate promoter. For our studies, the following constructs were co-expressed in the yeast strain, AH109: 1) a GAL4 DNA binding domain fused to a C-terminal Kir2.3 fragment in one multiple cloning site of the pBridge vector; 2) the σ2 AP subunit in the other multiple cloning site of pBridge; 3) the transcriptional activation domain (GAL4AD) fused to the αC AP subunit in pGADT7. The human immunodeficiency negative factor protein (Nef) was used as a positive control (56, 57) with the DNA binding domain alone (DB only) serving as a negative control. Survival in the absence of histidine was used to

![Figure 6. Internalization of Kir2.3 is dependent on the C-terminal di-isoleucine motif (II413). Rates of endocytosis were measured directly by surface biotinylation in HEK293 cells stably expressing Kir2.3 II413AA or the wild-type channel. A, representative biotin internalization experiment. In these studies, surface channels were labeled with sulfo-NHS-SS-biotin in the cold and then incubated at room temperature for 0–15 min to permit trafficking. Biotin remaining at the cell surface was cleared with NeutrAvidin-conjugated beads and detected along with the total surface pool in dot-immunoblots (IB) with anti-Kir2.3 antibodies. No proteins were detected in vector-only transfected cells (first lane), and capture on NeutrAvidin beads requires prior biotinylation (second lane). B, summary of the rates of internalization (mean ± S.E., n = 4). Rates were determined using best fit by linear regression to analyze densitometric data for separate experiments (R² > 0.99).

![Figure 7. The II413AA mutation does not affect recycling. Recycling rates were measured in HEK293 cells using a biotinylation assay (see “Experimental Procedures”). Briefly, cells were labeled with sulfo-NHS-SS-biotin and allowed to internalize channel for 30 min. After channels remaining at the surface were stripped of biotin with MesNa at 4 °C, cells were warmed to stimulate trafficking and the fate of the internalized, biotin-labeled channels was monitored in the presence and absence of extracellular MesNa. Biotinylated protein was recovered with NeutrAvidin-conjugated beads, and detected in immuno-dot blots with anti-Kir2.3. A, representative dot blot of the biotinylated (internalized) channels in the absence and presence of MesNa over the chase period. B, densitometric quantification of biotinylated channel (Kir2.3 wild-type and II413AA) over the entire chase period. Each point represents the difference between the mean optical densities of MesNa-treated and untreated samples point (mean ± S.E., n = 4). Lines are best fit by linear regression. C, recycling rates were calculated by subtracting the rate of disappearance of Kir2.3 channel in the absence of MesNa from the rate of disappearance of Kir2.3 channel in the presence of MesNa (mean ± S.E., n = 4).]
report interaction between the Kir2.3 di-isoleucine signal and the AP-2 hemichemplex, αC-σ2. As shown in Fig. 10 (A and B), Kir2.3 fusion proteins did not support growth in media lacking histidine. Interestingly, mutation of di-isoleucine to the canonical di-leucine did not create an αC-σ2 interaction site, as evidenced by failure of the Kir2.3 II413LL mutant to induce growth on histidine-deficient media (Fig. 10, A and B). To rule out conformational or steric effects that may prevent binding of the Kir2.3 fragment to the hemichemplex, we mutated the penultimate leucine residues in Nef to isoleucine as well as changed the entire Nef motif (ENTSLL) to match the Kir2.3 sequence (EEAGII). These substitutions completely disrupted binding between Nef and the AP-2 hemichemplex (Fig. 10, C and D), providing additional evidence that the αC-σ2 hemichemplex does not recognize the di-isoleucine motif.

**DISCUSSION**

Endocytosis influences the surface density and polarized expression of Kir2.3 channels (15, 17). In the present study, we elucidated the mechanism, revealing a new aspect of clathrin-dependent trafficking signals. Our observations that endocytosis of Kir2.3 can be abrogated by dominant-negative dynamin (K44A) and siRNA-mediated knockdown of α adaptin or clathrin heavy chain indicate that Kir2.3 is internalized from the cell surface through a clathrin-dependent process, requiring the AP-2 clathrin adaptor. Characterization of likely clathrin-adaptor interaction sequences in Kir2.3 revealed that a di-hydrophobic motif (II413), embedded with the extreme cytoplasmic C terminus, specifically controls the rate of endocytosis as well as interaction of the channel with AP-2. Chimeras, including the Kir2.3 di-isoleucine motif, displayed increased internalization relative to the CD4 reporter construct alone, highlighting the autonomous and transplantable nature of this signal. Thus, Kir2.3 channels are marked for clathrin-dependent internalization from the plasma membrane by a novel AP-2 interaction signal.

Identification of the di-isoleucine motif expands the repertoire of signals known to be recognized by the AP-2 complex. The motif shares an interesting degree of similarity with one type of di-leucine-based signal. Differing from “DXXLL” signals that are recognized by the Golgi-localized, γ-ear-containing, ADP-ribosylation factor-binding proteins and have a strict requirement for an aspartate residue at the conventionally defined −3 position relative to the first leucine residue (18), the motif in Kir2.3 (EEAGII413) is preceded by a glutamate residue at the −4 position, reminiscent of endocytic signals “(D/E)XXX(L/I)” that interact with classic AP-type clathrin adaptors. Consistent with this concept, we found disruption of the di-isoleucine motif with alanine substitution dually inhibits internalization of the Kir2.3 channel and decreases binding of the channel to the AP-2 complex. However, and to our great surprise, substitution of leucine residues in place of isoleucine also disrupted internalization. The di-isoleucine signal failed to bind to the
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AP-2 αC-α2 hemicomplex, validating that this signal was not simply a variant of di-leucine-based signals, but completely different. The portion of the AP-2 heterotetramer responsible for binding the di-isoleucine signal remains to be discovered, and the potential exists for an indirect association between the channel and the AP complex. Although the most solid evidence to date suggests that the di-leucine signals bind to the αC-α2 hemicomplex (56, 57), di-leucine-based signals have also been reported to interact with the μ (58–61) and β (62–64) subunits. Because di-leucine is unable substitute for the di-isoleucine signal and αC-AD, it would appear that recognition of both of these signals is highly contextually dependent. An intriguing possibility is that related signals have developed to interact with separate sites to control specificity of interaction with a subset of AP complexes.

Endocytic signals in the inward rectifying potassium channels appear to be individually tailored for specific physiological demands, allowing differential targeting in the endocytic pathway. In the renal collecting duct, for instance, the basolateral channel, Kir2.3, is thought to be controlled independently of the apical potassium secretory channel, Kir1.1 (ROMK). Distinct from the endocytic motif in Kir2.3, an “NPXY-type” signal mediates interaction of the ROMK channel with the clathrin-dependent endocytic pathway (65) to facilitate alteration of channel abundance in accordance with dietary potassium changes (66, 67). Even within the highly homologous Kir2.X subfamily of strong inward rectifiers, channel subtypes are equipped with different endocytic signals, providing a new and unappreciated mechanism for differential regulation. The di-isoleucine motif in Kir2.3, for example, is not present in the other Kir2.X members. Instead, a “YXXΦ” motif, homologous to the one at Kir2.3 position 233, has been reported to arbitrate endocytosis in the Kir2.1 channel (68) as well as regulate forward trafficking of Kir2.1 and Kir2.4 channels (10). This particular YXXΦ motif is conserved across the Kir2.X family, and interpretation of the trafficking motif, therefore, is presumably contextually driven by the neighboring amino acid residues. The contribution of these disparate endocytic motifs to physiological regulation of the Kir channels remains to be determined.

In summary, we have provided evidence that a novel di-isoleucine-containing motif mediates AP-2, clathrin-dependent internalization of the Kir2.3 channel. Kir2.X channels contain distinct internalization signals, facilitating differential regulation at the point of endocytic retrieval.

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