Identification and Characterization of a New Class of Trafficking Motifs for Controlling Clathrin-independent Internalization and Recycling*

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Plasma membrane proteins such as receptors and ion channels allow a cell to communicate with its environment and regulate many intracellular activities. Thus, the proper control of the surface number of these proteins is essential for maintaining the structural and functional homeostasis of a cell. Internalization and recycling plays a key role in determining the surface density of receptors and channels. Whereas the clathrin-mediated internalization and its associated recycling have been the focus of research in this field, recent studies have revealed that an increasing number of receptors and channels enter a cell via clathrin-independent pathways. However, little is known about the trafficking motifs involved in controlling clathrin-independent internalization and various associated recycling pathways. By using a potassium channel as a model system, we identified a class of trafficking motifs that function along a clathrin-independent pathway to increase the surface density of a membrane protein by preventing its rapid internalization and/or facilitating its recycling via the ADP-ribosylation factor 6-dependent recycling pathway. Moreover, our data suggest that these motifs may enhance the association of membrane proteins with the EFA6 family of guanine nucleotide exchange factors for ADP-ribosylation factor 6.

A cell needs to closely monitor and regulate the surface density of receptors and ion channels in its plasma membrane to maintain physiological homeostasis, sense environmental changes, and communicate with other cells. Failing to do so often causes serious consequences; for example, Bartter syndrome (a hypertension disorder), Andersen disease (a muscle disorder), or hyperinsulinism may be caused by mutations affecting the surface delivery of inwardly rectifying potassium (Kir) channel Kir1.1 (1), Kir2.1 (2), or the ATP-sensitive potassium channel composed of Kir6.2 and SUR1 (2). The importance of receptor and channel trafficking has been further emphasized by a recent report that 14 of 20 Kir1.1 mutations associated with Bartter syndrome display defective trafficking and fail to reach the cell surface (1).

Because internalization and recycling regulate the surface density of receptors and channels, they play crucial roles in the function of these proteins. The clathrin-dependent internalization pathway has been the focus of research in this field because it is mediated by specific trafficking motifs located within cargo proteins and thus allows a cell to selectively control its surface level of particular receptors and channels. Known motifs for clathrin-mediated internalization include the tyrosine-based motif, dileucine-based motif, NPYX (X is any amino acid), and monoubiquitination (3). Identification and characterization of these motifs have greatly facilitated our understanding of clathrin-mediated internalization. In contrast, no such trafficking motifs have been identified for clathrin-independent internalization pathways, and much less is known about these trafficking events. Given that clathrin-independent internalization can account for as much as 50% of the total endocytic activity in a cell (4) and an increasing number of membrane proteins are reported to be internalized independently of clathrin (5), our lack of knowledge of its mechanism and regulation represents a major gap in efforts to understand how a cell regulates the level of receptors and channels on its surface.

Most studies on recycling have been carried out on proteins internalized via the clathrin-mediated internalization pathway (6). Once internalized, the nascent clathrin-coated vesicles shed their coats and undergo homotypic fusion before fusing with early endosomes. From early endosomes, internalized proteins are either recycled to the cell surface (directly via recycling endosomes or via the trans-Golgi network) or targeted to degradative lysosomes via late endosomes (6). A small GTPase of the Rab subfamily, Rab5, mediates the formation of clathrin-coated vesicles, the fusion between internalized vesicles and early endosomes, the fusion among early endosomes, and endosomal cargo recruitment and endosomal motility (7, 8). Overexpression of the GTPase-defective Q79L mutant of Rab5 increases clathrin-mediated internalization, decreases the exit of internalized proteins from early endosomes (9), and promotes the homotypic fusion of early endosomes (10). As a con-
sequence, proteins internalized via the clathrin-mediated pathway are accumulated in the early endosomes in the presence of this mutant, which eventually leads to the formation of giant endosomal structures.

Recently Arf6, one member of the Arf subfamily of small GTPases, has been shown to regulate a non-conventional recycling pathway (11). Since its discovery, many cargo proteins have been identified, including the major histocompatibility complex class I protein, the interleukin-2 receptor α subunit (11), integrins (12), E-cadherin (13), and M2 muscarinic acetylcholine receptor (M2 mAChR) (14). In cell types such as HeLa and COS-7 cells, the cargo proteins entering the Arf6 recycling pathway are internalized by a clathrin-independent mechanism (12, 14). After internalization, these proteins are first transported to an intracellular Arf6-positive compartment that is distinct from the classical Rab5-positive early endosomes. From there, internalized proteins can be either directly recycled to the cell surface (11, 12) or redirected to the Rab5-positive sorting endosomes (14–16). Expression of the GTPase-defective Arf6Q67L mutant blocks the exit of cargo proteins from the Arf6 compartment and results in their intracellular accumulation in clusters of vacuoles (12). Direct surface recycling occurs via an array of tubules extending from the juxtanuclear region to the plasma membrane. Both Arf6 and EHD1, an Eps15 homology domain-containing protein, are found to be associated with these recycling tubules, and overexpression of EHD1 induces tubular formation and enhances Arf6-mediated recycling (17). With an increasingly growing list of cargo proteins, the Arf6 recycling pathway is likely to emerge as an important player in controlling the surface density of receptors and channels.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs and Molecular Cloning**—Plasmids pcDNA3-Kir3.4 and pcDNA3-Kir3.4ΔKAC have been described previously (18). For Kir3.4 constructs containing an extracellular hemagglutinin (HA) epitope, YPYDVPDYA was inserted between amino acid residues 121 (Val) and 122 (Gly). For Kir3.4 constructs containing a C-terminus of channel protein, vectors pcDNA3-HA, pcDNA3-4FACHA, pcDNA3-4EXHA, and pcDNA3-4EEHA were constructed by inserting an HA epitope (YPYDVPDYA), four copies of FAC motif (SDSEED-EGRG) plus an HA epitope, four copies of EX motif (AEEAKEAEAEHD) plus an HA epitope, and four copies of EE motif (AHDEEEEPNG) plus an HA epitope into the NotI-EcoRV sites and pcDNA3-4EX-HA, or pcDNA3-4EE-HA to generate pcDNA3-CD4ΔCfcd2A-HA, pcDNA3-CD4ΔCfcd2A-4FAC-HA, pcDNA3-CD4ΔCfcd2A-4EX-HA, and pcDNA3-CD4ΔCfcd2A-4EE-HA. Plasmids pGFPC2-Rab5 (or -Rab5Q79L) and pGFPN1-Arf6 (or -Arf6Q67L) were made from original constructs provided by Drs. C. Bucci and J. Donaldson, respectively. To generate HA-tagged TWIK1, the coding region of TWIK1 was PCR-amplified from a mouse brain cDNA library (Dr. D. Julius) and cloned into the pcDNA3 vector containing a C-terminal HA tag. The following constructs were kind gifts: FLAG-tagged EFA6A, the inactive mutant EFA6A(E246K), and EFA6C (Drs. H. Sakagami and J. Miyazaki); EFA6B and EFA6D (Dr. J. Donaldson); green fluorescent protein fusions of Eps15Δ95/295 and Eps15Δ3Δ2 (Drs. A. Dautry-Varsat and A. Benmerah); Myc-tagged AP180C (Dr. H. McMahon); and HA-tagged mACHR5 (UMR cDNA Resource Center).

**Antibodies and Reagents**—The primary antibodies used include mouse monoclonal anti-HA (HA.11, Covance), mouse monoclonal anti-CD4 (MAB1779, Chemicon), and mouse anti-FLAG (M2, Sigma) antibodies. Fluorophore-conjugated secondary antibodies were obtained from Molecular Probes or Jackson ImmunoResearch Laboratories. Biotin-XX-conjugated transferrin and dextran conjugate (molecular mass, 70 kDa; lysine-fixable) was obtained from Molecular Probes. Phorbol 12-myristate 13-acetate, chelerythrine, amiloride, and Wortmannin were purchased from Calbiochem.

**Cell Culture and Transfection**—COS-7 or HeLa cells were cultured in Advanced Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 4% fetal bovine serum, 2 mM glutamine, and 1× penicillin-streptomycin (Cellgro). HL-1 cells were cultured in Claycomb medium supplemented with 10% fetal bovine serum, 0.1 mM norepinephrine (Sigma), 2 mM glutamine, and 1× penicillin-streptomycin. Tissue culture flasks or coverslips used for growing HL-1 cells were coated with gelatin/fibroinectin overnight in a 37 °C incubator before use. FuGENE 6 (Roche Applied Science) or Lipofectamine 2000 (Invitrogen) was used for transfecting cells as needed.

**Quantitative Surface Chemiluminescence**—A detailed protocol for performing surface chemiluminescence measurement has been described previously (19). In brief, COS-7 cells were grown on 35-mm dishes until reaching a density of ~70–80% confluence and transfected with 1 μg of total DNA/dish. 24 h later, surface proteins containing an extracellular HA epitope or the extracellular domain of CD4 were labeled by primary antibodies against HA (0.1 μg/ml) or CD4 (0.1 μg/ml) followed by horseradish peroxidase-conjugated anti-mouse secondary antibody (1:1000 dilution, Jackson ImmunoResearch Laboratories) at the non-permeabilized conditions. Surface-bound horseradish peroxidase then oxidizes a chemiluminescent substrate (SuperSignal ELISA Femto Maximum Sensitivity Substrate, Pierce) and generates the chemiluminescence that can be measured using a quantitative luminometer (TD-20/20, Turner Design) at the following settings: sensitivity, 23.1; delay, 10 s; integration, 15 s.

**Internalization Assay**—Cells transfected with HA-tagged or CD4-fused proteins were blocked (in phosphate-buffered saline (PBS) containing 2% normal goat serum, 1 h) and incubated with anti-HA or anti-CD4 antibodies (0.1 μg/ml in PBS...
containing 2% goat serum, 1 h) at 0 °C to label the surface proteins. After removing the unbound antibodies by PBS wash at 0 °C (three times for 5 min each), cells were transferred into prewarmed culture medium and returned to 37 °C to allow internalization to proceed for the indicated periods of time before immunofluorescence study.

**Immunofluorescence**—Cells were grown on the 12-mm round glass coverslips (Warner Instruments) and transfected with FuGENE 6 (Roche Applied Science). After 24–48 h, transfected cells were fixed (in PBS containing 4% formaldehyde, 20 min), permeabilized (in PBS containing 0.1% saponin, 15 min), blocked (in PBS Casein Blocker (Pierce) supplemented with 5% goat serum and 0.1% saponin, 30 min), incubated with primary antibody (0.1 μg/ml in blocking buffer, 1 h), washed (in PBS, three times for 5 min each), incubated with fluorophore-conjugated secondary antibody (1:200 dilution in blocking buffer, 1 h), and washed (in PBS, two times for 5 min each). Coverslips were then allowed to air dry in the dark, mounted, and examined with an Olympus IX81 microscope.

**Immunoprecipitation**—24 h after transfection, COS-7 were solubilized in ice-cold Nonidet P-40 lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, and 1% Nonidet P-40 complemented with Complete protease inhibitors (Roche Applied Science) and 1 mM phenylmethylsulfonyl fluoride) and cleared by centrifugation. Immunoprecipitates were collected by a brief centrifugation after incubating the supernatant with 5 μg/ml mouse monoclonal anti-HA antibody for 1 h (Covance) followed by protein G-Sepharose (Invitrogen) for 2 h at 4 °C. The Sepharose beads were washed four times in ice-cold lysis buffer and once with PBS, and the bound proteins were eluted with SDS-PAGE sample buffer at 55 °C for 1 h or 90 °C for 10 min.

**GST Pulldown**—Four copies of EX or EE motifs were PCR-amplified using pcDNA3-4EXHA or pcDNA3-4EEHA as templates and cloned into pGEX4T2 (GE Healthcare). The plasmids pGEX4T2-4EX and pGEX4T2-4EE were utilized to transform an *Escherichia coli* strain BL21 for expressing the corresponding GST–KAC fusion proteins. Purification of GST fusion proteins bound to resins was carried out using a GST-Bind kit (Novagen) according to the manufacturer's instructions. COS-7 cells were transfected with FLAG-tagged EFA6 plasmids and solubilized 24 h later in ice-cold Nonidet P-40 lysis buffer. After clearing the cell debris by centrifugation at 4 °C, the supernatant was transferred to a new tube containing appropriate GST fusion proteins prebound to resins. The mixture was then incubated with shaking at 4 °C for 2 h and briefly centrifuged. After removing the supernatant, the resin was washed four times with ice-cold GST bind/wash buffer (Novagen) and eluted with SDS-PAGE sample buffer at 55 °C for 1 h or 90 °C for 10 min.

**SDS-PAGE and Western Blot Analysis**—Samples were separated by SDS-PAGE (6–15% gradient gels, Bio-Rad) and transferred to Immobilon 0.45-μm polyvinylidene difluoride membranes (Millipore) using a Semi Dry Electroblotting System (Owl). Membranes were incubated with appropriate primary antibody for 1 h in a 1:1 mixture of Odyssey blocking buffer (LI-COR) and PBS supplemented with 0.1% Tween 20, washed with PBS supplemented with 0.1% Tween 20 (three times for 5 min each), incubated with appropriate secondary antibody (Molecular Probes) for 1 h in a 1:1 mixture of Odyssey blocking buffer and PBS supplemented with 0.1% Tween 20 and 0.01% SDS, washed first with PBS supplemented with 0.1% Tween 20 (three times for 5 min each) followed by PBS for 5 min, and dried in the dark. Quantification was performed on an Odyssey Infrared Imaging System.

**RESULTS**

Our previous studies identified an acidic amino acid sequence (Fig. 1A) whose presence dramatically increases the surface density of one inwardly rectifying potassium channel, Kir3.4, in both COS-7 cells and primary hippocampal neurons (18). A similar sequence exists in another channel, Kir2.1, and its deletion also results in a lower surface level. Because these motifs are enriched in acidic residues, they are referred to as
KAC Motifs Are Sufficient to Increase the Surface Level of a Trafficking Reporter—A bona fide trafficking motif is expected to confer a trafficking phenotype in a manner that is not strictly dependent on its context. To determine whether this is the case for KAC motifs, we fused the EX or EE motif to an unrelated trafficking receptor, CD4ΔCFcd2A. CD4ΔCFCfd2A was derived from CD4AC (provided by Dr. M. von Zastrow), which contains the extracellular region, transmembrane region, and 10 amino acids of the cytoplasmic region of CD4. As a consequence, CD4AC lacks any specific intracellular trafficking motifs and is efficiently localized to the cell surface when expressed alone in HeLa cells (Fig. 2B). We then fused the mutated cytoplasmic domain of Furin, Fcd2A, to CD4AC to generate CD4ΔCFcd2A. The cytoplasmic domain of Furin has two clathrin-mediated internalization signals (YKGL and LI) and one acidic signal mediating the endosome-to-TGN retrieval of internalized proteins (21, 22). As a consequence, CD4ΔCFcd primarily resided at the TGN in transfected cells (Fig. 2B). When the endosome-to-TGN retrieval signal was inactivated by mutating two Ser residues to Ala in CD4ΔCFcd2A (23), the fusion protein was still internalized but localized to both endosomes and the TGN presumably due to inefficient retrieval from endosomes to the TGN (Fig. 2B) (21, 22). Given the endosomal localization of CD4ΔCFcd2A, we figured it may be used as a trafficking reporter to study the effects of EX or EE motif on internalization and recycling.

We first fused one copy of EX or EE motif to the reporter CD4ΔCFcd2A and examined the trafficking of either fusion protein in transfected HeLa cells. Analysis of more than 1500 transfected cells in three separate experiments showed that fusion of either motif resulted in an increased fraction of transfected cells exhibiting robust surface localization of the reporter (Fig. 3A, n > 1500). Fusing four copies of either EX or EE further enhanced the efficiency of each trafficking motif in stimulating surface expression (Fig. 3A, n > 1500). Compared with CD4ΔCFcd2A, both CD4ΔCFcd2A-4EX and CD4ΔCFcd2A-4EE displayed greatly elevated surface staining in more than 80% of transfected cells (Fig. 3B). Similar observations were made in transfected primary hippocampal neurons (Fig. 3C, n > 100). Thus, the presence of KAC motifs can enhance the surface expression of an unrelated membrane protein (i.e. CD4ΔCFcd2A), and these motifs function as bona fide trafficking signals.

KAC Motifs Increase the Surface Level of Membrane Proteins by Inhibiting Rapid Internalization and/or Facilitating Recycling—We next set out to determine which trafficking pathway or pathways may be controlled by KAC motifs. The
observation that KAC motifs increased the surface density of CD4ΔCFCd2A implied that it might function along the endocytic pathway. To explore this possibility further, we first transfected COS-7 cells with Kir3.4-HAM or KAC-deleted Kir3.4-HAM and labeled the surface channels using an anti-HA antibody at 0 °C (to block internalization during labeling) followed by warming the cells to 37 °C to allow the internalization to proceed for various periods of time. As shown in Fig. 4A, after warming to 37 °C, KAC-deleted channel was rapidly concentrated into vesicles within 15 min in about 40% of transfected cells (arrowheads); more than 200 transfected cells were analyzed, and representative images are shown. Surface localization is indicated by arrowheads.

**FIGURE 3.** KAC motifs are sufficient to promote the surface expression of the CD4ΔCFCd2A trafficking reporter. A, fusion of either EE or EX motif allowed the detection of a robust surface expression of CD4ΔCFCd2A in a higher fraction of transfected HeLa cells. This stimulatory effect was greatly enhanced by adding four copies of each motif to the reporter. More than 500 transfected cells were scored in each experiment, and the average result from three independent experiments is shown. B, representative images of CD4ΔCFCd2A containing four copies of either EE or EX in transfected HeLa cells (surface localization indicated by arrowheads). C, when expressed in primary hippocampal neurons, these fusion proteins exhibited distributions similar to those in HeLa cells. More than 100 transfected neurons were analyzed, and representative images are shown. Surface localization is indicated by arrowheads.

**FIGURE 4.** KAC motifs prevent the rapid internalization and/or promote the recycling of membrane proteins. Surface HA-tagged Kir3.4 channels or CD4ΔCFCd2A reporters in transfected COS-7 cells were labeled by an anti-HA or anti-CD4 antibody at 0 °C and allowed to be internalized for the indicated periods of time at 37 °C before fixation. A, the KAC-deleted mutant channel became rapidly concentrated in small vesicles in 40% of transfected COS-7 cells (arrowheads); more than 200 transfected cells were examined. B, in contrast, wild-type Kir3.4 in the plasma membrane did not accumulate in similar vesicles over the same period of time (n > 200). C, whereas CD4ΔCFCd2A became enriched in the vesicles containing internalized transferrin, fusion of EX or EE motif inhibited the enrichment of CD4ΔCFCd2A in similar transferrin-containing vesicles.

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In conclusion, KAC motifs inhibit internalization on the plasma membrane and/or facilitate the endosome-to-surface recycling.

**Surface Proteins Containing KAC Motifs Preferentially Enter the Arf6 Recycling Pathway via a Clathrin-independent Internalization Pathway**—The above result established a role of KAC motifs in internalization or recycling of surface proteins, but it did not address the nature of internalization and recycling pathways utilized by proteins carrying these motifs. Although most internalized membrane proteins are first transported to the Rab5-positive early endosomes (9), several proteins internalized independently of clathrin may bypass this conventional route and be targeted to the Arf6 compartment (12). To dissect the trafficking pathway of internalized Kir3.4 and its KAC-deleted mutant, we investigated the subcellular localization of both channels in HeLa cells transfected by Rab5Q79L or Arf6Q67L, two GTPase-defective mutants that block the exit
from the Rab5 early endosomes (9, 10) or Arf6 compartment (12), respectively. For this purpose, we utilized Kir3.4 containing a C-terminal HA tag (Kir3.4-HAC) because it generates less intracellular endoplasmic reticulum staining and thus would facilitate our imaging analysis of the channel along the endocytic pathway. Our previous study has demonstrated that trafficking of Kir3.4-HAC is affected by KAC motifs in a similar manner (18). As shown in Fig. 5, KAC-deleted channels were mainly trapped in the early endosomes of cells expressing Rab5Q79L (Fig. 5A, arrowheads), whereas the wild-type channels predominantly accumulated in the vacuolar structure of cells expressing Arf6Q67L (Fig. 5B, arrowheads). The Kir3.4-containing vacuolar structure was the Arf6 compartment because it also contained a high level of integrin β1 receptor, a cargo protein shown previously to be recycled via the Arf6 compartment (12). The localization of Kir3.4 to the Arf6 compartment in native cells was also confirmed using both the transfected primary hippocampal neurons (Fig. 5, C and D, arrowheads, n > 100) and HL-1 cells (24), which are a mouse neonatal cardiomyocyte cell line expressing endogenous Kir3.4 (25) (Fig. 5E, arrowhead). Analysis of 100 transfected HL-1 cells further indicated that endogenous Kir3.4 channel enters the Arf6 compartment in 90% of cells transfected by Arf6Q67L. In contrast, Kir3.4 could only be detected in about 20% of cells expressing Rab5Q79L (Fig. 5F). Finally when fused to CD4ΔFc2A, KAC was able to shift a significant fraction of internalized fusion protein from Rab5 to Arf6 compartment (Fig. 5G). Taken together, our data demonstrate that KAC motifs are both necessary and sufficient to promote membrane proteins to enter the Arf6 recycling pathway.

Recent studies suggest that a protein entering the Arf6 compartment can be either recycled to the plasma membrane or redirected to the con-

FIGURE 5. KAC motifs promote the entry into the Arf6 compartments. A, KAC-deleted Kir3.4 channel was trapped in classical Rab5-positive sorting endosomes (arrowheads) but not in the Arf6 compartment of transfected HeLa cells. B, on the other hand, wild-type Kir3.4 channel was trapped in the Arf6 compartment (arrowheads) but not in the classical Rab5 sorting endosomes. The channel was visualized using an anti-HA antibody. Integrin β1 was used as a marker of Arf6 compartment. C and D, as observed in HeLa cells, co-expression of Rab5Q79L (C) or Arf6Q67L (D) in the primary hippocampal neurons prevented the exit of KAC-deleted Kir3.4 from sorting endosomes (arrowheads) or wild-type Kir3.4 from Arf6 compartment (arrowhead), respectively. More than 100 transfected cells were examined, and representative images are shown. E, endogenous Kir3.4 also accumulated in the Arf6 compartment (arrowheads) of HL-1 cardiomyocyte cells in the presence of Arf6Q67L. F, endogenous Kir3.4 preferably trafficked via the Arf6 compartment over the classical Rab5 compartment in HL-1 cells. Kir3.4 in 100 transfected HL-1 cells was scored from three separate experiments for its co-localization with either Rab5Q79L or Arf6Q67L, and the average result is shown. G, fusing EX or EE to CD4ΔFc2A also led to the enrichment of either fusion protein in the Arf6 compartment upon the expression of Arf6Q67L in HeLa cells. H, wild-type Kir3.4, but not Kir3.4ΔKAC, was found associated with EHD1 recycling tubules (arrowheads) in transfected HeLa cells. GFP, green fluorescent protein; DAPI, 4',6-diamidino-2-phenylindole.
conventional Rab5 endosomes (14–16). Two lines of observations suggest that the internalized Kir3.4 is recycled back to the cell surface rather than being diverted to the Rab5 endosomes from the Arf6 compartment. First, little Kir3.4 accumulated in cells expressing Rab5Q79L mutant (Fig. 5B). Second, in the presence of overexpressed EHD1, which enhances the Arf6-mediated recycling (17), Kir3.4, but not its KAC-deleted mutant, was found to be associated with the EHD1-decorated recycling tubules (Fig. 5H).

Because an earlier study showed that proteins entering the Arf6 compartment are internalized through a clathrin-independent mechanism in HeLa and COS-7 cells (12), we decided to examine whether KAC motifs allowed Kir3.4 to enter the Arf6-mediated internalization pathway. For this purpose, we monitored the internalization of Kir3.4 in COS-7 cells transfected with a combination of either Kir3.4-HAM plus AP180C or Kir3.4-HAM plus the vector. AP180 is a protein normally involved in the formation of clathrin-coated pits on the plasma membrane, and the expression of its truncated mutant AP180C blocks the clathrin-dependent internalization (26). To facilitate the detection of internalized channel protein, we added anti-HA antibody to the growth medium, allowed the internalization to proceed for 1 h at 37 °C, and then stripped the surface-bound antibody using an acid wash at 0 °C. Under this condition, we were able to detect vesicles containing internalized Kir3.4 (Fig. 6A). These vesicles displayed little co-localization with internalized transferrin (Fig. 6A), a known cargo of clathrin-mediated endocytosis. Moreover internalization of Kir3.4 persisted in those cells where clathrin-dependent endocytosis of transferrin was efficiently inhibited by AP180C (Fig. 6B). More importantly, quantitative analyses showed that co-expression of AP180C led to a 50% reduction of relative surface to total protein ratio of Kir3.4 as well as a moderate increase (~40%) of relative internalization to surface ratio (Fig. 6C). These observations cannot be explained if Kir3.4 was internalized via the clathrin-dependent mechanism and supported our hypothesis that Kir3.4 was mainly endocytosed by a clathrin-independent pathway. The moderate increase of channel internalization in the presence of AP180C is also consistent with the previous report that clathrin-independent pinocytosis is induced upon the inhibition of clathrin-dependent internalization (27). Finally expression of AP180C in HL-1 cells did not significantly influence the ability of Kir3.4 to enter the Arf6 compartment. Kir3.4 could be still detected in up to 70% of cells co-expressing Arf6Q67L and AP180C (Fig. 6D, n = 100), a level similar to that (90%) observed in cells expressing Arf6Q67L alone (Fig. 5F). Our result suggests that Kir3.4 is targeted to the Arf6 compartment primarily through a clathrin-independent pathway.

KAC Motifs Enhance the Association with the EFA6 Family of Arf6 Guanine Nucleotide Exchange Factors (GEFs)—The observation that Kir3.4, but not its KAC-deleted mutant, enters the Arf6 compartment prompted us to explore whether our KAC motifs interact with regulators in the Arf6 pathway. We began our analysis with EFA6A because it is a plasma membrane-localized Arf6-specific GEF and participates in the Arf6-mediated endosomal recycling (28). Moreover it has been shown that EFA6 can interact with TWIK1, a potassium channel involved in regulating neuronal excitability and cell volume (29). Indeed HA-tagged Kir3.4 and FLAG-tagged EFA6A could be co-immunoprecipitated from co-transfected COS-7 cells, whereas deletion of the KAC motifs significantly reduced the efficiency of co-immunoprecipitation (Fig. 7A). Compared with TWIK1, Kir3.4 appeared to co-immunoprecipitate EFA6A more efficiently. To verify that the KAC motifs mediate the association of
Kir3.4 with EFA6A, we generated GST fusions of KAC and conducted a GST pulldown using the COS-7 lysate containing FLAG-tagged EFA6A (Fig. 7B). Indeed the GST fusion of either EX or EE motif was able to pull down EFA6A. In support of the above data, when co-transfected into the hippocampal neurons where EFA6A is endogenously expressed, Kir3.4 and EFA6A were co-localized in both the cell body and the dendrites (Fig. 7C).

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In addition to EFA6A, three other members of EFA family GEFs, EFA6B (30), -6C (30), and -6D (31) have been identified.
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To examine whether KAC motifs may potentiate the association of Kir3.4 with these isoforms, we compared the abilities of Kir3.4 and Kir3.4KAC to co-immunoprecipitate these isoforms from transfected COS-7 cells. The result shown in Fig. 7D implies that KAC motifs increase the association of channel proteins with EFA6B and -6C.

Phorbol 12-Myristate 13-Aacetate (PMA) Stimulation Induces the Internalization of Kir3.4 via Macropinocytosis—Given that Arf6 has been suggested to play a role in macropinocytosis (11, 12) and that surface Kir3.4 was occasionally observed in large internal vesicles (data not shown) in COS-7 cells, we investigated whether internalization of Kir3.4 can also be mediated by macropinocytosis. Because PMA is known to promote the macropinocytosis (32), we monitored the internalization of Kir3.4 after a brief treatment with PMA (100 nM, 30 min) in both COS-7 and hippocampal neurons (Fig. 8A). Whereas Kir3.4 appeared to be stable on the surface of resting cells, Kir3.4 channels were efficiently internalized into large vesicles in 80% of the PMA-stimulated cells (n > 100). The vesicles resembled macropinosomes because they were typically bigger than 1 μm in diameter (Fig. 8A), and their formation was blocked by the protein kinase C inhibitor chelerythrine (5 μM, data not shown).

To confirm the identity of these vesicles, we first measured the ability of specific endocytosis inhibitors to block the PMA-induced channel internalization in COS-7 cells, including overexpression of either Eps15(Δ95/295) (a truncated mutant of Eps15 (33)) or AP180C (26) either of which has been shown to specifically block clathrin-dependent internalization without affecting macropinocytosis, as well as amiloride (an inhibitor of Na+/H+ exchanger) and wortmannin (an inhibitor of phosphatidylinositol 3-kinase), which are known to selectively block macropinocytosis (32, 34). Our result indicated that amiloride or wortmannin, but not Eps15(Δ95/295) or AP180C, severely impaired the PMA-induced internalization of Kir3.4 (Fig. 8B). We next examined whether the PMA-induced Kir3.4-containing vesicles in COS-7 cells may be labeled by 70-kDa dextran added to the culture medium. High molecular weight dextran has been demonstrated previously to preferentially label macropinosomes (35). Indeed internalized dextran was enriched in channel-containing vesicles (Fig. 8C). Taken together, our data suggest that although the surface Kir3.4 channel is stable due to the existence of KAC motifs, its internalization may be induced by protein kinase C-mediated activation of macropinocytosis.

DISCUSSION

Our study using Kir3.4 channel demonstrated that two types of KAC motifs exist to maintain the surface expression of channel proteins on the plasma membrane. Because adding either motif to an unrelated reporter CD4ΔCFcd2A led to an enhanced surface level of fusion proteins, KAC motifs are bona fide trafficking signals. Given that the KAC-deleted mutant channel was present on the plasma membrane at a lower level yet found in endosomes to a much higher extent than wild-type Kir3.4 (18), KAC motifs inhibit the internalization and/or promote the recycling of surface proteins. Our observations that wild-type Kir3.4, but not its KAC-deleted mutant, preferentially entered the Arf6 recycling compartment via a clathrin-independent internalization pathway further suggest that KAC motifs either function at the cell surface to allow plasma membrane proteins to enter a slower clathrin-independent internalization and Arf6-dependent recycling pathway or act at an intracellular compartment to facilitate the Arf6-dependent recycling of internalized proteins. In the absence of KAC, mutant channel proteins failed to enter the Arf6 compartment.
and thus accumulated in the cell interior as a consequence of rapid internalization and/or inefficient recycling. Consistent with the above conclusion, Kir3.4, but not KAC-deleted mutant, was found associated with the Arf6 recycling tubules, and KAC motifs appeared to mediate the association of Kir3.4 with several members of the EFA6 family of Arf6 GEFs.

In the case that KAC motifs function on the plasma membrane, our data would indicate that they are able to prevent the rapid internalization of both clathrin-independent or -dependent pathways. This is because KAC motifs can promote the surface expression of both Kir3.4, a cargo primarily internalized via a clathrin-independent route, and CD4ΔCFCd2A, a cargo mainly endocytosed through a clathrin-dependent mechanism. KAC motifs may anchor a membrane protein from being rapidly internalized by interacting with EFA6 or another yet-to-be-identified protein on the plasma membrane.

How KAC motifs can affect both internalization and recycling under this condition is an intriguing question. We propose two possibilities. Accumulated surface proteins can slowly internalize and enter the constitutive Arf6 recycling pathway. In this scenario, how often a protein gets access to the Arf6 recycling pathway depends on its level on the surface, and no specific sorting signal is required. Alternatively KAC motifs may not only inhibit rapid internalization but also actively sort surface proteins into the Arf6 recycling pathway. This can be achieved, for example, if KAC motifs allow proteins to be enriched in a membrane subdomain where proteins are sequestered from rapid internalization pathways and predisposed to enter the Arf6 recycling pathway. This possibility is consistent with several recent studies showing that different subdomains on the plasma membrane are coupled to different internalization and recycling pathways. Light microscopy imaging studies have shown that clathrin-coated pits tend to assemble repeatedly at specific sites on the plasma membrane, called “coated pit zones” (36). On the other hand, interleukin 2 receptor associates with lipid rafts and is internalized independently of Eps15, a component of the clathrin machinery (37). Moreover transforming growth factor receptor β internalized via the clathrin-dependent pathway triggers signaling from the early endosomes; yet the same receptor internalized via caveolae is targeted to the caveosome for degradation (38).

Whereas the above model that KAC motifs function on the plasma membrane could explain our observations, our data are also consistent with an alternative model that KAC motifs function intracellularly to promote the recycling of internalized proteins to the plasma membrane. Because the results using the Arf6Q67L mutant suggest that the majority of internalized Kir3.4 channel, but not the KAC-deleted mutant, enters the Arf6 compartment, KAC motifs likely function to promote the correct sorting of internalized channel into the Arf6 compartment. Under this condition, KAC motifs may also facilitate recycling back to the surface from the Arf6 compartment. There is also evidence supporting that the Arf6 compartment may have a sorting function. Whereas most cargo proteins entering the Arf6 compartment are recycled to the cell surface, at least one receptor, M2 mAChR, has been shown to be targeted to the Rab5-positive early endosomes from this compartment (14). Future experiments such as fusing KAC motifs to M2 mAChR may allow us to test whether KAC motifs control the sorting of cargo proteins at the Arf6 compartment.

The sequence similarity between our KAC motifs and the well characterized Furin acidic cluster motif (Fig. 2A) raises the possibility that these motifs are functionally related. The Furin acidic cluster motif was originally found in the cytoplasmic region of Furin, a TGN protein shuttling between the TGN and the plasma membrane via endosomes (39). Subsequent studies have demonstrated that this motif promotes the TGN localization of Furin by facilitating the retrieval of internalized Furin proteins from endosomes to the TGN (39), and the phosphorylation of two serine residues by casein kinase II is essential for the function of Furin acidic cluster (23). Using the yeast two-hybrid system, Wan et al. (21) and Crump et al. (22) have identified that phosphofurin acidic cluster sorting protein-1 binds to the phosphorylated Furin acidic cluster and directs the endosome-to-TGN retrieval of Furin. It remains to be determined whether phosphofurin acidic cluster sorting protein-1 or similar proteins are involved in the KAC-mediated recycling through the Arf6 compartment.

Whether the Arf6 endosomal compartment exists in excitable cells such as neurons or cardiomyocytes has not been documented previously. In this study, we showed that overexpression of the Arf6 GTPase-defective mutant in the hippocampal neurons or HL-1 neonatal cardiomyocytes can trap transfected or endogenous Kir3.4 in the Arf6 endosomal compartments, respectively. Similar to what was observed in HeLa cells, deletion of KAC also caused the mutant channel to be diverted to the Rab5 endosomes in hippocampal neurons. These observations demonstrate the existence of an Arf6 endosomal compartment in neurons and cardiomyocytes and indicate that KAC motifs still mediate Arf6-dependent recycling in excitable cells.

Interestingly although Kir3.4 containing KAC was stable on the surface of resting cells, it could be efficiently internalized into macropinosomes following protein kinase C activation in COS-7 or HeLa cells. Whereas macroinocytosis is a constitutive process in hematopoietic cells such as macrophages and dendritic cells, it is relatively inactive in most other cell types but can be greatly stimulated by growth factors or activation of protein kinase C (32, 34). Such protein kinase C-induced internalization of Kir3.4 may confer a neuron or heart cell the ability to selectively regulate its membrane potential under certain physiological conditions. For example, protein kinase C activation in the heart during metabolic stress such as ischemia is known to lead to internalization of an ATP-sensitive potassium channel, KATP (40). This protein kinase C-mediated internalization of KATP has been suggested to play a role in preventing the excessive shortening of the action potential duration and thus the arrhythmias. In this regard, macroinocytosis of Kir3.4 may work synergistically with internalization of KATP to protect the metabolically stressed heart. Several recent studies have also discovered that formation of macropinosomes in frog motor neurons depends on the stimulus frequency associated with the onset of synaptic depression (41), suggesting a potential coupling between macroinocytosis and neural activities (42). Whether macroinocytosis of Kir3.4 or other KAC-con-
taining channel proteins is involved in the regulation of neuronal excitability is an intriguing possibility to be explored.

Acknowledgments—We thank the following individuals for plasmid constructs: Dr. H. Sakagami and Dr. J. Miyazaki for FLAG-tagged EFA6A, EFA6C, and the inactive mutant EFA6A(E246K); Dr. J. Donaldson for EFA6B, EFA6D, Arf6, and its mutants; Dr. A. Dautry-Varsat and Dr. A. Benmerah for the green fluorescent protein fusions of Eps15/95/295 and Eps15D3; Dr. H. McMahon for the CD45; Dr. C. Bucci for the Rab5 cDNA clone; Dr. M. von Zastrow for the CD44G; and Dr. G. Thomas for the Furin constructs. We are especially grateful to Dr. J. Donaldson for the antibodies against EFA6 and for invaluable suggestions.

REFERENCES
1. Peters, M., Ermert, S., Jeck, N., Derst, C., Pechmann, U., Weber, S., Schlingmann, K. P., Seyberth, H. W., Waldegger, S., and Konrad, M. (2003) Kidney Int. 64, 923–932
2. Kass, R. S. (2005) J. Clin. Investig. 115, 1986–1989
3. Mousavi, S. A., Malerod, L., Berg, T., and Kjeken, R. (2004) Biochem. J. 377, 1–16
4. Sandvig, K., and van Deurs, B. (1991) Cell Biol. Int. Rep. 15, 3–8
5. Kirkham, M., and Parton, R. G. (2000) Biochim. Biophys. Acta 1475, 273–286
6. Maxfield, F. R., and McGraw, T. E. (2004) Nat. Rev. Mol. Cell. Biol. 5, 121–132
7. Zerial, M., and McBride, H. (2001) Nat. Rev. Mol. Cell. Biol. 2, 107–117
8. Somsel Rodman, J., and Wandinger-Ness, A. (2000) J. Cell Sci. 113, 183–192
9. Bucci, C., Parton, R. G., Mather, I. H., Stunnenberg, H., Simons, K., Hoflack, B., and Zerial, M. (1994) EMBO J. 13, 1287–1296
10. Radhakrishna, H., and Donaldson, J. G. (1997) J. Cell Biol. 139, 49–61
11. Brown, F. D., Rozelle, A. L., Yin, H. L., Balla, T., and Donaldson, J. G. (2001) J. Cell Biol. 154, 1007–1017
12. Paterson, A. D., Parton, R. G., Ferguson, C., Stow, J. L., and Yap, A. S. (2003) J. Biol. Chem. 278, 21050–21057
13. Delaney, K. A., Murph, M. M., Brown, L. M., and Radhakrishna, H. (2002) J. Biol. Chem. 277, 33439–33446
14. Naslavsky, N., Weigert, R., and Donaldson, J. G. (2003) Mol. Biol. Cell 14, 417–431
15. Naslavsky, N., Weigert, R., and Donaldson, J. G. (2004) Mol. Biol. Cell 15, 3542–3552
16. Caplan, S., Naslavsky, N., Hartnell, L. M., Lodge, R., Polishchuk, R. S., Donaldson, J. G., and Bonifacino, J. S. (2002) EMBO J. 21, 2557–2567
17. Ma, D., Zerangue, N., Raab-Graham, K., Fried, S. R., Jan, Y. N., and Jan, L. Y. (2002) Neuron 33, 715–729
18. Zerangue, N., Schwappach, B., Jan, Y. N., and Jan, L. Y. (1999) Neuron 22, 537–548
19. Ma, D., Zerangue, N., Lin, Y. F., Collins, A., Yu, M., Jan, Y. N., and Jan, L. Y. (2001) Science 291, 316–319
20. Wan, L., Molloy, S. S., Thomas, L., Liu, G., Xiang, Y., Rybak, S. L., and Thoma, G. (1998) Cell 94, 205–216
21. Crump, C. M., Xiang, Y., Thomas, L., Gu, F., Austin, C., Tooze, S. A., and Thomas, G. (2001) EMBO J. 20, 2191–2201
22. Dittie, A. S., Thomas, L., Thomas, G., and Tooze, S. A. (1997) EMBO J. 16, 4859–4870
23. Claycomb, W. C., Lanson, N. A., Jr., Stallworth, B. S., Egeland, D. B., Delcarpio, J. B., Babinski, A., and Izzo, N. J., Jr. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 2979–2984
24. Dobrzynski, H., Marples, D. D., Musa, H., Yamanushi, T. T., Henderson, Z., Takagishi, Y., Honjo, H., Kodama, I., and Boyett, M. R. (2001) J. Histochem. Cytochem. 49, 1221–1234
25. Ford, M. G., Pearse, B. M., Higgins, M. K., Vallis, Y., Owen, D. J., Gibson, A., Hopkins, C. R., Evans, P. R., and McMahon, H. T. (2001) Science 291, 1051–1055
26. Damke, H., Baba, T., van der Bliek, A. M., and Schmid, S. L. (1995) J. Cell Biol. 131, 69–80
27. Franco, M., Peters, P. J., Boretto, J., van Donselaar, E., Neri, A., D’Souza-Schorey, C., and Chavrier, P. (1999) EMBO J. 18, 1480–1491
28. Decressac, S., Franco, M., Bendahhou, S., Warth, R., Knauer, S., Barhanin, J., Lazdunski, M., and Lesage, F. (2004) EMBO Rep. 5, 1171–1175
29. Derrien, V., Couillault, C., Franco, M., Martineau, S., Montcourrier, P., Houlgatte, R., and Chavrier, P. (2002) J. Cell Sci. 115, 2867–2879
30. Sakagami, H., Suzuki, H., Kamata, A., Owada, Y., Fukunaga, K., Mayanagi, H., and Kondo, H. (2006) Brain Res. 1093, 1–11
31. Swanson, J. A., and Watts, C. (1995) Trends Cell Biol. 5, 424–428
32. Benmerah, A., Bayrou, M., Cerf-Bensussan, N., and Dautry-Varsat, A. (1999) J. Cell Sci. 112, 1303–1311
33. Cardelli, J. (2001) Traffic 2, 311–320
34. Racoosin, E. L., and Swanson, J. A. (1993) J. Cell Biol. 121, 1011–1020
35. Santini, F., Gaidarov, I., and Keen, J. H. (2002) J. Cell Biol. 156, 665–676
36. Lamaze, C., Dujoncourt, A., Baba, T., Lo, C. G., Benmerah, A., and Dautry-Varsat, A. (2001) Mol. Cell 7, 661–671
37. Di Guglielmo, G. M., Le Roy, C., Goodfellow, A. F., and Wrana, J. L. (2003) Nat. Cell Biol. 5, 410–421
38. Thomas, G. (2002) Nat. Rev. Mol. Cell Biol. 3, 753–766
39. Hu, K., Huang, C. S., Jan, Y. N., and Jan, L. Y. (2003) Neuron 38, 417–432
40. Richards, D. A., Guatimosim, C., Rizzoli, S. O., and Betz, W. J. (2003) Neuron 39, 529–541
41. Wilkinson, R. S., and Lin, M. Y. (2004) Trends Neurosci. 27, 171–174